TRANSCRIPTIONAL REGULATION OF BRAIN-DERIVED NEUROTROPHIC FACTOR IN NEURONS

By

Fei Zheng

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ABSTRACT

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Neuroplasticity is an important feature of mammalian brains, referring to the functional or structural changes of the nervous system after learning or adapting to the changes of environment. Activity-dependent transcription is critical for neuroplasticity. Brain-derived neurotrophic factor, an activity-dependent gene, has emerged as a key molecule in synaptic plasticity, memory formation and neurological diseases. The regulation of activity-dependent BDNF transcription remains elusive.

Exon IV-containing BDNF mRNA (BDNF IV) is one of the major activity-responsive forms of the multiple BDNF mRNAs. Calcium influx via calcium channel or NMDA receptor stimulates BDNF IV transcription in cortical neurons, which requires activity of MAPK, PI3K, PKA and CaMKs. The three identified calcium-responsive elements (CaREs) in the BDNF IV promoter (promoter IV) mediate calcium-induced BDNF IV transcription coordinately. In mature neurons, only CaRE1 and CaRE3 are responsive to calcium influx and are regulated by MAPK, PI3K and CaMKs in a stimulation-selective manner.

BDNF IV is also induced by self-stimulation, which is regulated by MAPK activity, extra- and intracellular calcium level and the NMDA receptor. Both NR2A-

and NR2B-containing NMDA receptors, as well as synaptic and extrasynaptic NMDA receptor, are required for BDNF self-induction. Moreover, a synergistic effect of BDNF and NMDA stimulation has been found for BDNF IV upregulation.

In summary, we suggest a novel working model for the regulation of calcium- and BDNF-induced BDNF transcription.

ACKNOWLEDGEMENT

When I write this dissertation and summarize my work in the past five years, I notice that I will never forget the day when I entered Dr. Hongbing Wang's office and asked him, "May I do a rotation in your lab?" At that time, I really didn't know how far I could go on the road of science. However, in the following years, Dr. Wang, like a father more than a Project Investigator, spared no effort to help me, guide me and train me to realize my dream of being a scientist. He is not only an excellent researcher, but also absolutely a remarkable advisor. I appreciate, from the bottom of my heart, the fortune of being his first student in these five years, which have been the most rewarding time of my life. Additionally, I am glad to see this innovative and friendly lab growing up. The previous and current Wang lab members, Dr. Xianju Zhou, Dr. Ming Zhang, Dr. Yan Wang and Ferzin Sethna, all offered kind help to my research.

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Last but not least, I must express my gratitude and love to my family---my parents and my wife. I could have never done this without their support and encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

AC: adenylyl cylcase

AD: Alzheimer's disease

AMPA: 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid

Aβ: amyloid β-peptide

BD: bipolar disorder

BDNF: Brain-derived neurotrophic factor

bHLH: basic Helix-Loop-Helix

CaMK: calcium/calmodulin dependent kinase

CaRE; calcium responsive element

CaRF: calcium responsive factor

CFC: contextual fear conditioning

CRE: cAMP responsive element

CREB: CRE-binding protein

DAG: diacylglycerol

DKO: double knockout

Dn: dominant-negative

DNMT: DNA methyltransferase

GRB: growth factor receptor-bound protein

HAT: histone acetyltransferase

HDAC: histone deacetylases

HFS: high frequency stimulations

IEG: immediate early gene

IP3: inositol-1,4,5-trisphosphate

KA: kainic acid

KO: knock out

LTD: long-term depression

LTP: long-term potentiation

L-VGCC: L-type voltage-gated calcium channel

MAPK/ERK: mitogen-activated protein kinase /extracellular signal- regulated kinase

MEF: myocyte enhancer factor

mPFC: medial prefrontal cortex

MSK: mitogen- and stress-activated protein kinase

NFAT: nuclear factor of activated T cell

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells

NMDA: N-Methyl-D-aspartic acid

NRSE: neuron-restrictive silencer element

PD: Parkinson’s disease

PI3K: phosphatidylinositol 3-kinase

PIP2: phosphatidylinositol-4,5-bisphosphate

PKA: protein kinase A

PKB: protein kinase B

PKC: protein kinase C

PLC: phospholipase C

RACE: rapid amplification of cDNA ends

REST: RE1 silencing transcriptional factor

RSK: 90 kDa ribosomal S6 kinase

RT: reverse-transcribe

SOS: son of sevenless

tPA: tissue plasminogen activator

TrkB: receptor tyrosine kinase B

USF: upstream stimulatory factor

UTR: untranslated region

CHAPTER 1

BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) IN PLASTICITY, MEMORY AND DISEASE.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, plays critical roles in many aspects of neuronal events, including differentiation, survival and neuroplasticity in both developmental and adult brain (Poo, 2001; Lu, 2003; Reichardt, 2006; Bekinschtein et al., 2008a; Minichiello, 2009). In this chapter, I will first introduce some basic features of BDNF including its genetic structure, protein cleavage, and intracellular signals. Second, I will focus on BDNF functions in neuronal plasticity (mainly long-term potentiation or LTP), memory formation, and certain neurological diseases.

ACTIVITY-DEPENDENT BDNF SYNTHESIS AND BDNF-MEDIATED SIGNAL TRANSDUCTION

BDNF transcription

BDNF is involved and functions as a key molecule in synaptic plasticity, which is mainly due to its activity-dependent synthesis and release. BDNF expression is induced by several neuronal activities, both in vitro and in vivo. For example, calcium influx from L-type voltage-gated calcium channel (L-VGCC) (Tao et al., 1998) and activated NMDA (N-methyl-D-aspartic acid) receptors (Tabuchi et al., 2000) in cultured neurons, as well as electric stimulation in brain slices (Patterson et al., 1992) all evoke of BDNF mRNA expression. Moreover, the BDNF mRNA can also be induced in live animal brains after fear-associated memory training (Hall et al., 2000), physical exercise (Neeper et al., 1995), exposing to novel environment (Young et al., 1999), and kainic acid (KA)-induced seizure (Timmusk

et al., 1993).

Intriguingly, the activity-dependent BDNF transcription only produces certain BDNF mRNA forms (Timmusk et al., 1993; Lubin et al., 2008). BDNF gene consists of at least eight 5' untranslated exons (exon I to exon VIII) and one 3' coding exon (exon IX). Using 5' rapid amplification of cDNA ends (RACE), bioinformatics, RT-PCR and sequencing analysis, nine distinct transcriptional initiation sites were identified at the beginning of the eight 5' exons and in the intron preceding exon IX. As shown in Figure 1.1, each 5' exon is spliced to the common 3' exon to generate a BDNF mRNA form with one 5' noncoding exon and one 3' coding exon. Including the BDNF mRNA consisting of 5' extended coding exon (exon IXA) and exon IX, there are at least night different BDNF mRNA forms (Timmusk et al., 1993; Aid et al., 2007). Because of the alternative splice donor site within exon II, there are three exon II-containing transcript variants (IIA, IIB and IIC) (Timmusk et al., 1995). Moreover, in the 3' end of exon IX, there are two different poly-adenylation sites. Thus, each BDNF mRNA can use an alternative poly-A signal, resulting total 22 BDNF mRNA forms (Fig. 1.1). Considering the protein coding exon is common to all of these products, thus all isoforms are translated into the same BDNF protein.

RACE analysis and expression features of different exon-containing BDNF mRNA forms indicate that these transcripts are controlled by different promoters. It is hypothesized that the differential promoters of BDNF gene may provide

Figure 1.1

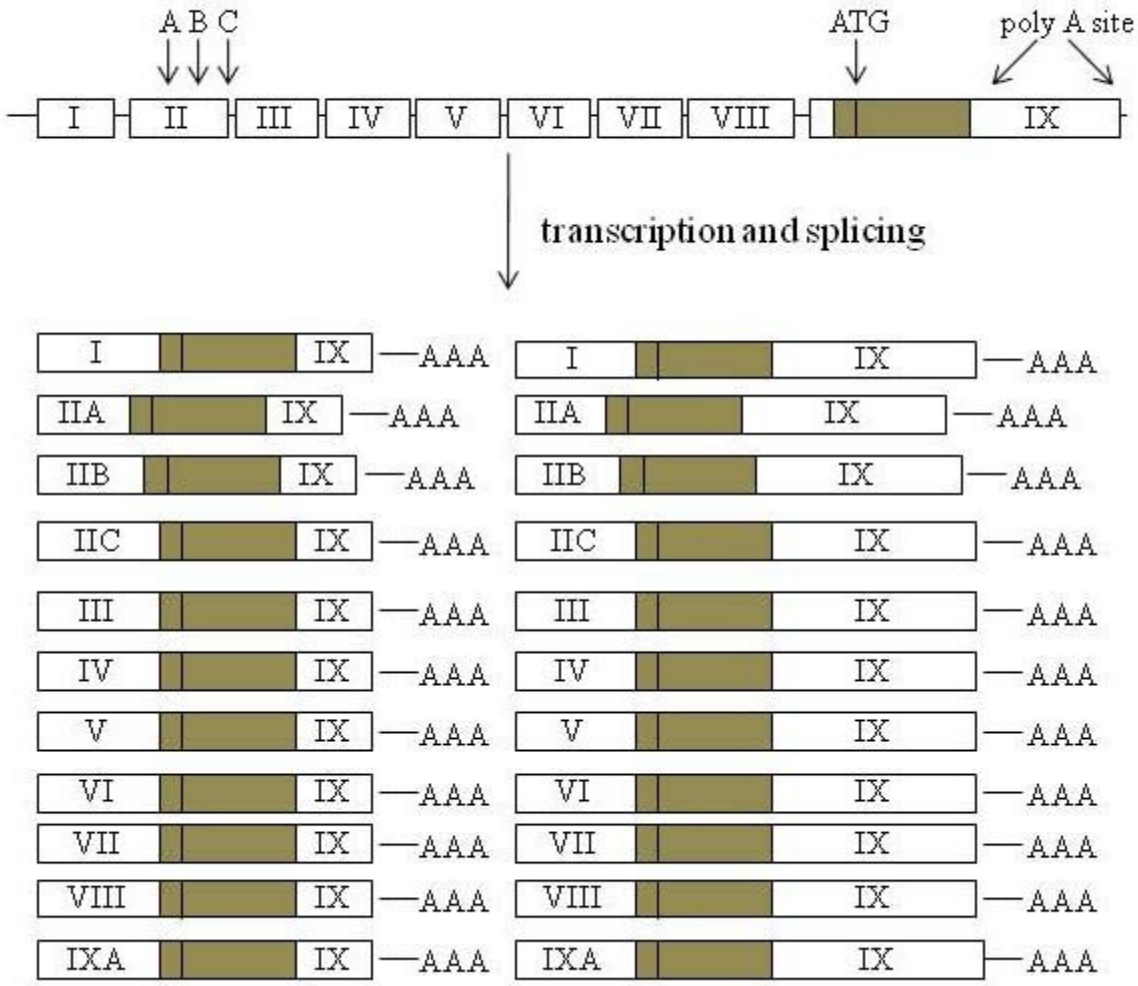


Figure 1.1 The scheme of mouse and rat BDNF gene. BDNF gene consists of eight 5' exons (I-VIII) and one 3' exon (IX). Introns in the gene are presented as straight lines. The protein coding regions are shown as dark boxes and the untranslated regions are shown as open boxes. Three alternative splicing sites (A, B and C) in exon II and the two poly-adenylation sites (polyA site) are indicated with arrows. Exon IXA is the 5' extended coding exon. Adapted from Aid et al., 2007. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

multiple layers of regulation by distinct modulators in different tissues. For example, in cortex, exon IV-containing BDNF mRNA (BDNF IV) is the major form induced by neuronal activity (Tao et al., 1998). Investigations on this specific transcript indicate that the 170-base-pair (bp) 5' flanking sequence of exon IV functions as the regulatory region for calcium-mediated BDNF IV transcription, in which three calcium responsive elements (CaRE1, 2 and 3) have been identified (Fig. 1.2).

CaRE1 is located at -73 to -64 relative to the exon IV transcriptional initiation site, which is conserved in the rat, mouse and human BDNF gene (Tao et al., 2002). A yeast one-hybrid screen purified a novel transcriptional activator, named calcium responsive factor (CaRF), as a CaRE1 binding protein. Computational analysis predicts several kinase-phosphorylation sites including mitogen-activated protein kinase/ extracellular signal regulated kinase (MAPK/ERK), calcium/calmodulin kinase II (CaMKII) and protein kinase C (PKC) sites, but the detailed regulation on CaRF/CaRE1 system is largely unknown. Recently, McDowell and colleagues generated mice with deletion of CaRF DNA binding domain (CaRF knockout/KO). The mice express reduced BDNF IV mRNA in the cortex but not hippocampus (McDowell et al., 2010), suggesting that the regulation of BDNF by CaRF may be tissue-selective.

CaRE2 has been identified as an E-BOX element, which is supposedly bound by a member of the basic helix-loop-helix (bHLH) family (Chen et al., 2003b).

Figure 1.2

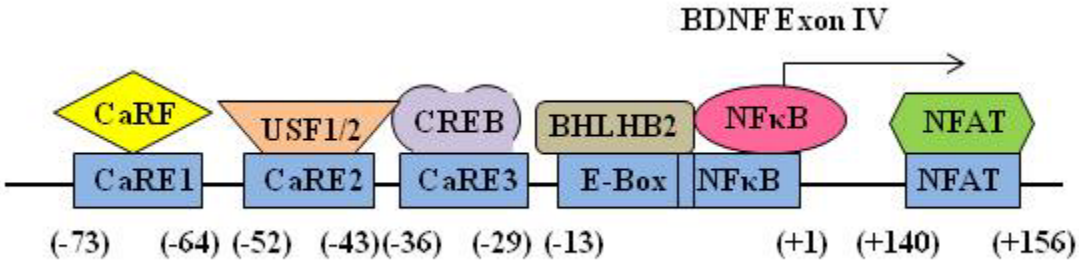


Figure 1.2 The transcriptional regulation on promoter IV. The currently identified *cis*-elements located in the 5' flanking region of exon IV and the NFAT sequence on the 3' of exon IV are presented as blue boxes. The relative location of the sequences to the transcriptional initialization site (+1) is labeled below. The E-Box and the NFκB sequence have one bp overlap.

Indeed, the results from yeast one-hybrid screen showed that CaRE2 is bound by USF1/2 (upstream stimulatory factor, belonging to the bHLH family) as homo- or heterodimers. USF1 and 2 regulate BDNF transcription independently because CaRE2-mediated transcription is intact in USF1 or USF2 single knockout neurons. Moreover, CaRE2-mediated transcription is elevated by membrane depolarization but not by activation of glutamate receptors, indicating that it is regulated in a stimulation-selective manner.

CaRE3 resembles a cAMP response element (CRE). Electrophoretic mobility shift assays (EMSA) provided strong evidence that CaRE3 is bound by CRE binding protein (CREB) (Tao et al., 1998). CREB is activated through phosphorylation at Ser-133 by either MAPK-RSK2/MSK (ribosome S6 kinase2/mitogen- and stress-activated kinase) or CaMKIV. The activated CREB recruits its co-activator CBP (CREB binding protein), which is associated with histone acetylation and RNA polymerase II complex recruitment, and facilitates transcription (Greer and Greenberg, 2008; Sakamoto et al., 2011). Furthermore, activation of CREB is necessary for BDNF IV transcription and sufficient for BDNF IV mRNA up-regulation. First, dominant-negative CREB blocks calcium-mediated BDNF IV transcription, and disrupted CREB binding by mutating CaRE3 sequence alone abolishes KCl- and sensory experience-induced BDNF IV transcription (Tao et al., 1998; Hong et al., 2008). Second, promoter IV-driven transcription can be elevated by a constitutively active CREB without calcium

stimulation.

However, accumulated evidence showed that the BDNF IV expression is also controlled by other *cis*-elements. For example, a 22-bp segment spanning from -21 to +1 contains overlapped sequence of a NFκB (nuclear factor κB) binding site and a class B E-BOX (Lipsky et al., 2001; Jiang et al., 2008). Although the direct evidence for the endogenous NFκB binding to promoter IV is lacking, NMDA-induced BDNF IV transcription does require NFκB activity and deletion of this NFκB site also abolishes up-regulation of BDNF IV mRNA by NMDA stimulation. The class B E-BOX is bound by BHLH2, a suppressor in the bHLH family. The reduced occupancy of BHLH2 after NMDA receptor activation is required for the upregulation of BDNF IV. Furthermore, either basal or KA-induced BDNFIV level is increased in BHLH2 knockout (KO) mice. Moreover, a composite NFAT/MEF2 (nuclear factor of activated T cell/ myocyte enhancer factor 2) consensus sequence exists at exon IV, which is bound by transcriptional factors NFATc4 and MEF2. Silencing NFATc4 decreases BDNF IV mRNA induced by NMDA stimulation and overexpression of NFATc4 enhances the induction, indicating that NFATc4 positively regulates BDNF IV transcription (Vashishta et al., 2009). Although the partner of NFATc4 varies in different cell types, it is suggested that MEF2, a transcriptional factor, which responses to neuronal activity, dissociates with histone deacetylases (HDAC) and facilitates transcription, is a strong candidate (Crabtree and Olson, 2002; Flavell et al., 2006). Moreover, a

recent study pointed out that a transcriptional factor, Npas4, responses slowly to neuronal activity but is required for sustained BDNF IV mRNA level after stimulation (Lin et al., 2008). Together with the CaRF, USF1/2 and CREB, these new-indentified transcriptional factors coordinately regulate activity-dependent BDNF IV transcription.

Beside BDNF IV, exon I-containing BDNF (BDNF I) is also an activity-responsive BDNF form, which is enhanced by calcium influx through L-VGCC (Tabuchi et al., 2000) or NMDA receptor (Tian et al., 2009). Intriguingly, this induction seems to be tissue-selective as BDNF I was evoked by NMDA in hippocamal but not cortical cultures. Furthermore, a CRE overlapped by a USF binding element (USFBE), which is required fro calcium-mediated BDNF I transcription, has been identified in Promoter I (Tabuchi et al., 2002). The role of CREB and USF1/2 on regulating BDNF I transcription has also been confirmed in cultured neurons.

In addition to the 5' UTRs (untranslated regions) of multiple exons, the 3' UTR with alternative poly-A sites has been implicated in subcellular translocation of BDNF mRNA (An et al., 2008). The BDNF transcript with short UTR is restricted to the soma, whereas the long UTR BDNF mRNA is able to be transported to dendrites. In mice with truncated long UTR, BDNF mRNA and protein are significantly reduced in the dendrites and the growth of dendritic spines is abnormal, suggesting that the localization and local translation of BDNF is

important for the formation of neural circuits.

Pro-BDNF and mature BDNF

The regulation of BDNF in nervous system occurs not only at mRNA level. The post-translational modification of BDNF protein is also essential for BDNF functions. The initially synthesized precursor protein pro-BDNF (32 kDa) undergoes a cleavage to form mature BDNF (mBDNF). It was thought that pro-BDNF is intracellular localized and the cleavage happens in either trans-Golgi (by the subtilisin-kexin family of endoproteases) or immature secretory granules (by proprotein convertases) (Mowla et al., 1999; Greenberg et al., 2009). However, reports from several laboratories have indicated that pro-BDNF is also releasable and physiological functional (Pang et al., 2004; Woo et al., 2005; Yang et al., 2009). In contrast to mBDNF, which facilitates long term potentiation (LTP, a cellular model for memory formation) and promotes cell survival by binding the receptor tyrosine kinase B (TrkB, see below), pro-BDNF binds to $p75^{NTR}$ receptor, which enhances long term depression (LTD) and induces apoptosis pathways (Teng et al., 2005; Woo et al., 2005). Additionally, the identification of tPA (tissue plasminogen activator)-plasmin regulated pro-BDNF cleavage also provided a novel pathway for pro-BDNF to mature BDNF conversion. High frequency stimulations (HFS) that normally induce LTP facilitate tPA secretion, and consistent with this pathway, tPA and plasmin are playing a functional role in late phase LTP (Pang et al., 2004; Nagappan et al., 2009).

BDNF- mediated signal transduction

How does BDNF function in the nervous system? Secreted mBDNF binds to the TrkB receptor, resulting in ligand-receptor dimerization and autophosphorylation of the tyrosine residue that is at the intracellular kinase domain of TrkB (Cunningham and Greene, 1998). The phosphorylation elicits recruitment of several proteins including adaptor protein Shc and phospholipase Cγ(PLCγ) (Kavanaugh and Williams, 1994; Minichiello, 2009). Activated TrkB triggers three major signaling cascades: the Ras-Raf-MAPK, PI3K (phosphatidylinositol 3-kinase)-Akt and PLCγ pathways. The recruited adaptor protein Shc is in turn bound by growth factor receptor-bound protein 2 (GRB2) and/or son of sevenless (SOS), and activates Ras-Raf-MAPK pathway (Reichardt, 2006). The activation of PI3K-Akt/PKB (protein kinase B) pathway is through the indirect binding of Ras and certain adaptor proteins like GRB associated binder 1 (GAB1) to TrkB receptor (Rodriguez-Viciana et al., 1994; Holgado-Madruga et al., 1997). PLCγ activated by the tyrosine residue of TrkB hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2), generating inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 activates IP3 receptor on ER (endoplasmic reticulum) to open the intracellular calcium stores, which induce calcium-dependent pathways such as CaMKs cascades. DAG is able to stimulate the PKC pathway (Reichardt, 2006). Notably, MAPK and CaMKIV have been indicated as the activators of transcriptional factor CREB, which regulates BDNF I

Figure 1.3

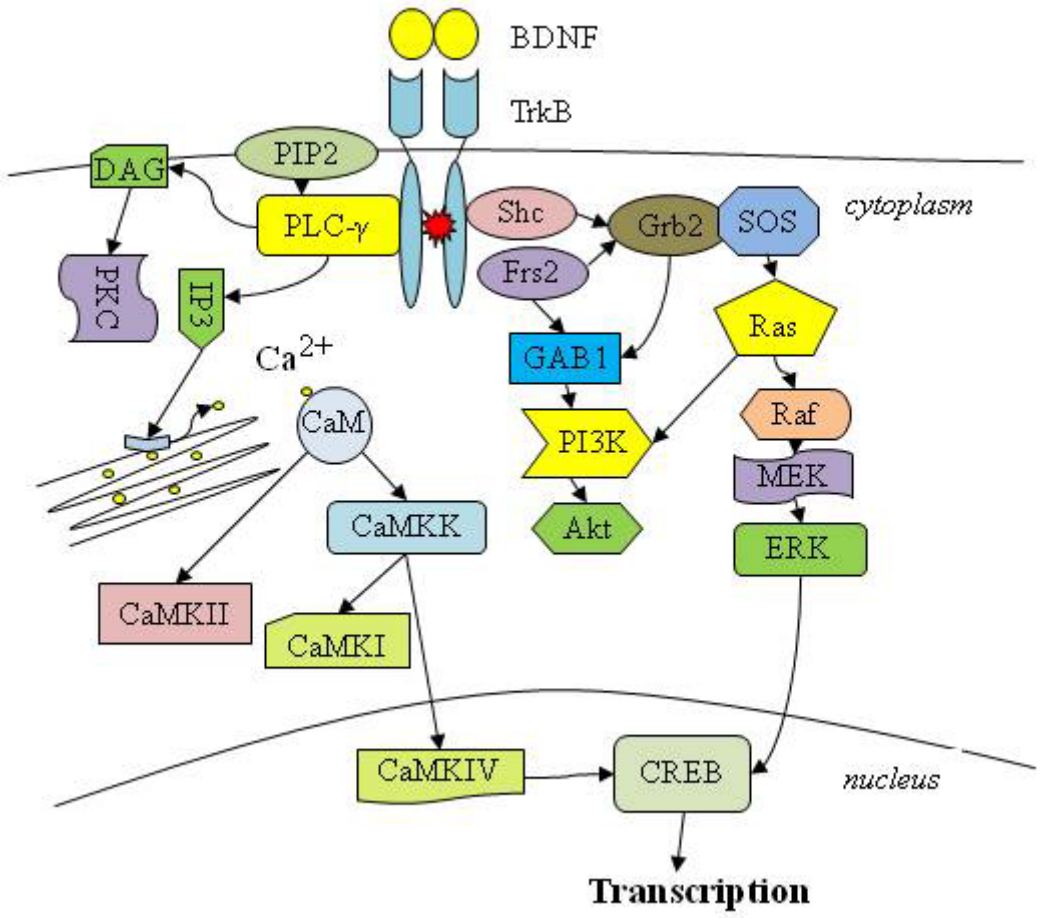


Figure 1.3 BDNF/TrkB-mediated signal transduction. When bound by BDNF, the TrkB receptor activates three major pathways: Ras-MAPK/ERK, PI3K-Akt, and PLCγ pathways. The direct docking sites on TrkB C-terminal are for adaptor protein Shc and PLCγ. Activated Shc recruits Grb and SOS and in turn activate Ras-MAPK pathway. The Grb2-GAB1 and Ras account for PI3K activation. Phophorylated PLCγ by TrkB converts PIP2 to IP3 and DAG. IP3 releases intracellular calcium and induces calcium/CaMK pathway. Elevated ERK and CaMKIV activity may phosphorylate CREB and contribute to gene transcription. Adapted from Reichardt, 2003.

and IV transcription. Thus, the induction of MAPK and PLCγ pathway may enhance BDNF mRNA, suggesting a positive feedback for neuronal activity-dependent BDNF transcription (Fig.1.3).

FUNCTION OF BDNF IN SYNAPTIC PLASTICITY, MEMORY FORMATION AND NEUROLOGICAL DISEASES

The mammalian brain is able to convert transient stimuli from the environment to a long-lasting morphological change or functional alternation, which is considered to be the basis of learning and adaption to the environment. This conversion process involves *de novo* gene transcription, protein synthesis and cell structure modification. BDNF, as described above, is an activity-dependent immediate early gene (IEG). It plays a critical role in neuronal plasticity, memory formation, mental disorders and neurodegenerative diseases.

BDNF and LTP

To study the molecular and cellular mechanism of learning and memory, a phenomenon named long term potentiation (LTP) is widely considered to be a cellular model of learning and memory. LTP is a persistent increase of synaptic strength resulting from synchronously stimulation of pre- and postsynaptic neurons. This enhancement can last from minutes (early-phase LTP or E-LTP) to hours or days (late-phase LTP or L-LTP). This process needs not only opening of calcium channels, activation of several enzymes, neural transmitter release, trafficking and insertion of glutamate receptors, but also, especially for the L-LTP,

gene transcription and protein synthesis (Sweatt, 1999; Malenka, 2003; Malenka and Bear, 2004; Minichiello, 2009).

BDNF has emerged as a key molecule in neuroplasticity. First, BDNF mRNA is dramatically increased by LTP induction, in both hippocampal neurons and brain slices (Patterson et al., 1992; Dragunow et al., 1993). BDNF release is also greatly enhanced during LTP induction, which is visualized by either overexpression of green fluorescence protein (GFP)-tagged BDNF or usage of a cell-based fluorescent indicator that selectively and efficiently detects BDNF (Brigadski et al., 2005; Nakajima et al., 2008). Second, BDNF is required for hippocampal LTP induction. For example, bath application of anti-BDNF antibodies or BDNF scavenger TrkB-IgG to block BDNF-TrkB signal attenuates LTP (Figurov et al., 1996; Kang et al., 1997). In addition, LTP induction is impaired in BDNF knockout mice, and this defect is able to be rescued by exogenous application of recombinant BDNF or a virus-reintroduced BDNF gene, ruling out BDNF deletion-caused developmental impairment (Korte et al., 1996; Patterson et al., 1996; Pozzo-Miller et al., 1999). BDNF can also facilitate LTP induction in an age-dependent manner: BDNF has no effect on potentiation in mice at postnatal day (P) 8-9, converts short-term LTP to long-term LTP at P12-13, and does not further increase the amplitude of LTP in P17-18 or adult brain. This variability may be due to the change of BDNF or TrkB expression levels during development (Maisonpierre et al., 1990; Figurov et al., 1996). Although one report indicated that

BDNF stimulation alone can induce LTP (Kang and Schuman, 1995), this result was not replicated by several other groups (Figurov et al., 1996; Patterson et al., 1996). Third, BDNF-TrkB signal is also required for L-LTP maintenance. Pretreatment with TrkB antiserum impaired L-LTP, and a similar effect was found in TrkB knockout mice (Korte et al., 1998; Xu et al., 2000; Minichiello et al., 2002). More surprisingly, bath application of BDNF can rescue impaired L-LTP in protein-synthesis blocked system, suggesting a key role of BDNF for L-LTP (Pang et al., 2004).

BDNF and memory formation

How is BDNF involved in learning and memory? BDNF expression is evoked by hippocampus-dependent learning, and it is necessary for short-term or long-term memory (STM or LTM) formation. First, BDNF mRNA is increased after hippocampus-dependent learning in several paradigms, such as Morris water maze, inhibitory avoidance and contextual fear conditioning (Kesslak et al., 1998; Ma et al., 1998; Hall et al., 2000). Second, Infusions of either BDNF antibodies or antisense oligonucleotides severely impair spatial and inhibitory avoidance memory in rats (Ma et al., 1998; Mu et al., 1999). In addition, heterozygous BDNF knockout mice with reduced BDNF expression fail to form spatial or contextual memory (Linnarsson et al., 1997; Liu et al., 2004). Moreover, conditional deletion of TrkB in the forebrain also impairs memory formation when tested using a Morris water maze or a radial arm maze (Minichiello et al., 1999). Third, BDNF is also

required for the storage of LTM. Infusion of BDNF antisense oligonucleotides 4 or 12 hours after training disrupts memory retention and blocks memory retrieval (Alonso et al., 2002; Bekinschtein et al., 2007; Bekinschtein et al., 2008b).

As mentioned above, there are multiple BDNF mRNA forms and only certain of them are responsive to neuronal activity. Thus, recent studies sought to understand whether this exon-specific effect is critical for BDNF function *in vivo*. There are two lines of BDNF IV-mutated mice generated by different groups (Hong et al., 2008; Sakata et al., 2009). Sakata and colleagues inserted GFP-STOP into the exon IV locus to prevent BDNF IV expression (the mouse is named BDNF-KIV), whereas Greenberg's group mutated the CREB binding site (CaRE3/CRE) in promoter IV to abolish the activity-dependent BDNF IV upregulation (the mouse is named CREmKI). Both mutants showed abnormal function of inhibitory synapses but not excitatory synapses. For example, less GABAergic synapses are formed in CREmKI cortex, whereas the medial prefrontal cortex (mPFC) of BDNF-KIV has reduced parvalbumin-positive GABAergic interneurons, and shows disrupted spike-timing-dependent potentiation. Interestingly, CaRF (the transcriptional factor regulating BDNF IV expression, see Section 1) knockout mice, which show significant decrease of BDNF IV in cortex, also express reduced GABAergic synapse markers (McDowell et al., 2010). It is still unknown whether the exon-specific mutation of BDNF affects learning and memory. However, the CaRF KO animals show disrupted

remote memory and impaired extinction. Thus, it would be interesting to study whether the two BDNF IV-mutated mice show similar phenotypes.

Mechanisms of BDNF function

How does BDNF regulate synaptic plasticity and memory formation? To be specific, it is important to know 1) where BDNF is released (presynapses or postsynapses) and 2) what signaling pathways are involved.

First, it has been suggested that the induction of either hippocampal LTP or striatal LTP only requires BDNF from presynaptic vesicles (Zakharenko et al., 2003; Jia et al., 2010). However, release of the preexisting BDNF is not sufficient to maintain its functional level at synaptic connections at the late phase of LTP. Additionally, there is no dispute that L-LTP requires new protein synthesis and sustained BDNF level, suggesting that enhanced BDNF transcription, which only occurs at postsynapses, accounts for the long-lasting synaptic change (Patterson et al., 1992). Moreover, postsynaptic BDNF secretion triggered by a spike-timing protocol at the single-cell level is required for the long-term structural change of spines, highlighting the role of postsynaptic BDNF release in plasticity (Tanaka et al., 2008). Taken together, it indicates that presynaptic and postsynaptic BDNF is released and functions at early and late stage of LTP separately (Fig. 1.4).

Second, the downstream cascades of BDNF involved in synaptic plasticity are largely unknown. It has been suggested that BDNF regulates neuroplasticity via modulating neurotransmission, as well as mediating transcription and translations.

Figure 1.4

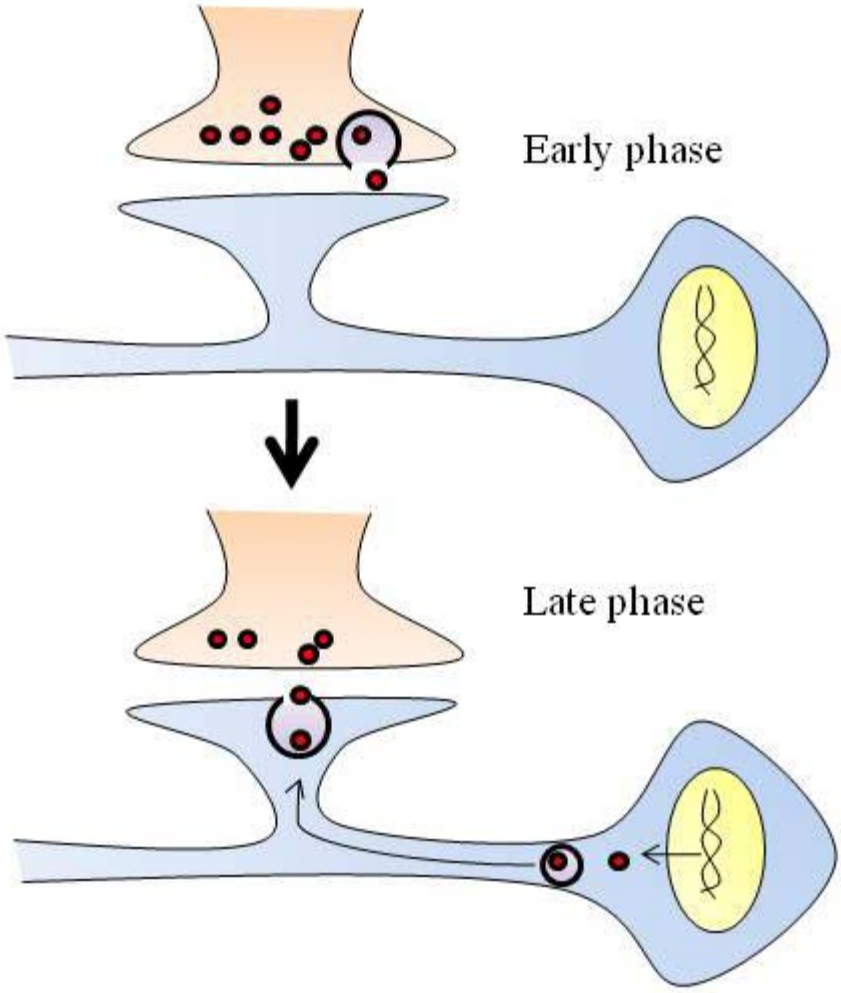


Figure 1.4 Secretion of BDNF from pre- and postsynapses accounts for the different phase of LTP. BDNF release from presynapses (pink color) may contribute to the early phase plasticity (e.g. E-LTP). Transcription and secretion of BDNF from postsynapses (blue color) may be necessary for the late phase plasticity (e.g. L-LTP). Adapted from Lu et, al., 2007.

BDNF triggers glutamate release and membrane depolarization (Poo, 2001; Tyler et al., 2002). For example, BDNF enhances glutamate release from synaptosomes and regulates a rapid recycling vesicle pool (Sala et al., 1998; Tyler et al., 2006). It has also been demonstrated that BDNF results in membrane depolarization through gating sodium channel $Na_v1.9$ (Kafitz et al., 1999; Blum et al., 2002) in a classical TrkB signal-independent manner. Additionally, BDNF modulates glutamate receptors. NMDA receptor as a major glutamate receptor consists of two NR1 subunits and two NR2 (2A, 2B, 2C and 2D) subunits. BDNF-activated TrkB interacts and phosphorylates NR2B subunit through Fyn, and in turn increases open possibility of NMDA receptor (Levine et al., 1998; Lin et al., 1998). Besides, BDNF also elevates the expression and regulates the trafficking of both NMDA and AMPA (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid) receptor. This process has been indicated as an essential step for the induction and maintenance of LTP (Caldeira et al., 2007b; Caldeira et al., 2007a; Nakata and Nakamura, 2007) (Fig. 1.5). Last but not least, BDNF stimulates gene transcription and protein translation that are required for L-LTP and LTM. This has been extensively reviewed elsewhere (Poo, 2001; Reichardt, 2006; Lu et al., 2008). Among the hundreds of BDNF-induced genes (Gokce et al., 2009) closely related to synaptic plasticity, learning and memory (e.g. *c-fos, arc*), it is notable that BDNF itself is also upregulated. This enhancement may interpret the sustained level of BDNF during the late phase of

LTP or LTM.

Further investigations have been performed to dissect the detailed pathways of BDNF-TrkB in plasticity and memory formation. For instance, inhibition of ERK by pharmacological drugs blocks the BDNF-induced ERK phosphorylation and abolishes BDNF-induced LTP (Ying et al., 2002). BDNF reverses memory deficit resulting from translation inhibition, and the ERK inhibitor blocks the rescue (Bekinschtein et al., 2008b). In contrast to the viewpoint that ERK mainly accounts for the effect of BDNF in neuroplasticity, Minichiello and colleagues showed discrepant results using mice with individual phosphorylation site-mutated TrkB receptor (Minichiello et al., 2002). They found that the docking site of PLCγ is important for LTP and calcium-dependent signals including CREB activation. The mutation of the Shc binding site impairs ERK activation without affecting LTP or CREB activity, however. This discrepancy may be attributed to different experimental set-up on different brain regions (mPFC vs. hippocampal CA3-CA1).

BDNF in neurodegenerative diseases and mental disorders

Beside its well-documented functions in regulating neuroplasticity, BDNF has also been implicated in many neurological diseases including Alzheimer's disease (AD) and Parkinson's disease (PD), as well as psychiatric disorders (Mattson et al., 2004). For example, AD patients have reduced BDNF mRNA in hippocampus and less catalytic TrkB in the temporal and frontal cortex (Phillips et al., 1991; Allen et al., 1999). Decreased BDNF has also been detected in

Figure 1.5

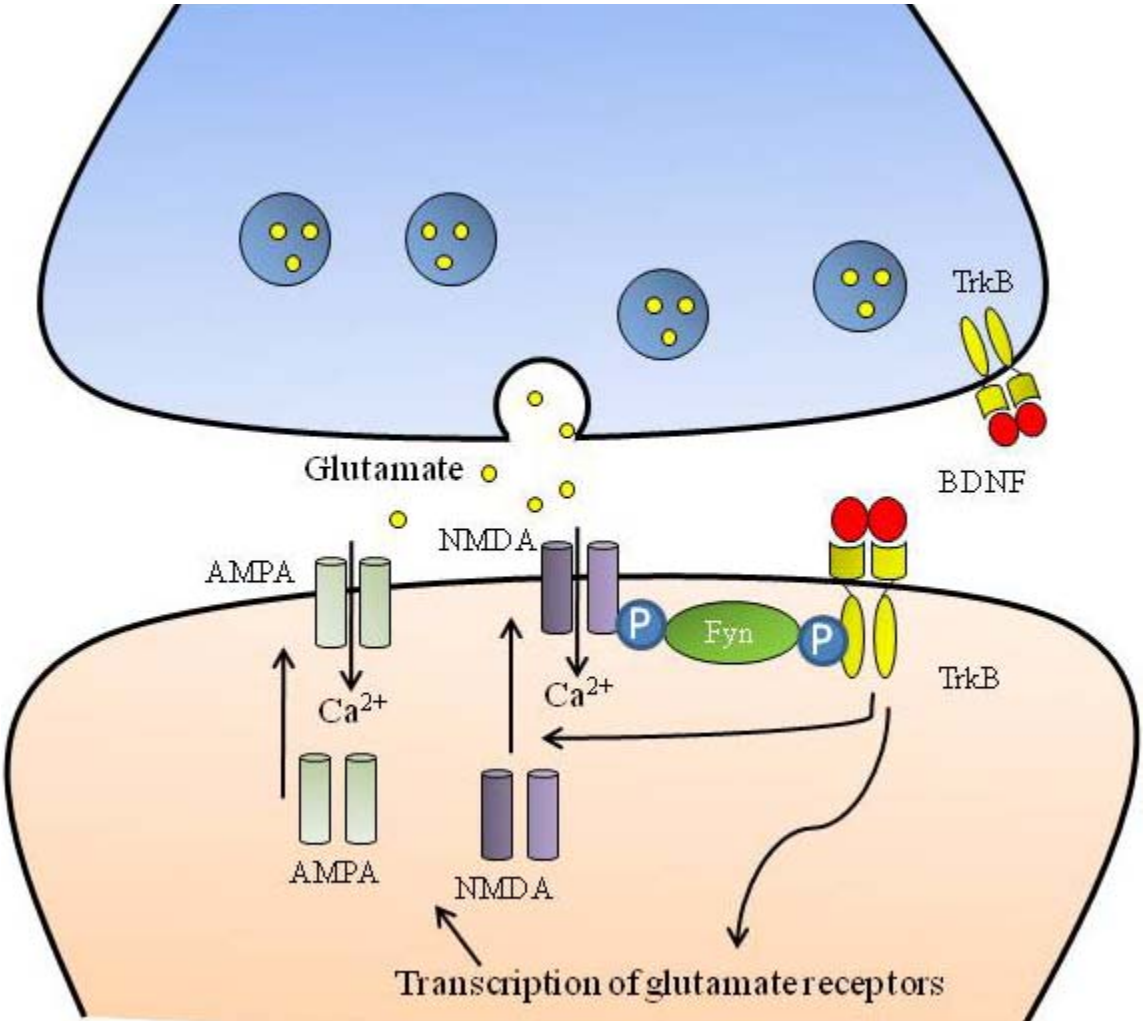


Figure 1.5 Modulation of glutamate receptors by BDNF-TrkB signals. Activated TrkB can modulate both NMDA and AMPA receptor. This modulation includes enhancing the transcription of the genes of NR1, NR2A, NR2B, GluR1 and GluR2; increasing the insertion of the receptors on postsynapses (pink); and phosphorylating NR1 and NR2B via Fyn to increase NMDA open possibility.

substantia nigra in PD patients (Mogi et al., 1999). Furthermore, administration of BDNF into the striatum of PD model animals prevents the degeneration of dopaminergic neurons (Levivier et al., 1995). Additionally, BDNF is involved in psychiatric disorders such as bipolar disorder (BD), depression and schizophrenia, which is supported by the following results: 1) reduced BDNF levels in the hippocampus of rats of depression and the plasma of patients suffering from depression, 2) decreased BDNF in BD and schizophrenia patients, 3) enhanced levels of BDNF induced by antidepressant treatment (Buckley et al., 2007; Post, 2007; Martinowich and Lu, 2008).

REFERENCES

Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. J Neurosci Res 85:525-535.

Allen SJ, Wilcock GK, Dawbarn D (1999) Profound and selective loss of catalytic TrkB immunoreactivity in Alzheimer's disease. Biochem Biophys Res Commun 264:648-651.

Alonso M, Vianna MR, Depino AM, Mello e Souza T, Pereira P, Szapiro G, Viola H, Pitossi F, Izquierdo I, Medina JH (2002) BDNF-triggered events in the rat hippocampus are required for both short- and long-term memory formation. Hippocampus 12:551-560.

An JJ, Gharami K, Liao GY, Woo NH, Lau AG, Vanevski F, Torre ER, Jones KR, Feng Y, Lu B, Xu B (2008) Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. Cell 134:175-187.

Bekinschtein P, Cammarota M, Izquierdo I, Medina JH (2008a) BDNF and memory formation and storage. Neuroscientist 14:147-156.

Bekinschtein P, Cammarota M, Igaz LM, Bevilaqua LR, Izquierdo I, Medina JH (2007) Persistence of long-term memory storage requires a late protein synthesis- and BDNF- dependent phase in the hippocampus. Neuron 53:261-277.

Bekinschtein P, Cammarota M, Katche C, Slipczuk L, Rossato JI, Goldin A, Izquierdo I, Medina JH (2008b) BDNF is essential to promote persistence of long-term memory storage. Proc Natl Acad Sci U S A 105:2711-2716.

Blum R, Kafitz KW, Konnerth A (2002) Neurotrophin-evoked depolarization requires the sodium channel Na(V)1.9. Nature 419:687-693.

Brigadski T, Hartmann M, Lessmann V (2005) Differential vesicular targeting and time course of synaptic secretion of the mammalian neurotrophins. J Neurosci 25:7601-7614.

Buckley PF, Mahadik S, Pillai A, Terry A, Jr. (2007) Neurotrophins and schizophrenia. Schizophr Res 94:1-11.

Caldeira MV, Melo CV, Pereira DB, Carvalho RF, Carvalho AL, Duarte CB (2007a) BDNF regulates the expression and traffic of NMDA receptors in cultured

hippocampal neurons. Mol Cell Neurosci 35:208-219.

Caldeira MV, Melo CV, Pereira DB, Carvalho R, Correia SS, Backos DS, Carvalho AL, Esteban JA, Duarte CB (2007b) Brain-derived neurotrophic factor regulates the expression and synaptic delivery of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons. J Biol Chem 282:12619-12628.

Chen WG, West AE, Tao X, Corfas G, Szentirmay MN, Sawadogo M, Vinson C, Greenberg ME (2003) Upstream stimulatory factors are mediators of Ca2+-responsive transcription in neurons. J Neurosci 23:2572-2581.

Crabtree GR, Olson EN (2002) NFAT signaling: choreographing the social lives of cells. Cell 109 Suppl:S67-79.

Cunningham ME, Greene LA (1998) A function-structure model for NGF-activated TRK. Embo J 17:7282-7293.

Dragunow M, Beilharz E, Mason B, Lawlor P, Abraham W, Gluckman P (1993) Brain-derived neurotrophic factor expression after long-term potentiation. Neurosci Lett 160:232-236.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381:706-709.

Flavell SW, Cowan CW, Kim TK, Greer PL, Lin Y, Paradis S, Griffith EC, Hu LS, Chen C, Greenberg ME (2006) Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. Science 311:1008-1012.

Gokce O, Runne H, Kuhn A, Luthi-Carter R (2009) Short-term striatal gene expression responses to brain-derived neurotrophic factor are dependent on MEK and ERK activation. PLoS One 4:e5292.

Greenberg ME, Xu B, Lu B, Hempstead BL (2009) New insights in the biology of BDNF synthesis and release: implications in CNS function. J Neurosci 29:12764-12767.

Greer PL, Greenberg ME (2008) From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function.

Neuron 59:846-860.

Hall J, Thomas KL, Everitt BJ (2000) Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. Nat Neurosci 3:533-535.

Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ (1997) Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. Proc Natl Acad Sci U S A 94:12419-12424.

Hong EJ, McCord AE, Greenberg ME (2008) A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. Neuron 60:610-624.

Jia Y, Gall CM, Lynch G (2010) Presynaptic BDNF promotes postsynaptic long-term potentiation in the dorsal striatum. J Neurosci 30:14440-14445.

Jiang X, Tian F, Du Y, Copeland NG, Jenkins NA, Tessarollo L, Wu X, Pan H, Hu XZ, Xu K, Kenney H, Egan SE, Turley H, Harris AL, Marini AM, Lipsky RH (2008) BHLHB2 controls Bdnf promoter 4 activity and neuronal excitability. J Neurosci 28:1118-1130.

Kafitz KW, Rose CR, Thoenen H, Konnerth A (1999) Neurotrophin-evoked rapid excitation through TrkB receptors. Nature 401:918-921.

Kang H, Schuman EM (1995) Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 267:1658-1662.

Kang H, Welcher AA, Shelton D, Schuman EM (1997) Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. Neuron 19:653-664.

Kavanaugh WM, Williams LT (1994) An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science 266:1862-1865.

Kesslak JP, So V, Choi J, Cotman CW, Gomez-Pinilla F (1998) Learning upregulates brain-derived neurotrophic factor messenger ribonucleic acid: a mechanism to facilitate encoding and circuit maintenance? Behav Neurosci 112:1012-1019.

Korte M, Kang H, Bonhoeffer T, Schuman E (1998) A role for BDNF in the late-phase of hippocampal long-term potentiation. Neuropharmacology 37:553-559.

Korte M, Staiger V, Griesbeck O, Thoenen H, Bonhoeffer T (1996) The involvement of brain-derived neurotrophic factor in hippocampal long-term potentiation revealed by gene targeting experiments. J Physiol Paris 90:157-164.

Levine ES, Crozier RA, Black IB, Plummer MR (1998) Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc Natl Acad Sci U S A 95:10235-10239.

Levivier M, Przedborski S, Bencsics C, Kang UJ (1995) Intrastriatal implantation of fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevents degeneration of dopaminergic neurons in a rat model of Parkinson's disease. J Neurosci 15:7810-7820.

Lin SY, Wu K, Levine ES, Mount HT, Suen PC, Black IB (1998) BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. Brain Res Mol Brain Res 55:20-27.

Lin Y, Bloodgood BL, Hauser JL, Lapan AD, Koon AC, Kim TK, Hu LS, Malik AN, Greenberg ME (2008) Activity-dependent regulation of inhibitory synapse development by Npas4. Nature 455:1198-1204.

Linnarsson S, Bjorklund A, Ernfors P (1997) Learning deficit in BDNF mutant mice. Eur J Neurosci 9:2581-2587.

Lipsky RH, Xu K, Zhu D, Kelly C, Terhakopian A, Novelli A, Marini AM (2001) Nuclear factor kappaB is a critical determinant in N-methyl-D-aspartate receptor-mediated neuroprotection. J Neurochem 78:254-264.

Liu IY, Lyons WE, Mamounas LA, Thompson RF (2004) Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. J Neurosci 24:7958-7963.

Lu B (2003) BDNF and activity-dependent synaptic modulation. Learn Mem 10:86-98.

Lu Y, Christian K, Lu B (2008) BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? Neurobiol Learn Mem 89:312-323.

Lubin FD, Roth TL, Sweatt JD (2008) Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. J Neurosci 28:10576-10586.

Ma YL, Wang HL, Wu HC, Wei CL, Lee EH (1998) Brain-derived neurotrophic factor antisense oligonucleotide impairs memory retention and inhibits long-term potentiation in rats. Neuroscience 82:957-967.

Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth ME, Lindsay RM, Yancopoulos GD (1990) NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. Neuron 5:501-509.

Malenka RC (2003) Synaptic plasticity and AMPA receptor trafficking. Ann N Y Acad Sci 1003:1-11.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Martinowich K, Lu B (2008) Interaction between BDNF and serotonin: role in mood disorders. Neuropsychopharmacology 33:73-83.

Mattson MP, Maudsley S, Martin B (2004) BDNF and 5-HT: a dynamic duo in age-related neuronal plasticity and neurodegenerative disorders. Trends Neurosci 27:589-594.

McDowell KA, Hutchinson AN, Wong-Goodrich SJ, Presby MM, Su D, Rodriguiz RM, Law KC, Williams CL, Wetsel WC, West AE (2010) Reduced cortical BDNF expression and aberrant memory in Carf knock-out mice. J Neurosci 30:7453-7465.

Minichiello L (2009) TrkB signalling pathways in LTP and learning. Nat Rev Neurosci 10:850-860.

Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, Korte M (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36:121-137.

Minichiello L, Korte M, Wolfer D, Kuhn R, Unsicker K, Cestari V, Rossi-Arnaud C, Lipp HP, Bonhoeffer T, Klein R (1999) Essential role for TrkB receptors in hippocampus-mediated learning. Neuron 24:401-414.

Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, Kuno S, Ichinose H, Nagatsu T (1999) Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease. Neurosci Lett 270:45-48.

Mowla SJ, Pareek S, Farhadi HF, Petrecca K, Fawcett JP, Seidah NG, Morris SJ, Sossin WS, Murphy RA (1999) Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J Neurosci 19:2069-2080.

Mu JS, Li WP, Yao ZB, Zhou XF (1999) Deprivation of endogenous brain-derived neurotrophic factor results in impairment of spatial learning and memory in adult rats. Brain Res 835:259-265.

Nagappan G, Zaitsev E, Senatorov VV, Jr., Yang J, Hempstead BL, Lu B (2009) Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. Proc Natl Acad Sci U S A 106:1267-1272.

Nakajima T, Sato M, Akaza N, Umezawa Y (2008) Cell-based fluorescent indicator to visualize brain-derived neurotrophic factor secreted from living neurons. ACS Chem Biol 3:352-358.

Nakata H, Nakamura S (2007) Brain-derived neurotrophic factor regulates AMPA receptor trafficking to post-synaptic densities via IP3R and TRPC calcium signaling. FEBS Lett 581:2047-2054.

Neeper SA, Gomez-Pinilla F, Choi J, Cotman C (1995) Exercise and brain neurotrophins. Nature 373:109.

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL, Lu B (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306:487-491.

Patterson SL, Grover LM, Schwartzkroin PA, Bothwell M (1992) Neurotrophin expression in rat hippocampal slices: a stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs. Neuron 9:1081-1088.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16:1137-1145.

Phillips HS, Hains JM, Armanini M, Laramee GR, Johnson SA, Winslow JW (1991) BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. Neuron 7:695-702.

Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2:24-32.

Post RM (2007) Role of BDNF in bipolar and unipolar disorder: clinical and theoretical implications. J Psychiatr Res 41:979-990.

Pozzo-Miller LD, Gottschalk W, Zhang L, McDermott K, Du J, Gopalakrishnan R, Oho C, Sheng ZH, Lu B (1999) Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. J Neurosci 19:4972-4983.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361:1545-1564.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370:527-532.

Sakamoto K, Karelina K, Obrietan K (2011) CREB: a multifaceted regulator of neuronal plasticity and protection. J Neurochem 116:1-9.

Sakata K, Woo NH, Martinowich K, Greene JS, Schloesser RJ, Shen L, Lu B (2009) Critical role of promoter IV-driven BDNF transcription in GABAergic transmission and synaptic plasticity in the prefrontal cortex. Proc Natl Acad Sci U S A 106:5942-5947.

Sala R, Viegi A, Rossi FM, Pizzorusso T, Bonanno G, Raiteri M, Maffei L (1998) Nerve growth factor and brain-derived neurotrophic factor increase neurotransmitter release in the rat visual cortex. Eur J Neurosci 10:2185-2191.

Sweatt JD (1999) Toward a molecular explanation for long-term potentiation. Learn Mem 6:399-416.

Tabuchi A, Sakaya H, Kisukeda T, Fushiki H, Tsuda M (2002) Involvement of an upstream stimulatory factor as well as cAMP-responsive element-binding protein in the activation of brain-derived neurotrophic factor gene promoter I. J Biol Chem 277:35920-35931.

Tabuchi A, Nakaoka R, Amano K, Yukimine M, Andoh T, Kuraishi Y, Tsuda M (2000) Differential activation of brain-derived neurotrophic factor gene promoters I and III by Ca2+ signals evoked via L-type voltage-dependent and N-methyl-D-aspartate receptor Ca2+ channels. J Biol Chem 275:17269-17275.

Tanaka J, Horiike Y, Matsuzaki M, Miyazaki T, Ellis-Davies GC, Kasai H (2008) Protein synthesis and neurotrophin-dependent structural plasticity of single dendritic spines. Science 319:1683-1687.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20:709-726.

Tao X, West AE, Chen WG, Corfas G, Greenberg ME (2002) A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. Neuron 33:383-395.

Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen ZY, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci 25:5455-5463.

Tian F, Hu XZ, Wu X, Jiang H, Pan H, Marini AM, Lipsky RH (2009) Dynamic chromatin remodeling events in hippocampal neurons are associated with NMDA receptor-mediated activation of Bdnf gene promoter 1. J Neurochem 109:1375-1388.

Timmusk T, Lendahl U, Funakoshi H, Arenas E, Persson H, Metsis M (1995) Identification of brain-derived neurotrophic factor promoter regions mediating tissue-specific, axotomy-, and neuronal activity-induced expression in transgenic mice. J Cell Biol 128:185-199.

Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H (1993) Multiple promoters direct tissue-specific expression of the rat BDNF gene. Neuron 10:475-489.

Tyler WJ, Perrett SP, Pozzo-Miller LD (2002) The role of neurotrophins in neurotransmitter release. Neuroscientist 8:524-531.

Tyler WJ, Zhang XL, Hartman K, Winterer J, Muller W, Stanton PK, Pozzo-Miller L (2006) BDNF increases release probability and the size of a rapidly recycling vesicle pool within rat hippocampal excitatory synapses. J Physiol 574:787-803.

Vashishta A, Habas A, Pruunsild P, Zheng JJ, Timmusk T, Hetman M (2009) Nuclear factor of activated T-cells isoform c4 (NFATc4/NFAT3) as a mediator of antiapoptotic transcription in NMDA receptor-stimulated cortical neurons. J Neurosci 29:15331-15340.

Woo NH, Teng HK, Siao CJ, Chiaruttini C, Pang PT, Milner TA, Hempstead BL, Lu B (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat Neurosci 8:1069-1077.

Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, Zang K, Wang D, Nicoll RA, Lu B, Reichardt LF (2000) The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. J Neurosci 20:6888-6897.

Yang J, Siao CJ, Nagappan G, Marinic T, Jing D, McGrath K, Chen ZY, Mark W, Tessarollo L, Lee FS, Lu B, Hempstead BL (2009) Neuronal release of proBDNF. Nat Neurosci 12:113-115.

Ying SW, Futter M, Rosenblum K, Webber MJ, Hunt SP, Bliss TV, Bramham CR (2002) Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. J Neurosci 22:1532-1540.

Young D, Lawlor PA, Leone P, Dragunow M, During MJ (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat Med 5:448-453.

Zakharenko SS, Patterson SL, Dragatsis I, Zeitlin SO, Siegelbaum SA, Kandel ER, Morozov A (2003) Presynaptic BDNF required for a presynaptic but not postsynaptic component of LTP at hippocampal CA1-CA3 synapses. Neuron 39:975-990.

CHAPTER 2

REGULATION OF BRAIN-DERIVED NEUROTROPHIC FACTOR EXON IV TRANSCRIPTION THROUGH CALCIUM RESPONSIVE ELEMENTS IN CORTICAL NEURONS

This manuscript has been submitted to *Biochemical Journal*. Dr. Xianju Zhou contributed to Figure 2.4, 2.5, Supplemental Figure 2.2 and 2.3.

Activation of immediate early gene transcription via Ca2+-stimulated signal transduction represents a major molecular mechanism of activity-dependent modification of neuronal function. Previous studies suggest that three calcium responsive elements (CaRE) in promoter IV may coordinate to regulate brain-derived neurotrophic factor (BDNF) exon IV transcription. However, it is not known how Ca2+-stimulated CaRE activation is regulated. Here, we report that CaRE1 and CaRE3, but not CaRE2 are activated by L-type voltage-gated calcium channel (L-VGCC) and NMDA receptor (NMDAR) activation. Ca2+-stimulation on CaRE1 activity was blocked by dominant negative (dn) constructs of MEK, PI3K, CaMKIV and CaMKIIN (an inhibitory construct for CaMKII). PKA and CaMKI activity were required for L-VGCC-, but not NMDAR-mediated CaRE1 activation. Sequence analysis suggests that CaRE3 resembles the previously identified cAMP responsive element (CRE). We found that CaRE3 and CRE activity were regulated by the same Ca2+-stimulated protein kinases. CaRE3 and CRE activity were blocked by dnMEK, dnPI3K, dnPKA and dnCaMKIV. CaMKI was required for NMDAR- but not L-VGCC-mediated CaRE3/CRE activation. Furthermore, overexpression of an active form of CREB (VP16-CREB) enhanced CaRE3 and CRE, but not CaRE1 activity. Surprisingly, the activation of the previously identified transcription factor for CaRE1 (i.e. CaRF) was only stimulated via L-VGCC but not NMDAR, and

inhibited by dnMEK, dnPI3K and CaMKIIN. These results suggest a new working model that BDNF exon IV expression may be coordinated by CaRE1 and CaRE3 activity, which show different responses to Ca2+-stimulated protein kinases.

Activity-dependent gene transcription is functionally relevant for animals to acquire information and adapt to their environments. One major molecule that transduces neuronal activity to regulate transcription is calcium. Ca2+ influx mediated by voltage-gated and ligand-gated Ca2+ channels triggers the activation of multiple protein kinases, and in turn regulates transcription factors and transcriptional initiation.

Previous studies on transcription factor CREB (cAMP responsive element binding protein) have demonstrated how transcriptional initiation can be tightly and specifically controlled by multiple signaling activities triggered by Ca2+ (Mayr and Montminy, 2001; West et al., 2001; Greer and Greenberg, 2008). Specifically, multiple phosphorylation sites in CREB are regulated by calmodulin (CaM)-dependent protein kinases, cAMP-dependent protein kinase (PKA) and Ras/Raf/MAPK/Rsk cascade. Further, lack of CREB-mediated transcription is implicated in mental disorders (Kawanishi et al., 1999; Newton et al., 2002), neurodegeneration (Steffan et al., 2000), apoptosis during development (Xing et al., 1996; Bonni et al., 1999), and impaired synaptic plasticity (Bourtchuladze et

al., 1994).

One important gene target of CREB is brain-derived neurotrophic factor (BDNF). BDNF transcription is very responsive to neural activity, and up-regulated by learning (Hall et al., 2000; Lubin et al., 2008), physical exercise (Neeper et al., 1995), and kindling or kainite-induced seizures (Dugich-Djordjevic et al., 1992). The induction of BDNF expression could theoretically exert further modification on synaptic functions, including regulating dendritic spine density (Ji et al., ; McAllister et al., 1995), enhancing both pre-synaptic and post-synaptic functions (Lessmann et al., 1994; Schinder et al., 2000), and mediating long-term potentiation (LTP) and memory formation (Figurov et al., 1996; Linnarsson et al., 1997).

Molecular studies have revealed that the BDNF gene consists of nine 5' exons (from exon I to IXA) and a common 3' encoding exon IX (Aid et al., 2007). After transcription and splicing, one and only one 5' exon is joined to exon IX, resulting nine BDNF mRNA forms each of which contains one 5' exon and the exon IX. In cultured cortical neurons, Ca2+ influx through L-type voltage-gated calcium channels (L-VGCC) and NMDA receptor (NMDAR) specifically stimulates transcription of exon IV-containing BDNF mRNA form or BDNF IV (Tao et al., 1998; Tabuchi et al., 2000; Zheng and Wang, 2009) (because of the recent discovery of new exons, exon IV was described as exon III in the earlier studies). The 1500bp

of the 5' flanking sequence of exon IV (defined as promoter IV) confers the full activity for Ca2+-stimulated transcription (Tao et al., 2002). Truncation and mutagenesis analysis identified three calcium responsive elements, namely CaRE1, CaRE2, and CaRE3 (Tao et al., 2002). It was shown that CaRE1 and CaRE2 activity is stimulated by membrane depolarization (Tao et al., 2002; Chen et al., 2003b). By using a yeast one-hybrid screening, transcription factors CaRF (Calcium responsive factor) and USF (upstream stimulatory factors) were found to bind CaRE1 and CaRE2, respectively (Tao et al., 2002; Chen et al., 2003b). Although sequence analysis of CaRF revealed putative phosphorylation sites for CaMKII and MAPK, how Ca2+ and these two Ca2+-activated protein kinases regulate CaRE1 and CaRF activity are not yet determined. Two-dimensional isoelectric focusing experiments also implicated that protein kinases might regulate USF activity and CaRE2-mediated transcription. The sequence of CaRE3 is similar to that of the CRE consensus sequence (Shieh et al., 1998; Tao et al., 1998). Although an *in vitro* study showed that an oligonucleotide containing CaRE3 binds CREB in neuronal extracts, it is not known whether CREB regulates the transcriptional activity of CaRE3. In addition to determining whether CREB is the physiological partner of CaRE3, it is important to determine whether the CREB-regulating protein kinases, such as CaMKs, PKA and MAPK, also mediated Ca2+-stimulated CaRE3 activity.

In the present study, we examined how Ca2+ and Ca2+-activated protein

kinases regulate BDNF promoter IV activity via the CaREs. We found that promoter IV activity and the up-regulation of exon IV-containing BDNF mRNA showed identical regulatory properties, indicating that the cloned 5' flanking sequence of exon IV recapitulates the activity of the endogenous promoter. We further found that CaRE1 and CaRE3, but not CaRE2 were activated by both L-VGCC and NMDAR. It appeared that L-VGCC- and NMDAR-mediated activation of CaRE1 and CaRE3 were differentially regulated by Ca2+-activated protein kinases. Moreover, our results also show that CaRE3 and CRE have the same regulatory property, indicating that CaRE3 is a CREB target. Furthermore, our study on CaRF regulation suggests mediation on CaRE1-driven transcription may not be all through this transcription factor. Our results demonstrate how BDNF expression is mediated by the complex protein kinase network via individual CaRE.

EXPERIMENTAL PROCEDURES

Reagents- All chemical reagents were purchased from Sigma (St. Louis, MO), unless otherwise stated. LY294002 (a PI3K inhibitor), PD98059 (a MEK inhibitor) and H89 (a PKA inhibitor) were purchased from Calbiochem (Gibbstown, NJ). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Cell culture and transfection reagents were from Invitrogen (Carlsbad, CA).

Plasmids and reporter constructs- The 911bp promoter IV region of BDNF was cloned from rat genomic DNA with the forward primer

(5'-ATGCTCGAGAAGAGGCTGTGGCACCGTGC-3') and the reverse primer (5'-CCCAAGCTTTCCCCAAGGTTCTAGACTC-3'). The fragment was inserted into XhoI/HindIII site of pGL3-basic firefly luciferase reporter vector (Promega, Madison, WI) to generate PIV-Luc. Three copies of CaRE1, or CaRE2, or CaRE3 (Tao et al., 2002) sequence in BDNF promoter IV were cloned into pGL3-promoter firefly luciferase reporter vector, which contains a minimal SV40 promoter, to generate plasmids CaRE1-Luc, CaRE2-Luc and CaRE3-Luc. The CRE-Luc reporter plasmid was described previously (Impey et al., 1998), and was a gift from Dr. Daniel Storm (University of Washington). Gal4, Gal4-CaRF, Gal4-CREB and UAS-Luciferase constructs (Tao et al., 2002) are generously provided by Dr. Michael Greenberg (Harvard University). The Renilla Luciferase reporter construct, TK-pRL (Renilla-luciferase), was from Dr. Richard Miksicek (Michigan State University). Expression plasmids for dominant negative PKA (dnPKA, cAMP binding positions mutated)(Impey et al., 1998), dominant negative MEK (dnMEK, S222A)(Impey et al., 1998), dominant negative PI3K (dnPI3K, p110ΔKIN)(Chen et al., 2005), dominant negative CaMKI (dnCaMKI, K49E, T177A, IHQS286EDDD and F307A, which mutated its catalytic residues, CaMKK phosphorylation domain and autoinhibitory domain), dominant negative CaMKIV (dnCaMKIV, mutations T196A, K71E and HMDT305DEDD are at ATP binding sites and autoinhibitory domain and a nuclear localization signal is attached)(Wayman et al., 2004) and CaMKIIN (which expresses a CaMKII inhibitory protein)(Chang et al., 2001) were

described previously. Dominant negative calmodulin (pJPA7/rCaM-DEF1234A)(Labasque et al., 2008) is from Dr. Philippe Marin (University of Montpellier, Germany). Plasmid expressing a constitutive active CREB (VP16-CREB, using transactivation domain of Herpes simplex virus VP16 to replace the first Gln-rich domain of CREB)(Barco et al., 2002) was from Dr. Karl Obrietan (Ohio State University).

Plasmids and reporter constructs- The 911bp promoter IV region of BDNF was cloned from rat genomic DNA with the forward primer (5'-ATGCTCGAGAAGAGGCTGTGGCACCGTGC-3') and the reverse primer (5'-CCCAAGCTTTCCCCAAGGTTCTAGACTC-3'). The fragment was inserted into XhoI/HindIII site of pGL3-basic firefly luciferase reporter vector (Promega, Madison, WI) to generate PIV-Luc. Three copies of CaRE1, or CaRE2, or CaRE3 (Tao et al., 2002) sequence in BDNF promoter IV were cloned into pGL3-promoter firefly luciferase reporter vector, which contains a minimal SV40 promoter, to generate plasmids CaRE1-Luc, CaRE2-Luc and CaRE3-Luc. The CRE-Luc reporter plasmid was described previously (Impey et al., 1998), and was a gift from Dr. Daniel Storm (University of Washington). Gal4, Gal4-CaRF, Gal4-CREB and UAS-Luciferase constructs (Tao et al., 2002) were generously provided by Dr. Michael Greenberg (Harvard University). The Renilla Luciferase reporter construct, TK-pRL (Renilla-luciferase), was from Dr. Richard Miksicek (Michigan State University). Expression plasmids for dominant negative PKA (dnPKA, which

harbors mutations at the cAMP binding sites) (Impey et al., 1998), dominant negative MEK (dnMEK, which harbors a S222A mutation) (Impey et al., 1998), dominant negative PI3K (dnPI3K, p110ΔKIN) (Chen et al., 2005), dominant negative CaMKI (dnCaMKI, which harbors multiple mutations in the catalytic residues, CaMKK phosphorylation domain, and autoinhibitory domain), dominant negative CaMKIV (dnCaMKIV, which harbors multiple mutations at ATP binding sites, and autoinhibitory domain with a nuclear localization signal attached) (Wayman et al., 2004) and CaMKIIN (which expresses a CaMKII inhibitory protein) (Chang et al., 2001) were described previously. Dominant negative calmodulin (pJPA7/rCaM-DEF1234A) (Labasque et al., 2008) was from Dr. Philippe Marin (University of Montpellier, Germany). Plasmid expressing a constitutive active CREB (VP16-CREB, in which the first Gln-rich domain of CREB is replaced by the transactivation domain of Herpes simplex virus VP16) (Barco et al., 2002) was from Dr. Karl Obrietan (Ohio State University).

Primary culture of cortical neurons- As described previously (Zhou et al., 2009), brain tissue was obtained from post-natal day 0 Sprague Dawley rats. Dorsal and frontal cortical regions were dissected and incubated in digestion buffer containing 10 units/ml papain (Worthington), 100 units/ml DNase I (Roche), and 5 mg/ml cystine (Sigma) in Hibernate A (BrainBits LLC). After 30 min digestion and dispersion, neurons were then seeded on poly-D-lysine (50 μg/ml, Sigma, St. Louis, MO)-coated plates at a density of 150k cells/cm^2. The culturing medium

was Neurobasal A with B27 supplement, penicillin/streptomycin and 0.5 mM glutamate.

Neuronal stimulation- Ca2+ influx triggered by membrane depolarization was achieved by KCl (at 50 mM) treatment. Neurons were first pre-treated with APV (100 μM) and CNQX (40 μM) for 30 min before the addition of KCl. Ca2+ influx triggered by NMDA receptor activation was achieved by NMDA (20 μM) and glycine (2 μM) treatment. Neurons were first pre-treated with nifedipine (10 μM) and CNQX (40 μM) for 30 min before NMDA. APV, nifedipine, and CNQX were used to block NMDAR, L-type voltage-gated calcium channel, and non-NMDA type glutamate receptors, respectively. To inhibit MEK, PI3K, and PKA, neurons were pre-treated for 30 min before stimulation with PD98059 (50 μM), LY294002 (30 μM), and H89 (10 μM), respectively. To stop transcription, a transcription inhibitor actinomycin D (ACD) was used at a concentration of 100 ng/ml.

Real-time PCR- Neurons were treated on 14 days *in vitro* (DIV). Control and stimulated neurons were lysed, and total RNA was extracted with TRIzol (Invitrogen). RNA concentration was measured by NanoDrop 1000 (Thermo, Waltham, MA), and 0.5 μg RNA was reverse transcribed to cDNA with SuperScript III kit (Invitrogen). Exon IV-containing BDNF (BDNF IV) mRNA and GAPDH mRNA were amplified by iQ SYBR Green real-time PCR system (Bio-Rad, Hercules, CA), and analyzed with the $2^{-\Delta\Delta Ct}$ method by normalizing BDNF IV signal to GAPDH. The annealing temperature for both genes was 55°C. The

primers were 5'-CTCCGCCATGCAATTTCCAC- 3' and 5'-GCCTTCATGCAACCG AAGTA-3' for BDNF exon IV. Primers 5'-TCCATGACAACTTTGGCATTG TGG-3' and 5'-GTTGCTGTTGAAGTCGCAGGAGAC-3' were used to amplify GAPDH.

Transfection and luciferase assay- Cultured neurons were transfected on 9 DIV by using Lipofectamine 2000 (Invitrogen). Following manufacturer's instruction, 0.1 μg luciferase reporter plasmid (or 0.1 μg Gal4 and 0.1 μg UAS-Luc) and 0.4 μg Renilla-luc plasmid were mixed with Lipofectamine 2000, and added to 0.3 million cells. For some experiments, neurons were also co-transfected with constructs that express regulatory proteins (such as dominant negative protein kinases). Forty-eight hours after transfection, neurons were stimulated by KCl or NMDA for 6 hours. Cells were lysed and the Luciferase activity of the lysate was analyzed with Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instruction. The luciferase activity was measured by a Veritas Microplate Luminometer. Firefly luciferase activity was normalized to Renilla luciferase activity. The activity of luciferase reporter plasmids (such as CaRE1, CaRE2, and CaRE3) was compared to that of pGL3-SV40-luciferase.

Western blotting- Neurons were pretreated with inhibitors for 30 minutes, followed by a 20-minute KCl stimulation. Cells were then collected in SDS lysis buffer (10 mM Tris–HCl buffer, 10% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol, pH 6.8) and proteins were separated on SDS-PAGE (Invitrogen). Separated proteins were transferred onto a nitrocellulose membrane,

and incubated by specific primary antibodies (1:1000 dilution for anti-pERK1/2 and 1:5000 dilution for anti-β-actin, Cell Signaling, MA) and then horseradish peroxidase (HRP)-labeled secondary antibodies (1: 5000 for both goat anti-rabbit IgG and goat anti-mouse IgG, Thermo). The protein signal was detected by an enhanced chemiluminescence system (Thermo). The signal intensity was determined by the Scion Image software (Scion Corp. Frederick, Maryland). The pERK1/2 level was normalized to β-actin.

Data analysis- All results were presented as average ± SEM, and statistically compared using one-way ANOVA and post hoc analysis ($p<0.05$ is considered to be statistically significant).

RESULTS

Regulation of BDNF exon IV transcription through promoter IV. Previous studies demonstrated that the route of Ca2+ influx matters for activity-dependent gene transcription of BDNF exon IV. Because co-application of nifedipine and APV ablates KCl-stimulated exon IV transcription, it was concluded that membrane depolarization triggers transcription through L-VGCC and NMDAR. Here, we examined L-VGCC-mediated transcription by co-application of KCl with NMDAR antagonist APV. The NMDAR-mediated transcription was stimulated by co-application of NMDA and L-VGCC antagonist nifedipine. In both cases, we included CNQX to block non-NMDA type glutamate receptors.

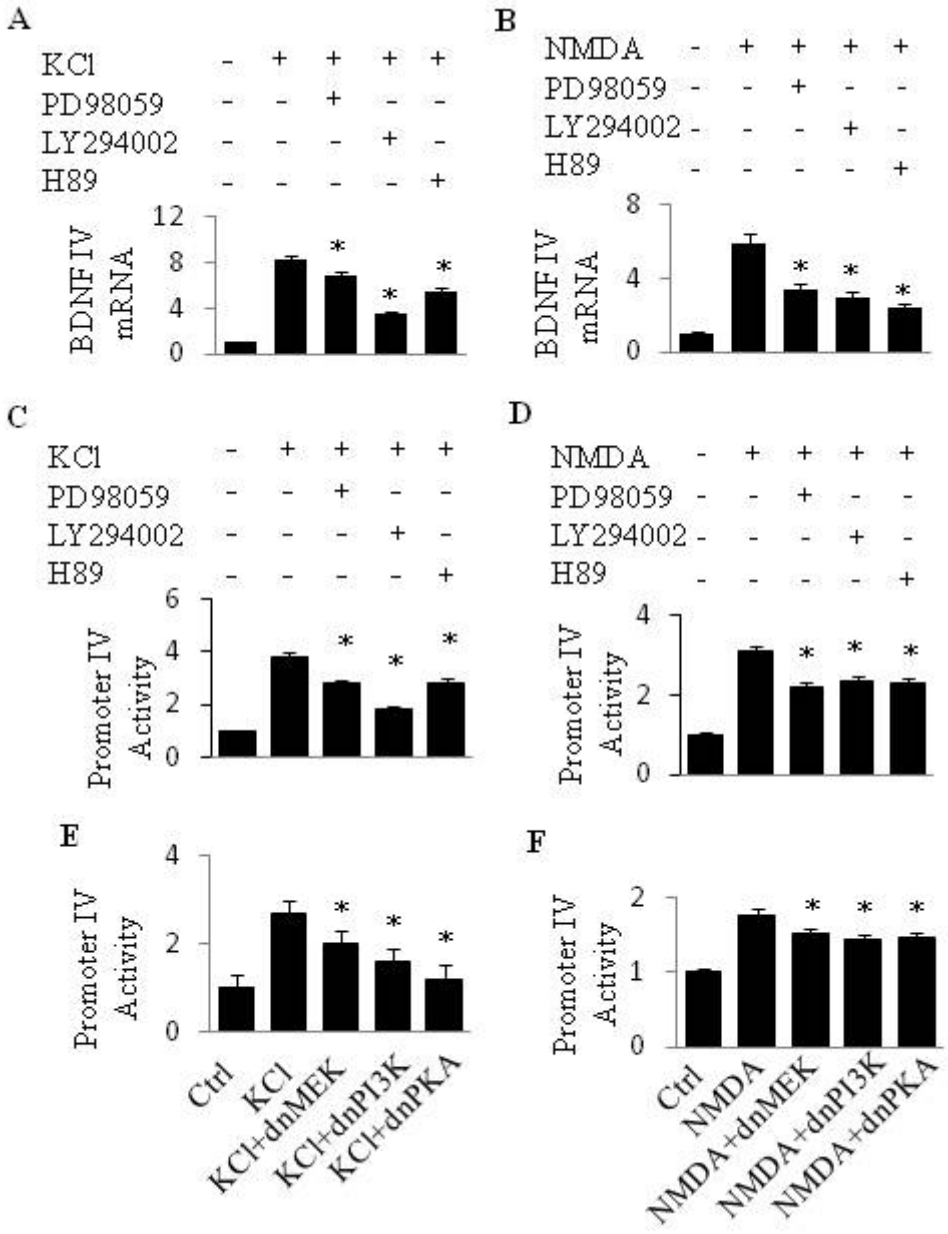
As shown in Fig. 2.1A and 1B, robust up-regulation of exon IV-containing

BDNF mRNA was stimulated by the activation of either L-VGCC or NMDAR in DIV 14 cortical neurons. By using pharmacological inhibitors, we blocked several Ca2+-stimulated protein kinases, which were implicated to regulate CREB activity. We found that both L-VGCC- and NMDAR-mediated exon IV transcription were effectively blocked by inhibiting MEK (by PD98059), PI3K (by LY294002), or PKA (by H89). Interestingly, acute inhibition of MEK with PD98059 suppressed the basal mRNA level of BDNF IV (Supplemental Fig. 2.1A). This result suggests that the MEK-ERK1/2 signaling may also be required for promoter IV-mediated transcription driven by spontaneous activity in resting neurons. It appeared that the effects of Ca2+ influx and the inhibitors on the mRNA level of exon IV were mainly due to transcription rather than altering mRNA stability, because there was no significant changes in neurons treated with transcription inhibitor actinomycin D (ACD) (Supplemental Fig. 2.2A and 2B).

Cloning of the 911 bp of the 5' flanking sequence of exon IV showed that this region is responsible for the Ca2+-stimulated promoter activity. Here, we sought for further evidence that this flanking sequence is a *bona fide* promoter for exon IV. We found that the luciferase activity driven by promoter IV (PIV) was stimulated by both L-VGCC and NMDAR. Further, pharmacological inhibition of MEK, PI3K and PKA blocked PIV activity in both KCl- and NMDA-stimulated neurons (Fig. 2.1C and D). Consistent with the function of MEK-ERK1/2 in regulating basal exon IV mRNA level, acute treatment with PD98059 also

Figure 2.1 Promoter IV activity correlates with the activation profile of exon IV-containing BDNF mRNA. For A and B, DIV 14 (days *in vitro* 14) cortical neurons were stimulated by 50 mM KCl (A) or 20 μM NMDA (B). To block the activity of MEK, PI3K and PKA, neurons were pre-treated with their inhibitors (i.e. PD98059, LY294002, and H89, respectively) for 30 min before stimulation. One hour after stimulation, total RNA was extracted. Extracted RNA was reverse transcribed to cDNA, and then amplified and analyzed by real-time PCR. Another set of cells were treated with inhibitors only to rule out the inhibitory effect on basal level. BDNF IV mRNA was normalized to GAPDH mRNA and the stimulator+inhibitor level is normalized to inhibitor-only level.. (n=3 for each treatment). From C to F, DIV 9 cortical neurons were transfected with promoter IV-luciferase reporter plus TK-pRL (C and D) or co-transfected with promoter IV-luciferase/TK-pRL and dominant negative construct of MEK, PI3K and PKA (E and F). Two days after transfection, neurons at DIV 11 were treated with 50 mM KCl (C and E) or 20 μM NMDA (D and F). To pharmacologically block MEK, PI3K and PKA, neurons were pre-treated with their inhibitors (i.e. PD98059, LY294002, and H89, respectively) for 30 min before stimulation (C and D). Six hours after stimulation, dual luciferase assay was performed. The firefly luciferase activity was normalized to Renilla-luciferase. Another set of cells were treated with inhibitors/dn constructs only to rule out the inhibitory effect on basal level. The effect of drugs/constructs is normalized to drugs/construct-only groups. *: statistically different to that of the KCl- or NMDA-treated groups ($p<0.05$, n=8 for all experiments in this figure).

Figure 2.1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PD98059 - - + - -
LY294002 - - - + -
H89 - - - - +
BDNF IV mRNA
12
8
4
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B
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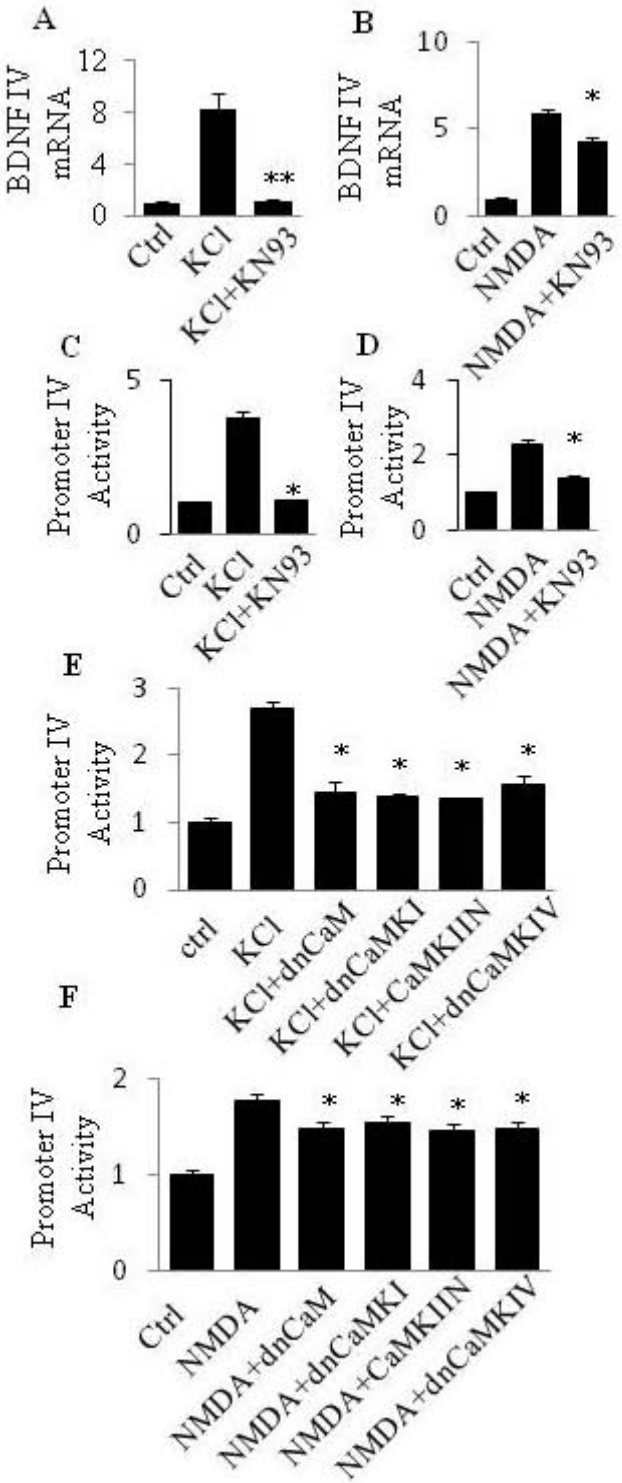
PD98059 - - + - -
LY294002 - - - + -
H89 - - - - +
BDNF IV mRNA
8
4
0
C
KCl - + + + +
PD98059 - - + - -
LY294002 - - - + -
H89 - - - - +
Promoter IV Activity
6
4
2
0
D
NMDA - + + + +
PD98059 - - + - -
LY294002 - - - + -
H89 - - - - +
Promoter IV Activity
4
2
0
E
Promoter IV Activity
4
2
0
Ctrl
KCl
KCl+dnMEK
KCl+dnPI3K
KCl+dnPKA
F
Promoter IV Activity
2
1
0
Ctrl
NMDA
NMDA+dnMEK
NMDA+dnPI3K
NMDA+dnPKA

Figure 2.2. Promoter IV activity is regulated by calmodulin (CaM)-dependent protein kinases I, II and IV (CaMKI, II, and IV). DIV 14 neurons were treated with KN93 for 30 min before stimulation (50mM KCl, A or 20 μM NMDA, B) to block the activity of CaMKI, II, and IV. Real-time PCR was performed to measure BDNF IV mRNA, which is normalized to GAPDH. DIV 9 cortical neurons were transfected with promoter IV-luciferase reporter plus TK-pRL (C and D) or co-transfected with promoter IV-luciferase/TK-pRL and dominant negative construct of CaM, CaMKI, CaMKIV, and CaMKIIN (E and F). Two days after transfection on DIV 11, neurons were treated with 50 mM KCl (C and E) or 20 μM NMDA (D and F). In C and D, KN93 was applied 30 min before stimulation. Six hours after stimulation, dual luciferase assay was performed. The firefly luciferase activity was normalized to Renilla-luciferase. Another set of cells were treated with inhibitors/dn constructs only to rule out the inhibitory effect on basal level. All the stimulator+inhibitor/dn plasmid level was shown after normalization to inhibitor/dn plasmid-only groups. *: statistically different to that of the KCl- or NMDA-treated groups ($p<0.05$, $n=8$ for all experiments in this figure).

Figure 2.2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BDNF IV mRNA
12
8
4
0
**
Ctrl
KCl
KCl+KN93
B
BDNF IV mRNA
10
5
0
*
Ctrl
NMDA
NMDA+KN93
C
Promoter IV Activity
5
0
*
Ctrl
KCl
KCl+KN93
D
Promoter IV Activity
4
2
0
*
Ctrl
NMDA
NMDA+KN93
E
Promoter IV Activity
3
2
1
0
*
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*
*
ctrl
KCl
KCl+dnCaM
KCl+dnCaMKI
KCl+CaMKIIN
KCl+dnCaMKIV
F
Promoter IV Activity
2
1
0
*
*
*
*
Ctrl
NMDA
NMDA+dnCaM
NMDA+dnCaMKI
NMDA+CaMKIIN
NMDA+dnCaMKIV

suppressed PIV-luciferase activity in non-stimulated neurons (Supplemental Fig. 2.1B). We next tested the function of these Ca2+-regulated protein kinases by co-transfecting their dominant negative (dn) constructs with PIV-luciferase. We found that dnMEK, dnPI3K and dnPKA blocked the up-regulation of PIV activity in both KCl- and NMDA-stimulated neurons (Fig. 2.1E and F) without significantly changing its basal level activity (Supplemental Fig. 2.1C). Because the MEK activity was suppressed for about 48 hours in neurons transfected with dnMEK, there might be some compensatory effects to counteract its regulation of basal PIV activity.

We next examined the function of calmodulin (CaM)-dependent protein kinases. CaMKI, II, and IV are abundant in neuronal system and implicated in regulating synaptic plasticity and gene transcription (Wayman et al., 2008). Our results showed that blocking these CaMKs by KN93 suppressed Ca2+-stimulated exon IV transcription (Fig. 2.2A and B). We also found that KN93 effectively blocked L-VGCC- and NMDAR-mediated up-regulation of PIV activity (Fig. 2.2C and D). Blocking CaM activity by dnCaM also blocked PIV activity. Furthermore, inhibition of these CaMKs by overexpressing dn constructs demonstrated that Ca2+-stimulated PIV activity requires CaMKI, CaMKII, and CaMKIV (Fig. 2.2E and F). In addition to regulating the Ca2+-stimulated transcription, acute treatment with KN93 also suppressed basal exon IV mRNA level (Supplemental Fig. 2.1A). Long-term expression of dnCaM and dnCaMKI caused significantly

reduction of basal PIV-Luc activity in non-stimulated neurons (Supplemental Fig. 2.1C). Further, we found that the CaMKs predominately regulated transcription rather than affecting exon IV mRNA stability (Supplemental Figure 2.2).

Calcium influx through L-VGCC and NMDAR up-regulates CaRE1 and CaRE3 activity. Three calcium responsive elements (CaRE) were identified within the 170 bp 5' flanking region of exon IV (see Fig. 2.3A). Previous studies demonstrated that membrane depolarization stimulates CaRE1 and CaRE2 activity through L-VGCC (Tao et al., 2002; Chen et al., 2003b). Mutation of CaRE3 in the context of the 170 bp sequence suppressed KCl-induced transcription. The sequence of CaRE3 is similar to the CRE consensus sequence (Fig. 2.3A), and a gel shift assay demonstrated that CaRE3 sequence binds to endogenous CREB. However, the activity of CaRE3 itself has not been examined. Surprisingly, we found that CaRE1-mediated transcription was up-regulated by activation of both L-VGCC and NMDAR (Fig. 2.3B). For CaRE2, we did not observe any significant stimulation by either L-VGCC or NMDAR (Fig. 2.3C). Next, we found that Ca2+ influx through either L-VGCC or NMDAR stimulated CaRE3-mediated transcription (Fig. 2.3D).

Regulation of CaRE1-mediated transcription by Ca2+-stimulated protein kinases. Although Ca2+ influx stimulated significant CaRE1 activity, the regulatory mechanism is unknown. As described earlier, we found that both exon IV transcription and PIV activity were regulated by the major Ca2+-activated protein

kinases, including MEK, PI3K, PKA and CaMKs. Here, we sought to determine the regulatory property of the individual CaREs. We found that CaRE1 activity induced by L-VGCC with membrane depolarization required MEK, PI3K, and PKA activity (Fig. 2.4A). Dominant negative MEK and PI3K also blocked NMDAR-activated CaRE1. Interestingly, blocking PKA by overexpressing dnPKA had no effect on NMDAR-activated CaRE1 (Fig. 2.4B). Further, basal CaRE1-luc level driven by spontaneous activity in non-stimulated neurons was suppressed by dnMEK and dnPI3K but not dnPKA (Supplemental Fig. 2.3A).

It has been shown that CaM physically interacts with L-VGCC, and the interaction may be functionally important for signal propagation. As for the function of NMDAR-mediated intracellular signaling, physical interaction between CaMKII and NMDAR is also significant for signaling triggered by NMDAR-mediated Ca2+ influx. Here, we show that inhibiting CaM by dnCaM blocked L-VGCC- and NMDAR-mediated CaRE1 activation. Further, overexpression of dnCaMKI, CaMKIIN, and dnCaMKIV blocked CaRE1 activation in KCL- -stimulated neurons (Fig. 2.5A). However, in NMDA-treated cells, CaRE1-mediated transcription was only blocked by CaMKIIN. Interestingly, all the CaMKs are also required for maintaining the basal activity of CaRE1 (Supplemental Fig. 2.3A).

Regulation of CaRE3-mediated transcription by Ca2+-stimulated protein kinases. As shown in Fig. 2.3D, we observed that CaRE3 activity is stimulated by both

Figure 2.3

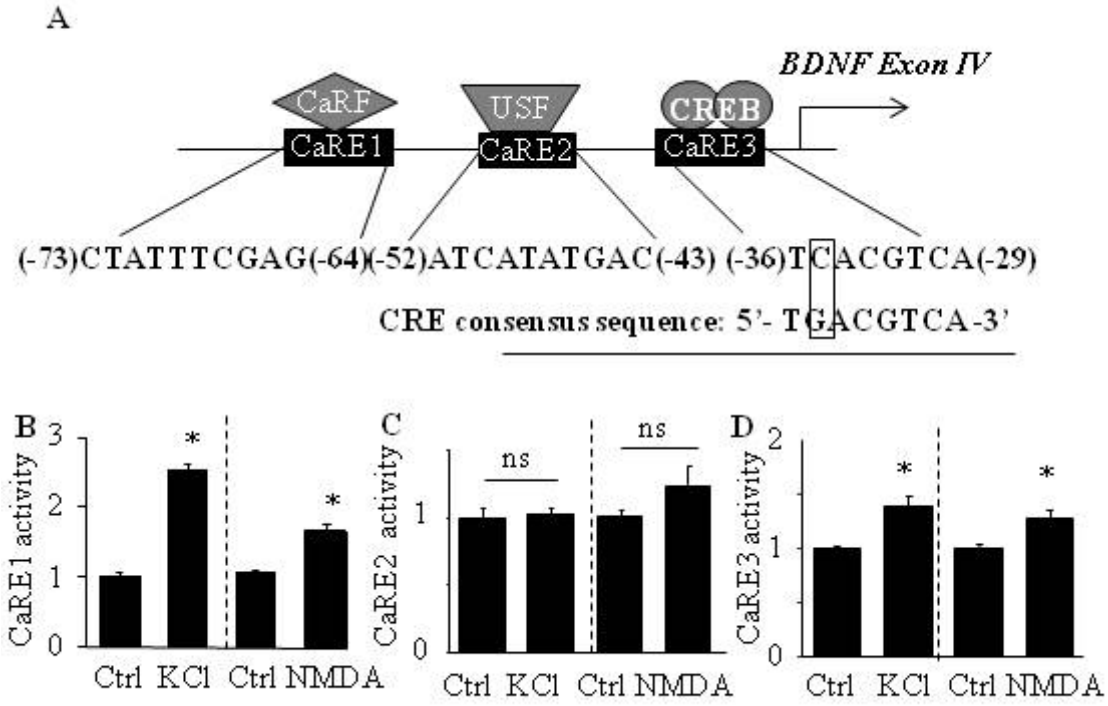

Figure 2.3. CaRE1 and CaRE3, but not CaRE2 activities are stimulated by calcium influx in DIV 11 cortical neurons. A. Schematic illustration of the relative positions of CaRE1, 2, and 3 in the promoter IV region of BDNF gene. These calcium responsive elements are binding sites for transcription factors CaRF, USF, and CREB. The nucleotide sequences of CaRE1, 2, 3, and CRE are also shown. From B to D, cortical neurons at DIV 9 were transfected with CaRE1, 2, or 3-luciferase reporter plus TK-pRL. As control, the backbone construct (SV40-luciferase) and TK-pRL were co-transfected for normalization purposes. On DIV 11, neurons were treated with 50 mM KCl or 20 μM NMDA. Dual luciferase assay was performed 6 hours after stimulation. The control groups for KCl treatment, neurons were pre-treated with APV and CNQX to block NMDAR and non-NMRA type glutamate receptors. The control groups for NMDA treatment, neurons were pre-treated with nifedipine and CNQX to block L-type calcium channels and non-NMRA type glutamate receptors. Similarly, APV and CNQX were always included in KCL stimulation. Nifedipine and CNQX were always co-applied with NMDA during stimulation. The firefly luciferase activity was normalized to Renilla-luciferase. Then, CaRE-mediated activity was normalized with SV40 activity. SV40 activity was obtained from neurons co-transfected with SV40-luc and TK-pRL. *: statistically different to that of control ($p<0.05$). ns: not statistically significant between stimulated and control neurons. n=16 for all experiments in this figure.

Figure 2.4

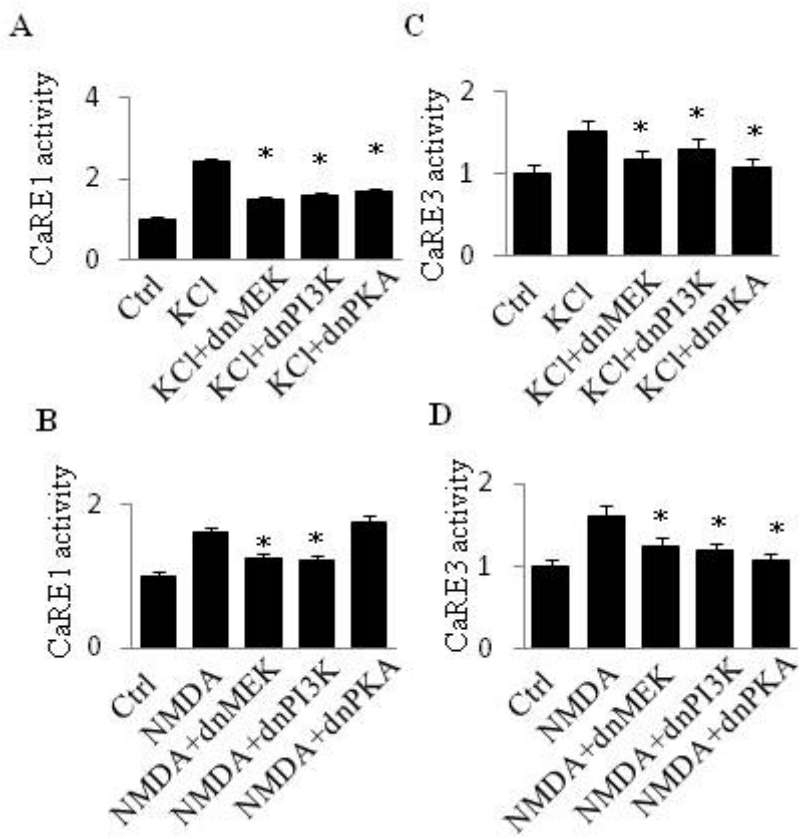

Figure 2.4 Regulation of Ca2+-stimulated CaRE1 and CaRE3 activity by MEK, PI3K, and PKA in cortical neurons. DIV 9 cortical neurons were transfected with CaRE1-luciferase (A and B) or CaRE3-luciferase (C and D) reporter plus TK-pRL and dominant negative MEK, PI3K and PKA as indicated. For normalization purpose, SV40-luciferase plus TK-pRL and dominant negative MEK, PI3K and PKA were transfected into different sets of neurons. On DIV 11, neurons were treated with 50 mM KCl (A and C) or 20 μM NMDA (B and D). Dual luciferase assay was performed 6 hours after stimulation. After normalized to Renilla luciferase activity, CaRE1- or CaRE-3 activity was further normalized to SV40 activity, and expressed as relative activities. Another set of cells were co-transfected dn constructs without simulation to rule out the inhibitory effect on basal level. All the stimulator+dn plasmid level was shown after normalization with dn plasmid-only groups. *: statistically different to that of stimulated neurons ($p<0.05$). n=12 for all experiments in this figure.

Figure 2.5

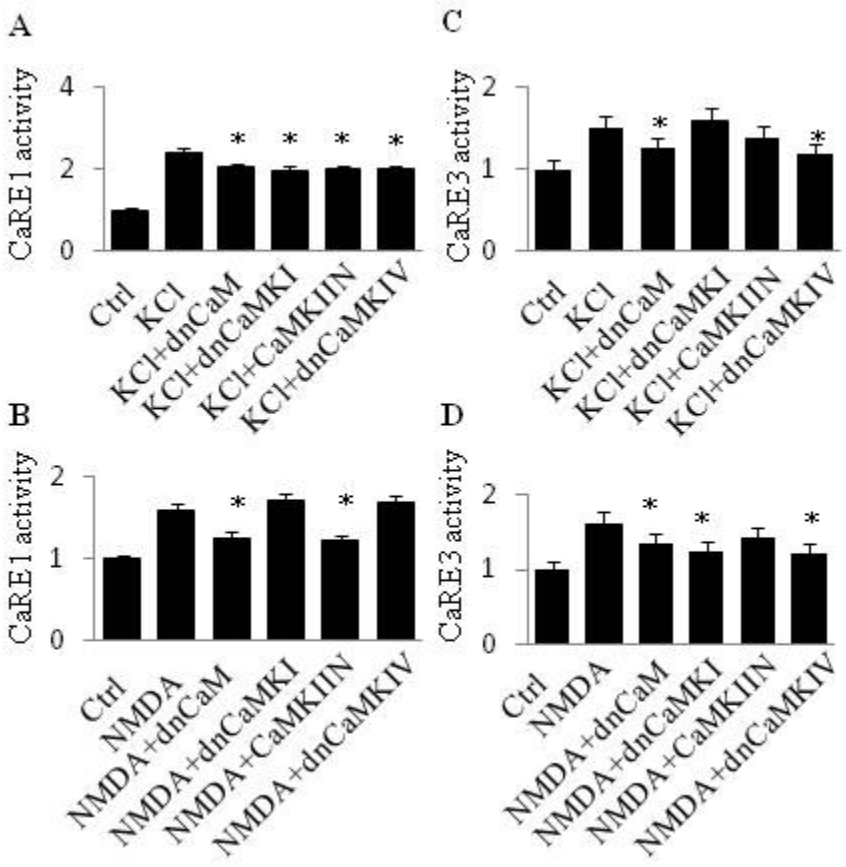

Figure 2.5 Regulation of Ca2+-stimulated CaRE1 and CaRE3 activity by CaM-dependent kinase I, II and IV. DIV 9 cortical neurons were transfected with CaRE1-luciferase (A and B) or CaRE3-luciferase (C and D) reporter plus TK-pRL and dominant negative CaM, CaMKI, CaMKIV, and CaMKIIN. For normalization purpose, SV40-luciferase plus TK-pRL and dnCaM, dnCaMKI, dnCaMKIV and CaMKIIN were transfected into another set of neurons. Two days after transfection, neurons were treated with 50 mM KCl (A and C) or 20 μM NMDA (B and D), and analyzed by dual luciferase assay 6 hours after stimulation. After normalized to Renilla luciferase activity, CaRE1 or CaRE3 activity was further normalized to SV40 activity, and expressed as relative activities. Another set of cells were co-transfected dn constructs without simulation to rule out the inhibitory effect on basal level. All the stimulator+dn plasmid level was shown after normalization with dn plasmid-only groups. *: statistically different to that of stimulated neurons ($p<0.05$). n=12 for all experiments in this figure.

L-VGCC- and NMDAR-mediated Ca2+ influx. We further examined how Ca2+-activated protein kinases regulate its activation. It appeared that L-VGCC- and NMDAR-mediated CaRE3 were similarly regulated by MEK, PI3K and PKA. Dominant negative MEK, dnPI3K, and dnPKA blocked CaRE3 activation in neurons stimulated by KCl and NMDA (Fig. 2.4C and D).

CaM activity was required for both L-VGCC- and NMDAR-mediated activation of CaRE3, because neurons co-transfected with CaRE3-luciferase and dnCaM did not show elevated luciferase activity in neurons stimulated with KCl or NMDA (Fig. 2.5C and D). It appeared that L-VGCC- and NMDAR-mediated CaRE3 up-regulation was differentially regulated by CaMKs. In KCl-stimulated neurons, dnCaMKIV, but not dnCaMKI and CaMKIIN blocked CaRE3 activity (Fig. 2.5C). In NMDA-stimulated neurons, dnCaMKIV and dnCaMKI, but not CaMKIIN blocked CaRE3 activity (Fig. 2.5D). In contrast to CaRE1, the basal level of CaRE3 in non-stimulated neurons was not affected by any of the dn constructs used in this study (Supplemental Fig. 2.3B).

CRE and CaRE3 activity are similarly regulated by Ca2+-stimulated protein kinases. Because the DNA sequence of CaRE3 is similar to that of CRE consensus sequence (Fig. 2.3A), it was suggested that CaRE3 and CRE activity should be regulated by the same mechanism. Here, we compared the activation profile of CRE to that of CaRE3. We found that CRE activity was regulated by the same set of Ca2+-activated protein kinases as those regulating CaRE3.

Figure 2.6

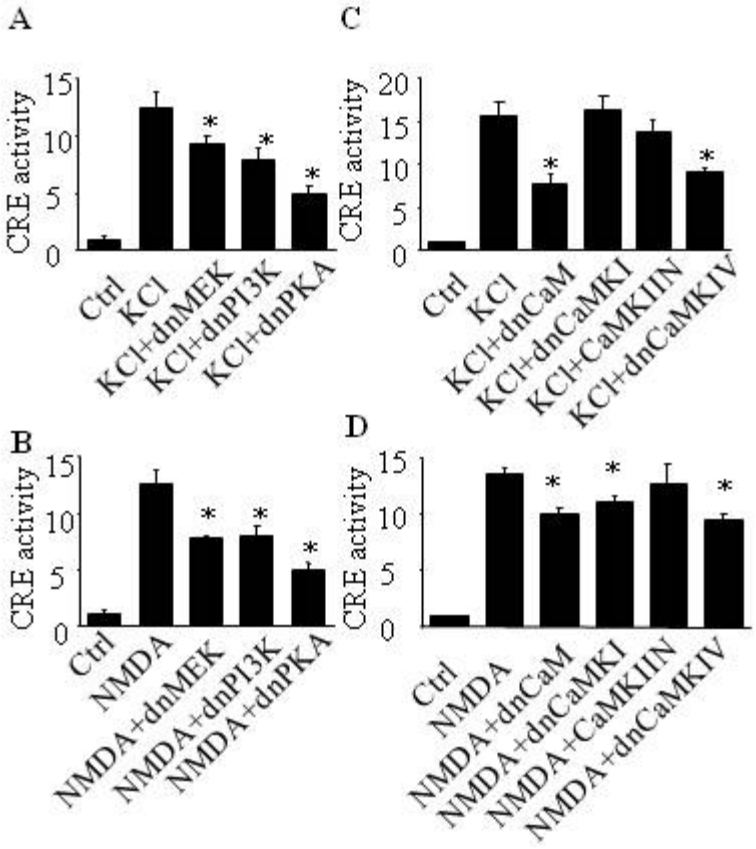

Figure 2.6 Regulation of CRE by Ca2+-stimulated protein kinases is similar to that of CaRE3. The CRE-luciferase reporter construct plus TK-pRL along with dominant negative form of Ca2+-stimulated protein kinases (dnMEK, dnPI3K, and dnPKA for A and B; dnCaM, dnCaMKI, CaMKIIN, and dnCaMKIV for C and D) into cortical neurons on 9 DIV. SV40-luciferase plus TK-pRL along with different dominant negative construct were transfected into neurons for normalization purposes. On 11 DIV, neurons were treated with 50 mM KCl (A and C) or 20 μM NMDA (B and D). Dual luciferase assay was performed 6 hours after stimulation. After normalized to Renilla luciferase activity, CRE activity was further normalized to SV40 activity, and expressed as relative activities. Another set of cells were co-transfected dn constructs without simulation to rule out the inhibitory effect on basal level. All the stimulator+dn plasmid level was shown after normalization with dn plasmid-only groups. *: statistically different to that of stimulated neurons ($p<0.05$). n=8 for all experiments in this figure.

Figure 2.7

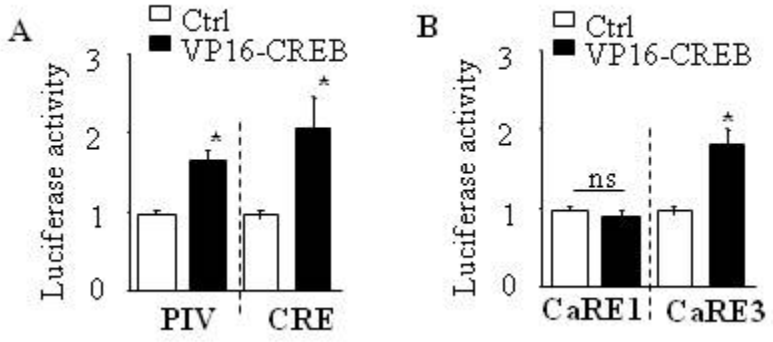

Figure 2.7 Constitutive active CREB enhances CaRE3 but not CaRE1 activity. A. Promoter IV- or CRE-luciferase reporter construct was co-transfected with TK-pRL and VP16-CREB expression plasmid into DIV 9 neurons. B. CaRE1- or CaRE3-luciferase reporter construct was co-transfected with TK-pRL and VP16-CREB expression plasmid into DIV 9 neurons. The activity of promoter IV, CRE, CaRE1 and CaRE3 was determined by dual luciferase assay on 11 DIV. After normalized to Renilla luciferase activity, CRE, CaRE1 or CaRE3 activity was further normalized to SV40 activity in neurons transfected with SV40-luciferase, TK-pRL and VP16-CREB. *: statistically different to that of controls ($p<0.05$). ns: not statistically significant. n=5 for all experiments in this figure.

Figure 2.8

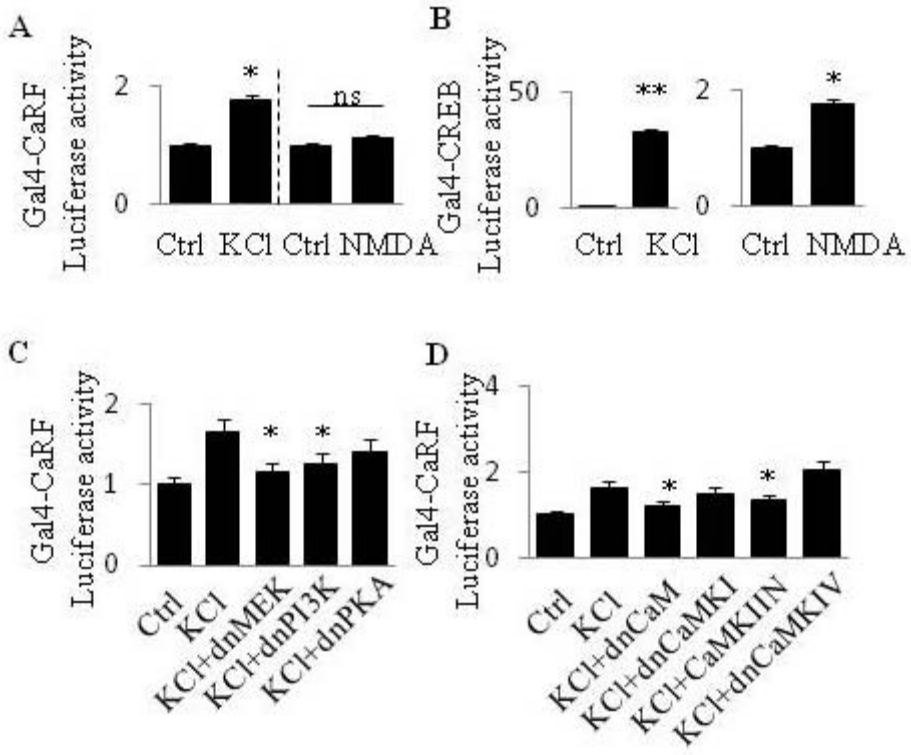

Figure 2.8 Regulation of CaRF transcriptional activity by calcium-mediated protein kinases. Neurons on 9 DIV were transfected with Gal4 only, Gal4-CaRF or Gal4-CREB together with Gal4-UAS-Luc and TK-pRL. After 48 hours, the neurons were treated with 50 mM KCl (A) or 20 μM NMDA (B) for 6 hours and submitted to luciferase assay. Dominant negative MEK, PI3K, PKA (C), CaM, CaMKI, CaMKIV and CaMKIIN (D) were co-transfected with or without KCl treatment. The data shown was normalized to TK-Renilla luciferase activity and then Gal4 only activity in Gal4 transfectd neurons. All the stimulator+dn plasmid level was further normalized with dn plasmid-only groups. *: statistically different ($p<0.05$) to that of controls (A and B) or stimulated neurons (C and D). ns: not statistically significant. n=12 for all experiments in this figure.

Specifically, both L-VGCC- and NMDAR-mediated CRE activation were blocked by dnMEK, dnPI3K, and dnPKA (Fig. 2.6A and B). CaM-mediated signaling was also required, because overexpression of dnCaM blocked L-VGCC- and NMDAR-mediated CRE activation (Fig. 2.6C and D). In NMDA-stimulated neurons, overexpression of dnCaMKIV, but not dnCaMKI or CaMIIN, blocked CRE-mediated luciferase transcription (Fig. 2.6D). In KCl- stimulated neurons, L-VGCC-mediated CRE activity was blocked by dnCaMKI and dnCaMKIV, but not by CaMKIIN (Fig. 2.6C).

We further tested whether activation of CREB could stimulate PIV and its calcium responsive elements. As shown in Fig. 2.7A, overexpression of a constitutive active form of CREB (VP16-CREB, which confers transcriptional factor activity in the absence of upstream CREB activators) significantly enhanced PIV- and CRE-mediated transcription. Consistent with the hypothesis that CaRE3 is a potential CRE, overexpression of VP16-CREB also enhanced CaRE3-mediated transcription (Fig. 2.7B). In contrast, activation of CREB was not sufficient to stimulate CaRE1 activity (Fig. 2.7B).

Regulation of CaRF transcriptional activity by Ca2+-stimulated protein kinases. Calcium responsive factor (CaRF) has been identified as a putative transcription factor for CaRE1, because it directly binds CaRE1 *in vitro* (Tao et al., 2002). We sought to understand how CaRF-mediated transcription is regulated. To measure CaRF activity, we transfected neurons with a plasmid expressing a fusion protein

consisting of the DNA binding domain of Gal4 and the transcriptional activation domain of CaRF together with Gal4-UAS driven luciferase reporter construct. Surprisingly, Gal4-CaRF activity was only induced by KCl but not NMDA (Fig. 2.8A). In contrast, Gal4-CREB activity was dramatically increased by both KCl and NMDA (Fig. 2.8B). Further, the regulatory property of CaRF was different from that of CaRE1. The L-VGCC-mediated activation of Gal4-CaRF activity was only blocked by dnMEK, dnPI3K, dnCaM and CaMKIIN (Fig. 2.8C and D). Although dnPKA did not affect KCl-induced activation (Fig. 2.8C), it caused significant decrease in basal CaRF activity (Supplemental Fig. 2.4). These data suggest that the regulation of CaRF is different from that of CaRE1. Other mechanisms, for example, how binding of CaRF to CaRE1 is regulated, may be involved.

Cross-talk between Ca2+-stimulated protein kinase pathways. It has been suggested that calcium-activated kinases may regulate each other. We focused on MAPK pathway, which is a critical pathway for exon IV transcription and has been found to be regulated by other signal transduction pathways (Chen et al., 2003b; Schmitt et al., 2005). Here, we showed that phospho-ERK1/2, which is correlated with ERK1/2 activation, was up-regulated by activation of L-VGCC. Both the basal level and the KCl-induced activation were suppressed by PI3K inhibitor LY294002, PKA inhibitor H89, and CaMK inhibitor KN93 (Fig. 2.9A, B and Supplemental Fig. 2.5). These data further confirmed that ERK1/2 activity might be significantly disrupted through inhibition of other pathways.

Figure 2.9

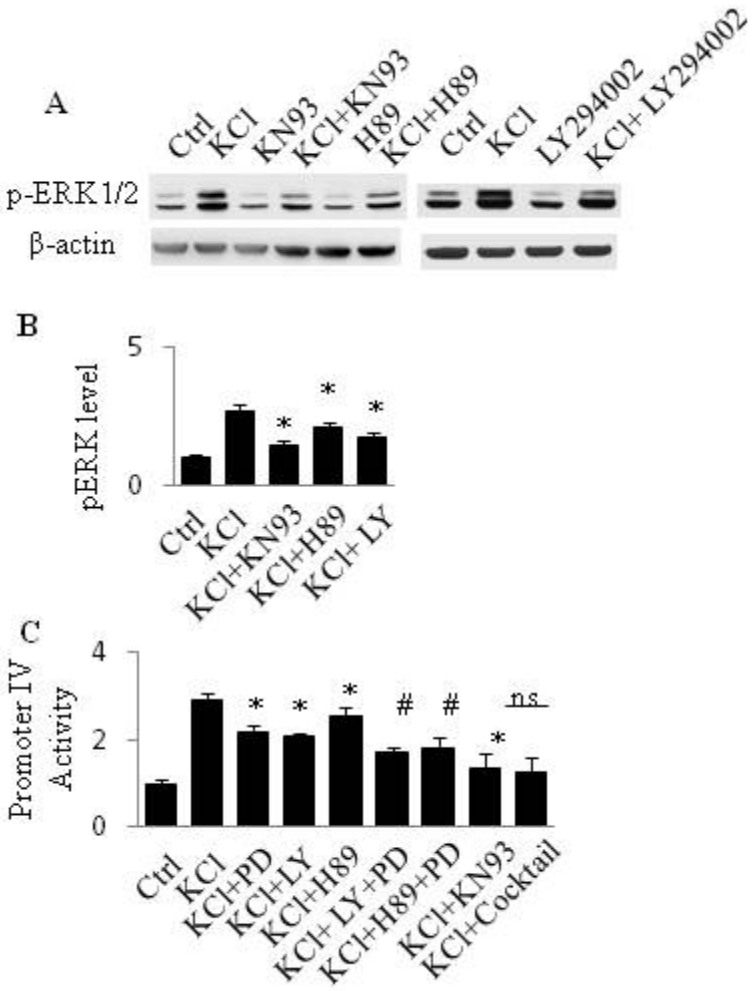

Figure 2.9. Crosstalk among calcium stimulated protein kinases and simultaneous inhibition on promoter IV mediated transcription. DIV 14 neurons were treated with KN93, LY294002 or H89 30 min before the KCl (50mM) stimulation. Protein level of phosphor-ERK1/2 and β-actin were examined 20 min after stimulation by western blotting (A). The density of pERK was quantified and normalized to β-actin level (B). DIV 9 neurons were transfected with PIV-Luc and TK-pRL for 2 days. Before the 6-hour KCl (50mM) treatment, the cells were treated with PD98059 (PD), LY294002 (LY), H89 and KN93 individually or a combination of PD+LY, PD+H89, PD+LY+KN+H89 (Cocktail) for 30 minutes. Another set of neurons were only received the inhibitor treatment. The luciferase activity was then measured (C). The stimulator+inhibitor level (B and C) was shown after normalization to inhibitor-only groups. *: statistically different to that of stimulated neurons ($p<0.05$); #: statistically different ($p<0.05$) to that of single inhibitors (C). n=4 for all experiments in this figure.

Because blocking a single pathway has shown partial inhibitory effects on PIV-mediated transcription, we tested whether further inhibition could be achieved by simultaneously blocking multiple pathways. Indeed, co-treatment with 2 inhibitors (as indicated in Fig. 2.9C) caused further more inhibition when compared to a single inhibitor. Interestingly, the CaMKs may play a more dominant role than other kinases. As shown in Fig. 2.2A and C, KN93 totally abolished L-VGCC-stimulated BDNF IV mRNA up-regulation. KN93 also showed more dramatic inhibition of KCl-induced PIV activity (Fig. 2.9C). Inclusion of more inhibitors (i.e. H89, PD98059, and LY294002) to KN93 did not cause further inhibition (Fig. 2.9C).

DISCUSSION

Many aspects of brain function depend on activity-dependent modifications of neuronal structure and property. One major molecular trigger for activity-driven signaling cascades is Ca2+ influx mediated by ligand- and voltage-gated Ca2+ channels. Crosstalk and coordination among Ca2+-stimulated protein kinases regulate the activity of transcription factors via phosphorylation and lead to gene transcription. Many lines of evidence have implicated that Ca2+-stimulated activation of cAMP responsive element (CRE) binding protein (CREB) and CRE-mediated transcription is functional relevant for neuronal survival (Riccio et al., 1999; Lonze et al., 2002), development (Pham et al., 1999), long-term synaptic potentiation (Impey et al., 1996), and memory formation (Athos et al.,

2002). As a CREB-regulated gene, BDNF transcription has been studied as a model to delineate how Ca2+-stimulated signaling cascade regulates the activity of its promoter through the coordination of multiple *cis*-elements and *trans*-factors (Tao et al., 1998; Tao et al., 2002; Chen et al., 2003b). In this study, we have examined the function of the major plasticity-related Ca2+-stimulated protein kinases in regulating promoter IV and CaREs.

It has been shown that Ca2+-activated BDNF exon IV transcription is mainly mediated by L-VGCC but not NMDAR (Ghosh et al., 1994). We and others have found that NMDA stimulation can stimulate robust CREB activation and exon IV transcription in younger (e.g. from DIV 3 to 7) but not in well-developed neurons (e.g. DIV 14) (Sala et al., 2000; Tabuchi et al., 2000; Zhou et al., 2009). Compared to DIV 3-7 neurons, the total expression of NMDAR is higher at DIV 14. Therefore, the same concentration of NMDA (e.g. 50 μM) may cause proper NMDAR activation in DIV 3 neurons, but lead to pathological overactivation and shut off the pro-survival CREB-BDNF signaling in DIV 14 neurons. It was further suggested that deactivation of CREB and BDNF transcription may be mediated by the activation of extrasynaptic NMDARs, because activation of synaptic NMDARs alone always activate CREB and CRE-mediated transcription (Hardingham et al., 2002). It appeared that lowering the concentration of NMDA significantly stimulated CREB activity and BDNF transcription, and was neural protective

(Soriano et al., 2006). Here, we show that NMDAR-mediated up-regulation of exon IV transcription, as well as PIV, CaRE1 and CaRE3 activity, was significant in DIV 11 and DIV 14 neurons stimulated with 20 μM NMDA. Consistent with the previous results, we observed significant L-VGCC-mediated exon IV transcription and PIV activity. Surprisingly, activation of NMDAR or L-VGCC only activated CaRE1 and CaRE3 but not CaRE2. It was reported that CaRE2 activity was triggered by membrane depolarization but not by glutamate stimulation (Chen et al., 2003b). The different results may be due to different experiment setups. The previous study stimulated DIV 5 neurons with KCl in the absence of CNQX and APV, but we used more mature neurons at DIV 9 and 14. We also excluded the involvement of glutamate receptors by co-treatment with CNQX and APV along with KCl. It is possible that CaRE2-mediated transcription is developmentally regulated. Indeed, we were able to detect KCl-mediated CaRE2 activation in DIV 5 neurons (data not shown). Future effort should be made to examine how CaRE2-mediated transcription is regulated in developing neurons.

Studies on the somatostatin promoter identified a CRE sequence 5'-TGACGTCA-3', which is stimulated by elevation of cAMP and PKA activity (Montminy and Bilezikjian, 1987). Mutational analysis with the BDNF promoter IV identified a CRE-like sequence 5'-TCACGTCA-3' (termed as B-CRE or CaRE3) (Shieh et al., 1998; Tao et al., 1998; Tao et al., 2002). Although it was shown that

endogenous CREB binds CaRE3 sequence *in vitro* (Shieh et al., 1998; Tao et al., 1998), it had not been clear whether the regulatory properties of CaRE3 are similar to CRE. Here, we report that CRE and CaRE3 showed similar activation profiles. Specifically, they were both stimulated by L-VGCC- and NMDAR-mediated Ca2+ influx. They also showed same regulation by MEK, PI3K, PKA, and CaMKs. Importantly, both CRE and CaRE3 activity were up-regulated by VP16-CREB. These data implicate that CaRE3 may be a *bona fide* CREB target.

CREB activity could be regulated by multiple Ca2+-stimulated protein kinases. It has been demonstrated that phosphorylation of CREB at Ser-133 facilitates the recruitment of coactivator CBP (CREB binding protein) (Chrivia et al., 1993), which is critical for CREB-mediated transcription (Shaywitz et al., 2000). Because CaMKIV is localized in the nucleus, it is considered as the major protein kinase to regulate Ser-133 phosphorylation. Supportively, inhibition of CaMKIV activity by antisense oligonucleotide or genetic deletion results in impaired Ca2+ stimulation on Ser-133 phosphorylation. In addition, CaMKIV activity is also required to phosphorylate CBP (Impey et al., 2002). Although CaMKII can phosphorylate Ser-133 *in vitro* (Sheng et al., 1991), it also phosphorylates Ser-142 (Sun et al., 1994). Because phosphorylation at Ser-142 antagonizes CREB-mediated transcription (Sun et al., 1994), the consequence of dual phosphorylation by

CaMKII is not clear. Intriguingly, membrane depolarization, which stimulates CREB-mediated transcription, up-regulates phosphorylation of both Ser-133 and 142 (West et al., 2001). Therefore, the function of CaMKIV and CaMKII in CRE-mediated transcription is unclear. Here, we show that blocking CaMKIV activity by dnCaMKIV suppressed both CRE and CaRE3. Inhibition of CaMKII by overexpressing CaMKIIN had no effects on Ca2+-stimulated CRE and CaRE3 activity.

Overexpression of constitutive active CaMKI causes the activation of CREB (Sun et al., 1996). Intriguingly, we found that dnCaMKI suppressed NMDAR- but not L-VGCC-mediated CRE and CaRE3 activity. It was demonstrated that NMDAR-mediated ERK1/2 activation requires the CaMKK-CaMKI cascade (Schmitt et al., 2005). Because ERK1/2-mediated activation of RSK2 and MSK1 may lead to phosphorylation of CREB at Ser-133 (Impey et al., 1998; Arthur et al., 2004; Vaidyanathan et al., 2007), dnCaMKI may suppress CREB activity and CRE/CaRE3-mediated transcription through the ERK1/2-RSK2/MSK1 cascade in NMDA-stimulated neurons. Although blocking CaMKs by KN93 inhibited KCL-stimulated p-ERK1/2, blocking CaMKI alone by dnCaMKI did not suppress L-VGCC-mediated CRE/CaRE3 activation.

The crosstalk among Ca2+-stimulated protein kinases expands beyond the interaction between CaMKI and ERK1/2. For example, PKA activity may directly

regulate Ser-133 phosphorylation of CREB, or indirectly through ERK1/2-RSK2 (Grewal et al., 2000). On one hand, cAMP elevation and PKA upregulate ERK1/2 through RAP (Vossler et al., 1997). On the other hand, PKA activity is required for nuclear translocation of ERK1/2 (Impey et al., 1998). Further, calcium activation of Ras-ERK1/2 cascade may directly regulate PI3K (Rodriguez-Viciana et al., 1994). It also appears that PI3K may be up-stream of ERK1/2 activation in the hippocampus during memory retrieval (Chen et al., 2005). During the induction of long-term synaptic potentiation, PI3K also affects high frequency stimulation-induced ERK1/2 activation (Opazo et al., 2003). Consistent with these previous studies, our data also showed that PI3K, PKA and CaMKs activity impinged on ERK1/2 activation. However, co-inhibition of multiple pathways caused more dramatic suppression of PIV-mediated transcription, suggesting a more elaborating regulation by these co-existing pathways. To add another layer of complication, blocking CaMKs fully abolished L-VGCC-mediated PIV activation. It is possible that CaMKs affect a broader spectrum of other signaling pathways (in addition to ERK1/2). Alternatively, some of these pathways may work in tandem rather than in parallel to control the sequential steps involved in transcriptional up-regulation.

Because cAMP-stimulated exon IV transcription is not as robust as Ca2+-stimulated transcription, it was hypothesized that additional *cis*-elements

other than CRE may exist in PIV. Indeed, a neuro-specific protein CaRF was identified to regulate L-VGCC-mediated CaRE1 activity (Tao et al., 2002). Although sequence analysis of CaRF implicates potential phosphorylation sites for ERK1/2 and CaMKII (Tao et al., 2002), how these protein kinases regulate CaRE1-mediated transcription is unknown. Here, we report that, similar to PIV, CRE and CaRE3, activation of either L-VGCC or NMDAR could stimulate CaRE1-mediated transcription. Interestingly, unlike CRE/CaRE3, Ca2+-stimulated CaRE1 activity was blocked by inhibiting CaMKII, PI3K or MEK. The requirement of PKA, CaMKI and CaMKIV, however, depended on the route of Ca2+ influx (i.e. L-VGCC- vs. NMDAR-mediated increase of intracellular Ca2+). Our data also showed that CaRE1 activity is independent of CREB, because VP16-CREB failed to stimulate CaRE1-mediated transcription. Furthermore, we found that the regulation of CaRF activity was quite different from CaRE1-mediated transcription. Supportively, a previous study showed that loss of CaRF did not fully phenocopy the mutation of CaRE1 [24, 61] (McDowell et al., 2010; Pfenning et al., 2010), suggesting that additional CaRE1 binding protein may exist. Further, it is possible that there are additional mechanisms to regulate binding of CaRF to CaRE1

It has been postulated that the property of Ca2+-activated signaling cascades depends on the route of Ca2+ entry. It was apparent that L-VGCC- and

NMDAR-mediated CaRE activation is differentially regulated. As described earlier, CaMKI activity is required for NMDAR- but not L-VGCC-mediated activation of CRE/CaRE3. For CaRE1 activity, PKA is required for L-VGCC- but not NMDAR-mediated transcription. Although it is not clear how Ca2+ entry through different channels specifies PKA-mediated CaRE1 activation, it has been demonstrated that L-VGCC and NMDAR interact with different intracellular signaling molecules. For example, NMDAR is tethered to scaffold proteins PSD95 and SynGap through the interaction with the cytoplasmic tails of NR2A and NR2B, respectively (Fagiolini et al., 2003; Kim et al., 2005). Functionally, disruption of these physical associations results in defective ERK1/2 activation and BDNF transcription (Kim et al., 2005; Chen et al., 2007). Other compartmentalized signaling cascades are shown to physically interact with L-VGCC. Among them, association of L-VGCC with PKA anchoring protein AKAP (AKAP15 and AKAP79/150) (Gray et al., 1998; Oliveria et al., 2007) and CaM may provide a tighter coupling between PKA and CaRE1-mediated transcription. Intriguingly, neuronal activity triggers the proteolysis of L-VGCC, resulting in the C-terminal tail's translocation to nucleus and direct activation of gene transcription in a Ca2+-dependent manner (Gomez-Ospina et al., 2006). It remains unclear how Ca2+-stimulated protein kinases regulates the nuclear translocation of the C-terminal tail.

How do CaRE1 and CaRE coordinate to regulate PIV activity? Mutation or deletion of either CaRE1 or CaRE3 ablates Ca2+-stimulated up-regulation of PIV activity (Tao et al., 1998; Tao et al., 2002). This indicates that activation of CaRE1 or CaRE3 alone in the context of flanking sequences of PIV is not sufficient to drive exon IV transcription. Our data is consistent with this possibility, and implicates that coordination and crosstalk between CaRE1 and CaRE3 may be required for the full activity of PIV. For example, although CaRE3 showed full activity, suppression of CaRE1 activity results in down regulation of PIV in CaMKIIN-expressing neurons. Similarly, dnPKA inhibited NMDAR-mediated PIV activation without affecting CaRE1.

In summary, we have found that the 5' 911bp flanking sequence of BDNF exon IV showed same regulatory properties the endogenous BDNF promoter IV. We systematically examined the function of the major Ca2+-stimulated CREB/CaRF kinases, and found that CaRE1 and CaRE3 were differentially regulated. Finally, based on our results and previous studies, we propose a working model to show how crosstalk between different signaling cascades and coordination of multiple CaREs regulate the activity-dependent transcription of BDNF (Fig. 2.10). In addition to the un-identified links and molecules, we suggest that necessary caution need to be taken. First, although the pharmacological inhibitors and dominant negative constructs were previously

characterized and the two approaches gave fairly consistent results in this study, their non-specific effects cannot be totally ruled out. For example, KN93 (similar to KN62) was previously used as a CaMKII antagonist, but it also has significant effects on CaMKI and CaMKIV. In addition to PKA, H89 can also affect CaMKII and PKC activity, though the Ki is about 3 orders of magnitudes higher. However, the use of acute pharmacological inhibition may be beneficial to avoid compensation, which is normally associated with long-term inhibition by using genetic knock down or knockout approaches.

ACKNOWLEDGMENTS

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Figure 2.10

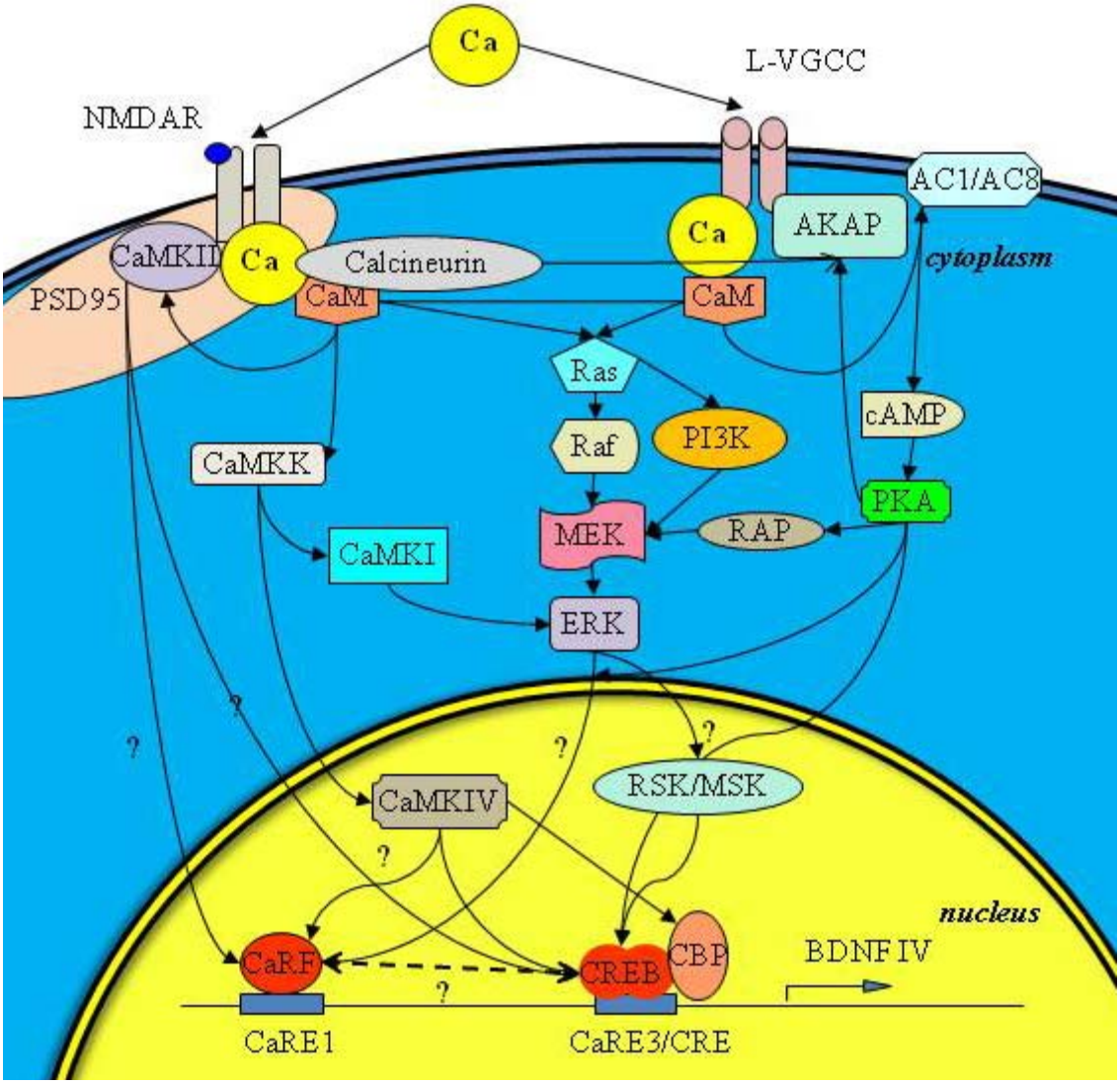
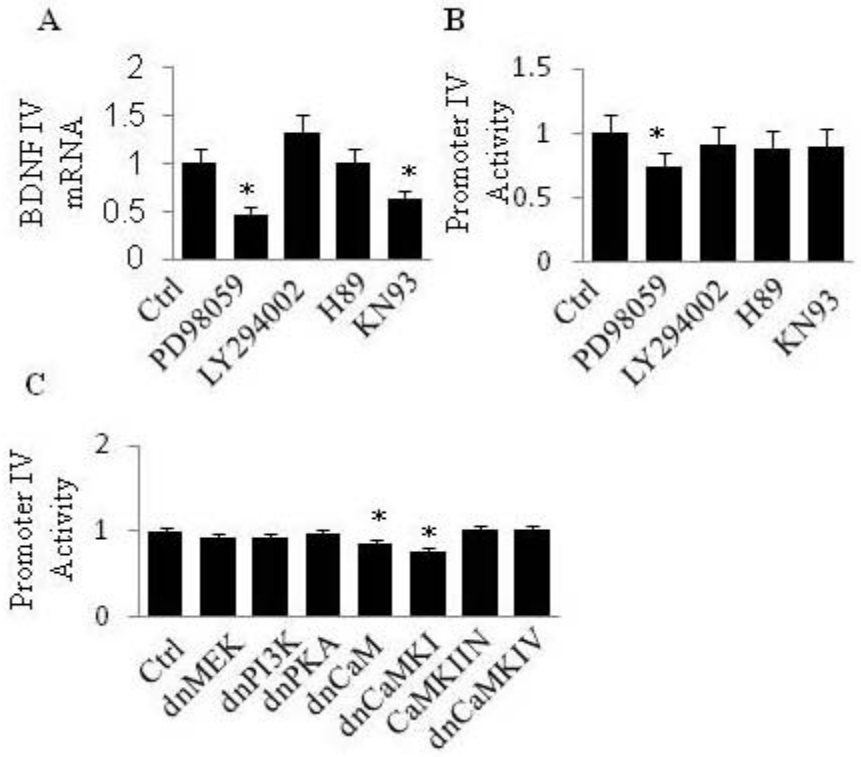


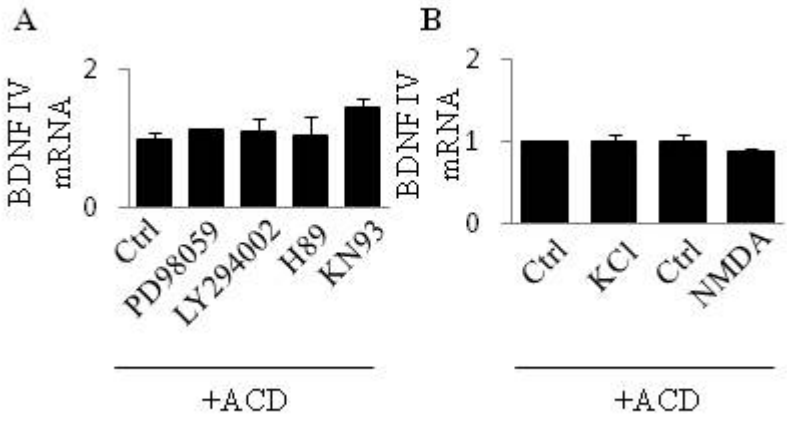
Figure 2.10 Regulation of BDNF promoter IV activity by Ca2+-stimulated signaling cascades. Based on the results of this study and existing literature, we propose that CaRE1 and CaRE3 are differentially regulated by protein kinase network that is stimulated by the calcium influx through NMDAR and L-VGCC. The activity of promoter IV is dictated by the coordination between CaRE1 and CaRE3. Different microdomains at the C terminal of NMDA receptors (CaMKII) and L-VGCC (AKAP15 and AKAP79/150) may result in different downstream pathways. Crosstalk among PKA, PI3K, CaMKs and MAPK pathways is complicated and the pathways may contribute to the transcription simultaneously.

Supplementary Figure 2.1



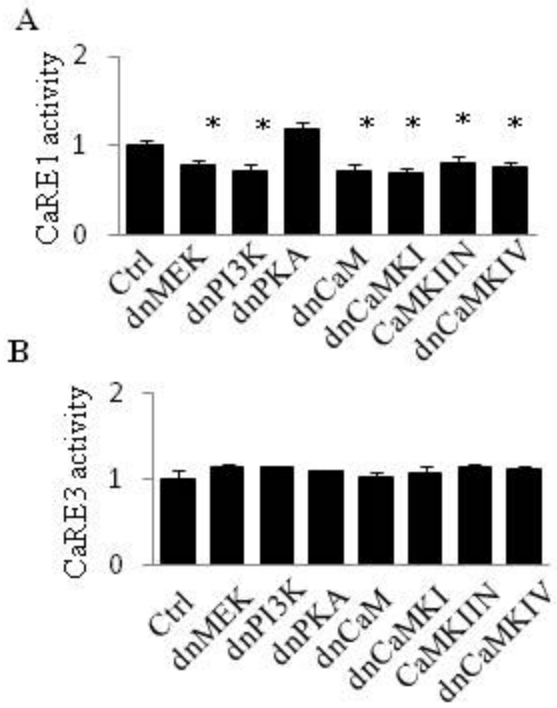
Supplementary Figure 2.1. Regulation of the basal level of BDNF IV mRNA and PIV-driven luciferase activity. The neurons were treated with the MEK inhibitor PD98059, the PI3K inhibitor LY294002, the PKA inhibitor H89 and the CaMKs inhibitor KN93 on 14 DIV, and the BDNF mRNA level was measure using real-time PCR. The presented mRNA level was normalized to GAPDH mRNA (A). DIV 9 neurons were transfected with PIV-Luc and TK-pRL (B) or cotransfected with dn constructs including dnMEK, dnPI3K, dnPKA, dnCaM, dnCaMKI, dnCaMKIV and CaMKIIN (C). The luciferase expression was assayed two days after transfection and for B, neurons were treated with inhibitors for 6.5 hours. Firefly luciferase activity was normalized to Renilla luciferase. *: statistically different to that of controls ($p<0.05$). n=8 for A and n=12 for B and C.

Supplementary Figure 2.2



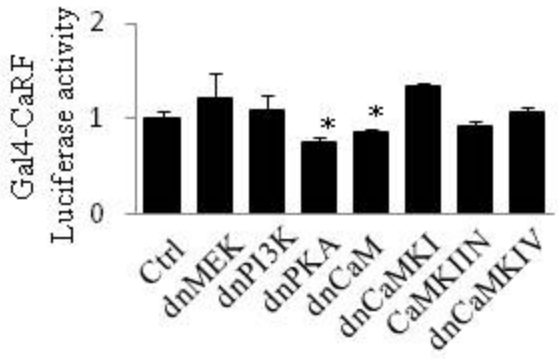
Supplementary Figure. 2.2. BDNF IV mRNA stability is not affected by simulation or inhibition. DIV 14 neurons were pre-treated with transcription inhibitor 100ng/ml actinomycin D (ACD) before either inhibited by PD98059, LY294002, H89 and KN93 or stimulated by 50mM KCl and 20 μM NMDA. Real-time PCR was performed to measure BDNF IV mRNA, which is then normalized to GAPDH. No significance was detected compared to the controls. n=8 for all experiments in this figure.

Supplementary Figure 2.3



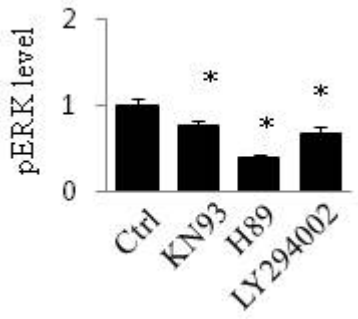
Supplementary Figure. 2.3. Regulation of basal transcription driven by CaRE1 and CaRE3. SV40/ CaRE1/ CaRE3-Luc with TK-pRL and dn constructs (dnMEK, dnPI3K, dnPKA, dnCaM, dnCaMKI, CaMKIIN and dnCaMKIV) were transfected into DIV 9 neurons. Dual-luciferase assay after two days of transfection examined CaRE1/CaRE3-Luc activity, which was first normalized to Renilla luciferase and then to SV40 activity. *: statistically different to that of controls ($p<0.05$). n=12 for all experiments in this figure.

Supplementary Figure 2.4



Supplementary Figure 2.4 Regulation of CaRF basal transcriptional activity by calcium-stimulated protein kinases. DIV 9 neurons were transfected with Gal4 only or Gal4-CaRF, Gal4-UAS-Luc, TK-pRL along with dnMEK, dnPI3K, dnPKA, dnCaM, dnCaMKI, CaMKIIN and dnCaMKIV. The luciferase assay was performed 48 hours after the transfection. Gal4-CaRF mediated luciferase activity was normalized to Renilla luciferase and then to Gal4-only. *: statistically different to that of controls ($p<0.05$). n=12 for all experiments in this figure.

Supplementary Figure 2.5



Supplementary Figure 2.5 ERK basal activity was suppressed by PI3K, PKA and CaMKs. Cultured cells at DIV 14 were treated by inhibitors KN93, H89 and LY294002 for 50 min. Cells were collected and pERK/β-actin levels were probed in western blotting. As presented in Fig. 9, basal pERK level change were quantified and normalized to β-actin. *: statistically different to that of controls ($p<0.05$). n=4 for all experiments in this figure.

REFERENCES

Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. J Neurosci Res 85:525-535.

Arthur JS, Fong AL, Dwyer JM, Davare M, Reese E, Obrietan K, Impey S (2004) Mitogen- and stress-activated protein kinase 1 mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins. J Neurosci 24:4324-4332.

Athos J, Impey S, Pineda VV, Chen X, Storm DR (2002) Hippocampal CRE-mediated gene expression is required for contextual memory formation. Nat Neurosci 5:1119-1120.

Barco A, Alarcon JM, Kandel ER (2002) Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. Cell 108:689-703.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286:1358-1362.

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ (1994) Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell 79:59-68.

Chang BH, Mukherji S, Soderling TR (2001) Calcium/calmodulin-dependent protein kinase II inhibitor protein: localization of isoforms in rat brain. Neuroscience 102:767-777.

Chen Q, He S, Hu XL, Yu J, Zhou Y, Zheng J, Zhang S, Zhang C, Duan WH, Xiong ZQ (2007) Differential roles of NR2A- and NR2B-containing NMDA receptors in activity-dependent brain-derived neurotrophic factor gene regulation and limbic epileptogenesis. J Neurosci 27:542-552.

Chen WG, West AE, Tao X, Corfas G, Szentirmay MN, Sawadogo M, Vinson C, Greenberg ME (2003) Upstream stimulatory factors are mediators of Ca2+-responsive transcription in neurons. J Neurosci 23:2572-2581.

Chen X, Garelick MG, Wang H, Lil V, Athos J, Storm DR (2005) PI3 kinase signaling is required for retrieval and extinction of contextual memory. Nat Neurosci 8:925-931.

Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365:855-859.

Dugich-Djordjevic MM, Tocco G, Willoughby DA, Najm I, Pasinetti G, Thompson RF, Baudry M, Lapchak PA, Hefti F (1992) BDNF mRNA expression in the developing rat brain following kainic acid-induced seizure activity. Neuron 8:1127-1138.

Fagiolini M, Katagiri H, Miyamoto H, Mori H, Grant SG, Mishina M, Hensch TK (2003) Separable features of visual cortical plasticity revealed by N-methyl-D-aspartate receptor 2A signaling. Proc Natl Acad Sci U S A 100:2854-2859.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381:706-709.

Ghosh A, Carnahan J, Greenberg ME (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263:1618-1623.

Gomez-Ospina N, Tsuruta F, Barreto-Chang O, Hu L, Dolmetsch R (2006) The C terminus of the L-type voltage-gated calcium channel Ca(V)1.2 encodes a transcription factor. Cell 127:591-606.

Gray PC, Johnson BD, Westenbroek RE, Hays LG, Yates JR, 3rd, Scheuer T, Catterall WA, Murphy BJ (1998) Primary structure and function of an A kinase anchoring protein associated with calcium channels. Neuron 20:1017-1026.

Greer PL, Greenberg ME (2008) From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. Neuron 59:846-860.

Grewal SS, Fass DM, Yao H, Ellig CL, Goodman RH, Stork PJ (2000) Calcium and cAMP signals differentially regulate cAMP-responsive element-binding protein function via a Rap1-extracellular signal-regulated kinase pathway. J Biol Chem 275:34433-34441.

Hall J, Thomas KL, Everitt BJ (2000) Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. Nat Neurosci

3:533-535.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405-414.

Impey S, Mark M, Villacres EC, Poser S, Chavkin C, Storm DR (1996) Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron 16:973-982.

Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR (1998) Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. Neuron 21:869-883.

Impey S, Fong AL, Wang Y, Cardinaux JR, Fass DM, Obrietan K, Wayman GA, Storm DR, Soderling TR, Goodman RH (2002) Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV. Neuron 34:235-244.

Ji Y, Lu Y, Yang F, Shen W, Tang TT, Feng L, Duan S, Lu B Acute and gradual increases in BDNF concentration elicit distinct signaling and functions in neurons. Nat Neurosci 13:302-309.

Kawanishi Y, Harada S, Tachikawa H, Okubo T, Shiraishi H (1999) Novel variants in the promoter region of the CREB gene in schizophrenic patients. J Hum Genet 44:428-430.

Kim MJ, Dunah AW, Wang YT, Sheng M (2005) Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46:745-760.

Labasque M, Reiter E, Becamel C, Bockaert J, Marin P (2008) Physical interaction of calmodulin with the 5-hydroxytryptamine2C receptor C-terminus is essential for G protein-independent, arrestin-dependent receptor signaling. Mol Biol Cell 19:4640-4650.

Lessmann V, Gottmann K, Heumann R (1994) BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultured hippocampal neurones. Neuroreport 6:21-25.

Linnarsson S, Bjorklund A, Ernfors P (1997) Learning deficit in BDNF mutant mice. Eur J Neurosci 9:2581-2587.

Lonze BE, Riccio A, Cohen S, Ginty DD (2002) Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. Neuron 34:371-385.

Lubin FD, Roth TL, Sweatt JD (2008) Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. J Neurosci 28:10576-10586.

Mayr B, Montminy M (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599-609.

McAllister AK, Lo DC, Katz LC (1995) Neurotrophins regulate dendritic growth in developing visual cortex. Neuron 15:791-803.

McDowell KA, Hutchinson AN, Wong-Goodrich SJ, Presby MM, Su D, Rodriguiz RM, Law KC, Williams CL, Wetsel WC, West AE (2010) Reduced cortical BDNF expression and aberrant memory in Carf knock-out mice. J Neurosci 30:7453-7465.

Montminy MR, Bilezikjian LM (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328:175-178.

Neeper SA, Gomez-Pinilla F, Choi J, Cotman C (1995) Exercise and brain neurotrophins. Nature 373:109.

Newton SS, Thome J, Wallace TL, Shirayama Y, Schlesinger L, Sakai N, Chen J, Neve R, Nestler EJ, Duman RS (2002) Inhibition of cAMP response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. J Neurosci 22:10883-10890.

Oliveria SF, Dell'Acqua ML, Sather WA (2007) AKAP79/150 anchoring of calcineurin controls neuronal L-type Ca2+ channel activity and nuclear signaling. Neuron 55:261-275.

Opazo P, Watabe AM, Grant SG, O'Dell TJ (2003) Phosphatidylinositol 3-kinase regulates the induction of long-term potentiation through extracellular signal-related kinase-independent mechanisms. J Neurosci 23:3679-3688.

Pfenning AR, Kim TK, Spotts JM, Hemberg M, Su D, West AE (2010) Genome-wide identification of calcium-response factor (CaRF) binding sites predicts a role in regulation of neuronal signaling pathways. PLoS One 5:e10870.

Pham TA, Impey S, Storm DR, Stryker MP (1999) CRE-mediated gene transcription in neocortical neuronal plasticity during the developmental critical period. Neuron 22:63-72.

Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 286:2358-2361.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370:527-532.

Sala C, Rudolph-Correia S, Sheng M (2000) Developmentally regulated NMDA receptor-dependent dephosphorylation of cAMP response element-binding protein (CREB) in hippocampal neurons. J Neurosci 20:3529-3536.

Schinder AF, Berninger B, Poo M (2000) Postsynaptic target specificity of neurotrophin-induced presynaptic potentiation. Neuron 25:151-163.

Schmitt JM, Guire ES, Saneyoshi T, Soderling TR (2005) Calmodulin-dependent kinase kinase/calmodulin kinase I activity gates extracellular-regulated kinase-dependent long-term potentiation. J Neurosci 25:1281-1290.

Shaywitz AJ, Dove SL, Kornhauser JM, Hochschild A, Greenberg ME (2000) Magnitude of the CREB-dependent transcriptional response is determined by the strength of the interaction between the kinase-inducible domain of CREB and the KIX domain of CREB-binding protein. Mol Cell Biol 20:9409-9422.

Sheng M, Thompson MA, Greenberg ME (1991) CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 252:1427-1430.

Shieh PB, Hu SC, Bobb K, Timmusk T, Ghosh A (1998) Identification of a signaling pathway involved in calcium regulation of BDNF expression. Neuron 20:727-740.

Soriano FX, Papadia S, Hofmann F, Hardingham NR, Bading H, Hardingham GE (2006) Preconditioning doses of NMDA promote neuroprotection by enhancing neuronal excitability. J Neurosci 26:4509-4518.

Steffan JS, Kazantsev A, Spasic-Boskovic O, Greenwald M, Zhu YZ, Gohler H, Wanker EE, Bates GP, Housman DE, Thompson LM (2000) The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. Proc Natl Acad Sci U S A 97:6763-6768.

Sun P, Lou L, Maurer RA (1996) Regulation of activating transcription factor-1 and the cAMP response element-binding protein by Ca2+/calmodulin-dependent protein kinases type I, II, and IV. J Biol Chem 271:3066-3073.

Sun P, Enslen H, Myung PS, Maurer RA (1994) Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev 8:2527-2539.

Tabuchi A, Nakaoka R, Amano K, Yukimine M, Andoh T, Kuraishi Y, Tsuda M (2000) Differential activation of brain-derived neurotrophic factor gene promoters I and III by Ca2+ signals evoked via L-type voltage-dependent and N-methyl-D-aspartate receptor Ca2+ channels. J Biol Chem 275:17269-17275.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20:709-726.

Tao X, West AE, Chen WG, Corfas G, Greenberg ME (2002) A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. Neuron 33:383-395.

Vaidyanathan H, Opoku-Ansah J, Pastorino S, Renganathan H, Matter ML, Ramos JW (2007) ERK MAP kinase is targeted to RSK2 by the phosphoprotein PEA-15. Proc Natl Acad Sci U S A 104:19837-19842.

Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 89:73-82.

Wayman GA, Lee YS, Tokumitsu H, Silva AJ, Soderling TR (2008) Calmodulin-kinases: modulators of neuronal development and plasticity. Neuron 59:914-931.

Wayman GA, Kaech S, Grant WF, Davare M, Impey S, Tokumitsu H, Nozaki N, Banker G, Soderling TR (2004) Regulation of axonal extension and growth cone motility by calmodulin-dependent protein kinase I. J Neurosci 24:3786-3794.

West AE, Chen WG, Dalva MB, Dolmetsch RE, Kornhauser JM, Shaywitz AJ, Takasu MA, Tao X, Greenberg ME (2001) Calcium regulation of neuronal gene expression. Proc Natl Acad Sci U S A 98:11024-11031.

Xing J, Ginty DD, Greenberg ME (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 273:959-963.

Zheng F, Wang H (2009) NMDA-mediated and self-induced bdnf exon IV transcriptions are differentially regulated in cultured cortical neurons. Neurochem Int 54:385-392.

Zhou X, Moon C, Zheng F, Luo Y, Soellner D, Nunez JL, Wang H (2009) N-methyl-D-aspartate-stimulated ERK1/2 signaling and the transcriptional up-regulation of plasticity-related genes are developmentally regulated following in vitro neuronal maturation. J Neurosci Res 87:2632-2644.

CHAPTER 3

REGULATION OF SELF-INDUCED BRAIN-DERIVED NEUROTROPHIC FACTOR EXON IV TRANSCRIPTION IN CORTICAL NEURONS: THE ROLE OF MAPK, CALCIUM AND NMDA RECEPTOR

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Neuronal activity-dependent transcription has been implicated in neuroplasticity, learning and memory. As an activity-dependent immediate early gene, brain-derived neurotrophic factor (BDNF) is involved in development, survival and plasticity in central nervous system. Here, we studied BDNF-induced transcription of exon IV-containing BDNF mRNA (BDNF IV) in cortical neurons. We found MAPK activity is required for BDNF self-induction, whereas PI3K or PLCγ activity is not involved. Moreover, extra- and intra-cellular calcium level and the activity of CaMKI, CaMKII and CaMKIV are necessary for self-induced BDNF IV. Moreover, MAPK activity is not affected by the calcium level. Furthermore, we identified NMDA receptor (NMDAR) as a mediator in BDNF self-induction. We found that both NR2A- and NR2B-containing NMDAR mediate BDNF IV transcription. We also showed that both synaptic and extrasynaptic NMDAR are required for BDNF self-induction. These findings suggest a novel model of BDNF self-induction parallel regulated by MAPK and calcium-CaMK, and identify the involvement of NMDAR in the regulation.

Activity-dependent transcription of brain-derived neurotrophic factor (BDNF) is involved in neural development, survival and neuroplasticity (Poo, 2001; Reichardt, 2006; Lu et al., 2008). In the adult brain, BDNF transcription is rapidly induced by neuronal stimulation, including high frequent stimulation (HFS)

(Patterson et al., 1992), seizure (Zafra et al., 1990), membrane depolarization (Tao et al., 1998), and activation of NMDA receptor (Tabuchi et al., 2000). BDNF mRNA is also elevated *in vivo* by experience from environmental enrichment (Young et al., 1999), voluntary exercise (Neeper et al., 1995), and fear-associated learning (Hall et al., 2000). The expression of BDNF is required for synaptic plasticity, learning and memory. For example, application of BDNF antibodies or BDNF antisense oligonucleotides impairs hippocampal long-term potentiation (LTP, a cellular model for synaptic plasticity) and memory formation (Figurov et al., 1996; Ma et al., 1998; Mu et al., 1999). Moreover, LTP is disrupted in homozygous BDNF knockout mice and this deficit can be rescued by exogenous application of recombinant BDNF (Patterson et al., 1996).

BDNF can further evoke the expression of several neuroplasticity-related genes, including Arc, c-fos and BDNF itself (Finkbeiner et al., 1997; Yashiro and Philpot, 2008; Zheng et al., 2009; Zheng and Wang, 2009). The self-induced BDNF transcription may contribute to the maintenance of activity-enhanced BDNF level, which is required in the late phase of synaptic plasticity (e.g. L-LTP or long term memory) (Poo, 2001; Pang et al., 2004; Bekinschtein et al., 2007).

The BDNF gene consists of at least eight 5' noncoding exons and one 3' coding exon. Each 5' exon is spliced to the 3' common exon to form a BDNF mRNA form (Aid et al., 2007). Among these BDNF mRNAs, exon IV-containing BDNF mRNA (BDNF IV) is responsive to neuronal activity in cortex (Tao et al.,

1998). Calcium-induced BDNF IV transcription is regulated by several protein kinases, including MAPK/ERK (mitogen-activated protein kinase / extracellular signal‐ regulated kinase), PI3K (phosphatidylinositol 3‐kinase), PKA (protein kinase A) and CaMKs (Ca2+/calmodulin- dependent kinase) (see Chapter 2). However, the regulation of self-induced BDNF transcription is largely unknown.

In this study, we used primary cultured cortical neurons to investigate the regulation on self-induced BDNF IV expression. When bound by BDNF, TrkB (receptor tyrosine kinase B) is activated by self-dimerization and transphosphorylation, triggering three downstream cascades: the Ras-MAPK/ERK pathway, the PI3K-Akt pathway and the PLCγ (phospholipase Cγ)-IP3 (inositol ‐ 1,4,5 ‐ trisphosphate)–calcium pathway (Reichardt, 2006; Minichiello, 2009). Moreover, BDNF may regulate synaptic plasticity by modulating glutamate receptors, including promoting glutamate release, and regulating expression and trafficking of AMPA and NMDA receptors (Kafitz et al., 1999; Poo, 2001; Blum et al., 2002; Tyler et al., 2002; Carvalho et al., 2008). However, the regulation of self-induced BDNF IV transcription is still largely unknown. We found that MAPK and calcium-CaMKs parallel regulate BDNF self-induction. Furthermore, we identified the specific regulatory role of NMDA receptor on self-induced BDNF IV. Both NR2A- and NR2B-containing NMDAR, both synaptic and extra-synaptic NMDAR are required for BDNF self-induction in mature neurons. We also found a synergistic effect on BDNF IV transcription by

co-activation of TrkB and NMDAR.

EXPERIMENTAL PROCEDURES

Plasmids, reagents and antibodies- PIV-Luc were generated by inserting the 911 bp of 5' flank region of BDNF exon IV and the whole exon IV into pGL3-Basic Firefly luciferase vector (Promega). Plasmid expressing dominant negative calmodulin (dnCaM) was a gift from Dr. Philippe Marin (University of Montpellier, Germany). The dnCaMKI, CaMKIIN (a specific CaMKII inhibitory peptide) and dnCaMKIV (nuclear localized form) expression constructs were generously provided by Dr. Gary Wayman (Washington State University). The Renilla Luciferase plasmid, TK-pRL, was from Dr. Richard Miksicek (Michigan State University). The plasmids expressing shRNA for NR2A and NR2B were from Dr. Oliver Schluter (European Neuroscience Institute, Germany). LY294002 (a PI3K inhibitor), U0126 (a MEK inhibitor), U73122 (a PLC inhibitor) and recombinant BDNF were purchased from Calbiochem (Gibbstown, NJ). All chemical reagents (KN93, APV, BAPTA-AM, and W13) were purchased from Sigma (St. Louis, MO). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). The primary antibodies (pERK, 1:1000; total ERK 1:1000) were from Cell Signaling and the secondary antibodies (HRP-labeled goat anti-rabbit IgG, goat anti-mouse IgG, and 1:5000) were bought from Thermo Scientific.

Primary cell culture- As described previously (Zheng et al., 2008), post natal day 0 rats were sacrificed and the frontal part of the cortex were dissected. The tissue

was digested in digestion buffer [10 units/ml papain (Worthington), 5 mg/ml cystine (Sigma), and 100 units/ml DNase I (Roche) in Hibernate A (BrainBits LLC)] and planted into 12-well or 24-well plates which were pre-coated by poly-D-lysine (50 μg/ml, Sigma) at a concentration of 0.6-0.8million/ml. The cells were maintained in Neurobasal A medium supplemented by B27 supplement, 0.5 mM glutamine and penicillin/ streptomycin (Invitrogen).

Semi-quantitative PCR and real-time PCR- Cells were treated on 7 or 14 days in vitro (DIV) as indicated. Total RNA was extracted using Trizol protocol (Invitrogen) following manufacturer's instruction. One μg RNA was then reverse-transcribed (RT) into cDNA using SuperScript III RT kit (Invitrogen). Semi-quantitative PCR was performed with Go-Taq system (Promega) on an Eppendorf thermocycler. The PCR products were separated on 1.2% agarose gels and their density was quantified by the Scion Image software (Scion Corp. Frederick, Maryland). BDNF IV was amplified by a forward primer 5'-CTCCGCCATGCAATTTCCAC-3' and a reverse primer 5'-GCCTTCATGCA ACCGAAGTA-3'. GAPDH was used as a reference gene, whose PCR primers are: 5'-TCCATGACAACTTTGGCATTGTGG-3' (forward) and 5'-GTTGCTG TTGAAGTCGCAGGAGAC-3' (reverse). The amplification cycles are 26 for BDNF IV and 20 for GAPDH, while the annealing temperature is 55℃ for all the genes. Real-time PCR for the genes used the same annealing temperature and was performed using iQ SYBR Green system (Bio-rad). The result of real-time PCR

was analyzed with the $2^{-\Delta\Delta Ct}$ method. All the genes were normalized to GAPDH in either semi-quantitative or real-time PCR.

Western blotting- The cultured neurons were collected and lysed in SDS buffer (10 mM Tris–HCl buffer, 10% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol, pH 6.8) on 7 or 14 DIV as mentioned. Proteins were separated on SDS-PAGE (Invitrogen) and transferred onto nitrocellulose membranes. The membrane was incubated with primary antibody overnight at 4 ℃ and then probed with the HRP-labeled secondary antibody. The ECL (chemiluminescence) kit (Thermo) is utilized to analyze the density of the protein.

Luciferase assay- The cultured neurons were tranfected with luciferase reporter plasmids and, if indicated, plasmids expressing shRNA of NR2A or NR2B. After 48 hours, the cells were pretreated with inhibitors or vehicles for 30 min, incubated with 5ng/ml BDNF for 6 hours and submitted to Duo-Glo Luciferase assay (Promega) according to the manufacturer's instruction. The luciferase activity was measured by a Microplate Luminometer (Veritas) and analyzed after normalizing firefly luciferase to Renilla luciferase.

Statistics- All results were shown as average ± SEM. Comparison among groups was statistically analyzed by one-way ANOVA and post hoc analysis ($p<0.05$ is considered to be significant).

RESULTS

Self-induced BDNF IV transcription requires MAPK activity. Because

BDNF-activated TrkB receptors may induce transcription through three pathways, including Ras-MAPK pathway, PI3K-Akt pathway and PLCγ pathway, we sought to investigate which pathway is involved in BDNF-induced BDNF IV transcription. Cortical neurons were pre-treated with MEK inhibitor U0126, PI3K inhibitor LY294002 or PLC inhibitor U73122 for 30 minutes followed by a one-hour treatment of 5ng/ml BDNF. The exon IV-containing BDNF mRNA (BDNF IV) and c-fos (another CRE-mediated gene) was examined. We found that the expression BDNF IV and c-fos were both upregulated, which is consistent with previous studies, and only U0126 blocked this induction. This indicates that BDNF-induced BDNFIV expression requires activation of MAPK but not PI3K or PLCγ (Fig. 3.1A). To further examine the function of ERK1/2 in BDNF transcription, we co-transfected PIV-reporter construct with dominant negative MEK (dnMEK). Consistent with the effect of pharmacological inhibition of ERK1/2, dnMEK also blocked BDNF-induced PIV activation (Fig. 3.1B).

Self-induced BDNF IV transcription requires calcium and Ca/Calmodulin-dependent kinases. Because the calcium signal is important for BDNF-induced CREB activation and CRE-mediated transcription (Finkbeiner et al., 1997; West et al., 2001), we next investigated the role of calcium in the BDNF self-induction. Before the BDNF simulation, neurons were pretreated with EGTA, which chelates the extracellular calcium, or BAPTA-AM, which is an intracellular Ca2+ chelator. It is shown that both extra- and intracellular calcium chelators

Figure 3.1

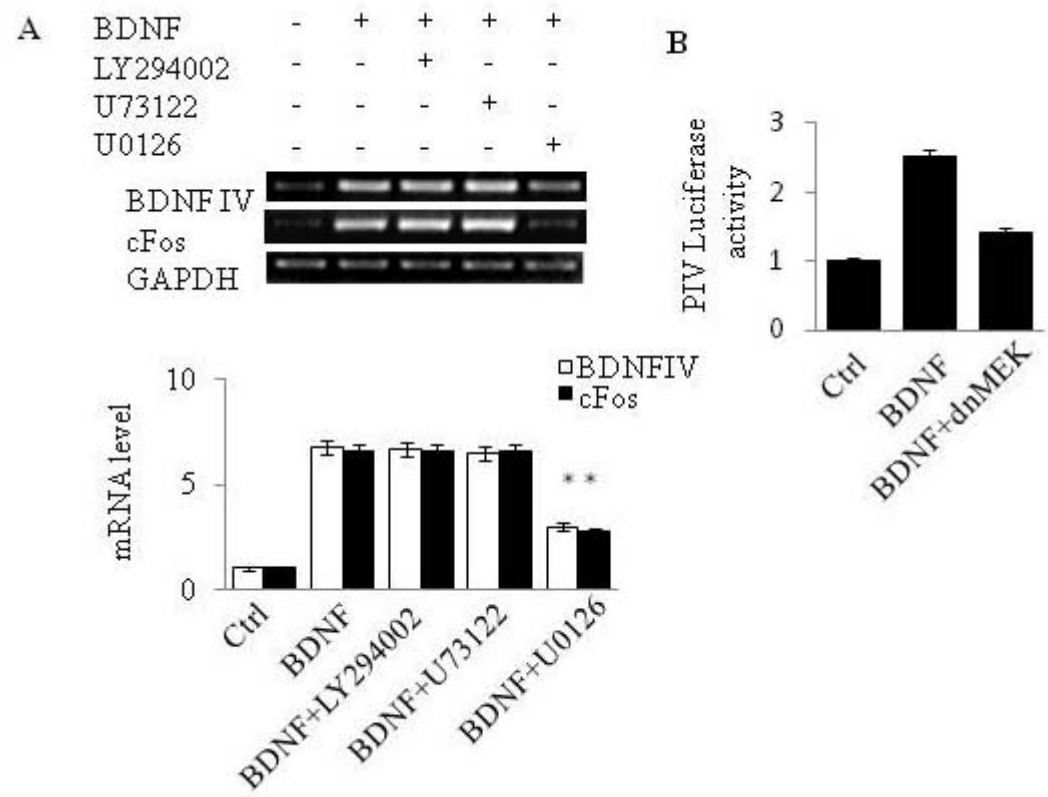

Figure 3.1 BDNF induced transcription requires MAPK activity. (A)Neurons on 14 DIV were pretreated with MEK inhibitor U0126, PI3K inhibitor LY294002 and PLC inhibitor U73122 for 30 min and followed by a one-hour BDNF (5 ng/ml) treatment. RT-PCR was performed with specific primers for BDNF IV, c-fos and GAPDH. The representative image is shown at the upper panel and the quantification after normalized to GAPDH is at bottom panel. (B) Neurons were transfected on 9 DIV with PIV-Luc, TK-pRL and dnMEK. Two days after transfection, neurons were treated with 5ng/ml BDNF for 6 hours. Firefly Luciferase activity was normalized to Renilla Luciferase activity and the effect of dnMEK was further normalized to vehicle-treated and dnMEK-transfected group. *: $p<0.05$ compared with the induction. n=3 for RT-PCR and n=4 for luciferase assay.

blocked self-induced BDNF IV transcription (Fig 3.2A). Moreover, the intracellular calcium is also necessary for BDNF-induced c-fos. However, extracellular chelator EGTA failed to block the induction of c-fos (Fig. 3.2A). These results indicate that calcium signal regulates BDNF-induced transcription in a gene-selective manner and extracellular calcium is specifically required for self-induced BDNF IV expression.

Furthermore, it has been demonstrated that calcium binds to calmodulin and activates Ca/calmodulin dependent kinases (CaMKs). Thus we asked whether calcium regulates the transcription via CaMK pathway. We blocked calmodulin with W13 and CaMKs with KN93 and KN62, which all reduced the BDNFIV transcription. Interestingly, c-fos expression was not affected by W13, KN93 or KN62 (Fig 3.2B), suggesting that calcium-calmodulin-CaMKs pathway regulates BDNF-induced BDNF IV specifically. Furthermore, dominant-negative (dn) calmodulin (dnCaM), dnCaMKI, dnCaMKIV and CaMKIIN (a plasmid that expresses a CaMKII inhibitory peptide) were co-transfected with PIV-Luc. As shown in Figure 3.2C, all the constructs suppressed the BDNF-induced PIV luciferase activity, which further confirmed that BDNFIV transcription requires calcium and CaMKs.

Activation of ERK1/2 does not depend on extra- or intracellular calcium. Because ERK belongs to Ca2+-stimulated protein kinases (Improta-Brears et al., 1999), we reasoned that EGTA or BAPTA-AM might suppress BDNF transcription through

inhibiting ERK1/2 activation. However, neither BAPTA-AM nor EGTA inhibited BDNF-induced ERK phosphorylation (Fig. 3.3A). Furthermore, although the Ca2+-mediated ERK phosphorylation (in KCl-stimulated neurons) was abolished by EGTA, BAPTA-AM did not inhibit KCl induced ERK phosphorylation, indicating that a transient influx of extracellular calcium is sufficient to activate ERK (Fig. 3.3B). Taken together, our data suggest that the calcium and ERK signaling pathways in BDNF stimulated neurons are parallel. Calcium regulation on BDNF IV and c-fos transcription is not mediated through ERK, and the activation of ERK alone is not sufficient for this BDNF-stimulated transcription.

NR2A and NR2B regulate BDNF IV transcription. It has been demonstrated that BDNF stimulation results in neurotransmitter release and phosphorylation of NMDA receptors, we sought to understand whether NMDA receptor functions in BDNF induced gene expression. We used two different mechanism-based NMDA receptor blockers APV and MK801. Both inhibitors blocked self-induced BDNF IV mRNA expression, without affecting BDNF-induced c-fos (Fig. 3.4A and B). APV also blocked PIV-mediated luciferase activity, indicating NMDA receptor is specifically required for BDNF self-induction.

Furthermore, NMDA receptor consists of two NR1 and two NR2 (2A-2D) subunits. It has been demonstrated that NR2A- and NR2B-containing NMDA receptors (the predominant NMDA receptor subtypes) develop and function distinctly in neurons. In order to dissect the roles of NR2A and NR2B in BDNF IV transcription, we used

Figure 3.2 BDNF self-induction requires calcium and CaMK activity. DIV14 neurons were pretreated with extra-cellular Ca2+ chelator EGTA or intracellular Ca2+ chelator BAPTA-AM (A), calmodulin inhibitor W13, and CaMK antagonists KN62 and KN93 (B) for 30 min and then exposed to 5ng/ml BDNF for one hour. RT-PCR was used to examine the mRNA level of BDNF IV, c-fos and GAPDH. The relative level of BDNF IV and c-fos were normalized to GAPDH level. The representative images are at left, whereas the quantifications are shown in the right panels. DIV 9 neurons were transfected with PIV-Luc, TK-pRL and plasmids expressing dnCaM, dnCaMKI, CaMKII and dnCaMKIV. On 11 DIV, the neurons were treated with 5ng/ml BDNF for 6 hours before submitted to a luciferase assay. The relative luciferase activity was shown after normalizing to Renilla Luciferase activity. The group which was transfected with dn plasmid but not treated with BDNF was used to normalize the effect of dn constructs on BDNF induction. *: $p<0.05$ compared with the induction. n=3 for RT-PCR and n=4 for luciferase assay.

Figure 3.2

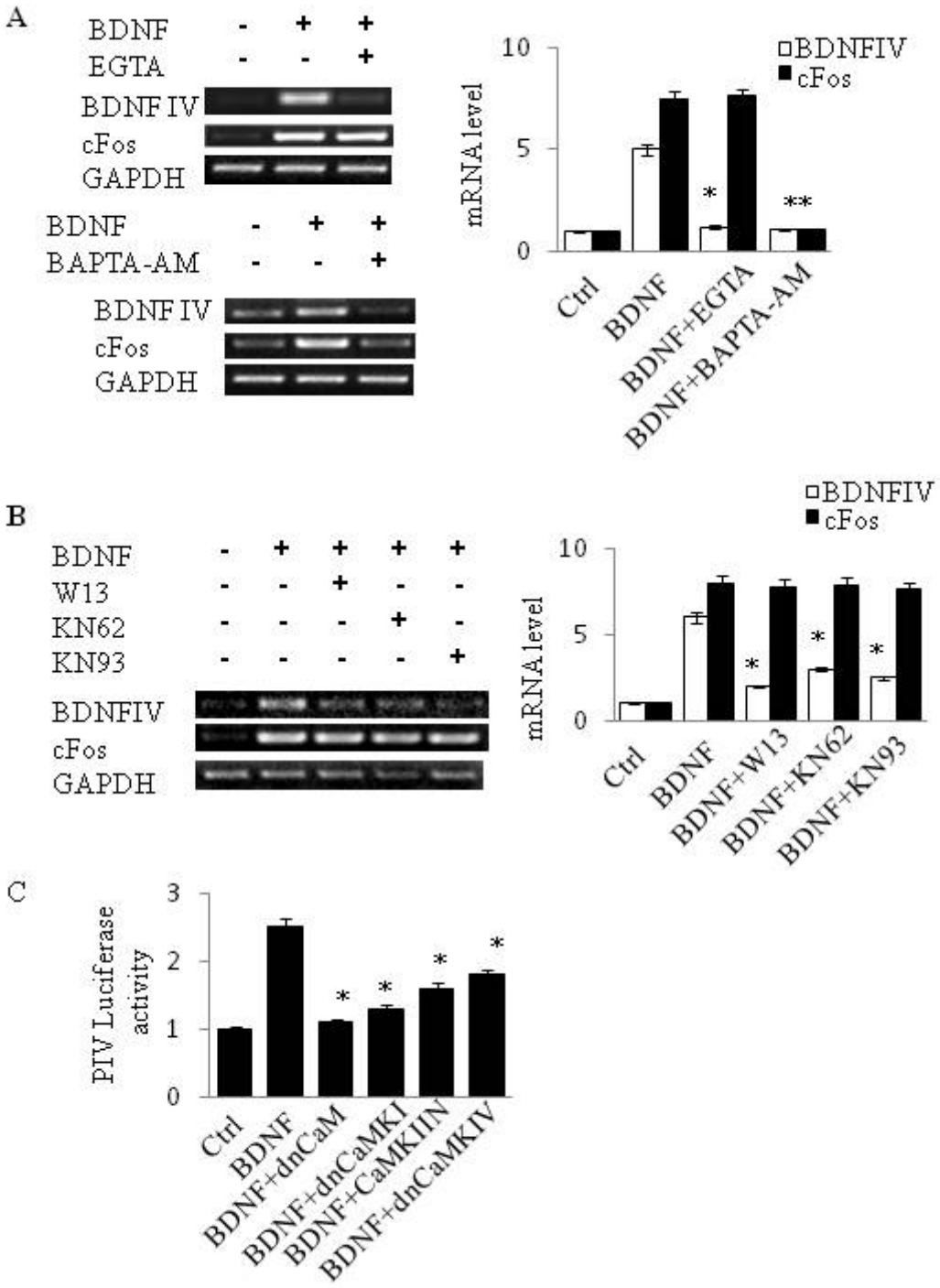
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Figure 3.3

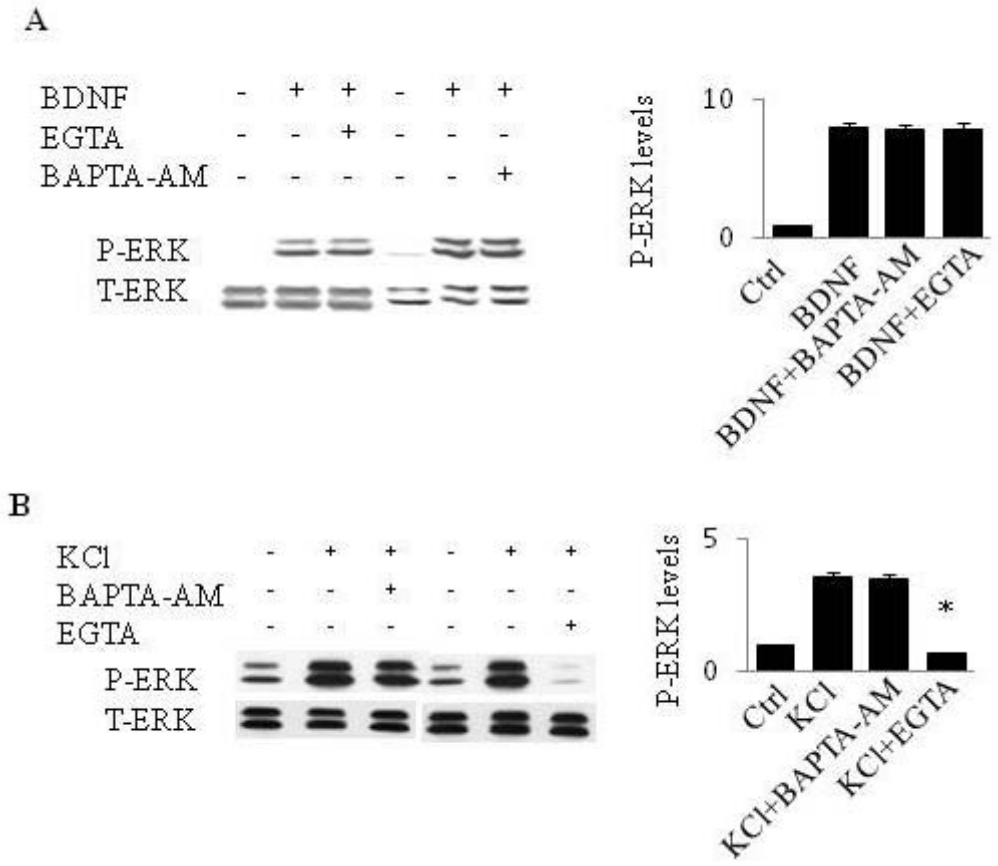
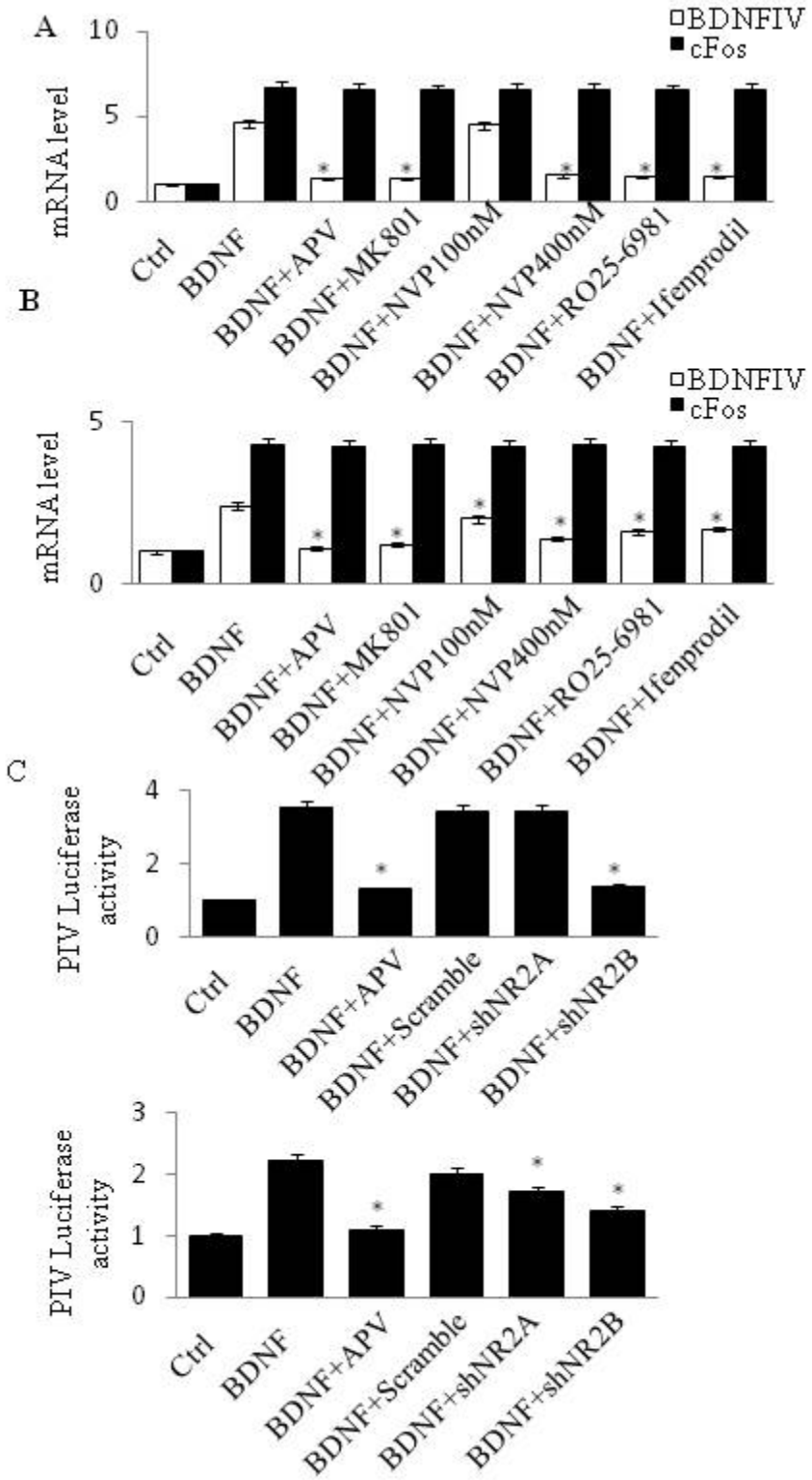


Figure 3.3 Activity-dependent ERK phosphorylation does not depend on intracellular calcium. Cortical neurons were stimulated by 50 mM KCl (B) or 5ng/ml BDNF (A). Pretreatments with EGTA (2.5 mM) or BAPTA-AM (30 μM) were applied 30 min before stimulation. Samples were collected 15 min after stimulation. The ERK phosphorylation was analyzed by Western blot. For quantification, the relative intensity of p-ERK was normalized to total ERK. Representative Western blots are shown at left and quantification at right (n=3 for each treatment group).

Figure 3.4 Both NR2A- and NR2B-containing NMDA receptor regulate BDNF self-induction. Neurons at DIV7 when only NR2B is expressed (A) or DIV14 when both NR2A and NR2B are expressed (B) were pretreated with NMDA antagonists APV or MK801, dose-dependent NR2A antagonist NVP-AAM077 (NVP) (100nM or 400nM), NR2B-preferable antagonist Ro25-6981 or Ifenprodil. Then the neurons were exposed to 5ng/ml BDNF for 1 hour. Real-time PCR was performed to examine the mRNA level of BDNF IV, c-fos and GAPDH. The relative level of BDNF IV and c-fos were analyzed with normalizing to GAPDH level. DIV 3 (C) or 9 (D) neurons were transfected with PIV-Luc, TK-pRL and plasmids expressing scrambled shRNA or shRNA specifically silencing NR2A or NR2B (shNR2A, shNR2B). On 7 (C) or 14 (D) DIV, the neurons were treated with 5ng/ml BDNF for 6 hours after pretreated with APV or vehicle. The relative luciferase activity was shown after normalizing to Renilla Luciferase activity. The group which was transfected with shRNA but not treated with BDNF was used for normalization. *: $p<0.05$ compared with the induction. n=3 for RT-PCR and n=4 for luciferase assay.

Figure 3.4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BDNF+RO25-6981
BDNF+Ifenprodil
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BDNF+shNR2A
BDNF+shNR2B

specific inhibitors for the two NMDA subunits. NVP-AAM077 selectively blocks NR2A at a dose of 100nM and blocks both NR2A and NR2B at a higher dose of 400nM. Ro25-6981 and Ifenprodil have been proved to block NR2B preferably without affecting NR2A activity. In DIV 7 neurons, NR2B is the predominant subunit for NMDA receptors and, as shown in Fig. 3.4A, 100nM NVP-AAM077 failed to block this BDNF self-induction. However, 400nM NVP-AAM077, NR2B blockers Ro25-6981 and Ifenprodil all suppressed the BDNF IV expression. On the other hand, both NR2A and NR2B are expressed in DIV 14 neurons. When pretreated with the inhibitors, BDNFIV transcription in DIV 14 neurons was significantly suppressed by NR2A inhibitor (100nM NVP-AAM077), NR2B inhibitors (Ro25-6981 and Ifenprodil), as well as antagonist for both NR2A and NR2B (400nM NVP-AAM077, APV and MK801) (Fig. 3.4B).

Moreover, we co-transfected specific shRNA to silence NR2A and NR2B with PIV-Luc in DIV 7 and 14 neurons. Similar to the effect of inhibitors, silencing NR2B suppressed PIV-driven luciferase expression on both 7 and 14 DIV, whereas shRNA for NR2A only reduced PIV-Luc on 14 DIV (Fig. 3.4C and D). The data indicate that when NR2A starts to be expressed during development, both NMDA subunits become essential for BDNF self-induction.

Synaptic NMDA receptors are necessary for BDNFIV expression. In addition to the diversity of subtypes, the distinct locations of the NMDA receptor also contributes to diverse function. For example, synaptic NMDA receptor is reported

to promote neuron survival, while extrasynaptic NMDA receptors may result in cell death. We sought to understand whether synaptic and/or extrasynaptic NMDA receptors are required for BDNF self-induction. As described previously (Hardingham et al., 2002), DIV 14 neurons, which have developed mature synapses, were treated with GABA(A) receptor antagonist bicuculline and competitive NMDA receptor antagonist MK801. During the 30-minute treatment, bicuculline-induced synaptic glutamate release region-specifically activates synaptic NMDA receptors, which were immediately blocked by MK801. After the treatment, medium containing the drugs were replaced with new medium, and these synaptic-NMDA-receptor-blocked neurons were treated with BDNF. Bicuculline/MK801-treated cells showed significantly lower induction fold of BDNF IV compared with control neurons (Fig. 3.5). However, this induction was further suppressed by application of APV, suggesting that both synaptic and extrasynaptic NMDA receptors contribute to the self-induced BDNF IV.

NMDA and BDNF synergistically stimulate BDNF IV transcription. Our data showed NMDA receptors are required for BDNF induction. On the other hand, we sought to know whether the activation of NMDA receptors and TrkB receptors have synergistic effect. We used 0.1, 0.5, 1, 5ng/ml BDNF and 5, 10, 20uM NMDA to stimulate the neurons transfected with PIV-Luc on 14 DIV. The luciferase assay showed a dose-dependent increase for PIV-mediated transcription (Fig. 3.6A and B). We selected the sub-peak doses, 2ng/ml BDNF and 10uM NMDA, to treat the

Figure 3.5

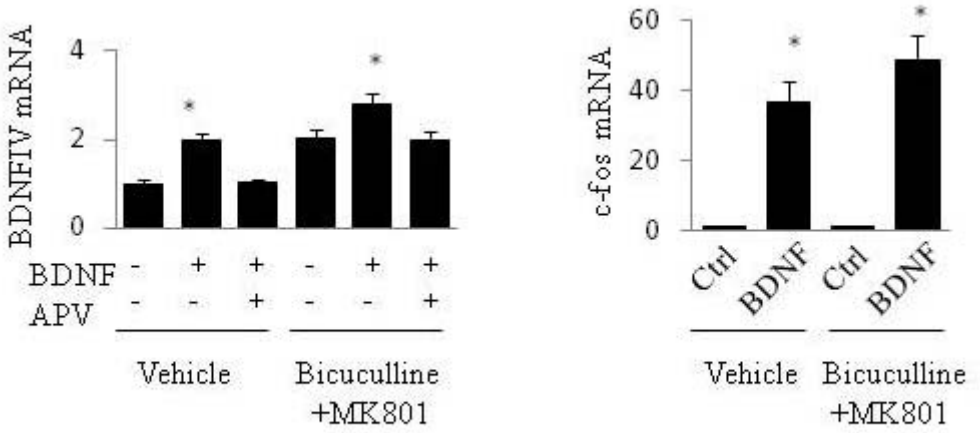

Figure 3.5 Synaptic NMDA receptor regulates BDNF self-induction. DIV 14 neurons were treated with vehicle or a mixture of GABA(A) antagonist bicuculline and competitive NMDA receptor blocker MK801 for 30 min. Then the medium containing drugs was removed and the neurons were washed and incubated in new medium for 15-min recovery. Then 5ng/ml BDNF was added to the cells and the neurons were lysed after one hour. The BDNF IV and c-fos mRNA level was measured by real-time PCR after normalizing to GAPDH level. The fold change of BDNF IV for vehicle group is significantly higher than that for Bicuculline/MK801 treated group (1.93 vs. 1.42, $p<0.05$, $n=12$). However, the Bicuculline/MK801-treated group still showed significantly increase after BDNF stimulation ($p<0.05$). There is no significant difference between the vehicle- and Bicuculline/MK801-treated groups for the fold change of BDNF-induced c-fos (38.47 vs. 40.12, $p<0.05$). *: $p<0.05$ compared to control groups.

Figure 3.6

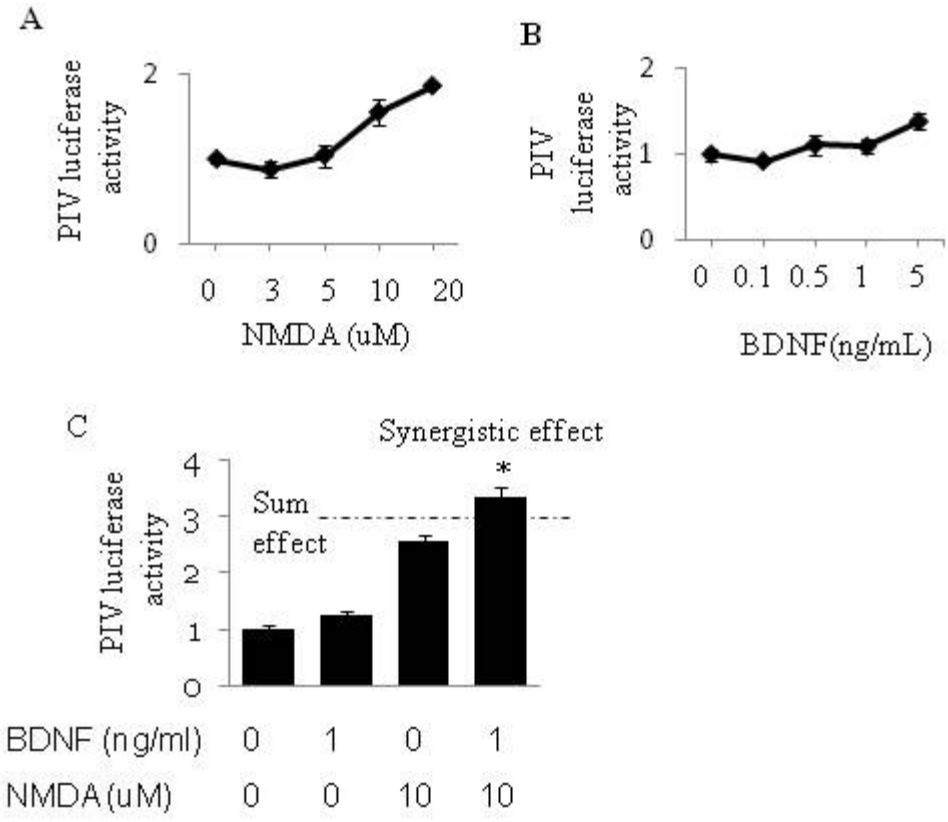

Figure 3.6 Synergistic effect of TrkB and NMDA receptor. DIV 9 neurons were transfected with PIV-Luc and pTK-RL and treated with NMDA (A, C) or BDNF (B, C) at doses as indicated 48 hours later. The PIV-luciferase activity is normalized to Renilla luciferase. The induction fold by a mixture of 1ng/ml BDNF and 10uM NMDA is significantly higher than the sum effect by adding the individual BDNF and NMDA induction amount (indicated by the dot line). *: $p<0.05$ compared to sum effect.

cells separately or together. We found a significant higher induction level of PIV-Luc by the BDNF/NMDA cocktail than the estimate sum effect (Fig. 3.6C), which indicates BDNF and NMDA synergistically increase BDNFIV transcription.

DISCUSSION

The BDNF transcription plays an important role in neuroplasticity and memory formation (Lu, 2003; Greer and Greenberg, 2008; Greenberg et al., 2009). Self-induced BDNF transcription has emerged as a mechanism to maintain BDNF concentration for the long-lasting changes at synapses. In this study, using cultured cortical neurons, we studied the regulation of the self-induced BDNF IV transcription, which is regulated by MAPK activity and calcium-CaMKs in parallel. BDNF self-induction is also mediated by NMDAR activity. To be specific, both NR2A and NR2B, both synaptic and extrasynaptic NMDAR are required for BDNF self-induction.

The Ras-MAPK pathway has been indicated to regulate CRE-mediated transcription by phosphorylating Ser133 at CREB, and the phosphorylation of CREB is critical for activity-dependent BDNF transcription (Finkbeiner et al., 1997; Impey et al., 1998; Tao et al., 1998; Wu et al., 2001; Hong et al., 2008). Similarly, we found that MAPK activity is required for BDNF self-induction. Although some reports showed that PI3K activity is required for MAPK activation in memory retrieval and LTP induction (Opazo et al., 2003; Chen et al., 2005) and the disrupted binding of PLCγ to TrkB attenuates CREB activation and LTP induction

(Minichiello et al., 2002), under our conditions, the PI3K and PLC activity is not required for BDNF self-induction. The cross-talk between the PI3K, PLCγ and MAPK pathway is stimulation-dependent.

Calcium signal and CaMK activity play important roles in BDNF transcription. For example, CaMKIV is another CREB activator, regulating CRE-mediated transcription (Ho et al., 2000; Impey et al., 2002). Moreover, CaMKI activity is required for MAPK activation in NMDA/KCl stimulated cells (Wayman et al., 2004; Schmitt et al., 2005). CaMKII is also capable of phosphorylating CREB (Finkbeiner et al., 1997; Wayman et al., 2008). In this study, we confirmed the function of calcium and CaMK activity in BDNF self-induction. However, we found that the CaMKs inhibitors failed to block CRE-mediated c-fos transcription, which seems to indicate that CaMKs regulate BDNF self-induction through a CREB-independent manner. However, it is notable that the sequence of CRE in promoter IV is slightly different from that in c-fos promoter (TCACGTCA vs. TGACGTTT) (Sheng et al., 1990; Tao et al., 1998). There is possibility that the binding affinity of CREB to CRE in promoter IV is different from that in c-fos promoter.

NMDAR, as an important glutamate-gated ion channel, is also implicated in BDNF-mediated plasticity. On one hand, BDNF modulates NMDAR directly or indirectly. For example, BDNF enhances glutamate release (Sala et al., 1998), elevates expression of NR1, NR2A and NR2B (Caldeira et al., 2007), and

phosphorylates NMDAR to increase the its open possibility (Levine et al., 1998). On the other hand, NR2B is required for BDNF-enhanced neurotransmission (Crozier et al., 1999). As well, the NMDAR activity regulates BDNF-induced Arc transcription and dendritic development (Rao et al., 2006; Finsterwald et al., 2010). Here we showed that NMDA also regulates BDNF self-induction, which suggests a regulation layer of BDNF signal by extracellular glutamate and calcium.

Self-induced BDNF transcription forms a positive feedback loop to continuously produce BDNF. Thus, a shut-off mechanism must exist to prevent the endogenous BDNF level from unstoppable increase. Indeed, after 24-hour incubation of BDNF, the mRNA level of BDNF IV returns to the basal level (Yasuda et al., 2007). Interestingly, 24-hour treatment of BDNF also results in the reduced size of dendritic spines in cultured hippocampal neurons (Zheng et al., unpublished data), which may suggest that long-time treatment by BDNF leads to smaller size of synapses, lower NMDA receptor expression and attenuated NMDA signals. Thus, the lack of NMDA activity terminates the self-inducted continuous BDNF expression and resets the balance of BDNF synthesis.

NMDA subunits have a variety of gating properties and physiological functions. For instance, NR2A promotes but NR2B inhibits GluR1 membrane insertion because NR2B associated with syn-GAP (synaptic Ras GTPase activating protein) blocks the GluR1 insertion-required Ras-ERK pathway (Kim et al., 2005).

Moreover, in hippocamal neurons, NR2A accounts for activity-induced BDNF transcription whereas NR2B is required for elevation of pERK (Chen et al., 2007). Our study showed that both NR2A and NR2B contribute in BDNF-induced transcription. Additionally, location of NMDA receptor is also important for its function. Synaptic NMDA receptor-mediated signal transduction activates CREB, whereas extrasynaptic NMDA receptor may oppose this function by triggering CREB shut-off (Hardingham et al., 2002). After blocking of synaptic NMDA receptors, we indeed detected a significantly suppression on BDNF self-induction. However, this induction can be further suppressed by APV. Thus, we believe that both synaptic and extra-synaptic are involved in this regulation. The discrepancy of our results with previous studies may be attributed to the different stimulations (NMDA vs. BDNF).

How does the NMDA receptor specifically regulate BDNF self-induction? Because we cannot detect any calcium influx in BDNF-treated neurons, it is highly possible that the clustering of NMDAR to TrkB receptor may affect its intracellular signal transduction. It has been shown that TrkB receptor is physically interacted with NMDA C-terminal through PSD95 (Ji et al., 2005). Activated TrkB receptor also phosphorylates NR1 and NR2B subunits (Lin et al., 1998). Consistent with this view, BDNF and NMDA synergistically increase BDNF IV transcription in our study. However, the downstream regulator of NMDAR needs to be determined. Besides CRE, several other *cis*-elements have been found in promoter IV,

including PasRE (Npas4 binding element), CaRE (calcium responsive element), USFBE (upstream stimulatory factor binding element), EBox, NFκB-RE (Nuclear factor kappa B binding element) and NFAT-RE (nuclear factor of activated T-cells binding element) (Lipsky et al., 2001; Tao et al., 2002; Chen et al., 2003; Jiang et al., 2008; Lin et al., 2008; Vashishta et al., 2009). Truncation and mutagenesis studies on BDNF promoter IV have indicated that PasRE, USFBE, EBox, and NFκB-RE are required for NMDA-induced promoter IV activity, and the activity of CaRE and NFAT-RE is increased by activation of NMDAR. It would be interesting to identify which element in promoter IV is regulated by NMDAR in BDNF self-induction.

In summary, we reported the novel regulatory features of BDNF self-induction and highlighted the role of calcium, MAPK and NMDA receptor. We also investigated the subtypes and locations of NMDA receptor in the regulation, which provides new insights on coordination of NMDA receptors and TrkB receptors in gene transcription.

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REFERENCES

Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. J Neurosci Res 85:525-535.

Bekinschtein P, Cammarota M, Igaz LM, Bevilaqua LR, Izquierdo I, Medina JH (2007) Persistence of long-term memory storage requires a late protein synthesis- and BDNF- dependent phase in the hippocampus. Neuron 53:261-277.

Blum R, Kafitz KW, Konnerth A (2002) Neurotrophin-evoked depolarization requires the sodium channel Na(V)1.9. Nature 419:687-693.

Caldeira MV, Melo CV, Pereira DB, Carvalho RF, Carvalho AL, Duarte CB (2007) BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons. Mol Cell Neurosci 35:208-219.

Carvalho AL, Caldeira MV, Santos SD, Duarte CB (2008) Role of the brain-derived neurotrophic factor at glutamatergic synapses. Br J Pharmacol 153 Suppl 1:S310-324.

Chen Q, He S, Hu XL, Yu J, Zhou Y, Zheng J, Zhang S, Zhang C, Duan WH, Xiong ZQ (2007) Differential roles of NR2A- and NR2B-containing NMDA receptors in activity-dependent brain-derived neurotrophic factor gene regulation and limbic epileptogenesis. J Neurosci 27:542-552.

Chen WG, West AE, Tao X, Corfas G, Szentirmay MN, Sawadogo M, Vinson C, Greenberg ME (2003) Upstream stimulatory factors are mediators of Ca2+-responsive transcription in neurons. J Neurosci 23:2572-2581.

Chen X, Garelick MG, Wang H, Lil V, Athos J, Storm DR (2005) PI3 kinase signaling is required for retrieval and extinction of contextual memory. Nat Neurosci 8:925-931.

Crozier RA, Black IB, Plummer MR (1999) Blockade of NR2B-containing NMDA receptors prevents BDNF enhancement of glutamatergic transmission in hippocampal neurons. Learn Mem 6:257-266.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381:706-709.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME

(1997) CREB: a major mediator of neuronal neurotrophin responses. Neuron 19:1031-1047.

Finsterwald C, Fiumelli H, Cardinaux JR, Martin JL (2010) Regulation of dendritic development by BDNF requires activation of CRTC1 by glutamate. J Biol Chem 285:28587-28595.

Greenberg ME, Xu B, Lu B, Hempstead BL (2009) New insights in the biology of BDNF synthesis and release: implications in CNS function. J Neurosci 29:12764-12767.

Greer PL, Greenberg ME (2008) From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. Neuron 59:846-860.

Hall J, Thomas KL, Everitt BJ (2000) Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. Nat Neurosci 3:533-535.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405-414.

Ho N, Liauw JA, Blaeser F, Wei F, Hanissian S, Muglia LM, Wozniak DF, Nardi A, Arvin KL, Holtzman DM, Linden DJ, Zhuo M, Muglia LJ, Chatila TA (2000) Impaired synaptic plasticity and cAMP response element-binding protein activation in Ca2+/calmodulin-dependent protein kinase type IV/Gr-deficient mice. J Neurosci 20:6459-6472.

Hong EJ, McCord AE, Greenberg ME (2008) A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. Neuron 60:610-624.

Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR (1998) Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. Neuron 21:869-883.

Impey S, Fong AL, Wang Y, Cardinaux JR, Fass DM, Obrietan K, Wayman GA, Storm DR, Soderling TR, Goodman RH (2002) Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV.

Neuron 34:235-244.

Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell DP (1999) Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc Natl Acad Sci U S A 96:4686-4691.

Ji Y, Pang PT, Feng L, Lu B (2005) Cyclic AMP controls BDNF-induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons. Nat Neurosci 8:164-172.

Jiang X, Tian F, Du Y, Copeland NG, Jenkins NA, Tessarollo L, Wu X, Pan H, Hu XZ, Xu K, Kenney H, Egan SE, Turley H, Harris AL, Marini AM, Lipsky RH (2008) BHLHB2 controls Bdnf promoter 4 activity and neuronal excitability. J Neurosci 28:1118-1130.

Kafitz KW, Rose CR, Thoenen H, Konnerth A (1999) Neurotrophin-evoked rapid excitation through TrkB receptors. Nature 401:918-921.

Kim MJ, Dunah AW, Wang YT, Sheng M (2005) Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46:745-760.

Levine ES, Crozier RA, Black IB, Plummer MR (1998) Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc Natl Acad Sci U S A 95:10235-10239.

Lin SY, Wu K, Levine ES, Mount HT, Suen PC, Black IB (1998) BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. Brain Res Mol Brain Res 55:20-27.

Lin Y, Bloodgood BL, Hauser JL, Lapan AD, Koon AC, Kim TK, Hu LS, Malik AN, Greenberg ME (2008) Activity-dependent regulation of inhibitory synapse development by Npas4. Nature 455:1198-1204.

Lipsky RH, Xu K, Zhu D, Kelly C, Terhakopian A, Novelli A, Marini AM (2001) Nuclear factor kappaB is a critical determinant in N-methyl-D-aspartate receptor-mediated neuroprotection. J Neurochem 78:254-264.

Lu B (2003) BDNF and activity-dependent synaptic modulation. Learn Mem 10:86-98.

Lu Y, Christian K, Lu B (2008) BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? Neurobiol Learn Mem 89:312-323.

Ma YL, Wang HL, Wu HC, Wei CL, Lee EH (1998) Brain-derived neurotrophic factor antisense oligonucleotide impairs memory retention and inhibits long-term potentiation in rats. Neuroscience 82:957-967.

Minichiello L (2009) TrkB signalling pathways in LTP and learning. Nat Rev Neurosci 10:850-860.

Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, Korte M (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36:121-137.

Mu JS, Li WP, Yao ZB, Zhou XF (1999) Deprivation of endogenous brain-derived neurotrophic factor results in impairment of spatial learning and memory in adult rats. Brain Res 835:259-265.

Neeper SA, Gomez-Pinilla F, Choi J, Cotman C (1995) Exercise and brain neurotrophins. Nature 373:109.

Opazo P, Watabe AM, Grant SG, O'Dell TJ (2003) Phosphatidylinositol 3-kinase regulates the induction of long-term potentiation through extracellular signal-related kinase-independent mechanisms. J Neurosci 23:3679-3688.

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL, Lu B (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306:487-491.

Patterson SL, Grover LM, Schwartzkroin PA, Bothwell M (1992) Neurotrophin expression in rat hippocampal slices: a stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs. Neuron 9:1081-1088.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16:1137-1145.

Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2:24-32.

Rao VR, Pintchovski SA, Chin J, Peebles CL, Mitra S, Finkbeiner S (2006) AMPA receptors regulate transcription of the plasticity-related immediate-early gene Arc. Nat Neurosci 9:887-895.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361:1545-1564.

Sala R, Viegi A, Rossi FM, Pizzorusso T, Bonanno G, Raiteri M, Maffei L (1998) Nerve growth factor and brain-derived neurotrophic factor increase neurotransmitter release in the rat visual cortex. Eur J Neurosci 10:2185-2191.

Schmitt JM, Guire ES, Saneyoshi T, Soderling TR (2005) Calmodulin-dependent kinase kinase/calmodulin kinase I activity gates extracellular-regulated kinase-dependent long-term potentiation. J Neurosci 25:1281-1290.

Sheng M, McFadden G, Greenberg ME (1990) Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron 4:571-582.

Tabuchi A, Nakaoka R, Amano K, Yukimine M, Andoh T, Kuraishi Y, Tsuda M (2000) Differential activation of brain-derived neurotrophic factor gene promoters I and III by Ca2+ signals evoked via L-type voltage-dependent and N-methyl-D-aspartate receptor Ca2+ channels. J Biol Chem 275:17269-17275.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20:709-726.

Tao X, West AE, Chen WG, Corfas G, Greenberg ME (2002) A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. Neuron 33:383-395.

Tyler WJ, Perrett SP, Pozzo-Miller LD (2002) The role of neurotrophins in neurotransmitter release. Neuroscientist 8:524-531.

Vashishta A, Habas A, Pruunsild P, Zheng JJ, Timmusk T, Hetman M (2009)

Nuclear factor of activated T-cells isoform c4 (NFATc4/NFAT3) as a mediator of antiapoptotic transcription in NMDA receptor-stimulated cortical neurons. J Neurosci 29:15331-15340.

Wayman GA, Lee YS, Tokumitsu H, Silva AJ, Soderling TR (2008) Calmodulin-kinases: modulators of neuronal development and plasticity. Neuron 59:914-931.

Wayman GA, Kaech S, Grant WF, Davare M, Impey S, Tokumitsu H, Nozaki N, Banker G, Soderling TR (2004) Regulation of axonal extension and growth cone motility by calmodulin-dependent protein kinase I. J Neurosci 24:3786-3794.

West AE, Chen WG, Dalva MB, Dolmetsch RE, Kornhauser JM, Shaywitz AJ, Takasu MA, Tao X, Greenberg ME (2001) Calcium regulation of neuronal gene expression. Proc Natl Acad Sci U S A 98:11024-11031.

Wu GY, Deisseroth K, Tsien RW (2001) Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology. Nat Neurosci 4:151-158.

Yashiro K, Philpot BD (2008) Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. Neuropharmacology 55:1081-1094.

Yasuda M, Fukuchi M, Tabuchi A, Kawahara M, Tsuneki H, Azuma Y, Chiba Y, Tsuda M (2007) Robust stimulation of TrkB induces delayed increases in BDNF and Arc mRNA expressions in cultured rat cortical neurons via distinct mechanisms. J Neurochem 103:626-636.

Young D, Lawlor PA, Leone P, Dragunow M, During MJ (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat Med 5:448-453.

Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D (1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. Embo J 9:3545-3550.

Zheng F, Wang H (2009) NMDA-mediated and self-induced bdnf exon IV transcriptions are differentially regulated in cultured cortical neurons. Neurochem Int 54:385-392.

Zheng F, Luo Y, Wang H (2009) Regulation of brain-derived neurotrophic factor-mediated transcription of the immediate early gene Arc by intracellular calcium and calmodulin. J Neurosci Res 87:380-392.

Zheng F, Soellner D, Nunez J, Wang H (2008) The basal level of intracellular calcium gates the activation of phosphoinositide 3-kinase-Akt signaling by brain-derived neurotrophic factor in cortical neurons. J Neurochem 106:1259-1274.

CHAPTER 4

CONCLUSIONS AND PROSPECTIVE

BDNF REGULATION: GENETIC AND EPIGENETIC MECHANISMS

In this dissertation, I showed how calcium-mediated BDNF IV transcription is regulated by several protein kinases, including MAPK, PI3K, PKA and CaMKs, and how self-induced BDNF is mediated by MAPK, calcium and NMDA receptors. Notably, this study not only showed a positive feedback centered by BDNF transcription, but also characterized the signaling pathways and elements in promoter IV involved in the transcription (Fig. 5.1).

Which signaling pathway is involved in BDNF transcription and is there any cross talk? This seems to be determined by the stimulation. For example, PI3K pathway is required for L-VGCC- and NMDAR-mediated BDNF IV transcription, but it has no effect on BDNF self-induction. Furthermore, although calcium influx and TrkB activation both elevates ERK phosphorylation, this phosphorylation depends on PI3K activity only in calcium-stimulated neurons but not in cells stimulated by BDNF.

In addition, several *cis*-elements have been identified in promoter IV. BDNF IV transcription is tightly regulated by the transcriptional factors via these elements. For example, CRE-mediated BDNF IV transcription depends on CREB function, including phosphorylation at Ser-133 of CREB, CREB binding to DNA, and recruitment of its coactivator (CBP/p300). Each step is regulated by different signals. First, CREB is phosphorylated by either MAPK or CaMKIV *in vitro*. However, their regulation on CREB activation *in vivo* is controversial. It is also

Figure 4.1

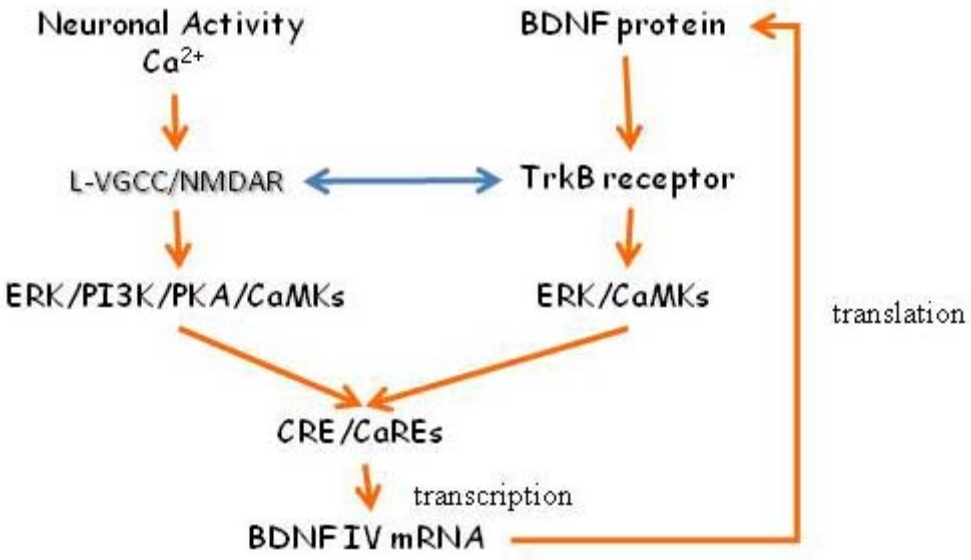

Figure 4.1 Activity-dependent BDNF generates a positive feedback. Calcium influx through L-VGCC or NMDAR mediates CRE/CaRE on promoter IV and evokes BDNF IV mRNA. This induction is regulated by ERK, PI3K, PKA and CaMKs. BDNF mRNA is translated to BDNF protein and activates TrkB signals, which also enhance BDNF IV transcription through ERK/CaMKs. NMDA receptor also functions in this TrkB signal.

unknown whether their activation on CREB is redundant or it depends on different time course(Impey and Goodman, 2001; Wu et al., 2001; Greer and Greenberg, 2008; Sakamoto et al., 2011). Second, although it was thought the binding of CREB to CRE was constitutive, a recent study identified nitric oxide as a regulator to control BDNF-enhanced CREB binding. This study suggests another layer of regulation for CRE-mediated transcription (Riccio et al., 2006). Third, the recruitment of CBP/p300 by CREB is a crucial step of CRE-mediate transcription. CBP/p300, with their intrinsic histone acetyltransferase (HAT) activity, can modify histone and chromatin structure, recruit RNA polymerase II complex and act as an adaptor for other transcriptional factor (e.g. calcium-responsive transactivator or CREST). CBP is also regulated by calcium signals. For instance, CaMKIV phosphorylates CBP at Ser-301 and enhances CBP function, whereas calcineurin-dependent CREST-CBP interaction also facilitates CRE-mediate transcription(Impey and Goodman, 2001; Qiu and Ghosh, 2008; Bedford et al., 2010).

Beside the regulation by transcriptional factors, accumulated evidence indicates that several epigenetic mechanisms are also involved in BDNF transcription, including both chromatin remodeling and DNA methylation.

Chromatin structure change results from the modification on histone residues, including acetylation, methylation and phosphorylation, by specific enzymes, such as histone acetyltransferases and histone deacetylase (HAT/HDAC) (Bird, 2007).

Interestingly, a recent paper first demonstrates the role of HDAC2 in synaptic plasticity and memory formation. HDAC2 knockout mice show facilitated memory formation, whereas HDAC2 transgenic animals expressing extra HDAC2 show a learning deficit (Guan et al., 2009). In addition, a decrease of lysine 9 dimethylation at Histone 3 is an essential step for NMDA-induced BDNF I transcription in hippocampal neurons(Tian et al., 2009).

DNA methylation is another important epigenetic mechanism for BDNF transcription and synaptic plasticity. For example, contextual learning can decrease methylation on the CpG islands (where the cytosine residues are followed by guanine residues and the methylation occurs on these cytosine residues) in promoter IV, which at least partially contribute to activity-dependent BDNF IV upregulation (Lubin et al., 2008). Cortical DNA hypermethylation, surprisingly, persists for as long as one month after learning to store remote memory (Miller et al., 2010). Additionally, DNA methyltransferase (DNMT) regulates synaptic plasticity both *in vitro* and *in vivo* (Levenson et al., 2006; Feng et al., 2010). The methylated CpGs on promoter IV selectively bind to a repressor named methyl-CpG binding protein 2 (MeCP2). Activity-dependent phosphorylation of MeCP2 results its release from promoter IV to facilitate BDNF IV expression(Chen et al., 2003). A novel identified molecule, Gadd45b, has also been implicated in regulating DNA methylation on promoter IX of BDNF gene (Ma et al., 2009).

All in all, the regulation on activity-dependent BDNF transcription is still elusive. The role of protein kinases in BDNF transcriptions shown in the dissertation may be expanded by further studies on these genetic or epigenetic modulations on BDNF expression.

BDNF TRANSCRIPTION IN LEARNING AND MEMORY: ROLES OF CALCIUM-STIMULATED ADENYLYL CYCLASES AND CAMKIV

In this dissertation, I reported the regulation of calcium-mediated and self-induced BDNF IV transcription. The experiments were performed in isolated cortical neurons. Thus, does the same regulation occur in live animals? To answer this question, we used contextual fear conditioning (CFC) system, a fear-associated learning and memory paradigm, which elevates BDNF transcription in animal brains. We examined BDNF mRNA in hippocampus, which is a critical region in brain for learning and memory, after an electric shock-conditioned training. Interestingly, we found that exon I-containing BDNF mRNA is the major form induced in hippocampus (Fig. 5.2), which is consistent with a previous study on NMDA-stimulated hippocampal cultures (Tian et al., 2009). In light of these results, I chose to study the role of calcium-stimulated adenylyl cylcases (AC) on BDNF transcription *in vivo*. It is because calcium-stimulated adenylyl cyclases couple two essential second messengers, calcium and PKA and play important roles in late phase LTP maintenance, memory formation, addiction and depression (Wong et al., 1999; Wang and Storm, 2003; Sindreu et al., 2007; DiRocco et al., 2009). In

the adenylyl cyclase family, only AC1 and AC8 are known as calcium-stimulated ACs. AC1 and AC8 double knockout mice (DKO) showed impaired ERK activation and failed to form long-term memory after CFC training (Wong et al., 1999; Sindreu et al., 2007). Since BDNF promoter contains CRE, which is regulated by ERK and PKA *in vitro*, BDNF expression may be regulated by AC1 and AC8 and the failure of BDNF induction may account for the impaired memory in DKO mice. Indeed, we found that BDNF I induction in hippocampus after contextual training was abolished in DKO mice, indicating AC1 and AC8 are required for learning-induced BDNF expression (Fig. 5.2). Following experiments should be performed to examine whether the failure of learning-induced BDNF is causal for the impaired memory in DKO mice. For example, can application of exogenous BDNF directly into hippocampi rescue the memory deficit in DKO mice?

Another interesting molecule in regulating BDNF IV transcription is CaMKIV. As introduced above and in Chapter 1, CaMKIV has been implicated in CRE-mediated transcription because it is a potential activator of CREB and its coactivator CBP. Several papers reported that calcium-mediated CREB phosphorylation is disrupted in CaMKIV knockout mice. The knockout mice also showed impaired LTP and fear memory (Ho et al., 2000; Wei et al., 2002; Wayman et al., 2008). I have shown that CaMKIV activity is required for both calcium- and BDNF-induced BDNF expression. Thus, CaMKIV may be another important BDNF regulator in learning process. We are going to use CaMKIV

Figure 4.2

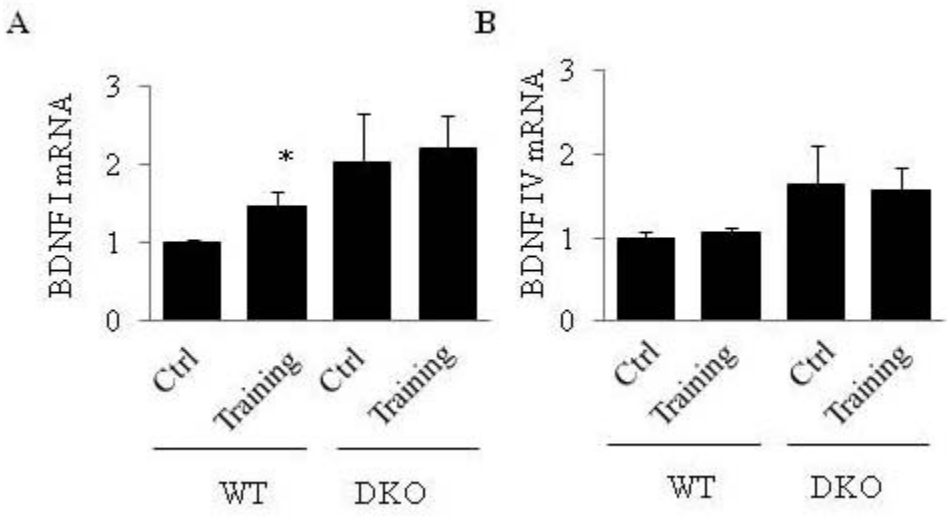

Figure 4.2 AC1 and AC8 are required for learning-induced BDNF I transcription. Wild type (WT) or DKO mice were trained by contextual fear conditioning. One hour after the training, the mice were anesthetized and both hippocampi were dissected and homogenized. BDNF I (A) and BDNF IV (B) mRNA were measured by real-time PCR and normalized to GAPDH. (A) Significant elevation of BDNF I was detected in WT group (*, $p<0.05$) but not in DKO group. (B) There is no increase of BDNF IV at both groups.

knockout mice to study CaMKIV-BDNF role in both cultured neurons and live animals.

BDNF IN DENDRITIC SPINE MODULATION: A ROLE OF NMDA RECEPTOR

BDNF regulation on synaptic plasticity is also reflected by its ability to alternate neuron synaptic structures, including axonal branching (Cohen-Cory and Fraser, 1995; Jeanneteau et al., 2010), dendritic growth (McAllister et al., 1999), synapse refinement (Cabelli et al., 1995) and dendritic spine formation (Ji et al., 2005; Ji et al., 2010). BDNF-induced gene expression may contribute to the morphological changes at synapses. However, the detailed regulation is still largely unknown. In Chapter 3, NMDA receptor was identified as an unexpected regulator for self-induced BDNF IV. Thus, it is interesting to know whether this specific regulation affects BDNF-mediated dendritic spine growth. Here, I showed that 24-hour exposure to 5ng/ml BDNF changed the dendritic spines from short, mushroom-shaped types to filopodia (long, thin, uniform-cabliber, headless protrusions from dendrites). Surprisingly, this change was blocked by NMDA antagonists APV and MK801 (Fig. 5.3). Although BDNF IV transcription may be a potential mechanism, it is unlikely to manipulate the dendritic spine structure by suppressing transcription of one single gene. Thus, future studies should be performed to understand more downstream targets of NMDA receptor in BDNF-stimulated neurons. For example, a gnome-wide screen may provide several valuable targets. Furthermore, more experiments are needed to solve the

problem: which pathway is involved in NMDA receptor-regulated dendritic spine formation?

Figure 4.3

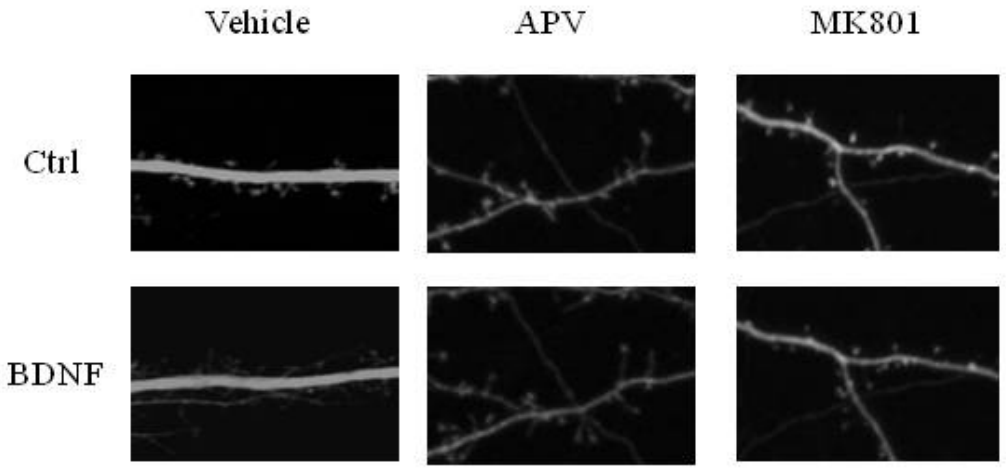

Figure 4.3 NMDA receptor is required for BDNF-induced dendritic spine change. DIV 14 neurons were transfected with pcDNA-EGFP and on 17-18 DIV neurons were confocal imaged. Then 10ng/ml BDNF were used to treat the neurons with or without APV/MK801 pretreatment. The same neurons were imaged after 24 hours. BDNF promoted dendritic spine changing was abolished by APV or MK801.

REFERENCES

Bedford DC, Kasper LH, Fukuyama T, Brindle PK (2010) Target gene context influences the transcriptional requirement for the KAT3 family of CBP and p300 histone acetyltransferases. Epigenetics 5:9-15.

Bird A (2007) Perceptions of epigenetics. Nature 447:396-398.

Cabelli RJ, Hohn A, Shatz CJ (1995) Inhibition of ocular dominance column formation by infusion of NT-4/5 or BDNF. Science 267:1662-1666.

Chen WG, Chang Q, Lin Y, Meissner A, West AE, Griffith EC, Jaenisch R, Greenberg ME (2003) Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science 302:885-889.

Cohen-Cory S, Fraser SE (1995) Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. Nature 378:192-196.

DiRocco DP, Scheiner ZS, Sindreu CB, Chan GC, Storm DR (2009) A role for calmodulin-stimulated adenylyl cyclases in cocaine sensitization. J Neurosci 29:2393-2403.

Feng J, Zhou Y, Campbell SL, Le T, Li E, Sweatt JD, Silva AJ, Fan G (2010) Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. Nat Neurosci 13:423-430.

Greer PL, Greenberg ME (2008) From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. Neuron 59:846-860.

Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, Gao J, Nieland TJ, Zhou Y, Wang X, Mazitschek R, Bradner JE, DePinho RA, Jaenisch R, Tsai LH (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459:55-60.

Ho N, Liauw JA, Blaeser F, Wei F, Hanissian S, Muglia LM, Wozniak DF, Nardi A, Arvin KL, Holtzman DM, Linden DJ, Zhuo M, Muglia LJ, Chatila TA (2000) Impaired synaptic plasticity and cAMP response element-binding protein activation in Ca2+/calmodulin-dependent protein kinase type IV/Gr-deficient mice. J Neurosci 20:6459-6472.

Impey S, Goodman RH (2001) CREB signaling--timing is everything. Sci STKE 2001:pe1.

Jeanneteau F, Deinhardt K, Miyoshi G, Bennett AM, Chao MV (2010) The MAP kinase phosphatase MKP-1 regulates BDNF-induced axon branching. Nat Neurosci 13:1373-1379.

Ji Y, Pang PT, Feng L, Lu B (2005) Cyclic AMP controls BDNF-induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons. Nat Neurosci 8:164-172.

Ji Y, Lu Y, Yang F, Shen W, Tang TT, Feng L, Duan S, Lu B (2010) Acute and gradual increases in BDNF concentration elicit distinct signaling and functions in neurons. Nat Neurosci 13:302-309.

Levenson JM, Roth TL, Lubin FD, Miller CA, Huang IC, Desai P, Malone LM, Sweatt JD (2006) Evidence that DNA (cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus. J Biol Chem 281:15763-15773.

Lubin FD, Roth TL, Sweatt JD (2008) Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. J Neurosci 28:10576-10586.

Ma DK, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N, Flavell RA, Lu B, Ming GL, Song H (2009) Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science 323:1074-1077.

McAllister AK, Katz LC, Lo DC (1999) Neurotrophins and synaptic plasticity. Annu Rev Neurosci 22:295-318.

Miller CA, Gavin CF, White JA, Parrish RR, Honasoge A, Yancey CR, Rivera IM, Rubio MD, Rumbaugh G, Sweatt JD (2010) Cortical DNA methylation maintains remote memory. Nat Neurosci 13:664-666.

Qiu Z, Ghosh A (2008) A calcium-dependent switch in a CREST-BRG1 complex regulates activity-dependent gene expression. Neuron 60:775-787.

Riccio A, Alvania RS, Lonze BE, Ramanan N, Kim T, Huang Y, Dawson TM, Snyder SH, Ginty DD (2006) A Nitric Oxide Signaling Pathway Controls CREB-Mediated Gene Expression in Neurons. Molecular Cell 21:283.

Sakamoto K, Karelina K, Obrietan K (2011) CREB: a multifaceted regulator of

neuronal plasticity and protection. J Neurochem 116:1-9.

Sindreu CB, Scheiner ZS, Storm DR (2007) Ca2+ -stimulated adenylyl cyclases regulate ERK-dependent activation of MSK1 during fear conditioning. Neuron 53:79-89.

Tian F, Hu XZ, Wu X, Jiang H, Pan H, Marini AM, Lipsky RH (2009) Dynamic chromatin remodeling events in hippocampal neurons are associated with NMDA receptor-mediated activation of Bdnf gene promoter 1. J Neurochem 109:1375-1388.

Wang H, Storm DR (2003) Calmodulin-regulated adenylyl cyclases: cross-talk and plasticity in the central nervous system. Mol Pharmacol 63:463-468.

Wayman GA, Lee YS, Tokumitsu H, Silva AJ, Soderling TR (2008) Calmodulin-kinases: modulators of neuronal development and plasticity. Neuron 59:914-931.

Wei F, Qiu CS, Liauw J, Robinson DA, Ho N, Chatila T, Zhuo M (2002) Calcium calmodulin-dependent protein kinase IV is required for fear memory. Nat Neurosci 5:573-579.

Wong ST, Athos J, Figueroa XA, Pineda VV, Schaefer ML, Chavkin CC, Muglia LJ, Storm DR (1999) Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. Neuron 23:787-798.

Wu GY, Deisseroth K, Tsien RW (2001) Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. Proc Natl Acad Sci U S A 98:2808-2813.