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DOCTORAL THESIS

Dissecting Stimulus-Dependent Transcription of Brain-Derived Neurotrophic Factor

Eli-Eelika Esvald

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

Eli-Eelika Esvald

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Aju-päritolu neurotroofse teguri stiimulsõltuva transkriptsiooni uuringud

ELI-EELIKA ESVALD

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

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CREB family transcription factors are major mediators of BDNF transcriptional autoregulation in cortical neurons

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II **Esvald EE**, Tuvikene J*, Moistus A*, Rannaste K, Kõomägi S, Timmusk T

Differential regulation of the BDNF gene in cortical and hippocampal neurons.

J Neurosci. 2022 Dec 7;42(49):9110-9128. doi: 10.1523/JNEUROSCI.2535-21.2022. Epub 2022 Oct 31.

III 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

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Author's Contribution to the Publications

Contribution to the papers in this thesis are as follows:

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

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

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

Introduction

Brain-derived neurotrophic factor (BDNF) is an important signalling molecule that plays a role in neuronal survival, differentiation, and synaptic plasticity, thereby influencing various cognitive functions such as behaviour, learning, and memory. The regulation of *Bdnf* gene expression during development and in response to different stimuli is a complex and highly regulated process. Given the involvement of BDNF in many functions of the nervous system, BDNF has great potential as a therapeutic tool for different neurodevelopmental and neurodegenerative diseases. However, the complexity of *Bdnf* gene regulation and BDNF-dependent signalling is still not completely understood.

In the present PhD thesis, we studied stimulus-dependent regulation of *Bdnf* expression. First, we focused on the mechanisms of BDNF-TrkB signalling-dependent *Bdnf* gene regulation in cultured cortical neurons. Next, we focused on brain region-specific activity-dependent regulatory mechanisms of *Bdnf*, focusing on cortical and hippocampal neurons. Finally, we studied distal regulatory regions of the *Bdnf* gene and revealed the first cell type-specific enhancer region potentiating *Bdnf* expression in neurons. The research underlying this thesis contributes to a comprehensive understanding of the molecular mechanisms governing *Bdnf* expression and lays the foundation for potential therapeutic interventions targeting neurodegenerative and neuropsychiatric disorders associated with aberrant *BDNF* expression.

Abbreviations

AP1	Activating protein 1
ATF1	Activating transcription factor 1
BDNF	Brain-derived neurotrophic factor
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
CRE	cAMP-response element
CREB	CRE-binding protein
CREM	CRE-modulator
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
CRTC	CREB-regulated transcription coactivator
CTX	Cortex
DIV	Days *in vitro*
E	Embryonic day
GFP	Green fluorescent protein
HC	Hippocampus
NGF	Nerve growth factor
NT	Neurotrophin
p75NTR	p75 neurotrophin receptor
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
TrkB	Tropomyosin-related kinase B
UTR	Untranslated region

1 Review of literature

1.1 The family of neurotrophic factors

Neurotrophins are a family of secreted signalling molecules that support neuronal survival, neurite growth, and neuronal maturation (Chao, 2003). NGF (*nerve growth factor*) was the first discovered neurotrophin that, at the time as an unknown compound in a tumour extract, stimulated neurite growth of different ganglia (Cohen et al., 1954; Levi-Montalcini & Hamburger, 1951). This marked the beginning of a new era in developmental neurobiology – the study of neurotrophic factors.

Almost 25 years after the discovery of NGF, it was discovered that a compound from the glioma cell culture could keep chicken embryonic sensory neurons alive (Y. A. Barde et al., 1978; Y.-A. Barde et al., 1980). In the following years, the active compound was purified from pig brain (Y. A. Barde et al., 1982) and shown to be a neuronal survival factor (Y. A. Barde et al., 1982; Johnson et al., 1986; Rodriguez-Tébar et al., 1989). Next, nucleotide sequence of the novel compound, named BDNF (*brain-derived neurotrophic factor*), was determined and the protein sequence was shown to be highly similar to NGF (Leibrock et al., 1989), starting the family of neurotrophic factors. Based on protein sequence similarity, neurotrophin 3 (NT-3) (Ernfors et al., 1990; Hohn et al., 1990) and neurotrophin 4 (NT-4) (Berkemeier et al., 1991; Hallböök et al., 1991) soon followed as members of the neurotrophic family. In fish, additional neurotrophins, NT-6 and NT-7, have been described (Götz et al., 1994; Lai et al., 1998; Nilsson et al., 1998). Altogether, NGF, BDNF, NT-3, and NT-4 form the family of neurotrophic factors in mammals (Chao, 2003).

1.2 *BDNF* gene structure

The *Bdnf* gene spans approximately 50 kbp and contains multiple promoters that direct tissue-specific expression of *Bdnf* (Nakayama et al., 1994; Timmusk et al., 1993). Initially, five exons were identified in the *Bdnf* gene (Timmusk et al., 1993), and further studies discovered additional 5' exons (Q.-R. Liu et al., 2005, 2006). Thus, the rodent *Bdnf* gene consists of eight 5' non-coding exons and one 3' coding exon (Aid et al., 2007), while the human *BDNF* gene contains ten 5' exons and one 3' coding exon (Figure 1) (Pruunsild et al., 2007). *BDNF* exon IIc contains three splice donor sites that could be used for alternative splicing (Aid et al., 2007; Pruunsild et al., 2007). In humans also exons V, VII, and VIII can undergo alternative splicing, and exons VIII and VIIIh can be used as internal exons and spliced together with exon V, further increasing the complexity of the *BDNF* gene (Pruunsild et al., 2007).

In humans an extra layer of regulation of *BDNF* mRNA levels is achieved by the non-coding antisense *BDNF* transcript that partially overlaps with the *BDNF* gene. Antisense *BDNF* has been shown to form duplexes with *BDNF* mRNA (Q.-R. Liu et al., 2005; Pruunsild et al., 2007). Knockdown of antisense *BDNF* RNA results in increased *BDNF* mRNA levels, indicating negative regulation of *BDNF* expression by the antisense transcript (Modarresi et al., 2012).

1.2.1 *BDNF* 5' exons

BDNF 5' exons are generally non-coding, although exon I in both humans and rodents (Aid et al., 2007; Pruunsild et al., 2007), and exons VII and VIII in humans contain an in-frame translation initiation codon and could be used to initiate translation (Pruunsild et al., 2007). Notably, the alternative in-frame AUG in *Bdnf* exon I is used more efficiently

for translation initiation than the start codon in the coding exon (Koppel et al., 2015). Furthermore, the exon I-containing transcripts are the most enriched *Bdnf* transcript in synaptoneurosomes (Lekk et al., 2023). It has also been shown that all *Bdnf* 5' UTRs (untranslated regions) repress translation, with the UTR resulting from exon II being the most repressive (Lekk et al., 2023).

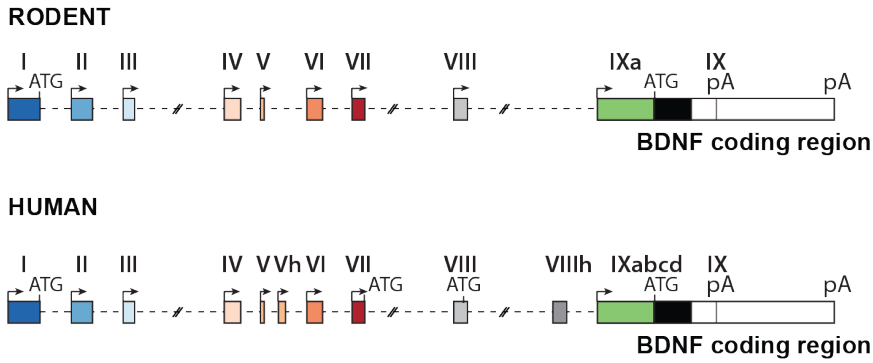

Figure 1. Schematical representation of rodent and human BDNF gene structure. *BDNF exons are shown with boxes with Roman numerals above the box indicating exon number. Dashed line represents introns. BDNF coding sequence in exon IX is shown in black. Arrows above the boxes indicate transcription start site, ATG indicates alternative translation start sites, and pA marks the locations of alternative poly-adenylation signal sequences that result in BDNF transcripts with either short or long 3' UTR. (Adapted from (Esvald et al., 2023)).*

The expression of the first cluster of *BDNF* exons (exons I, II, and III) is mainly brain-specific, whereas the second cluster (*BDNF* exons IV to VII) are expressed in both neural and non-neural tissues (Aid et al., 2007; Pruunsild et al., 2007; Timmusk et al., 1993). In the human brain, major *BDNF* transcripts contain exons I, IIc, or IV, while in rodents, *Bdnf* exon VI also remarkably contributes to the total *Bdnf* mRNA pool (Esvald et al., 2023). Some brain regions in the human brain exhibit specific expression patterns of different *BDNF* transcripts, e.g., exon I-containing transcripts contribute to total *BDNF* levels more in the amygdala and thalamus, whereas exon IIc-containing transcripts form a remarkable proportion of total *BDNF* mRNA in the cerebellum (Esvald et al., 2023). The usage of *Bdnf* transcripts varies between cell types, as majority of *Bdnf* transcripts in cortical neurons contain exon IV, whereas the major *Bdnf* transcripts contain exon VI in cortical astrocytes (Koppel et al., 2018).

Only certain cells, e.g., glutamatergic neurons, express *Bdnf* and are a major source of BDNF in the brain (Andreska et al., 2014; Hartmann et al., 2001). In peripheral tissues, for example, single-cell RNA sequencing data indicates that mesothelial cells in the lung and smooth muscle cells in musculature, spleen and vasculature are primary cells expressing *Bdnf* in these tissues (Esvald et al., 2023). It has been recently shown using single molecule fluorescent *in situ* hybridization that the expression levels of *Bdnf* varies between neurons (Maynard et al., 2020). Almost all neurons that express *Bdnf* exon I also express exon IV, whereas only around half of neurons that express *Bdnf* exon IV also express exon I transcripts at the same time (Maynard et al., 2020).

Membrane depolarization increases transport of *Bdnf* mRNA to dendrites (Tongiorgi et al., 1997) via interaction with translin (Chiaruttini et al., 2009) for local translation of *Bdnf* in the neurites (Baj et al., 2011). Translin participates in *Bdnf* trafficking after

neuronal activity, but not after BDNF treatment (Y.-C. Wu et al., 2011), implying the presence of several highly specific mechanisms governing *Bdnf* mRNA localization. It has been reported that the main *Bdnf* transcripts in distal dendrites contain exons II or VI, whereas other *Bdnf* transcripts are mostly restricted to the cell soma (Baj et al., 2011, 2013; Chiaruttini et al., 2009). More specifically, transcripts containing *Bdnf* exon IV remain in cell soma while exon VI-containing transcripts are localized in both soma and dendrites (Baj et al., 2013; Chiaruttini et al., 2008, 2009; Pattabiraman et al., 2005; Singer et al., 2018). Furthermore, specific localization of different *Bdnf* transcripts and local translation has been confirmed using knock-in of cyan or yellow fluorescent protein-encoding sequence to the *Bdnf* 5' exons both *in vitro* and *in vivo* (Matt et al., 2018; Singer et al., 2018). Although the exact mechanism of *Bdnf* mRNA compartmentalization remains to be studied, it has been reported that different *Bdnf* transcripts have distinct effects on dendrite complexity (Baj et al., 2011). In agreement with the localization and potential local translation of the transcripts, knockout of BDNF protein from exon II reduces dendrite branching and loss of BDNF protein from exon VI transcripts reduces spine morphology on apical dendrites (Maynard et al., 2017).

1.2.2 *BDNF* alternative polyadenylation signal

In addition to different 5' UTRs, *Bdnf* coding exon contains several polyadenylation sites, allowing for the formation of *Bdnf* transcripts with either short (~1.6 kbp) or long (~4.2 kbp) 3' UTR (Fukuchi & Tsuda, 2010; Maisonpierre et al., 1991; Ohara et al., 1992; Timmusk et al., 1993). *Bdnf* transcripts with short 3' UTR are generally more stable (Castrén et al., 1998) and more enriched to polysomes than transcripts with long 3' UTR (Timmusk et al., 1994). *Bdnf* long 3' UTR has been shown to decrease *Bdnf* mRNA stability (Will et al., 2013) due to miRNA binding sites that decrease *Bdnf* mRNA levels (Mellios et al., 2008; Varendi et al., 2014).

Different *Bdnf* 3' UTRs have been reported to result in distinct *Bdnf* transcript localisation patterns. The majority of *Bdnf* transcripts have a short 3' UTR and are located in the cell soma (An et al., 2008; Will et al., 2013), while transcripts with long 3' UTR localize in dendrites and promote maturation of dendritic spines (An et al., 2008). Notably, *Bdnf* transcripts with short and long 3' UTR have a slightly different role in dendrite branching, as they promote the branching of secondary and primary dendrites, respectively (O'Neill et al., 2018). *Bdnf* transcripts with different 3' UTRs are differentially transported to dendrites in response to various stimuli. For example, *Bdnf* transcripts with both short and long 3' UTR are transported to dendrites in response to membrane depolarization, only transcripts with short 3' UTR are transported after NT-3 signalling, and only transcripts with long 3' UTR are transported after BDNF signalling (Vicario et al., 2015).

The precise effect of *Bdnf* 3' UTR on translatability is still unclear. On one hand *Bdnf* long 3' UTR has been shown to repress translation at basal conditions, while neuronal activity leads to greater enrichment of *Bdnf* transcripts with long 3' UTR in polysomes compared to those with short 3' UTR (Lau et al., 2010). Also, it has been proposed that membrane depolarization increases the stability of *Bdnf* transcripts via formation of secondary structures in the *Bdnf* 3' UTR (Fukuchi & Tsuda, 2010). On the other hand, it has been demonstrated that the *Bdnf* 5' nor 3' UTRs do not affect the translatability of a reporter gene in response to membrane depolarization, but *Bdnf* long 3' UTR increases *de novo* synthesis of a reporter gene in HEK293 cells (Lekk et al., 2023).

The role of *Bdnf* 3' UTR has also been elucidated *in vivo*. Knockout of *Bdnf* long 3' UTR in hypothalamus decreases dendritic *Bdnf* mRNA levels and increases food intake and body weight (An et al., 2008). Decreasing the levels of *Bdnf* transcripts with long 3' UTR in cortex using shRNAs also results in lower dendritic *Bdnf* levels and more anxiety and depressive-like behaviour (Oh et al., 2018). *In vivo* deletion of a substantial part of the *Bdnf* 3' UTR with CRISPR-Cas9 also decreases total *Bdnf* mRNA levels in mouse brain (Mätlik et al., 2023). Similarly, removal of the whole *Bdnf* 3' UTR decreases the levels of BDNF protein in all brain regions, but increases BDNF levels in peripheral tissues (Lekk et al., 2023), implying unknown differences in the regulation of BDNF between the brain and peripheral tissues.

1.3 BDNF-regulated signalling pathways

1.3.1 Synthesis of BDNF protein

It has been proposed that genes encoding neurotrophins share a common ancestral origin and have undergone evolutionary changes over time (Hallböök, 1999). Similar to other neurotrophins, the BDNF protein consists of a signal peptide (pre region), pro region, and the mature BDNF region with conserved cysteine residues that form disulphide bridges (Hohn et al., 1990; Leibrock et al., 1989) (Figure 2). Notably, the amino acid sequence of mature BDNF is highly conserved during evolution, while the pro-domain has been under less selective pressure (Lucaci et al., 2022).

BDNF is synthesized as a prepro-precursor protein into the endoplasmic reticulum, with the pre-region cleaved co-translationally, and processed to mature BDNF either intracellularly in the Golgi complex by furin-like enzymes (Mowla et al., 1999, 2001) or extracellularly by plasmin and matrix metalloproteinases (Lee et al., 2001; Mizoguchi et al., 2011). In secretory vesicles, BDNF can be cleaved by pro-protein convertases (Seidah et al., 1996). Additionally, proBDNF protein, but not mature BDNF, can undergo N-glycosylation to increase stability (Mowla et al., 2001). As some *Bdnf* transcripts are transported away from the cell soma, BDNF can be locally translated in dendrites after neuronal activity, promoting spine maturation (Tanaka et al., 2008; Verpelli et al., 2010).

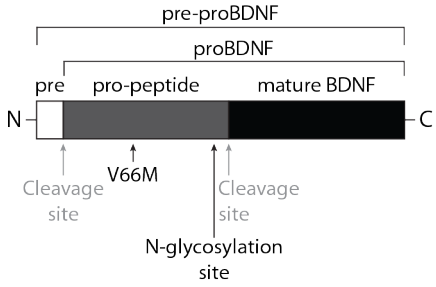

Figure 2. Schematical representation of BDNF protein structure. *Prepro-precursor BDNF is shown with boxes and grey arrows mark cleavage sites that produce proBDNF and mature BDNF protein from precursor protein. Val66Met substitution (V66M) and N-glycosylation site in the pro region are shown with black arrows.*

In neurons, BDNF is sorted within the Golgi complex into the regulated secretory pathway via interaction with sortilin (Z.-Y. Chen et al., 2005) and can be secreted from the cell in a neuronal activity-dependent manner (Mowla et al., 1999). Notably, a common valine to methionine substitution (Val66Met) in the BDNF pro-region impairs packaging

of BDNF into secretory granules resulting in reduced activity-dependent secretion of BDNF (Z.-Y. Chen et al., 2004; Egan et al., 2003). The prevalence of the Val66Met substitution varies among populations, being nearly absent in certain African groups and reaching up to 70% in specific Asian populations (Petryshen et al., 2010). The majority of secreted BDNF is in the mature form (Matsumoto et al., 2008; Mowla et al., 1999). Electron microscope studies using tissue sections and various BDNF antibodies suggest that BDNF is cleaved into the mature form soon after synthesis, with mature BDNF localized in presynaptic vesicles together with the BDNF pro-peptide (Dieni et al., 2012). Since then, it has been reported that the BDNF pro-peptide itself acts as a signalling molecule and has been associated with stress resilience (reviewed in Zanin et al., 2017) and depression (reviewed in Kojima et al., 2019).

1.3.2 BDNF-dependent signalling pathways

BDNF forms non-covalently associated dimers and binds two types of receptors: the p75 neurotrophin receptor (p75NTR) (Fayard et al., 2005; Rodriguez-Tebar et al., 1990) and the tropomyosin-related kinase B (TrkB) receptor (Klein et al., 1991; Squinto et al., 1991). Preferentially proBDNF binds p75NTR and mature BDNF binds TrkB (Reichardt, 2006). The outcomes of p75NTR and TrkB signalling are often opposing, e.g., p75NTR generally promotes apoptosis, whereas Trk receptors support cell survival (reviewed in Lu et al., 2005).

TrkB receptor is a member of the tropomyosin-related kinase family of tyrosine kinases, and in addition to BDNF, TrkB can also bind NT-3 and NT-4 (Reichardt, 2006). The binding of a neurotrophin dimer causes TrkB dimerization and trans-phosphorylation (Reichardt, 2006). Consequently, various adaptor proteins and enzymes can bind to the phosphorylated tyrosine residues in the TrkB intracellular domain and initiate different signalling pathways. The main pathways activated by TrkB are Ras-MAPK, PI3K-Akt, and PLCγ1-IP3/DAG (Reichardt, 2006). Since BDNF-dependent signals must reach the nucleus for long-term changes in the neuronal functions, the internalization and formation of signalling endosome is an important part of TrkB signalling (Barford et al., 2017; Moya-Alvarado et al., 2022). Recent findings have shown that TrkB dimerization can occur after endocytosis and initiate the PI3K-Akt pathway, whereas monomeric TrkB can activate the Ras-MAPK pathway already at the cell membrane (Zahavi et al., 2018).

TrkB is encoded by the *Ntrk2* gene, and several TrkB isoforms are generated through alternative splicing (Luberg et al., 2010; Stoilov et al., 2002). The most common TrkB isoforms are the full-length and C-terminally truncated isoform TrkB-T1, which lacks the intracellular kinase domain (Luberg et al., 2010; Stoilov et al., 2002). In different brain regions, both full-length TrkB and TrkB-T1 are expressed, whereas in non-neural tissues only TrkB-T1 is expressed (Esvald et al., 2023). In the brain, full-length TrkB is expressed in neuronal cells, whereas TrkB-T1 is expressed in both neuronal and non-neuronal cells, such as radial glia cells and oligodendrocytes (Pattwell et al., 2022). TrkB-T1 isoform often functions as a dominant-negative by sequestering BDNF and full-length TrkB (Haapasalo et al., 2002). However, TrkB-T1 has independent signalling roles (Baxter et al., 1997; Tessarollo & Yanpallewar, 2022), such as mediating calcium release in astrocytes (Rose et al., 2003) and cardiomyocytes (Fulgenzi et al., 2015).

The p75NTR, encoded by the *Ngfr* gene, belongs to the family of tumour necrosis factor receptor superfamily and can bind all proneurotrophins (Rodríguez-Tébar et al., 1992; Rydén et al., 1995). Lacking a catalytic domain, p75NTR initiates signalling pathways by interacting with other proteins, e.g., intracellularly with TRAF6, and extracellularly

with sortilin, Lingo-1, and Nogo-R as co-receptors (Kraemer et al., 2014). The binding of proneurotrophins to p75NTR initiates NF-kB, RhoA, or Jun kinase pathways, which can result in the promotion of apoptosis and the inhibition of axonal growth cones (Kraemer et al., 2014; Reichardt, 2006). Conversely, when interacting with members of the Trk family as co-receptors, p75NTR promotes cell survival instead (Kraemer et al., 2014; Zanin et al., 2019).

1.4 Roles of BDNF

1.4.1 *Bdnf* haploinsufficiency in humans

In humans, haploinsufficiency of *BDNF* has only been reported in a few cases. A mother and her 3-year-old child with *BDNF* deletion demonstrated delayed development and obesity, along with higher levels of cholesterol, insulin, and sodium in the serum, and displayed sedentary behavior (Harcourt et al., 2018). Haploinsufficiency of *BDNF* has further been described in a subpopulation of WAGR syndrome patients, and is associated with hyperphagia, obesity, lower IQ, diminished adaptive behaviour, and social impairments, as well as remarkably lower sensitivity to pain (Han et al., 2008, 2013; Sapio et al., 2019). Furthermore, deletions of the 11p14 region (distinct from those seen in WAGR syndrome patients), including *BDNF* among other genes, are associated with numerous issues, such as obesity, developmental delay, lower IQ and learning difficulties, attention deficiency disorder, and behavioural problems, including aggressiveness and hyperactivity (Ernst et al., 2012; Shinawi et al., 2011). Additionally, an 8-year old child with a *de novo* chromosomal inversion with a breaking point 850 kbp upstream from *BDNF* locus resulted in decreased levels of BDNF in the blood, and the child exhibited developmental delay, lower IQ, hyperphagia, obesity, extreme hyperactivity, and impaired nociception (Gray et al., 2006). Although decrease in BDNF levels has mainly been connected to obesity, low serum BDNF levels have also been associated with anorexia (Nakazato et al., 2003). Interestingly, a case of BDNF duplication has also been reported in an individual with developmental delay and dystonia (Ernst et al., 2012). Collectively, these case reports emphasize the importance of proper BDNF levels and suggest multiple roles, such as behavioural and metabolic control, for BDNF in humans.

1.4.2 Total *Bdnf* knockout animals

Mice with homozygous deletion of *Bdnf* coding exon show similar size and motor activity to wild-type mice at birth but generally die within the following days (Jones et al., 1994). Cardiac failure due to endothelial cell apoptosis leading to vascular hemorrage has been suggested to be the main cause of early death (Donovan et al., 2000). A few *Bdnf* $^{-/-}$ animals that survive are smaller than their littermates, experience movement difficulties, and display alternating periods of hyperactivity and inactivity (Jones et al., 1994). Older *Bdnf* $^{-/-}$ animals exhibit hunched posture, ataxia, and problems with breathing (Jones et al., 1994). In contrast, *Bdnf* $^{+/-}$ animals are fertile and can survive for over a year (Jones et al., 1994). Another research group described *Bdnf* $^{-/-}$ mice as smaller than wild-type mice, with death occurring during the second postnatal week (Ernfors et al., 1994). These mice also experience problems with movement and balance, and show periods of hyperactivity that are followed by immobility (Ernfors et al., 1994). At the cellular level, the absence of *Bdnf* leads to extensive death of sensory neurons (Ernfors et al., 1994; Jones et al., 1994). Subsequent studies of *Bdnf* knockout mice also report early death within two weeks after birth and extensive loss of sensory ganglia (Liebl et al., 1997). In addition, *Bdnf* $^{-/-}$ mice have a typical wide-based stance that is partially caused by the

dysfunction of cerebellum, as loss of *Bdnf* causes increased granule cell death, poor arborization of Purkinje cells, disrupted cerebellar layer formation and abnormal foliation (Schwartz et al., 1997).

Similar to humans, heterozygous *Bdnf* knockout rats display impaired sensitivity to pain (Sapio et al., 2019). Furthermore, heterozygous *Bdnf* knockout mice are more aggressive and impulsive, a phenomenon that can be relieved by the administration of fluoxetine, implying acute effects rather than developmental problems in *Bdnf* $^{+/-}$ animals (W. E. Lyons et al., 1999). In addition, *Bdnf* $^{+/-}$ mice, but not *NT*-3 $^{+/-}$ mice, have hyperphagia, higher insulin levels, and gradually become obese despite normal locomotor activity (W. E. Lyons et al., 1999). However, another study focusing on *Bdnf* haploinsufficiency reports the presence of two subgroups – fat and non-fat *Bdnf* heterozygous knockout animals (Kernie et al., 2000). The subgroup of fat *Bdnf* knockout animals gradually gains body weight and has adipose cell hypertrophy, higher body fat content, and higher levels of leptin and insulin in serum (Kernie et al., 2000). Meanwhile, the subgroup of non-fat *Bdnf* $^{+/-}$ animals are similar to wild type mice, except their locomotor activity is increased (Kernie et al., 2000). Notably, both increased body weight and food intake can temporarily be reduced to levels comparable to wild-type mice through BDNF or NT-4 infusion to the third ventricle, again suggesting acute BDNF effects rather than improper development of specific neuronal circuits (Kernie et al., 2000).

1.4.3 Conditional *Bdnf* knockout in neurons

Due to the low viability of *Bdnf* homozygous knockout animals, conditional knockout animals have been widely used to address the functions of BDNF in the adult organism. Mice with conditional embryonic *Bdnf* deletion in neocortex and hippocampus, driven by *Emx1*-Cre, display shorter lifespan, poor fecundity, increased aggressiveness in males, poor nesting behaviour in both males and females, impaired spatial learning, and decreased activity compared to wild-type mice (Gorski, Balogh, et al., 2003). These conditional *Bdnf* knockout mice are also slightly smaller than their wild-type littermates at a young age, but become mildly obese by the fourth month (Gorski, Zeiler, et al., 2003). Additionally, their cortex is thinner but with higher neuron density, smaller neuron soma and reduced dendrite arborization (Gorski, Zeiler, et al., 2003). Another study using *Emx1*-Cre recombinase-driven *Bdnf* knockout focusing on striatum reported smaller striatum, reduced dendrite complexity of striatal medium spiny neurons, and eventual loss of striatal neurons (Baquet et al., 2004).

Mice with conditional *Bdnf* knockout in postmitotic neurons driven by tau-dependent Cre survive for at least 8 months after birth and are indistinguishable from wild-type littermates despite lower BDNF protein levels in several brain regions (Rauskolb et al., 2010). Interestingly, the young *Bdnf* knockout animals are slightly smaller than their littermates and again exhibit periods of hyperactivity and inactivity in the first weeks of life (Rauskolb et al., 2010). These animals also display a clasping phenotype and abnormal exploratory behaviour (Rauskolb et al., 2010). Notably, by the sixth week after birth, female but not male conditional *Bdnf* knockout animals become obese (Rauskolb et al., 2010). At two months of age, these *Bdnf* knockout animals exhibit a reduction in brain size, particularly in the striatum, and a decrease in dendrite number and length among medium spiny neurons (Rauskolb et al., 2010). Interestingly, this conditional *Bdnf* knockout has a more pronounced effect on striatal neurons compared to hippocampal neurons (Rauskolb et al., 2010), suggesting a brain region-specific dependency on BDNF.

Using *CamKII*-driven Cre recombinase to postnatally knock out neuronal *Bdnf*, Rios et al., (2001) found reduced *Bdnf* mRNA levels at least in the cortex, hippocampus, and hypothalamus, leading to a range of behavioural and physiological changes in these mice. Specifically, these mice become hyperactive, aggressive, anxious, and eventually develop obesity and infertility (likely a secondary effect of obesity) (Rios et al., 2001). They also exhibit hyperphagia, elevated leptin, insulin, and glucose levels in serum, as well as an abnormal molecular response to starvation (Rios et al., 2001). Interestingly, treatment with fluoxetine alleviates hyperactivity phenotype in these *Bdnf* knockout mice, but does not decrease food intake and body weight (Rios et al., 2001), which is in contrast to total *Bdnf* knockout animals where food intake was partially suppressed (W. E. Lyons et al., 1999).

Early prenatal and postnatal knockouts of *Bdnf* have been achieved using *Nestin* or *CamKII*-driven Cre, respectively (Chan et al., 2006). These knockout animals display increased aggressiveness, anxiety, and depressive-like behaviour in various behavioural tests, with a more pronounced phenotypes observed in animals where *Bdnf* knockout occurred earlier in development (Chan et al., 2006). Using inducible Cre recombinase for neuronal *Bdnf* knockout has further enabled researchers to study the developmental roles of *Bdnf* (Monteggia et al., 2004). Early knockout of *Bdnf* during late embryogenesis leads to increased hyperactivity, while knockout in adult animals only results in impaired long-term potentiation and deficiencies in learning memory, without increased locomotor activity or aggression (Monteggia et al., 2004). In another study, direct injection of lentiviruses expressing Cre recombinase to knock out *Bdnf* in the hippocampus of young adult *Bdnf* $^{flox/flox}$ animals impairs novel object recognition and spatial learning (Heldt et al., 2007).

AAV-mediated *Bdnf* knockout in adult *Bdnf* $^{flox/flox}$ mice ventromedial and dorsomedial hypothalamus causes hyperphagia, weight gain, and increased levels of leptin, insulin, and glucose in serum (Unger et al., 2007), consistent with previous findings. Interestingly, these *Bdnf* knockout mice do not exhibit anxious, aggressive, or depressive behaviour, indicating that changes in complex neural circuits underlie these phenotypes (Unger et al., 2007). Taken together, the results obtained from the variety of *Bdnf* knockout animals suggest that the timing and brain region of *Bdnf* knockout are critical determinants of the outcome.

1.4.4 Conditional *Bdnf* knockout in non-neural cells

Astrocytes are an important cell type in the brain known to produce (Hisaoka-Nakashima et al., 2016; Jean et al., 2008, p. 200; Koppel et al., 2018; Zafra et al., 1992; Zhang et al., 2014) and respond to BDNF (Alderson et al., 2000; Holt et al., 2019; Rose et al., 2003). To investigate the role of astrocyte-derived BDNF, researchers have employed *Bdnf* knockout with *Gfap*-driven Cre recombinase to show that BDNF is important in myelination (Fulmer et al., 2014), oligodendrocyte maturation, and restoring myelin after injury (Miyamoto et al., 2015). Co-cultures of wild-type neurons with *Bdnf* deficient astrocytes have revealed that astrocytic BDNF also supports neuronal maturation (Pins et al., 2019). Additionally, deficiency in astrocytic BDNF production causes higher neuronal excitability in the hippocampus (Fernández-García et al., 2020).

In the heart, specific knockout of *Bdnf* using *Myh6*-Cre recombinase results in cardiomyopathy with a reduced thickness of the posterior ventricle wall (Fulgenzi et al., 2015). Detailed analysis of early knockout of heart-derived *Bdnf* shows cardiomyocyte death, myocardium degeneration, cardiomyocyte hypertrophy, decreased cardiac function,

increased inflammation, and metabolic disorders, ultimately leading to a shorter lifespan (L. Li et al., 2022). At the cellular level, loss of cardiac *Bdnf* increases expression of apoptosis-related genes (L. Li et al., 2022). Notably, heart-derived *Bdnf* knockout animals develop two subgroups, similar to heterozygous *Bdnf* knockout mice, with roughly 40% of animals developing edema (L. Li et al., 2022). The subgroup of animals with edema exhibits a higher body weight, increased cardiac dysfunction, and die shortly after the edema onset (L. Li et al., 2022).

Knockout of skeletal muscle-derived *Bdnf* using *Acta1*-Cre recombinase results in mice with higher body weight, more white adipose tissue, and impaired energy metabolism (Yang et al., 2019). These findings were later confirmed and extended, showing that animals with skeletal muscle-specific knockout of *Bdnf* have more dysfunctional mitochondria and mitophagy (Ahuja et al., 2021). Using *Myl1*-Cre recombinase, BDNF was shown to be secreted from muscles and to participate in glucose and insulin regulation in the pancreas (Fulgenzi et al., 2020).

1.4.5 Transcript-specific *Bdnf* knockout animals

Various *Bdnf* knockout animals have demonstrated the diverse roles of BDNF in an organism during development and in acute situations but have not elucidated the function of multiple promoters in the *Bdnf* gene. To expand upon the functional understanding of the intricate *Bdnf* gene structure, several *Bdnf* transcript-specific knockout animals have been generated. The first-generation of transcript-specific *Bdnf* knockout animals had a *Pgk*-Neo cassette and GFP coding sequence (with stop codon) inserted into *Bdnf* exon IV, resulting in normal transcription from *Bdnf* promoter IV but producing GFP protein instead of BDNF (Sakata et al., 2009). These animals, with disrupted *Bdnf* exon IV, exhibit a reduced number of interneurons, impaired GABAergic inhibition, and compromised synaptic plasticity in the cortex (Sakata et al., 2009). However, they display no significant effects on brain anatomy or dendrite morphology (Sakata et al., 2009), as would be anticipated from the studies using *Bdnf* conditional knockout animals. Additionally, disruption of *Bdnf* exon IV leads to impaired long-term potentiation, reversal learning and memory extinction (Sakata et al., 2013), depression, but not anxiety-like behaviour, defective response inhibition (Sakata et al., 2010) and sleep cycles (Martinowich et al., 2011). Notably, these animals have lower levels of all major *Bdnf* transcripts, at least in the cortex (Martinowich et al., 2011), and overall reduced levels of *Bdnf* mRNA might have contributed to some of the seen phenotypes.

It has been reported that *Pgk*-Neo cassette can impede the expression of the gene in the locus where it was inserted (Pham et al., 1996). Consistently, the *Pgk*-Neo cassette in the *Bdnf* gene of the first-generation knockout animals decreased the expression of other genes on the same chromosome (Maynard et al., 2016). Consequently, in the second generation of transcript-specific *Bdnf* knockout animals the *Pgk*-Neo cassette was removed and had only the GFP coding sequence in *Bdnf* exon I, II, IV, or VI (Maynard et al., 2016). All *Bdnf* transcript-specific knockout animals develop normally, with 2-week-old animals exhibiting typical gross morphology of the cortex and hippocampus, normal body weight, motor reflexes, and response to acoustic stimuli (Maynard et al., 2016).

In the second generation of *Bdnf* transcript-specific knockout animals, the expression of *Bdnf* transcripts is not uniformly decreased; rather, developmental and brain-region specific effects are observed. For instance, in young *Bdnf* exon I knockout animals, the levels of other *Bdnf* transcripts are not much affected. However, in adult *Bdnf* exon I knockout animals, all major *Bdnf* transcripts are diminished in the hypothalamus, only

Bdnf exon IV levels are increased in the hippocampus, and only *Bdnf* exon II and IV levels are increased in the cortex (Maynard et al., 2016). These findings emphasize that the regulation of *Bdnf* transcripts is subject to highly specific developmental and brain region-specific control. Remarkably, only animals with disrupted *Bdnf* exons I and II, but not exons IV or VI, exhibit aggression (Maynard et al., 2016, 2018), increased food intake, greater adipose tissue, and obesity (McAllan et al., 2018), despite similar reductions of BDNF protein levels in the hypothalamus of *Bdnf* exon I and IV knockout animals. Additionally, knockout of *Bdnf* exon I, but not exon IV or VI, results in impaired thermogenesis alongside obesity (You et al., 2020). *Bdnf* exon IV knockout animals display compromised fear memory retrieval (Hallock et al., 2020), abnormal sleep cycles and deficiencies in responding to sensory stimulus (Hill et al., 2016), in agreement with the previous studies of different *Bdnf* knockout animals (see Sections 1.4.2 and 1.4.3).

1.4.6 Molecular functions of BDNF in the nervous system

BDNF plays various cellular roles depending on the context, including promoting survival, maturation, and synaptic strength, and participates in forming neural circuits (Park & Poo, 2013). Its most well-established role is as a survival factor for sensory neurons during development (Ernfors et al., 1994; Hofer & Barde, 1988; Jones et al., 1994; Liebl et al., 1997). Although cultured sensory neurons initially require target-derived signals for survival, their dependency on BDNF decreases over time (Rodriguez-Tébar et al., 1989) and mature sensory neurons survive through autocrine BDNF signalling (Acheson et al., 1995).

BDNF has been suggested to promote adult neurogenesis by supporting neuronal precursor cell survival in the hippocampus (Sairanen et al., 2005) and forebrain (Kirschenbaum & Goldman, 1995). Direct BDNF infusion into adult hippocampus increases new-born granule cell numbers (Scharfman et al., 2005). However, adult *Bdnf* heterozygous knockout mice exhibit normal hippocampal progenitor cell proliferation and new cell survival but show compromised dendritic branching and migration (Chan et al., 2008). Additionally, *TrkB* knockout in adult animals impairs neurogenesis, dendrite arborization, spine density, and long-term potentiation in the hippocampus (Bergami et al., 2008). Notably, impairing BDNF signalling by *TrkB* knockout in progenitor cells reveals impaired neurogenesis and proliferation (Y. Li et al., 2008). In cultured cortical neurons, BDNF-TrkB signalling is essential for survival, proliferation, and differentiation, with outcomes determined by the specific TrkB-initiated pathway (Barnabé-Heider & Miller, 2003).

In many cases, BDNF functions as a maturation factor, inducing dendrite growth and branching in cortical neurons (McAllister et al., 1995; Singh et al., 2006). Through autocrine signalling, BDNF particularly increases the number of dendrites near the cortical neuron cell soma (Horch et al., 1999), and through paracrine signalling, it promotes dendrite branching in nearby cortical neurons (Horch & Katz, 2002). Autocrine signalling of BDNF also promotes the growth of hippocampal dendrites (L. Wang et al., 2015) and *Bdnf* knockout animals display impaired dendrite branching and spine morphology in the hippocampus (Maynard et al., 2017). BDNF can also promote axon initiation and differentiation (Shelly et al., 2007) and guide axon development through autocrine signalling (Cheng et al., 2011). In the postnatal hippocampus, BDNF knockout impairs dendritic arborization, spine maturation, synapse formation, and learning and memory (Gao et al., 2009). BDNF also has multiple roles in cerebellar granule neuron development, such as promoting survival, maturation (Schwartz et al., 1997), and migration (Borghesani et al., 2002).

Secreted in an activity-dependent manner, BDNF has local effects on dendritic structures and synapses (reviewed in Kuczewski et al., 2009), influencing long-term potentiation (Korte et al., 1995; Patterson et al., 1996), synaptic consolidation (Messaoudi et al., 2002; Wibrand et al., 2006), and proper formation of GABAergic networks (Bolton et al., 2000; Chan et al., 2008; Hong et al., 2008; Sakata et al., 2009; Yamada et al., 2002). Overall, BDNF promotes neurite differentiation, guidance, synapse formation, and neural circuit development (reviewed in Park & Poo, 2013).

1.4.7 BDNF in non-neural tissues

In addition to its role in the central nervous system, BDNF has been demonstrated to function in numerous peripheral tissues. For example, BDNF derived from cardio-myocytes is important for heart development and function (L. Li et al., 2022), and BDNF-TrkB-T1 signalling contributes to cardiac muscle contraction (Fulgenzi et al., 2015) and energy regulation (Yang et al., 2022). In addition, BDNF present in the blood promotes platelet aggregation (Boukhatem et al., 2021). In muscles, BDNF participates in muscle development (Delezie et al., 2019), regulates mitochondria (Ahuja et al., 2021), and modulates metabolism in adult organism (Fulgenzi et al., 2020; Yang et al., 2019). BDNF signalling also promotes repair mechanisms in the lungs following infection (Paris et al., 2020), facilitates innervation of mechanosensory neurons in skin (Rutlin et al., 2014), and regulates sensory innervation in mammary glands (Y. Liu et al., 2012; Sar Shalom et al., 2019). Furthermore, BDNF signalling is important for proper kidney development (Endlich et al., 2018; García-Suárez et al., 2006). Finally, TrkB signalling is required for oocyte survival (Dorfman et al., 2014), both p75NTR and TrkB signalling contribute to follicular development (Kerr et al., 2009), and activation of TrkB enhances fertility and offers potential treatment for ovarian failure (Qin et al., 2022).

1.4.8 BDNF in disease pathology and potential as therapy

BDNF has been associated with many neuropsychiatric diseases, such as depression, bipolar disorder, schizophrenia, and addiction, as well as neurodevelopmental disorders like Rett syndrome (Autry & Monteggia, 2012). BDNF-TrkB signalling underlies the effects of certain psychiatric treatments (C. S. Wang et al., 2022) and is essential for antidepressant effects (P.-Y. Lin et al., 2021; Saarelainen et al., 2003). It has even been reported that antidepressants like serotonin selective reuptake inhibitors (e.g., fluoxetine), and ketamine bind directly to TrkB and activate TrkB signalling (Casarotto et al., 2021).

BDNF has also been implicated in the pathogenesis of Huntington's, Alzheimer's, and Parkinson's disease, and increasing BDNF levels in the animal models of these diseases as a therapy has yielded some positive results (reviewed in Zuccato & Cattaneo, 2009). Furthermore, in Rett syndrome mouse models, *Bdnf* knockout worsened motor functions and decreased survival rates, while genetically overexpressing *Bdnf* rescued decreased synaptic activity and motor functions, and increased lifespan (Q. Chang et al., 2006). Direct injection of BDNF-expressing viral vectors into the hypothalamus reduced body weight and fat mass, and improved metabolism in Prader-Willi syndrome (Queen et al., 2022). Recently, intranasal delivery of BDNF was successful in improving learning and memory in mouse model of HIV/neuroAIDS (Vitaliano et al., 2022).

Haploinsufficiency of RAI1 (retinoic acid induced-1) leads to Smith-Magenis syndrome (SMS), characterized by common craniofacial features and behaviours (Bi et al., 2004; Slager et al., 2003). *Rai1* homozygous knockout mice, serving as a model of SMS, exhibit several problems, such as low viability, poor motor skills, clasping phenotype, and

learning difficulties (Bi et al., 2007) – all of which are also present in *Bdnf* knockout mice. SMS patients also commonly exhibit hyperphagia and become obese, with these phenotypes in the mouse model attributed to reduced hypothalamic *Bdnf* levels (Burns et al., 2010; Huang et al., 2016). In addition, point mutations in RAI1 cause autistic, hyperactive, and aggressive behaviours, suggested to result from improper regulation of *Bdnf* by RAI1 (Abad et al., 2018). As BDNF appears to be functionally relevant to SMS disease pathology, BDNF could potentially serve as a therapy for SMS patients. A recent study demonstrated that genetic overexpression of BDNF or acute BDNF injection reverses obesity, metabolic changes, and improves sociability in *Rai1* heterozygous knockout mice (Javed et al., 2022).

BDNF holds great promise as a potential therapeutic, and various methods of increasing BDNF in animal models have been tested, including genetically modified grafts, BDNF protein infusion with osmotic minipumps, injections of *Bdnf*-expressing adenoviruses, BDNF mimetics, low molecular weight compounds to increase *Bdnf* expression, diet, and environmental enrichment (Géral et al., 2013; Miranda-Lourenço et al., 2020; Zuccato & Cattaneo, 2009). However, ensuring safe and accurate spatiotemporal expression of BDNF faces several challenges due to the short half-life of BDNF protein and limited diffusion (Géral et al., 2013; Miranda-Lourenço et al., 2020). Therefore, sophisticated approaches such as polymer carriers and nanoparticles (Géral et al., 2013), and viral vectors encoding BDNF and TrkB for sustained BDNF signalling are being developed (Osborne et al., 2018).

1.5 Regulation of *Bdnf* gene expression

1.5.1 Stimuli inducing *Bdnf* expression

Bdnf expression is highly responsive to a wide range of external signals. Various environmental stimuli can either induce or repress *Bdnf* expression. Examples of stimuli that promote *Bdnf* expression include physical exercise, light exposure, sensory stimuli, insult, ischemia, and drug abuse, whereas stress generally decreases *Bdnf* levels (reviewed in West, 2008; West et al., 2014). Seminal studies demonstrating stimuli that induce *Bdnf* are listed in Table 1. Collectively, these findings provide evidence that *Bdnf* expression is regulated in a neuronal activity-dependent manner to promote neuronal functions.

Table 1. Different stimuli that induce Bdnf gene expression.

Stimulus	**Brain region or cell culture**	**Reference**
KCl treatment; KA treatment	Cultured rat HC neurons	(Zafra et al., 1990)
IP injection of KA	Rat HC *in vivo*	
KA injection to dorsal HC	Rat HC *in vivo*	(Ballarín et al., 1991)
Kindling epileptogenesis	Rat HC and CTX *in vivo*	(Ernfors et al., 1991)
Lesion-induced seizures; epileptiform afterdischarge	Rat HC, CTX, and amygdala *in vivo*	(Isackson et al., 1991)
Bicuculline treatment	Cultured rat HC neurons	(Zafra et al., 1991)
I.v. injection of bicuculline	Rat HC *in vivo*	
Light exposure	Rat visual cortex	(Castrén et al., 1992)
Tetanization (HFS) induced LTP	Rat HC slices	(Patterson et al., 1992)

Stimulus	**Brain region or cell culture**	**Reference**
FSK; KA; FSK with KA treatment	Cultured rat HC neurons	(Zafra et al., 1992)
FSK; norepinephrine; norepinephrine with glutamate; norepinephrine with quisqualic acid; epinephrine; dopamine treatment	Cultured rat HC astrocytes	
Quisqualic acid; KA; AMPA; NMDA treatment	Cultured rat cerebellar granule cells	(Bessho et al., 1993)
HFS-induced LTP	Rat HC *in vivo*	(Castrén et al., 1993)
Focal hippocampal injury	Rat HC *in vivo*	(Hughes et al., 1993)
Topical application of KCl to frontal cortex	Rat CTX *in vivo*	(Kokaia et al., 1993)
IP injection of KA; bicuculline; pilocarpine	Rat HC and CTX *in vivo*	(Metsis et al., 1993)
KCl; glutamate treatment	Cultured rat CTX neurons	(Ghosh et al., 1994)
GABA; KA treatment	Cultured rat immature HC neurons	(Berninger et al., 1995)
Bicuculline; KA treatment	Cultured rat mature HC neurons	
Electroconvulsive seizures; antidepressants	Rat HC and CTX *in vivo*	(Nibuya et al., 1995)
Immobilization stress	Rat HTH and pituitary *in vivo*	(Smith et al., 1995)
Physical exercise	Rat HC, CTX, and cerebellum *in vivo*	(Neeper et al., 1996)
Electrical stimulation induced paroxysmal discharge	Rat HC *in vivo*, HC slices	(Lauterborn et al., 1996)
Circadian rhythm	Rat HC and CTX *in vivo*	(Bova et al., 1998)
Contextual learning	Rat HC *in vivo*	(Hall et al., 2000)
Glutamate	Cultured rat hypothalamic neurons	(Marmigère et al., 2001)
Fear conditioning	Rat amygdala	(Rattiner, Davis, French, et al., 2004; Rattiner, Davis, & Ressler, 2004)
BDNF infusion-induced LTP	Rat HC *in vivo*	(Wibrand et al., 2006)
BDNF treatment	Cultured rat HC neurons	(Groth & Mermelstein, 2003)
	Cultured rat CTX neurons	(Yasuda et al., 2007) (Zheng & Wang, 2009) (Nakajima et al., 2015) (Tuvikene et al., 2016)
	Mouse maturing CGNs	(Ding et al., 2018)

Stimulus	Brain region or cell culture	Reference
NMDA treatment	Cultured rat CTX neurons	(Zheng & Wang, 2009)
Bicuculline; FSK treatment	Cultured mouse HC neurons	(Benito et al., 2011)
FSK treatment	Cultured rat CTX neurons	(Nakajima et al., 2015)

CGN – cerebellar granule neurons; CTX – cerebral cortex; HC – hippocampus; FSK – forskolin; HFS – high frequency stimulation; HTH – hypothalamus; IP – intraperitoneal; i.v. – intravenous; KA – kainic acid; LTP – long term potentiation.

1.5.2 Transcriptional regulation of *Bdnf*

Numerous transcription factors and transcriptional coregulators have been identified as regulators of *Bdnf* promoter activity (reviewed in West et al., 2014). Transcription factors that have been demonstrated to directly bind *Bdnf* promoters (as evidenced by chromatin immunoprecipitation (ChIP) or electrophoretic mobility shift assay (EMSA)) and modulate *Bdnf* mRNA levels or *Bdnf* promoter activity are listed in Table 2.

Bdnf promoters contain several calcium-response elements that contribute to activity-dependent *Bdnf* expression (Bishop et al., 1997; Tabuchi et al., 2002; Tao et al., 1998; West et al., 2014). After neuronal activity the best described regulator of *Bdnf* is cAMP-response element (CRE) binding protein (CREB) (Benito et al., 2011; Hong et al., 2008; Palomer et al., 2016; Pruunsild et al., 2011; Shieh et al., 1998; Tabuchi et al., 2002; Tai et al., 2016; Tao et al., 1998). Functional CRE sequences are present in *Bdnf* promoters I (Pruunsild et al., 2011; Tabuchi et al., 2002), IV (Hong et al., 2008; Pruunsild et al., 2011; Shieh et al., 1998; Tao et al., 1998), and IX (Pruunsild et al., 2011). The CRE element in *Bdnf* promoter IV has been shown to mediate the majority of neuronal activity-induced *Bdnf* expression *in vivo,* and disruption of this element impairs the formation of inhibitory neural circuits in the cortex (Hong et al., 2008). Additionally, important activity-dependent regulatory elements in *Bdnf* promoters are upstream stimulatory factor (USF)-binding element (UBE) in promoters I (Tabuchi et al., 2002) and IV (W. G. Chen, West, et al., 2003), neuronal PAS domain protein 4 (NPAS4)-binding element bHLH-PAS transcription factor response element (PasRE) in promoters I, IV and IX (Y. Lin et al., 2008; Pruunsild et al., 2011), and nuclear factor kappa B (NFkB)-binding site in promoter IV (Lipsky et al., 2001; Lubin et al., 2007). Furthermore, another calcium-response element in *Bdnf* promoter IV is bound by calcium response factor (CaRF) (McDowell et al., 2010; Tao et al., 1998) and myocyte enhancer factor 2 (MEF2) family (M. R. Lyons et al., 2012). Transcription factors mediating *Bdnf* transcriptional autoregulation are activating protein 1 (AP1) family members binding to *Bdnf* promoter I (Tuvikene et al., 2016), CCAAT-enhancer binding protein β (C/EBPβ) (Bambah-Mukku et al., 2014), nuclear factor of activated T-cells isoform c4 (NFATc4), nuclear factor 1 (NFI) (Ding et al., 2018; Groth & Mermelstein, 2003), and X-box binding protein 1 (XBP1) (Saito et al., 2018) binding to *Bdnf* promoter IV.

Many of the functional *cis*-elements identified within *Bdnf* promoters have been demonstrated to be capable of binding multiple transcription factors, suggesting a model of competitive binding. Furthermore, several important *cis*-regulatory elements in *Bdnf* promoters overlap. For instance, in *Bdnf* promoter I CRE, UBE, (Pruunsild et al., 2011; Tabuchi et al., 2002), and NRF2-binding element (Yao et al., 2021) partially overlap; in *Bdnf* promoter I AP1 family-binding site (Tuvikene et al., 2016) and NPAS4-binding site (Pruunsild et al., 2011) overlap; and in *Bdnf* promoter IV CaRF and MEF2 family-binding sites overlap (M. R. Lyons et al., 2012; Tao et al., 2002).

Table 2. Transcription factors regulating Bdnf gene expression.

Promoter | Transcription factor		Brain region or cell culture	Stimulus	Reference
pI	AP1	Rat cortical neurons	BDNF	(Tuvikene et al., 2016)
	CREB	Rat cortical neurons	KCl	(Tabuchi et al., 2002)
		Rat cortical neurons	KCl	(Pruunsild et al., 2011)
	NFkB	Rat hippocampus	Kainic acid	(Lubin et al., 2007)
	NPAS4	Rat and mouse cortical neurons	KCl	(Y. Lin et al., 2008)
	NPAS4, ARNT2	Rat cortical neurons	KCl	(Pruunsild et al., 2011)
	NRF2	Mouse hippocampus	SFN	(Yao et al., 2021)
		HEK293 cell line	None	
	USF1/2	Rat cortical neurons	KCl	(Tabuchi et al., 2002)
pII	REST	Mouse hippocampus	Kainic acid	(Timmusk et al., 1999)
		Rat cortical neurons	KCl	(Hara et al., 2009)
	SCIRR69	Rat cortical neurons	Mechanical injury	(Y. Liu et al., 2013)
pIV	β-actin	SH-SY5Y cell line	Forskolin	(Neasta et al., 2016)
	BHLHB2	Rat hippocampal neurons	NMDA	(Jiang et al., 2008)
	CaRF	Rat cortical neurons	KCl	(Tao et al., 2002)
		Mouse cortex	None	(McDowell et al., 2010)
		Mouse cortical neurons	KCl	
	C/EBPβ	Rat hippocampus	Inhibitory avoidance training	(Bambah-Mukku et al., 2014)
	CREB	Rat cortical neurons	KCl	(Tao et al., 1998)
		Rat cortical neurons	KCl	(Shieh et al., 1998)
		Differentiated NT2 cells	DbcAMP	(Fang et al., 2003)
		Rat cortical neurons	KCl	(Pruunsild et al., 2011)
		Mouse cortical neurons	NMDA	(Hong et al., 2008)
		Mouse visual cortex	Light exposure	
	CTCF	Mouse cortical neurons	KCl	(J. Chang et al., 2010)
	HSF1	Mouse hippocampus	Kainic acid, foot shock,	(Franks et al., 2023)
		Rat cortical neurons	None	
	MeCP2	Rat cortical neurons	KCl	(W. G. Chen, Chang, et al., 2003)
		Mouse cortical neurons	KCl	(Martinowich et al., 2003)
	MEF2	Rat cortical neurons	KCl	(M. R. Lyons et al., 2012)
	NFATc4	Rat cortical neurons	NMDA	(Vashishta et al., 2009)

Promoter | Transcription factor		Brain region or cell culture	Stimulus	Reference
pIV		Mouse cerebellar granule neurons	BDNF	(Ding et al., 2018)
		Mouse cerebellum	Development	
	NFI	Mouse cerebellar granule neurons	BDNF	(Ding et al., 2018)
		Mouse cerebellum	Development	
	NFkB	Rat cerebellar granule cells	NMDA	(Lipsky et al., 2001)
	NPAS4	Rat and mouse cortical neurons	KCl	(Y. Lin et al., 2008)
	NPAS4, ARNT2	Rat cortical neurons	KCl	(Pruunsild et al., 2011)
	PITX3	Midbrain dopaminergic neurons, HEK293 cells	None	(Peng et al., 2011)
	RACK1	SH-SY5Y cell line, rat hippocampal neurons	Forskolin	(He et al., 2010)
	RAI1	HEK293T cell line	None	(Burns et al., 2010)
		Mouse cortex and hypothalamus	None	(Huang et al., 2016)
	USF1/2	Rat cortical neurons	KCl	(W. G. Chen, West, et al., 2003)
		Rat cortical neurons	KCl	(Pruunsild et al., 2011)
	XBP1	Mouse hippocampus, HEK293, Neuro2A cell lines	None	(Martínez et al., 2016)
		Mouse hippocampal neurons	Glutamate, BDNF	(Saito et al., 2018)
pVI	C/EBPβ	NG108-15 cells	None	(Takeuchi et al., 2002)
	NR4A2	Rat cerebellar granule neurons	NMDA	(Barneda-Zahonero et al., 2012)
	SP1	NG108-15 cells	None	(Takeuchi et al., 2002)
pIX	CREB	Rat cortical neurons	KCl	(Pruunsild et al., 2011)
	NPAS4, ARNT2	Rat cortical neurons	KCl	(Pruunsild et al., 2011)
	NRF2	Rat visual cortex	None	(Nair & Wong-Riley, 2016)

AP1 – activating protein 1 (AP1) transcription factor family; ARNT2 – aryl hydrocarbon receptor nuclear translocator 2; BHLHB2 – basic helix-loop-helix domain containing, class B, 2; CaRF – calcium response factor; C/EBPβ – CCAAT-enhancer binding protein β; CREB – CRE-binding protein; CTCF – CCCTC-binding factor; HSF1 – heat shock factor 1; MeCP2 - Methyl-CpG Binding Protein 2; MEF2 – myocyte enhancer factor 2; NFATc4 – nuclear factor of activated T-cells isoform c4; NFI – nuclear factor 1; NFkB – nuclear factor kappa B; NPAS4 – neuronal PAS domain protein 4; NRF2 – nuclear factor-erythroid factor 2-related factor 2; NR4A2 – nuclear receptor subfamily 4 group A member 2 (also known as NURR1); p – BDNF promoter I, II, IV, VI, or IX; RACK1 – receptor for activated C kinase 1; RAI1 – retinoic acid induced 1; REST – RE1 silencing transcription factor (also known as NRSF); SFN – sulforaphane; SCIRR69 – spinal cord injury and regeneration-related gene #69; USF1/2 – upstream stimulatory factors 1 and 2; XBP1 – X-box binding protein 1.

In addition to numerous activators, several repressors bind to *Bdnf* promoters, making the balance between activators and repressors essential for *Bdnf* expression and induction after stimuli. For example, deletions of parts of *Bdnf* promoter I results in remarkable increase in basal activity and membrane depolarization-induced activity of the promoter (Tabuchi et al., 2002). This implies that the loss of binding of unknown repressors to *Bdnf* promoter I leads to promoter activation. Furthermore, repression of *Bdnf* promoters can also occur from distal regions, such as RE1 silencing transcription factor (REST, also known as NRSF) binding to *Bdnf* promoter II suppresses membrane depolarization-induced activity of both *Bdnf* promoters I and II (Hara et al., 2009). To alleviate the repression of *Bdnf* promoter IV, the binding of repressive transcription factor might decrease, as has been shown for methyl-CpG binding protein 2 (MeCP2) (W. G. Chen, Chang, et al., 2003; Martinowich et al., 2003), basic helix-loop-helix domain containing, class B, 2 (BHLHB2) (Jiang et al., 2008), and NFATc4 (Ding et al., 2018). Alternatively, external stimuli may trigger restructuring of repressive complexes present at *Bdnf* promoters. For instance, stimulus-induced SUMOylation of MeCP2 derepresses CREB and increases CREB binding on *Bdnf* promoter IV (Tai et al., 2016).

The cortex and hippocampus have been the primary focus in studies of *Bdnf* regulation, and years of research has revealed some brain region-specific regulators of *Bdnf* expression. For example, CaRF regulates *Bdnf* expression in the cortex but not in the hippocampus (McDowell et al., 2010), the MEF2 family regulates *Bdnf* in the hippocampus but not in the cortex (Flavell et al., 2008; M. R. Lyons et al., 2012), and nuclear receptor subfamily 4 group A member 2 (NR4A2, also known as NURR1) participates in *Bdnf* expression in midbrain neurons (Volpicelli et al., 2007), cerebellar granule neurons (Barneda-Zahonero et al., 2012), and hippocampal neurons, but not in cortical neurons (Abdollahi & Fahnestock, 2022). The specificity of these (and other) regulators of *Bdnf* expression to one or multiple brain regions warrants further research.

In addition to several transcription factors, proteins regulating chromatin structure bind to *Bdnf* promoters. For instance, a zinc finger transcription factor CCCTC-binding factor (CTCF) together with cohesin complex structures chromatin to activate or repress gene expression (Phillips & Corces, 2009). Following neuronal activity *Bdnf* gene forms long-range loops (Beagan et al., 2020; Calderon et al., 2022) and deletion of CTCF or cohesin complex decreases neuronal activity-induced *Bdnf* expression levels (Calderon et al., 2022; J. Chang et al., 2010). In the *Bdnf* locus, CTCF binds to regions in the first (exons I-III) and second cluster of exons (exons IV-VII) (J. Chang et al., 2010; Sams et al., 2016). Moreover, receptor for activated C kinase 1 (RACK1) functions as a scaffolding protein and participates in histone 4 acetylation and brings β-actin (Neasta et al., 2016) and 14-3-3 proteins (Neasta et al., 2012) to *Bdnf* promoter IV. The purpose of the scaffolding proteins in the regulation of *Bdnf* promoters is not yet fully understood.

2 Aims of the Study

In the current thesis we aimed to study stimulus-induced *Bdnf* gene regulation to gain a deeper understanding of how cells express *Bdnf*, which contributes to the development and maintenance of the nervous system.

The specific aims of the current thesis were as follows:

- Study the mechanism of *Bdnf* transcriptional autoregulation in primary cortical neurons.
- Investigate neuronal activity-induced *Bdnf* gene expression in cortical and hippocampal neurons.
- Determine enhancer regions regulating *Bdnf* expression.

3 Materials and Methods

In this study the following methods were used:

- Growing rat primary cell cultures (cortical and hippocampal neurons, cortical astrocytes) – Publications I, II, III
- Growing mouse embryonic stem cells and differentiating them into neurons – Publication III
- Molecular cloning, production, and usage of viral vectors – Publications I, II, III
- CRISPR genome and epigenome editing – Publications I, II, III
- RNA extraction, cDNA synthesis and qPCR – Publications I, II, III
- Transfection and luciferase reporter assay – Publications I, II, III
- Western Blot – Publications I, II
- Immunocytochemistry – Publication I
- Chromatin immunoprecipitation – Publications I, II, III
- *In vivo* electrophysiology – Publication I
- *In vitro* DNA pulldown and mass-spectrometry – Publications II, III
- Analysis of RNA-sequencing and ATAC-sequencing data – Publications II, III

4 Results

The main results of the current thesis are as follows:

Publication I

- *Bdnf* transcriptional autoregulation requires *de novo* protein synthesis.
- The CREB family transcription factors are the main mediators of the early phase of *Bdnf* transcriptional autoregulation in cortical neurons.
- CREB is rapidly phosphorylated and binds to *Bdnf* promoter IV after BDNF-TrkB signalling.
- CREB coactivators CBP/P300 are essential for *Bdnf* transcriptional autoregulation, while CRTCs are not.
- The CRE element in rat *Bdnf* promoter IV is necessary for the activation of the promoter after BDNF-TrkB signalling.
- Regulation of *Bdnf* promoters I and IV by the CREB family is partially conserved between rat and human.
- *Bdnf* promoter IX contains a primate lineage-specific CRE sequence that conveys the activation of the promoter after BDNF-TrkB signalling.
- All major *Bdnf* transcripts are induced in the adult rat hippocampus *in vivo* after BDNF infusion.

Publication II

- Total *Bdnf* mRNA and different *Bdnf* transcripts are more inducible in cortical neurons than in hippocampal neurons following membrane depolarization.
- Both proBDNF and mature BDNF protein levels show higher inducibility in cortical neurons than in hippocampal neurons upon membrane depolarization.
- Neuronal activity-induced *Bdnf* levels depend more on the CREB family and coactivators CBP and CRTC1 in cortical than in hippocampal neurons.
- CRTC1 knockdown increases both basal and induced levels of *Bdnf* exon I transcripts.
- CRTC1 knockdown decreases depolarization-induced proBDNF and mature BDNF levels in cortical neurons but increases them in hippocampal neurons.
- BDNF-TrkB signalling contributes to the late phase of membrane depolarization-induced *Bdnf* expression in cortical neurons but not in hippocampal neurons.
- The induction of CREM activator forms is similar in both cortical and hippocampal neurons, whereas the induction of ICER is faster and stronger in cortical neurons.
- *In vitro* DNA pulldown identified both known and novel regulators of *Bdnf* in the cortex and hippocampus.
- Several transcription factors that bind *Bdnf* promoters *in vitro* are differentially expressed in cultured cortical and hippocampal neurons, as well as in cortex and hippocampus *in vivo*.
- Transcription factors FOXP1, SATB2, RAI1, BCL11A regulate *Bdnf* expression in a brain region-specific manner.

Publication III

- The intronic region downstream of *Bdnf* exon III (named *Bdnf* +3 kb region) shows enhancer-associated characteristics in both mouse and human brain tissue.
- The +3 kb enhancer region is active in luciferase reporter assay in cultured cortical neurons and astrocytes, independent of orientation.
- The +3 kb enhancer region increases the activity of *Bdnf* promoters in a heterologous context in cultured cortical neurons but not in astrocytes.
- The +3 kb enhancer region regulates the expression of *Bdnf* transcripts starting from the first cluster of exons in cortical neurons. The enhancer is inactive in cortical astrocytes.
- Deletion of the +3 kb enhancer region in mouse embryonic stem cell-derived neurons decreases the basal and stimulus-induced levels of first cluster of *Bdnf* exons.
- The activity of the +3 kb enhancer region is regulated by CREB, AP1 family and E-box-binding transcription factors.

5 Discussion

5.1 Stimulus-dependent regulation of *Bdnf* expression

Here, we investigated *Bdnf* gene expression following BDNF-TrkB signalling (Publication I) and after KCl treatment (Publication II), which is commonly used *in vitro* to study neuronal activity-induced gene expression (reviewed in Rienecker et al., 2020). Our findings reveal that the dynamics of *Bdnf* expression after these stimuli differ remarkably. *Bdnf* expression following BDNF-TrkB signalling exhibits a bell-shaped curve with peak induction occurring ~3 hours after stimulation (Publication I, Tuvikene et al., (2016)), while *Bdnf* mRNA levels continuously increase even 8 hours after membrane depolarization (Publication II, Pruunsild et al., (2011)). The bell-shaped dynamics could potentially be explained by transient nature of BDNF-TrkB signalling due to TrkB internalization (Barford et al., 2017; Moya-Alvarado et al., 2022), resulting in a decrease in signal transduction over time. However, we acknowledge that global and prolonged treatment may not accurately represent physiological *in vivo* conditions. For instance, studies with KCl treatment have demonstrated that sustained global depolarization initiates the expression of different set of genes than local short-term depolarization (Tyssowski et al., 2018).

The question of whether *Bdnf* induction after neuronal activity requires *de novo* protein synthesis has been a subject of investigation. Some studies suggest that *Bdnf* is an immediate early gene and its induction does not depend on *de novo* protein synthesis (Castrén et al., 1998; Hughes et al., 1993; Sano et al., 1996; Tao et al., 1998), while others, including our work (Publication I) and Lauterborn et al., (1996) argue the opposite. This apparent discrepancy may be attributed to varying conditions, such as the type and duration of stimulus, cell type, timing of inhibited protein synthesis, and the use of different inhibitors at different concentrations. For instance, Hughes et al., (1993) and Tao et al., (1998) claim that protein synthesis is not involved in *Bdnf* induction. However, their studies examine the necessity of protein synthesis only 1 hour after focal hippocampal injury or KCl treatment, respectively, and both studies report maximum induction of *Bdnf* at a later time point. In contrast, *in vivo* study in the hippocampus show that the expression of *Bdnf* exons I and II depend on protein synthesis, while exons IV and VI do not after paroxysmal afterdischarge (Lauterborn et al., 1996). Our results demonstrate that after BDNF-TrkB signalling in cortical neurons, all major *Bdnf* transcripts need *de novo* protein synthesis 3 hours after the stimulus, when peak induction of *Bdnf* is reached. *Bdnf* exon IV, which is slightly induced 1 hour after treatment, maintains some inducibility despite impaired protein synthesis (Publication I). As shown in Table 2, several constitutive and inducible transcription factors regulate *Bdnf*, implying that *de novo* protein synthesis may be necessary or dispensable depending on the stimulus, time point, and cell type. Constitutively expressed transcription factors, such as CREB, could mediate basal and early induction of *Bdnf*, while inducible transcription factors, like AP1 family and NPAS4, provide stimulus-induced regulation of *Bdnf* expression.

Only AP1 family for *Bdnf* exon I (Tuvikene et al., 2016) and C/EBPβ (Bambah-Mukku et al., 2014), NFATc4, NFI (Ding et al., 2018), and XBP1 (Saito et al., 2018) for *Bdnf* exon IV have been identified to participate in *Bdnf* transcriptional autoregulation. To further elucidate the mechanism of *Bdnf* autoregulation, we examined the role of CREB, USF, C/EBP, and NFAT families, which have been shown to regulate *Bdnf* induction after

membrane depolarization (see Table 2). Our findings indicate that among the studied transcription factors, only the CREB family is involved in *Bdnf* transcriptional autoregulation in cortical neurons (Publication I). Previously, NFATc4 was shown to regulate *Bdnf* in cerebellar granule neurons (Ding et al., 2018) and C/EBPβ in the hippocampus *in vivo* (Bambah-Mukku et al., 2014), suggesting that cell type could lead to different mechanisms of *Bdnf* gene expression. Furthermore, given that BDNF-TrkB signalling and neuronal activity induce somewhat different sets of genes and transcription factors (Ibarra et al., 2022), it is plausible that neuronal activity and TrkB signalling-dependent sustained *Bdnf* expression are conveyed (at least partially) via different inducible transcription factors, providing stimulus-specific regulation. Supporting this theory, both KCl and BDNF treatment induce c-Fos expression, whereas only KCl treatment induces NPAS4 expression (Y. Lin et al., 2008). An AP1 family-binding sequence and NPAS4-binding element overlap in *Bdnf* promoter I and together with stimulus-specific expression this results in AP1 family regulating the promoter after BDNF-TrkB signalling (Tuvikene et al., 2016) and NPAS4 after membrane depolarization (Pruunsild et al., 2011).

5.2 CREB family-regulated *Bdnf* gene expression

Our investigation of *Bdnf* expression led us to focus on CREB family. The CREB family of transcription factors consists of three constitutively expressed basic leucine zipper transcription factors – CRE-binding protein (CREB), activating transcription factor 1 (ATF1), and cAMP response element modulator (CREM) (Mayr & Montminy, 2001). CREB family members bind to a DNA sequence named cAMP response element (CRE) (Andrisani et al., 1987; Comb et al., 1986; Craig et al., 2001; Montminy et al., 1986; Montminy & Bilezikjian, 1987; Schumacher et al., 2000; Short et al., 1986) and have numerous target genes that vary depending on the stimulus and brain region (Benito et al., 2011; Impey et al., 2004; Lesiak et al., 2013; Tanis et al., 2008). Our findings demonstrate that neither *Atf1, Creb1,* nor activator forms of *Crem* are notably induced after BDNF (Publication I) nor KCl treatment (Publication II), in line with the general understanding that these genes are constitutively expressed.

The CREM-encoding gene produces several CREM activator and repressor isoforms (Foulkes et al., 1991; Laoide et al., 1993), with the inducible cAMP early repressor (ICER) acting as an endogenous dominant negative protein for CREB family by competitively binding to CRE sequences (Mioduszewska et al., 2003; Molina et al., 1993; Walker et al., 1998). In contrast to activators of CREB family, *Icer*, is highly induced following BDNF-TrkB signalling in cortical neurons (Publication I) and after KCl treatment in both cortical and hippocampal neurons (Publication II). Interestingly, our results reveal that the induction of *Icer* after membrane depolarization largely depends on CREB in cortical neurons, but to a lesser extent in hippocampal neurons (Publication II). The negative autoregulatory loop formed by utilizing ICER serves as the foundation for transient CREB-regulated gene expression (Sassone-Corsi, 1998), and differences in these mechanisms warrant further investigation of CREB in different brain regions and cell types. It has been shown that CREB target genes are partially different in different brain regions (Tanis et al., 2008), and it is plausible that CREB family is more potent regulator of gene expression in cortical neurons. Importantly, compensation within the CREB family has been observed before (Hummler et al., 1994; Lemberger et al., 2008; Rafa-Zabłocka et al., 2017) and the expression dynamics of different CREB family members could contribute to brain region-specific CREB family-regulated gene expression. Lastly, our

results confirm that CREB family members strive to maintain homeostasis, as all members are induced after overexpressing A-CREB, dominant negative protein for the CREB family, and *Crem* is induced by decreased levels of *Creb1* gene expression (Publication I, II). Altogether, CREB-regulated gene expression is remarkably resilient to disruptions and capable of employing both positive and negative feedback mechanisms to preserve normal gene regulation.

In response to numerous stimuli, CREB family members are post-translationally modified, primarily by phosphorylation (Johannessen et al., 2004), and can then form stable interactions with coactivator CREB binding protein (CBP) (Campbell & Lumb, 2002; Chrivia et al., 1993; Dahal, Kwan, et al., 2017; Dahal, Shammas, et al., 2017; Parker et al., 1996; Radhakrishnan et al., 1997; Shaywitz et al., 2000; Thakur et al., 2014). CBP and its paralogue P300 enhance CREB-regulated transcription via histone acetyltransferase activity or interaction with transcriptional machinery (Bannister & Kouzarides, 1996; Kee et al., 1996; Kwok et al., 1994; Ogryzko et al., 1996). Another type of coactivators, CREB-regulated transcription coactivators (CRTCs) (Conkright et al., 2003; Iourgenko et al., 2003) are localized in the cytoplasm and are dephosphorylated and transported to the nucleus in response to stimulus (Bittinger et al., 2004; Ch'ng et al., 2012; Kovács et al., 2007; Nonaka et al., 2014). CRTCs bind to the CREB leucine zipper domain (Luo et al., 2012; Song et al., 2018) and stabilize CREB dimers and its interactions with general transcription factors (Conkright et al., 2003).

Our results show that in cortical neurons, the combination of CREB family and CBP is necessary for *Bdnf* gene expression following 1 hour and 3 hours of BDNF-TrkB signalling, but these factors become dispensable for late induction at 6 hours of treatment (Publication I). In contrast, CREB family along with coactivators CBP and CRTC1 are involved in basal and late (6 hours) induction of *Bdnf* after membrane depolarization in cortical neurons (Publication II). These results could be explained, for example, by CREB-induced transcription factors participating in late *Bdnf* gene regulation, or prolonged CREB activity following membrane depolarization but not after BDNF-TrkB signalling. Although CREB is mainly phosphorylated rapidly after membrane depolarization, it remains phosphorylated through the slower MAPK pathway (G. Y. Wu et al., 2001), potentially allowing sustained activity of CREB after neuronal activity. Regarding CREB family coactivators, CBP is required after both BDNF-TrkB signalling and membrane depolarization, whereas CRTC1 localizes less to the nucleus after BDNF-TrkB signalling compared to membrane depolarization, and thus CRTC1 does not participate in regulating *Bdnf* transcriptional autoregulation (Publication I). These findings show that different stimuli modulate CREB activity through the recruitment of either CBP, CRTCs, or both, leading to different regulation of *Bdnf* expression.

We also demonstrate that CREB with CBP is necessary for the early induction of *Bdnf* exons IV and IXa following BDNF-TrkB signalling (Publication I). Moreover, our ChIP-qPCR analysis of CREB and CBP show binding on *Bdnf* promoter IV and suggest potential binding on *Bdnf* promoters I and VI after BDNF-TrkB signalling (Publication I). Previous research has also demonstrated CREB and CBP binding on *Bdnf* promoters I, II, IV, and VI after NMDA treatment (Palomer et al., 2016). Functional CRE-like sequences have been described in *Bdnf* promoters I (Pruunsild et al., 2011; Tabuchi et al., 2002), IV (Pruunsild et al., 2011; Tao et al., 1998), and IX (Pruunsild et al., 2011). Our findings demonstrate that the activity of these CRE sequences in *Bdnf* promoters I and IV is evolutionarily conserved (Publication I). In contrast, we reveal that in primates, a mutation in promoter

IX has resulted in a functional CRE sequence that is solely responsible for the inducibility of *Bdnf* promoter IX following BDNF-TrkB signalling (Publication I).

Interestingly, mutating the CRE sequence in *Bdnf* promoter IV has stronger impact on the activity of the promoter than impairing the function of CREB family by overexpressing a dominant negative protein (Publication I). CREB binding to the CRE sequence in *Bdnf* promoter IV has been proposed to function as a nucleating protein to form a multifactor transcriptional complex required for proper activity-dependent induction of the promoter (Hong et al., 2008). Our results support the idea that the CRE element in *Bdnf* promoter IV is crucial; however, the CRE sequence may not be solely active through CREB binding. For instance, the HSF1-binding sequences in *Bdnf* promoters I, IV, and IX contain CRE sequences (Franks et al., 2023), and transcription factor XBP1 binds core sequence of CRE element (Clauss et al., 1996), competing with CREB for binding to *Bdnf* promoter IV (Martínez et al., 2016; Saito et al., 2018). Collectively, these findings suggest that the CRE sequences are essential *cis*-elements in *Bdnf* promoters but possibly function through various transcription factors, not only CREB. Further research is needed to determine which CRE-binding transcription factors regulate *Bdnf* transcription during development and in response to different stimuli.

Our results show potential binding of CREB and CBP on *Bdnf* promoter VI following BDNF-TrkB signalling (Publication I). However, a functional CRE element has not been described in *Bdnf* promoter VI. Notably, our results indicate that impairing the function of CREB family and reducing *Creb1* gene expression increases basal and induced levels of *Bdnf* exon VI after membrane depolarization (Publication II), but not after BDNF treatment (Publication I). The reason why CREB family seems to negatively regulate *Bdnf* exon VI after membrane depolarization remains unclear, but several possibilities could explain this phenomenon. First, CREB might induce the expression of an unknown repressor of *Bdnf* exon VI. Second, the expression of *Bdnf* exon VI might be hindered due to transcriptional interference arising from high transcriptional activity of *Bdnf* promoter IV. Supporting this hypothesis, deletion of the promoter IV or mutation of the CRE element in *Bdnf* promoter IV, which decreases the activity-dependent induction of *Bdnf* exon IV, both strongly upregulate depolarization-dependent induction of *Bdnf* exon VI (Hong et al., 2008). However, similar effect was not seen upon NMDA treatment in older cultures (Hong et al., 2008). A third possibility is that chromatin structure and various enhancer regions contribute to the regulation of *Bdnf* exon VI, and competitive looping determines which *Bdnf* transcript is expressed.

5.3 Brain region-specific regulation of *Bdnf* expression

BDNF has well-described roles in behaviour and memory processes (see section 1.4), which are attributed to modulation of neuronal circuits in cortical and hippocampal brain regions. However, gene expression in cortex and hippocampus differs significantly (Collado-Torres et al., 2019). Here, we set out to investigate *Bdnf* gene expression in cortical and hippocampal neurons, and our findings indicate that *Bdnf* is more inducible in response to neuronal activity in cultured cortical neurons than in hippocampal neurons (Publication II). The higher inducibility in cortical neurons could be due to stronger activation of CREB family transcription factors (as discussed above) or different regulatory mechanisms of *Bdnf* expression in the cortex and hippocampus.

BDNF is secreted in response to neuronal activation and then signals in an autocrine or paracrine manner (Cheng et al., 2011; Hartmann et al., 2001; Harward et al., 2016; Leschik et al., 2019; L. Wang et al., 2015). An *in vivo* study has shown that BDNF-TrkB

signalling contributes to *Bdnf* mRNA induction following kainic acid injection (Saarelainen et al., 2001). Here, we studied whether TrkB signalling contributes to membrane depolarization-dependent induction of *Bdnf* expression in cortical and hippocampal neurons (Publication II). Our findings reveal a TrkB signalling component in late *Bdnf* induction in cortical neurons, but not in hippocampal neurons (Publication II). It is possible that synthesis, secretion, and autocrine signalling leading to higher *Bdnf* mRNA levels take longer, and the contribution of BDNF-TrkB signalling would be even greater at later time points. Interestingly, we could not detect the component of autoregulation in hippocampal neurons (Publication II), although it has been previously demonstrated *in vivo* (Saarelainen et al., 2001). Together with difference in *Bdnf* inducibility between cortical and hippocampal neurons, it is plausible that *Bdnf* gene expression is more active in hippocampal neurons, resulting in higher basal levels of *Bdnf* and reduced effect of TrkB signalling after membrane depolarization.

The involvement of CREB and its coactivators in *Bdnf* gene regulation after neuronal activity is more pronounced in cortical neurons than in hippocampal neurons (Publication II). Additionally, CRTC1 is necessary for *Bdnf* induction, particularly for the late induction of first cluster of *Bdnf* exons in cortical neurons but seems to impair the induction of *Bdnf* exon I in hippocampal neurons (Publication II). Our findings show that *Crtc1* knockdown using CRISPR interference increases *Bdnf* exon I mRNA levels and BDNF protein levels, but not total *Bdnf* mRNA levels in the hippocampus (Publication II). These results can be explained by the use of an efficient translation initiation site in *Bdnf* exon I (Koppel et al., 2015) which leads to higher BDNF protein levels. Considering that *Bdnf* exon I knockout animals exhibit severe phenotypic effects (see section 1.4.5), *Bdnf* exon I mRNA levels reach similar levels to *Bdnf* exon IV after membrane depolarization (Pruunsild et al., 2011), and our results demonstrate that *Bdnf* exon I remarkably impacts BDNF protein levels after membrane depolarization (Publication II), it is crucial to investigate the regulation and levels of *Bdnf* exon I transcripts in future studies.

Considering the relatively modest participation of CREB in *Bdnf* gene regulation in hippocampal neurons, we employed an *in vitro* DNA pulldown assay coupled with mass-spectrometry to identify brain region-specific regulators of *Bdnf* (Publication II). We detected numerous known regulators of *Bdnf*, such as CREB, USF and AP1 family members, ARNT2, MEF2, and MeCP2 (for references see Table 2). Additionally, we discovered several novel regulators of *Bdnf*, including FOXP1, SATB2, BCL11A (also known as CTIP1), and RAI1. Brain region-specific regulation of *Bdnf* could stem from differential expression of the regulators across different brain regions. For instance, FOXP1, SATB2, BCL11A, and TBR1 are expressed in specific cortical layers and are widely used as marker genes for different cortical layers (Britanova et al., 2008; Fazel Darbandi et al., 2018; X. Li et al., 2015; Wiegreffe et al., 2015; Woodworth et al., 2016). In contrast, PROX1 determines dentate granule cell identity in the hippocampus and serves as a marker gene for hippocampal neurons (Iwano et al., 2012). Notably, these marker genes are also expressed in cultured neurons, exhibiting mostly non-overlapping expression pattern, with only a few cells expressing more than one of these marker genes simultaneously (Digilio et al., 2015). Our findings of the novel *Bdnf* regulators are intriguing, as it demonstrates that in addition to ubiquitously expressed transcription factors, cell type-specific transcription factors regulate *Bdnf* to possibly ensure specific expression of *Bdnf* during development. Furthermore, many of the discovered novel regulators of *Bdnf* are necessary for brain development and neuronal maturation (Braccioli et al., 2017; Dias et al., 2016; Fazel Darbandi et al., 2018; Iwano et al., 2012; Kaltezioti et al., 2020; Kennedy

et al., 2016; H. Li et al., 2019; McKenna et al., 2015; Page et al., 2018; Wiegreffe et al., 2015), functions similar to those of BDNF signalling. In summary, we propose that BDNF is a crucial effector molecule that participates in brain development by conveying signal from developmental transcription factors.

5.4 Distal regulatory regions governing *Bdnf* expression

Our findings using dominant-negatives for CREB family and CBP show decrease in the expression of all major *Bdnf* transcripts during BDNF-TrkB signalling, even though we only demonstrate direct binding of CREB to specific *Bdnf* promoters (Publication I). In addition, *Bdnf* proximal promoter regions are not as responsive in reporter assays as the mRNA expression is in endogenous context following BDNF-TrkB signalling (Publication I). Therefore, we hypothesize the existence of a CREB family-dependent enhancer for *Bdnf* that controls *Bdnf* transcriptional autoregulation. Furthermore, in astrocytes *Bdnf* proximal promoters are not inducible upon stimuli in reporter assays but are in the bacterial artificial chromosome context (Koppel et al., 2018). Additionally, CTCF binds to the first and second cluster of *Bdnf* exons (J. Chang et al., 2010; Sams et al., 2016), regulates chromatin structure in the *Bdnf* locus (Sams et al., 2016), and loss of CTCF or cohesin complex reduces *Bdnf* expression levels (Calderon et al., 2022; J. Chang et al., 2010). Given that CTCF together with cohesin complex remodel chromatin and form promoter-enhancer loops (Phillips & Corces, 2009) and *Bdnf* gene has been described to form long-range loops after neuronal activity (Beagan et al., 2020; Calderon et al., 2022), this data collectively suggests the existence of distal regulatory regions – enhancers – that participate in *Bdnf* gene regulation.

We began by searching for enhancer-associated chromatin marks and identified a potential intronic region located downstream from *Bdnf* exon III, referred to as +3 kb enhancer (Publication III). Our findings demonstrate that in both reporter assays and in endogenous context, this intronic region can potentiate the expression of *Bdnf* transcripts, particularly those originating from the first cluster of exons in cortical neurons. In contrast, the +3 kb enhancer region is inactive in astrocytes (Publication III). Our results highlight two noteworthy points. Firstly, we present the first evidence of cell type-specific enhancers of *Bdnf*. Secondly, our results provide evidence of an enhancer region regulating only some of the promoters in a multi-promoter gene. It appears that the regulation of *Bdnf* transcripts occurs via clusters and the +3 kb enhancer regulates the expression of transcripts starting from exons I to III.

To date, two additional enhancers of *Bdnf* have been described. First, a MEF2-regulated region located ~5 kbp upstream from the *Bdnf* exon I has been shown to potentiate the activity of *Bdnf* promoter I in hippocampal neurons but not in cortical neurons (Flavell et al., 2008; M. R. Lyons et al., 2012). The MEF2-dependent enhancer is the only currently known brain region-specific enhancer of *Bdnf*. Second, an enhancer ~210 kbp downstream from *Bdnf* exon I (and ~5 kbp upstream from *Lin7c* promoter; referred to as +170 kbp enhancer by the authors) has been reported to regulate all *Bdnf* transcripts during cortical neuron differentiation and following neuronal activity (Brookes et al., 2023). This is the only reported enhancer involved in the developmental expression of *Bdnf*. Furthermore, *Bdnf* locus has been shown to form neuronal activity-induced long-range interactions with upstream ~840 kbp (Beagan et al., 2020; Nott et al., 2019) and ~1.7 Mbp regions (Beagan et al., 2020; Calderon et al., 2022). These regions have not been shown to functionally modulate *Bdnf* expression and have not been validated as enhancer regions of *Bdnf*.

An intriguing question for future research is the mechanism of these enhancers and whether they function synergistically, competitively, or redundantly (mechanisms of enhancers are reviewed in Long et al., 2016). Our current understanding suggests that at least the ~5 kb upstream MEF2-dependent enhancer is not synergistic in cortical neurons, as it does not function in cortical neurons like the other two described *Bdnf* enhancers. Whether the +3 kbp or +210 kbp enhancer regions function in hippocampal neurons remains to be studied. In contrast, +3 kbp and +210 kbp enhancers could be either additive or competitive, as both function in cortical neurons during neuronal maturation and following neuronal activity. How these enhancers precisely interact and whether the 840 kbp and 1.7 Mbp regions also contribute to *Bdnf* gene regulation are interesting questions for future research. Given that enhancers play a crucial role in cell-type specific regulation of gene expression, we anticipate the discovery of more *Bdnf* enhancers, regulating *Bdnf* expression at different developmental stages in various brain regions and across different cell types.

We focused on the mechanism of regulation of the +3 kb enhancer region using *in vitro* DNA pulldown coupled with mass-spectrometry and we determined several E-box binding transcription factors, such as NeuroD family members, TCF4, USF family members, and MAZ (Publication III). Of these, NeuroD family participates in neuronal migration, maturation, signalling, and brain development (Bormuth et al., 2013; Guzelsoy et al., 2019; Ince-Dunn et al., 2006; Tutukova et al., 2021; Wilke et al., 2012); TCF4 participates in neuronal migration, functioning, and brain development (Kennedy et al., 2016; H. Li et al., 2019; Page et al., 2018; Schoof et al., 2020; Thaxton et al., 2018; Wittmann et al., 2021); and USF1 participates in the regulation of dendrite length and arborization (Sideromenos et al., 2022). Collectively, these transcription factors are important for proper neuronal functions. As the expression of *Bdnf* first cluster of exons is highly nervous-system specific (Aid et al., 2007; Esvald et al., 2023; Pruunsild et al., 2007), the +3 kb enhancer region could provide the neuron-specific expression pattern using these E-box binding transcription factors. Additionally, we determined several stimulus-induced transcription factors, such as AP1 and EGR family members binding to the +3 kb region (Publication III). As AP1 family members regulate the expression of *Bdnf* exon I (Tuvikene et al., 2016), it is possible that the expression of this transcript is potentiated by AP1 transcription factors also via the +3 kb enhancer region. Finally, we describe CREB binding to +3 kb region in cortical neurons and CBP binding after membrane depolarization. Although we did not study CREB binding after stimulus, +3 kb enhancer region could be one of our hypothesized CREB-dependent enhancer regions, participating in the regulation of the first cluster of *Bdnf* exons.

In conclusion, our results reveal that CREB family, along with its coactivators, participates in *Bdnf* gene regulation after BDNF-TrkB signalling and membrane depolarization through multiple promoters and an enhancer region. The presence of multiple promoters, and the formation of different transcripts with specific sub-cellular localizations and functional roles, raises the question why *Bdnf* gene regulation shows great redundancy. Functional CRE elements are found in *Bdnf* promoters I, IV, and IX (Pruunsild et al., 2011; Tabuchi et al., 2002; Tao et al., 1998), and CREB also binds the +3 kbp enhancer region (Publication III). In addition to CRE sequences, there are USF-binding elements in *Bdnf* promoters I and IV (W. G. Chen, West, et al., 2003; Pruunsild et al., 2011; Tabuchi et al., 2002); NPAS4-binding elements in *Bdnf* promoters I, IV, and IX (Pruunsild et al., 2011); NFkB-binding elements in *Bdnf* promoters I and IV (Lipsky et al., 2001; Lubin et al., 2007), and AP1 family-binding elements in *Bdnf* promoter

I and the +3 kbp enhancer region (Publication III, Tuvikene et al., (2016)). The reason of this extreme redundancy remains a mystery, but several intriguing possibilities might explain it. First, having the same regulatory elements in multiple *Bdnf* promoters might provide means to produce *Bdnf* from several promoters concurrently using the same set of transcription factors, thus amplifying the stimulus-dependent *Bdnf* expression levels. Second, it is possible that *Bdnf* promoters have evolved from one ancestral DNA sequence, explaining similar regulatory elements in multiple promoters. Third, different *Bdnf* promoters with similar transcription factor-binding sites could have evolved to ensure stable *Bdnf* expression in a variety of conditions.

6 Conclusion

The present thesis provides novel insights into the complex regulation of *Bdnf* expression. We show the role of various transcription factors, coactivators, and an enhancer region in modulating *Bdnf* mRNA levels in response to specific stimuli. The main findings of this study are the following:

- CREB family transcription factors together with coactivator CBP play a central role in *Bdnf* transcriptional autoregulation in cortical neurons (Publication I).
- The regulation of *Bdnf* expression differs between cortical and hippocampal neurons, with greater inducibility of *Bdnf* mRNA and protein levels after membrane depolarization in cortical neurons (Publication II).
- The involvement of CREB coactivators CBP and CRTC1 in the regulation of *Bdnf* expression after neuronal activity varies between cortical and hippocampal neurons, suggesting different regulatory mechanisms in different neuronal populations (Publication II).
- FOXP1, SATB2, RAI1, and BCL11A are novel brain region-specific regulators of *Bdnf* gene expression (Publication II).
- An intronic enhancer region downstream of *Bdnf* exon III specifically regulates the expression of *Bdnf* transcripts starting from the first cluster of exons in cortical neurons but not in astrocytes (Publication III).

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"In the realm of mind, where thoughts abound,
A great neurotrophin can be found,
BDNF, its name, a powerful guide,
Nurturing neurons far and wide.

Connections flourish, synapses mend,
In BDNF's presence, growth transcends,
A dance of life, a neural blend,
The great BDNF, our dearest friend."
- *ChatGPT4*

Abstract

Dissecting stimulus-dependent transcription of brain-derived neurotrophic factor

Neurotrophins are secreted signalling molecules that participate in various neuronal processes. One of the neurotrophins, brain-derived neurotrophic factor (BDNF), plays an important role in the central nervous system, supporting neuronal survival, differentiation, synapse formation and synaptic plasticity, thereby modulating both behaviour and memory functions. Abnormal BDNF levels are associated with many nervous system diseases, such as depression, and various neurodegenerative diseases, including Huntington's disease. Deeper understanding of *Bdnf* expression improves our understanding of both brain development and functioning. Furthermore, to extend the possibility of using BDNF in therapy, it is important to understand the mechanisms of *Bdnf* regulation.

In rodents, *Bdnf* gene consists of eight 5' non-coding exons and one 3' coding exon. The expression of different *Bdnf* transcripts is controlled by independent promoters that provide tissue and stimulus-specific expression. Different *Bdnf* transcripts vary in their subcellular localization, translatability, and participate in different physiological processes. Although the regulation of *Bdnf* expression after neuronal activity has been widely studied, little emphasis has been placed on brain region and cell type-specific regulatory mechanisms. Moreover, the mechanisms of *Bdnf* transcriptional autoregulation that amplifies and sustains BDNF-TrkB signalling are not well understood.

In the current thesis we studied the mechanisms of *Bdnf* transcriptional autoregulation in cortical neurons and the regulation of *Bdnf* expression after neuronal activity in cortical and hippocampal neurons. First, we focused on the cAMP-response element binding protein (CREB) family of transcription factors and their coactivators and show that CREB family together with the coactivator CREB-binding protein (CBP) are the main mediators of *Bdnf* transcriptional autoregulation. Second, we show that CREB family and its coactivators CBP and CREB-regulated transcription coactivator 1 (CRTC1) participate in neuronal activity-induced *Bdnf* expression in cortical neurons but contribute less to *Bdnf* induction in hippocampal neurons. To further study brain region-specific regulation of *Bdnf*, we performed *in vitro* DNA pulldown, and propose novel region-specific regulators of *Bdnf*, including FOXP1 and SATB2. Finally, we studied distal regulatory regions of the *Bdnf* gene and determined an intronic enhancer region that provides neuron-specific expression of the *Bdnf* transcripts starting from the first three *Bdnf* promoters.

Collectively, the findings presented in the current thesis highlight differences in *Bdnf* gene regulation after different stimuli and between cell populations. Our results shed light on the molecular mechanisms that govern *Bdnf* expression, thus contribute to our understanding of brain development and the maintenance of the nervous system. Our results could lead to the development of potential therapeutic interventions targeting neurodegenerative and neuropsychiatric diseases.

Lühikokkuvõte

Aju-päritolu neurotroofse teguri stiimulsõltuva transkriptsiooni uuringud

Neurotrofiinid on sekreteeritavad signaalmolekulid, mis toetavad mitmeid neuronite funktsioone. Üks neurotrofiinidest, aju-päritolu neurotroofne faktor (BDNF), signaliseerib kesknärvisüsteemis ja toetab neuronite ellujäämist, diferentseerumist, sünapside moodustumist ja sünaptilist plastilisust, mõjutades kokkuvõttes nii käitumist kui ka õppimist ja mälu. BDNF-i tasemete hälbimist on kirjeldatud paljude närvisüsteemi haiguste, näiteks depressiooni puhul, ja mitmesuguste neurodegeneratiivsete haiguste, sealhulgas Huntingtoni tõve korral. *Bdnf* geeni avaldumise parem mõistmine suurendab meie arusaamist aju arengust ja toimimisest. BDNF-i rakendamiseks ravivõimalusena on oluline mõista *Bdnf* geeni avaldumise regulatsiooni mehhanisme neuronites.

Närilistes koosneb *Bdnf* geen kaheksast 5' mittekodeerivast eksonist ja ühest 3' kodeerivast eksonist. Erinevate *Bdnf*-i transkriptide avaldumist kontrollivad sõltumatud promootorid, mis tagavad koe- ja stiimulspetsiifilise transkriptide avaldumise. *Bdnf*-i transkriptid erinevad oma lokalisatsiooni ja transleeritavuse poolest ning osalevad erinevates füsioloogilistes protsessides. Kuigi *Bdnf*-i avaldumise regulatsiooni pärast neuronaalset aktiivsust on laialdaselt uuritud, pole märkimisväärset rõhku pööratud erinevatele aju piirkondadele ega rakutüüpidele, kus võivad esinevad spetsiifilised regulatsioonimehhanismid. Veelgi enam, *Bdnf*-i transkriptsioonilist autoregulatsiooni, mis võimendab ja säilitab BDNF-TrkB signaliseerimist, pole märkimisväärselt uuritud.

Käesolevas väitekirjas uurisime *Bdnf* geeni transkriptsioonilise autoregulatsiooni mehhanisme kortikaalsetes neuronites ja *Bdnf*-i avaldumise regulatsiooni pärast neuronaalset aktiivsust kortikaalsetes ja hipokampaalsetes neuronites. Esmalt keskendusime cAMP vastuselementi siduva valgu (CREB) transkriptsioonifaktorite perekonnale ja nende koaktivaatoritele. Me näitame, et CREB-i perekond koos koaktivaatoriga CREB-siduva valguga (CBP) on *Bdnf*-i transkriptsioonilise autoregulatsiooni peamised vahendajad. Teiseks näitame, et CREB-i perekond ja selle koaktivaatorid CBP ja CREB-reguleeritud transkriptsiooni koaktivaator 1 (CRTC1) osalevad neuronaalse aktiivsuse poolt indutseeritud *Bdnf* geeni avaldumises kortikaalsetes neuronites, kuid osalevad märkimisväärselt vähem hipokampaalsetes neuronites. Selleks, et lähemalt uurida *Bdnf*-i spetsiifilist regulatsiooni erinevates aju piirkondades, määrasime DNA-le *in vitro* siduvad valgud ja pakume selle põhjal välja uued ajuregiooni spetsiifilised regulaatorid, sealhulgas FOXP1 ja SATB2 transkriptsiooni-faktorid. Viimaseks, uurisime *Bdnf* geeni distaalseid regulatoorseid alasid ehk enhanseralasid ja tuvastasime introonse ala *Bdnf* geenis, mis kontrollib esimese kolme *Bdnf* eksoni neuronspetsiifilist avaldumist.

Väitekirjas esitatud tulemused toovad esile erinevused *Bdnf* geeni regulatsioonis pärast erinevaid stiimuleid ja erinevates rakupopulatsioonides. Kokkuvõttes avavad meie tulemused *Bdnf* geeni ekspressiooni reguleerivaid molekulaarseid mehhanisme ning aitavad seega kaasa aju arengu ja närvisüsteemi toimimise mõistmisele. Meie tulemused võivad viia uute neurodegeneratiivsete ja neuropsühhiaatriliste haiguste ravimite väljatöötamiseni.

Publication I

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CREB family transcription factors are major mediators of BDNF transcriptional autoregulation in cortical neurons

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Cellular/Molecular

CREB Family Transcription Factors Are Major Mediators of BDNF Transcriptional Autoregulation in Cortical Neurons

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BDNF signaling via its transmembrane receptor TrkB has an important role in neuronal survival, differentiation, and synaptic plasticity. Remarkably, BDNF is capable of modulating its own expression levels in neurons, forming a transcriptional positive feedback loop. In the current study, we have investigated this phenomenon in primary cultures of rat cortical neurons using overexpression of dominant-negative forms of several transcription factors, including CREB, ATF2, C/EBP, USF, and NFAT. We show that CREB family transcription factors, together with the coactivator CBP/p300, but not the CRTC family, are the main regulators of rat *BDNF* gene expression after TrkB signaling. CREB family transcription factors are required for the early induction of all the major *BDNF* transcripts, whereas CREB itself directly binds only to *BDNF* promoter IV, is phosphorylated in response to BDNF-TrkB signaling, and activates transcription from *BDNF* promoter IV by recruiting CBP. Our complementary reporter assays with *BDNF* promoter constructs indicate that the regulation of *BDNF* by CREB family after BDNF-TrkB signaling is generally conserved between rat and human. However, we demonstrate that a nonconserved functional cAMP-responsive element in *BDNF* promoter IXa in humans renders the human promoter responsive to BDNF-TrkB-CREB signaling, whereas the rat ortholog is unresponsive. Finally, we show that extensive BDNF transcriptional autoregulation, encompassing all major *BDNF* transcripts, occurs also *in vivo* in the adult rat hippocampus during BDNF-induced LTP. Collectively, these results improve the understanding of the intricate mechanism of BDNF transcriptional autoregulation.

Key words: BDNF; CBP; CREB; positive feedback loop; transcription; TrkB

Significance Statement

Deeper understanding of stimulus-specific regulation of *BDNF* gene expression is essential to precisely adjust BDNF levels that are dysregulated in various neurological disorders. Here, we have elucidated the molecular mechanisms behind TrkB signaling-dependent *BDNF* mRNA induction and show that CREB family transcription factors are the main regulators of *BDNF* gene expression after TrkB signaling. Our results suggest that BDNF-TrkB signaling may induce *BDNF* gene expression in a distinct manner compared with neuronal activity. Moreover, our data suggest the existence of a stimulus-specific distal enhancer modulating *BDNF* gene expression.

Introduction

BDNF is a widely expressed neurotrophin in the CNS that has been described to promote the survival and differentiation of neurons and induce neurite outgrowth in the development of the brain (Bibel and Barde, 2000; Huang and Reichardt, 2001; Park and Poo, 2013). In the adult brain, the main function of BDNF is to enhance synaptic transmission, facilitate synaptic plasticity, and promote synaptic growth (Bramham and Messaoudi, 2005; Minichiello, 2009; Lu et al., 2013, 2014; Park and Poo, 2013). For example, BDNF signaling is important for synaptic consolidation (Wibrand et al., 2006; Panja et al., 2014) and memory consolidation (Bambah-Mukku et al., 2014). In addition, BDNF signaling

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in adult brain is involved in neurogenesis and dendrite formation of hippocampal granule neurons (Wang et al., 2015; Vilar and Mira, 2016).

BDNF gene expression is regulated in a sophisticated manner, with the transcription of every 5′ exon being controlled by a separate promoter (Timmusk et al., 1993; Aid et al., 2007; Pruunsild et al., 2007) and by numerous transcription factors (e.g., NPAS4, USF family, MeCP2, NFAT family, and CREB) in response to various stimuli (for review, see West et al., 2014). Interestingly, disruption of specific *BDNF* transcripts has proven important roles for different *BDNF* promoters in the formation of neural circuits underlying social behavior (Maynard et al., 2016, 2018).

The cAMP-response element-binding (CREB) family of transcription factors consists of three functionally redundant basic-leucine zipper transcription factors, namely, CREB protein, activating transcription factor 1 (ATF1), and cAMP-response element modulator (CREM) (Mayr and Montminy, 2001). The role of CREB family in the CNS has been investigated thoroughly (for review, see Barco and Marie, 2011), and CREB family has been shown to support neuronal survival (Mantamadiotis et al., 2002), regulate neuronal migration (Díaz-Ruiz et al., 2008), modulate synaptogenesis (Aguado et al., 2009), and contribute to the formation of LTP and long-term memory (Bourtchuladze et al., 1994). The CREB family has two types of coactivators. First, CREB binding protein (CBP) and its paralogue p300 (Lundblad et al., 1995), which interact with Ser-133-phosphorylated CREB and function via histone acetyl transferase activity (Bannister and Kouzarides, 1996) or interaction with basal transcriptional machinery (Kwok et al., 1994; Kee et al., 1996). Second, the CREB-regulated transcription coactivators (CRTC-s) that are localized in the cytoplasm, but in response to increase in cytoplasmic cAMP and Ca^{2+} levels can be dephosphorylated and transported to the nucleus (Bittinger et al., 2004) where they bind to the CREB leucine zipper domain to stabilize the CREB dimer (Luo et al., 2012; Song et al., 2018) and interact with general transcription factors (Conkright et al., 2003). The use of different coactivators allows for a differential activation of cAMP-responsive element (CRE)-containing promoters in response to diverse stimuli in different cell types.

BDNF can modulate its own mRNA expression via activation of the TrkB receptor and downstream MAPK signaling (Yasuda et al., 2007; Nakajima et al., 2015; Tuvikene et al., 2016). Furthermore, BDNF-TrkB signaling has been shown to induce the expression of all the *BDNF* transcripts in cultured rat cortical neurons (Tuvikene et al., 2016) and *BDNF* exon IV-containing transcripts in the rat hippocampus *in vivo* (Wibrand et al., 2006; Bambah-Mukku et al., 2014). The induction of *BDNF* exon I-containing transcripts is directly, while *BDNF* exon III- and VI-containing transcripts are indirectly, regulated by AP-1 transcription factors after BDNF-TrkB signaling (Tuvikene et al., 2016). The induction of *BDNF* exon IV-containing transcripts is mediated by C/EBPβ after inhibitory avoidance training-induced BDNF-TrkB signaling (Bambah-Mukku et al., 2014). Still, the complete repertoire of transcription factors and *cis*-elements involved in BDNF transcriptional autoregulation has not been described. Here, we have investigated the molecular mechanism of BDNF transcriptional autoregulation in rat cortical neurons and show that CREB family transcription factors together with coactivator CBP are the main regulators of TrkB signaling-dependent induction of all the major *BDNF* transcripts.

Materials and Methods

Primary cultures of rat cortical neurons. All animal procedures were performed in compliance with the local ethics committee. Primary cultures of cortical neurons were generated from Sprague Dawley rat male and female pups at embryonic day 20–21. Cerebral cortices together with hippocampi were dissected and preserved in Leibovitz L15 media (PAA Laboratories) until further processing. Cortices and hippocampi were cut into small pieces and incubated in 1 ml 0.25% Trypsin-EDTA 1× solution (Invitrogen) at 37°C for 10 min. Next, final concentrations of 0.5 mg/ml DNase I (Roche Diagnostics) and 12 mM $MgSO_4$ were added, and the mixture was again incubated at 37°C for 10 min. Then, 275 μl of 1% trypsin inhibitor (Invitrogen), 110 μl of 10% BSA (Pan-Biotech), and 50 μl DNase I (stock solution 5 mg/ml, Roche Diagnostics) were added and tissue was triturated 5 times using 1 ml pipette tip. After trituration, tissue suspension was diluted in 1× Hank's buffered salt solution and centrifuged at 200 × *g* for 30 s. Supernatant was transferred to a new tube and centrifuged again at 200 × *g* for 6 min. Finally, the supernatant was removed, and precipitated cells were resuspended in growth medium containing prewarmed Neurobasal A (PAA Laboratories or Invitrogen), 1× B27 Supplement (Invitrogen), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen), or 100 μg/ml Primocin (InvivoGen), 1 mM L-glutamine (Invitrogen). Cell suspension was distributed to plates previously coated with 0.2 mg/ml poly-L-lysine (Sigma Millipore) in 0.1 M borate buffer overnight at 4°C or at least 1 h at room temperature. Cells were grown at 37°C in 5% CO_2 atmosphere. At 2 DIV, half of the growth medium, or the whole media when the neurons were transduced with lentiviral particles, was changed and a final concentration of 10 μM mitotic inhibitor 5-fluoro-2′-deoxyuridine (Sigma Millipore) was added.

Neurons were transduced with lentiviral particles at 0 DIV, or with adeno-associated viral (AAV) vectors at 2 DIV after medium change at >3 multiplicity of infection. The cells were treated at 7–8 DIV with 50 ng/ml BDNF (Peprotech), 20 μg/ml cycloheximide (CHX, Tocris Bioscience), or 5 μM CBP-CREB interaction inhibitor (CCII, catalog #217505, lot 2758191, Merck Millipore). Control cells were treated with respective volume of vehicle (DMSO or water). For the lentivirus-mediated overexpression of E1A, neurons were transduced at 5 DIV, 72 h before lysing the cells after BDNF treatment, as prolonged overexpression of E1A led to neuronal death.

Viral vectors. The plasmids containing dominant-negative inhibitory protein coding sequences were obtained from Addgene: CMV500 A-CREB (plasmid #33371), CMV500 A-C/EBP (plasmid #33352), CMV500 A-C/EBPβ (plasmid #33363), CMV500 A-ATF2 (plasmid #33362), CMV566 A-USF (plasmid #33360), and GFP-VIVIT (plasmid #11106). The respective coding sequences were cloned to pAAV vector (AAV Helper-Free System, Agilent Technologies) under the control of human Synapsin I promoter. For A-USF, HA-tag was replaced with FLAG-tag. The coding sequences used for overexpression are listed in Tuvikene, 2018. Production of AAV particles and estimation of viral titer were performed as described previously (Koppel et al., 2015).

Lentivirus transfer plasmid pLV-hU6-sgRNA-hUbC-dCas9-KRAB-T2A-GFP coding for repressive dCas9-KRAB was obtained from Addgene (plasmid #71237), from which the gRNA expression cassette was removed using PacI restriction enzyme (Thermo Fisher Scientific). Specific guide RNA (gRNA) targeting sequences (Tuvikene, 2018) were designed using the Benchling Platform and cloned into pRRL-U6-gRNA-hPGK-EGFP lentiviral plasmid, where the expression of gRNA is under the control of U6 promoter and EGFP is under the control of human PGK promoter. Cloned plasmids were transformed into STBL3 competent cells (Invitrogen) and purified from bacteria using PureLink HiPure Plasmid Midiprep Kit (Invitrogen). Finally, all gRNA plasmids were verified by sequencing. For overexpressing EGFP, A-CREB, E1A, and dominant-negative CRTC, the respective coding regions (Tuvikene, 2018) were cloned into pRRL vector under the control of human PGK promoter, with A-CREB, E1A, and dominant-negative CRTC having an N-terminal FLAG-tag. Production of lentiviral particles was performed as described by Koppel et al. (2018). Relative titers of lentiviral particles were estimated by measuring provirus incorporation using qPCR. For further experiments, equal amounts of functional viral particles were

used to transduce neurons, and >95% transduction efficiency was verified by EGFP expression.

RNA extraction and qRT-PCR. For RNA extraction, the neurons were lysed at 7–8 DIV in RLT lysis buffer and RNA was purified using RNeasy Mini Kit (QIAGEN) with on-column digestion of genomic DNA using RNase-Free DNase Set (QIAGEN). Concentrations of the purified RNAs were determined with BioSpec-nano spectrophotometer (Shimadzu). An equal amount of RNA within an experiment was taken for complementary DNA (cDNA) synthesis using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) or SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific). qPCR was performed using LightCycler 480 SYBR Green I Master kit (Roche Diagnostics) or HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne) and LightCycler 480 Instrument II (Roche Diagnostics). Primers and annealing temperatures are listed in Tuvikene, 2018. All mRNA expression levels were normalized to *HPRT1* expression levels. Data were log-transformed, mean-centered and autoscaled as suggested by Willems et al. (2008) before statistical analysis.

Luciferase reporter assay. Plasmids containing different rat *BDNF* (r*BDNF*) and human *BDNF* (h*BDNF*) promoters (p) have been described previously as follows: pGL4.15 r*BDNF* pI (Pruunsild et al., 2011), pGL4.15 r*BDNF* pIV and pGL4.15 r*BDNF* pIV CRE site mutant (Koppel and Timmusk, 2013), pGL4.15 r*BDNF* pIII and pGL4.15 r*BDNF* pVI (Tuvikene et al., 2016); pGL4.15 h*BDNF* pI 5′, pGL4.15 h*BDNF* pIV, pGL4.15 h*BDNF* pIXa, and respective CRE site mutants (Pruunsild et al., 2011). pGL4.29 CRE reporter construct (Promega, #9PIE847) was used as a positive control. r*BDNF* pI 5′ region was generated from pGL4.15 r*BDNF* pI plasmid. r*BDNF* pII and pIXa sequences were generated from rat genomic DNA using Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific), and respective regions were cloned into pGL4.15 vector (Promega) in front of Firefly luciferase coding sequence and verified by sequencing. CRE site mutagenesis in r*BDNF* pI and pI 5′ was performed as published by Pruunsild et al. (2011). The genomic coordinates of *BDNF* proximal promoter regions and respective CRE site mutations used in the reporter constructs are shown in Tuvikene, 2018.

Cultured neurons were transfected at 6 DIV on 48-well plate in duplicate wells using ratio of 1:2 of total DNA and Lipofectamine 2000 Reagent (Invitrogen). Per well, 250 ng of effector plasmid (pAAV-h*SynI*-EGFP or pAAV-h*SynI*-A-CREB), 250 ng (or 500 ng if no effector was used) of Firefly luciferase reporter plasmid, and 20 ng of mouse *PGK*/pGL4.83 plasmid expressing *Renilla* luciferase for normalization were transfected. At 7 or 8 DIV, as indicated in the figure legend, neurons were lysed in 1× Passive lysis buffer (Promega). Luciferase signal was measured with Dual-Glo Luciferase Assay System (Promega) using Genios Pro Multifunction Microplate Reader (Tecan Group).

For data analysis, Firefly luciferase signal was background-corrected and normalized to background-corrected *Renilla* luciferase signal. The averages of duplicate wells were calculated, data were log-transformed, and statistical analysis was performed. For CRE reporter experiments using *CRTC1* knockdown and overexpression of dominant-negative CRTC, log-transformed data were also mean-centered and autoscaled before statistical analysis.

Western blot. Cultured neurons were lysed in 1× Laemmli buffer including 5% β-mercaptoethanol. Proteins were separated using 10% SDS-PAGE and transferred to PVDF membrane (Merck Millipore) in Towbin buffer at 100 V for 100 min using Mini Trans-blot Cell system (Bio-Rad). The membrane was blocked in 5% skimmed milk in TBST buffer (1× Tris-buffered saline, pH 7.4, and 0.1% Tween 20; Amresco), and then incubated with primary antibody α-CREB (1:1000, catalog #06-863, lot 2446851, Merck Millipore) and with secondary antibody goat anti-rabbit IgG conjugated with HRP (1:5000, Thermo Fisher Scientific) in 2% skimmed milk (AppliChem) in TBST at 4°C overnight and at room temperature for 1 h, respectively. After both incubations, the membrane was washed 3 times with TBST for 5 min at room temperature. Chemiluminescence signal was produced with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), and the membrane was exposed to imaging system ImageQuant Las 4000 (GE Healthcare Life Sciences).

For reprobing, the membrane was stripped with warm 0.1 M glycine solution, pH 2.0, blocked again as described previously, and incubated with primary antibody α-P-CREB (1:5000, catalog #9198, lot 0010, Cell Signaling Technology) in 5% BSA (Naxo) in TBST at 4°C overnight. Further procedure was performed as described previously. For loading control, the membrane was stained with Coomassie solution (0.1% Coomassie Brilliant Blue R-250 Dye, 25% ethanol, 7% acetic acid) for 15 min, followed by 2 washes with destaining solution (30% ethanol, 10% acetic acid) for 5 min and rinsing with tap water. The membrane was imaged using ImageQuant Las 4000 (GE Healthcare Life Sciences).

CREB and P-CREB signals and total protein staining were quantified using densitometric analysis on ImageQuant TL software (GE Healthcare Life Sciences). CREB protein levels were normalized to total protein levels, and P-CREB protein levels were normalized to CREB protein levels. Data were log-transformed, mean-centered, and autoscaled; and statistical analysis was performed.

Immunocytochemistry. Immunocytochemistry was performed as described by Koppel et al. (2018) using CRTC1 antibody (1:1500, catalog #2587, lot 3, Cell Signaling Technology) and goat anti-rabbit IgG conjugated with Alexa-488 (1:2000, Invitrogen) secondary antibody. Hoechst 33324 was used to visualize nuclei. Analysis was performed with LSM 510 META (Carl Zeiss). The specificity of the CRTC1 antibody in immunocytochemistry was verified using *CRTC1* knockdown with CRISPR interference in neurons (data not shown).

Chromatin immunoprecipitation (ChIP). Cultured neurons were grown on 100 mm dishes; and at 7 DIV, the cell growth media was reduced to 5 ml during BDNF treatment and proteins were crosslinked to DNA by adding 500 μl crosslinking buffer (11% formaldehyde, 100 mM NaCl, 0.5 mM EGTA, 50 mM HEPES, pH 8.0) and incubating at room temperature for 10 min for CREB and P-CREB ChIP. Crosslinking was stopped by adding final concentration of 0.12 M glycine and incubating at room temperature for 10 min. Subsequently, the cells were washed twice with 10 ml ice-cold 1× PBS and lysed in 500 μl lysis solution (1% SDS, 10 mM EDTA, pH 8.0, 50 mM Tris-HCl, pH 8.0, 1× cOmplete protease inhibitor cocktail, Roche Diagnostics; 1× PhosSTOP phosphatase inhibitor cocktail, Roche Diagnostics) for 5 min on ice. Cells were collected with cell scraper and transferred to an Eppendorf tube where lysates were sonicated on ice using VCX-130 sonicator (Sonics) until an approximate average fragment size of 250–500 bp was obtained. Following sonication, the lysates were centrifuged at 16 000 × *g* for 5 min at 4°C, and supernatant was transferred to a new tube.

Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific); and where necessary, the concentrations were equalized using lysis buffer. Equal amounts of protein were diluted 9 times in dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 8.0, 1× cOmplete protease inhibitor cocktail, Roche Diagnostics; 1× PhosSTOP phosphatase inhibitor cocktail, Roche Diagnostics). A total of 5 μg of α-CREB (catalog #06-863, lot 2446851, Merck Millipore) or α-P-CREB (catalog #9198, lot 0010, Cell Signaling Technology) was added to the diluted lysate and incubated overnight at 4°C. In parallel, 50 μl Dynabeads Protein G (Invitrogen) per immunoprecipitation reaction were washed twice with 1 ml dilution buffer for 10 min at 4°C and blocked overnight at 4°C in 1 ml dilution and lysis buffer solution (ratio of 9:1, respectively), containing 200 μg/ml BSA and 10 μg/ml salmon sperm DNA. The next day, the beads were washed twice with dilution buffer for 10 min at 4°C and finally diluted in dilution and lysis buffer solution (ratio of 9:1, respectively), distributed to samples, and incubated for ~5 h with rotation at 4°C. Then, the beads-antibody complexes were washed 4 times in 1 ml wash buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 8.0, 1× cOmplete protease inhibitor cocktail, Roche Diagnostics; 1× PhosSTOP phosphatase inhibitor cocktail, Roche Diagnostics) and once with final wash buffer (1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 8.0, 1× cOmplete protease inhibitor cocktail, Roche Diagnostics; 1× PhosSTOP phosphatase inhibitor cocktail, Roche Diagnostics) for 10 min per wash while rotating at 4°C. DNA-protein complexes were eluted from the beads 3 times with 50 μl elution buffer (1% SDS, 100 mM $NaHCO_3$, 1 mM EDTA) at 37°C for 10 min. The third elution was performed with an additional

incubation at 65°C for 5 min before combining with other eluates. Input samples were diluted with elution buffer to a final volume of 150 μl. Crosslinking was reversed by adding final concentration of 250 mM NaCl and incubating at 65°C overnight.

The next day, 125 μg/ml RNase A was added to the samples and incubated at 37°C for at least 30 min. After that, final concentrations of 6 mM EDTA and 240 μg/ml proteinase K were added and incubated at 45°C for at least 30 min. Finally, genomic DNA was purified using QIAquick PCR Purification Kit (QIAGEN), and DNA enrichment was analyzed using qPCR. Primers and annealing temperatures are listed in Tuvikene, 2018. The efficiencies of primer pairs were determined using serial dilutions of input DNA. DNA enrichment was calculated relative to the respective DNA levels in input sample considering primer pair efficiency. For statistical analysis, data were log-transformed.

CBP ChIP followed the described protocol, except for the following modifications: crosslinking with formaldehyde was performed for 20 min; sonication was performed with BioRuptor Pico (Diagenode); 7.5–10 μg of α-CBP (catalog #7389, lot 5, Cell Signaling Technology) antibody was used; phosphatase inhibitors were not used; washes with ice-cold solutions were performed quickly without rotation. For CBP ChIP, data were log-transformed and also mean-centered to account for variations in enrichment between different experiments.

Electrophysiology and intrahippocampal infusion of BDNF. Intrahippocampal BDNF infusion was performed as described previously (Ying et al., 2002; Messaoudi et al., 2007; Kuipers et al., 2016). All experiments were performed according to the ethical standards approved by the Norwegian Committee for Experiments on Animals. The experiments were performed on adult male rats of the Sprague Dawley strain (Taconic), weighing 250–350 g. Rats were anesthetized with urethane (1.5 g/kg, i.p.), and positioned in a stereotaxic frame, while the body temperature was maintained at 37°C using thermostatically controlled electric heating pad. A concentric bipolar stimulating electrode (Tip separation 500 μm; SNEX 100; Rhodes Medical Instruments) was lowered into the angular bundle for stimulation of the medial perforant path. Stereotaxic coordinates for stimulation were as follows (in mm): 7.9 posterior to bregma, 4.2 lateral to the midline, and 2.5 below the dura.

A Teflon-coated tungsten wire recording electrode (outer diameter of 0.075 mm; A-M Systems #7960) was glued to the infusion cannula (30 gauge). The electrode was then cut so that it extended 800 μm from the end of the cannula. Stereotaxic coordinates for recording in the dentate hilus were as follows (in mm): 3.9 posterior to bregma, 2.3 lateral, and 2.8–3.1 below the dura. The recording electrode was slowly lowered into the dorsal hippocampus until a positive-going fEPSP of maximum slope was obtained in the dentate hilus. The tip of the infusion cannula was located in the deep striatum lacunosum-moleculare of field CA1, 800 μm above the hilar recording site and 300–400 μm above the medial perforant synapses. The infusion cannula was connected via a polyethylene (PE50) tube to a 10 μl Hamilton syringe and infusion pump. After baseline recording for 20 min, the infusion of 2 μl of 1 μg/μl BDNF (Alomone Labs) over 25 min at a rate of 0.08 μl/min followed. cytochrome C (CytC; Sigma Millipore) was used as a control for BDNF infusion. Evoked responses were recorded for 120 min after infusion.

Biphasic rectangular test pulses of 150 μs duration were applied every 30 s throughout the experiment (0.033 Hz). Responses were allowed to stabilize for 1 h at a stimulation intensity that produced a population spike 30% of maximum. A stable 20 min baseline of evoked potentials was recorded (pulse-width 0.15 ms, at 0.033 Hz) before intrahippocampal drug infusion. Evoked responses were recorded for 120 min after infusion. Signals from the dentate hilus were amplified, filtered (0.1 Hz to 10 kHz), and digitized (25 kHz). Acquisition and analysis of field potentials were accomplished using DataWave Technologies. The maximum slope of the fEPSP and the averages of four consecutive responses were obtained. Changes in the fEPSP slope were expressed in percentage of baseline (20 min preceding infusion). After recordings were completed, the electrodes were removed, rats were decapitated, and dentate gyri from both ipsilateral and contralateral hemisphere were dissected, immediately frozen on dry ice, and stored at −80°C. RNA was purified using RNeasy Lipid Tissue Mini Kit (QIAGEN), and qRT-PCR was performed as stated previously.

Statistical analysis and graphical representation of the data. Statistical analysis was performed on log-transformed, and where applicable, mean-centered and autoscaled data from independent experiments: neuron cultures obtained from different litters and prepared separately; or independent animals for the *in vivo* experiments. Two-tailed paired or unpaired *t* tests, as indicated at each figure, were performed using Microsoft Excel 365. All tested hypotheses were specified before conducting the experiments. *p* values were adjusted for multiple comparisons with Holm–Sidak family-wise error rate correction method using Prism 7.03 (GraphPad Software). For graphical representation, means and means ± SEM of transformed data were calculated and data were back-transformed to linear scale with error bars indicating upper and lower limits of back-transformed mean ± SEM.

Results

BDNF transcriptional autoregulation requires protein synthesis

Currently, only AP-1 and C/EBPβ transcription factors have been shown to be involved in BDNF transcriptional autoregulation in neurons. To extend the understanding of BDNF transcriptional autoregulation, we decided to perform a thorough analysis of additional transcription factors involved. We first asked whether the transcription factors that regulate *BDNF* expression in primary cultures of cortical neurons are synthesized in response to BDNF-TrkB signaling (like the AP-1 proteins) or are present in the cell before activation of TrkB signaling. Therefore, we treated cultured rat cortical neurons with the protein synthesis inhibitor CHX before BDNF treatment and measured the expression levels of different *BDNF* transcripts using qRT-PCR (Fig. 1).

First, as a positive control of CHX treatment, we determined the expression levels of *c-Fos*, as it is known that the induction of *c-Fos* mRNA is largely independent of protein synthesis (Greenberg et al., 1986; Hughes et al., 1993). In cells pretreated with CHX, 1 h of BDNF treatment induced *c-Fos* expression level ~24-fold ($p = 0.0006$), and the expression levels remained high for 3 h (~31-fold, $p = 0.0056$) and 6 h (~14-fold, $p = 0.0116$) of BDNF treatment (Fig. 1). Next, we measured the induction of *BDNF* mRNA levels after BDNF-TrkB signaling. Without disrupting protein synthesis, the induction of total *BDNF* mRNA was ~3.6-fold ($p = 0.0737$) after 1 h, ~13-fold ($p = 0.0034$) after 3 h and ~6.7-fold ($p = 0.0152$) after 6 h of BDNF-TrkB signaling. Blocking protein synthesis reduced the induction of total *BDNF* mRNA to ~1.8-fold ($p = 0.0325$) after 1 h, to ~1.6-fold ($p = 0.2899$) after 3 h of BDNF treatment, and after 6 h of BDNF treatment the expression levels were approximately similar to the expression levels in cells not treated with BDNF. At different transcript level, blocking protein synthesis completely abolished the TrkB signaling-dependent induction of *BDNF* exon I-, III-, and IXa-containing transcripts. In contrast, the early induction of *BDNF* exon IV-containing transcripts remained after CHX treatment, in which case after 1 h of BDNF treatment a ~2.8-fold ($p = 0.0035$) induction was still observed, while the induction in cells not treated with CHX at the respective time point was ~6.3-fold ($p = 0.0618$). In CHX-treated cells, we also observed a minor ~1.4-fold ($p = 0.0379$) and ~1.3-fold ($p = 0.0417$) induction of *BDNF* exon IIc- and VI-containing transcripts, respectively, after 1 h of BDNF treatment, while the induction of these transcripts in cells not treated with CHX at the same time point was ~1.6-fold ($p = 0.2181$) and ~1.8-fold ($p = 0.0845$), respectively. Together, our results indicate that at least some transcription factors neces-

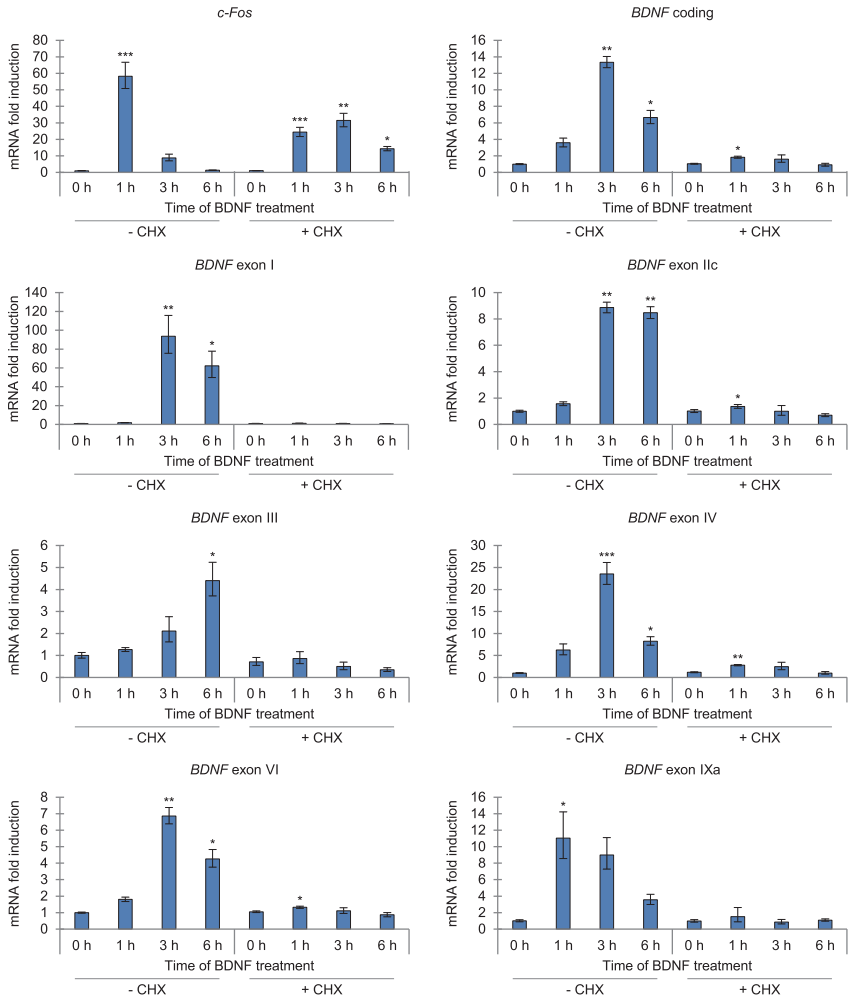

Figure 1. BDNF transcriptional autoregulation requires protein synthesis. At 7 DIV, rat primary cortical neurons were treated with 20 μg/ml CHX for 30 min where indicated and subsequently with 50 ng/ml BDNF for the indicated time periods. qRT-PCR was used to measure the levels of *c-Fos*, total *BDNF* (*BDNF* coding), and *BDNF* transcripts. The mRNA fold induction was calculated relative to the respective mRNA level in cells not treated with BDNF and either not treated or treated with CHX, respectively. Average of three independent experiments ($n = 3$) is shown. Error bars indicate SEM. Statistical significance relative to the respective transcript levels in cells not treated with BDNF: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

sary for the early induction of *BDNF* exon IIc-, IV-, and VI-containing transcripts after BDNF-TrkB signaling are already present in the cell, while the transcription factors that participate in later induction after BDNF-TrkB signaling or regulate other *BDNF* transcripts are mostly synthesized in response to BDNF-TrkB signaling.

CREB family of transcription factors is the main mediator of the early phase of BDNF transcriptional autoregulation

To investigate which transcription factors, in addition to AP-1, are involved in BDNF transcriptional autoregulation in cultured rat cortical neurons, we used dominant-negative forms of several transcription factor families to impair their potential regulatory role. We focused on transcription factors that have been shown to be involved in *BDNF* transcriptional regulation in other signaling pathways, particularly on those that are present in the neurons before TrkB signaling and are activated independent of translation. We created AAV vectors encoding the dominant-negative forms of CREB, C/EBPα and β, ATF2, USF, and NFAT families. The dominant-negative proteins A-CREB, A-C/EBPα, A-C/EBPβ, A-ATF2, and A-USF mediate their dominant-negative effect through dimerization with the respective endogenous transcription factors, thus preventing the transcription factor from binding to DNA and mediating transcriptional regulation (Krylov et al., 1995; Ahn et al., 1998; Qyang et al., 1999). To interfere with NFAT-regulated gene expression, we used overexpression of the VIVIT protein sequence (MAGPHPVIVITGPHEE) that blocks NFAT dephosphorylation by calcineurin and thus prevents NFAT translocation to the nucleus (Aramburu et al., 1999). We verified the expression of the dominant-negative proteins using immunocytochemistry and Western blot analysis (data not shown). Next, we infected neurons with the mentioned

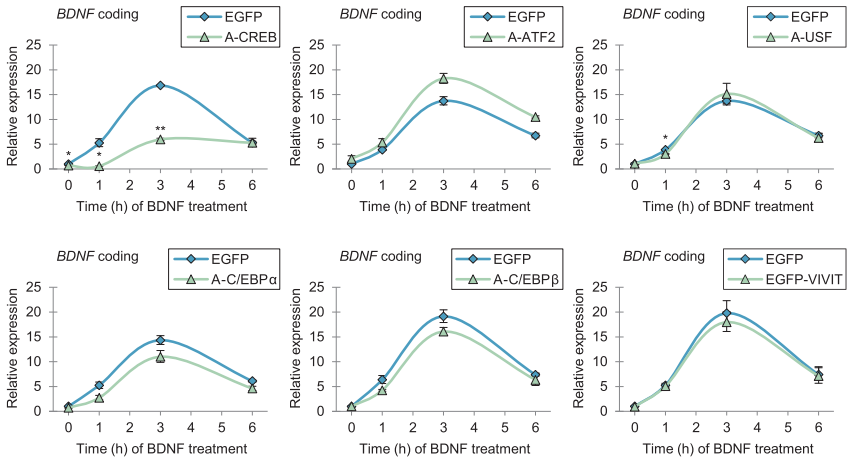

Figure 2. CREB family transcription factors are the main regulators of BDNF transcriptional autoregulation. Primary neurons were transduced at 2 DIV with AAV particles coding for indicated dominant-negative transcription factors or EGFP. At 8 DIV, neurons were treated with 50 ng/ml BDNF for the indicated time periods, and the expression levels of total *BDNF* mRNA (*BDNF* coding) were measured using qRT-PCR. The expression level of respective transcripts in cells transduced with EGFP-encoding AAV particles and not treated with BDNF was set as 1. Average of three independent experiments ($n = 3$) is shown. Error bars indicate SEM. Statistical significance between the respective transcript levels in EGFP and respective dominant-negative-overexpressing cells at respective time points: $^{**}p < 0.01$; $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

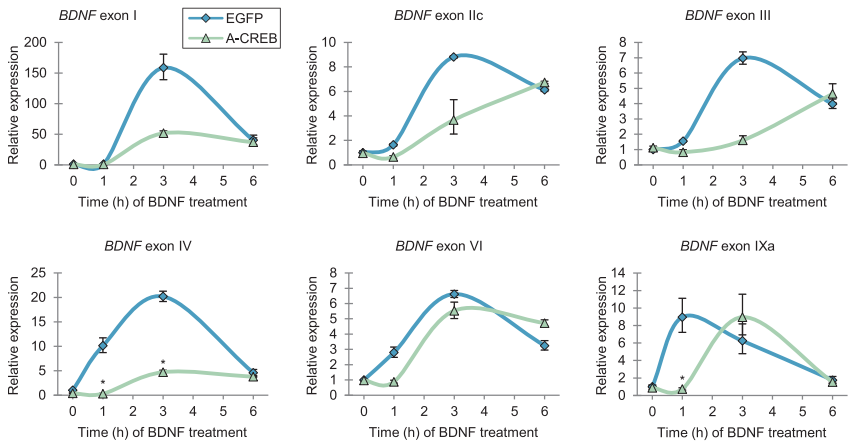

Figure 3. CREB family transcription factors are required for the early induction of all *BDNF* transcripts in response to BDNF-TrkB signaling. Primary neurons were transduced at 2 DIV with AAV particles coding for EGFP or A-CREB, a dominant-negative form of CREB family transcription factors. At 8 DIV, neurons were treated with 50 ng/ml BDNF for the indicated time periods; and the expression levels of different *BDNF* transcripts, as indicated above each graph, were measured using qRT-PCR. The expression level of respective transcripts in cells transduced with EGFP-encoding AAV particles and not treated with BDNF was set as 1. Average of three independent experiments ($n = 3$) is shown. Error bars indicate SEM. Statistical significance between the respective transcript levels in EGFP and A-CREB-overexpressing cells at respective time points: $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

AAV vectors at 2 DIV, treated the cells with BDNF at 8 DIV, and measured the expression levels of *BDNF* using qRT-PCR (Fig. 2).

The strongest effect on BDNF transcriptional autoregulation was seen in cells overexpressing A-CREB, where the basal expression level of total *BDNF* mRNA was reduced to 0.69-fold ($p = 0.0266$) compared with *BDNF* expression levels in neurons overexpressing EGFP. In BDNF-treated cells, the overexpression of A-CREB decreased the induced levels of total *BDNF* mRNA from 5.2-fold to 0.56-fold ($p = 0.0252$) after 1 h of BDNF treatment and from 16.8-fold to 6.0-fold ($p = 0.0054$) after 3 h of BDNF treatment. No significant difference between the induced levels of *BDNF* in EGFP and A-CREB-overexpressing cells was seen at 6 h of BDNF treatment.

Notably, we did not observe a noteworthy effect of overexpressing A-ATF2, A-USF, A-C/EBPα, A-C/EBPβ, or EGFP-VIVIT on the basal levels of *BDNF* mRNA or on TrkB signaling-induced *BDNF* levels, indicating that ATF2, USF, C/EBP, and NFAT family transcription factors do not significantly contribute to the BDNF autoregulatory loop in cortical neurons.

As A-CREB had the most prominent effect on BDNF autoregulation, we next investigated the effect of overexpressing A-CREB on the levels of different *BDNF* transcripts (Fig. 3). The induced levels of exon I-containing *BDNF* transcripts showed a remarkable decrease from 159-fold to 52-fold ($p = 0.1082$, uncorrected $p = 0.0282$) at 3 h of BDNF treatment. A similar effect was seen for exon IIc- and III-containing *BDNF* transcripts at 3 h of BDNF

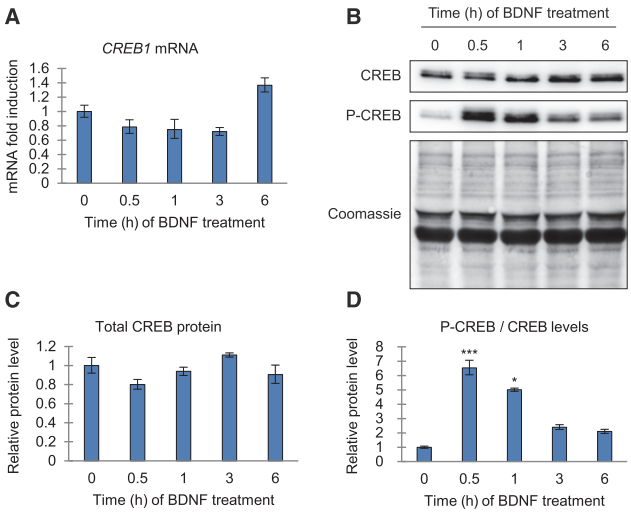

Figure 4. BDNF-TrkB signaling induces CREB phosphorylation but does not influence total CREB protein levels. Primary neurons were treated at 7 DIV with 50 ng/ml BDNF for the indicated time periods. ***A***, *CREB1* mRNA levels, measured using qRT-PCR, are represented as fold induction relative to *CREB1* mRNA levels in untreated cells. ***B***, Western blot analysis determining CREB and phospho-Ser-133 CREB (P-CREB) protein levels and Coomassie staining for loading control. ***C***, Quantification of total CREB protein levels. CREB protein levels were normalized using Coomassie staining, and normalized CREB protein level in nontreated cells was set as 1. ***D***, Quantification of CREB phosphorylation levels. P-CREB protein levels were normalized to total CREB protein levels, and the relative level of P-CREB protein in nontreated cells was set as 1. ***A***, ***C***, ***D***, Average of three independent experiments ($n = 3$) is shown. Error bars indicate SEM. Statistical significance relative to respective mRNA (***A***) or protein (***C***, ***D***) levels in untreated cells: $^{***}p < 0.001$; $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

CREB is rapidly phosphorylated in response to BDNF-TrkB signaling

To confirm BDNF signaling-dependent activation of CREB, as it has been described previously (Finkbeiner et al., 1997; Pizzorusso et al., 2000), in our primary cultures of neurons, we treated neurons with BDNF for different time periods and measured changes in the expression levels of *CREB1* mRNA using qRT-PCR and determined CREB and phospho-Ser-133 CREB protein levels using Western blot (Fig. 4).

BDNF treatment did not affect *CREB1* mRNA expression level (Fig. 4*A*). Although a small downregulation of *CREB1* mRNA level in response to BDNF-TrkB signaling was consistently observed after 3 h of BDNF treatment, the minor changes in *CREB1* mRNA levels were not reflected in CREB protein levels, which remained stable (Fig. 4*B*,*C*). In contrast, BDNF-TrkB signaling induced very rapid CREB phosphorylation at Ser-133 (Fig. 4*B*,*D*), as expected, considering that post-translational regulation of CREB is well established (for review, see Johannessen et al., 2004). P-CREB levels were ~6.5-fold ($p = 0.00004$) higher after 0.5 h, ~5.0-fold ($p = 0.0105$) higher after 1 h, and stayed ~2-fold higher after 3 and 6 h of BDNF treatment compared with P-CREB levels in untreated cells. Together, our results indicate that the activity of CREB after BDNF-TrkB signaling in our neuronal cultures is largely regulated through post-translational means and not by overall change in CREB protein levels.

treatment, when the induced levels of these transcripts decreased from 8.8-fold to 3.7-fold ($p = 0.2775$, uncorrected $p = 0.15002$) and from 7.0-fold to 1.6-fold ($p = 0.0776$, uncorrected $p = 0.0200$), respectively.

Overexpression of A-CREB decreased the basal levels of only *BDNF* exon IV-containing transcripts to 0.4-fold ($p = 0.0575$, uncorrected $p = 0.0292$) compared with the respective levels in neurons expressing EGFP. Remarkably, A-CREB also completely abolished the induction of exon IV-containing transcripts at 1 h ($p = 0.0119$) of BDNF-TrkB signaling and lowered the induced levels at 3 h from 20-fold to 4.7-fold ($p = 0.0119$). A similar tendency was observed for *BDNF* exon VI- and IXa-containing transcripts, where A-CREB abolished the induction completely at 1 h time point ($p = 0.1078$, uncorrected $p = 0.0281$, and $p = 0.0343$, respectively). However, at 6 h of BDNF treatment, the induced levels of all *BDNF* transcripts were mostly the same in A-CREB and EGFP-overexpressing cells, indicating that other transcription factors outside the CREB family mediate the late induction of *BDNF* expression in response to TrkB signaling.

As the human *Synapsin I* promoter used in our AAV vectors is slightly (~2-fold) upregulated in response to BDNF-TrkB signaling (data not shown), we verified the results of A-CREB overexpression using lentiviral vectors expressing EGFP or A-CREB under the control of human *PGK* promoter, which provides very stable expression in response to BDNF treatment (data not shown). The results obtained using lentivirus-mediated overexpression ($n = 3$, data not shown) were remarkably similar to those obtained with AAV vectors, further strengthening the notion that CREB family transcription factors are the main mediators of the early, but not late, induction of all *BDNF* transcripts upon TrkB signaling.

CREB coactivator CBP/p300 is necessary for the BDNF transcriptional autoregulation

As the overexpression of A-CREB mostly decreased the early induction, largely corresponding to the period where CREB is phosphorylated, but not the late induction of different *BDNF* transcripts in response to BDNF-TrkB signaling, we next investigated whether CREB coactivator CBP, which interacts with Ser-133 phosphorylated CREB, is involved in regulating the expression of *BDNF* transcripts after BDNF-TrkB signaling. For that, we used CBP-CREB interaction inhibitor (CCII), which binds to CBP's KIX domain (Best et al., 2004; B. X. Li and Xiao, 2009) and therefore prevents CBP interaction with CREB family members. As p300, a paralogue of CBP, has a highly similar KIX domain, CCII also inhibits CREB-p300 interaction (Breen and Mapp, 2018). We treated cultured rat neurons with CCII before treating the cells with BDNF for different time periods and then measured the levels of *BDNF* transcripts using qRT-PCR (Fig. 5).

First, we observed no remarkable effect of CCII on the expression of *c-Fos* mRNA in response to BDNF-TrkB signaling (Fig. 5). On the contrary, disturbing CBP/p300 recruitment decreased BDNF transcriptional autoregulation and affected the induced levels of most of the *BDNF* transcripts, whereas the basal expression levels of *BDNF* transcripts did not change. The induced levels of total *BDNF* mRNA were reduced from ~3.0-fold to ~2.0-fold ($p = 0.2236$, uncorrected $p = 0.0809$) after 1 h, and from ~16-fold to ~8.0-fold ($p = 0.0056$) after 3 h of BDNF

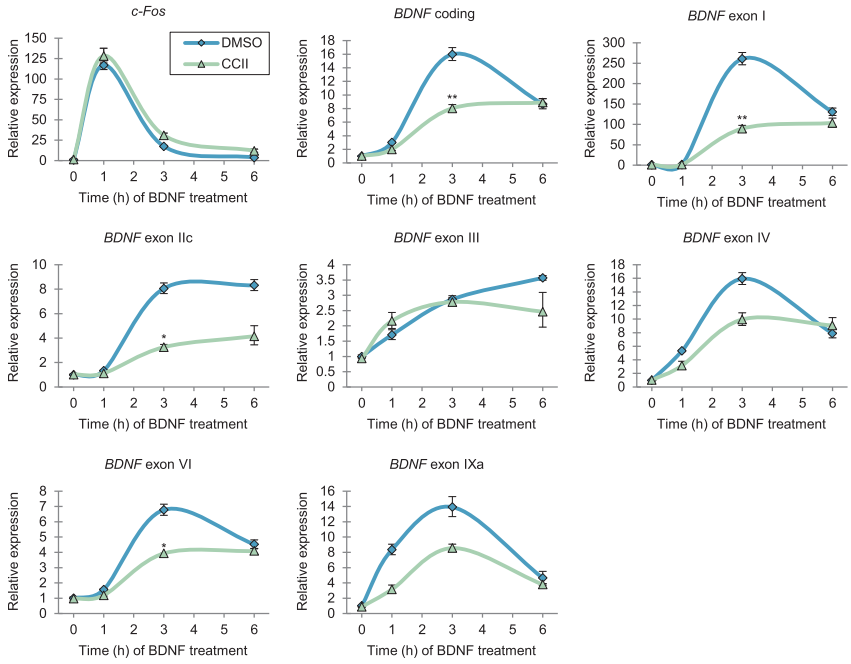

Figure 5. Early, but not late, phase of BDNF transcriptional autoregulation requires CREB-CBP/p300 interaction. At 8 DIV, primary neurons were treated with 5 μ_M CBP-CREB interaction inhibitor (CCII) or with DMSO as a control for 1 h before treating the cells with 50 ng/ml BDNF for the indicated time periods. qRT-PCR was used to measure *c-Fos*, total *BDNF* (*BDNF* coding), and *BDNF* transcript levels. The expression level of respective transcripts in vehicle-treated cells not treated with BDNF was set as 1. Average of three independent experiments ($n = 3$) is shown. Error bars indicate SEM. Statistical significance between respective transcript levels in DMSO and CCII-treated cells at respective time points: $^{**}p < 0.01$; $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

treatment in CCII-treated cells. In cells pretreated with CCII, the induced levels of *BDNF* transcripts containing exon IV and IXa were decreased from ~5.3-fold to ~3.2-fold ($p = 0.2972$) and from ~8.4-fold to ~3.1-fold ($p = 0.1486$), respectively, after 1 h of BDNF treatment, and from ~16-fold to ~9.9-fold ($p = 0.0647$) and from ~14-fold to ~8.6-fold ($p = 0.1707$), respectively, after 3 h of BDNF treatment. The induced levels of *BDNF* transcripts containing exon I, IIc, and VI were decreased by ~2-fold ($p = 0.0072$, $p = 0.0402$, and $p = 0.0359$, respectively) in cells pretreated with CCII after 3 h of BDNF treatment. The expression levels of *BDNF* exon III-containing transcripts did not depend on CBP/p300 recruitment. Interestingly, after 6 h of BDNF treatment, there were no differences in the induced levels of most *BDNF* transcripts, except for exon IIc-containing transcripts, in cells treated with DMSO or CCII, indicating that CREB-CBP/p300 interaction is largely dispensable for the later phases of BDNF transcriptional autoregulation.

To further elucidate the role of CBP/p300 in *BDNF* gene regulation, we overexpressed adenoviral *E1A* gene, which encodes proteins that are known to function as dominant-negative for CBP/p300 and impair CBP/p300-regulated transcription (Kalvakolanu et al., 1992; Lundblad et al., 1995; Nakajima et al., 1997). First, we noted a (Fig. 6) statistically insignificant ~3.3-fold increase ($p = 0.2957$, uncorrected $p = 0.1227$) in the basal expression level of *c-Fos*, and ~2-fold decrease ($p = 0.1963$, uncorrected $p = 0.0532$) in the induced levels of *c-Fos* mRNA after 1 h of BDNF treatment (Fig. 6). Notably, we also observed that the basal expression of most *BDNF* transcripts, except for transcripts containing *BDNF* exon VI, was upregulated 1.3-fold to 1.8-fold, and even 4.7-fold ($p = 0.1603$, uncorrected $p = 0.0786$) for exon I-containing *BDNF* transcripts, by E1A overexpression (Fig. 6). In contrast, the basal levels of exon VI-containing *BDNF* transcripts was decreased ~2-fold ($p = 0.1980$, uncorrected $p = 0.1494$) in E1A-expressing cells. The TrkB signaling-induced levels of total *BDNF* mRNA were reduced from ~4.4-fold to ~1.7 fold ($p = 0.0456$) after 1 h, from ~9-fold to ~2.6-fold ($p = 0.0458$) after 3 h, and from ~4.2-fold to ~2-fold ($p = 0.0458$) after 6 h of BDNF treatment. Correspondingly, the induced levels of all *BDNF* transcripts after BDNF-TrkB signaling were diminished after E1A overexpression. Notably, the induced levels of *BDNF* transcripts containing exon IV were reduced from ~6.9-fold to 2.1-fold ($p = 0.0064$) after 1 h, from 8.0-fold to 1.8-fold ($p = 0.0064$) after 3 h, and from 2.8-fold to 1.2-fold ($p = 0.0843$, uncorrected $p = 0.0431$) after 6 h of BDNF treatment by E1A overexpression. Together, our results show that CBP/p300 is absolutely required for the regulation of *BDNF* transcription in response to BDNF-TrkB signaling.

CREB coactivator CRTC does not participate in BDNF transcriptional autoregulation

Next, to further elucidate the mechanism of CREB family-regulated *BDNF* gene expression, we investigated whether other CREB coactivators are required for the BDNF transcriptional autoregulation. Three CRTCs have been described, but CRTC1 is the most abundantly expressed member of the CRTC family in the CNS (Watts et al., 2011; Saura and Cardinaux, 2017). It has also been shown that *CRTC1* KO mice have lower levels of differ-

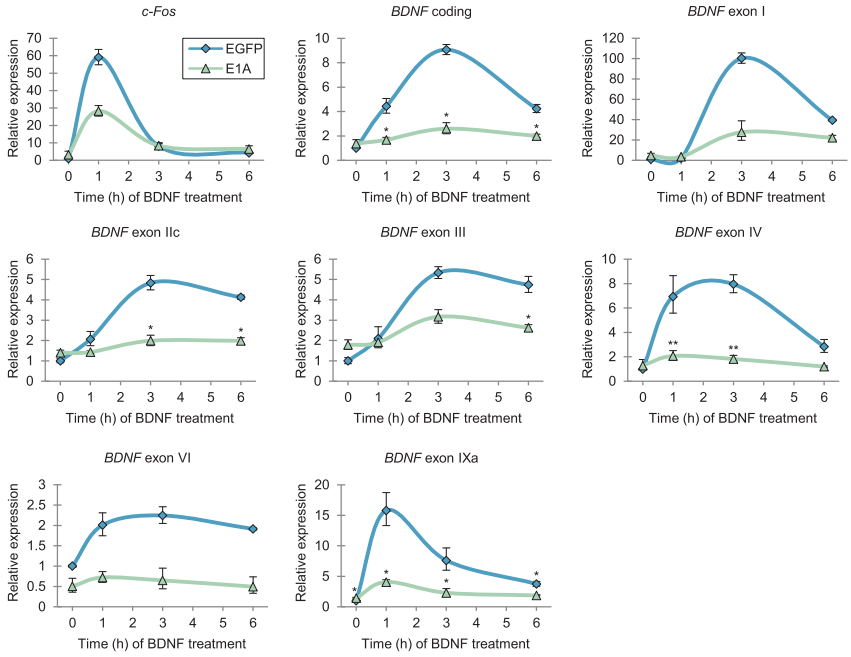

Figure 6. Activity of CBP/p300 is necessary for both the early and late induction of all *BDNF* transcripts after BDNF-TrkB signaling. Primary neurons were transduced at 5 DIV with lentiviral particles encoding EGFP, as control, or E1A, a dominant-negative for CBP/p300. At 8 DIV, neurons were treated with 50 ng/ml BDNF for the time indicated. qRT-PCR was used to measure c-Fos, total BDNF (BDNF coding), and BDNF transcript levels. The expression level of respective transcripts in cells expressing EGFP not treated with BDNF was set as 1. Average of three independent experiments ($n = 3$) is shown. Error bars indicate SEM. Statistical significance between respective transcript levels in EGFP and E1A-expressing cells at respective time points: $^{**}p < 0.01$; $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

ent *BDNF* transcripts both in PFC and in hippocampus (Breuillaud et al., 2012), suggesting a possible role for CRTC1 in *BDNF* gene regulation. Therefore, we first chose to investigate whether CRTC1 localizes to the nucleus in response to BDNF-TrkB signaling, as it has been established for various treatments (e.g., forskolin, membrane depolarization with KCl and NMDA) (Bittinger et al., 2004; Zhou et al., 2006; S. Li et al., 2009; Ch'ng et al., 2012; Fukuchi et al., 2014, 2015; Uchida et al., 2017). We analyzed CRTC1 localization in untreated neurons and neurons treated with BDNF using immunocytochemistry. We used KCl treatment as a positive control. Our results show that, in unstimulated cortical neurons, CRTC1 is mainly localized in the cytoplasm, whereas after 1 h KCl treatment almost all of CRTC1 is localized to the nucleus (Fig. 7). After BDNF treatment, the nuclear localization of CRTC1 increases in the majority of the cells; however, a detectable amount of CRTC1 also remains in the cytoplasm. Similar results were also observed after 3 and 6 h treatments (data not shown). Together, our data show that CRTC1 is translocated to the nucleus after BDNF treatment, although with lower efficiency than after membrane depolarization, and could possibly be involved in *BDNF* autoregulation.

Next, to investigate whether CRTC1 regulates *BDNF* gene expression after BDNF-TrkB signaling, we suppressed *CRTC1* gene expression using CRISPR interference with lentivirus-mediated overexpression of dCas9 protein coupled with KRAB repressor domain, and two gRNAs targeted to *CRTC1* promoter region. We transduced cultured rat neurons with lentiviral particles at 0 DIV, treated neurons with BDNF at 8 DIV, and used luciferase reporter assay to estimate CRE-reporter activity or qRT-PCR to

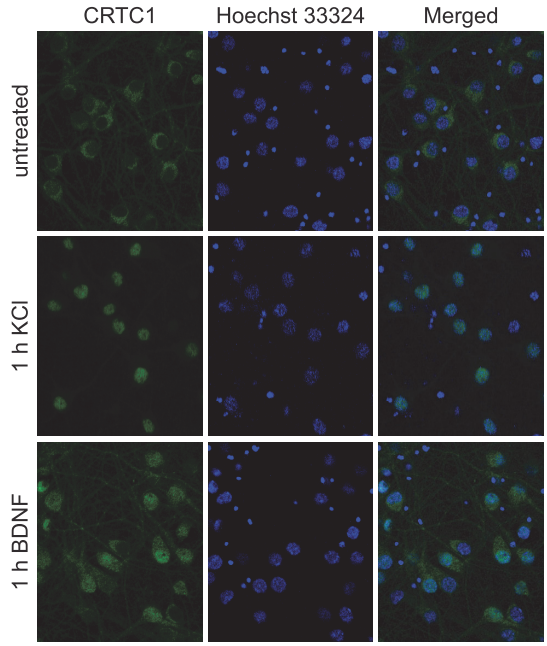

Figure 7. CRTC1 localizes to the nucleus after BDNF treatment. At 8 DIV, neurons were treated with 25 mM KCl, as a positive control, or 50 ng/ml BDNF. Immunocytochemistry was performed using anti-CRTC1 antibody (green). Hoechst 33324 was used to visualize nuclei (blue).

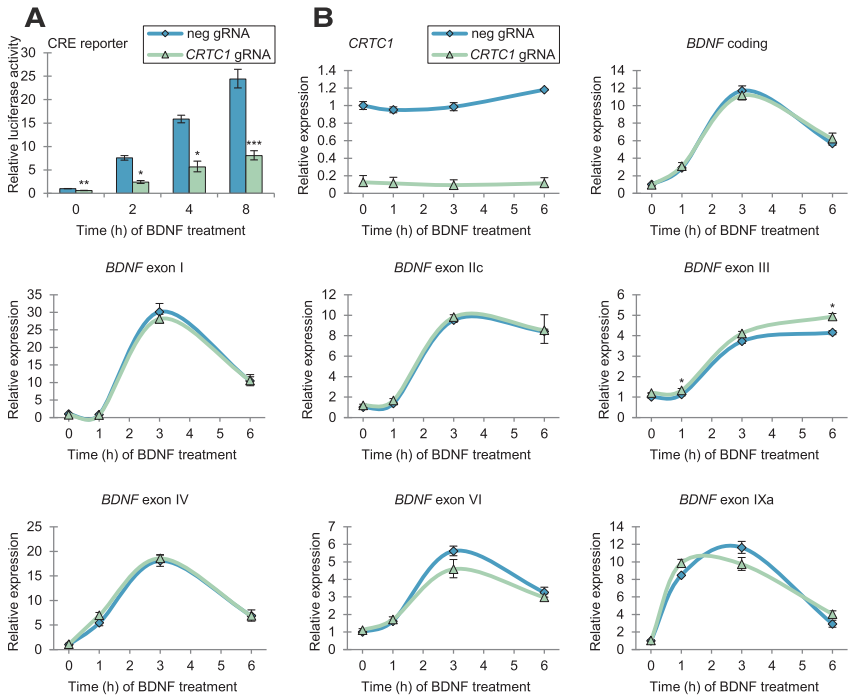

Figure 8. CREB coactivator CRTC1 does not participate in the induction of *BDNF* gene expression after BDNF-TrkB signaling. Primary neurons were transduced at 0 DIV with lentiviral particles coding for dCas9-KRAB, and a combination of two *CRTC1* gRNAs or negative gRNA (neg gRNA). ***A***, Transduced neurons were transfected with CRE-reporter at 6 DIV, and luciferase reporter activity was measured at 8 DIV after BDNF treatment ($n = 4$). Luciferase activity in cells transduced with dCas9-KRAB and negative gRNA-encoding lentiviral particles and not treated with BDNF was set as 1. ***B***, At 8 DIV, neurons were treated with 50 ng/ml BDNF for the indicated time periods, and the expression levels of *CRTC1*, total *BDNF* (*BDNF* coding), and different *BDNF* transcripts were measured using qRT-PCR. The expression level of respective transcripts in cells transduced with dCas9-KRAB and negative gRNA-encoding lentiviral particles and not treated with BDNF was set as 1. Average of three independent experiments ($n = 3$) is shown. Error bars indicate SEM. Statistical significance between the respective transcript levels in negative gRNA and *CRTC1* gRNA-overexpressing cells at respective time points: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

measure the expression levels of *BDNF* transcripts (Fig. 8). Using such CRISPR interference system, we succeeded in repressing *CRTC1* mRNA expression ~8-fold ($p = 0.1304$, uncorrected $p = 0.0413$) (Fig. 8*B*), which also resulted in ~2-fold to 3-fold decrease in the activation of CRE-reporter at different time points after BDNF-TrkB signaling (Fig. 8*A*). For example, *CRTC1* knockdown reduced CRE-reporter activation from 24-fold to 8-fold ($p = 0.0006$) at 8 h BDNF treatment. However, the reduction of *CRTC1* had no remarkable effect on the induction of any *BDNF* transcript after BDNF-TrkB signaling (Fig. 8*B*). Of note, no compensatory increase in the expression of *CRTC2* and *CRTC3* mRNA was detected in response to repressing the expression of *CRTC1* (data not shown).

Finally, we decided to take a wider approach in studying the role of CRTC family of CREB coactivators by overexpressing a dominant-negative protein for CRTC, which consists of the first 44 amino acids of CRTC1 protein. This dominant-negative CRTC binds to CREB and impairs the interaction of CREB with the members of CRTC family (used by Bittinger et al., 2004; Zhou et al., 2006; Kovács et al., 2007; Uchida et al., 2017). As a positive control, we first determined that overexpression of dominant-negative CRTC reduces BDNF-TrkB signaling-induced CRE-reporter activity ~2-fold at every measured time point, albeit statistically insignificantly (Fig. 9*A*). Meanwhile, overexpression of dominant-negative CRTC did not affect the basal expression nor BDNF-TrkB signaling-induced levels of any *BDNF* transcript (Fig. 9*B*). Together, our results show that CREB coactivators from the CRTC family do not participate in BDNF transcriptional autoregulation.

CRE element in rat *BDNF* promoter IV is required for the activation of the promoter after BDNF-TrkB signaling

Next, to determine the role of CREB family transcription factors in the activation of *BDNF* proximal promoter regions after BDNF-TrkB signaling, we used luciferase reporter assay. We transfected cultured rat neurons with rat *BDNF* (r*BDNF*) promoter I, II, III, IV, VI, and IXa constructs, or CRE reporter as a positive control, together with either EGFP or A-CREB overexpression vectors. In addition, we used a short version of r*BDNF* promoter I that contains only the 5′ transcription start site of the promoter I, designated here as r*BDNF* promoter I 5′, as it has been shown to be more inducible than the full-length promoter I after membrane depolarization (Pruunsild et al., 2011). Finally, we treated neurons with BDNF and measured promoter activity using luciferase assay (Fig. 10*A*).

First, we observed that overexpression of A-CREB reduced the basal activity of CRE reporter in unstimulated cells ~3.4-fold ($p = 0.0022$), and decreased the BDNF-TrkB signaling-induced activity of the CRE reporter by ~11-fold ($p = 0.0061$) and by ~15-fold ($p = 0.0114$) after 4 and 8 h of BDNF treatment, respectively (Fig. 10*A*), indicating the functionality of our reporter assay. Overexpression of A-CREB had no significant effect on the

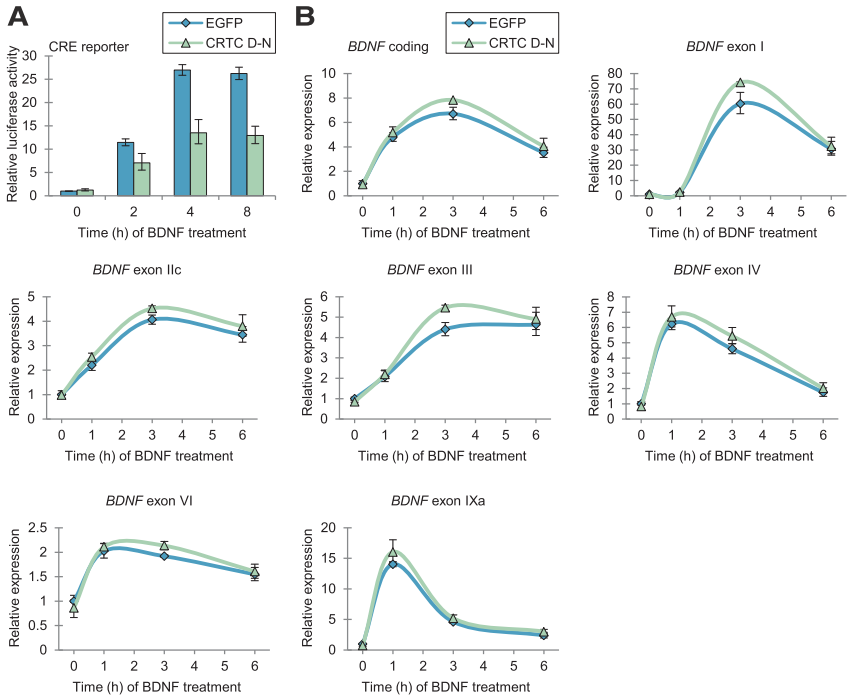

Figure 9. The CRTC family does not participate in the induction of *BDNF* transcripts after BDNF-TrkB signaling. Primary neurons were transduced at 0 DIV with lentiviral particles encoding EGFP, as control, or CRTC dominant-negative protein (CRTC D-N). ***A***, Transduced neurons were transfected with CRE-reporter at 6 DIV, and luciferase reporter activity was measured at 8 DIV after BDNF treatment for the time indicated ($n = 4$). Luciferase activity in cells transduced with EGFP-encoding lentiviral particles and not treated with BDNF was set as 1. ***B***, At 8 DIV, transduced neurons were treated with 50 ng/ml BDNF for the time indicated, and the levels of total *BDNF* (*BDNF* coding) and different *BDNF* transcripts were measured using qRT-PCR. The expression level of respective transcripts in cells expressing EGFP not treated with BDNF was set as 1. Average of 3 independent experiments ($n = 3$) is shown. Statistical significance between respective transcript levels in EGFP and CRTC D-N-expressing cells at respective time points: no statistically significant results (paired two-tailed t test, corrected for multiple comparisons using Holm–Sidak method). Error bars indicate SEM.

basal activity of r*BDNF* promoters I, I 5′, VI, and IXa. Notably, we saw a ~1.5-fold decrease ($p = 0.0366$) in the basal activity of r*BDNF* promoter IV in cells overexpressing A-CREB. Interestingly, A-CREB overexpression slightly increased the basal activity of r*BDNF* promoters II and III by ~1.3-fold ($p = 0.0304$) and ~1.1-fold ($p = 0.0348$), respectively.

Next, we determined that overexpression of A-CREB reduced the TrkB signaling-dependent activation of r*BDNF* promoters I, I 5′, and IV after 4 h of BDNF treatment from ~5.2-fold to ~4.0-fold ($p = 0.1937$), from ~7.4-fold to ~4.9-fold ($p = 0.0012$), and from ~2.4-fold to ~1.4-fold ($p = 0.0829$), respectively. Similar tendencies were observed after 8 h of BDNF treatment when overexpression of A-CREB reduced the BDNF-TrkB signaling-induced activity of r*BDNF* promoter I from ~6.6-fold to ~5.1-fold ($p = 0.2022$, uncorrected $p = 0.1068$), promoter I 5′ from ~12-fold to ~8.6-fold ($p = 0.0174$), and promoter IV from ~3.0-fold to ~1.7-fold ($p = 0.0303$). Overexpression of A-CREB enhanced the BDNF-induced activity of r*BDNF* promoter II and VI from ~2.6-fold to ~3.6-fold ($p = 0.0033$) and from ~1.4-fold to ~1.7-fold ($p = 0.0981$, uncorrected $p = 0.0473$), respectively, after 4 h, and from ~3.0-fold to ~4.3-fold ($p = 0.0030$) and from ~1.6-fold to ~2.0-fold ($p = 0.0981$, uncorrected $p = 0.0338$), respectively, after 8 h of BDNF-TrkB signaling. The TrkB signaling-induced activity of r*BDNF* promoter III was not changed in response to overexpressing A-CREB. Notably, the activity of r*BDNF* promoter IXa was not inducible in response to BDNF-TrkB signaling, and we could not detect any effect of A-CREB overexpression on the activity of this promoter region.

As it has been described that rat *BDNF* promoters I and IV have a functional CRE element (Shieh et al., 1998; Tao et al., 1998; Tabuchi et al., 2002), we next investigated the importance of these CRE elements by overexpressing r*BDNF* promoter constructs where the respective CRE element was mutated (Fig. 10*B*). Our results showed that mutating the CRE element slightly decreased the induction of r*BDNF* promoter I from ~7.4-fold to ~6.1-fold ($p = 0.1845$, uncorrected $p = 0.0657$) after 4 h, and from ~8.5-fold to ~7.1-fold ($p = 0.1331$, uncorrected $p = 0.0351$) after 8 h of BDNF treatment in cells expressing EGFP (Fig. 10*B*). In cells overexpressing A-CREB, the mutation in CRE element reduced the induction of r*BDNF* promoter I from ~5.5-fold to ~4.9-fold ($p = 0.3463$, uncorrected $p = 0.2550$) after 4 h, and from ~6.7-fold to ~5.4-fold ($p = 0.3463$, uncorrected $p = 0.1915$) after 8 h of BDNF treatment. A similar effect was observed for r*BDNF* promoter I 5′, in which case the mutation in CRE element slightly reduced the induction of the promoter activity. In cells expressing EGFP, r*BDNF* promoter IV lost all of its induction when CRE element was mutated, as promoter induction decreased from ~2.7-fold to ~1.2-fold ($p = 0.0317$) after 4 h, and from ~3.1-fold to ~0.84-fold ($p = 0.0016$) after 8 h of BDNF treatment. Similarly, in cells overexpressing A-CREB, the mutation in CRE site decreased the induction of r*BDNF* promoter IV from ~1.8-fold to ~1.4-fold ($p = 0.0317$) after 4 h, and

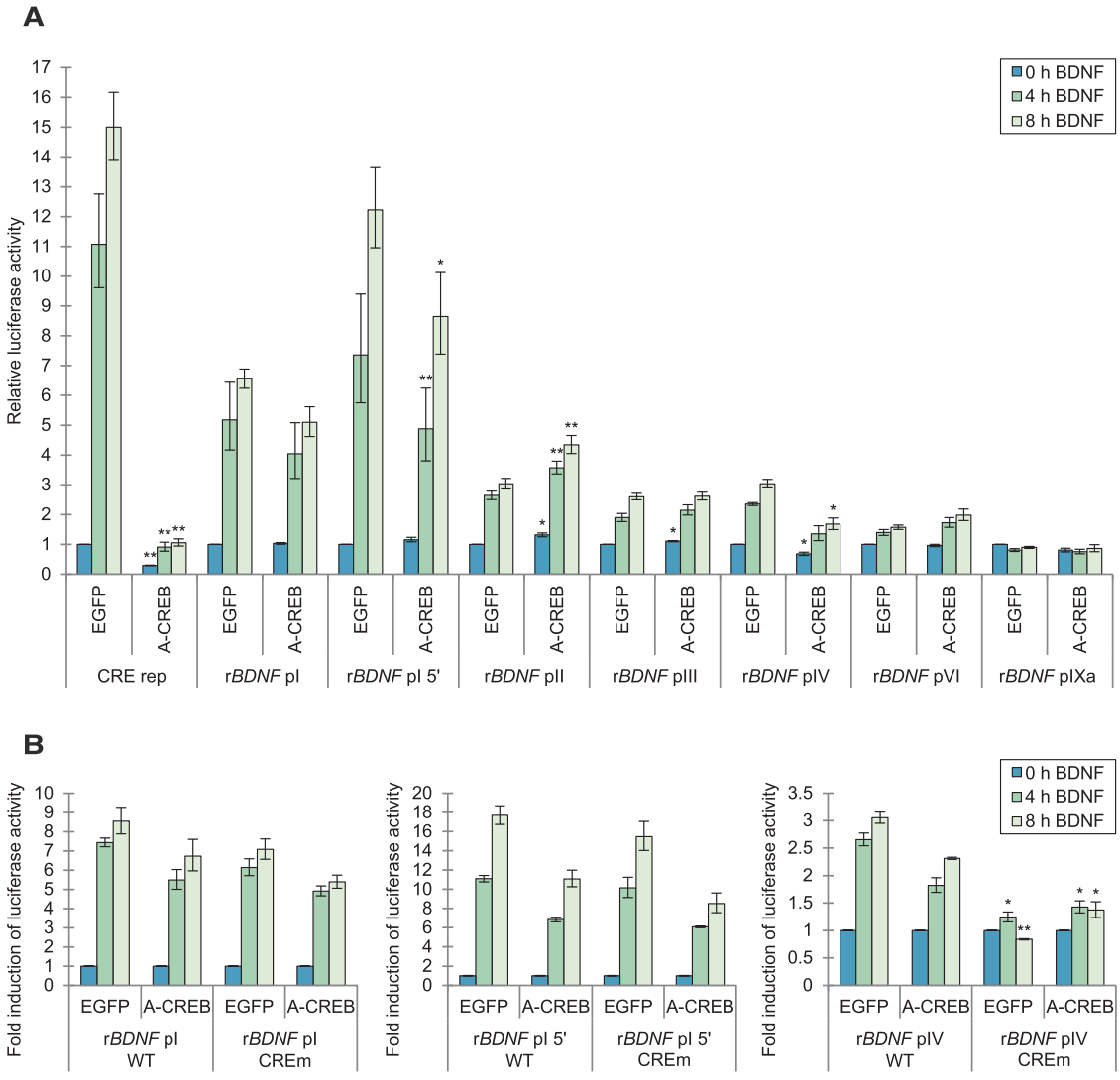

Figure 10. CREB family transcription factors are involved in the activation of rat *BDNF* promoters I and IV after BDNF-TrkB signaling. Primary neurons were transfected at 6 DIV with different rat *BDNF* promoter constructs or CRE reporter (CRE rep) together with EGFP or A-CREB overexpression constructs (***A***) or with wild-type (WT) rat *BDNF* promoter (r*BDNF* p) constructs and respective rat *BDNF* promoter constructs with mutated CRE site (CREm) together with EGFP or A-CREB overexpression constructs (***B***) as shown below the graph. At 7 DIV, neurons were treated with 50 ng/ml BDNF for 4 h ($n = 3$) or 8 h ($n = 4$) (***A***) and for 4 h ($n = 3$) or 8 h ($n = 3$) (***B***) or left untreated, and promoter activity was measured using luciferase reporter assay. Promoter activity was calculated relative to the respective promoter activity in cells transfected with EGFP and not treated with BDNF (***A***) or as fold induction relative to the respective promoter activity in untreated cells transfected with EGFP or A-CREB, respectively (***B***). Average of three or four independent experiments ($n = 3$ or 4) is shown. Error bars indicate SEM. Statistical significance between the activity of respective promoter regions in EGFP and A-CREB-overexpressing cells at respective time points (***A***) or between the induction of respective WT and CREm promoter regions in cells overexpressing EGFP or A-CREB, respectively, at respective time points (***B***). $^{**}p < 0.01$; $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

from ~2.3-fold to ~1.4-fold ($p = 0.0420$) after 8 h of BDNF treatment.

Collectively, our results show that CREB family proteins and the described CRE element in the *BDNF* promoter I region does not contribute significantly to the activation of the promoter after BDNF-TrkB signaling. In contrast, the induction of *BDNF* promoter IV activity after BDNF-TrkB signaling is mainly mediated by CREB family transcription factors through the CRE site in the promoter region.

TrkB signaling-dependent regulation of *BDNF* promoters I and IV, but not promoter IXa, by CREB family proteins is partially conserved between rat and human

Functional CRE elements, which participate in neuronal activity-dependent induction of the respective promoter activity, have been described in human *BDNF* promoters I, IV, and IXa (Pruunsild et al., 2011). We next decided to investigate whether the role of CREB family transcription factors in the induction of *BDNF* proximal promoter regions in response to BDNF-TrkB

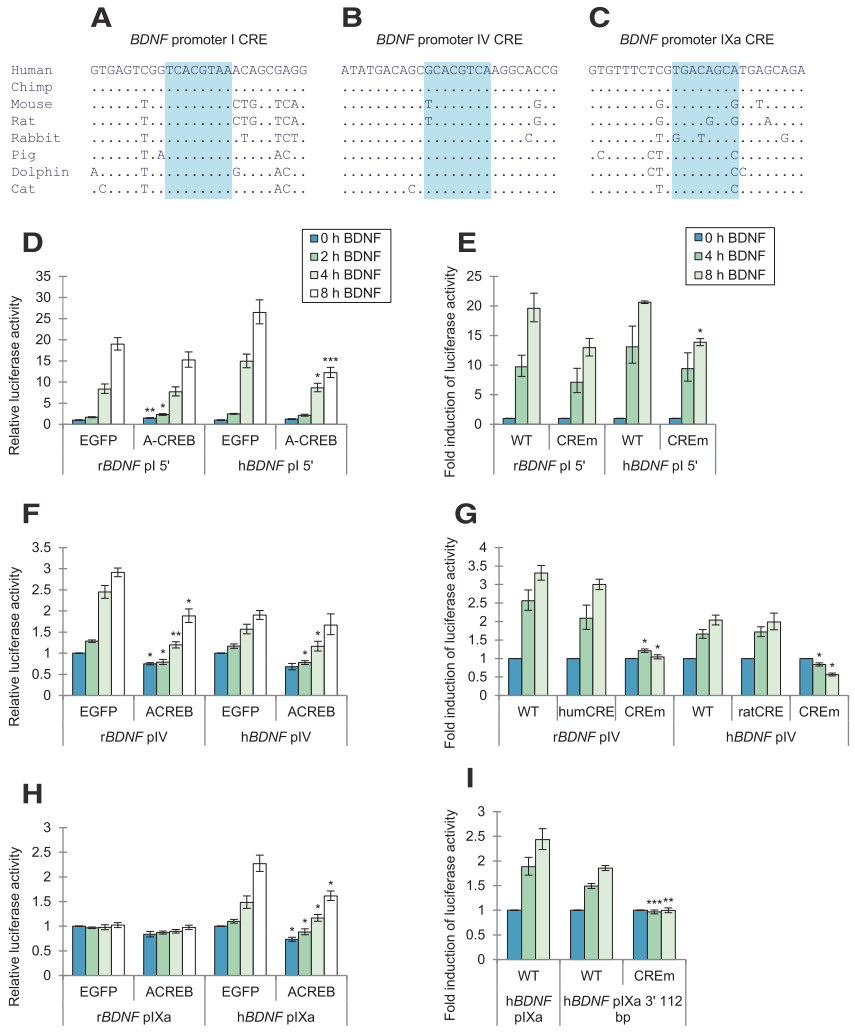

Figure 11. Regulation of *BDNF* promoters I and IV, but not promoter IXa, via CREB family after BDNF-TrkB signaling is partially conserved between rodents and humans. ***A–C***, Graphical representation of the conservation of previously described CRE elements in *BDNF* promoters I, IV, and IXa. Light blue shading represents the core sequence of the CRE element. ***D–I***, Primary neurons were transfected at 6 DIV with different rat and human *BDNF* promoter constructs (denoted as r*BDNF* and h*BDNF*, respectively) together with EGFP or A-CREB overexpression constructs (***D***, ***F***, ***H***) or with wild-type (WT) *BDNF* promoter construct or respective CRE-mutated promoter constructs (***E***, ***G***, ***I***) as shown below the graph. At 7 DIV, neurons were treated with 50 ng/ml BDNF for different time periods or left untreated as indicated, and promoter activity was measured using luciferase reporter assay. Promoter activity was calculated relative to the respective promoter activity in cells transfected with EGFP and not treated with BDNF (***D***, ***F***, ***H***) or as fold induction relative to the respective promoter activity in untreated cells (***E***, ***G***, ***I***). Average of three or four independent experiments (***D***, ***F***, ***H***: $n = 4$; ***E***, ***G***, ***I***: $n = 3$) is shown. Error bars indicate SEM. Statistical significance between the inductions of respective promoter constructs in EGFP and A-CREB-overexpressing cells at respective time points: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

signaling is similar between rat and human. For that, we used luciferase reporter assay with reporter constructs containing human or rat respective promoter regions (Fig. 11).

While the core CRE element in *BDNF* promoter I is conserved between rodents and human, its flanking region differs (Fig. 11*A*). Therefore, we first investigated the regulation of rat and human *BDNF* promoter I 5′ region in response to BDNF-TrkB signaling (Fig. 11*D*). Overexpression of A-CREB reduced the TrkB signaling-dependent activation of both rat and human *BDNF* promoter I 5′. Notably, the effect of A-CREB was more prominent on the activation of human *BDNF* promoter I 5′, in which case the activity of the promoter region decreased from ~15-fold to ~8.7-fold ($p = 0.0165$) after 4 h, and from ~26-fold to ~12-fold ($p = 0.0004$) after 8 h BDNF-TrkB signaling. In addition, we found that mutating the CRE site in the promoter I 5′ only slightly decreased the TrkB-induced levels of promoter activity of both human and rat *BDNF* promoter region (Fig. 11*E*). Together, our data show that the activation of human *BDNF* promoter I in response to BDNF-TrkB signaling is slightly more dependent on CREB family transcription factors than the rat ortholog, but not via CRE element located in the proximal promoter.

Next, we examined the regulation of *BDNF* promoter IV. The well-described CRE element in *BDNF* promoter IV has a murine

linage-specific single nucleotide difference between rat and human (GCACGTCA vs TCACGTCA; Fig. 11*B*), which could affect CREB family-dependent regulation of the promoter activity. Overexpression of A-CREB reduced the basal activity of both rat and human *BDNF* promoter IV ~1.3-fold ($p = 0.0100$) and ~1.5-fold ($p = 0.0793$), respectively (Fig. 11*F*). Interestingly, rat *BDNF* promoter IV was more inducible than the human ortholog, as both at 4 and 8 h of BDNF treatment the induction of rat *BDNF* promoter IV activity in EGFP-expressing cells was ~1.5-fold higher than its human counterpart. After BDNF-TrkB signaling, the overexpression of A-CREB greatly reduced the activity of rat *BDNF* promoter IV at all time points (Fig. 11*F*) similarly to the previous experiment (Fig. 10*A*). Likewise, although with a smaller effect, the activation of human *BDNF* promoter IV in response to BDNF-TrkB signaling was reduced from ~1.2-fold to ~0.78-fold ($p = 0.0103$) after 2 h, from ~1.6-fold to ~1.2-fold ($p = 0.0295$) after 4 h, and from ~1.9-fold to ~1.7-fold ($p = 0.3809$) after 8 h BDNF treatment by A-CREB overexpression.

To determine whether the difference in the activation of rat and human *BDNF* promoter IV results from the single-nucleotide difference in the CRE element, we made reporter constructs where we introduced human CRE element into rat *BDNF* promoter region and rat CRE element into human *BDNF* promoter region (Fig. 11*G*). We found that the single nucleotide variation in the CRE element did not significantly contribute to the difference in the inducibility of the promoter IV region. We also determined that mutating the CRE element in both rat and human *BDNF* promoter IV completely abolished the induction of the promoter in response to BDNF-TrkB signaling. Collectively, stronger activation of *BDNF* promoter IV in response to BDNF-TrkB signaling seems to be mediated by CREB family transcription factors, but the difference in the activation of rat and human respective promoters is not due to the single nucleotide difference in the CRE element.

In human *BDNF* promoter IXa, a functional CRE element responsible for neuronal activity-dependent induction of the promoter has been described (Pruunsild et al., 2011). However, there is a 2 nucleotide difference in the CRE element between rat and human (TGACGGCG vs TGACAGCA; Fig. 11*C*). We studied the induction of *BDNF* promoter IXa, and we found that while the rat promoter IXa region is not inducible in response to BDNF-TrkB signaling, the respective human promoter region is, with up to ~2.3-fold increase in the promoter activity after 8 h of BDNF treatment (Fig. 11*H*). Notably, overexpression of A-CREB decreased TrkB signaling-induced activity of the human *BDNF* promoter IXa by ~1.2-fold to 1.4-fold. We next localized the regulatory elements required for the induction of the human *BDNF* promoter IXa to the 112 bp 3′ region of the promoter, which showed similar activation in response to BDNF-TrkB signaling as the full-length 908 bp promoter region (Fig. 11*I*). Remarkably, mutating the CRE element completely abolished the activation of the promoter, indicating that the CRE element is the main mediator of TrkB signaling-dependent activation of human *BDNF* promoter IXa. Collectively, we found remarkable differences in the regulation of *BDNF* promoter IXa between rat and human, as the activity of the rat *BDNF* promoter IXa is not inducible in response to BDNF-TrkB signaling, whereas the activity of the human ortholog is through a primate lineage-specific CRE element.

CREB transcription factor mediates the early induction of exon IIc-, IV-, VI-, and IXa-containing *BDNF* transcripts after BDNF-TrkB signaling

Our results with overexpression of A-CREB and treatment with CCII indicated that CREB family is involved in the BDNF transcriptional autoregulation, but neither of the experiments was specific to CREB. Since the dimerization of A-CREB is based on CREB leucine zipper domain, it can form dimers with the transcription factors that also dimerize with CREB (e.g., possibly with all the members of the CREB family). Also, CBP/p300 can interact with several transcription factors via KIX domain. Therefore, we next wanted to determine the role of CREB in BDNF transcriptional autoregulation. We suppressed *CREB1* mRNA expression using CRISPR interference, treated neurons with BDNF at 8 DIV, and used qRT-PCR to measure the expression levels of *BDNF* transcripts (Fig. 12).

First, we determined the efficiency of *CREB1* knockdown in our cultured neurons (Fig. 12*A*). We found that dCas9-KRAB targeted to the *CREB1* promoter region reduced *CREB1* mRNA levels ~4.5-fold ($p = 0.00005$) (Fig. 12*A*), which also reflected in significant reduction of CREB protein level (data not shown). Next, we studied the influence of *CREB* knockdown on BDNF transcriptional autoregulation. Interestingly, suppressing *CREB1* expression abolished the early induction of total *BDNF* expression levels, as after 1 h of BDNF-TrkB signaling, the induced levels of total *BDNF* mRNA reduced from ~2.9-fold to ~1.2-fold ($p = 0.0001$) in *CREB1* knockdown cells. After 3 h of BDNF treatment, the induction of total *BDNF* mRNA decreased from ~11-fold to ~9-fold ($p = 0.0003$), but no difference in the induced levels was observed after 6 h of BDNF treatment. The induced levels of *BDNF* transcripts containing exons I and III were not significantly affected by the knockdown of *CREB1*. In contrast, CREB knockdown reduced the induced levels of BDNF transcripts containing exon IV and IXa from ~5.5-fold to ~1.5-fold ($p = 0.0002$) and from ~8-fold to ~3.6-fold ($p = 0.0035$), respectively, after 1 h of BDNF treatment. Also, *CREB1* knockdown reduced the induced levels of *BDNF* transcripts containing exon IV from ~13-fold to ~7.7-fold ($p = 0.00008$) after 3 h of BDNF treatment. Furthermore, *CREB1* knockdown also slightly decreased the induced levels of exon IIc- and VI-containing *BDNF* transcripts after 1 h of BDNF treatment: from ~1.4-fold to ~1.0-fold ($p = 0.0007$) and from ~1.8-fold to ~1.4-fold ($p = 0.0013$), respectively. Interestingly, we also observed a slight ~1.3-fold upregulation of the basal expression level of both exon III- and exon VI-containing *BDNF* transcripts ($p = 0.0256$ and $p = 0.0327$, respectively). Together, our results show that CREB transcription factor is required for the early induction of exon IIc-, IV-, VI-, and IXa-containing *BDNF* transcripts, but only for the later induction of exon IV-containing *BDNF* transcripts after BDNF-TrkB signaling.

In contrast to the specific effect of *CREB1* knockdown on *BDNF* exon IV-containing transcripts and only on the early induction of exon IIc, VI-, and IXa-containing transcripts, our results obtained with overexpression of A-CREB (Figs. 2, 3), and using CCII and E1A overexpression (Figs. 5, 6) indicated the involvement of CREB family in the regulation of most *BDNF* transcripts at 3 h BDNF treatment. Notably, it has been shown that the transcription factors within CREB family can compensate for each other (Hummler et al., 1994; Mantamadiotis et al., 2002; Díaz-Ruiz et al., 2008; Lemberger et al., 2008; Aguado et al., 2009; Gundersen et al., 2013), so it is possible that other CREB family members could have rescued the *BDNF* induction in *CREB1* knockdown neurons. Therefore, to investigate how

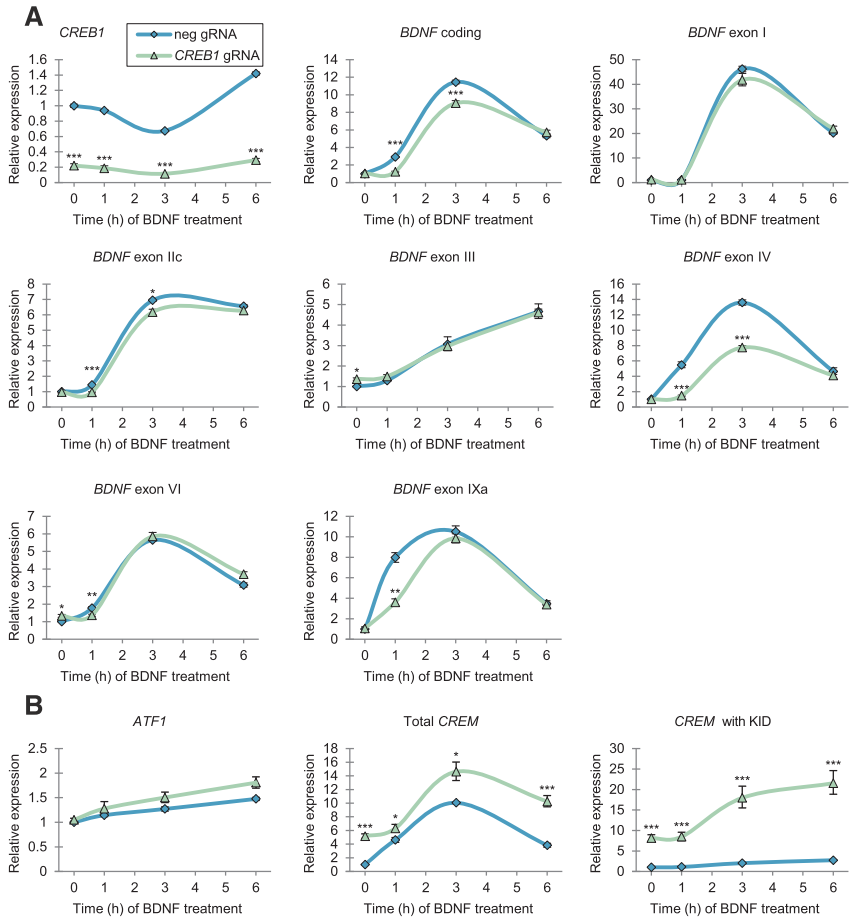

Figure 12. CREB is necessary for the early induction of exon IIc, IV-, VI-, and IXa-containing *BDNF* transcripts after BDNF-TrkB signaling. Primary neurons were transduced at 0 DIV with lentiviral particles coding for dCas9-KRAB, and *CREB1* gRNA or negative gRNA (neg gRNA). At 8 DIV, neurons were treated with 50 ng/ml BDNF for the indicated time periods. Expression levels of *CREB1*, total *BDNF* (*BDNF* coding), different *BDNF* transcripts (***A***) and other *CREB* family members (***B***) were measured using qRT-PCR. The expression level of respective transcripts in cells transduced with dCas9-KRAB and negative gRNA-encoding lentiviral particles and not treated with BDNF was set as 1. Average of six independent experiments ($n = 6$) is shown. Error bars indicate SEM. Statistical significance between the respective transcript levels in cells overexpressing negative gRNA and *CREB1* gRNA at respective time points: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

BDNF-TrkB signaling and suppressing *CREB1* levels influence the expression of *ATF1* and *CREM*, we measured their mRNA levels using qRT-PCR (Fig. 12*B*).

We found that *ATF1* was slightly induced in response to BDNF-TrkB signaling with maximal induction of ~1.5-fold after 6 h of BDNF treatment (Fig. 12*B*). Total *CREM* mRNA levels peaked at ~10-fold induction after 3 h of BDNF-TrkB signaling (Fig. 12*B*). In response to *CREB1* knockdown, the expression of *ATF1* mRNA did not change remarkably. However, the basal level of total *CREM* mRNA was ~5.2-fold ($p = 2 \times 10^{-6}$) higher in *CREB* knockdown neurons. Due to alternative splicing, the *CREM* gene produces several CREM protein isoforms, which can be transcriptional activators or repressors (Foulkes et al., 1991; Laoide et al., 1993). For example, ICER is a repressive CREM isoform that competes with CREB and provides a mechanism for downregulation of cAMP-dependent gene expression (Molina et al., 1993; Walker et al., 1998; Mioduszewska et al., 2003). To measure the levels of CREM activator forms, we used primers specific for *CREM* transcripts that contain KID domain-encoding sequence (denoted as *CREM* with KID in Fig. 12*B*) and observed moderate induction in response to BDNF-TrkB signaling, peaking at ~2.7-fold induction after 6 h of BDNF treatment. We also saw ~8.1-fold ($p = 0.00002$) increase in the *CREM* activator form mRNA levels in untreated *CREB* knockdown cells (Fig. 12*B*).

As *CREB1* knockdown greatly induced the expression of CREM activator forms, it is possible that the discrepancy between the results obtained with *CREB1* knockdown and with A-CREB and E1A overexpression and CCII treatment could originate from CREM activator forms binding to CRE sites and thus regulating CRE-dependent transcription in cells where *CREB1* is knocked down. Therefore, we next decided to elucidate the role of CREM in BDNF transcriptional autoregulation. For that, we knocked down the expression of *CREM* using CRISPR interference (Fig. 13). We determined that *CREM* mRNA expression level was reduced ~1.8-fold ($p = 0.0048$) and that the expression

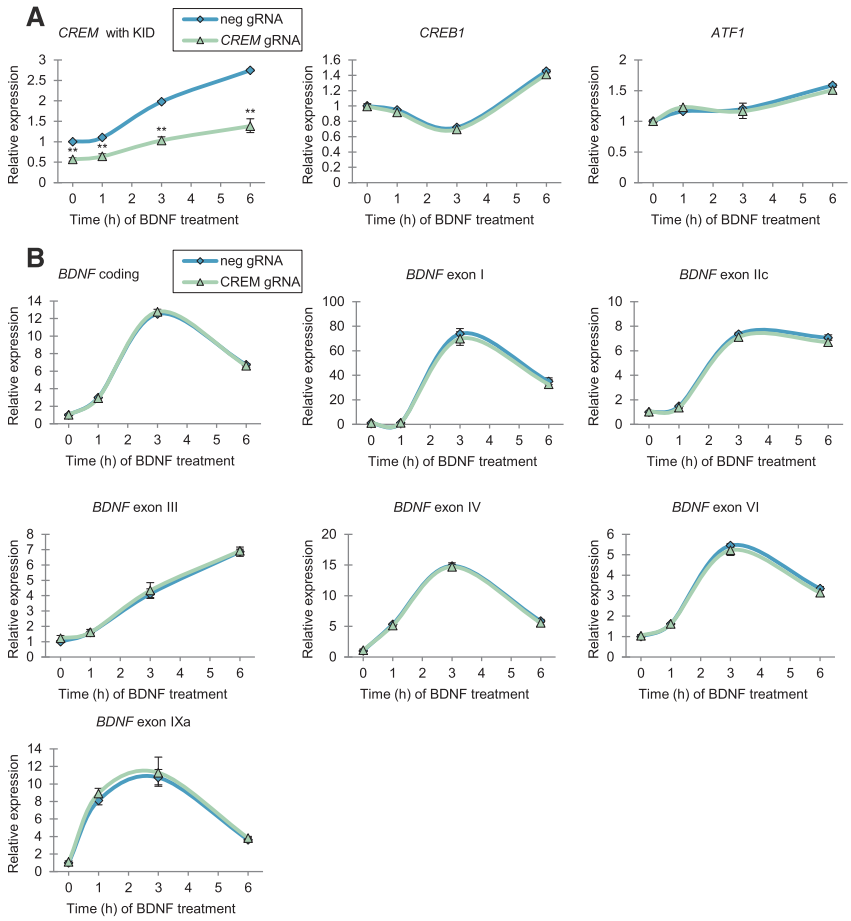

Figure 13. CREM is not involved in the induction of *BDNF* transcripts after BDNF-TrkB signaling. Primary neurons were transduced at 0 DIV with lentiviral particles coding for dCas9-KRAB and a combination of two *CREM* gRNAs or negative gRNA (neg gRNA). At 8 DIV, neurons were treated with 50 ng/ml BDNF for the indicated time periods. Expression levels of *CREM* with KID domain, *CREB1* and *ATF1* (***A***), and total *BDNF* (*BDNF* coding) and different *BDNF* transcripts (***B***) were measured using qRT-PCR. The expression level of respective transcripts in cells transduced with dCas9-KRAB and negative gRNA-encoding lentiviral particles and not treated with BDNF was set as 1. Average of five independent experiments ($n = 5$) is shown. Error bars indicate SEM. Statistical significance between the respective transcript levels in cells overexpressing negative gRNA and *CREM* gRNAs at respective time points: $^{**}p < 0.01$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

level of *CREB1* and *ATF1* did not change in response to *CREM* knockdown (Fig. 13*A*). Our data also showed that the induction of *BDNF* transcripts was not affected by *CREM* knockdown (Fig. 13*B*).

Finally, we investigated possible compensatory mechanisms between CREB and CREM by suppressing the expression of both *CREB1* and *CREM* simultaneously. If CREM really compensated for CREB in BDNF autoregulation after *CREB1* knockdown, one should observe a stronger effect on BDNF autoregulation in cells where both *CREB1* and *CREM* are knocked down. In contrast, we observed similar results as was seen with only *CREB1* knockdown; and no additional effect of *CREM* knockdown on *BDNF* gene expression after BDNF-TrkB signaling was detected (data not shown), indicating that CREM does not have compensatory effect on BDNF autoregulation in *CREB1* knockdown cells. Together, our data show that, while CREM does not participate in BDNF transcriptional autoregulation, CREB is indispensable for the early induction of *BDNF* exon IIc, IV-, VI-, and IXa-containing transcripts after BDNF-TrkB signaling.

Phosphorylated CREB binds to *BDNF* promoter IV after BDNF-TrkB signaling and recruits CBP

To determine whether CREB directly binds rat *BDNF* promoters in our cultured neurons and whether CREB binding changes after BDNF-TrkB signaling, we treated neurons with BDNF for 1 h and performed ChIP analysis with CREB and P-CREB antibodies. We then determined promoter enrichment using qPCR (Fig. 14).

As a positive control for CREB binding, we used *c-Fos* promoter, which showed ~0.31% ($p = 0.0130$) and ~0.16% ($p = 0.0172$) of input enrichment in untreated and BDNF-treated cells, respectively (Fig. 11*A*). Rat *BDNF* promoters I, II, III, and IXa showed no remarkable CREB binding compared with the negative control rat *BDNF* 3′ untranslated region. CREB binding to rat *BDNF* promoter IV showed 0.025% ($p = 0.2223$, uncorrected $p = 0.0410$) of input enrichment in nontreated cells, and 0.022% ($p = 0.2467$, uncorrected $p = 0.0461$) of input enrichment in BDNF-treated cells. Slight 0.019% ($p = 0.4438$, uncorrected $p = 0.1364$) and 0.015% of input enrichment ($p = 0.3647$, uncorrected $p = 0.2007$) was also detected for the rat *BDNF*

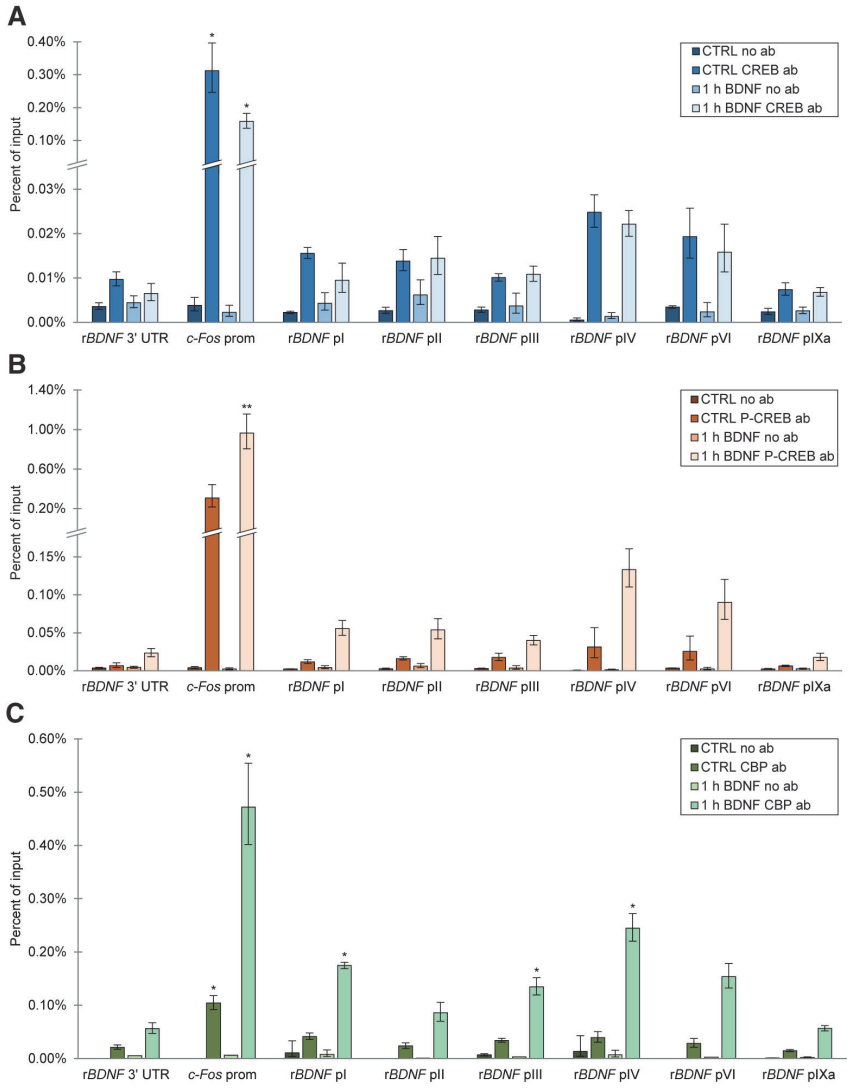

Figure 14. CREB binds to *BDNF* promoter IV, is phosphorylated after BDNF-TrkB signaling, and recruits CBP. Primary neurons were treated at 7 DIV with 50 ng/ml BDNF for 1 h or left untreated (CTRL) and then subjected to ChIP assay using CREB (***A***), phospho-Ser-133 CREB (P-CREB) (***B***), or CBP (***C***) antibodies (ab). DNA enrichment of *c-Fos* promoter, different rat *BDNF* (r*BDNF*) promoter (p) regions, and rat *BDNF* 3′ untranslated region (r*BDNF* 3′ UTR) was measured using qPCR. DNA enrichment is represented as percentage of input. Average of three or four (***A***, ***C***, $n = 4$; ***B***, $n = 3$) independent experiments is shown. Error bars indicate SEM. No antibody controls depict the same dataset in ***A*** and ***B***. Statistical significance is relative to the enrichment of r*BDNF* 3′ UTR region in respectively treated cells. Statistical analysis was not performed on no antibody controls. $^{**}p < 0.01$; $^{*}p < 0.05$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

promoter VI in untreated cells and after BDNF treatment, respectively.

Since we already established that CREB is phosphorylated after TrkB signaling (Fig. 4) we also investigated the binding of phospho-Ser-133 CREB using ChIP analysis (Fig. 14*B*). In untreated and BDNF-treated neurons, we observed ~0.31% ($p = 0.2553$) and ~0.97% ($p = 0.0063$) of input enrichment, respectively, at *c-Fos* promoter region for phospho-CREB. No remarkable binding of phospho-CREB was detected to rat *BDNF* promoters in nontreated cells. However, in response to BDNF-TrkB signaling, phospho-CREB binding increased ~3-fold to ~4-fold at every region measured, including at the negative control region. As we did not detect CREB binding to rat *BDNF* promoters I, II, III, and IXa, it is plausible that the observed increase in phospho-CREB binding to these promoters in BDNF-treated cells was an artifact due to overall higher phospho-CREB protein levels in neurons, and not a functional recruitment of phospho-CREB. In contrast, in BDNF-treated neurons, phospho-CREB binding showed notable 0.13% ($p = 0.1333$, uncorrected $p = 0.0339$) and 0.09% ($p = 0.1392$, uncorrected $p = 0.0787$) of input enrichment at rat *BDNF* promoters IV and VI, respectively. Together, our results indicate that CREB binds to *BDNF* pro-

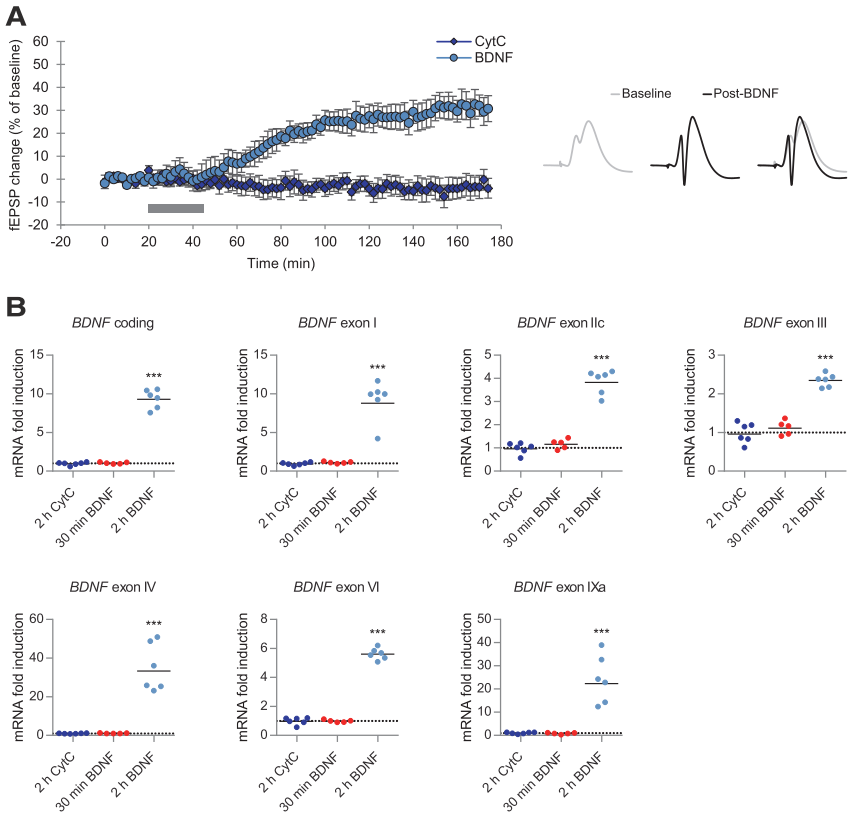

Figure 15. BDNF infusion into rat hippocampus elicits enhanced synaptic transmission and induces the expression of all *BDNF* transcripts in the hippocampal dentate gyrus region. ***A***, Time course plots of fEPSP slope recorded before and after BDNF or CytC infusion (left) and sample field potential traces (right). A total of 2 μl (1 μg/μl) of BDNF or CytC was infused over 25 min into the dentate gyrus over the period indicated by the gray bar. The evoked fEPSP slope change is expressed in percentage of baseline. Depicted values are mean ± SEM of 6 independent animals (n = 6 for both groups). ***B***, The expression levels of different *BDNF* transcripts was measured from dentate gyri of both ipsilateral and contralateral hemisphere using qRT-PCR. The expression levels of *BDNF* transcripts were normalized to the *HPRT1* expression levels and are depicted as fold induction compared with the respective transcript expression levels in the contralateral hemisphere. Dots represent data from all the experiments. Horizontal line indicates the geometric mean of 5 or 6 independent animals ($n = 5$ or 6). Dashed line indicates the level of respective transcripts in the contralateral side and was set as 1 (no induction). Statistical significance between the respective transcript levels in dentate gyri of ipsilateral and contralateral hemispheres: $^{***}p < 0.001$ (paired two-tailed *t* test, corrected for multiple comparisons using Holm–Sidak method).

moter IV and the binding of phosphorylated CREB increases after BDNF-TrkB signaling.

To clarify whether the requirement for CBP/p300 (Figs. 5, 6) is because of direct binding of CBP to *BDNF* proximal promoters, we also performed CBP ChIP with an antibody that does not cross-react with p300 and measured CBP binding to *BDNF* promoters with qPCR (Fig. 14*C*). We determined that CBP enrichment at *c-Fos* promoter was ~0.10% ($p = 0.0354$) of input in untreated neurons and ~0.47% ($p = 0.0377$) of input in BDNF-treated neurons. In untreated cells, CBP was not remarkably enriched at *BDNF* promoter regions compared with the enrichment at the negative control region *BDNF* 3′ UTR. However, after BDNF treatment we saw CBP binding to *BDNF* promoters I, III, and IV, and possibly to promoter VI, showing ~0.18% ($p = 0.0377$), ~0.14% ($p = 0.0377$), ~0.25% ($p = 0.0343$), and ~0.15% ($p = 0.0587$, uncorrected $p = 0.0199$) of input enrichment, respectively. Since we did not detect such extensive binding of P-CREB to *BDNF* promoters I and III (and possibly VI), it is plausible that CBP is recruited to these *BDNF* promoters via transcription factors other than CREB. Collectively, our results show that CREB binds to *BDNF* promoter IV, is phosphorylated after BDNF-TrkB signaling, and recruits CBP to increase *BDNF* expression.

All *BDNF* transcripts are induced in the adult rat hippocampus *in vivo* after BDNF-TrkB signaling

It has previously been shown that brief infusion of BDNF protein into adult rat hippocampus results in transcription-dependent LTP (Messaoudi et al., 2002), which requires MAPK pathway activation leading to CREB phosphorylation (Ying et al., 2002). Furthermore, infusion of BDNF into rat hippocampus induces the expression of *BDNF* exon IV-containing transcripts (Wibrand et al., 2006), whereas the expression of other *BDNF* transcripts has not been studied. To determine whether the BDNF transcriptional autoregulation pattern we have described here in cultured neurons is also present *in vivo*, we infused BDNF protein into adult rat hippocampus to elicit BDNF-LTP and measured the expression levels of different *BDNF* transcripts in the dentate gyrus region of the hippocampus using qRT-PCR (Fig. 15).

Local infusion of BDNF but not CytC into dentate gyrus elicited an ~30% increase in the fEPSP slope (Fig. 15*A*), indicating the functionality of our BDNF-induced LTP paradigm. In rats

infused with CytC, we did not detect induction of any *BDNF* transcripts 2 h after the infusion (Fig. 15). Likewise, 30 min after BDNF infusion, the expression levels of different *BDNF* transcripts were similar to the expression levels in the contralateral hemisphere. Notably, 2 h after the infusion of BDNF protein, the expression of all the *BDNF* transcripts was induced. Total *BDNF* mRNA level increased ~9.3-fold ($p = 5 \times 10^{-7}$), and the strongest inductions were seen for *BDNF* exon IV- and IXa-containing transcripts, which were induced ~33.3-fold ($p = 6{\cdot}10^{-6}$) and ~22.4-fold ($p = 4 \times 10^{-5}$), respectively, after 2 h of BDNF infusion. Moderate induction of ~8.8-fold ($p = 9 \times 10^{-5}$) and ~5.6-fold ($p = 7 \times 10^{-8}$) was seen for *BDNF* exon I- and VI-containing transcripts, respectively, and exon IIc- and III-containing *BDNF* transcripts showed slight but statistically significant ~3.8-fold ($p = 9 \times 10^{-6}$) and ~2.3-fold ($p = 3 \times 10^{-6}$) induction, respectively. Collectively, our results indicate that extensive BDNF transcriptional autoregulatory loop also occurs *in vivo*, encompassing all the major *BDNF* transcripts.

Discussion

The early induction of total *BDNF* mRNA levels in response to neuronal activity has been shown to be independent of protein synthesis (Hughes et al., 1993; Castrén et al., 1998). Additionally, the neuronal activity-dependent induction of *BDNF* exon IV-containing transcripts has been shown to be protein synthesis-independent (Lauterborn et al., 1996; Tao et al., 1998), while the induction of *BDNF* transcripts containing exon I and II depends on protein synthesis (Lauterborn et al., 1996). Here, we have determined the requirement for protein synthesis in BDNF transcriptional autoregulation. Our results show that the induction of most *BDNF* transcripts upon BDNF-TrkB signaling depends on protein synthesis, and only the early induction of *BDNF* exon IIc-, IV-, and VI-containing transcripts is partially protein synthesis-independent. The *de novo* protein synthesis-independent early induction of these *BDNF* transcripts could be explained by their regulation via CREB family proteins that are constitutively expressed and whose activity is regulated through post-translational mechanisms (for review, see Johannessen et al., 2004). Notably, the requirement for protein synthesis suggests that, in addition to constitutively expressed proteins, inducible transcription factors are necessary for the BDNF transcriptional autoregulation. For example, we have previously shown that AP-1 transcription factors regulate *BDNF* gene expression after TrkB signaling (Tuvikene et al., 2016).

It is well known that CREB transcription factor family regulates the transcription of *BDNF*, especially the activation of *BDNF* promoter IV (Shieh et al., 1998; Tao et al., 1998; Cha-Molstad et al., 2004; Hong et al., 2008; Pruunsild et al., 2011; West et al., 2014), but also *BDNF* promoter I (Tabuchi et al., 2002; Benito et al., 2011) in response to neuronal activity. Here, we have discovered that the CREB family is also a major regulator of *BDNF* gene expression after TrkB signaling. We show that USF and NFAT transcription factor families, which have previously been shown to be involved in neuronal activity-regulated *BDNF* gene expression (Chen et al., 2003; Vashishta et al., 2009; Pruunsild et al., 2011), do not significantly contribute to *BDNF* gene induction after TrkB signaling. This implies that the mechanisms of *BDNF* gene expression triggered by BDNF-TrkB signaling and neuronal activity differ. Notably, none of the dominant-negative proteins we used in our experiments completely abolished the induction of *BDNF* transcription after TrkB signaling, indicating that there are other, yet to be determined transcription factors involved in the BDNF autoregulation. Considering the profound effect of A-CREB overexpression on the expression of all major *BDNF* transcripts, one could hypothesize that important *de novo* synthesized transcription factors regulating *BDNF* gene expression are indeed CREB target genes. It is also plausible that CREB acts as a nucleating factor for other, *de novo* synthesized transcription factors to bind the *BDNF* promoters, as it has been proposed for *BDNF* promoter IV (Hong et al., 2008).

It is important to note the possible TrkB signaling-dependent component in the regulation of *BDNF* gene expression in paradigms using membrane depolarization. It has been described that neuronal activity induces BDNF secretion (for review, see Park and Poo, 2013), which could consequently activate TrkB receptors, possibly giving rise to the BDNF-TrkB autoregulatory loop. In the light of our results, it is plausible that neuronal activity induces *BDNF* expression and the subsequent BDNF-TrkB signaling maintains the expression of *BDNF* via an autonomous pathway. For example, the impairment of *BDNF* induction after kainic acid infusion in TrkB.T1 overexpressing mice implies that BDNF-TrkB signaling is required for proper *BDNF* expression in the brain after neuronal activity (Saarelainen et al., 2001).

It has been reported that CREB-mediated regulation of gene expression plays an important role in the proper development of the nervous system and neurons (Lonze and Ginty, 2002). Therefore, the effect of overexpressing dominan-negative CREB family protein, A-CREB, on the development and maturation of neurons in culture is an important question that should be kept in mind when interpreting our results. Results of our experiments with long-term overexpression of A-CREB are in good accordance with the results of our experiments with acute treatment of CREB-CBP interaction inhibitor (CCII), supporting the notion that CREB family regulates BDNF transcriptional autoregulation independent of potential developmental changes. Our results also showed the requirement for CREB family and its interaction with CBP/p300 for the early induction of *BDNF* gene after TrkB signaling. Notably, at a later phase, after 6 h of BDNF treatment, neither A-CREB nor CCII had an influence on *BDNF* gene induction. Furthermore, knockdown of *CREB1* expression also affected only the early induction of *BDNF* exon IIc-, IV-, VI-, and IXa-containing transcripts. This indicates that, while the early phase of TrkB signaling-dependent induction of *BDNF* in neurons is regulated by CREB-CBP/p300 complex, the late phase of induction is regulated in a CREB family-independent manner. In contrast, inhibition of CBP/p300 by overexpressing E1A strongly decreased both the early and late phase of *BDNF* gene induction after TrkB signaling. This could be explained by the fact that numerous other transcription factors in addition to CREB family proteins recruit CBP/p300 to regulate gene expression (Bedford et al., 2010). Furthermore, while we only noted CREB binding to *BDNF* promoter IV and possibly VI, we saw CBP binding to various *BDNF* promoters: promoter I, III, IV, and possibly VI. This further suggests that, while CREB recruits CBP to *BDNF* promoter IV and possibly to promoter VI, other transcription factors recruit CBP to other *BDNF* promoters.

The CREB family has been associated with the regulation of *BDNF* in both rodents and humans. Here, we found that, although the general activation of *BDNF* promoters I and IV in response to BDNF-TrkB signaling was conserved between human and rat, subtle differences existed in their regulation by CREB family proteins. The activation of human *BDNF* promoter I was slightly more dependent on CREB family transcription factors than the rat ortholog. Furthermore, we found that the human *BDNF* promoter IV region was less inducible than the

respective rat counterpart, and our results indicate potential CREB family-dependent differences in the regulation of this promoter between rat and human. It is notable that the murine-specific single nucleotide difference, which is absent in other major mammalian clades in *BDNF* promoter IV CRE element, does not remarkably change the activity of the promoter in cultured rat neurons. On the contrary, the two-nucleotide difference in the CRE element between human and rat *BDNF* promoter IXa seems to be responsible for the BDNF-TrkB signaling-dependent activation of human promoter, which is absent for the rat ortholog. As we have found a profound difference in the regulation of *BDNF* promoter IXa region in rat and human, the regulation of the human *BDNF* promoter IXa after BDNF-TrkB signaling in the endogenous context will need to be clarified in future research.

Importantly, our data suggest the existence of distal enhancer elements for *BDNF* gene regulation after TrkB signaling. First, overexpression of A-CREB did not decrease the induction from *BDNF* proximal promoters II, III, and VI in promoter assays, whereas overexpression of A-CREB reduced the induction of all these *BDNF* transcripts in the endogenous context. Second, the expression of rat endogenous *BDNF* exon IXa-containing transcripts is induced rapidly in response to BDNF-TrkB signaling, whereas the *BDNF* proximal promoter IXa was not inducible in our promoter activity assays. Third, functional CRE elements in rodents have only been described in *BDNF* promoters I and IV, and we also discovered that the CRE element in *BDNF* promoter IV was necessary for the activation of the proximal promoter region. On the contrary, our results using overexpression of A-CREB, treatment with CCII, and overexpression of E1A showed a strong effect on the induction of all the investigated *BDNF* transcripts. Moreover, CBP has been widely associated with enhancer regions (Kim et al., 2010). Notably, it has been reported that the binding of CREB coactivator CRTC1 is preferential to promoters containing a TATA box (Parra-Damas et al., 2017) for the interaction with general transcription factors (Conkright et al., 2003). As we found in our work that neither CRTC1 nor other CRTC family coactivators are required, while CBP is necessary for the BDNF transcriptional autoregulation, it could imply that *BDNF* gene expression is regulated by a distal enhancer region, not only by the proximal promoter regions. Together, these contradictions imply that important regulatory areas responsible for BDNF-TrkB signaling-dependent *BDNF* induction are located outside of the proximal promoter regions, possibly even outside the *BDNF* gene. As we noted an effect on all measured *BDNF* transcripts with A-CREB, CCII, and E1A, it is plausible that there is a locus control region affecting the expression of the whole *BDNF* gene, and the *cis*-elements in proximal promoter regions provide fine-tuning of the expression of different *BDNF* transcripts in response to TrkB signaling.

Dysregulation of *BDNF* expression in a brain region and cell type-specific manner has been implicated in various neurological and neurodevelopmental disorders and in various neurodegenerative diseases (Zuccato and Cattaneo, 2009; Autry and Monteggia, 2012; Tejeda and Díaz-Guerra, 2017). Although modulating BDNF levels could be successful in therapy, the challenge lies in controlling *BDNF* expression in a precise spatiotemporal manner. It has been shown that uniform overexpression of BDNF in the excitatory neurons of the forebrain, including hippocampus and amygdala, causes anxiety-like behavior (Govindarajan et al., 2006). Additionally, increased levels of BDNF protein in PFC and hippocampus impairs working memory and fear conditioning, increases susceptibility to seizures, and also causes an anxiety-like phenotype (Papaleo et al., 2011). This has raised the need to ultimately enhance the transcription of *BDNF* specifically in cells that express *BDNF* under physiological conditions, to minimize serious side effects. The knowledge of how *BDNF* promoters are regulated in response to BDNF-TrkB signaling added by this study could provide solutions to the challenges of precisely adjusting BDNF levels in both health and disease.

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Publication II

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Differential regulation of the BDNF gene in cortical and hippocampal neurons

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Cellular/Molecular

Differential Regulation of the BDNF Gene in Cortical and Hippocampal Neurons

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Brain-derived neurotrophic factor (BDNF) is a widely expressed neurotrophin that supports the survival, differentiation, and signaling of various neuronal populations. Although it has been well described that expression of BDNF is strongly regulated by neuronal activity, little is known whether regulation of BDNF expression is similar in different brain regions. Here, we focused on this fundamental question using neuronal populations obtained from rat cerebral cortices and hippocampi of both sexes. First, we thoroughly characterized the role of the best-described regulators of BDNF gene - cAMP response element binding protein (CREB) family transcription factors, and show that activity-dependent BDNF expression depends more on CREB and the coactivators CREB binding protein (CBP) and CREB-regulated transcriptional coactivator 1 (CRTC1) in cortical than in hippocampal neurons. Our data also reveal an important role of CREB in the early induction of BDNF mRNA expression after neuronal activity and only modest contribution after prolonged neuronal activity. We further corroborated our findings at BDNF protein level. To determine the transcription factors regulating BDNF expression in these rat brain regions in addition to CREB family, we used *in vitro* DNA pulldown assay coupled with mass spectrometry, chromatin immunoprecipitation (ChIP), and bioinformatics, and propose a number of neurodevelopmentally important transcription factors, such as FOXP1, SATB2, RAI1, BCL11A, and TCF4 as brain region-specific regulators of BDNF expression. Together, our data reveal complicated brain region-specific fine-tuning of BDNF expression.

Key words: BDNF cortex; CREB; hippocampus; neuronal activity

Significance Statement

To date, majority of the research has focused on the regulation of brain-derived neurotrophic factor (BDNF) in the brain but much less is known whether the regulation of BDNF expression is universal in different brain regions and neuronal populations. Here, we report that the best described regulators of BDNF gene from the cAMP-response element binding protein (CREB) transcription factor family have a more profound role in the activity-dependent regulation of BDNF in cortex than in hippocampus. Our results indicate a brain region-specific fine tuning of BDNF expression. Moreover, we have used unbiased determination of novel regulators of the BDNF gene and report a number of neurodevelopmentally important transcription factors as novel potential regulators of the BDNF expression.

Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that contributes to neuronal survival, differentiation and synaptic plasticity (Chao, 2003; Park and Poo, 2013). The expression of BDNF gene, consisting of numerous 5′ noncoding exons that are spliced together with the common protein-coding 3′ exon, is controlled by distinct promoter regions (Timmusk et al., 1993; Aid et al., 2007; Pruunsild et al., 2007) that are used for stimulus-specific, cell type-specific and brain region-specific BDNF expression. For example, in rodents BDNF transcripts containing BDNF exon II or exon IXa are highly expressed in the hippocampus, but at much lower levels in the cortex (Aid et al., 2007). Transgenic mice encompassing the whole rat or human BDNF locus revealed that the transgene could not recapitulate BDNF expression in hippocampal dentate granule cells (Koppel et al.,

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2009, 2010), implying differences in BDNF expression between cortex and hippocampus. The expression of different BDNF transcripts is dysregulated in a brain region-specific manner in patients with schizophrenia and in response to antidepressants (Wong et al., 2010), and in mice after chronic mild stress (P. Huang et al., 2018). Overall, the expression of BDNF varies in different brain regions in both health and disease.

It is acknowledged that BDNF has critical and distinguished roles in the cortex and hippocampus (Bergami et al., 2008; Hong et al., 2008; Sakata et al., 2009; Bambah-Mukku et al., 2014; Wang et al., 2015; Ortiz-López et al., 2017; Mudd et al., 2019). Understanding cell type-specific and brain region-specific stimulus-dependent regulation of BDNF expression serves as the basis for discerning how BDNF affects brain development and function. Therefore, we set out to compare the regulation of neuronal activity-dependent BDNF expression in cortical and hippocampal neurons.

To date, a number of transcription factors, including calcium-response factor (CaRF; Tao et al., 2002), Neuronal PAS Domain Protein 4 (NPAS4; Lin et al., 2008; Pruunsild et al., 2011), cAMP-response element binding protein (CREB; Shieh et al., 1998; Tao et al., 1998; Tabuchi et al., 2002; Hong et al., 2008; Benito et al., 2011; Pruunsild et al., 2011; Palomer et al., 2016; Tai et al., 2016), nuclear factor of activated T-cells (NFAT; Vashishta et al., 2009), and myocyte enhancer factor 2 (MEF2; Flavell et al., 2008; Lyons et al., 2012) families have been described in regulating neuronal activity-dependent BDNF expression (for review, see West et al., 2014). Notably, CaRF has been shown to regulate the levels of BDNF exon IV in cortex but not in hippocampus (McDowell et al., 2010) and MEF2 family transcription factors have been suggested to participate in the regulation of BDNF exon I levels in hippocampal but not in cortical neurons (Flavell et al., 2008; Lyons et al., 2012). Among the transcription factors that regulate activity-dependent BDNF expression, CREB transcription factor family is the best described. Notably, CREB binding to CRE-elements in the genome is cell-type specific (Cha-Molstad et al., 2004), and CREB target genes vary in different brain regions (Tanis et al., 2008). Although the role of CREB in the regulation of BDNF gene expression is well-established in both cortical and hippocampal neurons, it is not known whether the role of CREB in activity-dependent expression of BDNF is the same in both of these neuronal populations.

Here, focusing on the CREB transcription factor family we describe that the regulation of BDNF transcription by these factors and their coactivators CBP and CRTC1 is more prominent in cortical than in hippocampal neurons. Furthermore, our results indicate the role of CREB family in regulating the basal and early induced levels of BDNF mRNA after neuronal activity, whereas CREB family has a minor role in the late induction. We also show that CREB and coactivators CBP and CRTC1 are important for proper induction of BDNF protein expression after membrane depolarization in cortical neurons. We describe the component of BDNF-TrkB signaling in membrane-depolarization induced expression of BDNF in cortical but not in hippocampal neurons. Additionally, we used *in vitro* promoter pulldown coupled with mass spectrometric detection of the bound transcription factors and report novel regulators of BDNF, namely FOXP1, SATB2, RAI1, TCF4, BCL11A. Our results highlight the brain region-specific fine-tuning of BDNF expression.

Materials and Methods

Rat primary neuron culture and treatments

All animal procedures were performed in compliance with the local ethics committee. Cortical and hippocampal neuron cultures were generated from Sprague Dawley rat male and female pups of the same litter at embryonic day 20–21 as described in Esvald et al. (2020). For lentivirus-mediated overexpression, neurons were transduced at 0 days *in vitro* (DIV). All lentiviruses encoding EGFP, A-CREB, negative guide RNA (gRNA), CREB1 gRNA, CRTC1 gRNAs, and dCas9-KRAB-T2A-GFP were used as detailed by Esvald et al. (2020). At 2 DIV, half of the growth medium (for experiments with low molecular-weight inhibitors) or the whole media (in experiments where neurons were transduced with lentiviral particles) was changed and a final concentration of 10 μM mitotic inhibitor 5-fluoro-2′-deoxyuridine (Sigma) was added. For the Western blotting experiments, the neurons were plated in DMEM (Invitrogen) and 10% FBS (Pan Biotech) and the media was changed to supplemented NBA media after ~1.5 h. Half of the media was changed at 2 DIV and 5 DIV. In all the experiments, at 7 DIV (~24 h before the experiment), 1 μM tetrodotoxin (Tocris Bioscience) was added to the media to inhibit spontaneous neuronal activity. For experiments with low molecular-weight inhibitors, 5 μM CBP-CREB interaction inhibitor (CCII; in DMSO, Merck Millipore, catalog #217505, lot 2758191) or 25 nM Az23 (in DMSO, Axon Medchem) was added 1 h or 30 min, respectively, before KCl treatment at 8 DIV. Control cells were treated with the corresponding volume of DMSO. At 8 DIV, 25 mM KCl (Applichem; with 5 μM D-(2R)-amino-5-phosphonovaleric acid (D-APV, Cayman Chemical Company) to reduce excitotoxicity) was used to elicit membrane depolarization for 1, 3, or 6 h.

RNA extraction and RT-qPCR

After treatment, cultured neurons were lysed at 8 DIV in RLT lysis buffer (containing 1% β-mercaptoethanol (VWR Life Sciences) in experiments with lentivirus transduction) and RNA was purified with RNeasy Mini kit (QIAGEN) according to the manufacturer's recommendations with on-column digestion of genomic DNA using RNase-Free DNase set (QIAGEN). RNA concentration was measured with BioSpec-Nano (Shimadzu) or with Nanodrop 2000c (Thermo Scientific) spectrophomoteter.

To compare mRNA levels from cortical and hippocampal tissues, the lysates were prepared from the same pup and biological replicates were obtained from pups of different litters. The cortex and hippocampus of 8-day-old Sprague Dawley rat pups were dissected and frozen in 80°C. Tissue was weighed and homogenized in QIAzol lysis reagent (QIAGEN) at a ratio of 1 ml/100 mg of tissue (but not <500 µl). The amount of cortex was normalized to the amount of hippocampus by taking mass equivalent of homogenous lysate for RNA purification. Next, Qiazol was added to all lysates to achieve a volume of 1 ml and incubated for 5 min at room temperature. Then, 200 µl of chloroform was added, the mixture was shaken vigorously, and incubated at room temperature for 3 min. Finally, the solutions were centrifuged at 12,000 × *g* at 4°C for 15 min and the aqueous phase was transferred to a new tube. Lastly, equal volume of 70% ethanol was added, vortexed, and transferred to a Mini Spin column (EconoSpin). RNA purification was then performed as for cultured neurons using RNeasy Mini kit (QIAGEN) solutions and on-column digestion of DNA. The concentrations of RNA were measured with Nanodrop 2000c (Thermo Scientific) spectrophomoteter.

Equal amounts of RNA were taken to synthesize complementary DNA (cDNA) using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific) and oligo$(dT)_{20}$ primer. qPCR was performed using HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne) and LightCycler 480 Instrument II (Roche). Primers used in qPCR are listed in Tuvikene, 2021. All mRNA expression levels were normalized to HPRT1 expression levels.

Western blotting

The neurons were grown on 24-well plates, treated at 8 DIV with KCl, and lysed in 1× Laemmli buffer (without bromophenol blue and β-mercaptoethanol) followed by heating at 95°C for 5 min. The protein concentration was measured with Pierce BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's protocol. After measuring the concentration, bromophenol blue and β-mercaptoethanol (VWR Life Sciences, 5% final concentration) were added, lysates were heated at 95°C for 5 min, and centrifuged at 16,000 × *g* for 1 min.

Equal amount of total protein (16–20 µg) per experiment was loaded along with 100–200 pg of recombinant mature BDNF protein (Icosagen, catalog #P-105-100) and separated on 15% gel using SDS-PAGE. The proteins were transferred to a PVDF membrane using Trans-Blot Turbo Transfer system (Bio-Rad) high MW program (constant 1.3 A, 10 min). Next, the membranes were blocked for at least 1 h at room temperature in 5% skimmed milk in TBST buffer (1× Tris-buffered saline (pH 7.4) and 0.1% Tween 20), incubated overnight at 4°C with primary anti-BDNF antibody (Icosagen, catalog #327-100, clone 3C11, 1 mg/ml, 1:1000) in 2% milk in TBST, and then overnight at 4°C with secondary antibody anti-mouse IgG conjugated with horseradish peroxidase (HRP; Thermo Scientific, 1:5000) in 2% milk. After both incubations the membrane was washed three times with TBST for 5 min at room temperature. Chemiluminescence signal was produced with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) or SuperSignal West Atto Maximum Sensitivity Substrate (Thermo Scientific). The signal was measured using ImageQuant Las 4000 (GE Healthcare Life Sciences) imaging system.

To measure CREB protein levels, the anti-mouse IgG-HRP signal was quenched with 30% H_2O_2 for 20 min at 37°C, followed by three washes with TBST buffer. The membrane was blocked again for at least 1 h at room temperature in 5% skimmed milk in TBST buffer. Anti-CREB antibody (Cell Signaling, D76D11, rabbit mAb #4820, 1:2500) was incubated overnight at 4°C in 2% milk in TBST and secondary antibody anti-rabbit IgG-HRP (Thermo Scientific, 1:5000) in 2% milk for at least 1 h at room temperature. To measure FLAG-A-CREB levels, the previous signal was again quenched with H_2O_2, the membrane was washed and blocked as described above, and then reprobed with mouse monoclonal anti-FLAG M2-HRP antibody (Sigma-Aldrich, #A8592, 1:5000) overnight at 4°C in 2% milk.

CRTC1 protein levels were analyzed from a separate 10% gel. The proteins were transferred to PVDF membrane using Trans-Blot Turbo Transfer system (Bio-Rad) with modified high MW program (constant 1.3 A, 15 min). The membrane was blocked and washed as described for other membranes. Primary anti-CRTC1 antibody (Cell Signaling, C71D11, rabbit mAb #2587, 1:1000) was incubated overnight at 4°C in 2% milk and secondary antibody anti-rabbit IgG-HRP (Thermo Scientific, 1:5000) in 2% milk for at least 1 h at room temperature.

For loading control, the membranes were stained with Coomassie solution (0.1% Coomassie Brilliant Blue R-250 Dye, 25% ethanol, 7% acetic acid), followed by washes with destaining solution (30% ethanol, 10% acetic acid) and rinsing with tap water. The membranes were imaged using ImageQuant Las 4000 (GE Healthcare Life Sciences) system.

BDNF, CREB, CRTC1, and FLAG-A-CREB protein levels were quantified using densitometric analysis with ImageQuant TL software (GE Healthcare Life Sciences, version 7.0). A marginally small constant number was added to each densitometric value (to account for undetectable signal of pro-BDNF in untreated cortical neurons in some experiments), and the specific protein levels were then normalized using Coomassie staining values. Statistical analysis was performed as described below (Experimental design and statistical analysis).

In vitro DNA pulldown and mass spectrometry

Rat BDNF promoter I and BDNF promoter IV regions were amplified by PCR from luciferase reporter plasmids (published in Esvald et al., 2020). The primers used are listed in Tuvikene, 2021. The biotinylated PCR products were purified using DNA Clean and Concentrator-100 kit (Zymo Research).

Nine hippocampi and three cortices from 8-day-old Sprague Dawley rat pups of both sexes were dissected and snap-frozen in liquid nitrogen. Nuclear lysates were prepared and *in vitro* DNA pulldown was performed as described previously (Tuvikene et al., 2021). Here, 350 µg of nuclear proteins were bound to 75 pmol of biotinylated DNA in the pulldown assay. The subsequent mass spectrometric analysis and custom R-script are also described previously (Tuvikene et al., 2021). At least a 1.5-fold enrichment to one promoter respective to the other was considered as specific binding and tissue-specificity was calculated only for transcription factors passing the specific binding criteria. All proteins detected in mass spectrometry are listed in Extended Data Figure 7-1.

Analysis of RNA-sequencing (RNA-seq) data

Raw RNA-seq data of previously published RNA-seq experiments [SRA accession numbers ERP016406 (Dias et al., 2016), SRP049264 (Araujo et al., 2015), SRP058362 (McKenna et al., 2015), SRP068801 (Jaitner et al., 2016), SRP074487 (W.H. Huang et al., 2016), SRP102696 (Araujo et al., 2017), SRP166779 (Garay et al., 2020), SRP261474 (Carullo et al., 2020)] were downloaded directly as fastq files from European Nucleotide Archive with the assistance of SRA-explorer (www.sra-explorer.info). Adapter and quality trimming was done using BBDuk (part of bbmap version 38.79) using the following parameters: ktrim = r k = 23 mink = 11 hdist = 1 tbo qtrim = lr trimq = 10, and minlen = 50 (for SRP261474), minlen = 45 (for ERP016406, SRP102696), minlen = 30 (for SRP049264), minlen = 25 (for SRP166779, SRP068801, SRP058362) or minlen = 65 (for SRP074487). Reads were mapped to rn6 (annotation from ENSEMBL, release 101, for SRP261474), hg19 genome (annotation from Gencode release 34, for SRP049264), or mm10 (annotation from Gencode M25 primary assembly, for the rest of the datasets) using STAR aligner (version 2.7.3a). Detected splice junctions were combined, junctions on mitochondrial DNA and junctions with noncanonical intron motifs were removed. Novel splice sites with at least 10 reads (for ERP016406, SRP261474 and SRP074487), or at least five reads (for the rest of the datasets) in the whole dataset were added for 2nd pass alignment using STAR. Aligned reads were assigned to genes (to determine total BDNF expression levels) or exons (to determine the expression levels of BDNF exon I and exon IV) using FeatureCounts (version 2.0.0), with additional -B -C arguments for paired-end reads. Counts were normalized using DESeq2 R package (version 1.28.1) and visualized using ggplot2 R package (version 3.3.2). Statistical analysis was performed on log-transformed normalized counts using two-tailed equal variance *t* test in Microsoft Excel 365.

Chromatin immunoprecipitation (ChIP) assay

For ChIP assay from tissue, 10–12 hippocampi and six cortices from 8-day-old Sprague Dawley rat pups of both sexes from the same litter were dissected and snap-frozen in liquid nitrogen. Pups from different litters were considered biological replicates. Tissue pieces were weighed and homogenized in 1% formaldehyde (Cell Signaling, catalog #12606, diluted in PBS; the volume of the fixing solution was 20× the mass of the tissue) with Dounce tissue grinder (Wheaton) using loose pestle 20 times. After that, the homogenate was transferred to a 15-ml tube and incubated for 10 min at room temperature with rotation. Final concentration of 0.125 M glycine was added to quench formaldehyde and the mixture was rotated for another 10 min at room temperature. Next, the mixture was centrifuged at 2600 × *g* at 4°C for 5 min and the pellet was washed two times with ice-cold PBS (20× volume of the tissue weight). A protocol to obtain nuclear lysates was developed based on Vierbuchen et al. (2017). Briefly, the pellet was resuspended in 20× volume of ice-cold L1 [50 mM HEPES (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.25% Triton X-100, 0.5% NP-40, 10% glycerol, 1× cOmplete protease inhibitor cocktail (Roche)], rotated for 10 min at 4°C and centrifuged at 2600 × *g* at 4°C for 5 min. The pellet was next resuspended in L2 [10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1× cOmplete protease inhibitor cocktail (Roche)] in a volume of 20× the weight of the initial tissue piece, rotated for 10 min at 4°C and centrifuged at 2600 × *g* at 4°C for 5 min. Finally, the nuclear pellet was resuspended in 10× volume of L3 [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% N-lauroylsarcosine, 1× cOmplete protease inhibitor cocktail (Roche)], incubated on ice for a couple of minutes, and transferred to Diagenode 15 ml tubes onto prewashed sonication beads (0.2 g of beads per 0.5 ml of nuclear lysate was prepared by washing three times with 1 ml PBS, vortexing and spinning the beads down). After transferring the nuclear lysates onto the beads, the mixture was vortexed vigorously to improve sonication efficiency. Chromatin was sonicated using the BioRuptor Pico device (Diagenode) for five cycles 30 s on and 30 s off, vortexed vigorously and sonicated for another five cycles. Next, the lysate was transferred to a new tube, final concentration of 1% Triton X-100 was added, and centrifuged at 4500 × *g* at 8°C for 5 min in a swing-out rotor. The protein concentration was measured with BCA kit, and 200–275 µg of nuclear protein was used per IP; 10 µg of CREB (Cell Signaling, D76D11, rabbit mAb #4820) or TCF4 (CeMines) antibody or 4.8 µg of BCL11A (Developmental Studies

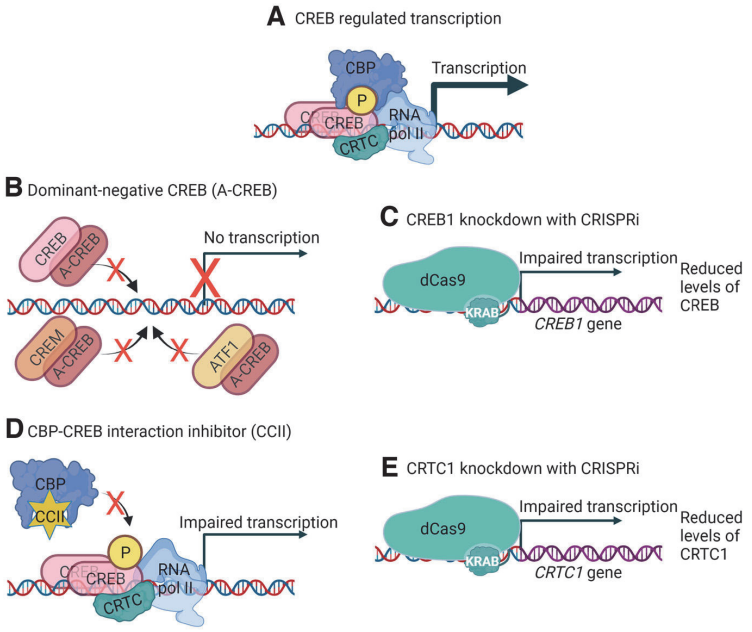

Figure 1. Schematical depiction of the tools used in the current study to elucidate the role of CREB family in BDNF gene regulation. ***A***, CREB regulates transcription of the target genes together with coactivators CBP (or P300) and CRTCs. ***B***, Overexpression of a dominant-negative form of CREB, named A-CREB, which dimerizes with CREB family members based on their basic leucine zipper domain (bZip) and inhibits binding to the DNA, therefore inhibiting CREB family-regulated transcription. ***C***, CRISPR interference (CRISPRi) system targeting dCas9-KRAB to the CREB1 gene promoter impairs the transcription of CREB1 gene, reducing the levels of CREB. ***D***, A low molecular weight compound CCII binds to the c-Myb-site on CBP and prevents CBP binding to CREB family members. ***E***, CRISPRi system targeting dCas9-KRAB to the CRTC1 gene promoter impairs the transcription of CRTC1, reducing the levels of CRTC1. Schematics were created with BioRender.com.

Hybridoma Bank, PCRP-BCL11A-1G10) antibody was used for overnight incubation at 4°C. At the same time, 50 µl Dynabeads Protein G (Invitrogen) per IP were prewashed 2 times with 0.02% Tween 20-PBS and blocked overnight with 200 µg/ml BSA (Thermo Scientific) at 4°C.

The next day, the Dynabeads were washed twice with 0.02% Tween 20-PBS and diluted in L3, containing Triton X-100 and protease inhibitor solution. Dynabeads were added to the lysates and incubated on a rotator for 5–7 h at 4°C. Finally, the beads were washed four times in 1 ml wash buffer [1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 1× cOmplete protease inhibitor cocktail (Roche)] and once with final wash buffer [1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 1× cOmplete protease inhibitor cocktail (Roche)]. DNA-protein complexes were eluted with 50 μl elution buffer (1% SDS, 100 mM $NaHCO_3$, 1 mM EDTA) at 37°C for 10 min for three times with the final elution performed at additional 65°C for 5 min. At the same time, 10% of input samples were also diluted with elution buffer to a final volume of 150 μl. Final concentration of 250 mM NaCl was added to IP and input samples and samples were decrosslinked overnight at 65°C.

Next day, 125 μg/ml RNase A (PanReac Applichem) was added and incubated at 37°C for 1–2 h. Then, final concentrations of 6 mM EDTA and 240 μg/ml proteinase K (Fisher Bioreagent) were added and incubated at 45°C for 2 h. The genomic DNA was purified using QIAquick PCR Purification kit (QIAGEN).

DNA enrichment was measured with qPCR using primers listed in Tuvikene, 2021 and LightCycler 480 SYBR Green I Master kit (Roche). The DNA enrichment was calculated as percent of input relative to the levels of unrelated region (URR).

Experimental design and statistical analysis

Sample size estimation was not performed, and randomization and blinding were not used. Statistical analysis was performed on log-transformed, mean-centered and autoscaled data (where applicable). For data in Figures 3 and 5 that show the direct comparison of the role of the effector in different cultures, the data were only log-transformed. All tested hypotheses were specified before conducting the experiments. Two-tailed paired *t* tests were performed using R 4.1.1 programming language or Microsoft Excel 365 and *p*-values were adjusted for multiple comparisons with Holm or Holm–Šídák family-wise error rate correction method using R p.adjust function or GraphPad Prism 7.03 (GraphPad Software), respectively. Exact *p*-values are shown in extended data files. All experiments were performed using three or four independent biological replicates (cultures obtained from different litters, $n = 3–4$). For graphical representation, transformed and scaled means and mean ± SEM were back-transformed to linear scale with error bars representing upper and lower limits of back-transformed mean ± SEM. For RNA-seq data from (Carullo et al., 2020), statistical analysis was performed using DESeq2 package in R.

Results

Activity-dependent BDNF gene regulation by CREB family transcription factors

The expression of BDNF is induced in response to various stimuli, of which induction by neuronal activity is one of the most well-studied (for review, see West et al., 2014). We cultured rat cortical and hippocampal neurons from pups of the same litter to reduce the role of biological variability and ensure the comparability of the two neuronal populations, and investigated the role of CREB family transcription factors in membrane depolarization-induced BDNF gene expression (Fig. 1*A*). CREB family consists of three basic-leucine zipper transcription factors – activating transcription factor 1 (ATF1), cAMP-response element-binding protein (CREB), and cAMP response element modulator (CREM; Mayr and Montminy, 2001). To investigate the involvement of CREB family transcription factors in membrane depolarization-dependent BDNF induction, we overexpressed a dominant-negative form of CREB, named A-CREB. The mechanism of function for A-CREB is based on dimerization with CREB basic leucine zipper (bZip) domain (Ahn et al., 1998). Since the bZip domain in ATF1, CREB, and CREM is highly similar, A-CREB can impair the DNA binding of all the CREB family members (Fig. 1*B*).

CREB family transcription factors have two main types of coactivators that potentiate CREB-regulated transcription: CREB-binding protein (CBP) and P300 (for review, see Mayr and Montminy, 2001) and CREB-regulated transcriptional coactivators or CRTCs (for review, see Saura and Cardinaux, 2017). Of these, CBP interacts with phosphorylated kinase-inducible domain (KID; Parker et al., 1996; Radhakrishnan et al., 1997; Shaywitz et al., 2000; Dahal et al., 2017a,b) and CRTCs bind the bZip domain of CREB family members (Luo et al., 2012; Y. Song et al., 2018). To determine the relevance of interaction between CREB family and its coactivator CBP, we used a low molecular weight CBP-CREB interaction inhibitor

(CCII; Best et al., 2004; Li and Xiao, 2009; Fig. 1*D*). To further elucidate the specific role of CREB transcription factor and CREB family coactivator CRTC1 in BDNF gene regulation, we silenced their expression with CRISPR interference (Fig. 1*C*,*D*, respectively).

Finally, we have previously described a BDNF-TrkB signaling-dependent positive feedback loop in rat cortical neurons where BDNF-TrkB signaling induces the expression of all major BDNF transcripts in a CREB-family and CBP-dependent manner (Esvald et al., 2020). As membrane depolarization induces both BDNF expression and BDNF secretion (for review, see Sasi et al., 2017; M. Song et al., 2017), it is plausible that at least part of the induction of BDNF expression after membrane depolarization depends on TrkB signaling. Therefore, to determine whether the BDNF-TrkB signaling component participates in depolarization-dependent BDNF gene regulation, we treated the cells with Trk receptor inhibitor Az23 (Thress et al., 2009) before KCl treatment. We measured the expression levels of total BDNF, major BDNF transcripts (exon I, IIc, III, IV, VI, IXa), and CREB family members using RT-qPCR to elucidate gene expression regulation after membrane depolarization in cortical and hippocampal neurons.

First, we determined the extent and dynamics of neuronal activity-dependent BDNF induction. Our results collectively showed that total BDNF expression is more inducible in response to membrane depolarization in cortical neurons than in hippocampal neurons (Figs. 2*A*, 3*A*). Our data also showed that all major BDNF transcripts are induced faster in cortical neurons than in hippocampal neurons as the inductions in cortical neurons after 1 h treatment were higher than in hippocampal neurons (Figs. 2*A*, 3*A*). Similarly, after prolonged depolarization all major transcripts except BDNF exon I transcripts showed higher inducibility in cortical neurons than in hippocampal neurons, whereas BDNF exon I transcripts showed similar induction between the two cultures (Figs. 2*A*, 3*A*).

Next, we focused on the role of CREB family in the regulation of BDNF gene expression. In cortical neurons, overexpression of a dominant-negative form of the CREB family, A-CREB, decreased the basal expression and both the early and late induction of the major BDNF transcripts (Figs. 2*B*, 3*B*). Meanwhile, in hippocampal neurons overexpression of A-CREB diminished mainly basal expression and early induction of the major BDNF transcripts, while the late induction was largely unaffected (Figs. 2*B*, 3*B*). In contrast to other BDNF transcripts, the levels of BDNF exon VI transcripts were increased in response to A-CREB overexpression both in unstimulated neurons and after depolarization in both cortical and hippocampal neurons (Figs. 2*B*, 3*B*). Collectively our results show that the neuronal activity-induced levels of BDNF are decreased by A-CREB overexpression more in cortical than in hippocampal neurons (Fig. 3*B*).

The specific knock-down of CREB1 gene expression (knock-down ~7- to 8-fold in both cortical and hippocampal untreated neurons, see Fig. 4*C*) revealed that CREB transcription factor participates in the early induction of BDNF exon I and exon IV transcripts in cortical neurons (Figs. 2*C*, 3*C*). In contrast, knock-down of CREB1 had only mild effect on BDNF expression in hippocampal neurons with the strongest effect on the early induction of BDNF exon IV (Figs. 2*C*, 3*C*). Notably, in both neuronal cultures we again detected increase in the expression of BDNF exon VI transcripts (Figs. 2*C*, 3*C*), showing an opposite regulation compared with other BDNF transcripts by CREB transcription factor.

Next, we focused on the coactivators of CREB family in the regulation of BDNF expression. Disrupting the interaction between coactivator CBP and CREB with low molecular weight inhibitor, CCII, showed that the late induction of BDNF exon I containing transcripts depends on CBP in both cortical and hippocampal neurons (Figs. 2*D*, 3*D*). Interestingly, in cortical neurons impairing CBP recruitment decreased both the early and late induction of BDNF exon IV and exon VI transcripts, whereas in hippocampal neurons the late induction of both transcripts was increased (Figs. 2*D*, 3*D*).

Knock-down of the coactivator CRTC1 in cortical neurons reduced CRTC1 levels ~7-fold (see Fig. 4*E*) and mostly decreased the early induction of major BDNF transcripts (Figs. 2*E*, 3*E*). In hippocampal neurons the CRTC1 knock-down was slightly more efficient and reduced the CRTC1 levels ~13-fold (see Fig. 4*E*) and this increased both the basal expression of BDNF transcripts of the first cluster of exons (exons I, IIc, and III) and induced levels of exon I and III transcripts (Figs. 2*E*, 3*E*). In contrast to the opposite effect on BDNF exon I transcripts in cortical and hippocampal neurons (Fig. 3*E*), CRTC1 knock-down decreased the induction of BDNF exon IV transcripts in both neuron cultures, although the effect was more prominent in hippocampal neurons (Figs. 2*E*, 3*E*). CRTC1 knock-down also decreased the early induction of BDNF exon VI transcripts in cortical neurons, whereas such effect was not seen in hippocampal neurons (Figs. 2*E*, 3*E*).

Lastly, we found that BDNF-TrkB signaling slightly contributed to the membrane depolarization-induced BDNF expression at later time points in cortical neurons, whereas no effect of inhibiting BDNF-TrkB signaling was seen in hippocampal neurons (Figs. 2*F*, 3*F*). The difference between cultures was consistently seen for all transcripts after prolonged membrane depolarization, although the difference was mostly not statistically significant (Fig. 3*F*).

Collectively our results show that BDNF exon I and IV transcripts, the most inducible BDNF transcripts, were consistently dependent on CREB family and their coactivators both in cortical and hippocampal neurons. Our results indicate that the induction of BDNF exon I in cortical neurons depends on CREB, CBP, and CRTC1, with CRTC1 being important throughout the measured timepoints of depolarization and CBP rather at later timepoints. The induction of BDNF exon IV in cortical neurons was also regulated by CREB, CBP, and CRTC1, whereas the induction in hippocampal neurons was regulated by CREB and CRTC1. The opposite effect of CRTC1 knock-down on BDNF exon I levels in cortical and hippocampal neurons and the opposite regulation of BDNF exon VI compared with other BDNF transcripts need further investigation. Overall, our results suggest that the activity-dependent regulation of BDNF transcripts in cortical neurons relies more on the CREB family and coactivators than in hippocampal neurons. Our results also suggest that the faster inducibility of BDNF in cortical neurons could be a result of the increased regulation by CREB family.

Activity-dependent expression and regulation of CREB family members

To further elucidate the mechanism of CREB family-dependent gene expression in different neuronal populations, we measured the activity-dependent expression levels of all the CREB family members, CREB1, ATF1, and CREM, in cortical and hippocampal neurons. CREB1 is known to be a constitutively expressed gene and CREB protein activity is mainly regulated by posttranslational mechanisms (for review, see Johannessen et al., 2004), as phosphorylation of CREB in the

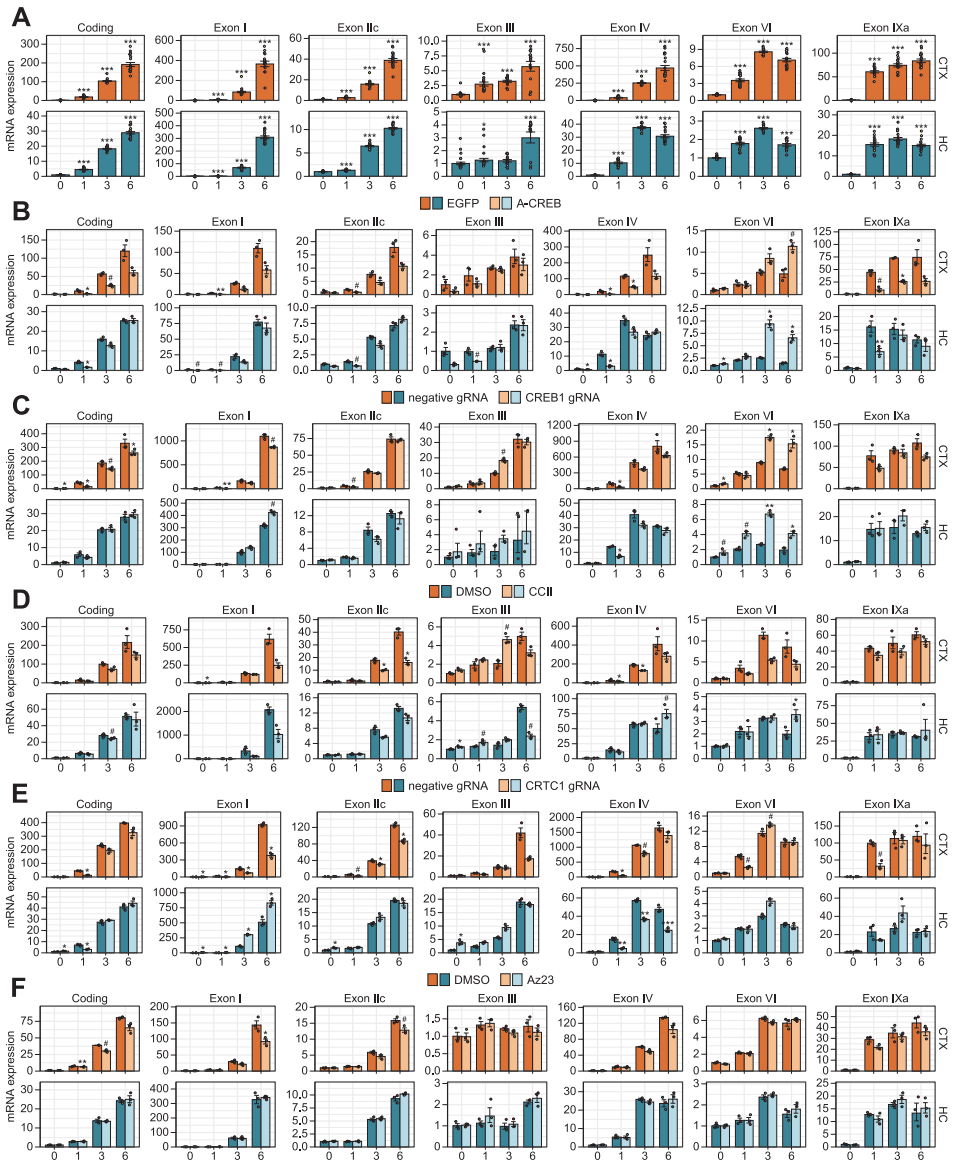

Figure 2. Regulation of activity-dependent BDNF gene expression by CREB family transcription factors and their coactivators in cortical and hippocampal neurons. Cultured rat cortical (CTX) or hippocampal (HC) neurons were left untreated (0 h) or treated with 25 mM KCl at 8 DIV for the indicated time (1, 3, or 6 h). Total BDNF levels (Coding) or different BDNF transcripts (indicated by the respective 5′ exon name) were measured using RT-qPCR. ***A***, Induction of BDNF in response to membrane depolarization with KCl. Regulation of activity-dependent BDNF expression by various effectors: (***B***) overexpression of dominant-negative for the CREB family (A-CREB), (***C***) knock-down of CREB1 gene expression using CRISPRi, (***D***) disruption of the interaction between CBP and CREB (CCII), (***E***) knock-down of coactivator CRTC1 with CRISPRi, and (***F***) inhibition of Trk receptor signaling with Az23. Data are shown relative to the transcripts' levels in untreated cells in the respective culture (***A***) or in the control cells [expressing EGFP, or negative guide RNA (gRNA) and dCas9-KRAB, or treated with DMSO] at the indicated time point in the respective culture (***B–F***). The bar graphs show the average fold changes for $n = 18$ (***A***, 3 independent experiments plus the combined data from all control cells shown on panels ***B–F***) or $n = 3$ (***B–F***) independent biological experiments. All data points are shown as dots, and each biological replicate (i.e., cultures derived from animals of different litters) is denoted with the same color. Error bars represent mean ± SEM. Statistical significance is shown compared with the expression levels in untreated cells in the respective culture (***A***) or in control cells at the respective time point of KCl treatment in the respective culture (***B–F***). Group averages and exact *p*-values are shown in Extended Data Figure 2-1. #$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, paired two tailed *t* test, corrected for multiple comparisons using Holm method.

kinase-inducible domain (KID) stabilizes its interaction with the transcriptional coactivator CBP (Dahal et al., 2017a, b). Our results showed that membrane depolarization slightly decreases CREB1 levels after 3 h membrane depolarization in both cortical and hippocampal neurons (Fig. 4*A*). However, in hippocampal but not in cortical neurons there was a slight but consistent upregulation of both CREB1 and ATF1 mRNA levels at the 6-h time point (Figs. 4*A*, 5*A*).

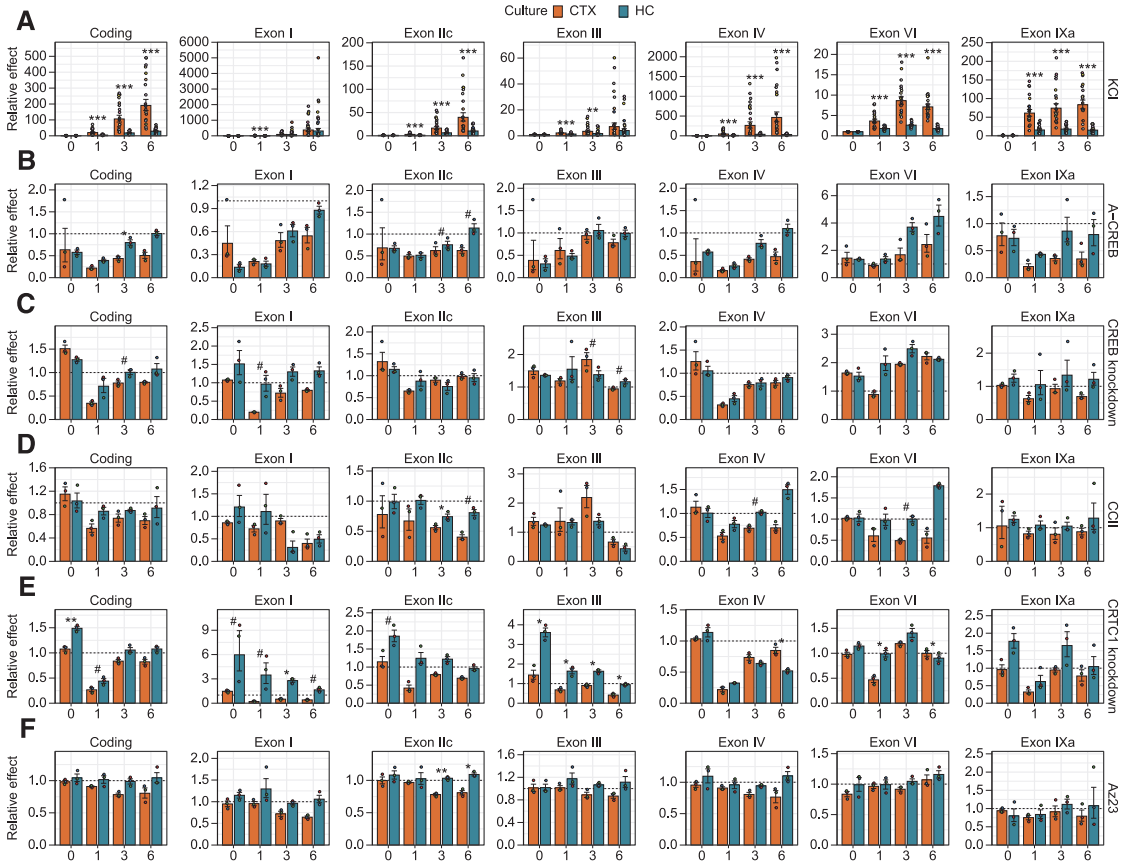

Figure 3. Comparison of the effect of impairing CREB family and coactivators in cortical and hippocampal neurons on activity-dependent BDNF gene expression. Cultured rat cortical (CTX) or hippocampal (HC) neurons were left untreated (0 h) or treated with 25 mM KCl at 8 DIV for the indicated time (1, 3, or 6 h). Total BDNF levels (Coding) or different BDNF transcripts (indicated by the respective 5′ exon name) were measured using RT-qPCR. Data from Figure 2 are shown relative to the transcripts' levels in the control cells [cells expressing EGFP, or negative guide RNA (gRNA) and dCas9-KRAB, or treated with DMSO] at the respective treatment time point. The bar graphs show the average fold changes of the effector, all data points are shown as dots, and each biological replicate [i.e., cultures derived from animals of different litters, $n = 18$ (***A***) or $n = 3$ (***B–F***)] is denoted with the same color. Error bars represent mean ± SEM. Statistical significance is shown compared with the effector fold change between cortical and hippocampal neurons at the respective time point of KCl treatment. Group averages and exact *p*-values are shown in Extended Data Figure 3-1. #$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, paired two tailed *t* test, corrected for multiple comparisons using Holm method.

Next, we focused on the expression of CREM and inducible cAMP early repressor (ICER). The expression of ICER inside the CREM gene is controlled by an intronic promoter with four tandemly placed CRE elements, making it highly inducible by CREB family transcription factors (Molina et al., 1993). ICER contains only the CREM DNA binding domain and competes with the CREB family members in binding to DNA, therefore attenuating CREB family-regulated gene expression (Molina et al., 1993; Walker et al., 1998; Mioduszewska et al., 2003). Since CREM gene can also produce various repressor and activator forms of CREM via alternative splicing and exon shuffling (Foulkes et al., 1991; Laoide et al., 1993), we specifically measured the expression levels of CREM activator forms that contain the KID domain. Our data showed that the levels of the activator forms of CREM were slightly and the negative regulator ICER were remarkably increased in response to membrane depolarization (Fig. 4*A*). Notably, while the induction of CREM activator forms was relatively similar in both cortical and hippocampal neurons, the induction of ICER was faster and reached a higher induced level in cortical neurons than in hippocampal neurons (Figs. 4*A*, 5*A*).

Next, we analyzed the expression of CREB family members after overexpression of A-CREB, knock-down of CREB1 and CRTC1 and inhibition of CBP recruitment by CCII. Notably, overexpression of A-CREB strongly induced the expression of CREB1 and CREM, with the effect being stronger in cortical neurons, and also slightly induced ATF1 expression in both cultures (Figs. 4*B*, 5*B*), indicating that CREB family members try to normalize their functional levels in response to impairing CREB family-dependent transcription. In agreement with Esvald et al. (2020), knock-down of CREB1 expression remarkably induced the expression of CREM in both neuronal cultures (Fig. 4*C*), but more strongly in cortical neurons (Fig. 5*C*), indicating the existence of a compensatory mechanism between CREB and CREM, and also providing a possible explanation why A-CREB, which inhibits all the members of the CREB family, tends to have a more profound effect on BDNF gene expression than specific silencing of CREB1. Neither inhibition of CBP recruitment nor knock-down of CRTC1 affected the expression of CREB1 and ATF1 in cortical neurons (Fig. 4*D*, *E*). Interestingly, the late induction of CREB1 in hippocampal neurons seems to slightly

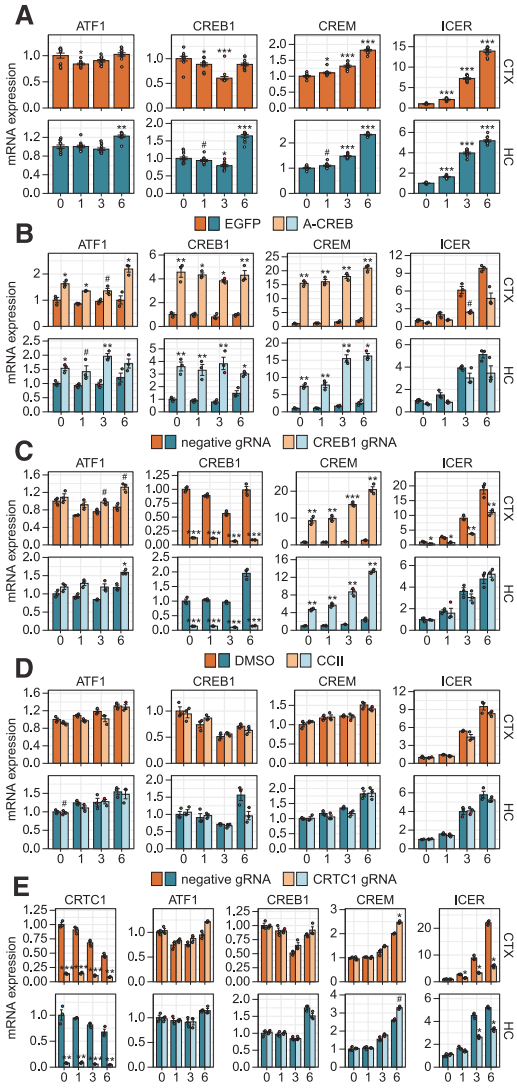

Figure 4. Regulation of ATF1, CREB1, and CREM by CREB family transcription factors and their coactivators in cortical and hippocampal neurons. Cultured rat cortical (CTX) or hippocampal (HC) neurons were left untreated (0 h) or treated with 25 mM KCl at 8 DIV for the indicated time (1, 3, or 6 h). Levels of different CREB family members (ATF1, CREB1, and CREM activator forms containing the KID domain (CREM) or CREM negative regulator ICER) were measured using RT-qPCR. ***A***, Regulation of CREB family members in response to membrane depolarization with KCl. Regulation of activity-dependent CREB family expression by various effectors: (***B***) overexpression of dominant-negative for the CREB family (A-CREB), (***C***) knock-down of CREB1 gene expression using CRISPRi, (***D***) disruption of the interaction between CBP and CREB (CCII), (***E***) knock-down of coactivator CRTC1 with CRISPRi. Data are shown relative to the transcripts' levels in untreated cells in the respective culture (***A***) or in the control cells [expressing EGFP, or negative guide RNA (gRNA) and dCas9-KRAB, or treated with DMSO] at the indicated time point in the respective culture (***B–E***). The bar graphs show the average fold changes for $n = 12$ (***A***, combined data from all control cells shown on panels ***B–E***) or $n = 3$ (***B–E***) independent biological experiments. All data points are shown as dots, and each biological replicate (i.e., cultures derived from animals of different litters) is denoted with the same color. Error bars represent mean ± SEM. Statistical significance is shown compared with the expression levels in untreated cells in the respective culture (***A***) or in control cells at the respective time point of KCl treatment in respective culture (***B–E***). Group averages and exact *p*-values are shown in Extended Data Figure 4-1. $\#p < 0.1$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, paired two tailed *t* test, corrected for multiple comparisons using Holm method.

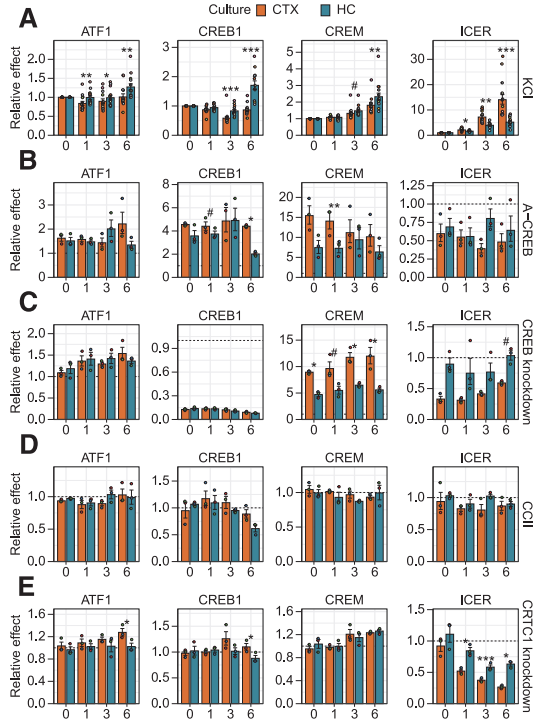

Figure 5. Comparison of impairing CREB family and coactivators in cortical and hippocampal neurons on the gene expression of CREB family members. Cultured rat cortical (CTX) or hippocampal (HC) neurons were left untreated (0 h) or treated with 25 mM KCl at 8 DIV for the indicated time (1, 3, or 6 h). Levels of different CREB family members [CREB1, ATF1, CREM activator forms containing the KID domain (CREM) or CREM negative regulator ICER] were measured using RT-qPCR. Data from Figure 4 are shown relative to the transcripts' levels in the control cells [expressing EGFP, or negative guide RNA (gRNA) and dCas9-KRAB, or treated with DMSO] at the respective treatment time point. The bar graphs show the average fold changes of the effector, all data points are shown as dots and each biological replicate [i.e., cultures derived from animals of different litters, $n = 12$ (***A***) or $n = 3$ (***B–E***)] is denoted with the same color. Error bars represent mean ± SEM. Statistical significance is shown compared with the effector fold change between cortical and hippocampal neurons at the respective time point of KCl treatment. Group averages and exact *p*-values are shown in Extended Data Figure 5-1. $\#p < 0.1$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, paired two tailed *t* test, corrected for multiple comparisons using Holm method.

depend on CBP and CRTC1 (Fig. 4*D*, *E*). The late induction of CREM was increased upon CRTC1 knock-down similarly in both cultures (Figs. 4*E*, 5*E*).

The regulation of ICER by CREB family members was different in cortical and hippocampal neurons. The expression of ICER was more inducible in cortical neurons (Figs. 4*A*, 5*A*) and this activity-dependent induction of ICER was impaired by CREB1 knock-down in cortical neurons but not in hippocampal neurons (Figs. 4*C*, 5*C*). The knock-down of CRTC1 decreased the induced levels of ICER in both cultures (Fig. 4*E*), but the effect on ICER levels was stronger and also apparent earlier after membrane depolarization in cortical neurons compared with hippocampal neurons (Fig. 5*E*). It appears that the induction of ICER is lower and less dependent on CREB in hippocampal neurons, suggesting that the ICER-dependent CREB family negative feedback loop is also less potent in hippocampal neurons than in cortical neurons.

Activity-dependent regulation of BDNF protein levels

Next, we investigated whether BDNF protein levels are also affected by impairing CREB family transcription factors and coactivators. For that we used the same tools as for qPCR analysis (Fig. 1), treated cortical and hippocampal neurons at 8 DIV with KCl and analyzed the protein levels using Western blotting. To detect BDNF we used a highly sensitive BDNF antibody that has been validated on BDNF knock-out animal brain tissue (Wosnitzka et al., 2020) and can detect even 15 pg of recombinant BDNF in Western blotting (our unpublished data).

In agreement with the BDNF mRNA levels, our data show that both pro-BDNF and mature BDNF protein levels are more inducible in cortical neurons than in hippocampal neurons (Fig. 6*A–D*). Our results also indicate that the BDNF protein induction is faster in cortical than in hippocampal neurons, and that an increase in pro-BDNF levels can be detected before mature BDNF levels rise (Fig. 6*A–D*). The overexpression of A-CREB abolished the activity-dependent expression of mature BDNF protein in cortical neurons, while in hippocampal neurons only a minor effect on mature BDNF protein levels was seen (Fig. 6*A*). Similarly, the overexpression of A-CREB almost completely abolished the induction of pro-BDNF in cortical neurons, and decreased the induction in hippocampal neurons (Fig. 6*A*). In accordance with the CREB1 mRNA levels, the overexpression of A-CREB increased CREB protein levels in both cortical and hippocampal neurons, but the effect on protein levels was more prominent in hippocampal neurons (Fig. 6*A*).

We next focused on the specific role of CREB transcription factor. The knock-down of CREB1 gene decreased CREB protein levels 5- to 8-fold in both cortical and hippocampal neurons, and decreased the protein induction of mature BDNF in cortical neurons at both 3 and 6 h of KCl treatment, and in hippocampal neurons only at the 6 h time point (Fig. 6*B*). A decrease in the levels of pro-BDNF was detected in both cortical and hippocampal neurons, with the effect being smaller in hippocampal neurons (Fig. 6*B*).

Next, we focused on the coactivators CBP and CRTC1 in the regulation of BDNF expression levels. By impairing CBP recruitment the induction of pro-BDNF and mature BDNF induction was greatly inhibited in cortical neurons, and was again mostly evident in hippocampal neurons only at the 6 h time point (Fig. 6*C*). Interestingly, CCII treatment slightly increased CREB protein levels in cortical neurons (Fig. 6*C*), although we did not detect any effect on the CREB1 mRNA levels (Fig. 4*D*).

Finally, CRTC1 knock-down decreased CRTC1 protein levels ~7- and ~15-fold in cortical and hippocampal neurons, respectively (Fig. 6*D*). We also detected depolarization-dependent dephosphorylation of CRTC1 as a shift in the mobility in Western blot analysis. Interestingly, CRTC1 knock-down had an opposing effect on the BDNF protein levels in cortical and hippocampal neurons – CRTC1 knock-down decreased the depolarization-induced levels of pro-BDNF and mature BDNF in cortical neurons but increased their levels in hippocampal neurons (Fig. 6*D*). Although the CRTC1 knock-down did not increase total BDNF mRNA levels, it increased BDNF exon I mRNA levels in hippocampal neurons (Fig. 2*E*), and thus the increased BDNF protein levels in hippocampal neurons could be explained by more efficient translation of BDNF exon I-containing transcripts as shown previously (Koppel et al., 2015). The lower levels of CRTC1 did not affect the CREB protein levels (Fig. 6*D*).

Collectively, our data show that CREB family and coactivators CBP and CRTC1 are the main regulators of activity-dependent induction of BDNF in cortical neurons, whereas they play a less prominent role in the induction of BDNF levels in hippocampal neurons.

Identification of tissue-specific transcription factors binding to BDNF promoters I and IV *in vitro*

Since, to our surprise, the role of CREB family in BDNF gene regulation was smaller than we expected, especially for the late induction and in the hippocampus, we next aimed to decipher which transcription factors additionally regulate BDNF gene expression in the cortex and hippocampus. For that we employed *in vitro* DNA pulldown assay with promoter I and IV fragments using 8-day-old rat cortical and hippocampal tissue nuclear lysates and identified the bound proteins using label-free liquid chromatography-coupled mass spectrometry (LC-MS; Fig. 7*A*). A similar approach has previously been used to determine transcription factors that bind to an obesity-related SNP region within the BDNF gene (Mou et al., 2015) and to an intronic enhancer region inside the BDNF gene (Tuvikene et al., 2021). Here, we selected transcription factors from mass spectrometric analysis according to Gene Ontology classification and compared their signal between pulldowns using either BDNF promoter I or IV. We set a ratio of 1.5 as a threshold for specific binding between the two promoter regions and then determined the brain region preference of the identified promoter-specific transcription factors (Fig. 7*B*).

For BDNF promoter I, we detected binding of several factors that are widely acknowledged to regulate the activity of the promoter, i.e., cAMP-response element binding protein (CREB; Tabuchi et al., 2002; Pruunsild et al., 2011), aryl hydrocarbon receptor nuclear translocator 2 (ARNT2; Pruunsild et al., 2011; Bloodgood et al., 2013) and activator protein 1 (AP1) family transcription factors JunB and JunD (Tuvikene et al., 2016). For BDNF promoter IV, our assay also revealed numerous transcription factors previously reported to regulate the activity of the promoter (Fig. 7*B*), including myocyte enhancer factor 2 (MEF2) family members MEF2a and MEF2c (Flavell et al., 2008; Lyons et al., 2012; Chen et al., 2020), USF1 and USF2, the members of upstream stimulatory factor (USF) family of basic helix-loop-helix leucine zipper proteins (Chen et al., 2003b; Pruunsild et al., 2011), and methyl CpG binding protein 2 (MeCP2; Chen et al., 2003a; Martinowich et al., 2003; Yazdani et al., 2012; Tai et al., 2016). Moreover, several of the factors have a predicted binding site (Fig. 7*B*) according to Jaspar in silico prediction (Fornes et al., 2020). Based on the rediscovery of many known regulators of BDNF, we conclude that our *in vitro* pulldown assay is reliable and could therefore possibly also determine novel regulators of BDNF. We discovered numerous potential regulators of BDNF promoter I that might confer brain region-specific expression. For example, nuclear factor 1 protein NFIA, homeobox protein PROX1, and zinc finger proteins ZFP37 and KLF12 showed enriched binding to BDNF promoter I in the hippocampal lysate and zinc finger protein BCL11A and T-box family transcription factor TBR1 in the cortical lysate (Fig. 7*B*). Meanwhile, homeobox proteins SATB1 and SATB2, and forkhead box protein FOXP1, identified by us as novel regulators of BDNF promoter IV, showed cortex-specific binding and zinc finger protein RAI1 and helix-loop-helix protein TCF4 showed hippocampus-specific binding to BDNF promoter IV (Fig. 7*B*).

Differential expression of the *in vitro* bound factors in different neuronal populations

Next, we asked whether the transcription factors that showed brain region-preference in the *in vitro* promoter pulldown assay

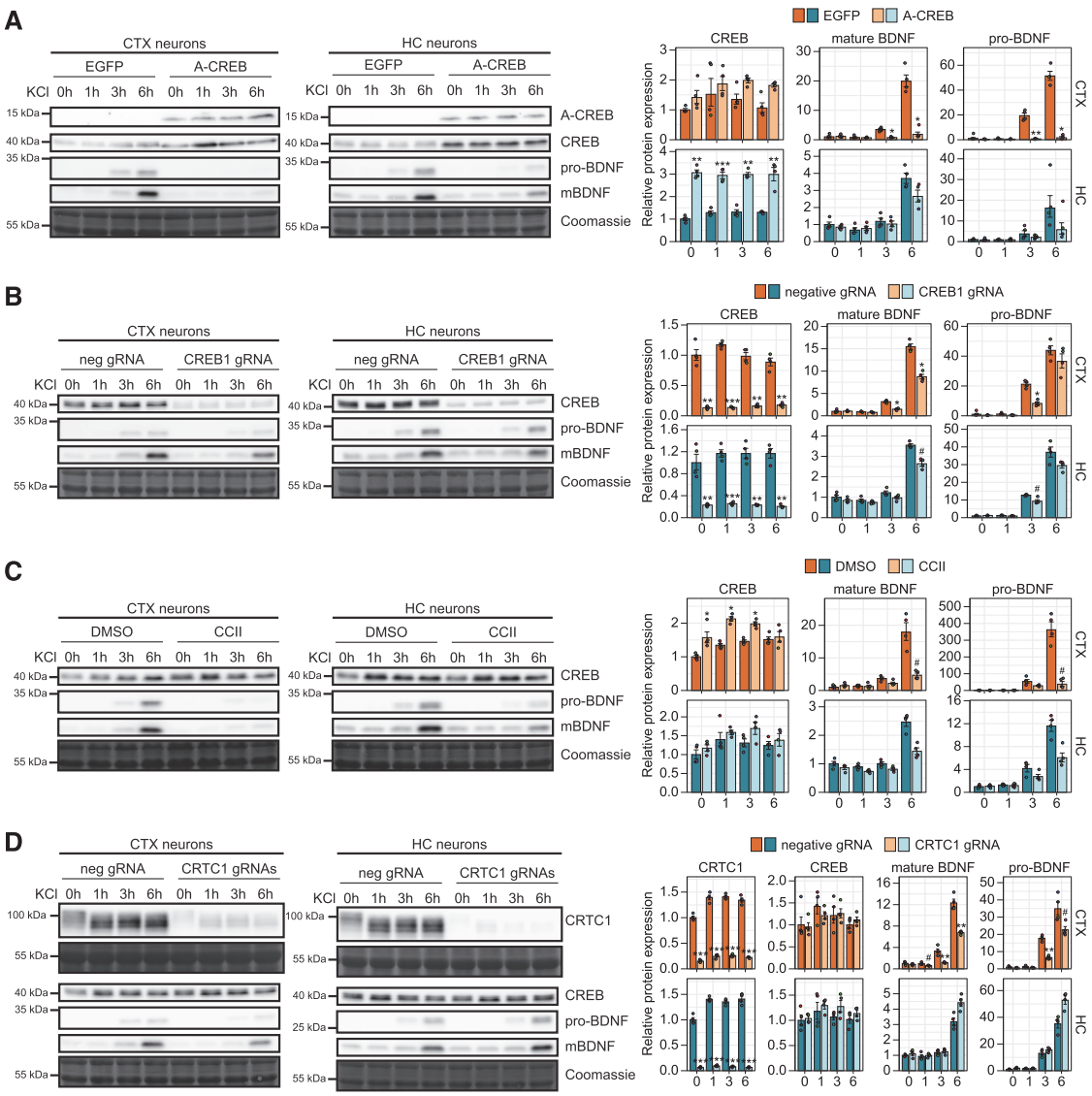

Figure 6. Regulation of activity-dependent BDNF protein levels by CREB family transcription factors and coactivators in cortical and hippocampal neurons. Cultured rat cortical (CTX) or hippocampal (HC) neurons were left untreated (0 h) or treated with 25 mM KCl at 8 DIV for the indicated time (1, 3, or 6 h). Regulation of activity-dependent BDNF protein expression was analyzed using lentiviruses encoding EGFP- or A-CREB (***A***), dCas9-KRAB along with negative control guide RNA (neg gRNA) or gRNA targeting CREB1 promoter (CREB1 gRNA; ***B***), or preteated with CBP-CREB interaction inhibitor (CCII; ***C***), or with dCas9-KRAB along with gRNA targeting CRTC1 promoter (CRTC1 gRNA; ***D***). A-CREB, CREB, CRTC1, pro-BDNF, and mature BDNF (mBDNF) protein levels were measured using Western blotting and representative Western blotting images in cortical and hippocampal neurons are shown on the left, with Coomassie staining as a loading control. The pro-BDNF and mature BDNF levels were measured from the same membrane and their protein levels are shown using the same exposition time. The bar graphs on the right show densitometric analysis of the expression levels of CREB, CRTC1, pro-BDNF, and mature BDNF normalized with Coomassie signal. The protein levels in untreated control neurons (EGFP- or neg gRNA-expressing neurons or neurons treated with DMSO) were taken as 1. Error bars represent mean ± SEM of four biological replicates ($n = 4$), and individual data points of each biological replicate are shown with dots. Statistical significance is shown relative to the respective protein levels in control cells at the respective time point of KCl treatment in respective culture. Group averages and exact *p*-values are shown in Extended Data Figure 6-1. #$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, paired two tailed *t* test, corrected for multiple comparisons using Holm method.

are expressed in a brain region-specific manner which could possibly explain their binding to BDNF promoters in certain brain regions. For that we used a previously published RNA-sequencing dataset (Carullo et al., 2020) to determine whether the identified transcription factors are differentially expressed in cultured neurons originating from different brain regions. First, we analyzed the levels of BDNF and determined that the levels were slightly higher in the cultured cortical neurons than in hippocampal neurons (Fig. 8*A*). Next, of the 88 transcription factors that we determined by *in vitro* pulldown assay, 17 showed at least

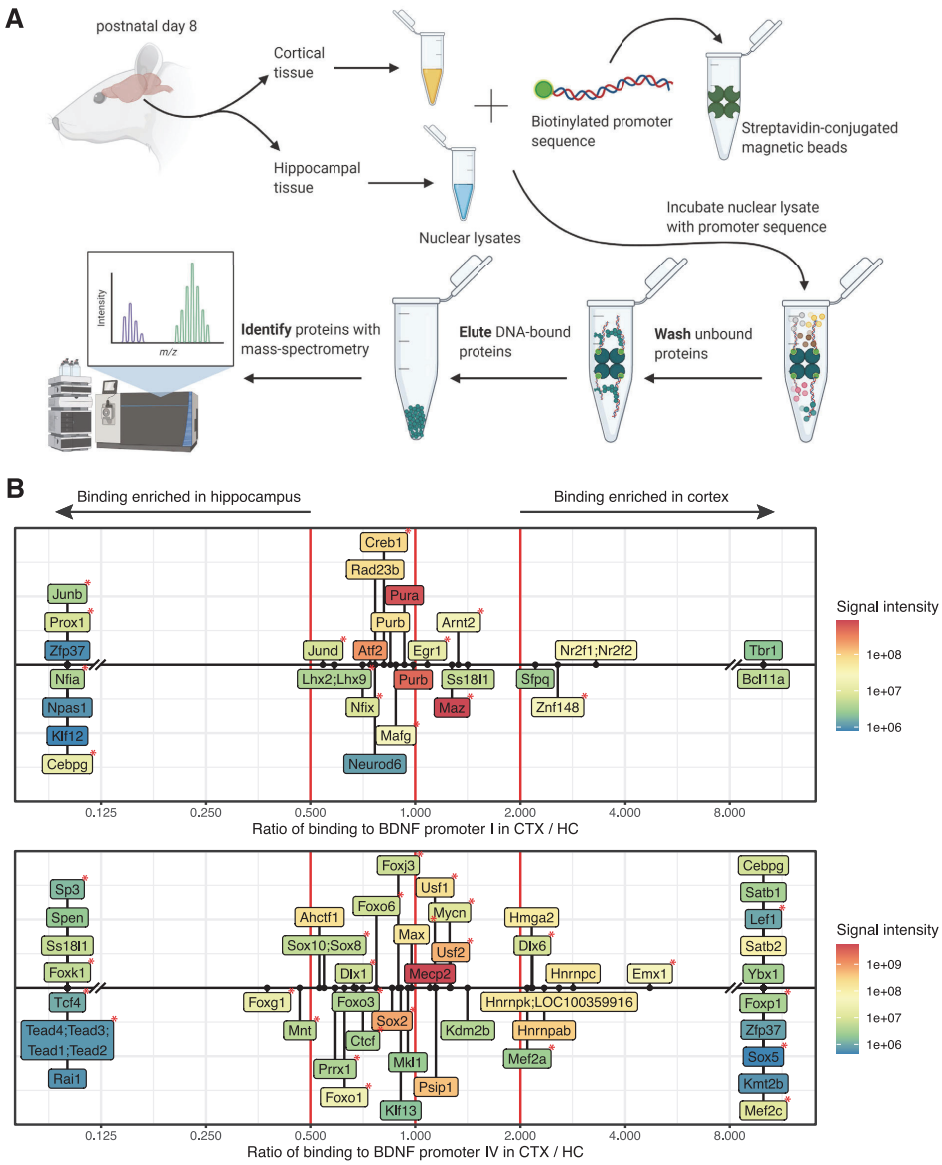

Figure 7. Numerous novel and previously reported transcription factors bind to BDNF promoters I and IV *in vitro*. ***A***, Schematic of the setup of the *in vitro* DNA pulldown experiment (schematics created with BioRender). Cortical and hippocampal tissues were isolated from 8-day-old Sprague Dawley rat pups, nondenaturing nuclear lysates were prepared and subjected to *in vitro* promoter pulldown. Transcription factors bound to BDNF promoters I and IV were determined by LC-MS/MS. All peptides, proteins and transcription factors detected in mass spectrometry are listed in Extended Data Figure 7-1. ***B***, Transcription factors showing promoter-specific binding to BDNF promoters I (upper panel) and IV (lower panel). Specific binding was determined as $\geq$1.5 ratio of binding signal to one promoter relative to the other promoter in the respective tissue sample. The figure depicts only transcription factors that were found to bind specifically to the respective promoter in either cortical or hippocampal sample. List of transcription factors with semicolons between gene names mark uncertainty in assigning the detected peptides to proteins. The red lines represent zero (no tissue-specific difference) and borders of 2-fold difference in label free quantification (LFQ) intensity between the two tissues. Colors indicate the maximal LFQ signal intensity for the indicated transcription factor in either cortical or hippocampal samples. The red asterisks mark transcription factors that have a binding site in the respective promoter according to Jaspar database (Fornes et al., 2020).

2-fold difference in expression levels between cultured cortical and hippocampal neurons (Fig. 8*B*; Extended Data Fig. 8-1). Of the novel factors that showed specific binding to BDNF promoter I, Prox1 has remarkably higher expression levels in hippocampal neurons, and Tbr1 is more highly expressed in cortical neurons (Fig. 8*B*), in agreement with our *in vitro* pulldown assay. Foxp1, Mef2c, Satb1, Satb2, and the high-mobility group (HMG) domain-containing Sox5, which showed preferential binding to BDNF promoter IV in cortical lysates, also have higher expression levels in cortical neurons than in hippocampal neurons (Fig. 8*B*).

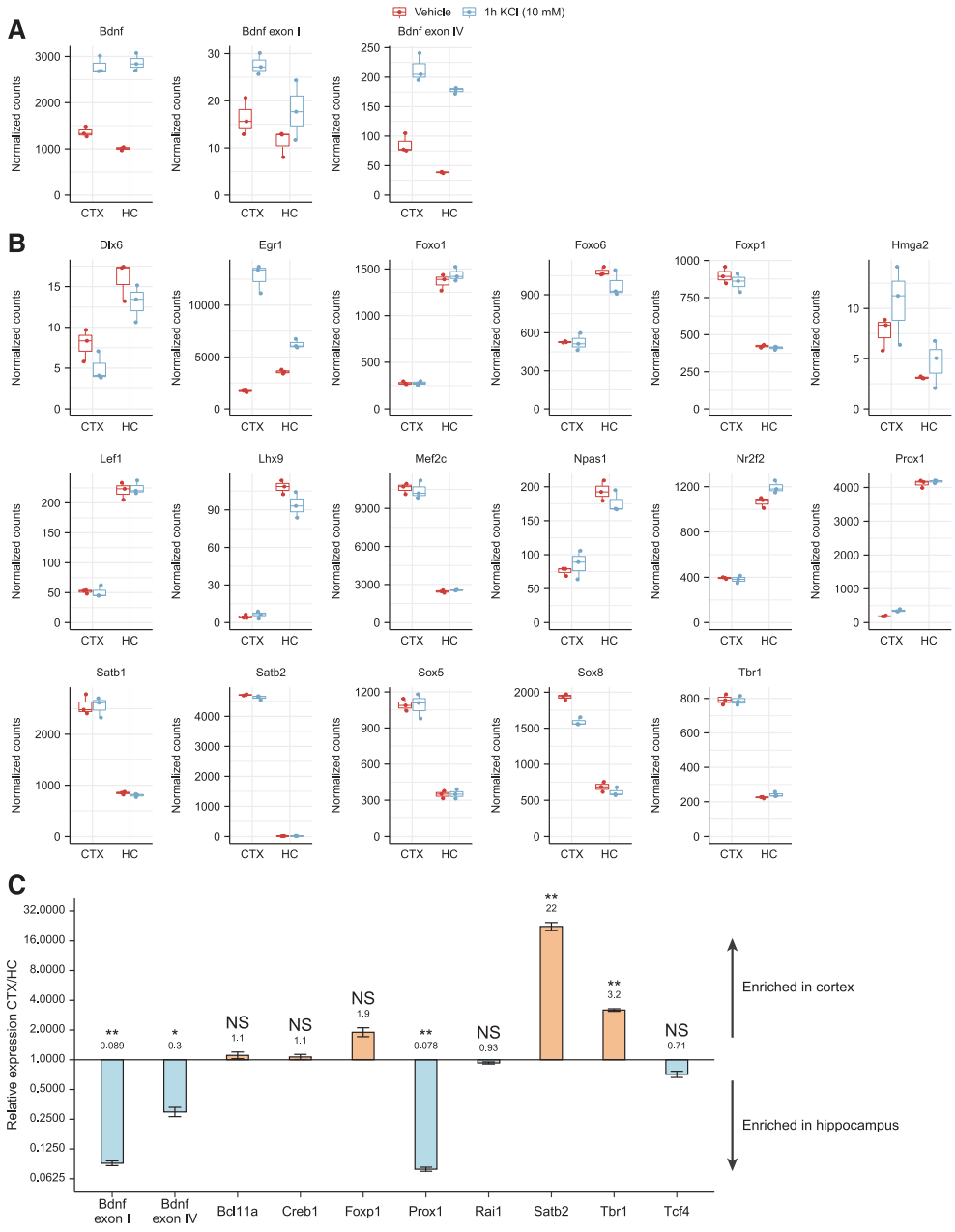

Figure 8. Various transcription factors that bind BDNF promoters *in vitro* are differentially expressed in cortical and hippocampal neurons and in cortex and hippocampus *in vivo*. ***A***, ***B***, RNA-sequencing (RNA-seq) data from embryonic day 18-derived rat cultured cortical (CTX) or hippocampal (HC) neurons treated with 10 mM KCl or vehicle for 1 h at 11 DIV (from Carullo et al., 2020) was used to analyze the expression levels of BDNF (***A***) and the transcription factors that we determined as specifically bound to BDNF promoter regions in the *in vitro* promoter pulldown experiment (***B***). Only transcription factors showing at least 2-fold expression difference between cultured cortical and hippocampal neurons in either untreated or KCl-treated neurons are shown. RNA-seq counts were normalized with DESeq2 and are shown as box plots, where the hinges show 25% and 75% quartiles, the horizontal line shows the median value, the upper whisker extends from the hinge to the largest value no further than 1.5× interquartile range from the hinge, the lower whisker extends from the hinge to the smallest value at most 1.5× interquartile range of the hinge. All data points are shown with dots. Expression data and statistical analysis for all the transcription factors detected to bind BDNF promoters in the *in vitro* pull-down assay are listed in Extended Data Figure 8-1. ***C***, mRNA levels of the selected transcription factors in cortex and hippocampus of P8 Sprague Dawley pups shown as relative to the expression levels in cortex measured by RT-qPCR (group averages and exact *p*-values are listed in Extended Data Fig. 8-2). The expression levels of the determined transcription factors in the human cortex based on the GTEx portal are shown in Extended Data Figure 8-3. Error bars represent upper and lower limits of back-transformed mean ± SEM of three different litters ($n = 3$). Statistical significance is shown compared with the expression levels in cortex. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, paired two tailed *t* test, corrected for multiple comparisons using Holm–Šídák method.

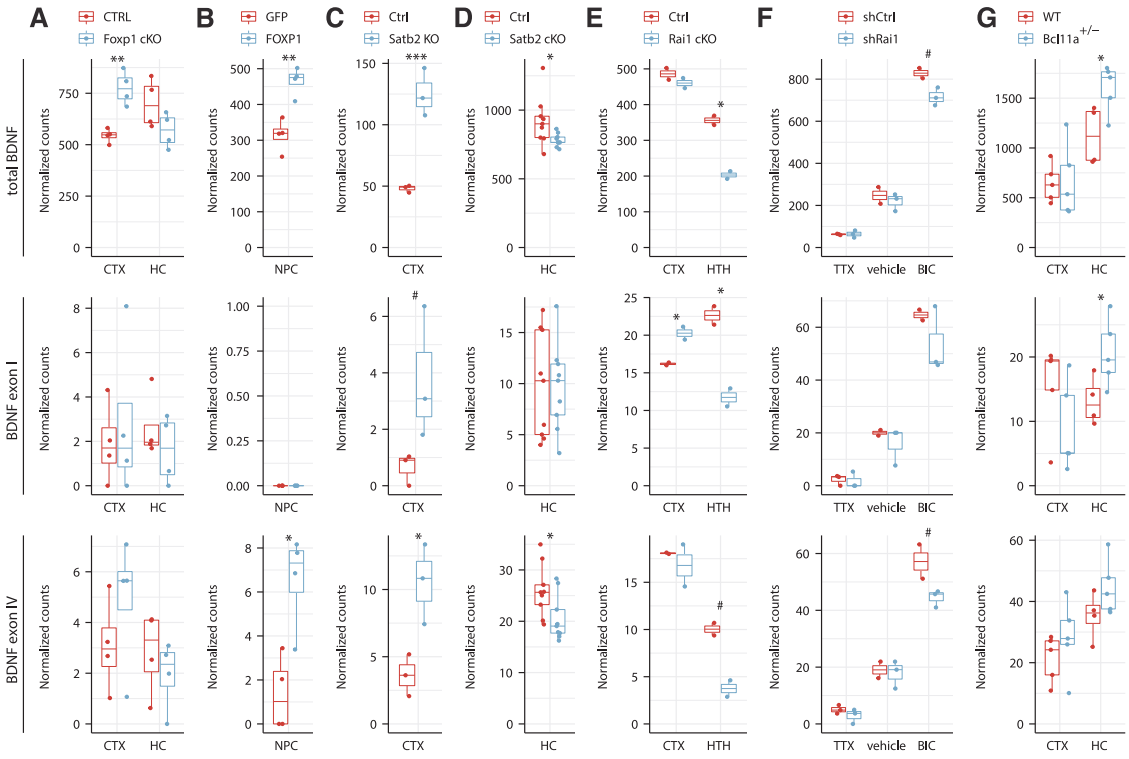

Figure 9. Transcription factors FOXP1, SATB2, RAI1, BCL11A regulate BDNF expression in a brain region-specific manner. Total BDNF levels (upper panels), levels of BDNF exon I (middle panels), and BDNF exon IV (lower panels) were analyzed from previously published RNA-seq experiments: (***A***) postnatal day 47 adult male mice cortex (CTX) and hippocampus (HC) in pyramidal neuron-specific Foxp1 conditional knock-out (cKO) animals using Emx1-Cre driver (Araujo et al., 2017); (***B***) human neural progenitor cells (NPC) overexpressing GFP or FOXP1 (Araujo et al., 2015); (***C***) postnatal day 0 CTX of Satb2 knock-out animals (McKenna et al., 2015); (***D***) adult mice HC CA1 region in Satb2 conditional knock-out in adult neurons using Camk2a-Cre driver (Jaitner et al., 2016); (***E***) three-week-old Rai1 flox/flox (Ctrl) and Rai1 cKO using Nestin-Cre driver in CTX and eight-week-old Rai1 cKO using Vglut2-Cre driver in hypothalamus (HTH; W.H. Huang et al., 2016); (***F***) Bru-seq to determine nascent transcripts in cultured cortical and hippocampal neurons derived from embryonic day 18 mice, grown 17 DIV and infected with lentiviruses expressing shRNA against Rai1 (shRai1) and treated with either tetrodotoxin (TTX), vehicle, or bicuculline (BIC) for 4 h (Garay et al., 2020); (***G***) CTX and HC of 16-week-old male wild-type (WT) or Bd11a heterozygous knock-out mice (Dias et al., 2016). RNA-seq counts were normalized with DESeq2 and are shown as box plots, where the hinges show 25% and 75% quartiles, the horizontal line shows the median value, the upper whisker extends from the hinge to the largest value no further than 1.5× interquartile range from the hinge, the lower whisker extends from the hinge to the smallest value at most 1.5× interquartile range of the hinge. All data points are shown with dots. Statistical analysis was performed on log-transformed data. #$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, unpaired equal variance two tailed *t* test.

Next, we decided to validate the expression levels of a selection of transcription factors found in our *in vitro* pulldown assay in the cerebral cortex and hippocampus of 8-day-old rat pups using RT-qPCR (Fig. 8*C*; Extended Data Fig. 8-2). Interestingly, we discovered that *in vivo*, both BDNF exon I and exon IV-containing transcripts are substantially more expressed in the hippocampus than in the cortex (Fig. 8*C*). Of the transcription factors, we determined that Prox1 was markedly more expressed in the hippocampus and Satb2 and Tbr1 were more expressed in the cortex (Fig. 8*C*), which is in agreement with the preference shown in the pulldown assay. Meanwhile, in agreement with no preference in binding in the pulldown assay, the expression of Creb1 was similar in both brain regions. Interestingly, Rai1 and Tcf4, that showed a strong preference in the *in vitro* pulldown assay, did not show remarkable differences in their expression levels between different brain regions. Finally, we verified the expression of the identified transcription factors in the human cortex and hippocampus using the data from the Genotype-Tissue Expression (GTEx) portal (Extended Data Fig. 8-3). Collectively, we determined that many of the novel region-specific transcription factors binding to BDNF promoter regions, e.g., TBR1, FOXP1, SATB1, SATB2, and PROX1, are differentially expressed, possibly explaining their brain region-specific binding to BDNF promoters and suggesting their potential role in tissue-specific regulation of BDNF expression.

Validation of brain region-specific regulators of BDNF promoters I and IV

Next, to study whether the determined transcription factors identified by us are brain region-specific regulators of BDNF, we searched Gene Expression Omnibus (GEO) database for previously published RNA-seq datasets investigating the factors that were determined by us to bind BDNF promoters in the *in vitro* pulldown assay. First, in Foxp1 conditional knock-out (cKO) animals (Araujo et al., 2017), total BDNF levels were slightly increased in the cortex but not in hippocampus (Fig. 9*A*). When analyzing BDNF expression at a transcript level, we determined no change in the levels of BDNF exon I-containing transcripts, however, in agreement with our *in vitro* DNA pulldown experiment, we determined a slight increase of BDNF

exon IV-containing transcripts in the FOXP1 cKO animals in cortex but not in hippocampus. Furthermore, overexpressing FOXP1 in human neural progenitor cells (Araujo et al., 2015) increased the levels of both total and BDNF exon IV-containing transcripts, whereas the levels of BDNF exon I-containing transcripts were undetectable in both control and FOXP1 overexpressing cells (Fig. 9*B*). These results suggest that FOXP1 is a novel regulator of BDNF exon IV-containing transcripts, with the exact outcome depending on the brain region and/or cell type.

Second, we investigated the role of Satb family transcription factors in the regulation of tissue-specific BDNF expression. It has been reported that total BDNF mRNA levels are lower in Satb1 knock-out mice and that SATB1 binds to the BDNF locus *in vivo* in cerebral cortex (Balamotis et al., 2012). Here, we found that in the cortex of postnatal day 0 Satb2 knock-out mice (McKenna et al., 2015), total BDNF levels were strongly increased (Fig. 9*C*). Similarly, the levels of BDNF exon I and exon IV-containing transcripts were both increased (Fig. 9*C*). In contrast, in the hippocampus of adult conditional knock-out of Satb2 mice (Jaitner et al., 2016) the BDNF expression levels were the same as in wild type (Fig. 9*D*). While these results are in agreement with our *in vitro* pulldown assay (Fig. 7*B*) and higher Satb2 expression levels in cortex (Fig. 8*B*,*C*), we acknowledge that different effects in different brain regions could also be because of the use of different genetic models of Satb2 knock-out or because of the difference in the age of the animals at the time of the analysis. Additional experiments are needed to confirm the role of SATB2 as a tissue-specific regulator of BDNF expression.

Third, we focused on RAI1, which showed binding to BDNF promoter IV preferably in the hippocampus in our *in vitro* pulldown experiment (Fig. 7*B*), and which has previously been shown to regulate BDNF expression in the hypothalamus (Burns et al., 2010; W.H. Huang et al., 2016). To decipher the role of RAI1 in the regulation of BDNF, we analyzed BDNF expression levels in various Rai1 conditional knock-out (cKO) animals (W.H. Huang et al., 2016). Our results indicate that cKO of Rai1 had minor or no effect on total BDNF expression in cortex of three-week-old animals, whereas a strong decrease in the levels of total BDNF, exon I and exon IV-containing transcripts was observed in the hypothalamus of eight-week-old animals (Fig. 9*E*). Although no data were available on the effect of Rai1 knock-out in the hippocampus, these results indicate that RAI1 regulates BDNF expression in a brain region-specific manner, although it is not possible to rule out developmental stage-specific effects. As RAI1 has been described to regulate activity-dependent gene expression (Garay et al., 2020), we also analyzed BDNF expression levels in cultured neurons where the expression of Rai1 was silenced (Garay et al., 2020), and noted a slight decrease in the BDNF expression levels in bicuculline-treated neurons on silencing Rai1 expression (Fig. 9*F*). However, this effect was similar for total BDNF levels and BDNF exon I and exon IV-containing transcripts (Fig. 9*F*). Our results suggest that RAI1 could be a stimulus-specific regulator of BDNF gene expression.

Finally, we investigated the role of BCL11A (also known as CTIP1) that bound to BDNF promoter I and showed stronger binding in the cortex in our promoter pulldown assay (Fig. 7*B*). In the heterozygous Bcl11a knock-out mice (Dias et al., 2016) total BDNF levels were increased in the hippocampus, but remained the same in the cortex (Fig. 9*G*). At the transcript level, Bcl11a knock-out slightly reduced BDNF exon I levels in the cortex, but increased the levels in the hippocampus (Fig. 9*G*). However, the levels of BDNF exon IV were increased in both cortex and hippocampus of Bcl11a knock-out mice (Fig. 9*G*). Collectively, these results indicate that BCL11A has different effect on BDNF gene expression in different brain regions, however, the exact mechanism needs further clarification.

Finally, we focused on the promising candidates of region-specific regulators of BDNF and investigated whether they bind *in vivo* to BDNF promoters using ChIP assay. We focused on the binding of CREB, BCL11A, and TCF4 and our results show that all these factors bind *in vivo* to a BDNF intronic enhancer that is located 3 kb downstream from BDNF promoter I (Fig. 10) as was previously described for CREB and TCF4 in cultured neurons (Tuvikene et al., 2021). Furthermore, all the factors bind to BDNF promoters, however, not as efficiently as to the BDNF +3 kb enhancer region (Fig. 10). First, while BCL11A does bind to BDNF promoters, it only displays brain region-preference for the +3-kb region. Next, CREB binding to both BDNF promoters (and +3 kb region) was very stable in both cortex and hippocampus, indicating a lack of brain region-dependent binding specificity that is in agreement with our *in vitro* pulldown assay. Finally, TCF4 showed a slight preference in binding to BDNF promoter I and strong binding to the +3-kb enhancer region. Unfortunately, as we could not find antibodies suitable for ChIP assay for FOXP1, SATB2, and RAI1, we could not analyze their binding *in vivo*. Collectively, our ChIP experiment suggests that BCL11A, CREB, and TCF4 bind to BDNF promoters *in vivo*, however, with no notable differences in binding between cortex and hippocampus.

Discussion

In this study, we investigated comparatively BDNF gene expression in response to neuronal signaling in cortical and hippocampal neurons and compared the role of CREB transcription factor family. Our results show that, as expected, CREB family transcription factors and specifically CREB in combination with its coactivators CBP and CRTC1 regulate the early induction of BDNF exon I and IV transcripts after neuronal signaling in cortical neurons. To our surprise, impairing the activity of CREB family or its coactivators had remarkably smaller effects on BDNF mRNA levels after prolonged stimulation than during the early time points of membrane depolarization. The rapid activation of CREB via posttranslational mechanisms and the lower levels of ICER, the endogenous negative regulator of the CREB family, could explain the faster inducibility of BDNF mRNA in cortical neurons. Although the early induction of BDNF mRNA seems to be strongly regulated by CREB family, the much higher expression levels of BDNF that are reached after prolonged neuronal signaling, seem to be mediated by other potent transcription factors that are synthesized as a second wave in response to neuronal activity.

Notably, the involvement of CREB family in the regulation of BDNF gene expression in hippocampal neurons appears to be complex. We made several observations on the regulation of BDNF exon I transcripts in hippocampal neurons: (1) overexpression of dominant-negative CREB and CREB knock-down had opposing effects on the basal levels of BDNF exon I transcripts, (2) inhibiting CBP recruitment had a profound effect on the late induction of BDNF exon I transcript, although neither overexpression of A-CREB nor CREB knock-down had such strong effect, and (3) expression of BDNF exon I transcripts was increased after CREB or CRTC1 knock-down, indicating their potential (probably indirect) repressor activities. Notably, the effect of CRTC1 knock-down on increased BDNF exon I mRNA levels also seems to result in higher BDNF protein levels in

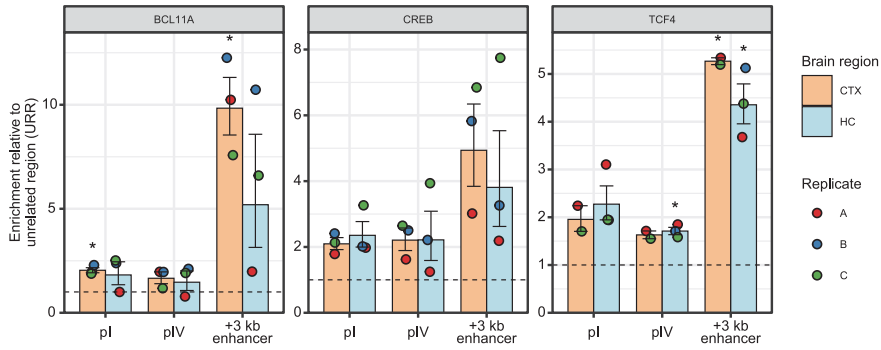

Figure 10. BCL11A, CREB, and TCF4 bind to BDNF promoters *in vivo*. ChIP-qPCR analysis of BCL11A, CREB, and TCF4 binding to BDNF intronic enhancer located +3 kb downstream of BDNF promoter I (+3 kb enhancer), BDNF promoters I (pI) and IV (pIV) in the cortex and hippocampus of 8-d-old Sprague Dawley pups. Data are shown as percent of input relative to the binding of respective factor to unrelated region (URR). All data points are shown as dots, and each biological replicate (i.e., animals from different litters) is denoted with the same color. Error bars represent mean ± SEM of two or three different litters ($n = 2$–3). Statistical significance is shown compared with the binding of the respective factor to URR in the respective brain region. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, paired two tailed *t* test, corrected for multiple comparisons using Holm–Šídák method.

hippocampal neurons. Compared with cortical neurons where both CRTC1 and CBP participated in the induction of BDNF exon IV transcripts, CRTC1 was clearly the dominant coactivator in hippocampal cells. Collectively, this data show that in cortical and hippocampal neurons BDNF gene regulation is different at the level of CREB transcription factor family.

An interesting implication of our results is that the feedback loop regulating CREB family-dependent gene expression is different between cortical and hippocampal neurons. While the regulation of CREM activator forms by CREB family transcription factors was similar in both cortical and hippocampal neurons, we noted differences in the regulation of the endogenous negative regulator of CREB family, ICER, between the two neuronal populations. Namely, in cortical neurons neuronal activity-dependent induction of ICER was stronger and CREB knock-down had a more profound effect on its neuronal activity-dependent expression than in hippocampal neurons. Furthermore, knock-down of CRTC1 had stronger effect on the induction of ICER in cortical than in hippocampal neurons although the knock-down itself was stronger in hippocampal neurons. It would be of interest to investigate how the differences in the CREB family negative feedback loop affect CREB-dependent signaling.

Our results indicate that CREB family is a positive regulator of the expression of BDNF exon IV, but is a negative regulator of BDNF exon VI in neurons after membrane depolarization. In contrast, CREB family is a positive regulator of both transcripts after BDNF-TrkB signaling (Esvald et al., 2020). It could be explained by suppression of the expression of BDNF exon VI by transcriptional interference arising from transcription starting from BDNF promoter IV. Also, as CBP is highly associated with enhancer regions and impairing CBP recruitment decreased the induced levels of BDNF exon VI, it is also plausible that BDNF gene expression relies on a specific 3D chromatin structure and enhancer region(s) that are regulated by CBP recruitment. Competitive looping between specific promoter and enhancer regions would explain the opposite effects seen on BDNF exon IV and VI expression levels. We have previously shown that an intronic enhancer region governs the regulation of first cluster of BDNF exons, but not exons IV or VI (Tuvikene et al., 2021), showing that enhancers could potentiate only specific BDNF promoter regions. Further work will delineate the opposite effects of CREB family on BDNF exon IV and VI levels.

Neuronal signaling is known to cause BDNF secretion (Sasi et al., 2017; M. Song et al., 2017) and the released BDNF can signal the cells in an autocrine or paracrine manner by binding to TrkB receptor to induce BDNF mRNA production (Tuvikene et al., 2016; Esvald et al., 2020). Previous reports have shown that infusion of BDNF to the adult rat hippocampus can elicit the expression of different BDNF transcripts (Wibrand et al., 2006; Esvald et al., 2020), implying that BDNF-TrkB signaling is also relevant for BDNF expression in the hippocampus. Our previous work has suggested that BDNF transcriptional autoregulation and membrane depolarization-induced expression of BDNF are regulated via distinct mechanisms in cortical neurons (Esvald et al., 2020). Here, we note that TrkB signaling slightly contributes to neuronal activity-dependent expression of BDNF in cortical neurons, but not in hippocampal neurons. Further work is necessary to understand why the TrkB signaling participates in cortical but not in hippocampal neurons in activity-regulated expression of BDNF, and what is the biological implication of this difference.

Finally, we focused on deciphering brain region-specific regulators of BDNF and determined numerous transcription factors that are involved in BDNF transcriptional regulation. Interestingly, we detected transcription factors from all the transcriptional waves of neuronal maturation during cortical neurogenesis (Telley et al., 2016) and many of the determined transcription factors are important for neuronal differentiation and formation of specific brain regions. For example, FOXP1 is needed for the differentiation of neural stem cells (Braccioli et al., 2017), SATB2 for the differentiation of subcerebral projection neurons (McKenna et al., 2015), BCL11A for brain development (Dias et al., 2016) and the migration and formation of polarity of cortical neurons (Wiegreffe et al., 2015), TCF4 for neuronal migration and cortical layering (Page et al., 2018; Li et al., 2019; Wittmann et al., 2021) and other processes of brain development (Kennedy et al., 2016; Thaxton et al., 2018; Phan et al., 2020; Schoof et al., 2020), TBR1 for synapse formation in cortical layer 6 (Fazel Darbandi et al., 2018), and PROX1 for suppression of neurite outgrowth (Kaltezioti et al., 2020) and determining granular cell identity in the dentate gyrus (Iwano et al., 2012). Collectively, this array of different transcription factors implies that BDNF has a complex spatiotemporal regulation depending on the developmental stage and brain

region. In support of this, it has also been shown that NURR1 transcription factor regulates BDNF expression in midbrain (Volpicelli et al., 2007) but not in cortical neurons (Abdollahi and Fahnestock, 2022). It is plausible that BDNF is an effector molecule guiding the development of the nervous system and formation of neuronal circuits. Moreover, many of the transcription factors we identified as novel regulators of BDNF gene expression have been linked to various neurodevelopmental diseases. For example, in humans mutations in Foxp1 gene cause severe forms of autism spectrum disorder and intellectual disability (Hamdan et al., 2010; Horn et al., 2010) and in mice FOXP1 regulates genes implicated in autism (Araujo et al., 2015) and Foxp1 deletion causes anxiety-like behavior, impaired social communication and spatial learning (Bacon and Rappold, 2012; Araujo et al., 2017), alterations of Satb2 gene cause SATB2-associated syndrome characterized by delayed development and intellectual disability (Zarate and Fish, 2017), mutations of Bcl11a gene lead to intellectual disability and autism spectrum disorders (Simon et al., 2020), TCF4 haploinsufficiency causes syndromal mental retardation – Pitt-Hopkins syndrome (Amiel et al., 2007; Brockschmidt et al., 2007; Zweier et al., 2007), Tbr1 is one of the highly associated genes in autism (O'Roak et al., 2012) and its knock-out causes increased anxiety and aggression in adult mice (Fazel Darbandi et al., 2018) as well as behavioral abnormalities that resemble autism (T.N. Huang et al., 2014). Notably, many of the phenotypes caused by defects in these transcription factors resemble those obtained with altered levels of BDNF, e.g., hyperactivity, aggressiveness, obesity, memory problems, lower intellect, and autism (Chao, 2003; Gray et al., 2006; Han et al., 2013; Zheng et al., 2016; Skogstrand et al., 2019). It is tempting to speculate that some of the phenotypes observed when these transcription factors are perturbed could arise from the insufficiency of BDNF. For example, haploinsufficiency of RAI1, which is required for motor functions and learning (Bi et al., 2007; W.H. Huang et al., 2016), causes Smith-Magenis syndrome (SMS; Slager et al., 2003; Bi et al., 2004). Some phenotypic effects of SMS, including obesity, have been explained by decreased levels of BDNF in the hypothalamus (Burns et al., 2010; W.H. Huang et al., 2016) and overexpression of BDNF has been shown to be an effective therapeutic approach for SMS (Javed et al., 2021). However, further work is needed to confirm this hypothesis for other novel BDNF regulators.

Our results deepen the understanding of BDNF gene expression, highlighting the complex regulation of BDNF in different brain regions. This data could give insight for understanding various neurodevelopmental disorders but also for developing therapeutics to modulate the expression of BDNF in a brain region and cell type-specific manner. However, a substantial amount of work is still required to understand the exact mechanisms that govern BDNF gene expression, including chromatin conformation, the set of transcription factors expressed in a given cell, their posttranslational modifications and the different participating signaling pathways in different neuronal populations.

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Publication III

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

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RESEARCH ARTICLE

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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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.

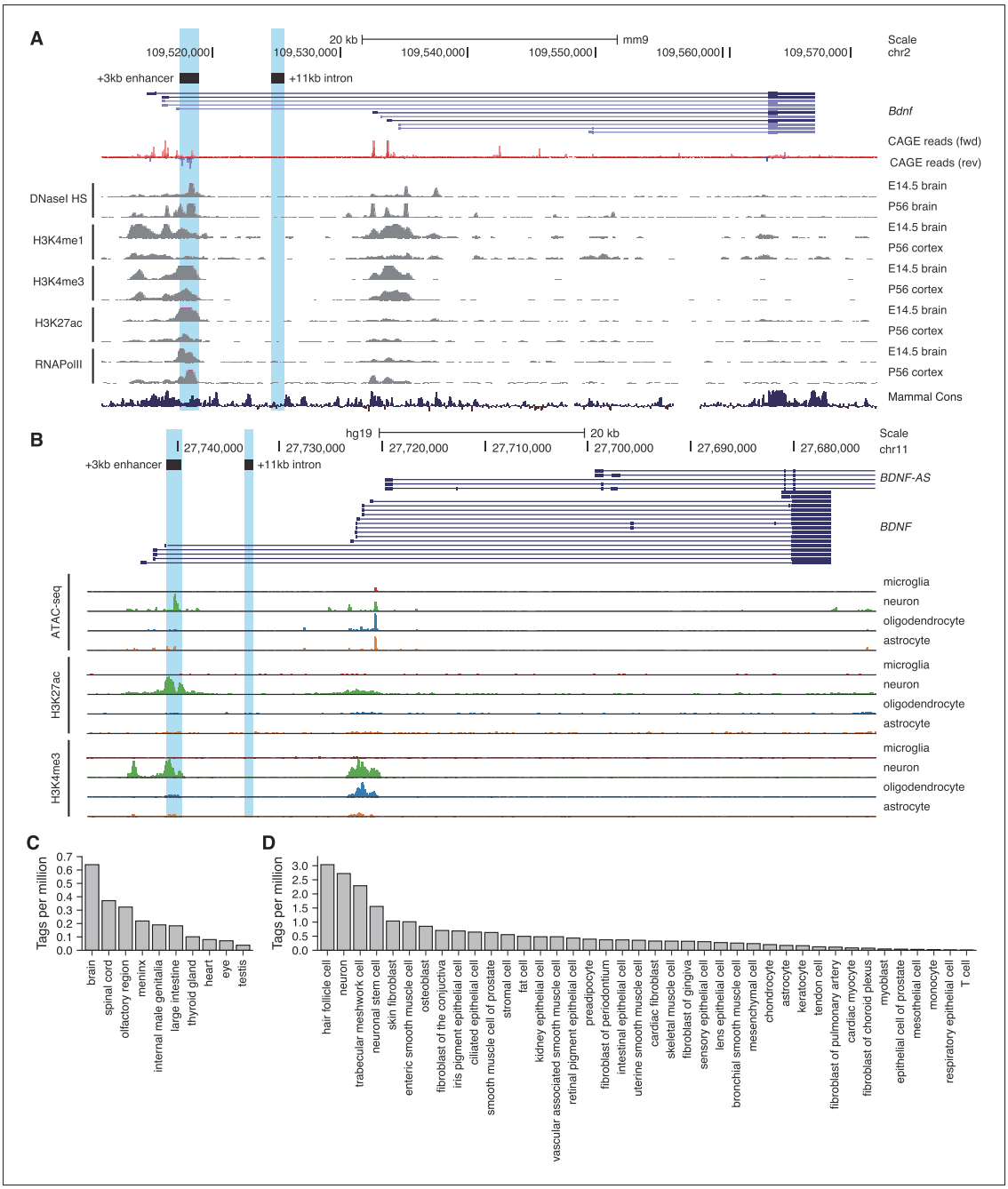

Figure 1. Region downstream of *Bdnf* exon III shows enhancer-associated characteristics in mouse and human neural tissues. UCSC Genome browser was used to visualize (**A**) DNaseI hypersensitivity sites and ChIP-seq data from the ENCODE project in mouse brain tissue, CAGE data of transcription start 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.

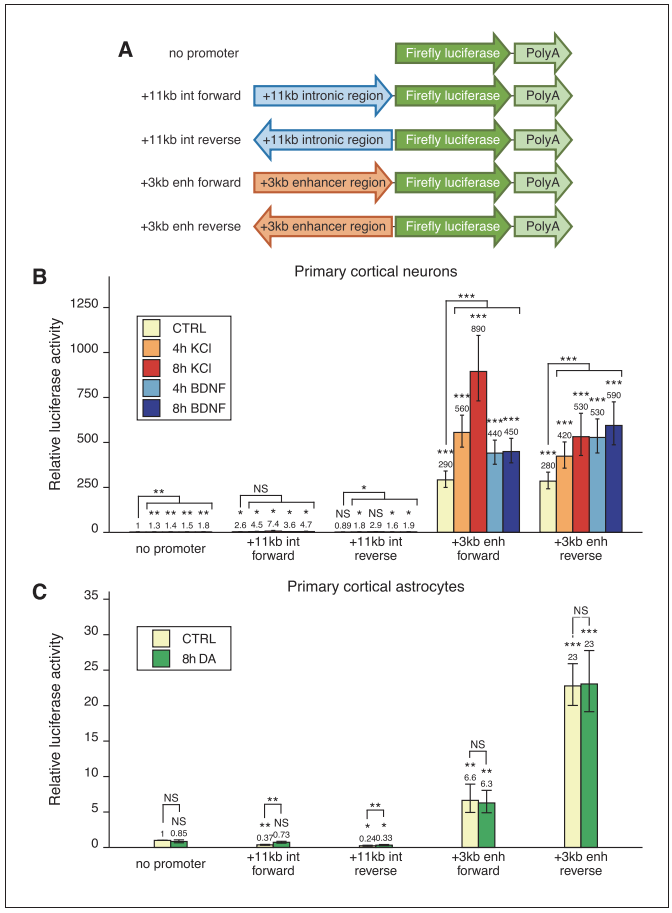

Figure 2. The +3 kb enhancer region shows bidirectional transcription in luciferase reporter assay in rat cultured cortical neurons and astrocytes. (**A**) Reporter constructs used in the luciferase reporter assay where the +3 kb enhancer region and the +11 kb control region were cloned in either forward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

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 (respective 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

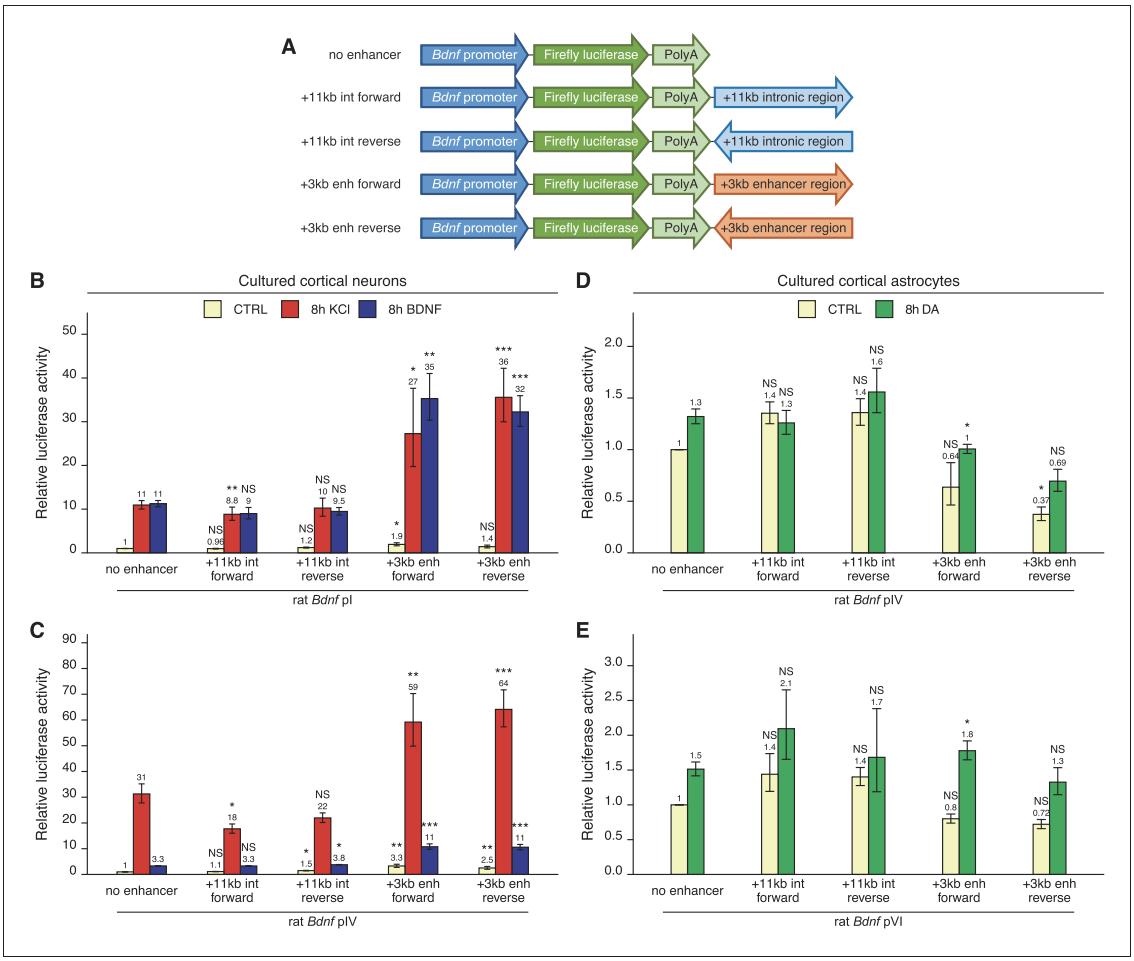

Figure 3. The +3 kb enhancer region potentiates the activity of *Bdnf* promoters in luciferase reporter assay in rat cortical neurons but not in astrocytes. (**A**) A diagram of the luciferase reporter constructs used in this experiment, with a *Bdnf* promoter in front of the firefly luciferase coding sequence and the +3 kb enhancer or +11 kb intronic region in either forward or reverse orientation (respective to the rat *Bdnf* gene) downstream of the luciferase expression cassette. Rat cortical neurons (**B, C**) or astrocytes (**D, E**) were transfected with the indicated reporter constructs at 6 and 13 DIV, respectively. Two days post transfection, neurons were left untreated (CTRL) or treated with 25 mM KCl (with 5 µM D-APV) or 50 ng/ml brain-derived neurotrophic factor (BDNF) for 8 hr (**B, C**); astrocytes were treated with 150 µM 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

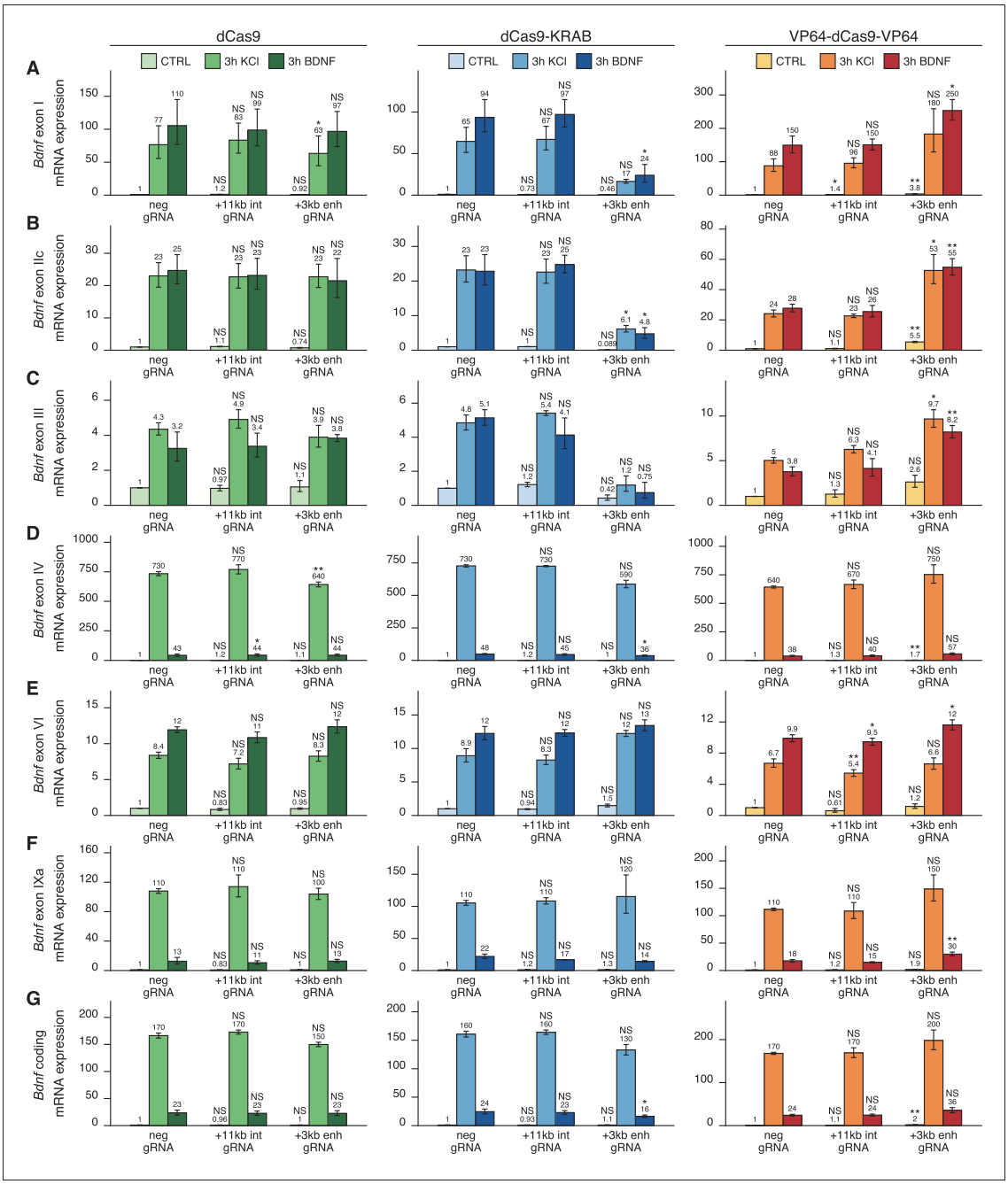

Figure 4. The +3 kb enhancer is a positive regulator of *Bdnf* exon I-, IIc-, and III-containing transcripts in rat cortical neurons. Rat cultured cortical neurons were transduced at 0 DIV with lentiviral particles encoding either catalytically inactive Cas9 (dCas9, left panel, green), dCas9 fused with 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 μM 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.

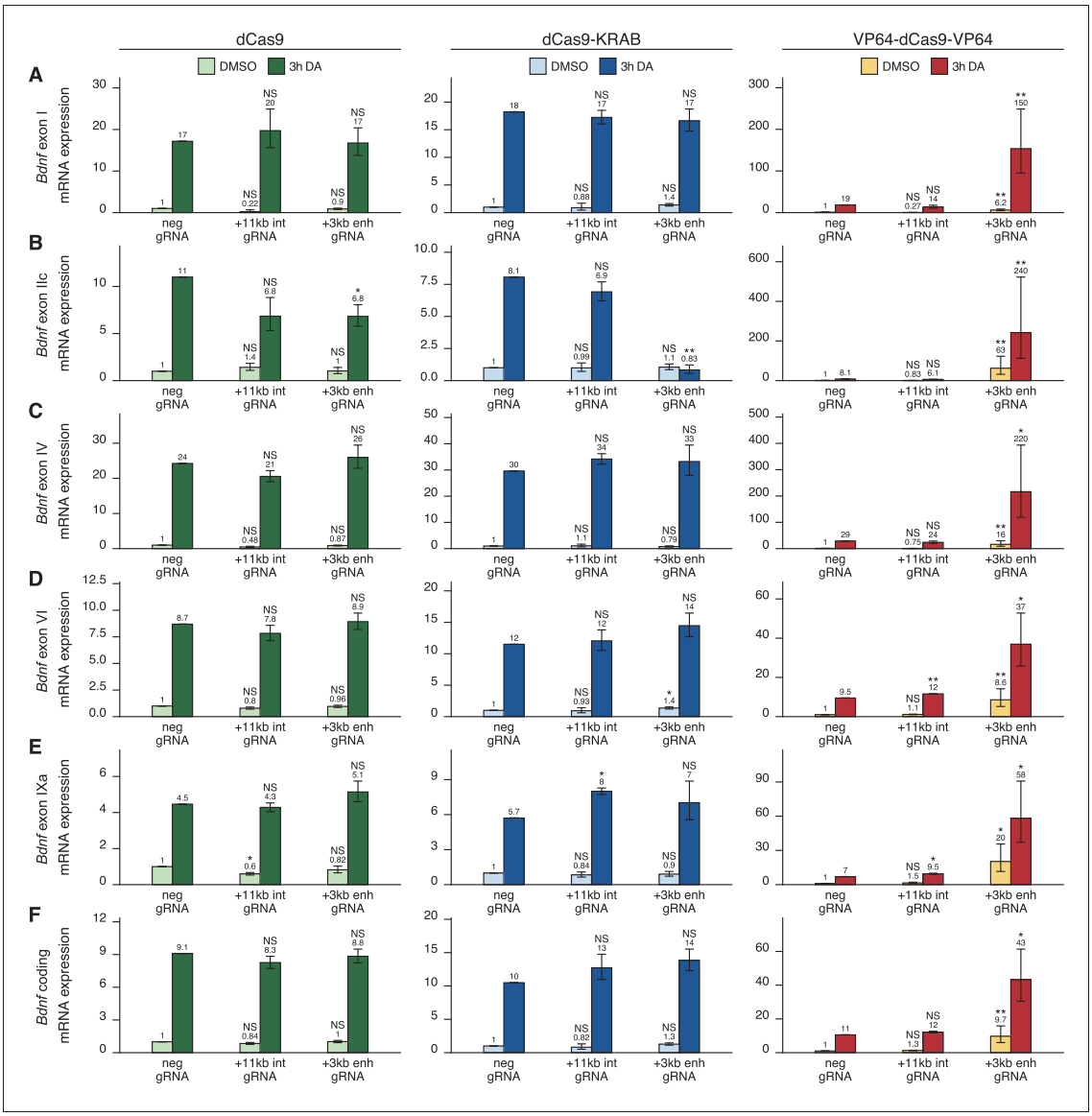

Figure 5. The +3 kb enhancer region is mainly inactive in rat cortical astrocytes. Rat cultured cortical astrocytes were transduced at 7 DIV with lentiviral particles encoding either catalytically inactive Cas9 (dCas9, left panel, green), dCas9 fused with Krüppel-associated box domain (dCas9-KRAB, middle 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 µM 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-, IIc-, and III-containing transcripts (***Figure 7A–C***). Notably, the effect was more prominent in clones containing homozygous deletion compared to heterozygous clones. We also noted a slight, albeit not statistically significant decrease in the expression of

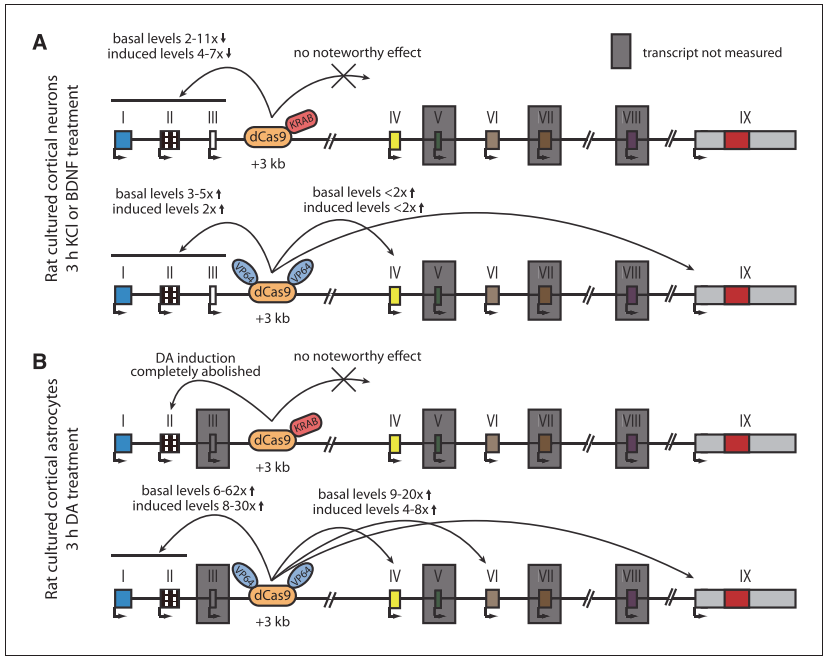

Figure 6. Summary of the CRISPRi and CRISPRa experiments with *Bdnf* +3 kb enhancer region in rat cultured cortical neurons and astrocytes. Graphical representation of the main results shown in ***Figures 4*** (**A**) and ***5*** (**B**). Different *Bdnf* exons are shown with boxes, and red box in exon 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.

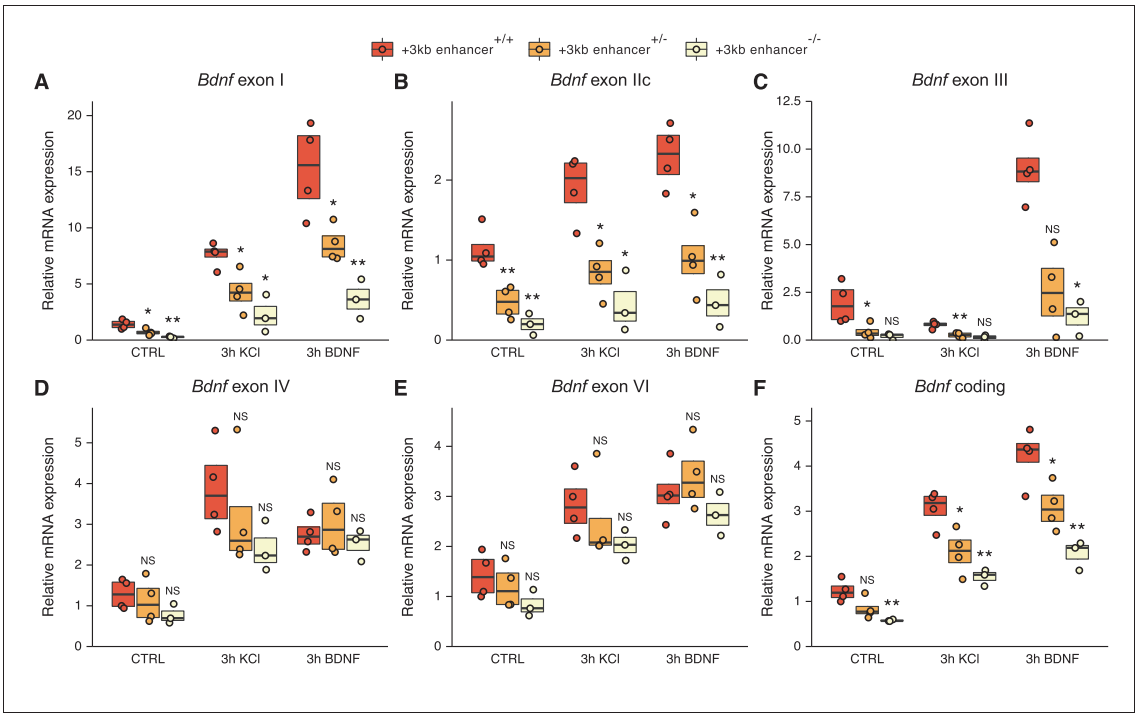

Figure 7. The deletion of the +3 kb enhancer region decreases the expression of *Bdnf* exon I-, IIc-, and III-containing transcripts in mouse embryonic stem cell (mESC)-derived neurons. CRISPR/Cas9 system was used to generate mESC cell lines with ~300–500 bp deletions of the conserved core region of 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 μM 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***).

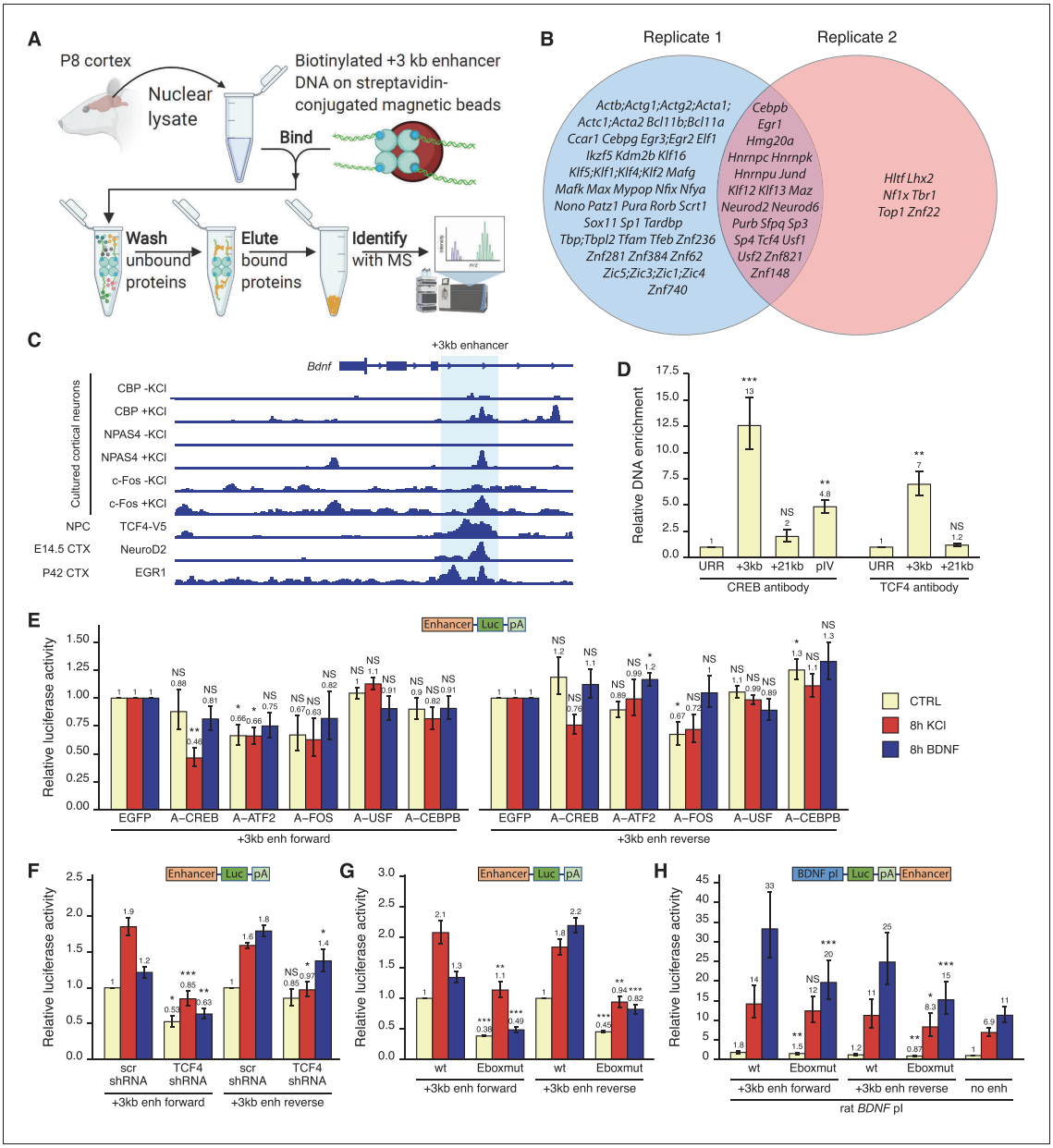

Figure 8. Various transcription factors, including CREB, AP-1 proteins, and E-box-binding transcription factors, regulate the activity of the +3 kb enhancer region. (**A**) Schematic overview of the in vitro DNA pulldown assay to determine transcription factors binding to the +3 kb enhancer region. The illustration was created with BioRender.com. (**B**) Gene names of the transcription factors identified in the in vitro DNA pulldown 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 µM 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 (**Esvald 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_2 environment. At 2 days in vitro (DIV), half of the medium was replaced with fresh supplemented NBA or the whole medium was replaced for cells transduced with lentiviruses. To inhibit the proliferation of non-neuronal cells, a mitotic inhibitor 5-fluoro-2′-deoxyuridine (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^2 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_2 environment. At 1 DIV, the medium was replaced with fresh growth medium to remove loose tissue clumps. At 6 DIV, the flasks were placed into a temperature-controlled shaker Certomat BS-1 (Sartorius Group, 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 (respective to the rat *Bdnf* gene). The *Bdnf* promoter regions were obtained from rat *Bdnf* promoter constructs (***Esvald et al., 2020***) and cloned in front of the firefly luciferase coding sequence. Plasmids encoding control and TCF4 shRNA have been published previously (***Sepp et al., 2017***). Coding regions of different dominant negative transcription factors were subcloned from AAV plasmids (***Esvald et al., 2020***) into pRRL vector backbone under the control of human *PGK* promoter.

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

Cultured cortical neurons were transfected as described previously (***Jaanson et al., 2019***) with minor modifications. Transfection was carried out in unsupplemented NBA using 500 ng of the luciferase reporter construct and 20 ng of a normalizer plasmid pGL4.83-mPGK-hRLuc at 5–6 DIV using Lipofectamine 2000 (Thermo Scientific) with DNA to Lipofectamine ratio of 1:2. Transfection was terminated by replacing the medium with conditioned medium, which was collected from the cells before transfection.

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

The cells were lysed with Passive Lysis Buffer (Promega, 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$(dT)_{20}$ or a mixture of oligo$(dT)_{20}$ 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 µl volume in triplicates with 1× 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 µM 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 $MgCl_2$, 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_3VO_4 [ChemCruz, Dallas,TX], and 1 mM $Na_4P_2O_7$ [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 µm nylon cell strainer (VWR) to remove tissue debris and the flow-through was centrifuged at 2600 × *g* at 4˚C for 1 min to pellet nuclei. The supernatant (cytoplasmic fraction) was discarded and the nuclear pellet was resuspended in 1 ml per 1 g of tissue ice-cold nuclear lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 2.5% glycerol, 1x cOmplete Protease Inhibitor Cocktail [Roche, 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 × *g* at 4˚C for 10 min. The supernatant was collected as nuclear fraction, and protein concentration was measured with BCA Protein Assay Kit (Pierce).

In vitro DNA pulldown was performed as follows. Two biological replicates were performed using nuclear lysates of cortices from pups of different litters. Pierce Streptavidin Magnetic Beads (50 µl per pulldown reaction) were washed 2 times with 1× binding buffer (BB, 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl, 0.05% Tween-20), resuspended in 2× BB, and an equal volume of 50 pmol biotinylated DNA (in 10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA) was added and incubated at room temperature for 30 min with rotation. To remove the unbound probe, the beads were washed three times with 1× BB. Finally, 400 µg of nuclear proteins (adjusted to a concentration of 1.6 mg/ml with nuclear lysis buffer) and an equal volume of buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 8% glycerol, 1x cOmplete Protease Inhibitor Cocktail [Roche, 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× 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***.

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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.

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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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Additional files

Supplementary files

- 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	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21161	NCBI Gene Expression Omnibus, GSE21161
Nord AS, Visel A, Pennacchio LA	2013	Rapid and Pervasive Changes in Genome-Wide Enhancer Usage During Mammalian Development	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52386	NCBI Gene Expression Omnibus, GSE52386
Malik AN	2014	Genome-wide identification and characterization of functional neuronal activity-dependent enhancers	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60192	NCBI Gene Expression Omnibus, GSE60192
Dunn GI	2015	NeuroD2 ChIP-SEQ from embryonic cortex	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67539	NCBI Gene Expression Omnibus, GSE67539
Brandsma JH, Moen MJ, Poot RA	2016	A protein interaction network of mental disorder factors in neural stem cells	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70872	NCBI Gene Expression Omnibus, GSE70872
Sun Z, Sun M, He J, Xie H	2019	Genome-wide maps of EGR1 binding in mouse frontal cortex	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67482	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"	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150589	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"	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150499	NCBI Gene Expression Omnibus, GSE150499

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Curriculum vitae

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Education

2017–2023	Tallinn University of Technology, PhD
2014–2017	Tallinn University of Technology, MSc (Gene technology), diploma *cum laude*
2010–2041	Tallinn University of Technology, BSc (Applied chemistry and biotechnology), diploma *cum laude*
2007–2010	Gustav Adolf Grammar School, with honours

Language competence

Estonian	Native
English	Fluent

Employment

September 2017– ...	Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology, Early-Stage Researcher
2018–2022	Protobios LLC, Researcher
November 2018– February 2018	King's College London, England, visiting scientist
February 2014– June 2014	Tallinn University of Technology, Faculty of Science, Department of Gene Technology, Other staff
May 2014– May 2015	East Tallinn Central Hospital, Laboratory Assistant
February 2013– January 2014	Republic of Estonia Health Board, Laboratory Specialist
2011–2013	AHHAA Science Centre, Presenter of science theatre
June 2011– August 2012	Estonian Environmental Research Institute, Laboratory technician in fuels quality control, environmental, and air quality laboratory

Awards

2022	Kristjan Jaak scholarship and Cajal's stipend to participate in course "Neuroepigenetics: writing, reading and erasing the epigenome"
2022	FENS-IBRO/PERC travel grant to FENS Forum 2022

2021	Ann Mihkelson's scholarship, Estonian National Culture Foundation
2021	Erkki Truve Doctoral study scholarship, Tallinn University of Technology
2017	The best scientific publication of Tallinn University of Technology in the field of natural, exact, and medical sciences in 2016
2017	Estonian National Contest for University Students 2017, II prize in the field of bio- and environmental sciences

Research training

November 2022–December 2022	Cajal Neuroscience Training Course "Neuroepigenetics: writing, reading and erasing the epigenome", Bordeaux, France
March 2022	Participating in virtual conference "Experience-Dependent Transcription – From Genomic Mechanisms to Neural Circuit Function"
November 2021	Neuroscience 2021 virtual conference
August 2020	Virtual conference "Epigenetics in Neurobiology"
August 2019	Course "Baltic summer school on behavioural characterization of rodent models of major brain disorders", Pühajärve, Estonia
September 2018	Course "Developmental Biology Minisymposium", Tallinn, Estonia
September 2018	Workshop "From Nanotechnology to Nanomedicine 2018", Tallinn, Estonia
January 2018	Conference "Teaching for Learning – the University Perspective", Tartu, Estonia

Presentations at conferences

2022	FENS Forum 2022, Paris, France, poster presentation "Novel stimulus-dependent regulators of BDNF gene expression"
2022	Sigrid Juselius Symposium, Espoo, Finland, poster presentation "Novel stimulus-dependent regulators of BDNF gene expression"
2021	Virtual NGF webinar series, oral presentation "Novel brain region- and stimulus-specific regulators of the BDNF gene"
2020	FENS virtual conference, poster presentation "Differential regulation of the BDNF gene by CREB family transcription factors in cultured cortical and hippocampal neurons"

2019	Nordic Neuroscience 3, Helsinki, Finland, poster presentation "CREB and BDNF transcriptional autoregulation"
2018	SZTest mini-symposium, Tallinn, Estonia "Gene Expression in Health and Disease", Tallinn, Estonia, oral presentation "CREB and BDNF transcriptional autoregulation"
2018	Neuroscience 2018, San Diego, USA, poster presentation "CREB and BDNF transcriptional autoregulation"
2018	International Conference on Neurotrophic Factors, Salamanca, Spain, poster presentation "CREB and BDNF transcriptional autoregulation"

Supervision

February 2022– June 2023	Supervised Erasmus exchange student Enrico di Marco. Thesis title: *Investigating potential neuronal activity-regulated enhancers*
November 2021– June 2023	Supervised bachelor student Annabel Parkman. Thesis title: *Characterization of marker gene expression in cultured neurons and astrocytes*
September 2020– …	Co-supervising master student Susann Kõomägi on creating biotin-tagged transcription factors and determining their interaction partners using co-immunoprecipitation and mass-spectrometry
September 2020– June 2021	Supervised bachelor student Neeme Raidvere. Thesis title: *Chromatin affinity purification using dCas9-based CAPTURE and CASPEX systems*
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March 2018– June 2019	Supervised bachelor student Käthy Rannaste. Thesis title: *The role of the transcription factor CREB and its coactivator CRTC1 in the activity-dependent transcription of the neurotrophin BDNF in cortical and hippocampal neurons*
September 2017– June 2018	Supervised bachelor student Susann Kõomägi. Thesis title: *Neuronal activity-dependent regulation of BDNF gene in cortical and hippocampal neurons*

Publications

Esvald EE*, Tuvikene J*, Kiir CS*, Avarlaid A, Tamberg L, Sirp A, Shubina A, Cabrera-Cabrera F, Pihlak A, Koppel I, Palm K, Timmusk T. (2023). Revisiting the expression of BDNF and its receptors in mammalian development. Frontiers in Molecular Neuroscience, 16.

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Esvald EE, Tuvikene J, Moistus A, Rannaste K, Kõomägi S, Timmusk T. (2022). Differential regulation of the BDNF gene in cortical and hippocampal neurons. Journal of Neuroscience, 42, 9110−9128.

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, 10, e65161.

Esvald EE*, Tuvikene J*, Sirp A, Patil S, Bramham CR, Timmusk T. (2020). CREB family transcription factors are major mediators of BDNF transcriptional autoregulation in cortical neurons. Journal of Neuroscience, 40, 1405−1426.

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

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Kontaktandmed

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Hariduskäik

2017–2023	Tallinna Tehnikaülikool, PhD
2014–2017	Tallinna Tehnikaülikool, MS (geenitehnoloogia), diplom *cum laude*
2010–2014	Tallinna Tehnikaülikool, BSc (rakenduskeemia ja biotehnoloogia), diplom *cum laude*
2007–2010	Gustav Adolfi Gümnaasium, kuldmedal

Keelteoskus

Eesti keel	emakeel
Inglise keel	kõrgtase

Teenistuskäik

September 2017– ...	Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut, doktorant-nooremteadur
November 2018–Veebruar 2019	King's College London, Inglismaa, külalisteadlane
2018–2022	Protobios OÜ, teadur
Veebruar 2014–Juuni 2014	Tallinna Tehnikaülikool, Loodusteaduskond, Geenitehnoloogia instituut, insener
Mai 2014–Mai 2015	Ida-Tallinna keskhaigla, laboriassistent
Veebruar 2013–Jaanuar 2014	Terviseamet, laborant
2011–2013	AHHAA teaduskeskus, teadusteatri läbiviija
Juuni 2011–August 2012	Eesti Keskkonnauuringute Keskus, laborant kütuselaboris, keskkonna ja analüütilise keemia laboris ning õhukvaliteedi osakonnas

Stipendiumid ja tunnustused

2022	Kristjan Jaagu ja Cajali instituudi stipendiumid, et osaleda kursusel "*Neuroepigenetics: writing, reading and erasing the epigenome*"
2022	FENS-IBRO/PERC stipendium, et osaleda FENS Forum 2022 konverentsil

2021	Ann Mihkelsoni stipendium, Eesti Rahvuskultuuri Fond
2021	Erkki Truve nimeline doktoriõppe stipendium, Tallinna Tehnikaülikool
2017	Tallinna Tehnikaülikooli 2016. aasta parim teadusartikkel loodus-, täppis- ja terviseteaduste valdkonnas
2017	Üliõpilaste teadustööde riiklik konkurss 2017, II preemia bio- ja keskkonnateaduste valdkonnas

Kursused ja konverentsid

November 2022– Detsember 2022	Cajali instituudi praktiline kursus "*Neuroepigenetics: writing, reading and erasing the epigenome*", Bordeaux, Prantsusmaa
Märts 2022	Virtuaalne konverents "*Experience-Dependent Transcription – From Genomic Mechanisms to Neural Circuit Function*"
November 2021	Neuroscience 2021 virtuaalne konverents
August 2020	Virtuaalne konverents "*Epigenetics in Neurobiology*"
August 2019	Kursus "*Baltic summer school on behavioural characterization of rodent models of major brain disorders*", Pühajärve, Estonia
September 2018	Kursus "*Developmental Biology Minisymposium*", Tallinn, Eesti
September 2018	Kursus "*From Nanotechnology to Nanomedicine 2018*", Tallinn, Eesti
Jaanuar 2018	Konverents "*Teaching for Learning – the University Perspective*", Tartu, Eesti

Ettekanded konverentsidel

2022	FENS Foorum 2022, Pariis, Prantsusmaa, posterettekanne "*Novel stimulus-dependent regulators of BDNF gene expression*"
2022	Sigrid Juselius Sümpoosium, Espoo, Soome, posterettekanne "*Novel stimulus-dependent regulators of BDNF gene expression*"
2021	Virtuaalne NGF miitingute sari, suuline ettekanne "*Novel brain region- and stimulus-specific regulators of the BDNF gene*"
2020	FENS virtuaalne konverents, posterettekanne "*Differential regulation of the BDNF gene by CREB family transcription factors in cultured cortical and hippocampal neurons*"

2019	Nordic Neuroscience 3, Helsinki, Soome, posterettekanne "*CREB and BDNF transcriptional autoregulation*"
2018	SZTest mini-sümpoosium, Tallinn, Eesti "*Gene Expression in Health and Disease"*, Tallinn, Eesti, suuline ettekanne "*CREB and BDNF transcriptional autoregulation*"
2018	Neuroscience 2018, San Diego, USA, posterettekanne "*CREB and BDNF transcriptional autoregulation*"
2018	*International Conference on Neurotrophic Factors*, Salamanca, Hispaania, posterettekanne "*CREB and BDNF transcriptional autoregulation*"

Juhendamised

Veebruar 2022– Juuni 2023	Erasmuse vahetustudeng Enrico di Marco, magistritöö "*Investigating potential neuronal activity-regulated enhancers*"
November 2021– Juuni 2023	Annabel Parkman, bakalaureusetöö "*Markergeenide ekspressiooni iseloomustamine rakukultuuris kasvatatud neuronites ja astrotsüütides*"
September 2020– …	Magistritudengi Susann Kõomägi kaasjuhendamine biotiini märgisega transkriptsioonifaktorite kloneerimise ja interaktsioonipartnerite määramise teemal
September 2020– Juuni 2021	Neeme Raidvere, bakalaureusetöö "*dCas9 valgul põhinevate Capture ja Caspex süsteemide kasutamine kromatiini afiinsuspuhastamisel*"
September 2019– Juuni 2021	Andra Moistus, magistritöö "*BDNF geeni uute regulaatorite tuvastamine*"
Märts 2018– Juuni 2019	Käthy Rannaste, bakalaureusetöö "*Transkriptsioonifaktori CREB ja tema koaktivaatori CRTC1 roll neurotrofiin BDNF geeni aktiivsusest sõltuvas transkriptsioonis ajukoore ja hipokampuse neuronites*"
September 2017– Juuni 2018	Susann Kõomägi, bakalaureusetöö "*Neuronaalsest aktiivsusest sõltuv BDNF geeni regulatsioon kortikaalsetes ja hipokampaalsetes neuronites*"

Publikatsioonid

Esvald EE*, Tuvikene J*, Kiir CS*, Avarlaid A, Tamberg L, Sirp A, Shubina A, Cabrera-Cabrera F, Pihlak A, Koppel I, Palm K, Timmusk T. (2023). Revisiting the expression of BDNF and its receptors in mammalian development. Frontiers in Molecular Neuroscience, 16.

Lekk I*, Cabrera-Cabrera F*, Turconi G*, Tuvikene J, **Esvald EE**, Rähni A, Casserly L, Garton DR, Andressoo JO*, Timmusk T*, Koppel I*. (2023). Untranslated regions of brain-derived neurotrophic factor mRNA control its translatability and subcellular localization. Journal of Biological Chemistry, 299, 102897.

Esvald EE, Tuvikene J, Moistus A, Rannaste K, Kõomägi S, Timmusk T. (2022). Differential regulation of the BDNF gene in cortical and hippocampal neurons. Journal of Neuroscience, 42, 9110−9128.

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, 10, e65161.

Esvald EE*, Tuvikene J*, Sirp A, Patil S, Bramham CR, Timmusk T. (2020). CREB family transcription factors are major mediators of BDNF transcriptional autoregulation in cortical neurons. Journal of Neuroscience, 40, 1405−1426.

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

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