Neurotrophins: Transcription and Translation

A.E. West, P. Pruunsild, and T. Timmusk

Abstract

Neurotrophins are powerful molecules. Small quantities of these secreted proteins exert robust effects on neuronal survival, synapse stabilization, and synaptic function. Key functions of the neurotrophins rely on these proteins being expressed at the right time and in the right place. This is especially true for BDNF, stimulus-inducible expression of which serves as an essential step in the transduction of a broad variety of extracellular stimuli into neuronal plasticity of physiologically relevant brain regions. Here we review the transcriptional and translational mechanisms that control neurotrophin expression with a particular focus on the activity-dependent regulation of BDNF.

Keywords

NGF • NT3 • NT4/5 • BDNF • Transcription • Translation • Activity-dependent • Plasticity

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1 Introduction

Transcriptional regulation is mediated by the association of DNA binding proteins with gene regulatory elements, which confer developmental, cell-type-specific, and stimulus-dependent regulation on gene transcription. Protein–DNA interactions influence transcription by modulating the recruitment and/or activation of RNA polymerase II at nearby genes. Gene regulatory elements are defined by their function, and although many closely neighbor genes, regulatory elements can also act over long distances. Many regulatory elements are found in promoters, which are broadly defined as the region of genomic DNA immediately proximal to and up to about 2 kb upstream of the transcription start site (TSS) for a given gene. By contrast enhancer elements can be located at very great distances on either side of the TSS. Gene transcription can also be influenced by protein–DNA interactions at insulator and silencing elements, which impact transcription over large regions of the surrounding genome. All of these elements are subject to an additional level of regulation by the secondary and tertiary structure of chromatin, which can be modulated by modifications of both genomic DNA and its associated histone proteins. Once synthesized, mRNA is subject to several modes of posttranscriptional regulation that can impact levels of gene expression through regulation of RNA stability, transport, and translation. In addition to protein–RNA interactions, there is a growing awareness of the role of noncoding RNAs as mediators of these processes.

Here we describe the characterization of the transcriptional and translational processes that regulate expression of the neurotrophins. Though all the neurotrophins play important roles in neuronal physiology, commensurate with the importance of stimulus-dependent regulation of *Bdnf* mRNA expression for neural plasticity, the mechanisms underlying the dynamic regulation of this gene have received substantial attention and will be reviewed in the greatest detail.

2 Nerve Growth Factor

2.1 Expression Pattern and Regulation

Nerve growth factor (NGF) is expressed in both neuronal and non-neuronal cells of the peripheral and central nervous systems (Sofroniew et al. 2001). NGF is highly expressed in the target tissues of TrkA expressing neurons, which include dorsal root ganglia (DRG), cranial sensory neurons that mediate pain and temperature, sympathetic neurons, basal forebrain cholinergic neurons, striatal cholinergic neurons, and certain thalamic and brainstem neurons. Hippocampal and cortical neurons that are targets of cholinergic innervation express the highest levels of NGF mRNA in the brain (Lauterborn et al. 1993, 1995; Rocamora et al. 1996a), and interestingly, the majority of these NGF-positive neurons are GABAergic interneurons. In the striatum, NGF is also expressed by a population of small GABAergic interneurons (Bizon et al. 1999). In non-neuronal cells of the adult mouse, the highest levels of NGF mRNA are present in the salivary gland, vas

deferens, and heart. Expression of NGF mRNA in salivary gland is sex specific; in male animals the levels are much higher than in females (Sofroniew et al. 2001). Immature Schwann cells produce NGF during development, but in adults, NGF expression is undetectable in mature myelinating Schwann cells. However, after nerve injury the expression is induced in reactive and dedifferentiated Schwann cells (Heumann et al. 1987; Lindholm et al. 1987).

Expression of NGF is sensitive to regulation by both neuronal activity and stimuli related to inflammation. Limbic seizures induce *Ngf* expression by 1 h in the dentate gyrus, whereas expression appears in the neocortex and olfactory forebrain some hours later (Gall and Isackson 1989). Consistent with enhanced transcription as a mechanism underlying the activity-dependent increases in *Ngf* mRNA levels, membrane depolarization of cultured embryonic cortical neurons induces the association of RNA PolII with the *Ngf* gene promoter (Kim et al. 2010). CNS induction of *Ngf* is responsive to both glutamate (Zafra et al. 1990) and acetylcholine (da Penha Berzaghi et al. 1993). In addition, *Ngf* expression can be upregulated by glucocorticoids (Mocchetti et al. 1996; Barbany and Persson 1992) and activation of β2 adrenergic receptors (Colangelo et al. 1998). The interleukin IL-1 strongly induces expression of *Ngf* in non-neuronal cells of the peripheral nervous system after injury (Lindholm et al. 1987). Intraventricular injection of IL-1 also induces *Ngf* expression in the hippocampus, but it is not clear whether this induction is in neuronal or non-neuronal cells (Spranger et al. 1990).

2.2 Promoter Structure and Elements

The *Ngf* gene is found on chromosome 3qF2.2 in mouse, chromosome 2q34 in rat, and chromosome 1p13.2 in human. The mammalian *Ngf* gene contains several 5′ exons encoding the 5′ untranslated region (UTR) and one 3′ exon encoding the NGF protein (Metsis 2001) (Fig. 1). The structure of the mammalian *Ngf* gene and its transcripts has been studied most extensively in the mouse (Edwards et al. 1986; Selby et al. 1987). In mouse the *Ngf* gene comprises five exons, exons IA, IB, II, III, and IV covering about 50 kb. According to current knowledge, exons IA, IB, II, and III encode 5′ UTRs and exon IV the NGF pre-protein. Although exons IA and II both contain additional putative ATG codons, their usage for translation initiation of NGF protein has not been established. Four different splicing patterns have been described for the mouse *Ngf* gene leading to the following transcripts: transcripts containing exons IA, III, and IV, transcripts containing exons IB, III, and IV, transcripts containing exons IB, II, III, and IV, and transcripts containing 5′ extended exon III and exon IV. Exon IA-III-IV transcripts are the most abundant *Ngf* mRNAs in the submandibular gland comprising about 90 % of the pool of *Ngf* mRNAs. In other tissues, including heart, kidney, and brain, the most abundant transcript is exon IB-III-IV followed by exon IA-III-IV transcripts. The levels of exon IB-II-III-IV transcripts and exon III-IV transcripts are much lower. One major transcription initiation site has been determined both for exon IA and IB by primer extension and S1 nuclease protection assay showing that exons IA and IB, separated by only 142 bp, are linked to separate promoters. It has also been shown that the 5′

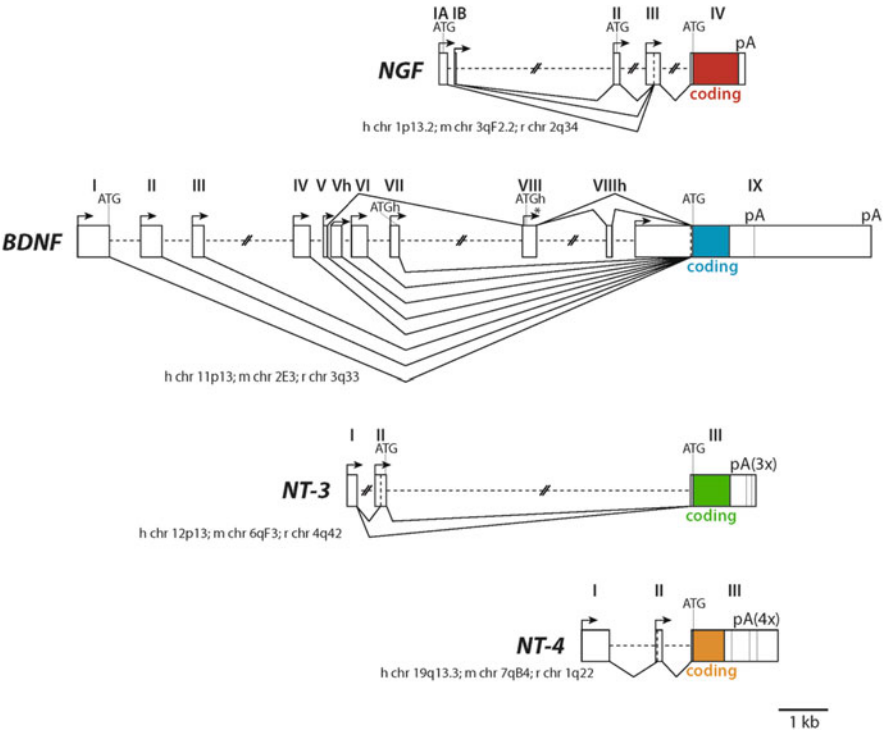

Fig. 1 Structures of mammalian neurotrophin genes. The structures of the genes include data published on the human, mouse, and rat neurotrophins. All neurotrophin genes consist of multiple 5′ exons linked to promoters that initiate transcription of distinct mRNAs. As a common feature, the 3′ exon that is included in all different transcripts of each neurotrophin comprises the open reading frame (ORF, *colored box*) encoding the respective prepro-neurotrophin. The beginning of the ORF is marked by the translation initiation codon ATG. There are variant upstream ATGs in all neurotrophin genes except *Ntf4*, but the usage of these translation initiation sites has not been verified. For all except the *Ngf* gene, usage of at least two alternative polyadenylation sites (pA, *thin vertical line*) has been detected. In the case of *Bdnf*, human-specific exons that are not present in rodent *Bdnf* are marked with the letter "h" following the Roman numeral representing the name of the exon brought above the box designating the exon. *Horizontal dashed lines* represent introns. *Vertical dashed lines* inside exons indicate alternative splicing acceptor sites used within that exon. Splicing patterns of neurotrophin mRNAs are shown by *lines* linking exons. The most upstream transcription start site (TSS) is indicated by an *arrow* for each exon. The *asterisk* marking the TSS of *BDNF* exon VIII stands for a rodent-specific transcription initiation site that has not been detected to be used in human. The genomic locations of human (h), mouse (m), and rat (r) genes are shown adjacent to each schematic. *Scale bar* is for exons and introns shown with uninterrupted *dashed lines*. Introns that are interrupted with *double slash* are longer and out of this scale

region of mouse exon IB is able to drive reporter gene expression when transiently expressed in cultured cells showing that this exon is linked to a functional promoter (Zheng and Heinrich 1988; D'Mello and Heinrich 1991). The putative promoter of mouse *Ngf* exon IA has not been studied.

The rat and human *NGF* genes have not been characterized in detail; however, bioinformatic analysis of GenBank suggests that, similar to mouse, exon IB-III-IV transcripts are the most abundant in several tissues, including brain. The transcription initiation site of exon IB has been determined for rat exon IB by S1 nuclease protection (Zheng and Heinrich 1988) and the 5′ region of both rat and human exon IB is able to direct reporter gene expression in various cultured cells using transient expression assays (Zheng and Heinrich 1988; Cartwright et al. 1992). In addition, transgenic mice expressing reporters under control of human and mouse *NGF* promoter regions have been characterized that partially recapitulate expression of the endogenous gene (Alexander et al. 1989; Kaisho et al. 1999; Kawaja et al. 2011).

The function of regulatory elements in the *Ngf* exon IB promoter has been studied in non-neuronal cells. Following cloning of the *Ngf* gene, attention focused on an AP-1 site found at +35 bp, mutation of which reduces activity of an *Ngf* promoter reporter plasmid in heterologous expression assays (D'Mello and Heinrich 1991). AP-1 elements are bound by members of the Fos/Jun family of transcription factors, and lesion of the sciatic nerve was known to induce both Fos protein and *Ngf* mRNA expression. Using a fibroblast line in which Fos could be inducibly overexpressed, it was shown that Fos increases *Ngf* mRNA expression through a mechanism that supports DNAse protection of the AP-1 containing fragment, suggesting that Fos binding to this AP-1 may contribute to lesion-induced increases in *Ngf* mRNA (Hengerer et al. 1990). By contrast elements 5′ to the TSS have been implicated in transcriptional regulation of *Ngf* in response to β2 adrenergic receptor activation. In C6-2B glioma cells, *Ngf* expression can be induced by addition of the β2 adrenergic receptor agonist clenbuterol. Activation requires an element mapped by DNAse footprinting and reporter transactivation to a region −90 to −70 bp relative to the TSS (Colangelo et al. 1998). Binding and reporter studies identified CCAAT/enhancer-binding protein δ (C/EBPδ) as a putative regulatory transcription factor for this site, and further studies showed that CREB binds to a CRE half-site at −65 bp. Importantly C/EBPδ knockout mice have significantly reduced β2 adrenergic receptor-induced NGF expression in the cortex, suggesting that similar transcriptional mechanisms may contribute to *Ngf* regulation in the brain (McCauslin et al. 2006).

2.3 Regulation of mRNA Stability

In addition to transcriptional regulation, *Ngf* mRNA is subject to stimulus-dependent changes in its stability. In cultured rat fibroblasts, in addition to a change in the transcriptional rate of *Ngf* synthesis as revealed by nuclear run-on, RNase protection assays demonstrate that IL-1 increases the half-life of *Ngf* mRNA (Lindholm et al. 1988). In smooth muscle cells, the secreted factors PDGF and TGFβ increase NGF secretion in the presence of the transcriptional inhibitor Actinomycin D and elevate the ratio of NGF protein to *Ngf* mRNA again suggesting an effect on RNA stability and/or processing (Sherer et al. 1998). AU-rich regions in the 3′UTR often serve as instability elements, and AU-rich regions of the *Ngf*

3′-UTR have been identified that appear to contribute to mRNA stability (Tang et al. 1997). However, the specific signaling mechanisms and proteins that regulate stability of *Ngf* mRNA under basal or stimulus-induced conditions remain unknown.

3 Neurotrophin-3

3.1 Expression Pattern and Regulation

Neurotrophin-3 (NT-3) is widely expressed in non-neuronal tissues during development and, in general, the levels are lower in the adult. In the adult rat the highest NT-3 protein levels have been detected in the pancreas and spleen (Katoh-Semba et al. 1996). In the nervous system, NT-3 is most highly expressed in the immature CNS when proliferation, migration, and differentiation of neuronal precursors are ongoing. NT-3 expression dramatically decreases with maturation of these regions (Maisonpierre et al. 1990b; Ernfors et al. 1992; Friedman et al. 1991b). The factors that regulate expression of NT-3 have been most highly studied in the developing cerebellum where expression of NT-3 is required for proper cerebellar development (Bates et al. 1999). Brain-derived neurotrophic factor (BDNF) can drive NT-3 expression in the cerebellum as can thyroid hormone T3 (Leingärtner et al. 1994). Strikingly, unlike BDNF and NGF, expression of NT-3 is not induced by traditional stimuli that increase neural activity in the CNS. For example, NT-3 shows no induction in the hippocampus following pilocarpine-induced seizures (da Penha Berzaghi et al. 1993) and reduced expression levels following kainate-induced seizure (Katoh-Semba et al. 1999).

3.2 Promoter Structure and Elements

NT-3 is encoded by the *Ntf3* gene on mouse chromosome 6qF3, rat chromosome 4q42, and human chromosome 12p13. In all mammals studied (mouse, rat, and human) the *Ntf3* gene comprises three exons giving rise to multiple *Ntf3* mRNA transcripts (Fig. 1). Several TSSs in both upstream exons and three different polyadenylation sites in exon III have been mapped by RNase protection assays and by RACE. Alternative promoter usage upstream of exons I and II leads to expression of transcripts that differ in the putative translation initiation ATGs (Leingärtner and Lindholm 1994; Kendall et al. 2000). Exon I-III transcripts contain an ATG in the beginning of exon III suggesting that it is used for initiation of protein translation. Exon I-II-III and exon II-III transcripts have two potential translation initiation codons; however, it has not been determined which of the ATGs is used for protein translation. Exon II-III transcripts appear to be the predominant transcripts in most tissues, including brain, and exon I-II-III transcripts have been demonstrated only in a few tissues in rat (Kendall et al. 2000). Both promoters are active when fused to reporter genes and transfected into cerebellar granule neurons (Leingärtner and Lindholm 1994). Transcripts

initiating from both promoters have been detected in cerebellar granule neurons; however, only promoter II is transcriptionally upregulated by tri-iodothyronine (T3).

In reporter assays, both promoters I and II of the *Ntf3* gene contain regions that function as enhancer and repressor elements (Leingärtner and Lindholm 1994; Katoh-Semba et al. 1996). One family of regulators that contributes to regulation of promoter II are the related zinc-finger transcription factors Sp4, Sp1, and Sp3. Sp4 and Sp1 bind directly to *Ntf3* promoter II in cerebellar granule neurons as shown by chromatin immunoprecipitation (Ramos et al. 2009). Knockdown of Sp4 expression leads to increased *Ntf3* expression in these cells suggesting that this interaction is required for *Ntf3* repression. However, the effects of Sp4 on NT-3 regulation may be context or cell-type dependent because mice with reduced Sp4 expression show reduced NT-3 in the hippocampus (Zhou et al. 2005). By contrast, BDNF-dependent activation of *Ntf3* promoter II in cerebellar granule cells is mediated by members of the MEF2 and CREB families of transcription factors (Shalizi et al. 2003). BDNF drives phosphorylation and activation of the MAP kinase family member Erk5, which then induces phosphorylation and activation of MEF2. BDNF-dependent induction of *Ntf3* requires a region −1087 to −838 bp relative to the TSS of exon II. Both MEF2 and CREB bind sequences within this region, and knockdown of MEF2 or overexpression of dominant-negative CREB inhibits BDNF-dependent induction of *Ntf3* suggesting that the two factors cooperate to mediate the regulation of this element (Shalizi et al. 2003). Finally the POU-domain transcription factor Brn-3c (POU4F3) has been implicated in *Ntf3* regulation in a cell line derived from organ of Corti (Clough et al. 2004). However, unlike the other factors, Brn-3c appears to be an activator of *Ntf3* promoter I.

4 Neurotrophin-4

4.1 Expression Pattern and Regulation

Although neurotrophin-4 (NT-4) (also called NT-4/5 or NT-5) binds and activates the TrkB receptor, regulation of NT-4 expression shares few similarities with the other TrkB ligand, BDNF. In the rat NT-4 is widely expressed in non-neuronal tissues both during embryonic and postnatal development and also in the adult. Highest NT-4 levels have been detected in early postnatal testis (Timmusk et al. 1993b). NT-4 is highly expressed in embryonic and adult skeletal muscle and it is strongly expressed by both neuronal and non-neuronal cells of the spinal cord (Ip et al. 1992; Scarisbrick et al. 1999). By contrast it is expressed at much lower levels in the CNS (Ip et al. 1992), both during development and in the adult animal (Timmusk et al. 1993b). Compared with *Bdnf* knockout mice, *Ntf4* null mice show minimal neurological phenotypes (Liu et al. 1995; Conover et al. 1995). NT-4 expression is induced in muscle by electrical stimulation (Funakoshi et al. 1995) and in spinal cord by systemic administration of the excitotoxic stimulus kainic acid (Scarisbrick et al. 1999). Analysis of *Ntf4* knockout mice has demonstrated that

muscle-derived NT-4 is required for maintenance of postsynaptic acetylcholinergic receptor clustering, normal muscular electrophysiological responses, and resistance to muscle fatigue. Thus, NT-4 is involved in activity-dependent feedback mechanisms involved in the maintenance of neuromuscular connections and muscular performance (Belluardo et al. 2001). Surprisingly, in the brain *Ntf4* is not activity regulated since there is no change in NT-4 expression in the hippocampus after pilocarpine-induced seizure (Mudo et al. 1996), a common method for inducing activity-regulated gene transcription.

4.2 Promoter Structure and Elements

NT-4 is encoded by the *Ntf5* gene on mouse chromosome 7qB4, the *Ntf4* gene on rat chromosome 1q22, and the *NTF4* gene on human chromosome 19q13.3. We refer here to the gene in all three species as "*Ntf4*". The *Ntf4* gene comprises three exons with two alternative promoters upstream of exons I and II (Fig. 1). The transcription initiation sites have been determined for rat *Ntf4* gene in newborn testis and adult skeletal muscle; however, there has been no comprehensive analysis of alternative promoter usage in other tissues and cell types in vivo. In cell lines promoter II confers significantly stronger transcriptional activity on a reporter plasmid than promoter I (Salin et al. 1997). Generation of transgenic mice that contain the full *Ntf4* gene plus 1.4 kb of additional upstream sequence show high levels of *Ntf4* expression in muscle and low but detectable expression in brain and thymus, indicating that this region is largely sufficient to confer proper expression of *Ntf4*. Importantly this transgene also recapitulates the activity-regulated expression of *Ntf4* in muscle (Funakoshi et al. 1995; Salin et al. 1997), suggesting that activity-responsive elements lie within this fragment. However, the position of these elements and their associated transcription factors has not yet been identified.

5 Brain-Derived Neurotrophic Factor

5.1 Expression Pattern and Regulation

Bdnf has a widespread expression pattern that is conserved among mammalian species (Maisonpierre et al. 1990a, b, 1991; Conner et al. 1997; Katoh-Semba et al. 1997). During development, *Bdnf* expression is more abundant in the nervous system compared with other tissues and its levels are dramatically increased in the brain postnatally (Kaisho et al. 1991; Katoh-Semba et al. 1997). In the adult nervous system, *Bdnf* displays a wide distribution pattern, with the highest levels of mRNA and protein in the hippocampus, amygdala, cerebral cortex, hypothalamus, and septum in the brain and in the dorsal root ganglia in the PNS. *Bdnf* mRNA expression is mostly confined to neurons and there are only a few brain areas where *Bdnf* transcripts are not detected (Ernfors et al. 1990; Hofer et al. 1990; Timmusk et al. 1994b; Conner et al. 1997; Katoh-Semba et al. 1997; Phillips et al. 1990;

Friedman et al. 1991a; Webster et al. 2006). *Bdnf* expression in adult tissues is also detectable outside of the nervous system. Similar *Bdnf* mRNA levels to those found in the brain have been detected in the heart and lung and lower levels in the thymus, liver, spleen, and muscle (Ernfors et al. 1990; Maisonpierre et al. 1990a; 1991; Katoh-Semba et al. 1997; Yamamoto et al. 1996).

Regulation of transcription is a major contributor to the pleiotropic functions of BDNF. Accordingly, *Bdnf* expression levels in neurons are regulated by many stimuli including ischemic and hypoglycemic insults (Lindvall et al. 1992), peripheral nerve axotomy (Michael et al. 1999), immobilization stress (Smith et al. 1995a, b), antidepressant treatment (Nibuya et al. 1995; Dias et al. 2003), drug craving after cocaine withdrawal (Grimm et al. 2003), and chronic social defeat stress (Tsankova et al. 2006). However, the best studied and probably the most potent *Bdnf* transcription-inducing stimulus is neuronal activity. Neuronal activity in the brain and *Bdnf* mRNA expression are both evoked by excitatory stimulus-evoked seizures by kainic acid treatment (Zafra et al. 1990; Ballarin et al. 1991; Metsis et al. 1993), electrical stimulation resulting in epileptogenesis (Ernfors et al. 1991), lesion-induced recurrent limbic seizures (Isackson et al. 1991), exposure to light as sensory input (Castren et al. 1992), electrical stimulation inducing LTP of synaptic transmission (Patterson et al. 1992; Castren et al. 1993), enriched environment (Falkenberg et al. 1992; Young et al. 1999), application of KCl to the cortical surface inducing spreading depression (Kokaia et al. 1993), mechanical stimulation of mystacial whiskers (Rocamora et al. 1996b; Nanda and Mack 2000), physical activity (Neeper et al. 1996; Russo-Neustadt et al. 2000), singing in birds (Li et al. 2000), hippocampus-dependent contextual learning (Hall et al. 2000), and amygdala-dependent learning (Rattiner et al. 2004). On the other hand, treatments or conditions that reduce neuronal activity, for example, inhibition of neuronal activity by gamma-aminobutyric acid (GABA) (Berninger et al. 1995) and monocular deprivation (Bozzi et al. 1995; Rossi et al. 1999), have been demonstrated to decrease *Bdnf* mRNA levels. Furthermore, expression of *Bdnf* undergoes circadian oscillation, mirroring variations in physiological activity (Bova et al. 1998; Berchtold et al. 1999). Thus, environmental stimuli that produce excitatory inputs onto neurons and increase their intracellular Ca^{2+} concentration, i.e., induce neuronal activity, have been found to be the key regulators of *Bdnf* transcription. The significance of this activity-regulated transcription of *Bdnf* is emphasized by the fact that BDNF is one of the major regulators of neuronal activity-dependent neurotransmission and plasticity in the brain (Schinder and Poo 2000; Poo 2001; Lu 2003; Bramham and Messaoudi 2005).

5.2 Promoter Structure

The *Bdnf* gene comprises nine exons that span 52.3 kb of chromosome 2qE3 in mouse, chromosome 3q33 in rat, and chromosome 11p14.1 in human. All three species appear to have at least eight homologous exons that contribute to alternate 5′ UTRs, each of which is linked to a separate promoter and can be spliced to form a

bipartite transcript and in some rare cases also a tripartite or a quadripartite transcript (V-VIII-VIIIh-IX, V-VIIIh-IX, and VI-IXb-IXd) with a common ninth exon that contains the coding sequence and 3′-UTR (Fig. 1) (Liu et al. 2005, 2006; Aid et al. 2007; Pruunsild et al. 2007). An ATG in exon I provides an alternative putative translation start site for exon I-IX variants (Timmusk et al. 1993a). Expression constructs encoding a human BDNF-GFP fusion protein containing both the exon I ATG and exon IX ATG are translated when transiently expressed in primary hippocampal neurons. However, it was not studied which of these two ATGs was used for translation initiation (Jiang et al. 2008). The pufferfish and zebrafish *Bdnf* genes preserve a similar multi-exon organization suggesting that this genomic structure may have a conserved function through evolution (Heinrich and Pagtakhan 2004). Rat and human BDNF genomic regions recapitulating tissue-specific, neuronal activity-, and axotomy-induced expression of rat *Bdnf* (Timmusk et al. 1995; Koppel et al. 2010) and human *BDNF* (Koppel et al. 2009) have been characterized in transgenic mice.

The functional importance of the multi-promoter organization of *Bdnf* is incompletely understood; however, it appears that the stimulus-selective activation of the distinct sets of transcription factors bound at each of these promoters serves to make BDNF expression responsive to a very diverse range of stimuli. Different 5′ *Bdnf* exons are induced by distinct kinds of stimuli (West 2008), consistent with the idea that transcription originating at each promoter may be differentially important for the myriad biological functions of BDNF. It should be noted that while the importance of *Bdnf* exon IV containing mRNA transcription in the development of GABAergic inhibition in the cortex has been studied relatively well in vivo using specific genetic manipulations that disrupt basal and activity-responsive *Bdnf* exon IV-derived production of BDNF protein (Hong et al. 2008; Sakata et al. 2009), the in vivo role of *Bdnf* exon I- and II-containing transcripts has not been addressed. In the light of the findings that exon I mRNAs of *Bdnf* are among the most strongly induced *Bdnf* transcripts upon neuronal activity (Metsis et al. 1993; Timmusk et al. 1993a) and that overexpression of *Bdnf* exon I, II, and III mRNAs without increasing other *Bdnf* transcripts is associated with enhanced LTP in mice (Barco et al. 2005), it would be especially interesting to elucidate the roles of all the multiple exons of *Bdnf*.

5.3 Promoter Regulation

5.3.1 Promoter I

The levels of *Bdnf* exon I increase markedly in the brain after kainic acid-induced seizures (Metsis et al. 1993; Timmusk et al. 1993a) and other experimental conditions that produce neuronal activity. The first transcription factors that were shown to contribute to this Ca^{2+}-mediated activation of *Bdnf* promoter I were the activating transcription factor (ATF)/cAMP/Ca^{2+}-response element binding protein (CREB) family basic leucine zipper protein CREB and the basic helix-loop-helix (bHLH) proteins upstream stimulatory factor (USF) 1 and USF2 (Fig. 2a) (Tabuchi

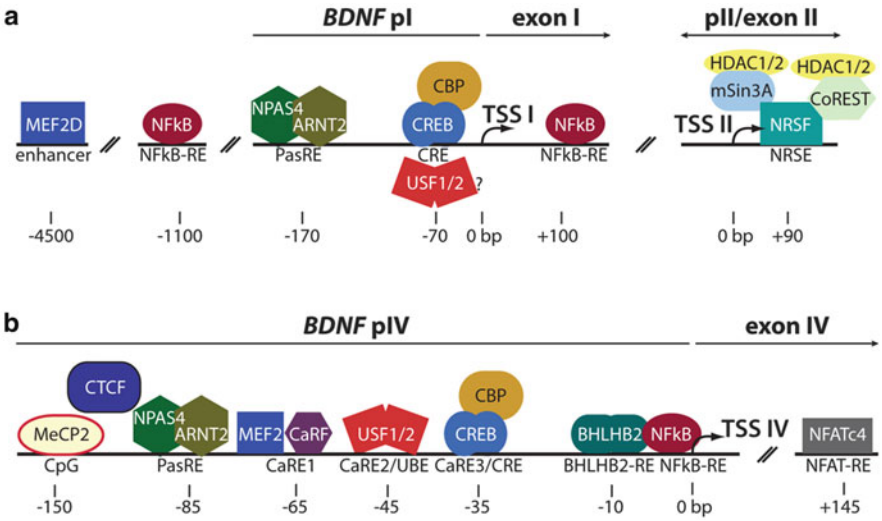

Fig. 2 Transcription factors and regulatory elements involved in the regulation of activity-dependent transcription from *BDNF* promoters I, II, and IV. *Bdnf* promoters (p) I, pII, and pIV are bound by multiple transcription factors (TFs) that regulate neuronal activity-dependent induction of *Bdnf* exon I, II, and IV mRNA transcription. (**a**) The TFs and *cis*-elements that have been shown to regulate activity-dependent *Bdnf* exon I or II transcription are myocyte-specific enhancer factor 2D (MEF2D); nuclear factor kappa B (NF-κB); neuronal PAS domain protein 4 (NPAS4); aryl hydrocarbon receptor nuclear translocator 2 (ARNT2); cAMP/Ca^{2+}-response element binding protein (CREB) bound by CREB binding protein (CBP); upstream stimulatory factors 1 and 2 (USF1/2); neuron-restrictive silencing factor (NRSF) bound by mSin3A (histone deacetylase complex subunit Sin3A), RE1-silencing transcription factor (REST) co-repressor 1 (CoREST), and histone deacetylase 1 and 2 (HDAC1/2); NF-κB response element (NF-κB-RE); bHLH-PAS transcription factor response element (PasRE); cAMP/Ca^{2+}-response element, in pI, a CRE-like element (CRE); and neuron-restrictive silencing element (NRSE). (**b**) The TFs and *cis*-elements that have been shown to regulate activity-dependent *Bdnf* exon IV transcription are methyl-CpG binding protein (MeCP2); CCCTC-binding factor (CTCF); NPAS4; ARNT2; MEF2; Ca^{2+}-response factor (CaRF); USF1 and USF2; CREB and CBP; basic helix-loop-helix domain containing, class B, 2 (BHLHB2); NF-κB; nuclear factor of activated T-cells cytoplasmic 4 (NFATc4); PasRE; Ca^{2+}-response element 1, 2 (UBE, USF-binding element), and 3 (CRE) (CaRE1, 2, and 3); and BHLHB2-RE, NF-κB, and response elements for the respective TFs (NFAT-RE). All factors that have been shown to bind specific *cis*-regulatory DNA elements in the promoters are depicted on the *line* representing DNA. The *cis*-elements are specified below the factors. The TFs that have been shown to contribute to regulation, but for which the binding site is not known, are depicted above the promoter. The *question mark* adjacent to the USF1/2 factors that are drawn below pI indicates that although USFs have been shown to regulate the rat promoter, regulation of human pI by the USF factors has not been confirmed and the regulatory element that has been found to bind USF in the rat promoter is not conserved in human. Transcription start sites (TSSs) are designated by *arrows*. Only the most upstream TSS for each promoter is shown. Distance in base pairs (bp) relative to the TSS is shown below the line representing DNA. This figure shows all the TFs and regulatory elements that have been shown by different groups, although data about some TFs are contradictory (for example, USF1/2 and CREB for pI, see text for details)

et al. 2002). Tabuchi et al. (2002) studied rat *Bdnf* promoter I regulation and found that Ca^{2+}-responsive DNA elements in *Bdnf* promoter I are located in two promoter regions: in a proximal and in a distal region that are located at approximately −70 to −100 bp and −180 to −280 bp, respectively, relative to the most 5′ transcription start site of rat exon I. In the proximal region, a cAMP/Ca^{2+}-response element (CRE)-like element overlapping with a USF-binding site was identified. These *cis*-elements were shown to be bound in vitro by CREB and USF1/USF2, correspondingly, and mutations in the CRE and USF-binding sites were shown to reduce rat *Bdnf* promoter I-dependent transcriptional activity in response to membrane depolarization of primary neurons. In addition, overexpression of dominant-negative forms of CREB and USF proteins in neurons was found to interfere with activity-dependent transcription from rat *Bdnf* promoter I (Tabuchi et al. 2002).

The transcription factors and *cis*-element that contributed to the Ca^{2+} responsiveness of the distal region of promoter I were identified when the regulation of the human *BDNF* promoter I in primary neurons was analyzed (Pruunsild et al. 2011). The deletion of these distal elements was even more potent in reducing the inducibility of *Bdnf* promoter I than deletions in the proximal region (Tabuchi et al. 2002), It was shown that the human as well as the rat *BDNF* promoter I is induced by neuronal activity by the bHLH-Per-Arnt-Sim (bHLH-PAS) transcription factors aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) and neuronal PAS domain protein 4 (NPAS4), which dimerize and bind to a Ca^{2+}-responsive element termed bHLH-PAS transcription factor response element (PasRE) located approximately −170 bp relative to the most 5′ transcription start site of human *BDNF* promoter I (Pruunsild et al. 2011). Pruunsild et al. demonstrated that mutating the PasRE drastically reduces neuronal activity-responsive induction of *BDNF* promoter I-dependent transcription. Also, it was shown that expression of dominant-negative ARNT2 and NPAS4 almost completely blocks and overexpression of ARNT2 and NPAS4 strongly enhances activity-responsive exon I transcription, respectively, in primary neurons. Moreover, ARNT2 binds *BDNF* promoter I in human brain in vivo (Pruunsild et al. 2011). In a separate study, NPAS4 has been detected to be bound on the mouse *Bdnf* promoter I region in mouse brain by chromatin immunoprecipitation (ChIP) (Lin et al. 2008), further strengthening involvement of the bHLH-PAS proteins in *BDNF* exon I regulation.

Some different results have been seen between the regulation of rodent and human *Bdnf* promoter I. Despite evidence for its use in rodents, the USF-binding element is not conserved in the human *BDNF* promoter I and the USF proteins have been found not to contribute to the neuronal activity-dependent regulation of the human promoter I (Pruunsild et al. 2011). Furthermore, the CRE-like element, although conserved, has been found in transient transfection assays of reporter constructs to be more important for basal transcription than for the activity-dependent induction of the human promoter I (Pruunsild et al. 2011). Nonetheless in vivo, a constitutively active form of CREB is able to enhance promoter I-dependent transcription suggesting the physiological relevance of this interaction (Barco et al. 2005). The potential importance of ARNT2 and NPAS4 factors is strongly supported by a study showing that neuronal activity-dependent

transcription of *BDNF* exon I transcripts is sensitive to protein synthesis inhibitors, indicating that immediate-early gene products are involved in activating promoter I (Lauterborn et al. 1996). As NPAS4 is one of the most strongly induced immediate-early genes by neuronal activity (Lin et al. 2008), it is conceivable that the ARNT2 and NPAS4 heterodimers, which would upregulate exon I transcription, form after the first wave of immediate-early genes have been transcribed and translated in response to the activating stimulus. This would also explain why the rise in the levels of *BDNF* exon I transcripts takes place with a delay compared to *BDNF* exon IV transcripts (Kokaia et al. 1994; Lauterborn et al. 1996; Pruunsild et al. 2011) that are predominantly under the control of the CREB/CRE system (Hong et al. 2008).

In addition to the factors described above, two other transcription factors have been implicated in mediating the neuronal activity-dependent induction of *Bdnf* exon I transcription: (1) nuclear factor kappa B (NF-κB), through binding two pairs of NF-κB response elements in proximity of *BDNF* promoter I (Lubin et al. 2007), and (2) myocyte-specific enhancer factor (MEF) 2D via binding a far upstream enhancer element (Flavell et al. 2008). Lubin et al. (2007) showed that pharmacological inhibition of the NF-κB pathway in rats decreased kainate-induced *Bdnf* exon I mRNA expression and that NF-κB was detectable on *Bdnf* promoter I with ChIP. MEF2D was shown to bind a far upstream *Bdnf* enhancer element with ChIP as well. Additionally, by mutation and deletion analyses of a *Bdnf* promoter I construct, the MEF2D binding site was demonstrated to significantly contribute to *Bdnf* promoter I neuronal activity-dependent induction in primary neurons, providing evidence that the *cis*-element whereby MEF2D augments *Bdnf* promoter I activity-responsive induction is the enhancer element approximately 4,500 bp upstream of *Bdnf* exon I (Flavell et al. 2008). The *cis*-elements for NF-κB in the activity-dependent regulation of *Bdnf* promoter I still need verification. Further insights into the role of NF-κB, as well as more understanding of the role of CREB and the possible rodent-specific function of the USFs in promoter I regulation, may reveal important new aspects of transcriptional control of *BDNF* expression.

5.3.2 Promoter II

Although to a lesser extent than *Bdnf* mRNAs containing exon I, BDNF exon II transcript levels also rise in response to neuronal activity in the brain (Metsis et al. 1993; Timmusk et al. 1993a). However no Ca^{2+}-responsive *cis*-elements or transcription factors have yet been described for *Bdnf* promoter II. Nonetheless promoter II-regulated transcripts have a unique role in the regulation of *Bdnf* expression since they are under the control of a neuron-restrictive silencer element (NRSE) (Palm et al. 1998; Timmusk et al. 1999). This element binds the zinc-finger protein neuron-restrictive silencer factor (NRSF) that recruits transcriptional co-repressors mSin3A and CoREST and in turn interacts with several other proteins, including HDACs, to regulate transcription (Fig. 2a) (Andres et al. 1999; Huang et al. 1999; Roopra et al. 2000). In transgenic mice with wild-type or mutated NRSE sequences, it has been shown that the *Bdnf* NRSE is involved in the repression of basal and kainic acid-induced transcription from *Bdnf* promoter II and, interestingly, also promoter I in neurons in vivo, indicating a role for this

element in modulating activity-dependent expression of *Bdnf* (Timmusk et al. 1999). One of the causes for Huntington's disease has been proposed to be mutant huntingtin-mediated NRSF-dependent decreases in *Bdnf* gene transcription, leading to reduced trophic support for striatal neurons (Zuccato et al. 2003). The remote effect of the NRSE in *Bdnf* promoter II on *Bdnf* exon I transcription suggests that *Bdnf* exons I and II, which are separated only by approximately 630 bp in the human genome, could be co-regulated as a single cluster. Although indications in this direction have been obtained by using reporter constructs encompassing the genomic region covering both promoters I and II of *Bdnf* (Timmusk et al. 1999; Hara et al. 2009), this hypothesis, and especially the role of Ca^{2+}-dependent *cis*-elements in front of *Bdnf* exon I in the activity-responsive induction of exon II, has yet to be proved by using additional control experiments where the expression of not only the reporter protein but also exon-specific mRNA is analyzed.

5.3.3 Promoter IV

Exon IV-containing *Bdnf* transcripts are broadly expressed and strongly stimulus responsive in the CNS. Exon IV-containing *Bdnf* transcripts are also found in some non-neuronal cells including those of the heart and lung (Timmusk et al. 1993a). Promoter IV is the most active of the inducible *Bdnf* promoters in the developing brain (Pattabiraman et al. 2005; Metsis et al. 1993) and its regulation has been strongly correlated with activity-regulated neuronal and synapse development (Hong et al. 2008; Sakata et al. 2009). RNAse protection and RACE assays have identified two major clusters of TSSs for promoter IV separated by about 80 bp. Both clusters are used for transcription initiation in all seven regions of the adult rat brain that have been analyzed (cerebral cortex, hippocampus, cerebellum, midbrain, thalamus, pons/medulla, and striatum). Also both TSSs are used under control conditions and 3 h after kainic acid treatment (Timmusk et al. 1993a, 1994a).

Sequences in the proximal region of *Bdnf* promoter IV (e.g., <250 bp upstream of the exon IV TSS) are sufficient to confer about a 5–6-fold induction on a luciferase reporter gene following KCl-induced membrane depolarization, suggesting that important calcium-response elements are found within region. Indeed promoter-luciferase reporter mutagenesis studies have led to the identification of several calcium-response elements (CaREs) within the proximal *Bdnf* promoter that are required for cooperative regulation of calcium-induced transcription of *Bdnf* exon IV (Chen et al. 2003b; Shieh et al. 1998; Tao et al. 1998; Pruunsild et al. 2011; Jiang et al. 2008). However, it is important to note that expression of endogenous exon IV-containing *Bdnf* transcripts shows over a 100-fold induction in response to the same stimulus (Tao et al. 2002). These data suggest that additional features of the endogenous *Bdnf* locus, such as epigenetic modifications of chromatin (Bird and Wolffe 1999) or the action of distant enhancers (Kim et al. 2010; Flavell et al. 2008), are likely to make a major contribution to activity-regulated *Bdnf* transcription.

A key insight from studies of *Bdnf* promoter IV is that the tight temporal, spatial, and stimulus-specific regulation of this single promoter is achieved by a complex

interplay between multiple activity-regulated transcriptional factors. At least eight different transcription factors have been shown to bind to CaREs in *Bdnf* promoter IV (Fig. 2b). Starting at the most upstream element, these factors include (1) the activity-inducible transcription factor NPAS4, heterodimerized with ARNT2, which has been shown to bind a PasRE in human *Bdnf* promoter IV (Pruunsild et al. 2011), (2) members of the myocyte enhancer factor 2 (MEF2) family of stimulus-regulated transcription factors, which bind to the upstream half of the element called CaRE1 (Hong et al. 2008; Lyons et al. 2012; Tao et al. 2002), (3) the unique transcription factor calcium-response factor (CaRF), which binds the downstream half of CaRE1 (Tao et al. 2002), (4) the upstream stimulatory factors USF1/2, which are basic helix-loop-helix family members that bind an E-box element referred to as CaRE2 (Chen et al. 2003b), (5) members of the CREB family, which bind a CRE half-site also called CaRE3 (Shieh et al. 1998; Tao et al. 1998), (6) the basic helix-loop-helix factor BHLHB2 which binds immediately upstream of the first TSS (Jiang et al. 2008), (7) the nuclear factor κB (NF-κB) which binds a site overlapping the first TSS (Lipsky et al. 2001), and (8) the nuclear factor of activated T cells (NFAT) which associates with an intragenic element +140 relative to the second TSS (Vashishta et al. 2009).

Distinct requirements for these transcription factors in the regulation of *Bdnf* promoter IV have been revealed through molecular genetic approaches that include RNA interference, the generation of transcription factor knockout mice, and the generation of transgenic mice that block the ability of specific factors to regulate *Bdnf*. For example, mice lacking *Bhlhb2* expression show enhanced hippocampal *Bdnf* exon IV expression under both basal and activity-induced conditions, implicating this protein as a repressor of *Bdnf* promoter IV (Jiang et al. 2008). Interestingly knockdown of *Npas4* or overexpression of dominant-negative forms of the PAS domain proteins ARNT2 and NPAS4 selectively impairs *Bdnf* exon IV expression at late time points after membrane depolarization (Lin et al. 2008; Pruunsild et al. 2011). *Npas4* is an immediate-early gene that shows very little expression prior to membrane depolarization, but very rapid and robust protein synthesis following stimuli that induce calcium influx into neurons (Lin et al. 2008). Recruitment of newly synthesized NPAS4 to *Bdnf* promoter IV appears to prolong the activation of transcription, allowing amplification of the initial transcription-inducing stimulus.

Despite the fact that CaRF binds the calcium-response element CaRE1 and is broadly expressed throughout the brain, studies in mice CaRF revealed that this factor appears to play a brain region-specific role in basal regulation of *Bdnf* transcription (McDowell et al. 2010). *Carf* knockout mice show reduced levels of *Bdnf* exon IV-containing mRNA transcripts and reduced BDNF protein in the frontal cortex compared with their wild-type littermates; however, *Bdnf* expression is unchanged in the hippocampus and striatum of the knockout mice (McDowell et al. 2010). Furthermore, although CaRE1 is required for activity-dependent transcription of *Bdnf* exon IV, CaRF is selectively required for the activity-*in*dependent regulation of *Bdnf* promoter IV activity (McDowell et al. 2010). By contrast, the MEF2 family transcription factor MEF2C appears to be selectively

required for the membrane depolarization-dependent activity of CaRE1 (Lyons et al. 2012). These data demonstrate that differential transcription factor binding to single gene regulatory elements can confer stimulus specificity upon the regulation of target genes.

By contrast with CaRF, the binding of CREB to CaRE3 is selectively required for the activity-dependent regulation of *Bdnf* exon IV transcription. The functional importance of this interaction was elegantly demonstrated by generation of a mouse strain bearing a mutation knocked into *Bdnf* promoter IV that selectively mutates the CRE/CaRE3 site (Hong et al. 2008). Neurons from CaRE3 mutant mice have normal basal levels of BDNF but lack activity-inducible transcription from promoter IV, validating the requirement for this CaRE in activity-dependent *Bdnf* gene regulation in vivo. Interestingly, disruption of CaRE3 is associated with impaired *Bdnf* promoter IV recruitment of other transcriptional regulators including MEF2, which binds to a DNA sequence distinct from CaRE3. These data provide experimental support for the role of a multifactor transcriptional complex at *Bdnf* promoter IV and suggest a function for CREB in nucleating the assembly of this complex.

5.4 Chromatin Regulation

In addition to the binding of sequence-specific transcription factors to gene regulatory elements, transcription is both gated and modulated by the secondary and tertiary structure of genomic DNA and its associated architectural proteins, which are collectively called chromatin. The core unit of chromatin is the nucleosome, which comprises ~146 bp of DNA wrapped around an octamer of histone proteins with two copies each of histone H2A, H2B, H3, and H4. The positioning and stability of nucleosomes impact transcription by modulating the accessibility of gene regulatory elements for transcription factor binding. Chromatin structure is sensitive to modifications of both genomic DNA and histone proteins. Differences in chromatin structure are a major determinant of cell-type-specific programs of gene transcription, and as we will discuss below, stimulus-dependent changes in chromatin regulation are emerging as an important mechanism that contributes to the plasticity of *Bdnf* transcription.

5.4.1 Posttranslational Histone Modifications

Dynamic acetylation of specific lysine (K) residues on the N-terminal tails of histones H3 (at K9 and K14) and H4 (at K5, K8, K12, and K16) bound to gene promoters is highly associated with transcriptional activation (Roh et al. 2004). A wide variety of environmental stimuli that induce *Bdnf* transcription have been demonstrated to drive increased acetylation of histones selectively at induced *Bdnf* promoters in physiologically relevant brain regions. Stimuli that have been shown to induce histone acetylation in conjunction with *Bdnf* transcription include seizure (Tsankova et al. 2004; Huang et al. 2002), membrane depolarization (Chen et al. 2003a; Martinowich et al. 2003), antidepressant treatment (Tsankova

et al. 2006), cocaine administration (Kumar et al. 2005), forced cocaine abstinence (Sadri-Vakili et al. 2010), dopamine D1 receptor agonist administration (Schroeder et al. 2008), and extinction of conditioned fear (Bredy et al. 2007). Among the molecular mechanisms that mediate steady-state changes in histone acetylation at *Bdnf*, the histone acetyltransferase CBP has been shown to be recruited to the CREB binding site of *Bdnf* promoter IV in an activity-dependent manner (Hong et al. 2008), and the histone deacetylase HDAC2 has been found to be preferentially associated compared to HDAC1 with *Bdnf* promoters I and II in vivo (Guan et al. 2009).

Activity-dependent regulation of histone methylation has also been observed on *Bdnf* promoters, implicating an additional set of regulatory enzymes in transcriptional control. Histone methylation has been associated with both transcriptional activation and repression depending on the particular lysine that is methylated, with H3K4 and H3K36 correlating with transcriptionally active genes, whereas H3K9, H3K27, and H4K20 correlate with transcriptionally repressed genes (Barski et al. 2007; Lachner and Jenuwein 2002). Furthermore, the mono-, di-, or tri-methylation (me1, me2, or me3) of lysines can mediate differential recruitment of methyl-sensitive binding partners to histones (Shi et al. 2006). On *Bdnf* promoter IV, chronic membrane depolarization of cultured cortical neurons drives increased H3K4me2, a modification associated with transcriptional activation (Martinowich et al. 2003), while on the same promoter, repressive methylation events including H3K9me2, H3K9me3, and H3K27me3 are reduced by acute membrane depolarization (Chen et al. 2003a) or exposure to an enriched environment (Kuzumaki et al. 2011). Large families of enzymes mediate the site-specific methylation and demethylation of histones suggesting a potential source of specificity for the regulation of histone methylation (Shi 2007). However, which specific enzymes act at *Bdnf* promoters and how their function and/or recruitment is coupled to neuronal activity remain largely unknown.

The observation that histone modifications are subject to stimulus-dependent plasticity at *Bdnf* promoters is intriguing because the persistent nature of many chromatin structural changes suggests that these changes could provide a mechanism of molecular memory. To address this possibility, a growing number of studies are examining correlations between histone modifications and *Bdnf* gene expression in chronic stimulation paradigms. For example, in vivo, downregulation of *Bdnf* exons III and IV is seen in hippocampus in a paradigm of chronic social defeat stress in mice (Tsankova et al. 2006). This decrease in *Bdnf* transcription is correlated with an increase in repressive histone H3K27me2 on both promoters III and IV. Interestingly, acute treatment of defeated mice with the antidepressant imipramine restores *Bdnf* expression and induces the activating mark H3K4me2 *without* diminishing the "repressive" H3K27me2 mark. A similar dissociation between H3K27 methylation and *Bdnf* gene expression has also been observed following light deprivation in mice. One week of light deprivation leads to reduced expression of multiple *Bdnf* isoforms in the visual cortex while *Bdnf* expression in the hippocampus remains unchanged (Karpova et al. 2010). However, H3K27me3 levels rise on *Bdnf* promoter IV in both brain regions and are elevated at all active

Bdnf promoters in the hippocampus. One hypothesis suggested by these data is that persistent histone modifications may chronically alter the transcriptional state of *Bdnf* in subtle ways that modulate but do not eliminate stimulus-dependent promoter regulation. Future studies that address more subtle aspects of transcriptional regulation such as the kinetics of gene activation or the cell-type specificity of induction may yield more insight into the functional relevance of these long-lasting changes in histone modifications.

5.4.2 DNA Methylation

In mammalian cells, genomic DNA is extensively modified by the addition of methyl-groups, predominantly at cytosine residues in CpG dinucleotides (Lister et al. 2009). DNA methylation of gene promoters has traditionally been associated with the persistent transcriptional repression that characterizes X-chromosome inactivation, gene imprinting, and long-term silencing of retrotransposons (Bird 2002). More recently, genome-wide studies have shown that substantial DNA methylation is also found over active gene bodies and in intergenic regions (Hellman and Chess 2007; Meissner et al. 2008), where it is thought to modulate gene expression by influencing diverse processes that include maintenance of active chromatin states, alternative promoter choice, and RNA splicing (Luco et al. 2011; Maunakea et al. 2010; Wu et al. 2010).

DNA methylation can be very persistent. For example, the differential methylation of imprinting regions can impact selective parent-of-origin gene expression for the life of a cell (Reik 2007). However a growing body of data indicates that DNA methylation is also subject to neuronal activity-regulated changes suggesting that modulation of DNA methylation may impact the transcriptional regulation of plasticity genes. Consistent with this possibility, stimulus-dependent changes in DNA methylation at *Bdnf* promoters have been correlated with regulation of *Bdnf* mRNA expression. Martinowich et al. (Martinowich et al. 2003) were the first to suggest that chronic membrane depolarization of cortical neurons in culture could lead to activity-regulated loss of methylation in *Bdnf* promoter IV. Subsequent studies have shown changes in the level of DNA methylation that are negatively correlated with *Bdnf* mRNA expression following contextual fear conditioning (Lubin et al. 2008), exercise (Gomez-Pinilla et al. 2011), and early life adversity (Roth et al. 2009).

Intriguing data suggest that the stimulus-regulated demethylation of DNA in the CNS is mediated by activation of DNA repair mechanisms. In the hippocampus, seizure drives rapid, transient demethylation of a highly methylated region of the *Bdnf* gene that is found just upstream of and overlapping the coding exon, exon IX (Ma et al. 2009). Loss of DNA methylation is maximal 4 h following seizure initiation but returns to baseline by 24 h. Seizure-induced DNA demethylation of *Bdnf* requires the enzyme Tet1, and demethylation fails to occur when *Tet1* expression is knocked down (Guo et al. 2011). In the absence of Tet1, seizure also fails to induce *Bdnf* expression, suggesting the causal importance of this demethylation reaction for *Bdnf* gene expression. Tet1 is part of a family of enzymes that mediate the conversion of 5-methyl-cytosine (5mC) to the

intermediate 5-hydroxymethyl-cytosine (5hmC) (Ito et al. 2010). Once induced by Tet1, 5hmC is a substrate for demethylation by the *Aid*/*Apobec* family of Zn^{2+}-dependent cytidine deaminases. Overexpression of *Aid* in the dentate gyrus demethylates *Bdnf* exon IX and induces *Bdnf* mRNA expression, whereas knockdown of *Apobec* reduces seizure-induced DNA demethylation at *Bdnf* and impairs stimulus-dependent *Bdnf* induction (Guo et al. 2011). It will be of great interest in the future to understand how neural activity modulates the activity of this demethylation pathway.

DNA methylation impacts transcription by inhibiting or recruiting the association of DNA binding proteins with methylated regions of the genome (Klose and Bird 2006). Two methyl-DNA-sensitive proteins implicated as effectors of DNA methylation for the regulation of *Bdnf* are the methyl-CpG binding protein 2 (MeCP2) (Chen et al. 2003a; Martinowich et al. 2003) and the insulator protein CTCF (Chang et al. 2010). MeCP2 is of particular interest in the CNS because loss-of-function mutations in human *MECP2* cause the neurodevelopmental disorder Rett syndrome (RTT) (Chahrour and Zoghbi 2007; Amir et al. 1999). Several lines of evidence suggest that MeCP2 modulates both synapse development and function (Deng et al. 2010; Medrihan et al. 2008; Nelson et al. 2006; Dani et al. 2005; Chao et al. 2007; Tropea et al. 2009; Armstrong 2005), and loss of MeCP2-dependent regulation of *Bdnf* expression has been suggested to make a major contribution to these defects. Although MeCP2 has been shown to associate with both a histone deacetylase and a histone H3-K9 methyltransferase (Fuks et al. 2003; Nan et al. 1998), and traditionally has been studied for its role in transcriptional repression, adult *Mecp2* null mice show impaired expression of *Bdnf* suggesting a more complex role for MeCP2 in regulation of this and likely other genes. Unlike classic transcriptional regulators, which bind discrete gene regulatory elements, MeCP2 is bound widely across the genome in a pattern that closely tracks the distribution of DNA methylation. This binding pattern suggests that MeCP2 is a global regulator of chromatin, perhaps via effects of chromosome architecture or long-distance genomic interactions. How global chromatin regulation of this kind would affect *Bdnf* expression in particular and activity-regulated gene transcription in general is an exciting question that remains to be understood.

5.5 Translational Regulation

Although transcriptional regulation is thought to make the major contribution to determining the expression levels of BDNF, several lines of evidence suggest that once synthesized, *Bdnf* mRNA is subject to additional modes of regulation that refine the spatial and temporal synthesis of BDNF protein. Neuronal activity may also play a role in sculpting translational regulation. The *Bdnf* 3′-UTR has been shown to confer activity-regulated stability on a luciferase reporter gene, and elements mediating this effect have been mapped in the 3′-UTR though the regulatory mechanisms remain to be determined (Fukuchi and Tsuda 2010). Another way that neuronal activity may influence the translation of *Bdnf* has been shown for

Exon VI-containing *Bdnf* transcripts, for which RNAse protection analyses have revealed that in response to membrane depolarization of neurons, a different TSS is activated that is well downstream of the primary TSS (Timmusk et al. 1994a). The shorter transcript generated from this new TSS lacks a GC-rich region near the 5′ end of Exon VI and is predicted to be more easily translated, potentially enhancing the activity-dependent expression of BDNF protein. Here we review described mechanisms that may modulate the stability and/or translation of *Bdnf* mRNA as well as regulatory pathways that direct *Bdnf* mRNA trafficking in the cell.

5.5.1 MicroRNAs Targeting the *Bdnf* 3′-UTR

MicroRNAs (miRNAs) are short noncoding RNA molecules encoded within conserved regions of the genome. These regulatory RNAs bind to complementary sequences that are usually located in the 3′ untranslated region (UTR) of their target messenger RNAs. Although miRNA binding can regulate protein expression by repressing translation, miRNA–mRNA pairs most often lead to degradation of the target messenger RNA (Guo et al. 2010).

Bioinformatics-based in silico analyses of putative miRNA binding sites have suggested that multiple miRNAs may be capable of binding the *Bdnf* 3′ UTR (Konopka et al. 2010; Lewis et al. 2003). For example, one panel of prediction algorithms identified potential binding sites for 26 different miRNAs in the 3′ UTR of human *BDNF* (Mellios et al. 2008). Five of these miRNA families were shown to be highly expressed in the prefrontal cortex, a brain region where control of *BDNF* levels is important for cognitive function. The authors of this study demonstrated that overexpression of either of two of these miRNAs, miR-30a-5p and miR-195, was sufficient to reduce the expression of luciferase when transfected into heterologous cells along with a luciferase construct fused to the *BDNF* 3′-UTR (Mellios et al. 2008). Interestingly, overexpression of the miR-30a-5p precursor in cultured rat forebrain neurons was shown to reduce BDNF protein levels without changing levels of *Bdnf* mRNA (Mellios et al. 2008). These data raise the possibility that miR-30a-5p may modulate *Bdnf* translation rather than inducing degradation of *Bdnf* mRNA; however, the mechanisms of this effect remain to be determined. Other studies have started with screens for miRNAs of relevance to a biological phenomenon and then addressed *Bdnf* as a potential target gene. For example, the miR-22 gene contains a single-nucleotide polymorphism that is linked to panic disorder (Muiños-Gimeno et al. 2011). *Bdnf* was identified bioinformatically as a potential target of miR22 and overexpression studies in heterologous cells were used to demonstrate that miR-22 can degrade a luciferase report fused to the *BDNF* 3′-UTR. In another study miR-15-a was identified as a miRNA genetically required for inner ear development, which is a process that is highly sensitive to BDNF levels (Ernfors et al. 1995). *Bdnf* was again identified and tested as a candidate target of regulation using a combination of in silico analysis and heterologous expression assays (Friedman et al. 2009).

As is apparent from these examples, the challenge that remains for miRNA studies is to demonstrate the physiological relevance of endogenous miRNA–target gene interactions for the modulation of gene expression levels in vivo. In support of

a role for endogenous miRNAs in the regulation of BDNF, expression levels of BDNF have been shown to be elevated in the hippocampus of *Camk2a*-Cre conditional *Dicer* knockout mice (Konopka et al. 2010). However this observation does not demonstrate that the effect on BDNF protein is the result of a direct interaction between miRNAs and the *Bdnf* 3′-UTR. Future studies of miRNA knockout strains and/or targeted knockin mutations of miRNA binding sites in the 3′-UTRs of *Bdnf* will enhance our understanding of the functional relevance of this regulatory mechanism for BDNF expression during neuronal development and plasticity.

5.5.2 Natural Antisense *BDNF* Transcripts

In humans, the opposite strand of the *BDNF* gene encodes a variably spliced, apparently noncoding transcript spanning 11 exons transcribed in reverse orientation to *BDNF* (Pruunsild et al. 2007; Liu et al. 2005). This *antiBDNF* gene spans ~191 kb and consists of ten exons with no evidence of open reading frames. *5′* RACE indicates that there is a single promoter upstream of exon I (Pruunsild et al. 2007). Exons I–IV of *antiBDNF* are located 3′ to the *BDNF* gene, and exons VII–X overlap *BDNF* introns. However, exons V and VI of the *antiBDNF* transcript overlap the coding exon of *BDNF*. *AntiBDNF* mRNA is expressed in many tissues where *BDNF* is also expressed, raising the possibility that these two RNAs could form complementary double-stranded RNA species. Consistent with this model, RNAseA/T1 treatment of RNA harvested from human cerebellum supports recovery of double-strand RNA templates of the *BDNF* coding exon for cDNA synthesis (Pruunsild et al. 2007). Natural antisense transcripts are a heterogeneous class of regulatory RNAs that can form sense–antisense RNA duplexes to lead to RNA degradation or translational repression (Faghihi and Wahlestedt 2009). Although *AntiBDNF* was first reported to be expressed only in humans by two groups (Liu et al. 2006; Aid et al. 2007), there has been a recent identification of an antisense *Bdnf* transcript in mice (Modarresi et al. 2012). Inhibition of this *Bdnf* antisense transcript leads to increased expression of BDNF protein; however, this appears to be through a mechanism that is independent of changes in *Bdnf* transcript stability (Modarresi et al. 2012). Although both mouse and human *Bdnf* antisense transcripts overlap the coding region of the *Bdnf* gene, the transcription start sites and exon organization of these transcripts are otherwise entirely different. One hypothesis of the origin of species-specific antisense transcripts is that insertion of the long-terminal repeats of human-specific endogenous retroviruses may create new promoters that drive the formation of these antisense transcripts (Gogvadze et al. 2009). Regardless, this evidence for a species-specific mechanism that may modulate expression of BDNF adds a new and interesting dimension to the intricate complexity of this highly regulated gene.

5.5.3 Dendritic Trafficking of *Bdnf* mRNA

At synapses, BDNF is hypothesized to activate local signaling cascades that modulate synaptic strength and structure (Poo 2001). Though not as robustly targeted to dendrites as the classic dendritic RNAs (*Camk2a*, *Mtap2*, and *Arc*)

(Schuman 1999) the evidence that *Bdnf* mRNA can be even weakly detected in dendrites (Tongiorgi et al. 1997, 2004) raised intense interest in the possibility that regulated trafficking and localized synthesis of *Bdnf* might impact the specificity of neuronal plasticity.

Expression analyses suggest that multiple regions of the *Bdnf* mRNA contribute to its dendritic localization. Most commonly, RNA targeting determinants have been mapped to 3′-UTRs. Through the use of two different alternative polyadenylation sites, *Bdnf* transcripts fall into two categories with either a short or long 3′-UTR (Hofer et al. 1990; Timmusk et al. 1993a). RNAs containing the long UTR are preferentially localized to dendrites and genetic truncation of the long 3′-UTR of *Bdnf* leads to impaired dendritic *Bdnf* mRNA localization, consistent with a localization of a positive dendritic target sequence to this region (An et al. 2008). However the coding sequence and 5′-UTRs of *Bdnf* appear to contribute to cellular mRNA localization as well. In situ analyses show that *Bdnf* mRNAs with different 5′-UTRs are differentially localized in the cell. For example, exon VI-containing forms of *Bdnf* are targeted to dendrites after stimulation of visual cortical neurons, whereas exon IV-containing forms of *Bdnf* are localized only to the somata of the same cell (Pattabiraman et al. 2005). Furthermore, in the hippocampus, exon II and exon VI probes detect *Bdnf* mRNA in apical dendrites after kainate-induced seizure, whereas exon I- and IV-containing transcripts remain restricted to the somata despite being strongly induced in levels by the stimulus (Chiaruttini et al. 2008). Overexpression analyses in hippocampus neurons show that when fused to GFP alone, the coding sequence of *Bdnf* is trafficked to the dendrites whereas addition of exon I or exon IV sequences to the 5′-UTR of the reporter construct leads to retention of *Bdnf* in the somata. These data raise the possibility that competing dendritic targeting and somatic retention signals may be found in the coding sequence and 5′-UTRs of the *Bdnf* mRNA, respectively (Chiaruttini et al. 2008).

The identification of *Bdnf* mRNA binding proteins is just beginning to yield insights into the regulation of its trafficking. Using bioinformatics, Chiaruttini et al. (2008) identified a putative binding site for the RNA binding/trafficking protein Translin (Li et al. 2008) in the coding sequence of *Bdnf*. Intriguingly, this binding site overlaps the sequence encoding the common nonsynonymous Val66Met SNP in BDNF, which has been shown to impact BDNF synthesis and secretion (Egan et al. 2003; Chen et al. 2004).There is reduced dendritic targeting of *Bdnf* mRNA in the apical dendrites of the hippocampus following pilocarpine seizure in *Bdnf* Met/met mice compared with Val/Val (Chiaruttini et al. 2008). Translin and its associated protein Trax are in dendrites, and Translin knockouts do show moderately reduced levels of dendritic *Bdnf* mRNA under baseline conditions. However these mice show robust dendritic trafficking of *Bdnf* mRNA following pilocarpine seizure demonstrating that Translin expression is not required for trafficking under these conditions (Wu et al. 2011). Another RNA binding protein that may influence *Bdnf* mRNA trafficking and/or translation is the heterogeneous nuclear ribonucleoprotein CArG box binding factor A (CBF-A) (Raju et al. 2011). CBF-A is found in dendrites and synaptosomes as well as somata

and nuclei, suggesting that it could have functions in regulation of dendritic mRNAs. CBF-A coimmunopreciptiates with *Bdnf*, *Arc*, and *Camk2a* RNA from synaptosomes, and electrophoretic mobility shift assays demonstrate that CBF-A can form a direct interaction with hnRNP A2 response elements (RTS) located in the 3′ untranslated regions of all three mRNAs. However, rather than selectively inhibiting dendritic localization of these mRNAs, knockdown of CBF-A reduces overall NMDA-R-dependent induction of *Bdnf*, *Arc*, and *Camk2a* mRNAs, suggesting a more general role for CBF-A in stability and/or processing of mRNAs including *Bdnf* (Raju et al. 2011).

Despite the presence of *Bdnf* mRNA in dendrites, it remains to be determined whether *Bdnf* is actually locally translated in dendrites or at synapses. Nonetheless, several lines of evidence suggest the importance of translational regulation of BDNF expression for its functions at synapses. For example, truncation of the long 3′-UTR of *Bdnf* not only reduces dendritic *Bdnf* levels but also causes defects in pruning of dendritic spines and a selective impairment of long-term potentiation at synapses onto the dendrites of hippocampal neurons (An et al. 2008). *Bdnf* transcripts with the long 3′-UTR are more likely to be recovered in the polysome fraction from cells, suggesting that they are more readily translated (Lau et al. 2010; Timmusk et al. 1994a). Under basal conditions, addition of the long 3′-UTR of *Bdnf* to a reporter suppresses translation, whereas following neuronal activity the long 3′-UTR enhances reporter translation, raising the possibility that stimulus-sensitive translational regulatory elements lie within this domain (Lau et al. 2010). One signaling pathway that has been shown to modulate neuronal BDNF translation in a stimulus-regulated fashion is the eukaryotic elongation factor 2 kinase (eEF2K, also known as CaMKIII). eEF2 is a critical component of the translational machinery that promotes ribosomal translocation during protein synthesis. Under resting conditions in neurons, basal activity of NMDA receptors promotes phosphorylation of eEF2 by eEF2K, which inhibits general translation (Sutton et al. 2007). However, upon NMDA receptor blockade, reduced activity of eEF2K permits dephosphorylation of eEF2 that promotes translation of target mRNAs including *Bdnf* (Autry et al. 2011). Intriguingly, translation induction of BDNF by the NMDA-receptor antagonist ketamine is positively correlated with the antidepressant actions of this drug (Autry et al. 2011). Thus these data raise the possibility that translational regulation of BDNF could contribute to the modulation of complex cognitive and emotional behaviors.

Conclusions

Two decades of research into the transcriptional and translational mechanisms that control expression of the neurotrophins have yielded a wealth of molecular information about fundamental regulatory pathways that contribute to neuronal development and plasticity. These regulatory pathways offer promising targets for the development of therapeutics that could be used to extrinsically regulate neurotrophin levels for the correction of neurological disorders. The challenge for the future is to understand how these pathways are integrated in vivo to sculpt

subtle aspects of the gene expression program that underlies the complexity of the mammalian brain.

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