THE ROLES OF CYPIN IN BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)-PROMOTED NEURONAL DENDRITE BRANCHING AND IN THE REGULATION OF POSTSYNAPTIC DENSITY-95 (PSD-95) PROTEIN LEVELS

By

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and approved by

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New Brunswick, New Jersey

May, 2012

ABSTRACT OF THE DISSERTATION

THE ROLES OF CYPIN IN BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)-PROMOTED NEURONAL DENDRITE BRANCHING AND IN THE REGULATION OF POSTSYNAPTIC DENSITY-95 (PSD-95) PROTEIN LEVELS

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Dissertation Director:

Bonnie L. Firestein, Ph.D

Alterations in dendrite branching and morphology are present in many neurodegenerative diseases. These variations disrupt postsynaptic transmission and affect neuronal communication. Thus, it is important to understand the molecular mechanisms that regulate dendritogenesis and how they go awry during disease states. Previously, our laboratory showed that cypin, a guanine deaminase, increases dendrite number by regulating microtubule assembly. Cypin interacts with postsynaptic density-95 (PSD-95) protein, and reduces its synaptic clustering in cultured neurons when overexpressed. Brain-derived neurotrophic factor (BDNF) is one of most studied neurotrophins, which play a role in regulating neurite growth and branching in neurons. Here we examined the complex actions of cypin in BDNF-promoted dendrite branching and in regulating PSD-95 protein levels. First, we show the expression patterns of cypin and its new isoform in the developing rat brain. Second, we report that treatment of neurons with BDNF results

in different effects, depending on how it is administered. Global administration of BDNF increases cypin mRNA and protein levels to regulate dendrite number only in the region proximal to the cell body. BDNF increases cypin levels via cyclic adenosine 3’5’-monophosphate (cAMP) response element-binding protein (CREB)-dependent transcriptional regulation by mitogen-activated protein kinase (MAPK) signaling pathways. Furthermore, localized BDNF application to the dendritic arbor also increases dendrite branching at sites of stimulation. Interestingly, local administration of BDNF increases dendrite branching not only proximal to the soma but also at distal regions in the arbor. Taken together, these studies suggest that distinct sources of BDNF regulate dendritic arborization via different mechanisms. Finally, we investigate the role of cypin on the expression of its binding partner, PSD-95. Overexpression of cypin increases total cellular PSD-95 levels, and this effect is occluded by treatment with a proteasome inhibitor. Since PSD-95 is important for assembling signaling complexes and mediating synaptic activity, a role for cypin at synaptic sites in proteasome-dependent regulation of PSD-95 is proposed. Therefore, we suggest new roles of cypin in BDNF-promoted dendrite branching and in the alteration of PSD-95 protein levels at synaptic sites.

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Chapter 1. Introduction

Dendrite development

Neurons send and receive information through their highly specialized extensions, axons and dendrites. Axons transport signals from neurons to other neurons, while dendrites receive signals from the other neurons. Dendrites have highly branched structures that integrate various inputs from presynaptic neurons. Appropriate dendrite patterning determines how information is received and processed by a neuron. The branching pattern and the extent of dendrite branching are directly associated with the number and distribution of inputs that the neuron receives and processes. There are abnormalities in dendritic arbors in many neurodegenerative diseases. Changes in dendrite branching patterns, including retraction or loss of dendrite branching, extended dendritic trees, fragmentation of dendrites, are found in neurological disorders, such as Alzheimer's disease, schizophrenia, Down syndrome, autism spectrum disorders (ASDs), Fragile X syndrome, Rett syndrome (reviewed in (Kaufmann and Moser, 2000), depression, and anxiety (Eiland and McEwen, 2010; Soetanto et al., 2010).

Synapses

Communication between neurons occurs at specialized junctions called synapses. Appropriate formation of synapses is also important for normal brain function. Synapses consist of a presynaptic component, synaptic cleft, and a postsynaptic component. The presynaptic component is the axon terminal, the

synaptic cleft is the space between two neurons, and the postsynaptic component is usually a dendritic spine. Neurotransmitter is released into the cleft and binds to the receptors on the postsynaptic neurons to transmit signals from presynaptic to postsynaptic neurons. There are two types of synapses. Inhibitory synapses use GABA or glycine as a neurotransmitter, and excitatory synapses usually use glutamate as a neurotransmitter. Neurons, in particular excitatory neurons, have protrusions from the main dendritic shaft, called dendritic spines. Spines are structures that are specialized for neurons to receive and process signals efficiently by increasing the surface area of the synapse. Spines usually contain organelles, such as smooth endoplasmic reticulum for calcium storage and polyribosomes for local protein synthesis. Various numbers, shapes, and sizes of spines exist in neurons. The regulation of spine morphology is also very important for synaptic signaling and neuronal plasticity. Abnormal spine density and morphology cause a loss or defect in synaptic signaling (Blanpied and Ehlers, 2004; Bourne and Harris, 2008) in many neurological diseases, such as schizophrenia (Kauer and Malenka, 2007; Lau and Zukin, 2007), Fragile X syndrome (Antar et al., 2004; Dolen and Bear, 2008), and autism (Sudhof, 2008). Spines usually have dense regions adjacent to plasma membrane, called postsynaptic densities (PSDs). In order to receive and process signals properly, accurate localization of postsynaptic components is needed. The PSD has a number of signaling proteins, including receptors and effectors, and cytoskeletal elements, especially actin.

Cypin and PSD-95

Cypin (cytosolic PSD-95 interactor) was first identified as a protein involved in decreased localization of PSD-95 at the postsynaptic density by binding to the PDZ domains of PSD-95 via its carboxyl terminus (Firestein et al., 1999). Cypin is a guanine deaminase and consists of three important domains, which include a zinc-binding motif, CRMP (collapsin-response mediator protein) homology domain, and a PDZ-binding domain (Figure 1-1). Our laboratory has shown that cypin plays an important role in regulating dendrite number in rat hippocampal neurons. Overexpression of cypin results in an increase in dendrite branching, and this increase is correlated with cypin's guanine deaminase activity (Akum et al., 2004). Cypin binds directly to tubulin heterodimers and promotes microtubule assembly, suggesting that cypin regulates dendrite branching via cytoskeletal rearrangement. Our laboratory also found that RhoA controls dendrite branching by regulating cypin protein levels (Chen and Firestein, 2007). Recently, we showed that cypin regulates changes in the microtubule cytoskeleton during exposure to sublethal concentrations of NMDA and is also involved in regulating the density of spines and filopodia (Tseng and Firestein, 2011).

PSD-95 is a membrane-associated guanylate kinase (MAGUK), and it consists of three PDZ domains, Src homology 3 domain, and a guanylate kinase homology domain (Figure 1-2). PSD-95 is one of most studied postsynaptic proteins, and it serves as a scaffold that links various receptors to signaling complexes (Cho et al., 1992; Kistner et al., 1993; Kim and Sheng, 2004; Sheng and Hoogenraad, 2007). PSD-95 regulates synaptic localization of membrane proteins by inhibiting their lateral diffusion or internalization (Roche et al., 2001; Prybylowski et al., 2005; Bats et al., 2007) and the

functional properties of interacting proteins (Nehring et al., 2000; Sheng and Kim, 2002; Lin et al., 2006). It also contributes to changes in synaptic strength and plasticity (El-Husseini et al., 2000; Ehrlich and Malinow, 2004). PSD-95 undergoes dynamic changes in localization at the synapse (Okabe et al., 1999; Marrs et al., 2001; Inoue and Okabe, 2003; Gray et al., 2006). Synaptic localization of PSD-95 is regulated by neuronal activity and mechanisms, including protein palmitoylation (el-Husseini Ael and Bredt, 2002), expression (Bao et al., 2004), and degradation (Colledge et al., 2003; Bingol and Schuman, 2004). Our laboratory has shown that PSD-95 also has a non-synaptic roles in dendrite branching. Overexpression of PSD-95 reduces the number of dendrites and acts as a stop signal for dendrite branching by disrupting microtubule organization (Charych et al., 2006; Sweet et al., 2011).

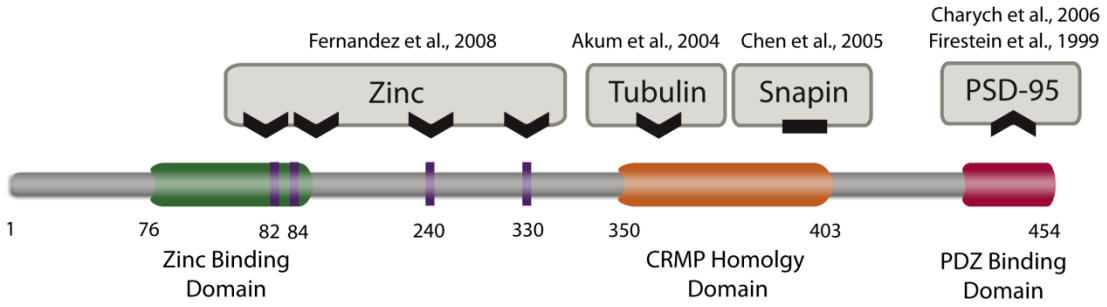

Figure 1-1. Schematic representation of the cypin protein domains and the proteins that interact with the domains. (Figure was originally made by Eric Sweet)

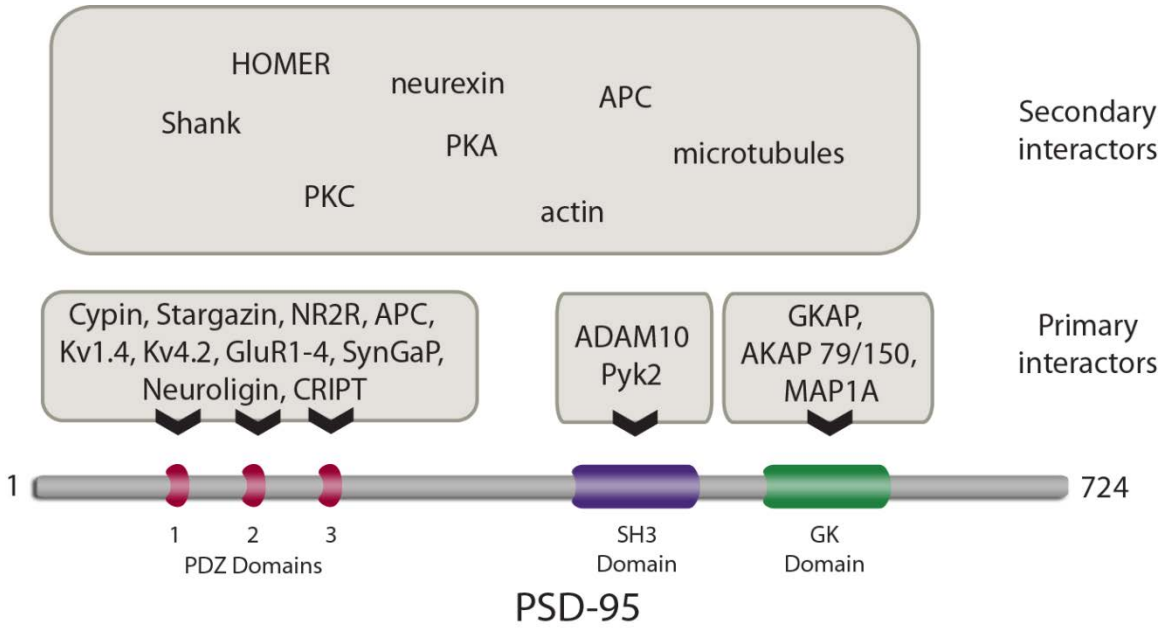

Figure 1-2. Schematic representation of the PSD-95 protein domains and the proteins that interact with the domains. (Figure was originally made by Eric Sweet)

Brain-Derived Neurotrophic Factor

Neurotrophins are proteins that are essential for regulating neurite outgrowth and branching in neurons of the central nervous system. Each neurotrophin signals through different types of receptors. Two major cell surface receptors are the Trk receptor tyrosine kinase family and the p75 neurotrophin receptor ($p75^{NTR}$). BDNF exists in two states, proteolytically processed, which is the active form and can bind TrkB receptor, or unprocessed, which allows it to bind with high affinity to $p75^{NTR}$. BDNF is one of the most critical factors regulating dendrite outgrowth and branching. Previous research has shown that application of BDNF greatly enhances dendritic growth and increases dendrite number in pyramidal neurons (McAllister et al., 1995). Overexpression of BDNF adds more branches close to the soma (Horch et al., 1999). BDNF also regulates the dynamic stability of dendrite patterning (Horch et al., 1999; Horch and Katz, 2002). Recent studies show that local BDNF release from donor cells acts directly on nearby recipient neurons to increase dendrite branching in a distance-dependent manner (Horch and Katz, 2002). These data suggest that the study of local BDNF application, in addition to global exogenous application, is also important for understanding how dendritic arborization is regulated. TrkB receptors have three different isoforms, a full length, and two truncated forms, T1 and T2. Truncated isoforms lack the cytoplasmic tyrosine kinase domains, which are responsible for autophosphorylation and clustering of the receptor when it is activated (Chao, 2003; Segal, 2003). Different isoforms show different effects on dendrite branching. When overexpressed in cultured neurons, full length TrkB activation results in increased proximal dendrite branching, whereas truncated T1 promotes net elongation of distal dendrites (Yacoubian and Lo, 2000). It is suggested that

neurons have different mechanisms to regulate their dendrite branching patterns in proximal and distal regions from the soma.

BDNF binds to the full length TrkB isoform and triggers a cascade of phosphorylation events (Figure 1-3). Phosphotyrosines bind the signaling molecules Shc and phospholipase C (PLC). These molecules activate three different signaling pathways, the Ras/MAPK cascade, PI3 kinase/Akt pathway, and IP_3-dependent calcium release. These pathways also promote transcriptional changes for various proteins that are involved in cell survival, death, cytoskeletal changes, and neurite outgrowth (Chao, 2003; Segal, 2003). Local administration of BDNF to dendrites results in mTOR-dependent activation of protein synthesis (Takei et al., 2004).

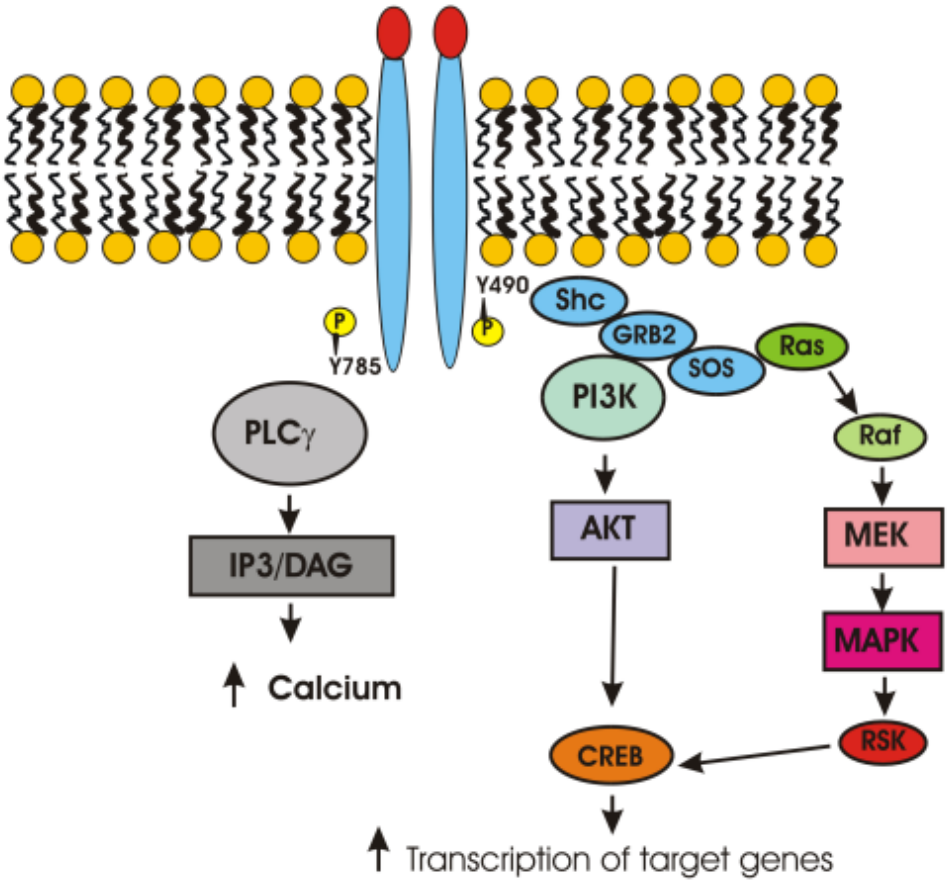

Figure 1-3. TrkB full length signaling pathway by BDNF activation. (Adapted from (Chao, 2003; Segal, 2003))

Thesis overview

Dendritic morphology is regulated by the interplay between extrinsic factors and intrinsic factors. It is of importance to understand how these factors coordinate each other to regulate dendritic arbors and synaptic functions. Cypin is one of the key regulators of dendrite branching that our laboratory has studied. This thesis will explore how dendritic morphology is regulated by the interplay between BDNF and cypin, and furthermore, how cypin regulates the content of the PSD by changing protein levels of PSD-95, its binding partner. To begin with, Chapter 2 will focus on the expression pattern of cypin and its alternative splice variant in the developing brain. In Chapter 3, we will investigate how BDNF regulates cypin expression to mediate proximal dendrite branching. Furthermore, we will show how local BDNF stimulation affects dendrite morphology in a different manner than global stimulation in Chapter 4. Finally, Chapter 5 will study the effect of cypin on PSD-95 protein levels and synaptic localization.

Chapter 2. The Expression of Cypin and A Novel Alternative Splice Variant in The Developing Brain

INTRODUCTION

Cypin (cytosolic PSD-95 interactor) was first identified as a protein involved in decreased localization of PSD-95 at the postsynaptic density by binding to the PDZ domains of PSD-95 via its carboxyl terminus (Firestein et al., 1999). Cypin is a guanine deaminase, thus catalyzing the breakdown of guanine to xanthine and ammonia (Yuan et al., 1999; Paletzki, 2002; Akum et al., 2004; Fernandez et al., 2008). The rat, human, and mouse guanine deaminase orthologues contain 454 amino acids with a nine-residue metal-binding motif and a four-residue PDZ binding motif at the C-terminus, which is responsible for interaction with PSD-95 neurons (Firestein et al., 1999). In addition, cypin's CRMP (collapsin-response mediator protein) homology domain is important for binding to tubulin heterodimers thus promoting microtubule assembly. We have shown that cypin regulates the number of primary and secondary dendrites in cultured rat hippocampal neurons and that this regulation correlates with guanine deaminase activity (Akum et al., 2004). We also found that RhoA shapes the dendritic arbor by regulating cypin protein levels (Chen and Firestein, 2007). Recently, we found that cypin regulates BDNF-promoted increase of proximal dendrites (Kwon et al., 2011), and cypin is involved in microtubule-mediated changes to dendrites in response to sublethal stimulation of the NMDA receptor (Tseng and Firestein, 2011).

Guanine deaminases is expressed as four alternative splice variants (Nedasin S, V1,V2, and V3) (Kuwahara et al., 1999), and the standard (S) form is cypin. These splice variants have different C-terminal structures. Unlike cypin, the variant forms do not have the consensus X(S/T)XV motif at their C-teminal ends, representing the PDZ-binding motif, which is crucial for binding to PSD-95 and SAP-102 for the regulation of dendrite branching and synaptogenesis. The V1 transcript does not have a 68 bp exon of the cypin transcript, which causes a frameshift in translation, resulting in the addition of 17 amino acids at the C-terminal end. The V2 transcript lacks an additional 23 bp exon of the V1 transcript, which extends of translation, generating a 505 amino acid protein. Furthermore, the V3 transcript lacks a 44 bp exon of the V2 transcript, encoding a 460 amino acid protein (Figure 2-1). The expression patterns of these 4 splice variants are slightly different. The standard isoform (cypin) is predominantly expressed in neuronal tissues, and the V1 isoform is expressed in non-neuronal tissues (Kuwahara et al., 1999). This suggests that cypin has important role in neuronal function, while the other isoforms most likely play roles in non-neuronal cells.

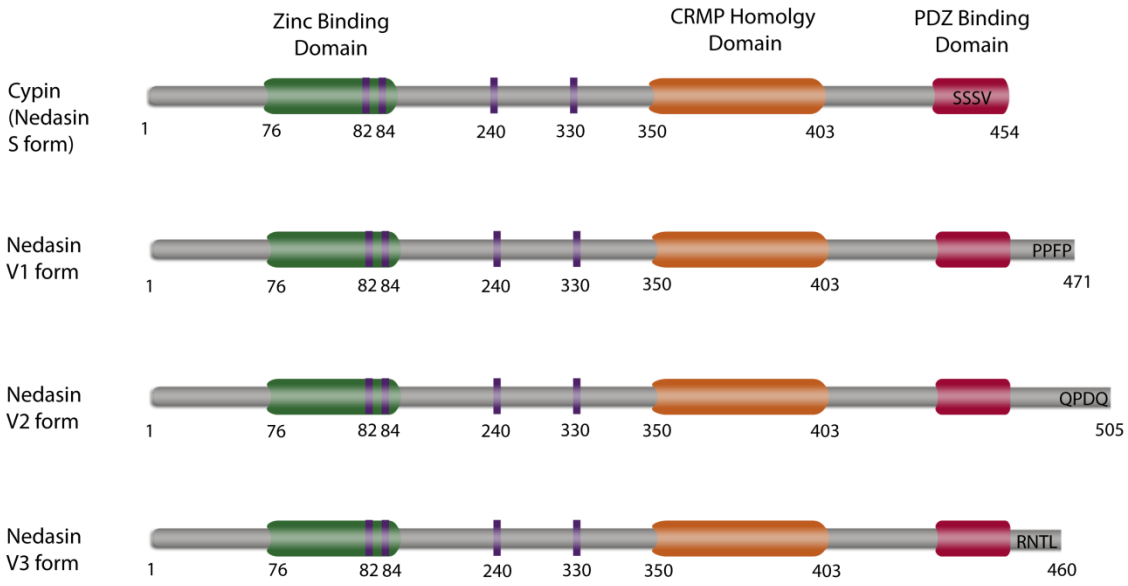

Figure 2-1. Schematic representation of the four variant forms of guanine deaminase/nedasin generated by alternative splicing. Cypin is Nedasin S. (Modified from (Kuwahara et al., 1999))

Alternatively spliced variants have been reported to have different subcellular localization, function, and binding partners. Importantly, alternative splicing of neuronal proteins is involved in regulating dendritic spine morphology (Chen et al., 2011) and membrane receptor trafficking (Hanley and Henley, 2010). Interestingly, many ion channels and membrane receptors use this type of splicing for their diversity and ligand specificity, respectively (Tsien et al., 1991; Coetzee et al., 1999; Cull-Candy et al., 2001; Grabowski and Black, 2001; Lipscombe et al., 2002; Chih et al., 2006). It is suggested that proteins produced by alternative splicing support complex functions in the brain.

Understanding expression patterns of proteins in the developing brain is important for the understanding of protein function in development. Specifically, differential activation of gene expression at certain time points and in specific tissues results in of the production of diverse cell types. Cypin is one of the key regulators of dendritic development, and its expression is tightly regulated by neurotrophin and signaling pathways. Thus, elucidating where in the developing brain cypin is expressed will give us information on what types of neurons utilize cypin as a regulator of dendrite development.

Here, we report and characterize a novel isoform of cypin, which is produced by alternative splicing. In this chapter, we also show cypin protein expression patterns in the developing rat brain.

MATERIALS AND METHODS

Animals

Sprague-Dawley (SD) rats were purchased from Taconic Farms (Taconic, PA). The date of conception was established by the presence of a vaginal plug and recorded as embryonic day zero (E0), and the day of birth was designated as P0. All animals were housed with continuous access to food and water and were maintained on a 12:12 hour light-dark cycle. All experiments were performed in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and approved by the Animal Research Committees of Rutgers University. All efforts were made to minimize animal suffering and the number of animals used in the study.

Tissue processing

Rat pups were lethally anesthetized with a mixture of ketamine/xylazine and perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in PBS. The embryos were removed by Caesarean section and fixed by immersion in the paraformaldehyde solution. Brains were dissected, postfixed overnight at 4°C, and cryoprotected in 30% sucrose solution in PBS at 4°C. Tissues were embedded in OCT compound and stored at -80°C. Brain sections were cut sagittally at a thickness of 20 µm with a cryostat and mounted on Super Frost slides (Fisher Scientific).

Antibodies

Characterization of the rabbit polyclonal antibody raised against cypin has been described previously (Firestein et al., 1999; Akum et al. 2004; Charych et al., 2006). Cyanine (Cy)

2- and Cy 3-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Immunohistochemistry

The slides with cryosections were dried overnight at 37˚C. Nonspecific binding was reduced by incubating sections in 0.1% Triton X-100, and 10% normal goat serum in PBS (pH 7.4) for 1 hour at room temperature. The sections were then incubated with primary antibodies diluted in 0.1% Triton X-100 and 5% normal goat serum in PBS (block solution) overnight at 4˚C. After three washes in PBS containing 0.1% Triton X-100, sections were incubated with secondary antibodies in block solution. For control sections, the cypin primary antibody was omitted. The immunostained tissues were visualized by immunofluorescence using an Olympus IX50 microscope with a Cooke Sensicam charge-coupled device cooled camera, fluorescence, imaging system, and Image Pro software.

Western Blotting

Brains were homogenized using a Potter-Elvehjem tissue grinder (20 strokes) in TEE (25mM Tris-HCl, 1mM EDTA, 1mM EGTA, pH 7.4), or hippocampal neurons or astrocytes were lysed in TEE by passing the extract through a 25 gauge needle twenty times. After solubilizing with Triton X-100, lysates were centrifuged at 14,000 x *g* for 10 minutes at 4°C. Proteins were resolved on a 10% SDS polyacrylamide gel and transferred to PVDF membrane. The blot was probed with the indicated antibodies.

Guanine Deaminase Assay

Heterologous COS-7 cells were plated on gridded coverslips (Bellco; Vineland, NJ). Cells were transfected with cDNA encoding GFP, GFP-Cypin or GFP-Cypin variant. Forty eight hours after transfection, cells were washed with ice-cold PBS, and images were taken on a fluorescence microscope. The protocol for guanine deaminase assay was modified from Paletzki et al. (Paletzki, 2002). Cells were then fixed for 1hr in 2.5% glutaraldehyde in 0.05M Sodium cacodylate buffer, pH 7.8. Endogenous xanthine was removed by incubating the coverslips in 2.5U/ml xanthine oxidase in bicine buffer for 15min at 37°C followed by two 5min rinses in bicine buffer. Cells were incubated in substrate solution (8mM guanine, 0.625U/ml xanthine oxidase, 9% 4-nitro blue tetrazolium chloride (NBT) in 0.1M bicine pH 7.8) for 1-3hr at 37°C. The reaction was stopped by washing in distilled water twice for 2min, dehydrated and mounted on to slides. Control reactions were performed without guanine. Brightfield images of cells in same grid box with fluorescence image were taken with.

Reverse transcription PCR

Rat brains from indicated ages were homogenized in TRIzol reagent (Invitrogen). Insoluble material was removed by centrifugation at 12,000 x *g* for 10 min at 4°C. The supernatant was incubated for 5min at room temperature, followed by the addition of chloroform. After incubation at 30°C for 3min, samples were centrifuged at 12, 000 x *g* for 15min at 4°C. RNA containing aqueous phase was subjected to precipitation with isopropyl alcohol. The pellet was washed with 75% ethanol and re-dissolved in RNase-free water.

Specific primers for full-length cypin and the alternative splice variant were designed and used for reverse transcription reaction using SuperScript reverse transcriptase (Invitrogen).

RESULTS

Identification of new cypin alternative splice variant

During the cloning of a mouse cypin construct for the targeting vector for the creation of conditional cypin knockout mice, we identified a novel alternative splice variant. The nucleotide sequence of new isoform, designated cypin short form (CypinS), was identical to the NCBI reference sequence (NM_010266) except for the first exon. As depicted in Figure 2-2, cypinS has alternative exon, and this exon exists upstream of the first exon of full-length cypin. This alternative splicing produces an mRNA which has a start codon in exon3, and this mRNA encodes a short protein isoform. Interestingly, unlike the other C-terminal splice variants, this variant has variation in the N-terminus. This variant contains PDZ binding motif (-SSSV) in C-terminus, making it capable of binding to MAGUK proteins.

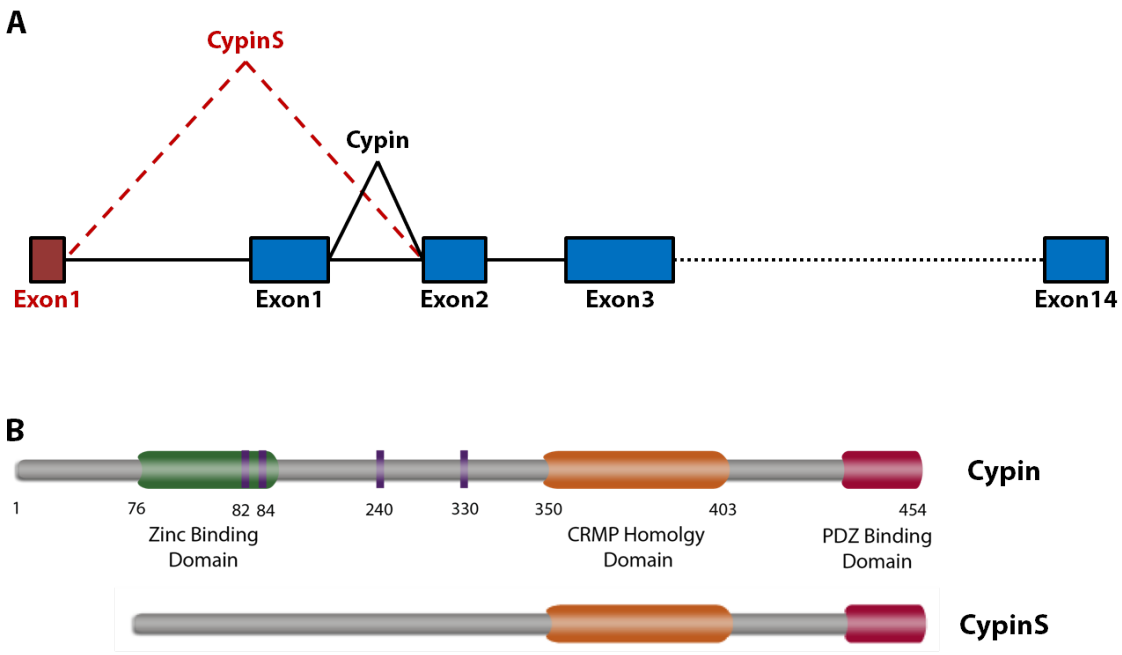

Figure 2-2. Schematic illustration of Cypin gene and proteins

A, Diagram of alternative splicing for cypin gene. Alternative exon1 indicated as red.

B, Cypin proteins. Zinc binding motif is shaded green, CRMP homology domain is shaded orange, and PDZ binding domain shaded magenta.

CypinS does not act as a guanine deaminase.

To determine whether the short form has guanine deaminase activity as does cypin, we performed a guanine deaminase assay in heterologous COS-7 cells. There was no guanine deaminase activity for CypinS when overexpressed COS-7 cells (Figure 2-3). Previous deletion mutant studies showed that zinc-binding and the CRMP-homology domains are required to maintain guanine deaminase activity in full-length cypin (Akum et al., 2004). Cypin shares a 9 residue motif (amino acid 76-84 in cypin) with the metal-binding aminohydrolase, and histidines 82, 84, together with histidines 240, 279 and aspartic acid 330 are important for zinc ion binding (Yuan et al., 1999; Fernandez et al., 2008). Although Cypin S is homologous to full length cypin at amino acid 76 and beyond, it is possible that CypinS is unable to bind to zinc ions properly because it folds differently, altering the accessibility of the zinc-binding motif.

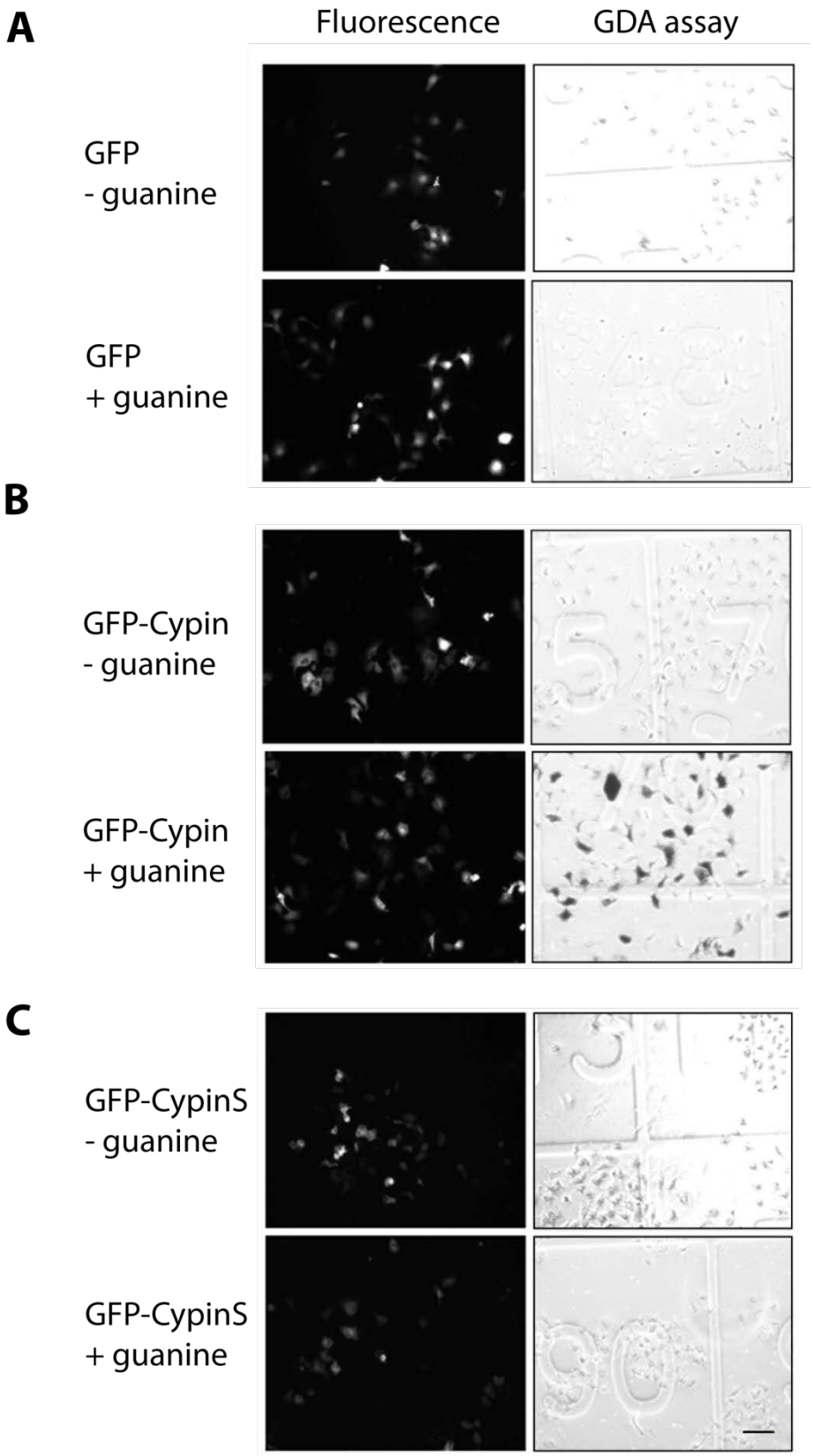
A
Fluorescence
GDA assay
GFP
- guanine
GFP
+ guanine
B
GFP-Cypin
- guanine
GFP-Cypin
+ guanine
C
GFP-CypinS
- guanine
GFP-CypinS
+ guanine

Figure 2-3. Cypin short form does not have guanine deaminase activity.

A, COS-7 cells were transfected with pEGFP vector, ***B***, with pEGFP-Cypin, ***C***, with pEGFP-CypinS and were subjected to guanine deaminase assay. –guanine is negative control. CypinS does not have guanine deaminase activity, while Cypin shows guanine deaminase activity. Scale bar, 25μm.

To determine the expression pattern for both Cypin and CypinS, we performed immunoblotting of cypin in different tissues and brain regions in adult rat. Since cypin is expressed highly in brain, the mRNA levels were also determined using RT PCR on tissue from developing brains. Figure 2-4 shows that the full-length and short forms of cypin have different tissue distributions, and the short form is highly enriched in liver, heart, and pancreas in adult. CypinS is not expressed in lung, kidney, ovary, and spleen although the full-length form is. Brain regions show similar patterns of both isoforms. Cypin is highly expressed in cortex, hippocampus, striatum, and olfactory bulb, but not in cerebellum and spinal cord. CypinS is expressed at lower levels than full-length Cypin in all brain regions, suggesting that the short form might have a tissue-specific function in tissues other than the brain.

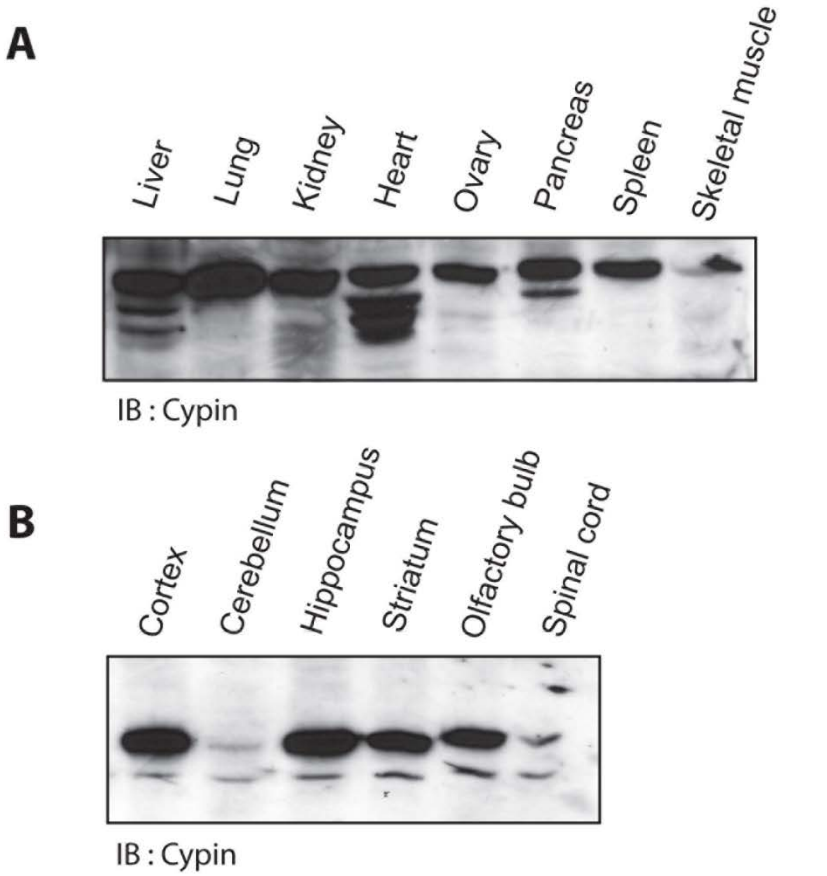
A
Liver
Lung
Kidney
Heart
Ovary
Pancreas
Spleen
Skeletal muscle
IB : Cypin
B
Cortex
Cerebellum
Hippocampus
Striatum
Olfactory bulb
Spinal cord
IB : Cypin
C
E16 E18 E21 P0 P6 P10 Adult
Cypin
CypinS
IB : Cypin

Figure 2-4. Expression of cypin and its variant in various tissues and brain regions

A, Tissue distribution of cypin in adult rats. Protein extracts from various tissues (20µg protein per lane) were subjected to immunoblotting using anti-cypin after SDS-PAGE. The cypin antibody recognizes both cypin (50kDa) and spliced variants (smaller bands) ***B***, Distribution of cypin in adult rat brain. ***C***, Developmental expression of cypin. RT-PCR analysis was performed from mRNAs from brains of indicated ages with primers either specific for cypin full-length (top panel) or specific for cypin short form (middle panel). Immunobloting of cypin in brains as indicated (bottom panel). E, embryonic; P, postnatal

Immunohistochemical study on the expression of cypin

Cypin is highly expressed in developing brains as well as in adult brain and olfactory bulb (Figure 2-4). To characterize the expression pattern of cypin in the developing brain in more detail, we performed cypin immunohistochemistry in sagittal sections of embryonic and postnatal rat brains. Cypin was detected as early as E18 (Figure 2-5), consistent with our Western blotting data. Since the cypin antibody used was produced by immunizing rabbits with a GST- full-length cypin fusion protein, this antibody is may be immunoreactive with cypin alternative splice variants. However, it is more likely that the antibody recognizes full-length cypin better than the short form because short form mRNA was detected as early as E16 and cypin protein was not (Figure 2-4C).

Almost every region of developing brains show cypin expression. Cypin regulated dendrite branching in hippocampal neurons (Akum et al., 2004), and it is highly expressed in adult hippocampus (Firestein et al., 1999). As shown in Figure 2-5, cypin is expressed in the hippocampus and cortex at E18, and increased expression is detected at later ages. In addition, we found that developing hippocampus shows high cypin expression (Figure 2-6), suggesting that cypin is required for neuronal development in hippocampus. Interestingly, cypin expression in olfactory bulb is very high from E22 to P0, but disappears by (Figure 2-7). This expression coincides with the time period, E21 to P0, that juxtaglomerular (JG) neurons begin to migrate into the region surrounding glomeruli, specialized structure where synapses form between the axons of olfactory receptor neurons and the dendrites of JG, mitral, and tufted cells (Bailey et al., 1999).

These data suggest that cypin might be involved in the migration, differentiation, and/or neurite outgrowth of JG neurons.

It is surprising to find that cypin is expressed in the developing cerebellum, and in specific, the purkinje cell layer (Figure 2-8), since both cypin mRNA and protein were not detected in adult cerebellum ((Firestein et al., 1999); Figure 2-4). Cerebellar cortex is postnatally formed (Wolf, 2000), and purkinje cell dendritogenesis occurs during the first few postnatal weeks. Purkinje cells have elaborate dendritic arbors, and purkinje cells undergo dramatic changes in development (Hendelman and Aggerwal, 1980; Armengol and Sotelo, 1991). Although most of the mature purkinje cells have one primary dendrite, these cells have several primary dendrites in first postnatal week while they are developing. After this first week, the dendrites extend and branch and form synapses with parallel and climbing fibers and interneurons within the next couple weeks (Tanaka, 2009). Since cypin expression appears around P6, the data suggest that cypin may play a role in dendritic development in purkinje cells.

To investigate in which cell types cypin is expressed, we subjected lysates from mixed cultures of hippocampal neurons and astrocyes and pure astrocyte cultures to SDS-PAGE and immunoblotting with antibody against cypin, along with co-immunostaining of cell type markers, such as MAP2 and GFAP (data not shown). Cypin is expressed in both neurons and astrocytes (Figure 2-9), supporting the idea that cypin is important for neuronal development, not only in neurons, but also in glial-supporting cells.

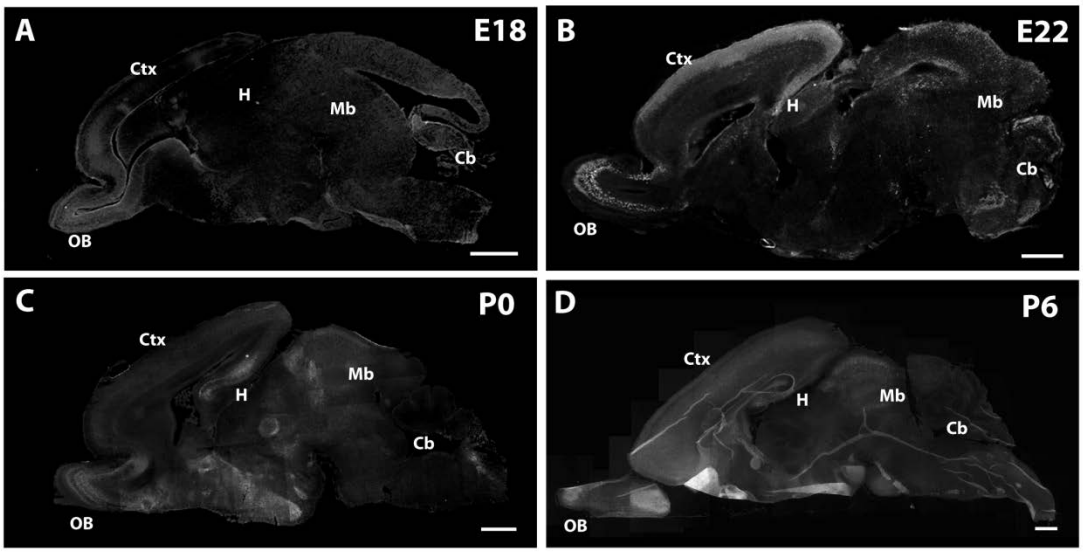

Figure 2-5. Distribution of cypin immunoreactivity in sagittal sections of developing brains.

A-D, Sagittal sections of rat brains at the indicated ages were immunostained with cypin antibody. Scale bar = 200μm

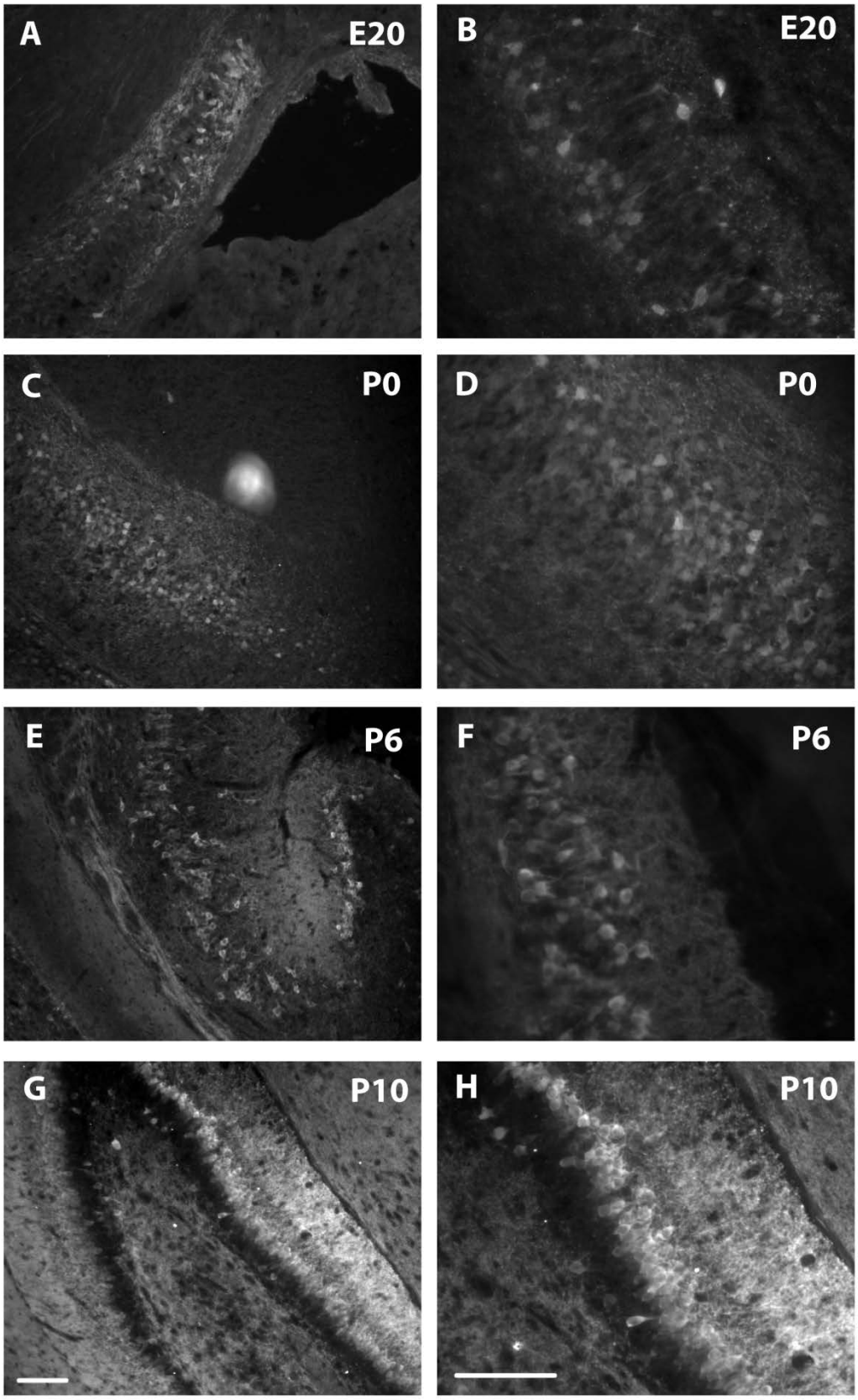
A
E20
B
E20
C
P0
D
P0
E
P6
F
P6
G
P10
H
P10

Figure 2-6. Cypin is expressed in the developing hippocampus.

Cypin immunoreactivity was detected in the hippocampal formation. ***A-H,*** Sagittal brain sections at the level of the hippocampus at the indicated ages are shown. Right panels are higher magnifications of regions in the adjacent left panels in ***B, D, F, H***. Scale bar = 100μm

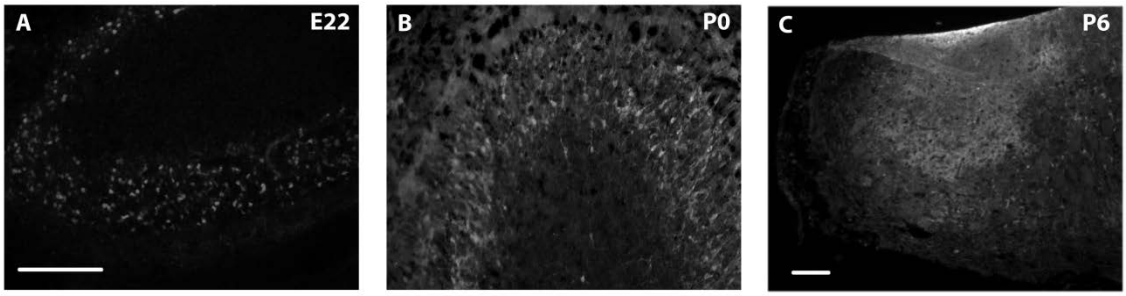

Figure 2-7. Cypin is expressed in the developing olfactory bulb.

Cypin immunoreactivity was detected in the olfactory bulb. ***A-C,*** Olfactory bulb in sagittal sections are shown at the indicated times. Scale bar = 100μm

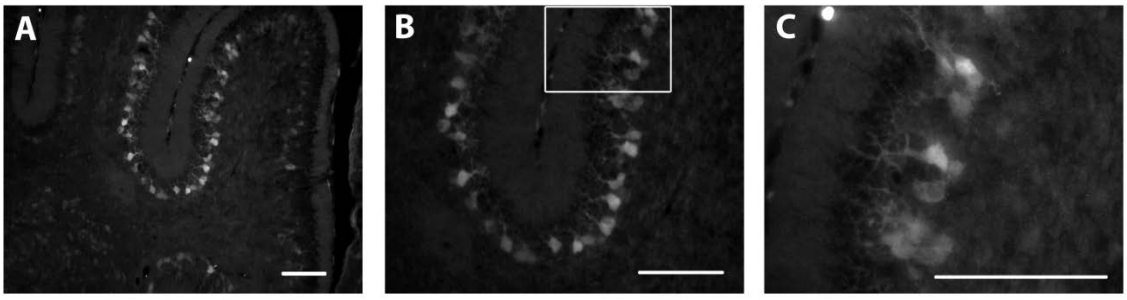

Figure 2-8. Cypin is expressed in the developing cerebellum.

Cypin immunoreactivity was detected cerebellum. ***A-C,*** Cerebellar regions in P6 sagittal brain sections are shown. Higher magnification view of left panel or boxed region in ***B*** or ***C***. Scale bar = 100μm

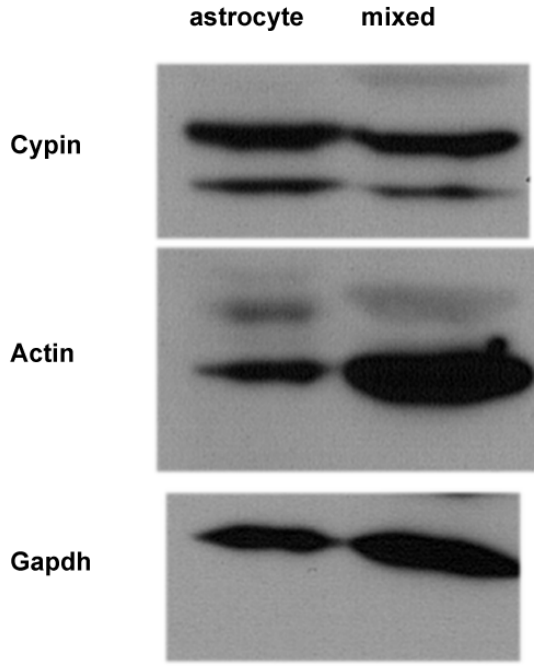

Figure 2-9. Cypin and its variant are expressed in both neurons and astrocytes.

Lysates from hippocampal mixed culture and cortical astrocyte culture were subjected to SDS-PAGE and immunoblotting using cypin antibody. Both mixed culture and astrocyte cultures have high cypin expression.

DISCUSSION

Alternative splicing is a mechanism for eukaryotic organisms to make multiple proteins from a single gene. Choosing which splice sites determines which exons will be encoded. Slight changes in peptide sequences can alter protein localization, enzymatic activity, and binding partners (Black, 2003). In the first part of this chapter, we showed a novel isoform of cypin produced by alternative splicing. This splice variant does not have guanine deaminase activity (Figure 2-3) and an effect on dendrite number and branching (data not shown), suggesting that this short form may have another cellular function. CypinS is expressed in the developing rat brain and its expression pattern in brain regions is pretty similar to that of the full-length form of cypin (Figure 2-4B and C). In contrast, CypinS is highly expressed in liver, heart, and pancreas, whereas the other organs including kidney, ovary and spleen do not have short form expression (Figure 2-4B). Interestingly, even forms shorter than cypinS were detected in liver and heart. This suggests that cypinS may have distinct tissue-specific roles.

Unlike the other splice variants previously reported (Kuwahara et al., 1999), this new short form has PDZ-binding motif, suggesting a possible function for regulating membrane-associated guanylate kinase (MAGUK) proteins or other PDZ-containing proteins in neuronal or non-neuronal cells. Synaptic clustering of receptors and signaling molecules is mainly accomplished by the MAGUK protein family in neurons. Some MAGUK proteins are reported to function in signaling pathways in cell adhesion and proliferation (Woods and Bryant, 1991; Matsumine et al., 1996). Taken together, CypinS may play a role in the modulation of synaptic clustering, growth, and/or cell-cell contact signals in neuronal or non-neuronal cells by regulating PDZ-associated signaling.

Proper placement and maturation of neurons and supporting cells in the brain is important for brain development and function. The temporal and spatial regulation of gene expression is required in this process. Understanding gene expression in these tightly regulated timed events helps us to elucidate the mechanism underlying development. Cypin is expressed in the developing brain, and its expression increased as the animal ages. This suggests that an important role for cypin in developing brains. During postmigratory differentiation, new dendrites grow and progressively develop complex branching. Dendrite morphology, which is characterized by dendritic outgrowth, branching, and spine formation, influence the function of neurons by determining synaptic function and plasticity (Matus, 2000; Hering and Sheng, 2001; Blanpied and Ehlers, 2004).

We found that cypin is expressed in the purkinje cell layer in developing cerebellum, but not in adult, supporting the idea that cypin is a key regulator of dendrite branching in various cell types. We believe the story to be similar in olfactory bulb because cypin is expressed in the developing olfactory bulb, when neuronal migration, differentiation, and neurite outgrowth occurs. Interestingly, purkinje cells are a class of GABAergic neurons and cypin is mainly expressed in nNOS-positive inhibitory neurons (Akum et al., 2004), suggesting major role of cypin in inhibitory interneurons.

Cypin expressed as early as E18 and increases through P10, where it reaches levels seen in adulthood. Cypin was originally characterized by its function of altering in synaptic clusters of PSD-95 and SAP102 (Firestein et al., 1999; Kuwahara et al., 1999), which are the key scaffolding molecules in neurons. It is expected that cypin mainly regulates dendrite branching in early stages and begins to influence synaptic formation in

later stages of development. Interestingly, a recent founding in our laboratory that cypin is involved in morphological changes in dendrites following sublethal NMDA exposure by altering microtubule and acts as a neuroprotective agent (Tseng and Firestein, 2011) can somehow explain function of cypin in the adult neurons.

ACKNOWLEDGEMENTS

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Chapter 3. BDNF-promoted Increases in Proximal Dendrites Occurs via CREB-dependent Transcriptional Regulation of Cypin

INTRODUCTION

Dendrite patterning and development are responsible for determining how input signals are processed in the central nervous system. The degree of dendrite branching can regulate the electrical properties of a neuron (Miller and Jacobs, 1984), and dendrite development is regulated by intrinsic and extrinsic factors (Landgraf and Evers, 2005; Libersat, 2005). Extrinsic factors can modulate specific patterns of dendrite growth and branching by activating intrinsic cues that directly affect the cytoskeleton or the transcriptional regulation of gene expression (Whitford et al., 2002). Neurotrophic factors play important roles in regulating neurite growth and branching in neurons. The major neurotrophic factors are the neurotrophins: nerve growth factor (NGF; (Levi-Montalcini, 1987; Shooter, 2001), brain-derived neurotrophic factor (BDNF; (Barde et al., 1982)), neurotrophin-3 (NT-3; (Maisonpierre et al., 1990)), and neurotrophin-4/5 (Hallbook et al., 1991). These factors are secreted from neurons and glial cells to exert their effects through the tyrosine receptor kinase family (Trk) in response to neuronal activity (Chao et al., 1992; Levine et al., 1995; Muller et al., 1995). BDNF is one of the most studied extrinsic factors regulating dendrite outgrowth and branching (Segal et al., 1995; McAllister et al., 1996, 1997; Schwartz et al., 1997; Baker et al., 1998; Jin et al., 2003) and increases proximal dendrite growth and number in pyramidal neurons (McAllister et al., 1995; Baker et al., 1998; Horch et al., 1999).

Cypin (cytosolic PSD-95 interactor) was first identified as a protein that decreases the synaptic localization of PSD-95 (Firestein et al., 1999). Cypin is a guanine deaminase and contains a zinc-binding motif, CRMP (collapsin response mediator protein) homology domain, and a PDZ-binding motif (Fernandez et al., 2008). Our laboratory has reported that cypin plays an important role in regulating dendrite number in rat hippocampal neurons. Overexpression of cypin results in an increase in dendrite branching, which correlates with cypin's guanine deaminase activity (Akum et al., 2004). Cypin binds directly to tubulin heterodimers and promotes microtubule assembly (Akum et al., 2004), suggesting that cypin regulates dendrite branching via cytoskeletal rearrangement.

Here, we report a novel signaling pathway by which BDNF increases proximal dendrite branching. BDNF activates the MAPK signaling pathway, resulting in increased cypin mRNA and protein levels. Furthermore, this increase is only seen after 72 hours of BDNF treatment, suggesting that cypin may mediate the effects of BDNF under chronic exposure. We also show that CREB binds to the cypin promoter to increase cypin transcription, and this binding is increased upon exposure to BDNF. Most importantly, we show that BDNF signals through CREB and cypin to increase proximal dendrite number. Our work links an important extrinsic regulator of dendrite branching to an important intrinsic factor that increases dendrite number, further uncovering molecular mechanisms that shape dendrites during development.

MATERIALS AND METHODS

Antibodies and reagents

Rabbit polyclonal antibody raised against cypin has been previously described (Firestein et al., 1999). Mouse monoclonal antibody recognizing β-actin was purchased from Sigma (St. Louis, MO). Mouse anti-MAP2 was from BD-Pharmingen (San Diego, CA), and chicken anti-GFP was purchased from Rockland Immunochemicals (Gilbertsville, PA). Mouse monoclonal antibody to CREB was from Cell Signaling Technology (Danvers, MA). Rabbit monoclonal antibodies recognizing phospho-Akt, Akt, phospho-p70S6 kinase, p70S6 kinase, phospho-Erk1/2, and Erk1/2 were from Cell Signaling Technology (Danvers, MA). Cyanine (Cy) 2-, Cy3- and Cy5-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). rhBDNF, mNGF, and U0126 were purchased from Promega (Madison, WI) and myristoylated PKA inhibitory peptide 14-22 amide, wortmannin, rapamycin were from Calbiochem/EMD chemicals (Gibbstown, NJ) . Actinomycin D was purchased from Sigma (St. Louis, MO). PKA kinase activity kit was from Enzo Lifesciences (Plymouth Meeting, PA).

RNA interference and DNA constructs.

pSuper GFP vector (Oligoengine, Seattle, WA) containing shRNA against the cypin transcript and an unrelated sequence, as a negative control, were used as described previously (Chen and Firestein, 2007). cDNA encoding rat CREB isoform A (NCBI Accession # NM_114443.1) was subcloned into pEGFP-C1 or dsRed2-C1 vector (Clontech Inc.). A dominant negative (DN) mutant of CREB and a constitutively active (CA) mutant of CREB were constructed using the QuickChange II site-directed

mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. The serine 119 residue of CREB was replaced with alanine or aspartate using the following primers, respectively: 5′- CCT TTC AAG GAG GCC TGC CTA CAA GAA AAT CTT GAA TGA CTT ATC-3′ or 5′- CTT TCA AGG AGG CCT GAC TAC AAG AAA ATC TTG AAT GAC TTA TC-3′.

Primary culture of hippocampal neurons

Neuronal cultures were prepared from hippocampi of rat embryos at 18 days gestation as described previously (Firestein et al., 1999). The hippocampi were dissociated, and cells were plated on poly-D-lysine-coated glass coverslips (12 mm diameter) at a density of 1800 cells/mm^2 or 1 X 10^6 cells on 35mm culture dishes. Cultures were maintained in Neurobasal media (Gibco) supplemented with B27 (Gibco), penicillin, streptomycin, and Glutamax (Gibco). Cells were grown for 7 days *in vitro* (DIV) and used for specific experiments as indicated below.

Western blotting

Hippocampal neurons were plated 1 x 10^6 cells per dish. Neurons were treated with neurotrophins, kinase inhibitors, or DMSO vehicle (0.01% final concentration) at the indicated concentrations for 72 h. Neurons were washed with ice-cold PBS and lysed in TEE (25 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, pH 7.4). Cells were further lysed by passing the extract through a 26 gauge needle 20 times and solubilized using Triton X-100 at a final concentration of 1%. Insoluble material was pelleted at 12,000 x *g* at 4°C for 15 min. Proteins were resolved on a 10% SDS polyacrylamide gel and transferred to

PVDF membrane. The blot was probed with the indicated antibodies. Experiments were repeated three times. Blots were scanned and intensities of bands were quantitated using ImageJ software (NIH, Bethesda, MD) as we have previously performed (Chen and Firestein, 2007; Carrel et al., 2009). An area close to the bands was used as a reference for background intensity. The difference between intensities of the background and the band is the absolute intensity of the band. The number of pixels for the bands was normalized to the intensity of the internal control (β-actin) and then compared with that of the control condition.

Quantitative RT-PCR

Neurons were plated as above. At 7 DIV, neurons were treated with indicated concentrations of neurotrophins, kinase inhibitors, or DMSO vehicle. At 10 DIV, RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total cDNA was then generated using the high-capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) using 1μg of total RNA and following the manufacturer's protocol. We used a Stratagene Mx3000P QPCR system (Stratagene, La Jolla, CA) to perform multiplex assays using 50 ng of total cDNA for cypin/GDA and GAPDH as an internal control. The TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA) containing primers and probes were used in our experiments. The cypin/GDA probe contained the FAM490 fluorophore, and the GAPDH probe contained the HEX fluorophore, both with the MGB quencher. Results were analyzed following the $2^{-\Delta\Delta Ct}$ method using GAPDH as an internal control and non-treated or vehicle control.

PKA kinase activity assay

Cultured hippocampal neurons (7 DIV) were treated with PKA inhibitory peptide for 72 h. Neurons were washed with ice-cold PBS and lysed in buffer containing 20 mM 3-(N-morpholino) propanesulfonic acid, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM DTT, and 1 mM PMSF. Insoluble material was pelleted at 12,000 x *g* at 4°C for 15 min. Lysates were assayed following the manufacturer's instructions. Samples were added to plates pre-coated with PKA substrate, and ATP was added to the reactions. After 90 min incubation at 30°C, phospho-specific substrate antibody was added, and the plate was incubated for 1 h. The plate was washed four times, and secondary antibody conjugated with HRP was applied. After 30 min incubation, the plate was washed and TMB substrates were added followed by the addition of Stop solution. The absorbance was measured at 450 nm using a SpectraMax 250 microplate reader (Molecular Devices, Inc., CA).

Transfection of cultured cells

Cultured hippocampal neurons were transfected at 5 DIV for shRNAs and 6 DIV for cDNAs using LIPOFECTAMINE LTX with PLUS reagent following the manufacturer's protocol (Invitrogen).

Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde in PBS for 15 minutes and then incubated in blocking solution (PBS containing 0.1% Triton X-100, 2% normal goat serum, and 0.02% sodium azide) for 1 hour. All antibodies used were diluted in blocking solution, and

dilutions of 1:500 for chicken anti-GFP, mouse anti-MAP2, and rabbit anti-cypin were used. Neurons were incubated in primary antibody solution at 4^{o}C overnight. Neurons were then washed with PBS three times. The secondary antibody solution consisted of a 1:250 dilution of Cy2-conjugated anti-chicken IgY, Cy3-conjugated anti-mouse IgG, and Cy-5 conjugated anti-rabbit IgG. Coverslips were then mounted onto frosted glass microscope slides using Fluoromount G (Southern Biotechnology, Birmingham, AL). Labeled cells were visualized by immunofluorescence on an Olympus Optical (Tokyo, Japan) IX50 microscope with a Cooke Sensicam charge-coupled device cooled camera fluorescence imaging system and Image Pro software (Media Cybernetics, Silver Spring, MD).

Assessment of dendrite number

Dendrite morphology was assessed as described (Kutzing et al., 2010; Langhammer et al., 2010) using custom scripts written in MATLAB (MathWorks, Natick, MA). The axon was excluded based on the absence of MAP2 immunostaining. We performed Sholl analysis with a 6 µm ring interval starting at 9.3 µm from the soma. The experimenter was blinded to conditions during all data analysis. Dendrites less than 3 µm in length were not counted (Yu and Malenka, 2004; Charych et al., 2006).

Chromatin Immunoprecipitation

At 7 DIV, neurons were treated with 25 ng/ml of rhBDNF and DMSO for 24 h. Cells were treated with 1% formaldehyde at 37°C for 10 min to cross-link histones to DNA. After washing the cells twice with ice-cold PBS containing protease inhibitors, cells were

scraped and pelleted at 1,000 x g at 4°C for 4 min. The pellet was resuspended in SDS lysis buffer (1% SDS, 10mM EDTA and 50mM Tris, pH 8.1), and the lysate was sonicated. After centrifuging the lysates at 12,000 x *g* at 4°C for 10 min, the supernatant was diluted in ChIP dilution buffer (Millipore, Billerica, MA). Lysate was pre-cleared with salmon sperm DNA/protein A agarose (50% slurry) for one hour, then subjected to immunoprecipitation with monoclonal CREB antibody or mouse IgG at 4°C overnight. Salmon sperm DNA/protein A agarose slurry was added for one hour incubation, and beads were washed one time each with Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer, and then twice with TE (all from Millipore). Immunoprecipitated histone complex was eluted with elution buffer (1% SDS, 0.1M $NaHCO_3$). NaCl (5M) was added to the eluates, followed by heating at 65°C for 4 hours. After one hour incubation with Proteinase K, the DNA was recovered by phenol/chloroform extraction and ethanol precipitation. PCR was performed with following primers: Forward 5'- GAG GAC TTT AGA CTG GAA ACT TGC AAT TGG-3', Reverse 5'- TTC CTG AGT GTG AGG GAT GCT GAC TAT G-3'.

Quantification of fluorescence intensity

Hippocampal neurons were prepared, cultured, and transfected as above. The neurons were immunostained using our polyclonal antibody against cypin. Fluorescence intensities of cypin were measured using ImageJ software as we have previously described (Chen and Firestein, 2007). Briefly, the cell bodies for each neuron were traced, and intensities were measured as integrated pixel intensity within the selected region corrected for background. Fluorescence was visualized using an Olympus LCPlanFL

20X/0.4 (air) objective. To quantitate the fluorescence levels of endogenous proteins, images of neurons were captured by CCD as above using a constant gain and exposure time for all samples. Images were corrected for coverslip fluorescence by subtracting a background image generated using an 11 x 11 erosion filter. The experimenter was blinded to the condition when taking images and assaying fluorescence intensities.

RESULTS

BDNF increases cypin protein levels in hippocampal neurons

To investigate the possibility that neurotrophic factors may modulate cypin expression to promote increases in dendrite number, we treated cultures of primary hippocampal neurons with three neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). These neurotrophins have a degree of receptor specificity when applied at low levels, with NGF binding to TrkA, BDNF and NT-4/5 binding to TrkB, and NT-3 preferentially binding to TrkC (Reichardt, 2006). We investigated the role of these neurotrophins in promoting changes in cypin expression by examining mRNA levels with quantitative RT-PCR (qRT-PCR) and protein levels with Western blotting. Since different concentrations of neurotrophins can have different affinities for Trk receptors (Davies et al., 1993; Mahadeo et al., 1994), we used two different concentrations of each neurotrophin. Only exposure to BDNF significantly increases cypin mRNA and protein levels after 72 hours of treatment (Fig. 3-1A, B and C). This increase is dose-dependent and is due to TrkB activation since at the concentrations tested, no apparent cross-stimulation of TrkA, TrkC, or p75 should occur (Ji et al., 2005). Furthermore, since it has been reported that exposure of cortical neurons to BDNF for as little as 5 hours is sufficient to increase the number of primary dendrites (Dijkhuizen and Ghosh, 2005), and TrkB is maximally phosphorylated in 5 min by 25ng/ml of BDNF (Ji et al., 2005), we tested whether a shorter exposure of hippocampal neurons to BDNF results in this increase in cypin expression. As seen in Figure 3-1D, 72 hours is the minimum time of BDNF exposure needed to see the increase in cypin expression. It should be noted that cypin levels increase as neurons mature in

culture (Kuwahara et al., 1999), and thus, we observed this baseline increase in cultures treated with DMSO. Thus, we used a 72 h incubation time for the rest of our studies, acknowledging the fact that we are studying pathways involved in longer term exposure to BDNF rather than a short pulse of BDNF exposure.

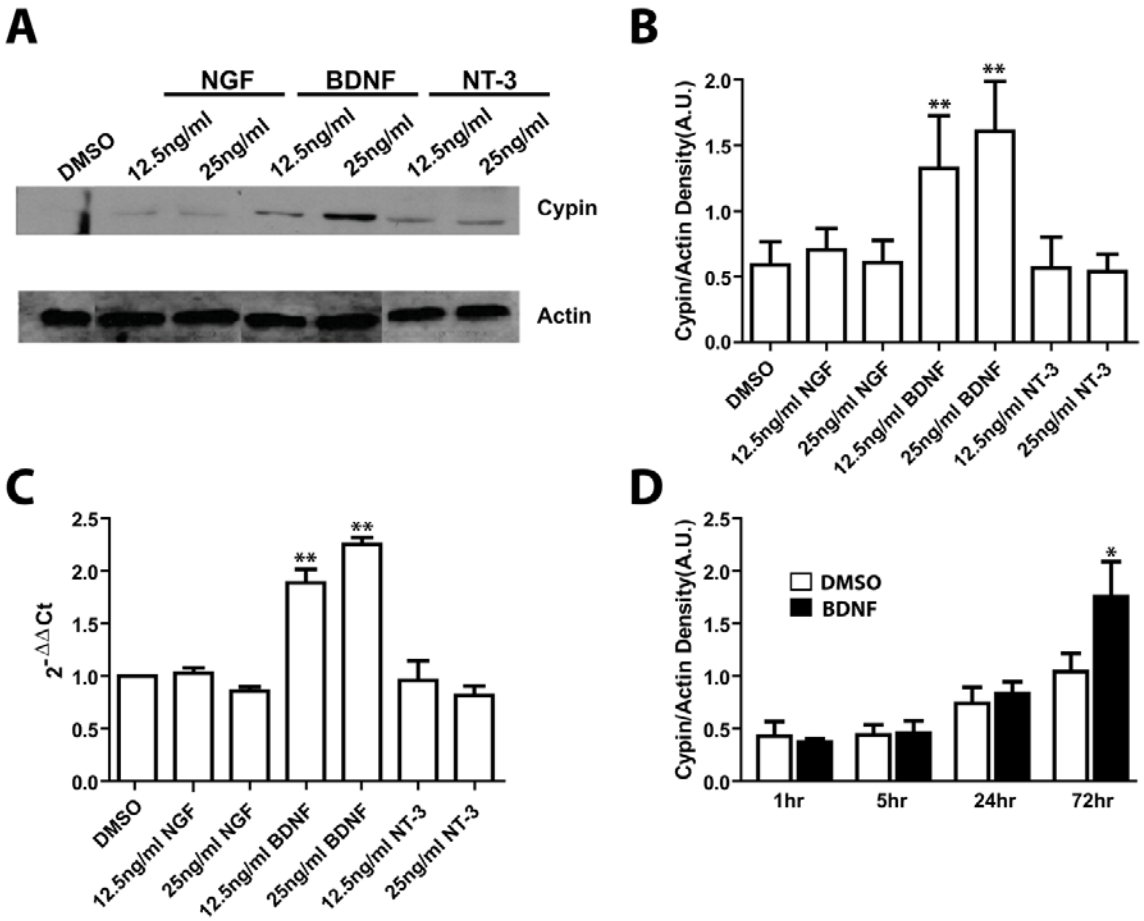

A
NGF
BDNF
NT-3
DMSO
12.5ng/ml
25ng/ml
12.5ng/ml
25ng/ml
12.5ng/ml
25ng/ml
Cypin
Actin
B
Cypin/Actin Density(A.U.)
2.0
1.5
1.0
0.5
0.0
**
**
DMSO
12.5ng/ml NGF
25ng/ml NGF
12.5ng/ml BDNF
25ng/ml BDNF
12.5ng/ml NT-3
25ng/ml NT-3
C
2^-ΔΔCt
2.5
2.0
1.5
1.0
0.5
0.0
**
**
DMSO
12.5ng/ml NGF
25ng/ml NGF
12.5ng/ml BDNF
25ng/ml BDNF
12.5ng/ml NT-3
25ng/ml NT-3
D
Cypin/Actin Density(A.U.)
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
BDNF
*
1hr
5hr
24hr
72hr

Figure 3-1. Endogenous cypin expression increases in response to BDNF but not NGF or NT-3.

A, Cells were treated with the indicated concentrations of neurotrophins beginning at 7 DIV for 72 hours. Extracts from untreated and treated cultures of hippocampal neurons were analyzed by SDS-PAGE and Western blotting using antibodies that recognize cypin or actin. Representative blot is shown. ***B***, Densitometry analysis of cypin normalized to actin expression. A.U., arbitrary units. ***C***, Quantitative RT-PCR assay for the rat cypin gene (GDA) after indicated treatments of cultured hippocampal neurons. $^{**}p < 0.01$ by ANOVA followed Student-Newman-Keuls multiple comparison test compared to DMSO as control. Cypin mRNA and protein levels are upregulated by increasing doses of BDNF. ***D***, Exposure of hippocampal neurons to BDNF for 72 hours, but not 24 hour or less, results in increased cypin levels. Cells were treated with BDNF at 7 DIV for each indicated duration. Cypin protein levels were compared by Western blotting. ns, no significance, $^{*}p<0.05$ by two-tailed t-test compared to control. Error bars indicate SEM. A.U., arbitrary units. n=3 experiments for all panels.

BDNF signals through cypin to increase dendrite numbers

To determine whether BDNF signals via cypin to regulate dendrite patterning, we used an shRNA against cypin as we have previously described and shown to be specific for cypin by rescue experiments (Chen and Firestein, 2007). Consistent with results from other laboratories (Dijkhuizen and Ghosh, 2005), BDNF increases primary and secondary dendrite numbers in neurons treated during DIV7-10 in the presence of the expression of a control shRNA. However, we did not see changes in dendrite number in our hippocampal neurons in response to shorter exposures of BDNF as has been seen in cortical neurons (Dijkhuizen and Ghosh, 2005), in line with a previous report that pathways that control dendrite outgrowth differ between cortical and hippocampal neurons (Ko et al., 2005). As seen in Figure 3-2A and B, knockdown of cypin blocks increases in primary and secondary dendrite numbers in response to 72 h treatment with BDNF. We also observed some pruning in primary dendrite number from DIV7-10, consistent with previous results (Cline, 2001; Wong and Ghosh, 2002; Charych et al., 2006). In addition, we performed Sholl analysis by measuring the number of dendrites that cross ellipsoids at different radial distances from the cell body to analyze the roles of BDNF and cypin in higher-order dendritic branching (Figure 3-2D). Sholl analysis shows that treatment with BDNF for 72 h results in an increase in dendrite numbers proximal to the soma, the 75 μm closest to the cell body (Figure 3-2E). In contrast, this treatment with BDNF does not increase dendrites when cypin is knocked down, strongly suggesting that BDNF promotes dendrite branching via cypin at DIV7-10. We also noted that knocking down cypin at an earlier time window, DIV5-7 (for the 5 h treatment; Fig. 3-2

D and E), has no effect on dendrite numbers, suggesting that cypin may not regulate neurite outgrowth in early stages of development.

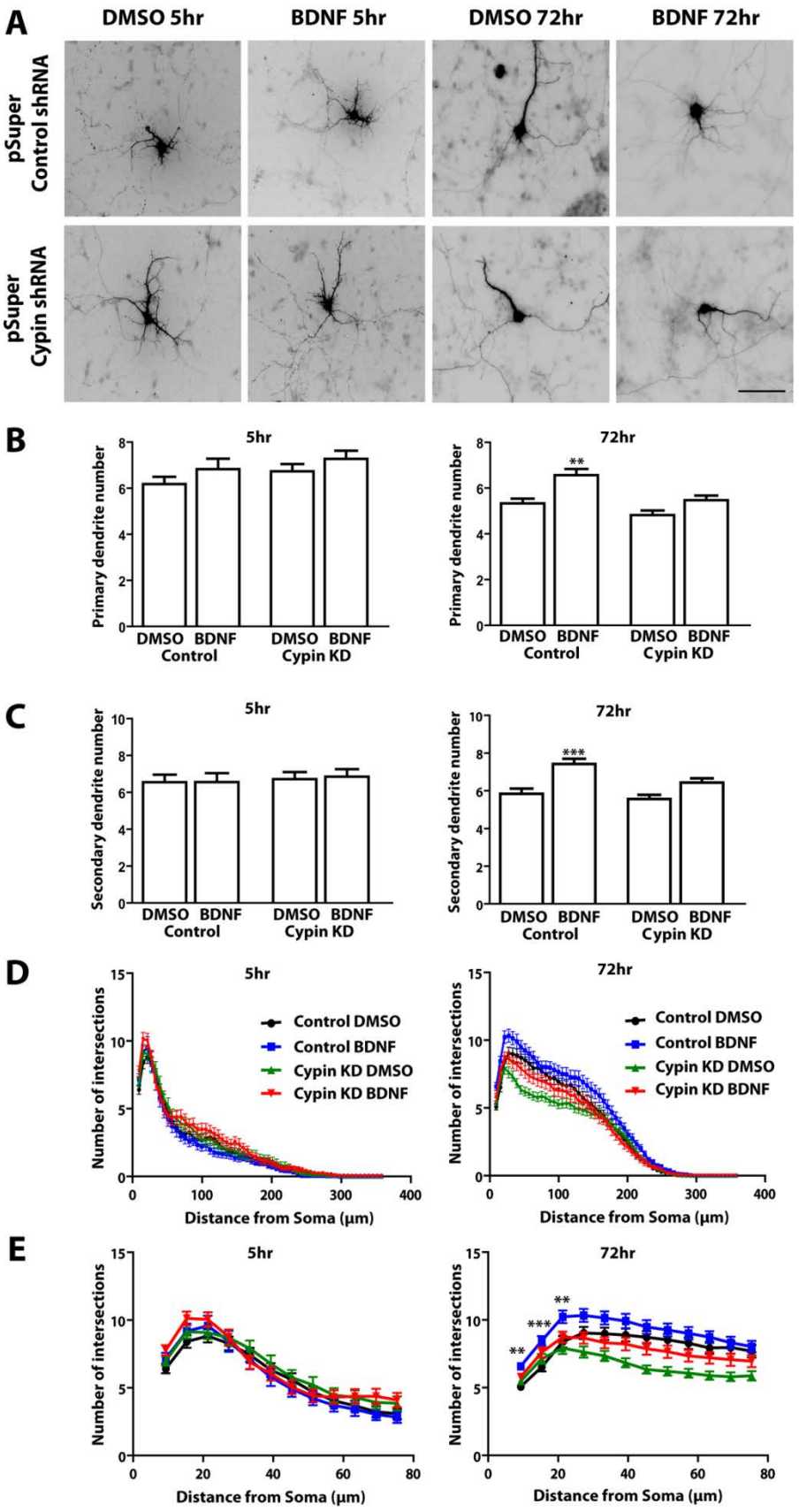

A
DMSO 5hr
BDNF 5hr
DMSO 72hr
BDNF 72hr
pSuper Control shRNA
pSuper Cypin shRNA
B
5hr
72hr
Primary dendrite number
DMSO BDNF Control
DMSO BDNF Cypin KD
**
C
5hr
72hr
Secondary dendrite number
***
D
5hr
72hr
Number of intersections
Control DMSO
Control BDNF
Cypin KD DMSO
Cypin KD BDNF
Distance from Soma (µm)
E
5hr
72hr
Number of intersections
Distance from Soma (µm)
**
***

Figure 3-2. BDNF treatment results in increased proximal dendrites via a cypin-dependent pathway.

A, Hippocampal neurons were transfected with shRNA against GST (control) or with shRNA against cypin at 5 DIV and treated with either DMSO (vehicle) or BDNF (25 ng/ml) at 7 DIV either for 5hr or for 72 hrs. Dendrite number was assessed at 7 DIV or 10 DIV. Scale bar, 100 μm. ***B***, Cypin knockdown blocks BDNF-promoted increases in primary dendrites, and ***C***, secondary dendrites with 72 hour treatment (Right panels). BDNF treatment for 5 hours does not increase dendrite numbers (Left panels). ***$p<0.001$, **$p<0.01$ comparing control shRNA + BDNF 72hr with control shRNA with DMSO 72hr treatment and no significance comparing cypin knockdown + DMSO 72hr and cypin knockdown + BDNF 72hr. *p* values were determined by Kruskal-Wallis test followed by Dunn's multiple comparison test. ***D***, Sholl analysis. BDNF treatment for 72 hours increases the number of intersections close to the soma (proximal dendrites) ***E***, Proximal Sholl analysis (from ***D***) within the first 75 μm of the soma. (*$p<0.05$ at 9 μm, ***$p<0.001$ at 15 μm,**$p<0.01$ at 33 μm when comparing control shRNA + BDNF 72hr and control shRNA + DMSO 72hr, and no significance comparing cypin knockdown + DMSO 72hr and cypin knockdown with BDNF 72hr treatment. Cypin knockdown results in a significant decrease (***$p < 0.001$) in intersections in comparison to control shRNA, consistent with our previous work. *p* values were determined by two-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate SEM. n=51 neurons, control shRNA + DMSO 5hr; n=40, control shRNA + BDNF 5hr; n=48, cypin knockdown + DMSO 5hr; n=52, cypin knockdown + BDNF 5hr; n=76, control shRNA +

DMSO 72hr; n=79, control shRNA + BDNF 72hr; n=90, cypin knockdown + DMSO 72hr; and n=87, cypin knockdown + BDNF 72hr.

The cAMP/PKA pathway is not required for BDNF-promoted increases in cypin expression

We performed experiments to further identify the downstream pathways that mediate BDNF-promoted cypin gene expression. TrkB receptors dimerize in response to BDNF and phosphorylate one another. These phosphotyrosines generate binding sites for Shc and phospholipase C, which can recruit the signaling molecules to trigger activation of major pathways, such as cAMP/PKA, PI3-Kinase, mTOR, and MAPK signaling pathways (Chao, 2003; Segal, 2003; Kumar et al., 2005; Spencer et al., 2008). Therefore, we first tested the possibility that BDNF-mediated increases in cypin expression are mediated by the cAMP/PKA pathway. We treated cultured hippocampal neurons with a myristoylated membrane-permeable PKA inhibitor peptide and assessed BDNF action on cypin expression. Inhibition of PKA (Figure 3-3A, bottom panel) does not block BDNF-promoted increases in cypin mRNA or protein (Figure 3-3A, B, and C). These results suggest that the activation of PKA by BDNF is not required for increased cypin expression. Interestingly, the appearance of a lower molecular weight band in our Western blot analysis suggests that cAMP signaling pathway may alter expression of a lower molecular weight cypin variant (data not shown).

The PI3K-Akt-mTOR pathway is not required for BDNF-promoted increases in cypin expression

Given that the cAMP/PKA pathway appears not to be involved in BDNF-promoted expression of cypin in neurons, we further tested other signaling pathways reported to be activated by BDNF. PI3 kinase/Akt pathways are triggered by BDNF to regulate primary dendrite formation in cortical neurons (Dijkhuizen and Ghosh, 2005). In addition, there are several studies that support the idea that BDNF can regulate dendrite branching through the PI3 kinase pathway by activating the mammalian target of rapamycin (mTOR; (Schratt et al., 2004; Jaworski et al., 2005; Kumar et al., 2005)). Therefore, we tested the possibility that BDNF uses these pathways to increase cypin expression. We treated cultured neurons with Wortmannin and rapamycin, potent and specific PI3 kinase and mTOR inhibitors, respectively. We then measured cypin mRNA and protein expression. Inhibition of PI3 kinase (Figure 3-3D, bottom panels) or the mTOR (Figure 3-3G, bottom panels) does not affect BDNF-mediated increases in cypin mRNA or protein expression (Figure 3-3 D-F, and G-I), suggesting that the activation of PI3K/Akt and mTOR by BDNF is not required for increased cypin expression.

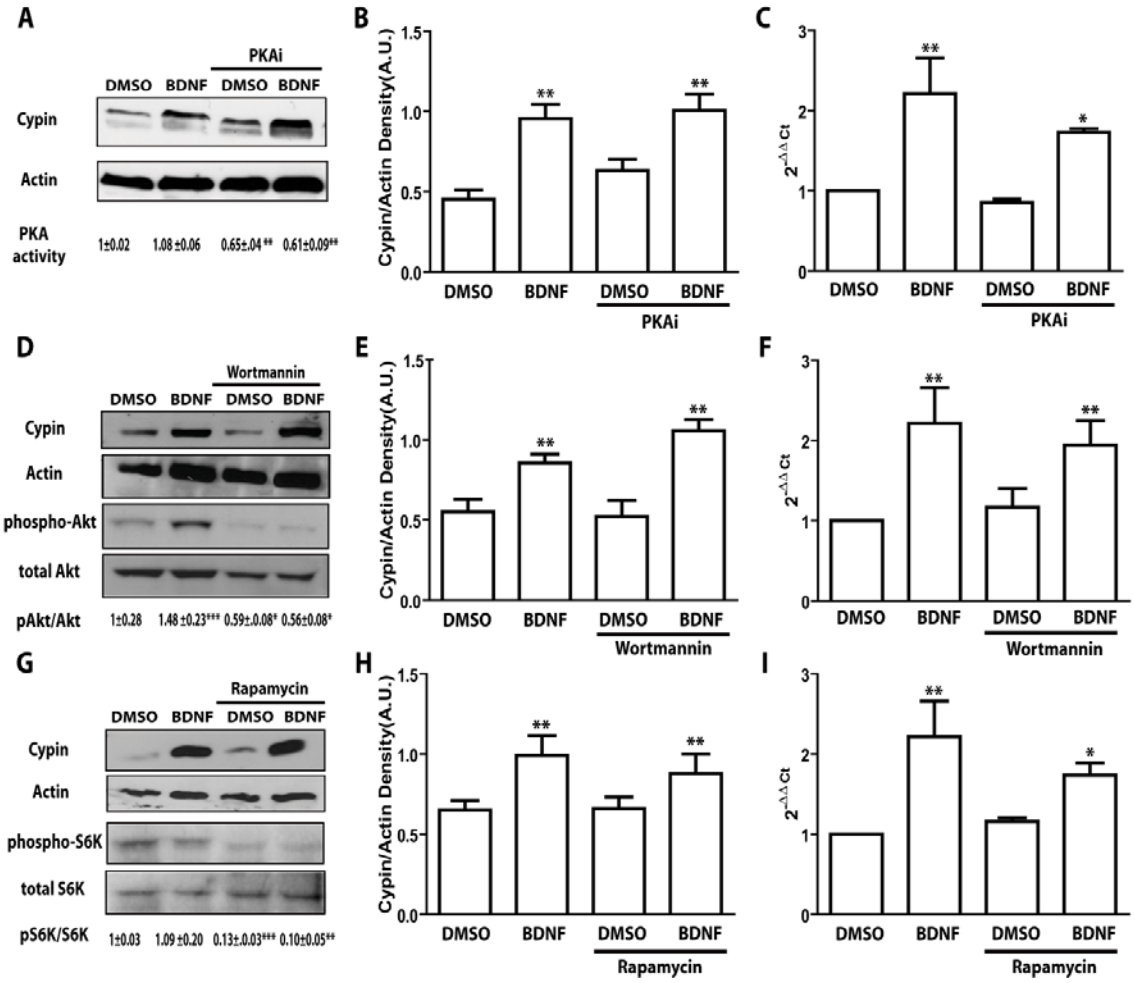

A
PKAi
DMSO BDNF DMSO BDNF
Cypin
Actin
PKA activity
1±0.02 1.08±0.06 0.65±.04** 0.61±0.09**
B
Cypin/Actin Density(A.U.)
DMSO BDNF DMSO BDNF
PKAi
C
2^-ΔΔCt
DMSO BDNF DMSO BDNF
PKAi
D
Wortmannin
DMSO BDNF DMSO BDNF
Cypin
Actin
phospho-Akt
total Akt
pAkt/Akt
1±0.28 1.48±0.23*** 0.59±.0.08* 0.56±0.08*
E
Cypin/Actin Density(A.U.)
DMSO BDNF DMSO BDNF
Wortmannin
F
2^-ΔΔCt
DMSO BDNF DMSO BDNF
Wortmannin
G
Rapamycin
DMSO BDNF DMSO BDNF
Cypin
Actin
phospho-S6K
total S6K
pS6K/S6K
1±0.03 1.09±0.20 0.13±.0.03*** 0.10±0.05**
H
Cypin/Actin Density(A.U.)
DMSO BDNF DMSO BDNF
Rapamycin
I
2^-ΔΔCt
DMSO BDNF DMSO BDNF
Rapamycin

Figure 3-3. The cAMP/PKA, PI3K/Akt/PKB, and mTOR pathways are not involved in BDNF-mediated increases in cypin expression.

Hippocampal neurons were treated with ***A-C,*** membrane-permeable PKA inhibitory peptide (25 ng/ml; Myr-N-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2), ***D-F,*** Wortmannin (100 nM), or ***G-I,*** rapamycin (25 ng/ml) at 7 DIV and treated with BDNF for 72 hours. Cytoplasmic protein extracts were resolved by SDS-PAGE and Western blotting using antibodies that recognize indicative proteins. PKA activity was measured by absorbance at 450nm with an ELISA-based PKA activity assay. PKA activity is shown as mean normalized absorbance±SEM. Densitometric analysis of active phospho-proteins normalized to total proteins are indicated as mean±SEM. Densitometric analysis of cypin is normalized to actin protein expression. Extracts from treated cultures were then analyzed by quantitative RT-PCR using specific rat cypin/GDA primers. *p< 0.05, **p< 0.01, ***p< 0.001 by ANOVA followed Student-Newman-Keuls multiple comparison test compared to DMSO as vehicle control. Error bars indicate SEM. n=3 experiments for all panels with representative blots shown in panels A,D,G. S6K, p70S6 kinase.

The MAPK signaling pathway is responsible for increased cypin expression in hippocampal neurons

The Ras/MAPK pathways have also been reported to mediate BDNF-mediated increases in primary dendrite number in cortical neurons (Kumar et al., 2005). To test the involvement of MAPK in BDNF-mediated increases in cypin expression, we used the highly selective MAP kinase kinase (MAPK/MEK) inhibitor U0126 (Favata et al., 1998). As seen in Figure 3-4, U0126 inhibits BDNF-promoted increases in cypin mRNA and protein overexpression. These results suggest that BDNF specifically increases cypin protein expression in hippocampal neurons via activation of the MAPK signaling pathway.

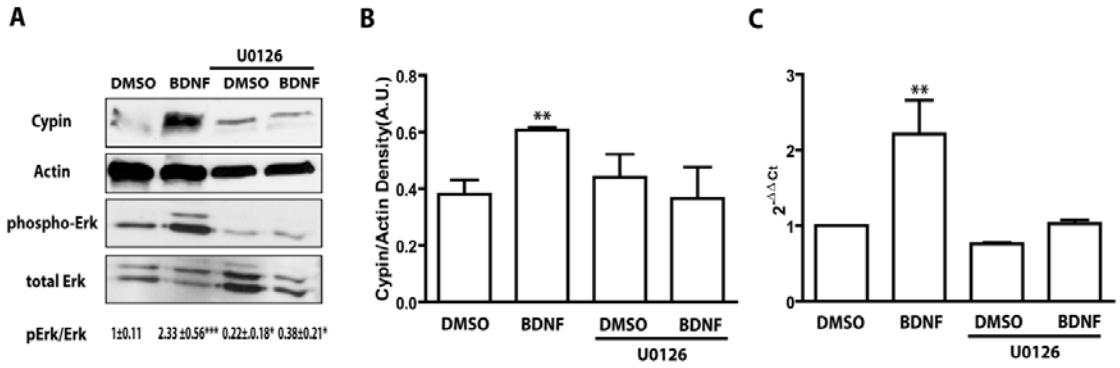


Figure 3-4. BDNF increases cypin expression via the MAPK signaling pathway.

A, Hippocampal neurons were treated with DMSO or 10 nM U0126, a MEK inhibitor, which consequently inhibits MAPK, in the absence or presence of 25 ng/mL BDNF on DIV 7 for 72 hours. Representative Western blots using antibodies that recognize cypin, actin, phospho-Erk1/2, and total Erk1/2 are shown. Densitometric analysis of active MAPK normalized to total MAPK is indicated as mean±SEM. ***B***, Densitometric analysis of cypin is normalized to actin protein expression. ***C***, Quantitative RT-PCR using specific rat cypin primers. *p< 0.05, **p< 0.01, ***p< 0.001 by ANOVA followed Student-Newman-Keuls multiple comparison test compared to DMSO as vehicle control. n = 3 experiments for all panels.

BDNF treatment increases cypin expression via a transcription-dependent mechanism

Since BDNF not only increases cypin protein levels but also cypin mRNA levels, and MAPK pathways can trigger the activation of transcription factors to perform various mechanism of cellular actions, we hypothesized that BDNF promotes cypin expression in a transcription-dependent manner. We used a pharmacological drug, actinomycin D, to inhibit transcription by binding DNA at the transcription initiation complex and preventing elongation of transcription by the RNA polymerase (Sobell, 1985). As shown in Figure 3-5, actinomycin D treatment blocks BDNF-mediated increases in cypin mRNA and protein levels. We then tested the possibility that actinomycin D acts by decreasing MAPK activity, which would lead to decreased cypin levels, rather than by directly blocking BDNF-promoted transcription of cypin. This is not the case as we found the opposite effect of actinomycin D. Actinomycin increases MAPK activity (Figure 3-5A and D), suggesting that actinomycin D may inhibit the transcription of inhibitors of MAPK and that MAPK activity is upstream of BDNF-promoted increases in cypin transcription. Taken together with our other results, these data suggest that BDNF increases dendrite number by upregulating cypin transcription, and ultimately, cypin translation.

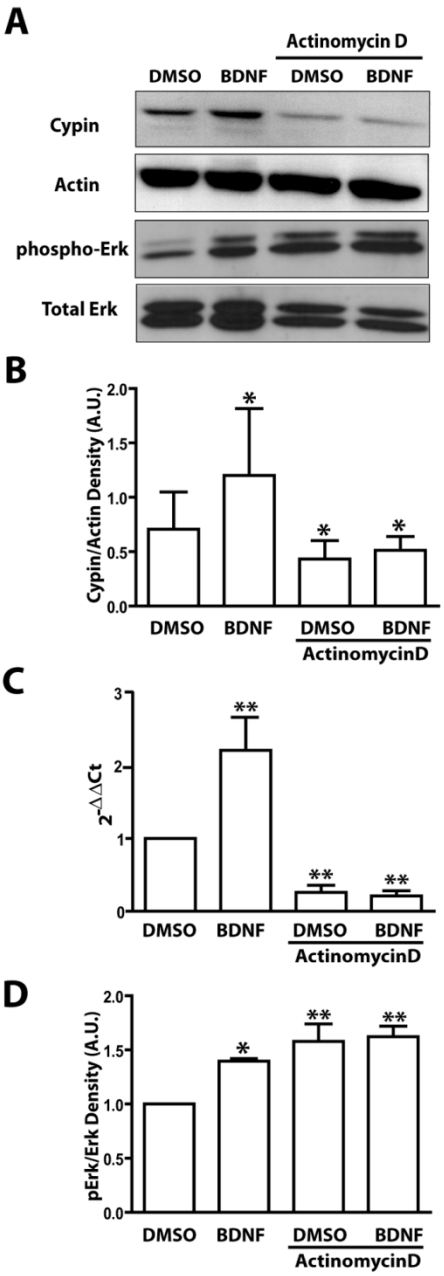

A
Actinomycin D
DMSO
BDNF
DMSO
BDNF
Cypin
Actin
phospho-Erk
Total Erk
B
Cypin/Actin Density (A.U.)
2.0
1.5
1.0
0.5
0.0
*
*
*
DMSO
BDNF
DMSO
BDNF
ActinomycinD
C
2-ΔΔCt
3
2
1
0
**
**
**
DMSO
BDNF
DMSO
BDNF
ActinomycinD
D
pErk/Erk Density (A.U.)
2.0
1.5
1.0
0.5
0.0
*
**
**
DMSO
BDNF
DMSO
BDNF
ActinomycinD

Figure 3-5. BDNF promotes cypin expression via a transcription-dependent mechanism.

A, Cultured hippocampal neurons were treated with 25 ng/mL BDNF concurrent with or without 5 μM actinomycin D at 7 DIV for 72 hours. Proteins were extracted at 10 DIV, and Western blotting was performed using a cypin antibody. A representative blot is shown. ***B***, Densitometric analysis of cypin protein expression normalized to actin protein expression is shown. *$p < 0.05$ by Kruskal-Wallis test followed by Dunnett's multiple comparison test compared to untreated control. ***C***, Quantitative RT-PCR using specific rat cypin/GDA primers. ***D,*** Densitometric analysis of phospho-Erk1/2 protein levels normalized to total Erk1/2 protein levels is shown. **$p < 0.01$ by ANOVA followed Student-Newman-Keuls multiple comparison test compared to DMSO as control. n = 3 experiments.

BDNF increases CREB binding to the cypin gene promoter

To identify possible cis-acting regulators of the cypin gene promoter, we performed a bioinformatics-based analysis to predict regulatory binding regions. Since the minimal promoter region of the rodent or human promoter region of cypin has not yet been characterized, we analyzed a vast region upstream of the first exon start region (10 Kb). We compiled a list of predicted transcription factors, consisting of AP-1, Sp-1, E2F, NF-kappaB, and CREB, that may bind to a regulatory region of the cypin gene (Figure 3-6A). Both NF-kappaB and CREB obtained higher binding probability than the other factors, and thus, we analyzed whether there is conservation of these regions between rodent and human genes. CRE regions were conserved between the mouse, rat, and human cypin genes, as shown in Figure 3-6B, although the positions of these regions differed within the gene. In addition, distinct TATA box domains were also found in this region and in proximity to the CRE elements, suggesting that together, these domains may represent an important regulatory region of the cypin gene. To assess whether CREB is involved in BDNF-promoted increases in cypin transcription, we performed chromatin immunoprecipitation assay. We found that CREB binds to the cypin promoter under basal conditions, and this binding is enhanced by extracellular treatment of BDNF (Figure 3-6C). Taken together, these data suggest that BDNF increases CREB binding to the cypin promoter to promote transcription of the cypin gene.

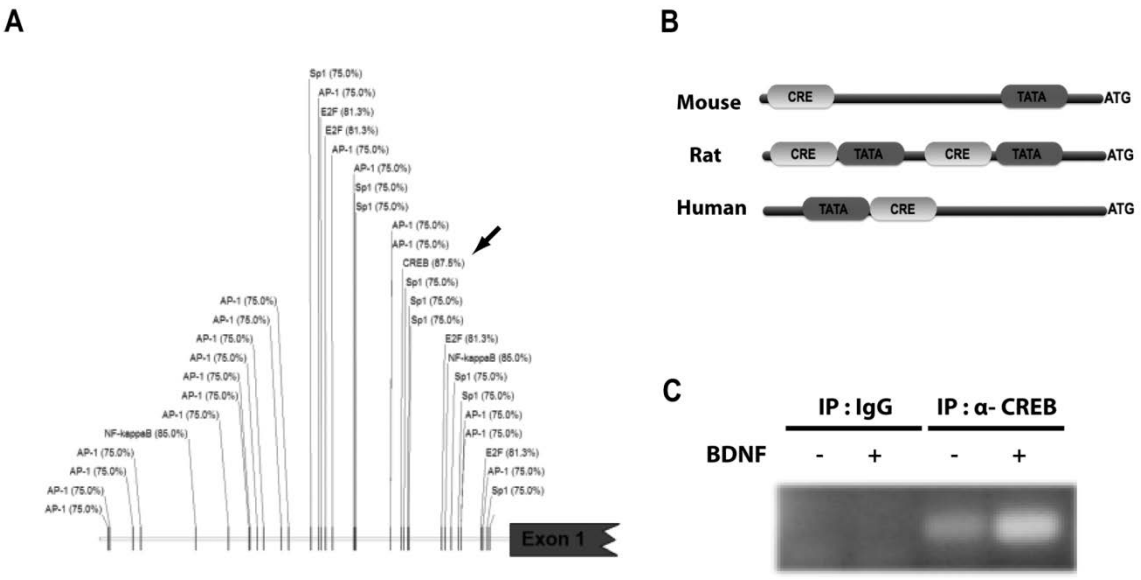


Figure 3-6. BDNF increases CREB binding to the cypin promoter.

A, Prediction of transcription factor binding sites in the promoter region of rat cypin. The 10 Kb upstream sequence of the first exon of cypin was used to predict consensus-binding sequences using the TF Search algorithm and TRANSFAC database (Heinemeyer et al., 1998). Each position is represented by the transcription factor and the binding probability (expressed as percentage of identity) is indicated as predicted by TFSearch-specific matrices. ***B,*** Conserved cAMP responsive element (CRE) regions are present in rodent and human cypin gene promoters. Genomic sequences from mouse, rat, and human were retrieved from the NCBI database up to 2 Kb upstream from the first exon of cypin. These sequences were analyzed for the occurrence of conserved CRE and TATA box regions. ***C***, Hippocampal neurons were treated with 25 ng/ml BDNF on DIV 7 for 24 hours. Chromatin was isolated on DIV 8, and ChIP was performed using specific primers to the cypin promoter. n = 3 experiments. A representative ChIP is shown.

BDNF activates CREB to increase cypin expression and dendrite branching

CREB is activated by its phosphorylation at Ser119 (equivalent position to Ser 133 in CREB-341 isoform B, NCBI accession number NP112279.1) to promote cellular gene expression, and this Ser119 phosphorylation of CREB is sufficient to recruit CBP-RNA polymerase II complexes and activate transcription of gene expression (Nakajima et al., 1997). We used a dominant negative mutant of CREB (CREB DN), in which serine 119 is mutated to alanine and cannot be phosphorylated, to further investigate the role of CREB in BDNF-mediated increases in cypin and dendrite number. Wildtype CREB (CREB WT) was used as a control, and BDNF treatment increased dendrite numbers in neurons expressing CREB WT (Figure 3-7A and B) as in other control neurons (i.e. Figure 3-2). Expression of CREB DN blocked BDNF-promoted increases in cypin expression (Figure 3-7A and B). We then tested whether CREB activation is sufficient to increase cypin levels. We used a constitutively active mutant of CREB (CREB CA) in which serine 119 is mutated to aspartate to mimic the phosphorylated state. Neurons that express CREB CA showed increased cypin protein levels. Furthermore, expression of CREB CA occludes BDNF-induced increases in cypin expression. Our data strongly suggest that BDNF activates CREB, which in turn increases cypin expression. We further investigated whether BDNF regulates dendritogenesis via CREB activation. Neurons were co-transfected with a dsRed-tagged CREB DN construct and eGFP containing vector to assess dendrite number. Sholl analysis shows that neurons that express CREB DN do not show increased proximal dendrite numbers when exposed to BDNF (Figure 3-8C). Our data support the idea that BDNF activates CREB to increase the expression of cypin, and as a result, proximal dendrite numbers.

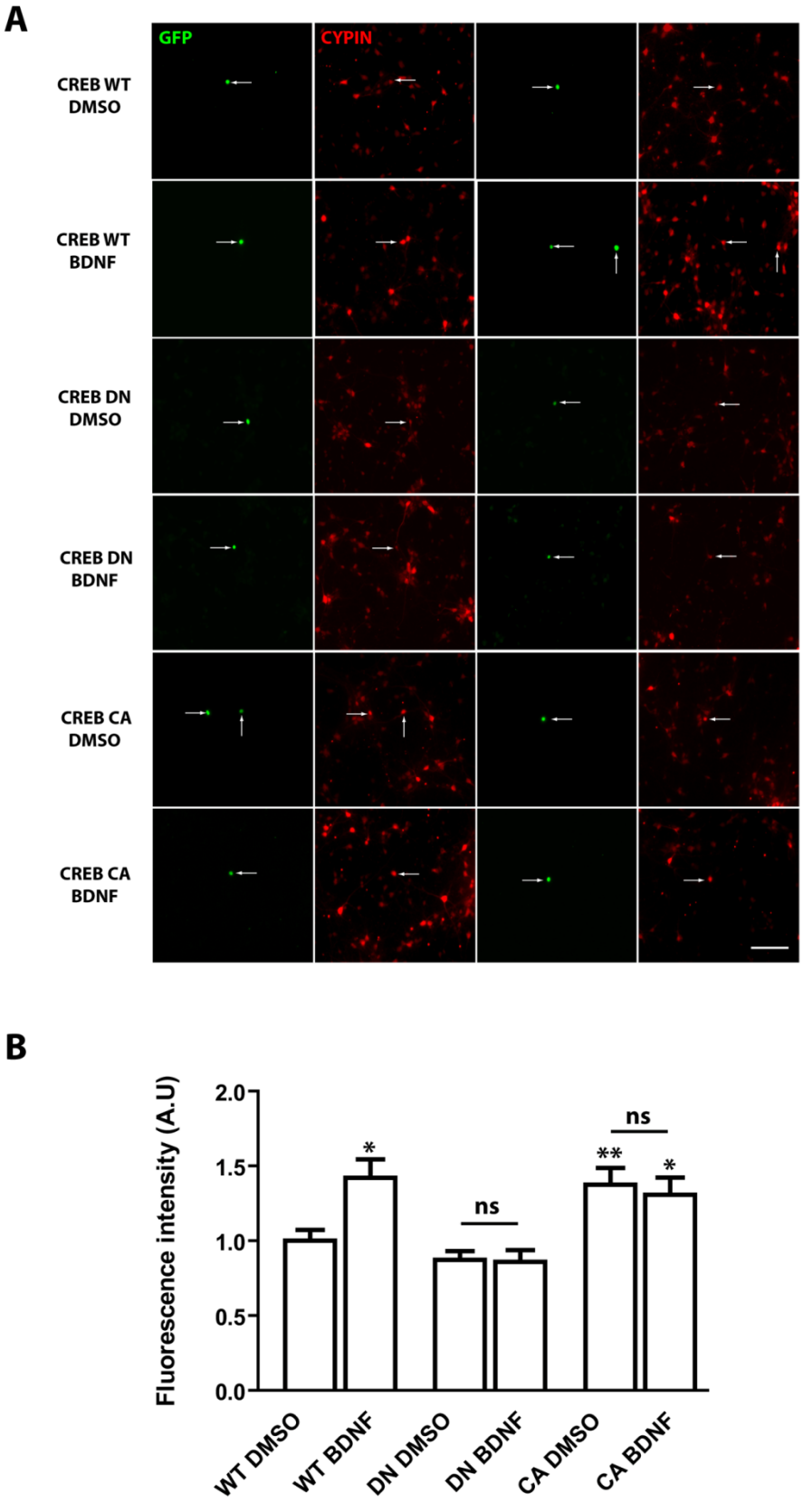
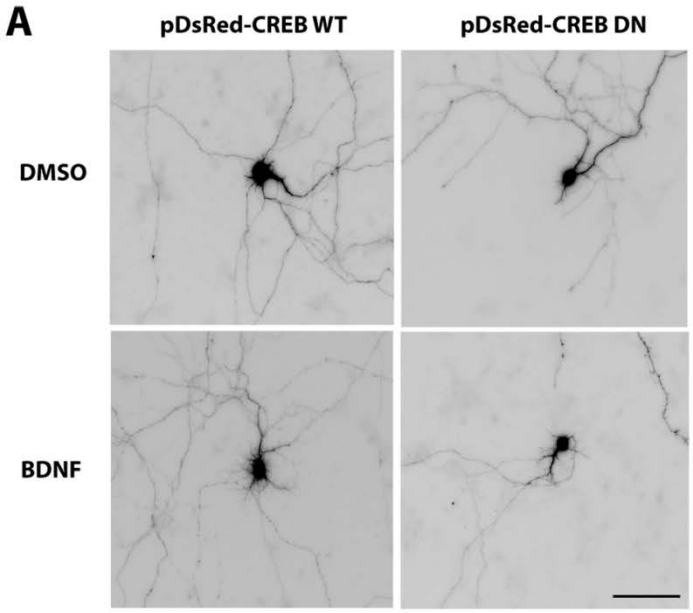
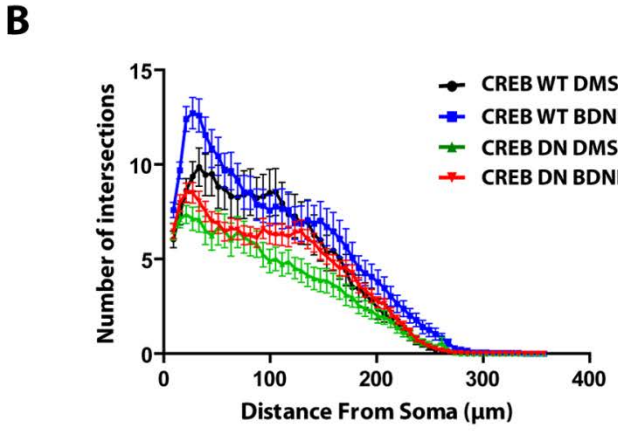

A
GFP
CYPIN
CREB WT DMSO
CREB WT BDNF
CREB DN DMSO
CREB DN BDNF
CREB CA DMSO
CREB CA BDNF
B
Fluorescence intensity (A.U)
2.0
1.5
1.0
0.5
0.0
*
ns
**
*
ns
WT DMSO
WT BDNF
DN DMSO
DN BDNF
CA DMSO
CA BDNF

Figure 3-7. Expression of a dominant negative mutant or a constitutively active mutant of CREB attenuates BDNF-promoted increases in cypin expression.

A, Hippocampal neurons were transfected with pEGFP-CREB WT (wild type, control)vector (control),with pEGFP-CREB DN (dominant negative form, Ser 119 Ala) or with pEGFP-CREB CA (constitutively active form, Ser 119 Asp) at 6 DIV and treated with either DMSO (vehicle) or BDNF (25 ng/ml) at 7 DIV for 72 hrs. Neurons were immunostained using a polyclonal cypin antibody. Arrows point to transfected neurons. Scale bar, 100 μm. ***B,*** Average fluorescence intensity (arbitrary units) of cypin immunostaining was assessed in individual cells. Expression of CREB DN blocks and expression of CREB CA occludes BDNF-promoted increases in cypin expression. n=20 neurons for each condition, $*p<0.05$, $**p<0.01$ by ANOVA followed Student-Newman-Keuls multiple comparison test compared to DMSO-treated neurons expressing CREB WT. ns, no significance compared to DMSO-treated neurons.


A
pDsRed-CREB WT
pDsRed-CREB DN
DMSO
BDNF


B
CREB WT DMSO
CREB WT BDNF
CREB DN DMSO
CREB DN BDNF
Number of intersections
Distance From Soma (µm)
0
5
10
15
0
100
200
300
400

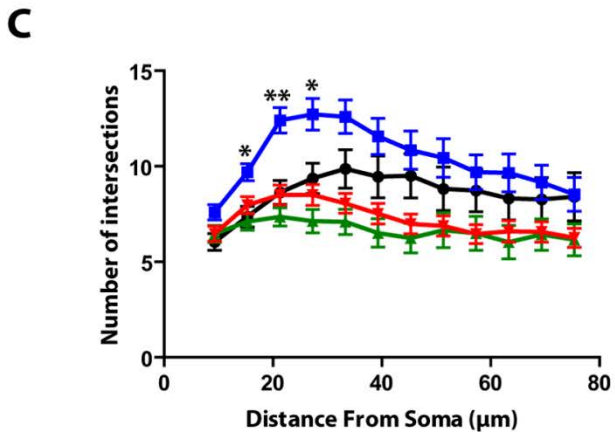

C
Number of intersections
Distance From Soma (µm)
*
**
*
0
5
10
15
0
20
40
60
80

Figure 3-8. Expression of a dominant negative mutant of CREB attenuates BDNF-promoted increases in dendrite number.

A, Hippocampal neurons were co-transfected with pEGFP and either dsRed-CREB WT(control) or dsRed-CREB DN at 6 DIV and treated with either DMSO (vehicle) or BDNF (25 ng/ml) at 7 DIV for 72 hrs. Representative GFP images are shown. Dendrite number was assessed at 10 DIV. Scale bar, 100 μm. ***B***, Sholl analysis. CREB DN overexpression blocks BDNF-promoted increases in the number of intersections close to the soma (proximal dendrites). ***C***, Proximal Sholl analysis (from ***B***) within the first 75 μm of the soma. *p<0.05 at 15 μm and 27 μm, **p<0.01 at 21 μm when comparing CREB WT + BDNF to CREB WT + DMSO and no significance when comparing CREB DN + DMSO to CREB DN + BDNF treatment. p values were determined by two-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate SEM. n = 22 neurons, CREB WT + DMSO; n=32, CREB WT + BDNF; n=33, CREB DN + DMSO; n=42, CREB DN + BDNF.

DISCUSSION

Dendrite number and branching are regulated by the interplay between extrinsic factors and intrinsic factors. Much progress has been made in identifying the key players and elucidating the signaling mechanisms that regulate dendrite morphology and patterning. There are a long list of external factors, including neurotrophins (Reviewed in (McAllister et al., 1996; McAllister, 2000)), estrogen (Audesirk et al., 2003; Sakamoto et al., 2003), and electrical activity (Vaillant et al., 2002). Some of the known intrinsic factors are the small GTPases RhoA, Rac1, and Cdc42 (Threadgill et al., 1997; Ruchhoeft et al., 1999; Li et al., 2000; Chen and Firestein, 2007), calcium/calmodulin-dependent protein kinase II (Fink et al., 2003), β-catenin (Yu and Malenka, 2004), CREST (Aizawa et al., 2004), Dishevelled (Rosso et al., 2005), some novel genes in *Drosophila* (Gao et al., 1999; Moore et al., 2002; Grueber et al., 2003; Emoto et al., 2004), cypin (Akum et al., 2004) and PSD-95 (Charych et al., 2006). Our laboratory has focused on cypin, a protein that regulates dendrite formation by promoting microtubule assembly and negatively regulates trafficking of PSD-95, which is associated with signaling networks at excitatory synapses and decreases dendrite branching. Our current studies are the first to demonstrate that extracellular factors, specifically BDNF, can regulate cypin gene expression.

Our results suggest that the MAPK pathway regulates BDNF-mediated cypin expression in hippocampal neurons. This signaling cascade is involved in promoting neuritogenesis and neurite outgrowth via CREB-mediated gene expression (Tojima et al., 2003). Therefore, we suggest that cypin is a candidate gene that is regulated by this signaling pathway via activation of CREB. Our results support this hypothesis since

pharmacological data confirm that a MEK-specific inhibitor and expression of a dominant negative form of CREB block BDNF-mediated increases in cypin expression.

Since BDNF is an important molecule for dendrite growth, much work has focused on elucidating BDNF signaling pathways. Ligand binding to TrkB triggers a cascade of phosphorylation events. Phosphorylated tyrosine residues on the receptor allow for Shc and phospholipase C (PLC) to attach. PLC becomes phosphorylated and activated (Segal, 2003). In addition to PLC, TrkB can signal via the PI3 kinase pathway. Tyrosine phosphorylation allows SHC and Grb2 to recruit PI3 kinase as well as SOS. PI3 kinase activates the AKT pathway, and SOS activates the Ras/MAPK cascade (Segal, 2003). In contrast, there are also several studies that support the idea that BDNF can regulate dendrite branching through the PI3 kinase pathway by activating the mammalian target of rapamycin (mTOR; (Jaworski et al., 2005; Kumar et al., 2005)), which can then activate the translation of a group of mRNAs (Schratt et al., 2004). In fact, local administration of BDNF to dendrites of cultured hippocampal neurons results in mTOR-dependent phosphorylation of S6, which is involved in local protein synthesis (Takei et al., 2004). While this increased phosphorylation was observed within 5 minutes of stimulation, our studies were performed using longer treatment times, suggesting the possibility that the duration of BDNF influences the activation of different signaling pathways. Recent studies support this idea that there would be the temporal and spatial regulation of signaling by BDNF in neurons and have shown that acute and gradual increases in BDNF give rise to differential expression of Homer1 and Arc in cultured hippocampal neurons (Ji et al., 2010). Moreover, mRNAs with the same BDNF coding sequence but distinct 3’ UTRs have distinct cellular localizations and function (An et al.,

2008), increasing the possibility that BDNF regulates neuronal morphology and function via several distinct cellular mechanisms. Our studies support the idea that increases in proximal dendrites resulting in response to BDNF do not depend on the activation of either PI3 kinase or mTOR (Figure 2-3), suggesting an alternative pathway of activation for BDNF-mediated increases in cypin expression and proximal dendrites in 72 hours.

The MAPK signaling cascade, which can be activated by BDNF, is involved in promoting neuritogenesis and neurite outgrowth via CREB-mediated gene expression (Tojima et al., 2003). Similarly, in cortical neurons, primary dendrite number is regulated by BDNF via MAPK signaling pathways, and this effect is independent of nascent protein synthesis (Dijkhuizen and Ghosh, 2005). This is in contrast to our results in hippocampal neurons, whereby BDNF increases cypin transcription and translation to regulate proximal dendrite number. These differences may be due to the fact that different neuronal types were studied. This idea is supported by recent evidence that neurite outgrowth is regulated differently in cortical and hippocampal neurons (Ko et al., 2005).

Differences in signal transduction pathways activated by hippocampal versus cortical neurons may also due to the BDNF receptor that is activated. Like other neurotrophins, BDNF exists in two states, proteolytically processed, which is the active form and can bind Trk receptors, or unprocessed, which allows BDNF to bind with high affinity to $p75^{NTR}$. Thus, the complex actions of BDNF on the dendritic arbor may be mediated, in part, by multiple receptors, resulting from alternative splicing of TrkB. Three alternative splice variants of TrkB have been identified. The full-length TrkB.FL contains a cytoplasmic tyrosine kinase domain, which is responsible for

autophosphorylation and clustering of the receptor when activated by BDNF (Chao, 2003; Segal, 2003). Two truncated isoforms, termed TrkB.T1 and TrkB.T2, lacking the cytoplasmic tyrosine-kinase domains, have also been identified (Barbacid, 1994). TrkB.FL and TrkB.T2 are expressed exclusively in neurons, and TrkB.T1 is expressed in both neurons and nonneuronal cells (Frisen et al., 1993; Rudge et al., 1994; Armanini et al., 1995; Biffo et al., 1995; Wetmore and Olson, 1995). Importantly, in rat, expression of the TrkB.FL in the hippocampus is barely detectable at El5 but increases throughout development to reach adult levels by P5 (Muragaki et al., 1995). Conversely, TrkB.FL reaches adult levels in cortex by P0. In addition, TrkB.T1 is expressed in the hippocampus at low levels until birth and reaches adult levels by P10-P15. The expression of TrkB.T1 in cortex is similar, but adult levels are reached between P10 and P20 (Muragaki et al., 1995). This difference in developmental expression pattern, which encompasses the developmental time points of our cultures, coupled with the fact that TrkB.FL controls proximal branching while TrkB.T1 increases elongation of distal dendrites in cortical culture (Yacoubian and Lo, 2000) may explain the different pathways activated by BDNF in our study and other studies. In addition, TrkB.FL can inhibit the effects of TrkB.T1 and vice versa when both receptors are overexpressed, adding an additional layer of receptor crosstalk (Yacoubian and Lo, 2000; Hartmann et al., 2004).

MAPK phosphorylates extracellular signal-regulated kinases (ERKs), and these activated ERKs phosphorylate CREB (Bonni et al., 1999). CREB has been characterized to be a critical molecule for the transcriptional regulation of dendritic complexity (Redmond and Ghosh, 2005; Wayman et al., 2006). Loss of CREB function has been

shown to impair dendrite growth and arborization of newborn hippocampal neurons (Jagasia et al., 2009). In line with these reports, we have found that cypin is a target gene for CREB regulation and that BDNF enhances the binding of CREB to the cypin promoter. Thus, we have demonstrated, by pharmacological and cell biological methods, that BDNF, an important extrinsic factor needed for normal neuronal development, activates CREB-mediated increases in the expression of cypin, an essential intrinsic regulator of microtubule assembly and dendrite arborization. Since BDNF is thought to play a role in the development of cognitive disorders (Zuccato et al., 2001; Neves-Pereira et al., 2002; Sklar et al., 2002; He et al., 2004; Schumacher et al., 2005; Strauss et al., 2005; Lynch et al., 2007; Nagahara et al., 2009; Peng et al., 2009), our results will add to our understanding of how dendrite arborization is disrupted in these disorders.

ACKNOWLEDGEMENTS

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Chapter 4. Local Administration of BDNF Regulates Dendrite Branching in a Different Manner than does Global Administration.

INTRODUCTION

The precise patterning of dendrites is important for the way that neurons receive information. There is tight regulation of the dendritic arborization process, and this regulation often occurs locally, such as at small regions of the dendrite. BDNF is one of well-studied extrinsic factors regulating dendrite development (McAllister et al., 1996; Baker et al., 1998; Jin et al., 2003; Segal, 2003). Recent studies demonstrate that BDNF is a mediator of activity-dependent dendritic branching (McAllister et al., 1996; Shooter, 2001). Treatment with BDNF causes dendrites to be more active; that is, dendrites are both gained and lost more quickly than when no treatment is present. Importantly, BDNF release from single cells elicits local dendritic growth in nearby neurons, and the source of BDNF has to be within 4.5 μm to induce dendritic growth in the recipient neuron (Horch and Katz, 2002), suggesting that application of global versus local BDNF may result in different effects on dendritic arbor. Furthermore, the proximal and distal dendrites are regulated by different mechanisms.

Primary dendrite number is regulated by BDNF via MAPK signaling pathways, independent of nascent protein synthesis, in cortical neurons (Shooter, 2001). In contrast, BDNF regulate dendrite branching through the PI3 kinase pathway by activating mTOR (Jaworski et al., 2005; Kumar et al., 2005) , which is involved in local translation of a group of mRNAs (Schratt et al., 2004). Local puff application of BDNF to dendrites of

hippocampal neurons in culture phosphorylates S6, which is the mediator of mTOR-induced local protein synthesis (Takei et al., 2004).

BDNF exists in two different states and binds multiple receptors to mediate signaling throughout the cells. Pro-BDNF, an unprocessed state, can bind to $p75^{NTR}$ with high affinity, while proteolytically processed active BDNF can bind to TrkB receptors. There are two truncated isoforms of TrkB receptors, TrkB.T1 and TrkB.T2. Truncated TrkB isoforms do not have cytoplasmic tyrosine kinase domains (Barbacid, 1994). There is a difference in developmental expression patterns of these isoforms (Muragaki et al., 1995), suggesting the possibility of differential control of dendritic development mediated by different receptors. In fact, full length TrkB increases proximal dendrite branching and TrkB.T1 increases elongation of distal dendrites when overexpressed in culture of cortical neurons (Yacoubian and Lo, 2000).

Little is known about the signaling pathways activated by truncated isoforms of TrkB, TrkB. T1 and TrkB.T2. Although TrkB.T1 is known to negatively regulate full length TrkB signaling (Eide et al., 1996; Ninkina et al., 1996; Fryer et al., 1997), TrkB.T1 has its own function in neurite outgrowth (Haapasalo et al., 1999; Yacoubian and Lo, 2000) and in cytoskeletal changes in astrocytes (Ohira et al., 2007) and glioma cells (Ohira et al., 2006) by regulating Rho GTPase activity (Ohira et al., 2005). TrkB.T1 has both ligand-independent (Yacoubian and Lo, 2000) and ligand-dependent activity (Hartmann et al., 2004). TrkB.T1 mediates inositol-1,4,5-trisphosphate-dependent calcium release (Rose et al., 2003) and does not affect gene expression (Offenhauser et al., 2002). BDNF activation of either TrkB.T1 or TrkB.T2 increases the rate of acidic

metabolite release from the cell (Baxter et al., 1997), suggesting that these receptors may activate known signal transduction pathways.

It is of importance to understand how neurotrophins activate signaling pathways at the site of dendritic stimulation. Here, we show how BDNF act differentially when they applied globally versus locally. By using beads coupled with BDNF, we revealed that local BDNF stimulation can affect changes in dendrites in a different manner than global BDNF application does.

MATERIALS AND METHODS

Primary culture of hippocampal neurons

Neuronal cultures were prepared from hippocampi of rat embryos at 18 days gestation as described previously (Firestein et al., 1999). The hippocampi were dissociated, and cells were plated on poly-D-lysine-coated 24-well plate at a density of 1800 cells/mm^2. Cultures were maintained in Neurobasal media (Gibco) supplemented with B27 (Gibco), penicillin, streptomycin, and Glutamax (Gibco). Cells were grown for 7 days *in vitro* (DIV) and used for specific experiments as indicated below.

Transfection of cultured cells

In order to visualize neurons, cultured hippocampal neurons were transfected at 6 DIV for cDNA encoding GFP using LIPOFECTAMINE LTX with PLUS reagent following the manufacturer's protocol (Invitrogen).

Preparation of BDNF-coated beads

Either monodisperse latex particles or fluorescent microspheres (641 nm for excitation wavelength; Polysciences, Inc. Warrington, PA) were used. Monodisperse latex particles that contain primary amine surface functional groups and a glutaraldehyde kit (Polyscience, Inc. Warrington, PA) was used for coupling BDNF to beads. After washing with PBS, beads (2.5% aqueous suspension) were incubated in 8% glutaraldehyde solution for 4-6 hours at room temperature. After washing with PBS, BDNF (200 μg) was coupled to the beads overnight at room temperature with gentle end-to-end mixing.

BDNF protein can bind 11-12 carbon atoms from the surface of the beads. BDNF-coupled beads were resuspended in 0.2 M ethanolamine to block unreacted sites on the microparticles. Beads were stored in a solution containing BSA to block any remaining nonspecific protein binding sites. Alternatively, carboxylate groups on fluorescent monodispersed polystyrene microspheres were activated for covalent coupling of BDNF proteins. BDNF was coupled using Protein Coupling Kit (Polyscience, Inc. Warrington, PA) following the manufacturer's protocol. Briefly, microparticles were resuspended in coupling buffer (50 mM MES, pH 5.2, 0.05% Proclin-300) and then activated by adding 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) solution. BDNF (200 µg) was incubated with activated microparticles for 1 hour at room temperature with gentle end-to-end mixing. After washing with wash buffer (10 mM Tris, pH 8.0, 0.05% Bovine Serum Albumin, 0.05% Proclin-300), BDNF-coupled particles were resuspended in wash buffer and stored at 4°C. BSA was used as control protein for coupling to both particles.

Treatment with BDNF-coated beads

To mimic local exposure to BDNF, neurons were treated with 1 or 3 µm latex beads or 1.75 µm fluorescent beads. Beads (10 or 20 µl of a 0.0625% aqueous suspension) either coupled to BDNF or prepared in the same manner without BDNF in the coupling solution (BSA-coated, control) were used for each well of a 24-well plate (10^7 beads/well). Each well contained 0.7 ml of medium. Cultures were treated with BDNF-coated beads for indicated duration at day *in vitro* (DIV) 7 prior to imaging.

Dendrite assessment

Images were taken as stated in Chapter 3 using an Olympus Optical (Tokyo, Japan) IX50 microscope with a Cooke Sensicam charge-coupled device cooled camera fluorescence imaging system and Image Pro software (Media Cybernetics, Silver Spring, MD). The plate containing the cultures remained on a heated stage during the duration of imaging. Bright-field images were taken for neurons treated with latex particles for visualizing the location of beads. Fluorescent images were taken to assess neuron and bead location for fluorescent beads. Dendrites were assessed in the following ways:

1) Percentage of sites stimulated with BDNF-coated beads or control beads that shows dendrite branching within approximately 10 μm from the bead.
2) Number of dendrite branches extending out from the stimulated region.
3) Order of dendrites that were stimulated by BDNF-coated beads or control beads.
4) Overall branching pattern of dendrites compared to the pattern following global BDNF administration.

We performed Sholl analysis with a 6 μm ring interval starting at 9.3 μm from the soma. The experimenter was blinded to conditions during all data analysis. Dendrites less than 3 μm in length were not counted (Yu and Malenka, 2004; Charych et al., 2006).

RESULTS

Local BDNF source can be mimicked by treatment of BDNF-coated microbeads.

To mimic a local BDNF source at dendrites, we seeded microbeads with covalently bound BDNF within the neuronal culture and allowed then to make a contact with a dendritic segment. Microbeads were spread throughout the neurons from proximal to distal area. As seen in Figure 4-1, microbeads made multiple contacts with dendrites. Dendrite branching at bead sites increases from approximately 20-25% in control to over 60% in dendrites contacting BDNF-coated beads (Figure 4-1B). Interestingly, neurons treated for 24 hours or less do not show increased branching, while the percentage of branching at sites stimulated with BDNF versus control is much higher in neurons treated for 48 hours and 72 hours (Figure 4-2A). To understand how this localized application affects dendrite order, we analyzed the percentage of branching by the beads for each order of dendrites. Increased branching induced by localized beads does not appear to be specific for any order of dendrites (Figure 4-2B). These results suggest that local stimulation by BDNF increases local dendrite branching at proximity of stimulated sites independent of branch order.

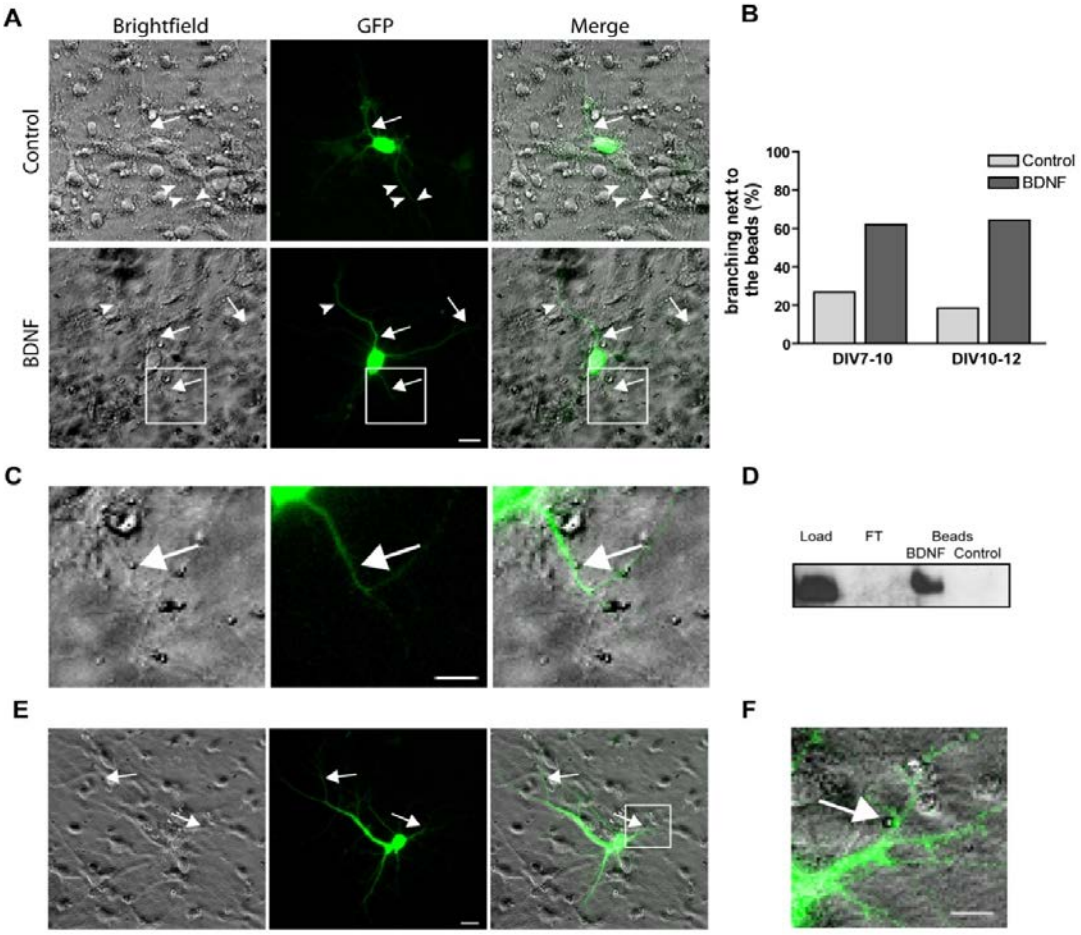
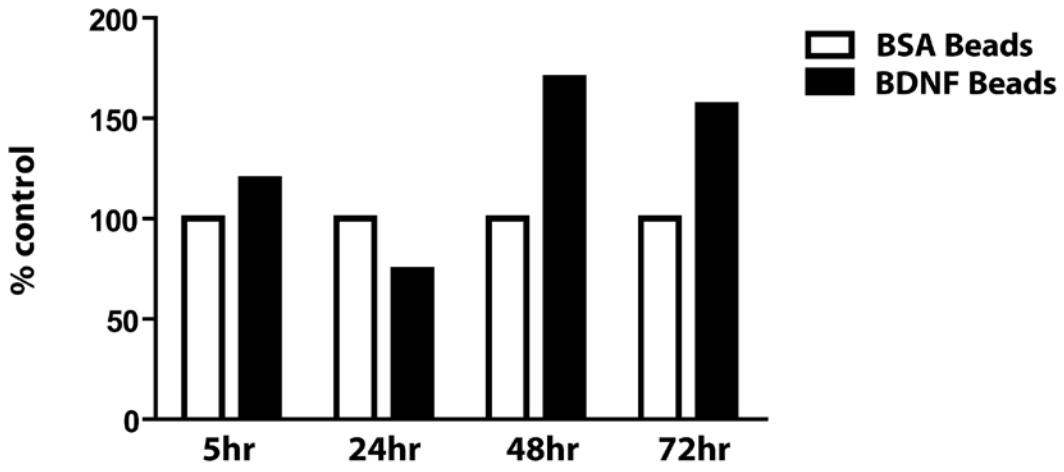

A
Brightfield
GFP
Merge
Control
BDNF
B
branching next to the beads (%)
100
80
60
40
20
0
Control
BDNF
DIV7-10
DIV10-12
C
D
Load
FT
Beads
BDNF
Control
E
F

Figure 4-1. Local treatment of dendrites with BDNF by use of BDNF-coated latex microbeads.

Hippocampal neurons overexpressing GFP were treated with BDNF-coated or control microbeads. ***A***, Representative brightfield and GFP fluorescence pictures of neurons treated from DIV 10 to DIV 12 with 1 μm beads. Arrows point to bead-dendrite contact site close to a branching point. Arrowheads point to sites without branching point. ***B***, percentage of sites stimulated with control versus BDNF-coated 1 μm beads that show dendrite branching within approximately 10 μm from the bead (for DIV 7-10, n=41 control sites, n=45 BDNF sites; for DIV 10-12, n=27 control sites, n=28 BDNF sites). ***C***, Region of ***A*** at a higher magnification, showing a BDNF-coated bead touching a dendrite and in proximity of a branching point. ***D***, Western blot analysis of BDNF coating efficiency. BDNF found in load control is also present in the beads lane but is absent in the flow-through (FT) and in the control beads lane. ***E***, Brightfield and fluorescence pictures of neurons treated from DIV10 to 12 with BDNF-coated 3 μm beads. ***F***, Region of E at a higher magnification, showing a BDNF-coated bead touching a dendrite and in proximity of a branching point. Scale bars, 20 μm (***A***, ***C***) and 10 μm (***E***).

A


200
150
100
50
0
% control
5hr
24hr
48hr
72hr
BSA Beads
BDNF Beads

B

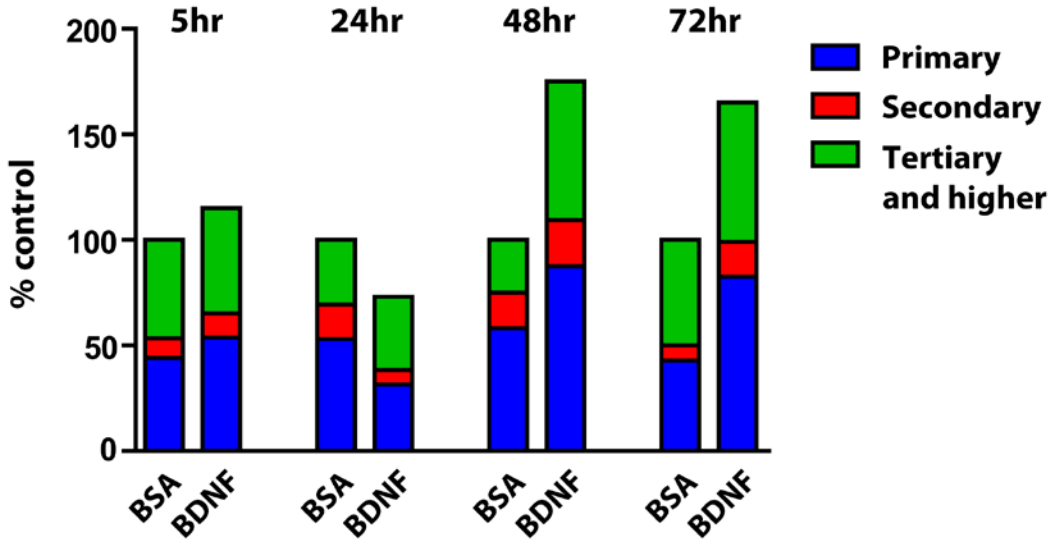

5hr
24hr
48hr
72hr
200
150
100
50
0
% control
BSA
BDNF
Primary
Secondary
Tertiary
and higher

Figure 4-2. Local BDNF increases local dendrite branching.

A, Percentage of sites stimulated with BSA-coated (control) versus BDNF-coated (1.75 μm) beads that show dendrite branching within approximately 10 μm from the bead. ***B***, Order-specific distribution of percentage branching next to the bead. n=300 sites (beads), 5hr BSA beads; n= 358, 5hr BDNF beads; n =286, 24hr BSA beads; n=580, 24hr BDNF beads; n = 347, 48hr BSA beads; n=428, 48hr BDNF beads; n = 120, 72hr BSA beads; n=104, 72hr BDNF beads. Chi-squared analysis shows that 24 hour treatment, but not 5, 48, or 72 hour, results in a greater effect on primary and secondary dendrites versus branching in control cultures.

Localized BDNF treatment increases overall dendrite branching in a different manner than does global treatment.

We performed Sholl analysis on neurons treated with control (BSA-coated) or BDNF-coated beads for 5 hours, 24 hours, 48 hours and 72 hours starting on DIV7 to investigate how local treatment of BDNF changes overall dendritic arbor shape. We found that treatment for 24 hours or less does not have a significant effect on the overall dendritic arbor; however, there is a significant increase in dendrites when neurons are treated for 48 hours and more, in line with branching percentage data shown in Figure 4-2. As seen in Figure 4-3C, intersections in the distal area (93 μm to 159 μm from soma) increase with treatment with BDNF-coated beads for 48 hours, an effect not seen with global treatment with BDNF. Interestingly, 72 hour treatment with BDNF-coated beads increases proximal dendrites as does global treatment (Figure 3-2); however, the beads also significantly increase distal dendrites (Figure 4-3D) unlike that seen with global BDNF treatment. We also investigated how the percentage branching next to the beads is distributed along the distance from the soma. As shown in Figure 4-4, neurons treated with BDNF-coated beads for 48 hours or more have increased branching sites at distal regions, 100 μm and further from the soma. This suggests that the two treatments, global versus beads, signal differently to increase dendrites.

Interestingly, when we performed order-specific Sholl analysis, we found that 24 treatment of neuron with BDNF beads for 24 hours or less significantly increases proximal primary dendrites (Figure 4-5A, D, G, J). Furthermore, an increase in secondary dendrites 50-75 μm from the cell body was seen at all time points (Figure 4-5B, E, H, K). Lastly, increases tertiary and higher order dendrites 100-200 μm from the cell body was

seen only when neurons were treated with BDNF beads for 48 hours (Figure 4-5C, F, I, L). To assess the mechanism that underlies changes in order-specific increases in dendrite branching, we plotted the data differently, with dendrite order-specific Sholl analysis grouped for each condition. As seen in Figure 4-6, primary dendrites are not pruned for either condition. Secondary, tertiary, and higher order dendrites are pruned when comparing control treatment at 24 hours and later versus 5 hours; however, it appears that treatment with BDNF-coated beads blocks this pruning. In fact, it has been shown that neurons undergo pruning during development (Cline, 2001; Wong and Ghosh, 2002; Charych et al., 2006), and BDNF-$p75^{NTR}$ is involved in axonal pruning (Singh et al., 2008). These data suggest that local treatment with BDNF increases dendrite number not by adding dendrites but by blocking pruning.

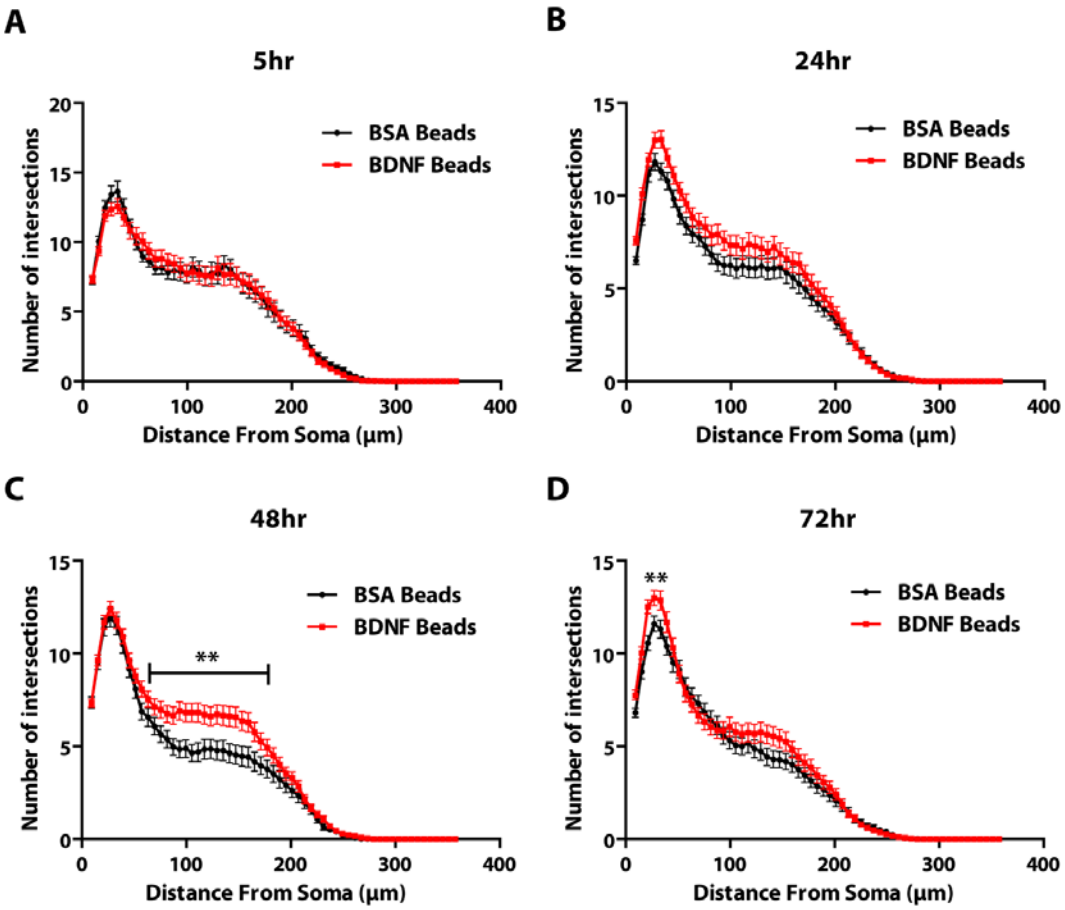

A
5hr
BSA Beads
BDNF Beads
Number of intersections
Distance From Soma (µm)
B
24hr
BSA Beads
BDNF Beads
Number of intersections
Distance From Soma (µm)
C
48hr
BSA Beads
BDNF Beads
**
Number of intersections
Distance From Soma (µm)
D
72hr
**
BSA Beads
BDNF Beads
Number of intersections
Distance From Soma (µm)

Figure 4-3. Local BDNF treatment increases dendrite branching.

A-D, Sholl analysis. Neurons were treated with either BSA-coated beads (control) or BDNF-coated beads at DIV7 for the indicated durations. Treatment with BDNF-coated beads increases the number of intersections not only close to the soma (proximal dendrites) but also distal from soma (distal dendrites) when treatment occurs for 48 hours or more. $^{**}p<0.01$ from 93 μm to 159 μm for 48 hr treatment (***C***) when comparing neurons treated with BDNF-coated beads to those treated with BSA-coated beads. $^{**}p<0.01$ from 69 μm to 93 μm and at 147 μm, and $^{*}p<0.05$ from 153 μm to 165 μm for 72 hr treatment (***D***). *p* values were determined by two-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate SEM. n = 40 neurons, 5hr BSA beads; n=50, 5hr BDNF beads; n = 71, 24hr BSA beads; n=75, 24hr BDNF beads; n = 64, 48hr BSA beads; n=93, 48hr BDNF beads; n = 56, 72hr BSA beads; n=75, 72hr BDNF beads.

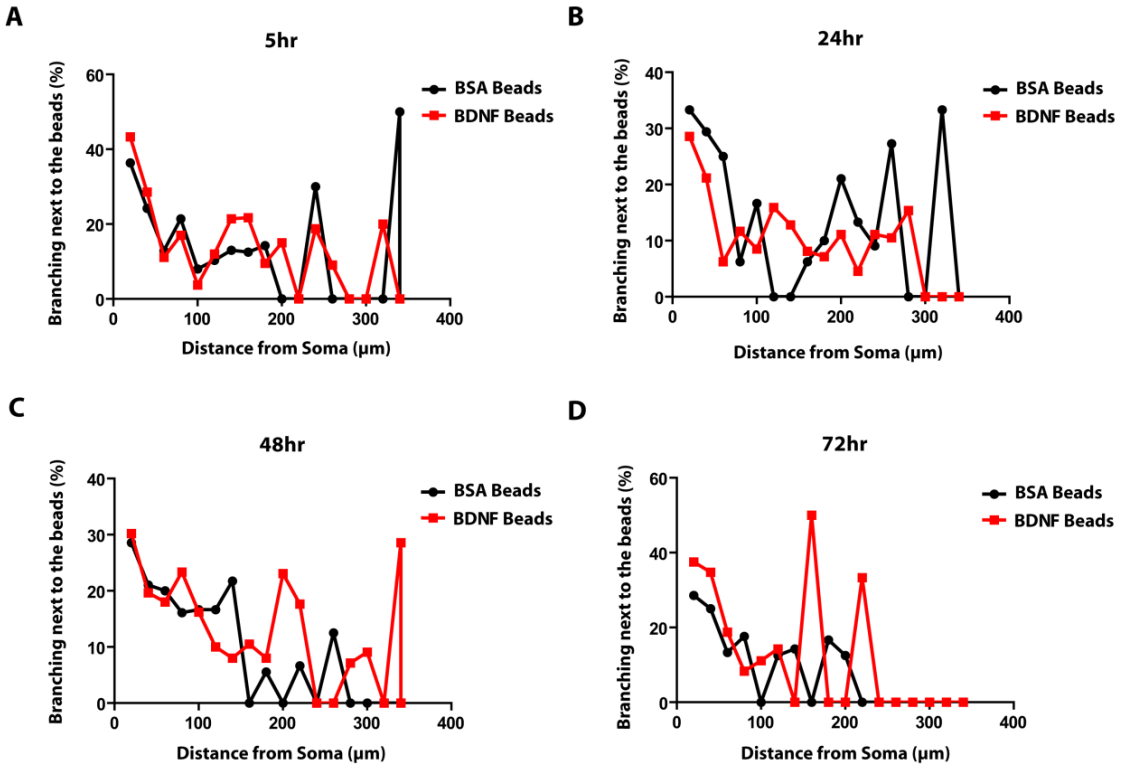


Figure 4-4. Localized BDNF treatment increases dendrite branching distally from the soma.

A-D, Percentage of sites with dendrite branching when stimulated with BSA-coated (control) versus BDNF-coated beads as a function of distance from the soma. After 48hr treatment with BDNF-coated beads, more branching occurs in distal dendrites (further than 100 µm). *** $p<0.001$ for 48 hr treatment, ** $p<0.01$ for 5 hr treatment, and * $p<0.05$ for 24 hr and 72 hr treatment when p values were determined by Chi-square test for trend. n=300 sites (beads), 5hr BSA beads; n= 358, 5hr BDNF beads; n =286, 24hr BSA beads; n=580, 24hr BDNF beads; n = 347, 48hr BSA beads; n=428, 48hr BDNF beads; n = 120, 72hr BSA beads; n=104, 72hr BDNF beads.

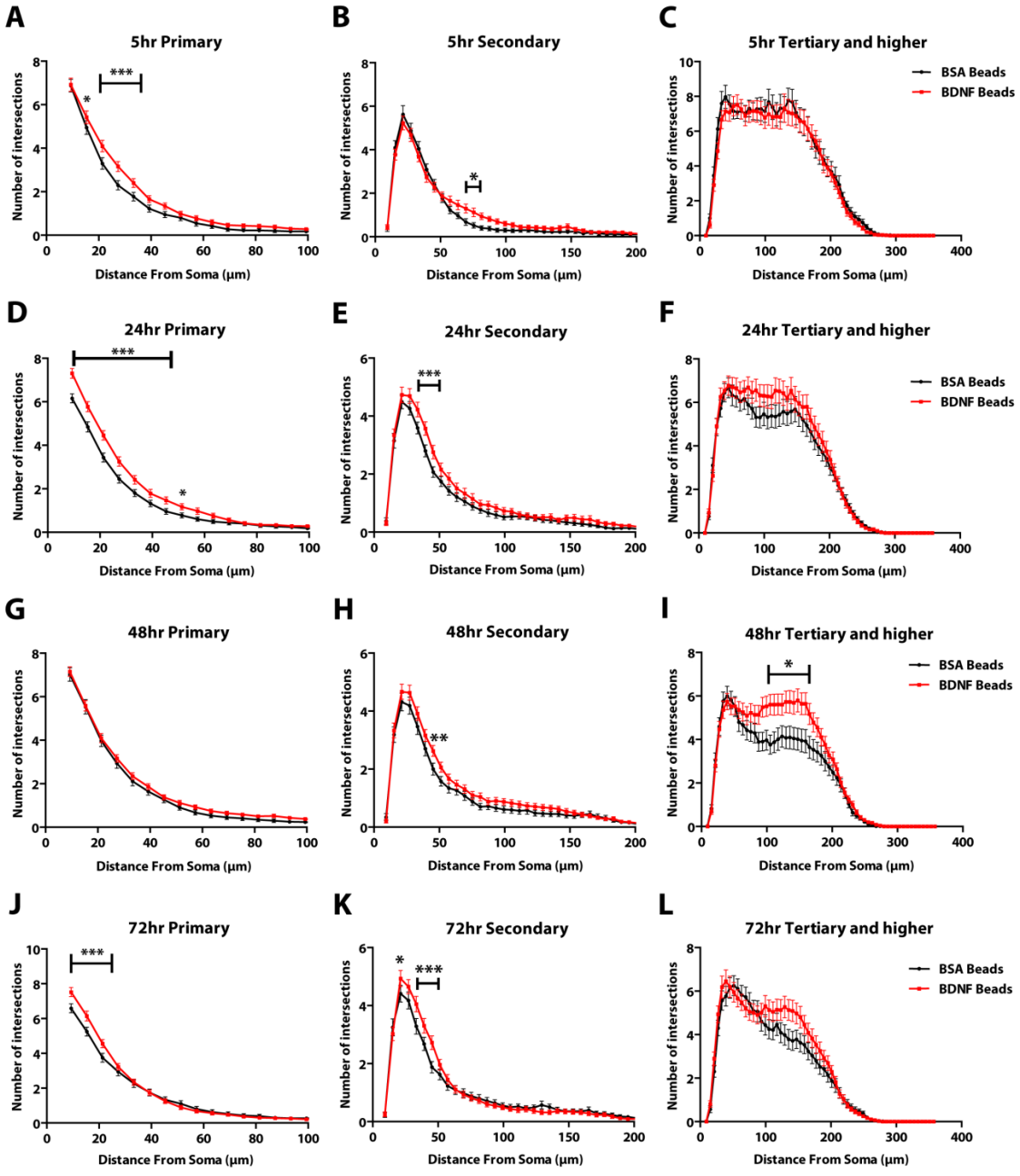

A
5hr Primary
B
5hr Secondary
C
5hr Tertiary and higher
BSA Beads
BDNF Beads
D
24hr Primary
E
24hr Secondary
F
24hr Tertiary and higher
G
48hr Primary
H
48hr Secondary
I
48hr Tertiary and higher
J
72hr Primary
K
72hr Secondary
L
72hr Tertiary and higher
Number of intersections
Distance From Soma (µm)

Figure 4-5. Local BDNF treatment increases dendrite branching in an order-specific manner.

A-L, Sholl analysis of specific branch orders in the dendritic field. Neurons were treated with either BSA-coated beads (control) or BDNF-coated beads at DIV7 for the indicated durations. Treatment with BDNF-coated beads increases the number of all orders of dendrites. *p<0.05 at 15 μm for primary and from 63 μm to 69 μm for secondary, and ***p<0.001 from 21 μm to 33 μm for primary in 5 hr treatment (***A-C***) when comparing neurons treated with BDNF-coated beads to those treated with BSA-coated beads. ***p<0.001 from 9 μm to 45 μm for primary, and 39 μm to 45 μm for secondary in 24 hr treatment, **p<0.01 at 39 μm for primary and at 33 μm for secondary for secondary , and *p<0.05 at 51 μm for primary in 24 hr treated neurons (***D-F***). **p<0.01 at 45 μm for secondary and at 105 μm for tertiary and higher, and *p<0.05 at 111 μm and from 129 μm to 159 μm for tertiary and higher in 48 hr treatment (***G-I***). ***p<0.001 from 9 μm to 21 μm for primary and 33 μm and 45 μm for secondary, **p<0.01 at 39 μm, and *p<0.05 at 21 μm for secondary in 72 hr treated neurons (***J-L***). p values were determined by two-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate SEM. n = 40 neurons, 5hr BSA beads; n=50, 5hr BDNF beads; n = 71, 24hr BSA beads; n=75, 24hr BDNF beads; n = 64, 48hr BSA beads; n=93, 48hr BDNF beads; n = 56, 72hr BSA beads; n=75, 72hr BDNF beads.

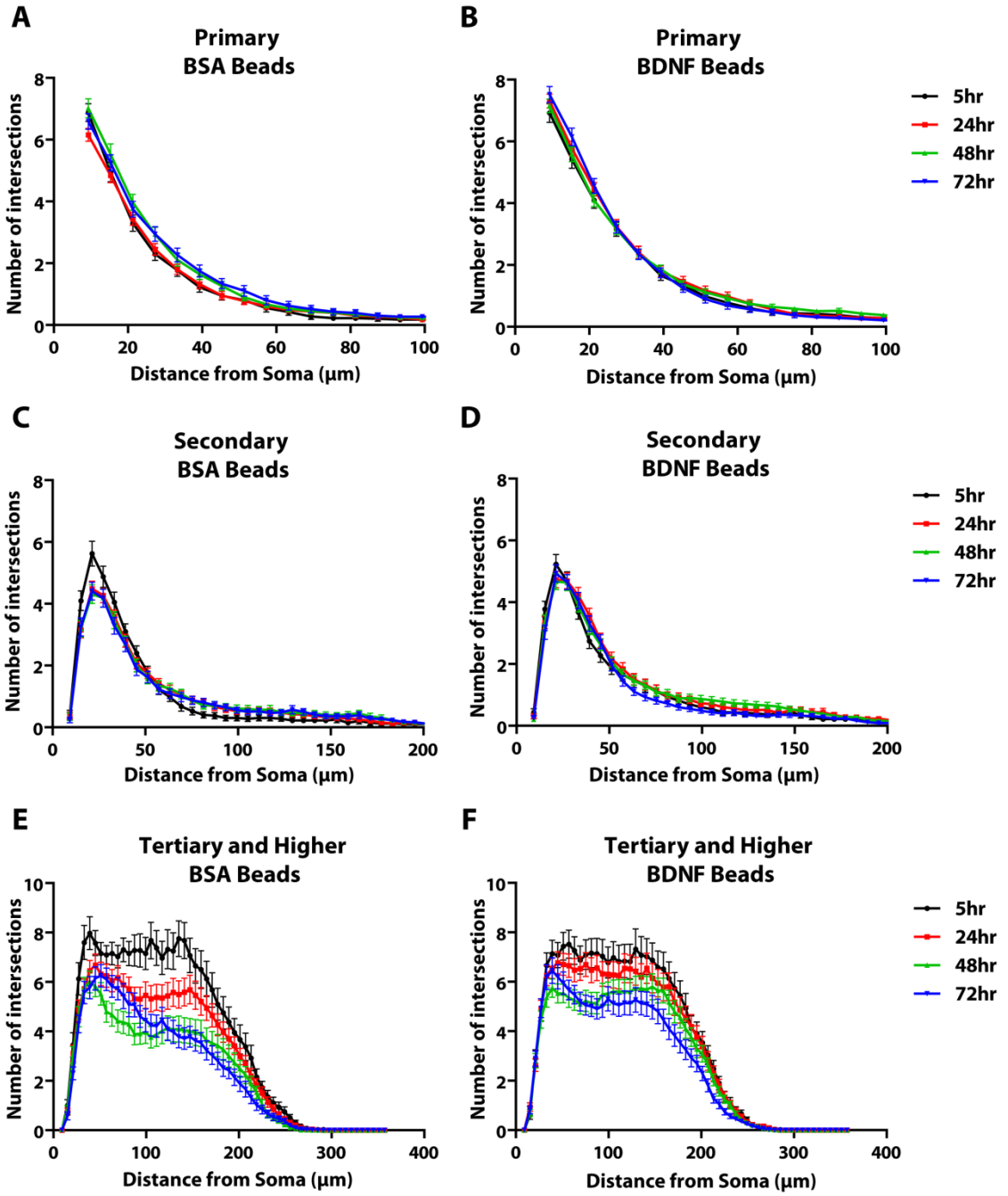

A
Primary
BSA Beads
Number of intersections
Distance from Soma (µm)
B
Primary
BDNF Beads
Number of intersections
Distance from Soma (µm)
5hr
24hr
48hr
72hr
C
Secondary
BSA Beads
Number of intersections
Distance from Soma (µm)
D
Secondary
BDNF Beads
Number of intersections
Distance from Soma (µm)
E
Tertiary and Higher
BSA Beads
Number of intersections
Distance from Soma (µm)
F
Tertiary and Higher
BDNF Beads
Number of intersections
Distance from Soma (µm)

Figure 4-6. Local beads treatment blocks pruning of dendrites.

A-F, Sholl analysis of specific branch orders in the dendritic field by duration of bead treatment. Neurons were treated with either BSA-coated beads (***A***, ***C***, ***E***) or BDNF-coated beads (***B***, ***D***, ***F***) at DIV 7 for the indicated durations. Neurons undergo massive pruning of dendrites from DIV7 to DIV9 in secondary and tertiary and higher when treated with control (BSA-coated) beads, while little pruning occurs when treated with BDNF-coated beads. Statistics not shown on graph (see Table 4-1). Error bars indicate SEM. n = 40 neurons, 5hr BSA beads; n=50, 5hr BDNF beads; n = 71, 24hr BSA beads; n=75, 24hr BDNF beads; n = 64, 48hr BSA beads; n=93, 48hr BDNF beads; n = 56, 72hr BSA beads; n=75, 72hr BDNF beads.

		* $p<0.05$	** $p<0.01$	*** $p<0.001$
Primary BSA beads (A)	5 hr vs. 24 hr			9 μm
	5 hr vs. 48 hr		15 μm	21, 27 μm
	5 hr vs. 72 hr	33, 39 μm		27 μm
	24 hr vs. 48 hr		27 μm	9 -21 μm
	24 hr vs. 72 hr	9-21, 39 μm	27, 33 μm	
	48 hr vs. 72 hr	9 μm		
Primary BDNF beads (B)	5 hr vs. 24 hr			
	5 hr vs. 48 hr			
	5 hr vs. 72 hr	21 μm		9-15 μm
	24 hr vs. 48 hr			
	24 hr vs. 72 hr			27 μm
	48 hr vs. 72 hr	9 μm	21 μm	15 μm
Secondary BSA beads (C)	5 hr vs. 24 hr	27 μm		15, 21 μm
	5 hr vs. 48 hr	33 μm	27 μm	15, 21 μm
	5 hr vs. 72 hr		27 μm	15, 21,33 μm
	24 hr vs. 48 hr			
	24 hr vs. 72 hr			
	48 hr vs. 72 hr			
Secondary BDNF beads (D)	5 hr vs. 24 hr			39 μm
	5 hr vs. 48 hr	21 μm		
	5 hr vs. 72 hr			15 μm
	24 hr vs. 48 hr			
	24 hr vs. 72 hr			
	48hr vs. 72 hr			
Tertiary and higher BSA beads (E)	5 hr vs. 24 hr	9, 87, 93,111,123 μm	105, 135, 145 μm	
	5 hr vs. 48 hr	39 μm	57, 171 μm	63-165 μm
	5 hr vs. 72 hr	33, 81, 189 μm	39, 87, 177, 183 μm	93-171 μm
	24 hr vs. 48 hr	69, 147 μm		
	24 hr vs. 72 hr	135-141, 153-165 μm	147 μm	
	48 hr vs. 72 hr			
Tertiary and higher BDNF beads (F)	5 hr vs. 24 hr			
	5 hr vs. 48 hr	51, 63, 75-87 μm	57 μm	
	5 hr vs. 72 hr	75, 93, 129-147, 159-177 μm	81, 87 μm	
	24 hr vs. 48 hr			
	24 hr vs. 72 hr	165 μm		
	48 hr vs. 72 hr			

Table 4-1. *p* values in Figure 4-6.

p values were determined by two-way ANOVA followed by Bonferroni multiple comparisons test.

DISCUSSION

Dendrite morphology regulates how a postsynaptic neuron receives information from presynaptic neurons. The specific patterning of dendrite branches is controlled by extrinsic and intrinsic factors that trigger the activation of functional signaling pathways. Current research shows that extrinsic factors can modulate specific patterns of dendritic growth and branching by activating intrinsic cues. Multiple studies were performed by treating or altering protein expression in the entire neuron. *In vivo*, however, local regulation occurs in the small region of neurites. To understand how dendrite patterning is regulated globally and locally, we examined the role of BDNF, one of the critical extrinsic factors regulating dendritic arborization, in hippocampal culture.

Our results suggest the possibility that distinct mechanisms are involved in regulating neurite outgrowth and patterning by different sources of BDNF. Our current data shows that different types of stimulation with BDNF affect different regions of dendrites, and others have shown that local BDNF application in cultured hippocampal neurons phosphorylates S6 protein, which is involved in mTOR-dependent local protein synthesis (Takei et al., 2004). As seen in Chapter 3 and published data (Kwon et al., 2011), global BDNF treatment increases proximal dendrite branching by activating the MAPK signaling pathway. Phosphorylated CREB activated by this signaling pathway binds to the cypin promoter to increase cypin transcription and protein levels, resulting in increased dendrite number close to the soma. In contrast, localized treatment using BDNF-coated microbeads showed increased branching in distal areas of the dendrite, suggesting that distinct regulation between proximal and distal regions exists.

Recent studies show that different transcript variants of BDNF, which have

distinct 3'UTRs, show different localizations and functions (An et al., 2008), and acute versus chronic exposure of neurons to BDNF influences activation of different transcription factors (Ji et al., 2010). BDNF signals through multiple receptors, including TrkB.FL, truncated isoforms, and $p75^{NTR}$, suggesting that BDNF can activate distinct signal transduction pathways. There are variations in expression and localization of these receptors in brain regions (Fryer et al., 1996; Ohira et al., 1999; Ohira and Hayashi, 2003), suggesting that these distributions might regulate localized BDNF function. BDNF is secreted from neurons and astrocytes (Rudge et al., 1994), and these different sources, form of BDNF (pro- form vs. mature form), and location of BDNF release, could be involved in mediating the complex actions of BDNF.

Interestingly, our data suggest that local BDNF treatment reduces the pruning of secondary and higher order dendritic branches during DIV7-9, when compared to treatment with control BSA-coated beads. There is a report that BDNF-p75NTR is important for developmental axonal pruning (Singh et al., 2008), supporting the idea that different signaling pathway is involved in BDNF-dependent regulation of dendrite branching.

The characterization of signaling pathways using pharmacological agents and overexpression and knockdown studies are needed to understand how localized BDNF regulates dendritic arborization. Furthermore, these studies will help us to understand the molecular mechanisms that underlie learning and memory mediated by plasticity in neuronal dendrite branching.

Chapter 5. Cypin Regulates Postsynaptic Density-95 (PSD-95) protein levels.

INTRODUCTION

Neurons transfer electrical or chemical signals to other neurons via synapses. The appropriate number and type of connections are necessary for normal brain function. Defects in synaptogenesis cause a number of cognitive disorders, including autism and Alzheimer's disease. Postsynaptic density protein-95 (PSD-95), member of membrane-associated guanylate kinase family, is an essential scaffolding protein, which assembles signaling complexes at excitatory synapses (Cho et al., 1992; Kistner et al., 1993; Brenman et al., 1996; Kim et al., 1998). Its PDZ domains, protein-protein interaction motifs (Kim and Sheng, 2004), bind to other PDZ domains and to various receptors and ion channels (Kim et al., 1995; Kornau et al., 1995; Cohen et al., 1996) that contain the C-terminal PDZ binding consensus sequence Thr/Ser-X-Val/Ile-COOH (Kornau et al., 1995; Sheng and Wyszynski, 1997). Thus, by aggregating neurotransmitter receptors and downstream enzymes, these proteins mediate subcellular protein compartmentalization, ensuring selective activation of different signal-transduction cascades within a single cell (Scott and Zuker, 1997, 1998). PSD-95 also promotes spine formation and maturation at synaptic sites (Okabe et al., 1999; El-Husseini et al., 2000; Prange and Murphy, 2001). The regulation of PSD-95 expression/degradation, clustering, and localization may underlie the regulation of spine morphogenesis and density, which are required for the

establishment and maintenance of proper synaptic connections during development and throughout life (Matus, 2000).

In addition to its synaptic function, we have recently found that PSD-95 also exhibits a nonsynaptic function during hippocampal neuron dendritic development (Charych et al., 2006). PSD-95 acts as a stop signal for proximal dendrite branching, resulting in fewer dendrites when overexpressed in cultured hippocampal neurons. Conversely, attenuation of endogenous PSD-95 expression, by a mutated 5' end U1 snRNA or by antisense oligonucleotide knockdown, results in an increase in the number of dendrites. Furthermore, this effect of PSD-95 on proximal dendrite branching is activity-independent (Charych et al., 2006), and PSD-95 inhibits dendrite branching by disrupting microtubule organization (Sweet et al., 2011). The src homology 3 (SH3) domain of PSD-95 directly interacts with a proline-rich region of end-binding protein 3 (EB3), protein that mediates interactions between the plus-ends of microtubules, organelles, and protein complexes, and alters microtubule stability (Nakagawa et al., 2000; Komarova et al., 2005; Lansbergen and Akhmanova, 2006). Taken together, the data suggest that PSD-95 shapes dendritic morphology in young neurons and is involved in the formation and/or stabilization of dendritic spines in more mature neurons. In addition, PSD-95 participates in the construction of the postsynaptic density by assembling synaptic signaling complexes with various receptors and signaling molecules.

Cypin (cytosolic PSD-95 interactor) was described as a protein that decreases the synaptic localization of PSD-95 and other related proteins called MAGUKs (Firestein et al., 1999)). When cypin is co-expressed with PSD-95 or the related SAP-102, the number of synaptic clusters of these proteins significantly decreases. Expression of a cypin

mutant, lacking the PDZ-binding motif, does not influence the clustering of PSD-95 or SAP-102. Cypin may be important for the establishment of synaptic NMDA receptor signaling complexes by blocking protein localization at the PSD. Cypin is also a guanine deaminase, an enzyme that catalyzes the breakdown of guanine to xanthine and ammonia (Yuan et al., 1999; Paletzki, 2002; Fernandez et al., 2008). It contains 454 amino acids with a zinc-binding motif and a four-residue PDZ binding motif at the C-terminus, which is responsible for its interaction with PSD-95 in neurons (Firestein et al., 1999). In addition, cypin regulates the number of primary and secondary dendrites in cultured rat hippocampal neurons by promoting microtubule assembly (Akum et al., 2004). Importantly, both the binding of cypin to PSD-95 and cypin's guanine deaminase activity are important for regulating dendrite branching (Akum et al., 2004; Charych et al., 2006; Fernandez et al., 2008). However, we have not yet determined whether cypin's guanine deaminase activity is necessary for cypin's role in PSD-95 localization.

The postsynaptic density undergoes structural remodeling during synaptic maturation, and this occurs by a number of molecular changes, including the addition of new proteins (Steward and Schuman, 2001) and protein turnover (Okabe et al., 1999; Marrs et al., 2001). One of the mechanisms underlying protein turnover is ubiquitination, followed by degradation by the proteasome (Glickman and Ciechanover, 2002). Dynamically regulated protein degradation occurs in the postsynaptic density, and this can alter synaptic signaling to the downstream effectors (Ehlers, 2003). Indeed, PSD-95 is ubiquitinated and rapidly disappears from synaptic sites when the NMDA receptor stimulated (Colledge et al., 2003). In addition, PSD-95 levels decrease with AMPA-stimulated GluR endocytosis, which itself is regulated by the ubiquitin-proteasome

system (Bingol and Schuman, 2004). In fact, Bingol and Schuman were unable to detect ubiquitinated PSD-95, thus suggesting that either polyubiquitinated PSD-95 proteins are extremely transient or the possibility of the existence of an intermediate protein to accomplish proteasome-dependent regulation of PSD-95 levels. Since cypin also reduces PSD-95 levels at synaptic sites (Firestein et al., 1999), here, we examined the mechanism underlying cypin's role in the regulation of PSD-95 cellular protein levels and synaptic localization. Although further investigation is needed, we found that cypin increases total PSD-95 cellular protein levels and that the interaction between the two proteins is required for this increase. Cypin also binds to the proteasome β7 subunit, increasing the possibility that cypin regulates synaptic localization of PSD-95 via a proteasome-dependent pathway. It is possible that cypin blocks proteasome-dependent degradation of PSD-95, resulting increased cellular PSD-95 levels.

MATERIALS AND METHODS

Antibodies and reagents

Rabbit polyclonal antibody raised against cypin has been previously described (Firestein et al., 1999). Mouse monoclonal GAPDH antibody and ubiquitin antibody were purchased from BD-Pharmingen (San Diego, CA), and goat anti-GFP was purchased from Rockland Immunochemicals (Gilbertsville, PA). Sheep polyclonal antibody to PSD-95 was used (Brenman et al., 1996; Christopherson et al., 2003) for immunoprecipitation, and either mouse monoclonal PSD-95 antibody from Thermo scientific (Waltham, MA) or rabbit anti-PSD-95 from Abcam (Cambridge, MA) was used for immunoblotting. Mouse antibody against HA was purchased from Millipore (Billerica, MA). MG132 was from Tocris (Minneapolis, MN), and actinomycin D and cycloheximide were purchased from Sigma (St. Louis, MO).

Expression or knockdown Constructs

cDNAs encoding rat cypin and indicated deletion mutants were subcloned into pEGFP-C1 vector (Clontech). The dsRed-tagged PSD-95 construct in dsRed2-N1 vector and the HA-ubiquitin construct in pCMV was used for COS-7 cell transfection. 5’ end mutated U1 snRNA constructs previously described (Akum et al., 2004) were used for knocking down cypin.

Primary culture of hippocampal neurons

Neuronal cultures were prepared from hippocampi of rat embryos at 18 days gestation as described previously (Firestein et al., 1999). The hippocampi were dissociated, and cells were plated on poly-D-lysine-coated glass coverslips (12 mm diameter) at a density of 1800 cells/mm^2 or 1 X 10^6 cells on 35mm culture dishes. Cultures were maintained in Neurobasal media (Gibco) supplemented with B27 (Gibco), penicillin, streptomycin, and Glutamax (Gibco). Cells were grown for 12-17 days *in vitro* (DIV) and used for specific experiments as indicated below.

Transfection of cultured neurons

Primary cultures of neurons were transfected with the appropriate constructs at DIV0 using AMAXA nucleofection (Lonza; Basel, Switzerland) with Amaxa Nucleofector kit for Rat Neurons. For clustering analysis, cultured hippocampal neurons were transfected at 10 DIV using LIPOFECTAMINE LTX with PLUS reagent following the manufacturer's protocol (Invitrogen).

Western blotting

AMAXA transfected either cortical or hippocampal neurons were plated at a density of 1 x 10^6 cells per 35 mm dish. Neurons were washed with ice-cold PBS and lysed in modified RIPA (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS). Cells were further lysed by passing the extract through a 25 gauge needle 20 times, and insoluble material was pelleted at 12,000 x *g* at 4°C for

15 min. Proteins were either subjected to immunoprecipitation or resolved on a 8 or 10% SDS polyacrylamide gel and transferred to PVDF membrane. The blot was probed with the indicated antibodies. Experiments were repeated more than three times. Blots were scanned and intensities of bands were quantitated using ImageJ software (NIH, Bethesda, MD) as we have previously performed (Chen and Firestein, 2007; Carrel et al., 2009). An area close to the bands was used as a reference for background intensity. The difference between intensities of the background and the band is the absolute intensity of the band. The number of pixels for the bands was normalized to the intensity of the internal control (GAPDH) and then compared with that of the control condition.

Rat Brain Co-immunoprecipitation

Rat brain was homogenized in TEE (25mM Tris-HCl, pH 7.4, 1mM EDTA, 1mM EGTA, 1mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)) and incubated at 4°C for 1 hour. Detergent-insoluble material was pelleted by centrifugation at 12,000 x *g* at 4°C for 15 min. Lysate was pre-cleared with protein A agarose 50% slurry (GE Healthcare, Piscataway, NJ) for one hour and then subjected to immunoprecipitation with polyclonal cypin antibody or rabbit IgG at 4°C overnight. Immunoprecipitates were washed, and bound proteins were eluted in SDS-PAGE sample buffer (63 mM Tris-HCl, pH 6.8, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue). Eluates were subjected to SDS-PAGE and immunoblotting.

COS-7 cell culture, transfection, and co-immunoprecipitation

COS-7 cells were plated at 70-80% confluence and maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere. Cells were transfected with 1.5µg of plasmids encoding the indicated proteins using LipofectAMINE 2000 (Invitrogen) following the manufacturer's protocol. Cells were lysed 48hr after transfection in modified RIPA (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) and subjected to immunoprecipitation using 5µg of antibody. Immunoprecipitated complexes were incubated with protein A agarose and eluted in protein loading buffer (63 mM Tris-HCl, pH 6.8, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue). Proteins were analyzed by SDS-PAGE and immunoblotting.

Immunocytochemistry

Neurons were fixed in ice-cold methanol for 15 minutes and then incubated in blocking solution (PBS containing 0.1% Triton X-100, 2% normal goat serum, and 0.02% sodium azide) for 1 hour. All antibodies used were diluted in blocking solution, and dilutions of 1:500 for chicken anti-GFP, mouse anti-PSD-95, and rabbit anti-MAP2 were used. Neurons were incubated in primary antibody-containing solution at 4^oC overnight. Neurons were then washed with PBS three times. The secondary antibody solution consisted of a 1:250 dilution of Cy2-conjugated anti-chicken IgY, Cy3-conjugated anti-mouse IgG and Cy-5 conjugated anti-rabbit IgG. Coverslips were then mounted onto frosted glass microscope slides using Fluoromount G (Southern Biotechnology, Birmingham, AL). Labeled cells were visualized by immunofluorescence on an Olympus

Optical (Tokyo, Japan) IX50 microscope with a Cooke Sensicam charge-coupled device cooled camera fluorescence imaging system and Image Pro software (Media Cybernetics, Silver Spring, MD).

Assessment of synaptic clustering

To quantify PSD-95 clusters, images of neurons were captured by CCD with a 60x oil objective using a constant gain and exposure time (filling the 12-bit dynamic range). Cluster outlines were calculated for fluorescent signals that are two standard deviations above the unlocalized baseline using a macro written for ImagePro (Charych et al., 2006). Cluster area was measured for each outlined cluster. Cluster number was calculated by counting the average number of clusters per μm^2 of dendritic area.

RESULTS

Cypin increases total PSD-95 protein levels.

Since we previously described that overexpression of cypin decreases synaptic clustering of PSD-95 (Firestein et al., 1999), we investigated whether alterations in cypin protein levels change total PSD-95 cellular protein levels. Surprisingly, cypin overexpression increases total PSD-95 protein levels in cortical cultured neurons (Figure 5-1A and B). We did not see any significant changes in PSD-95 levels when the cypin mutant lacking the PDZ-binding motif (CypinΔPDZ) was expressed, suggesting that interaction between cypin and PSD-95 is necessary for mediating this change in PSD-95 levels. More dramatic effects are shown in heterologous COS-7 cells (Figure 5-1C and D), when both cypin and PSD-95 were overexpressed. The fact that COS-7 cells do not have synaptic structures may be the reason that these dramatic changes in PSD-95 levels are seen, and hence, PSD-95 levels are more easily altered. Interestingly, although not significant, cypinΔPDZ slightly increases PSD-95 levels in both neurons and COS-7 cells. It is also possible that other domains of cypin might be also involved in regulating PSD-95 levels.

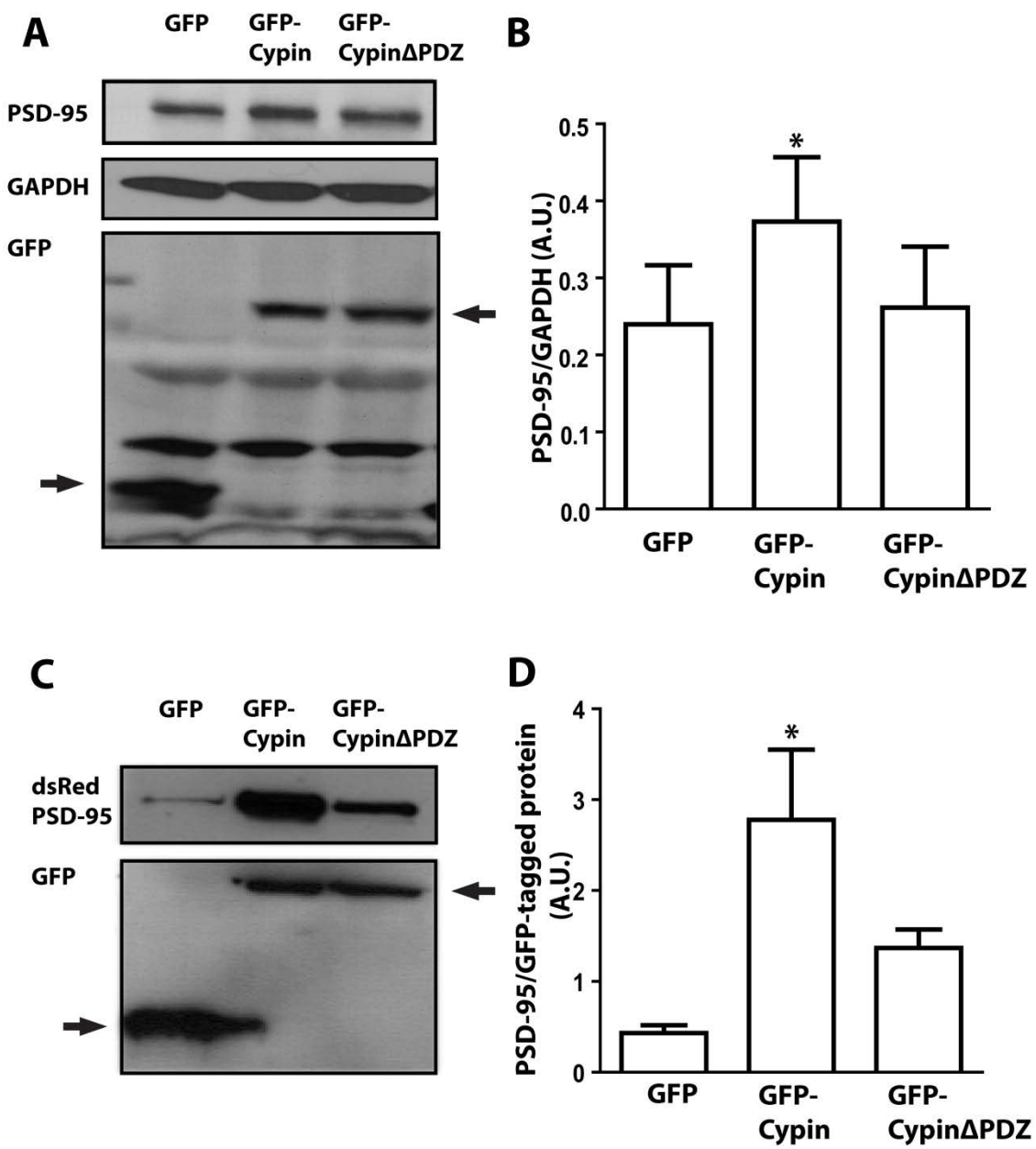

A
GFP
GFP-Cypin
GFP-CypinΔPDZ
PSD-95
GAPDH
GFP
B
PSD-95/GAPDH (A.U.)
0.5
0.4
0.3
0.2
0.1
0.0
*
GFP
GFP-Cypin
GFP-CypinΔPDZ
C
GFP
GFP-Cypin
GFP-CypinΔPDZ
dsRed PSD-95
GFP
D
PSD-95/GFP-tagged protein (A.U.)
4
3
2
1
0
*
GFP
GFP-Cypin
GFP-CypinΔPDZ

Figure 5-1. Cypin increases total PSD-95 protein levels

A, Neurons were transfected by AMAXA with the plasmids encoding the indicated proteins at 0 DIV. Western blotting was performed at 17 DIV. The blot was probed with anti-GFP (bottom panel) to show transfection and expression efficiencies. ***B***, Densitometric analysis of PSD-95 protein normalized to GAPDH protein expression. ***C***, COS-7 cells were transfected with plasmids encoding indicated proteins and analyzed 48 hours later. ***D***, Densitometry of PSD-95 is normalized to GFP or GFP-tagged proteins. Arrows indicate predicted sizes of GFP or GFP-tagged proteins (***A***,***C***). *$p<$ 0.05, by ANOVA followed Student-Newman-Keuls multiple comparison test compared to cells expressing GFP. n >= 3 experiments for all panels.

Cypin regulates PSD-95 synaptic clustering

We previously reported that overexpression of cypin decrease PSD-95 synaptic clusters, and that the PDZ-binding motif is required for this effect (Firestein et al., 1999). To further investigate the roles of the other cypin domains in this process, we overexpressed plasmids encoding cypin with residues 76-84 deleted, a region required for zinc-binding and guanine deaminase activity, or cypin with the CRMP domain deleted, a region responsible for binding tubulin heterodimers and for promoting microtubule assembly. Neither mutant was able to decrease the number of PSD-95 clusters, while wildtype cypin significantly changes cluster number (Figure 5-2A). Consistent with data previously published by our laboratory (Firestein et al., 1999), cypin lacking the PDZ-binding motif does not decrease PSD-95 clustering. Consistent with our overexpression results, cypin knockdown increases PSD-95 clustering when compared with GFP control. Co-expression of a 5' end mutated U1 snRNA resistant cypin completely rescues the effect of knockdown, while cypin ΔPDZ does not (Figure 5-2B). Interestingly, although not significant, the cypin mutant lacking the PDZ-binding motif results in a slight decrease in PSD-95 clusters when overexpressed and trends toward rescue when co-expressed with the knockdown construct. The same results were found with Western blotting (Figure 5-1), suggesting that PDZ-binding motif is necessary for mediating the effects of cypin.

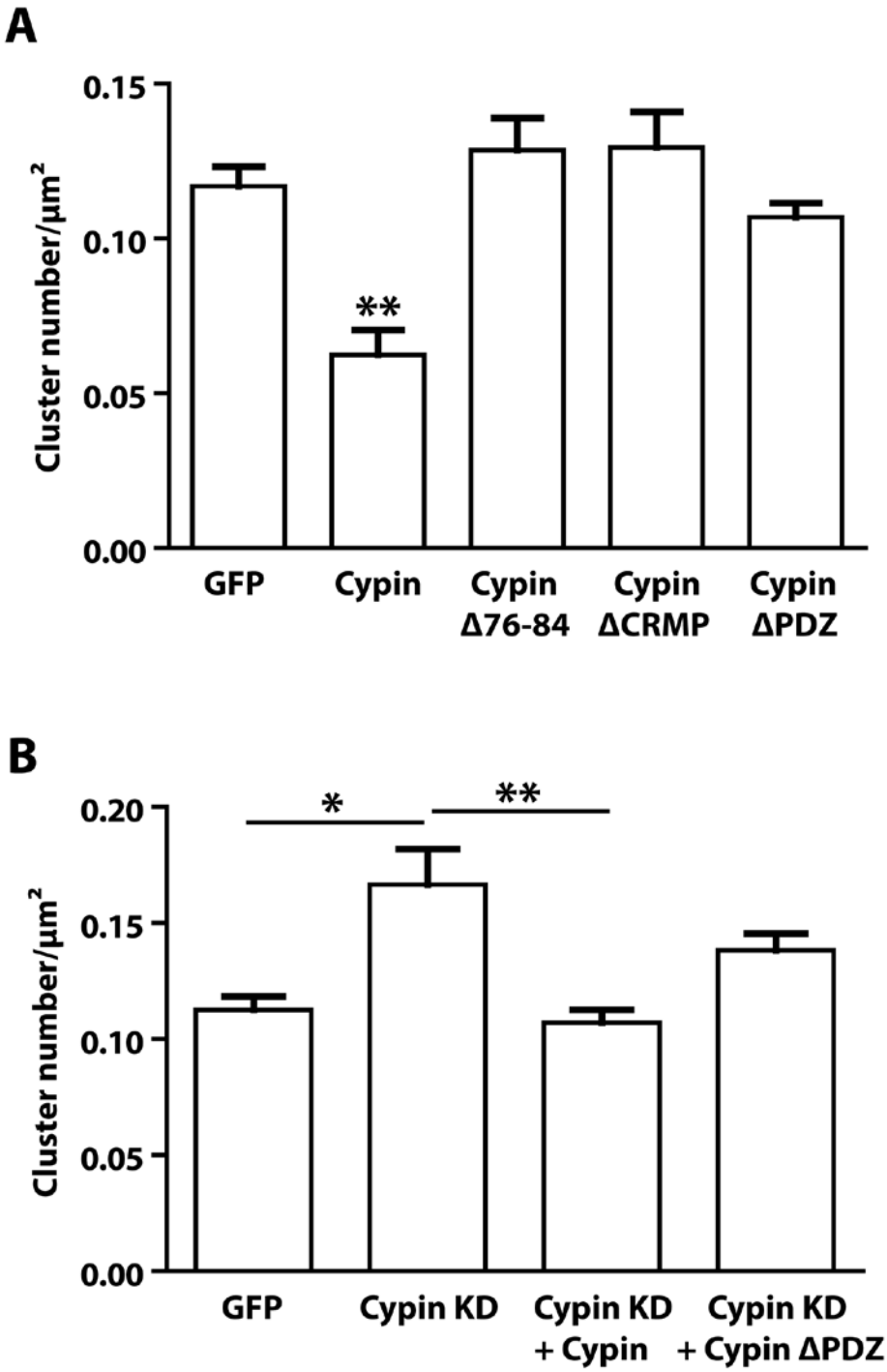

A
0.15
0.10
0.05
0.00
Cluster number/µm²
**
GFP
Cypin
Cypin Δ76-84
Cypin ΔCRMP
Cypin ΔPDZ
B
0.20
0.15
0.10
0.05
0.00
Cluster number/µm²
*
**
GFP
Cypin KD
Cypin KD + Cypin
Cypin KD + Cypin ΔPDZ

Figure 5-2. Cypin regulates PSD-95 synaptic clustering

A, Neurons were transfected with plasmids encoding the indicated proteins, or ***B***, with 5' end mutated U1 snRNA for knockdown together with plasmids for rescue experiments. Cypin KD indicates cypin knockdown. PSD-95 clusters were quantified at DIV17 using Image Pro. Clusters were normalized to dendrite area. $^{**}p < 0.01$ and $^{*}p < 0.05$, by Kruskal-Wallis test followed by Dunn's multiple comparison test compared to GFP expressing cells. n=20, GFP (A); n=22, Cypin; n=23, CypinΔ76-84; n=24, CypinΔCRMP; n=16, CypinΔPDZ; n=14, GFP; n=20, Cypin knockdown; n=16, Cypin knockdown + Cypin and Cypin knockdown + CypinΔPDZ.

Cypin regulates PSD-95 protein levels via a proteasome-dependent pathway

To investigate whether cypin-promoted increases in PSD-95 protein levels are dependent on increased transcription, increased protein synthesis, or inhibition of the proteasome, we performed experiments in the presence of inhibitors of each of these processes. We transfected neurons with same plasmids as above at DIV0 and treated neurons with the proteasome inhibitor MG132 (20μM for 5 hours), transcription inhibitor actinomycin D (5μM for 8 hours), or protein synthesis inhibitor cycloheximide (5.5 μg/ml for 6 hours) at DIV 17. Inhibition of either transcription or translation has no effect on cypin-mediated increases in PSD-95 expression (Figure 5-3 C and D). However, inhibition of the proteasome by MG132 occludes the effect of cypin on PSD-95 expression (Figure 5-3 B). These results suggest that the proteasome is involved in cypin-promoted increases in PSD-95 protein levels. Since the proteasome is required for protein degradation, this result suggests that cypin may act to inhibit proteasomal degradation of PSD-95.

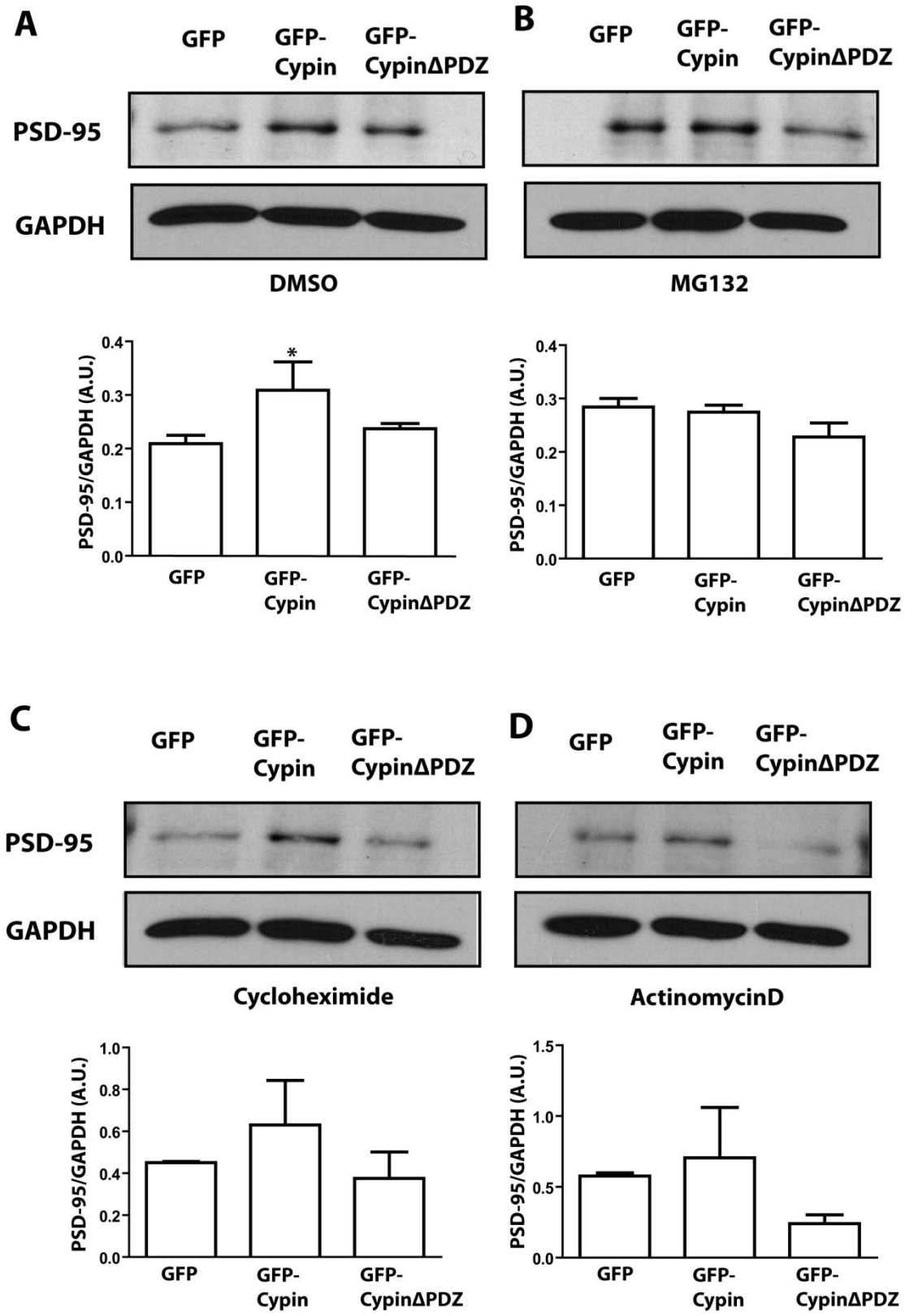

A
GFP
GFP-Cypin
GFP-CypinΔPDZ
PSD-95
GAPDH
DMSO
PSD-95/GAPDH (A.U.)
0.4
0.3
0.2
0.1
0.0
*
GFP
GFP-Cypin
GFP-CypinΔPDZ
B
GFP
GFP-Cypin
GFP-CypinΔPDZ
MG132
PSD-95/GAPDH (A.U.)
0.4
0.3
0.2
0.1
0.0
GFP
GFP-Cypin
GFP-CypinΔPDZ
C
GFP
GFP-Cypin
GFP-CypinΔPDZ
PSD-95
GAPDH
Cycloheximide
PSD-95/GAPDH (A.U.)
1.0
0.8
0.6
0.4
0.2
0.0
GFP
GFP-Cypin
GFP-CypinΔPDZ
D
GFP
GFP-Cypin
GFP-CypinΔPDZ
ActinomycinD
PSD-95/GAPDH (A.U.)
1.5
1.0
0.5
0.0
GFP
GFP-Cypin
GFP-CypinΔPDZ

Figure 5-3. Proteasome inhibitor MG132 occludes effect of cypin-mediated increase of PSD-95.

Neurons were transfected by AMAXA nucleofection with plasmids encoding the indicated proteins at DIV 0 and treated with ***A***, DMSO as a vehicle, ***B***, MG132 (20μM for 5 hours), ***C***, cycloheximide (5.5 μg/ml for 6 hours), or ***D***, actinomycin D (5μM for 8 hours) at DIV12. Protein extracts were resolved by SDS-PAGE and subjected to Western blotting using antibodies that recognize indicative proteins. Densitometric analysis of PSD-95 protein normalized to GAPDH protein expression (bottom panel). *$p < 0.05$, by Kruskal-Wallis test followed by Dunn's multiple comparison test compared to cells expressing GFP. n=4 experiments for DMSO and MG132 treatment, n=2 experiments for cycloheximide and actinomycin D treatments.

Cypin interacts with a subunit of the proteasome

We performed a yeast two hybrid screen using cypinΔPDZ as a bait to identify interactors with cypin. One of the potential interactors is the beta-7 subunit of proteasome. To confirm that this interaction occurs *in vivo*, we performed co-immunoprecipitation from rat brain lysate and successfully pulled down proteasome β-7 subunit when cypin was immunoprecipitated (Figure 5-4).

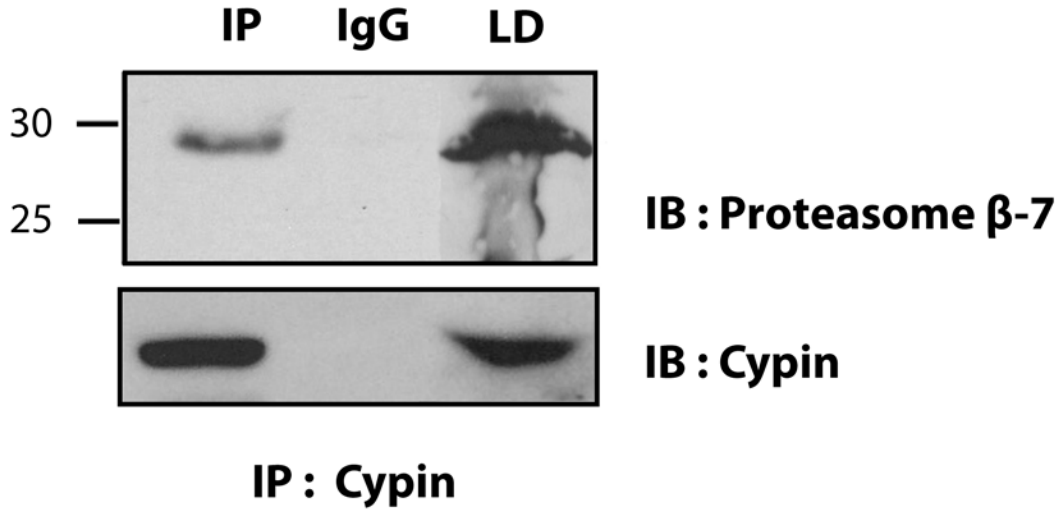
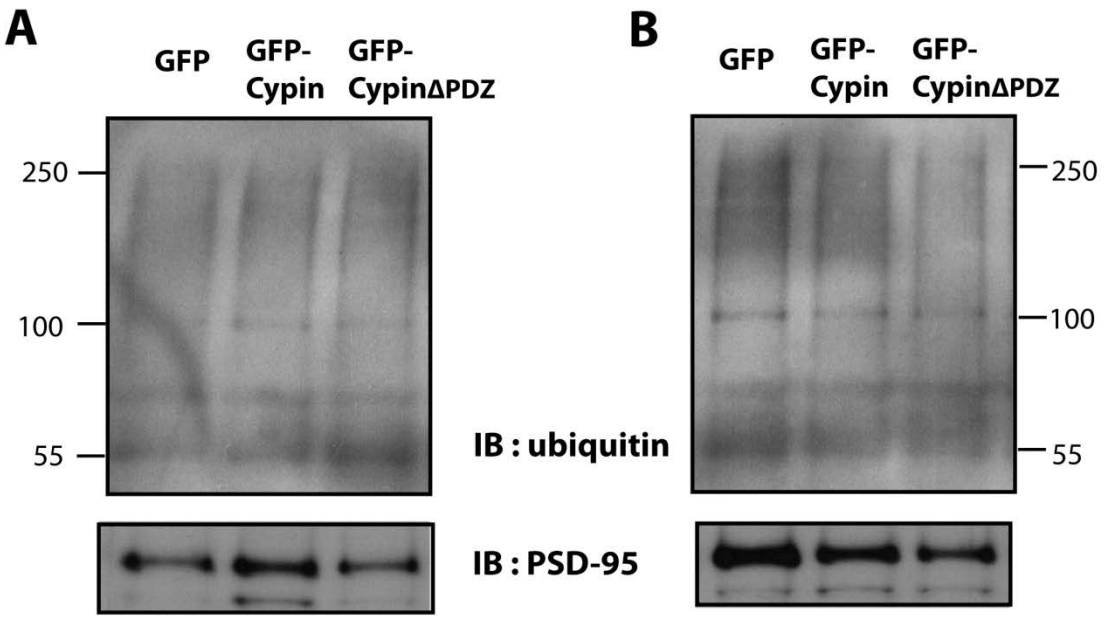


Figure 5-4. The β7 subunit of the proteasome co-immunoprecipitates with cypin in brain extracts

Co-immunoprecipitation assays of detergent extracts of brain using anti-cypin demonstrates that β7 subunit of proteasome co-immunoprecipitates with cypin (first lane). Neither cypin nor β7 was immunoprecipitated by the rabbit preimmune sera (middle lane). LD indicates loading control of brain lysate.

Cypin does not change the ubiquitination status of PSD-95

Polyubiquitinated proteins are the main targets for protein degradation by the proteasome. Furthermore, it has reported that PSD-95 is targeted for polyubiquitination when neuronal cultures are stimulated with NMDA (Colledge et al., 2003). Since our data suggest that the proteasome is involved in cypin-mediated increases in PSD-95 protein levels and decreases in PSD-95 clusters at synaptic sites, we investigated whether polyubiquitination of PSD-95 is changed by cypin overexpression. We performed immunoprecipitation of PSD-95 from extracts of cells that overexpress GFP or cypin and examined ubiquitination with Western blot analysis to determine whether PSD-95 polyubiquitination is altered. We were unable to see any changes in polyubiquitination of PSD-95 in any cell type studied, including cortical neurons, hippocampal neurons, and COS-7 cells when cypin was overexpressed. In fact, it is difficult to detect polyubiquitinated PSD-95 in our cultured neurons and COS-7 cell lysates under baseline conditions. This result is consistent with several studies in which PSD-95 polyubiquitination was not detected in hippocampal culture or whole hippocampal lysates (Bingol and Schuman, 2004) and in which GST fusions of the polyubiquitin-binding proteasome subunit S5a did not pull-down PSD-95 from cortical culture lysates (Ehlers, 2003). Taken together, our data and other studies suggest that polyubiquitinated PSD-95 may have a very short half-life or that PSD-95 binding partners regulating protein levels and targeting to proteasome independent of ubiquitination.


A
GFP
GFP-Cypin
GFP-CypinΔPDZ
250
100
55
IB : ubiquitin
IB : PSD-95
B
GFP
GFP-Cypin
GFP-CypinΔPDZ
250
100
55

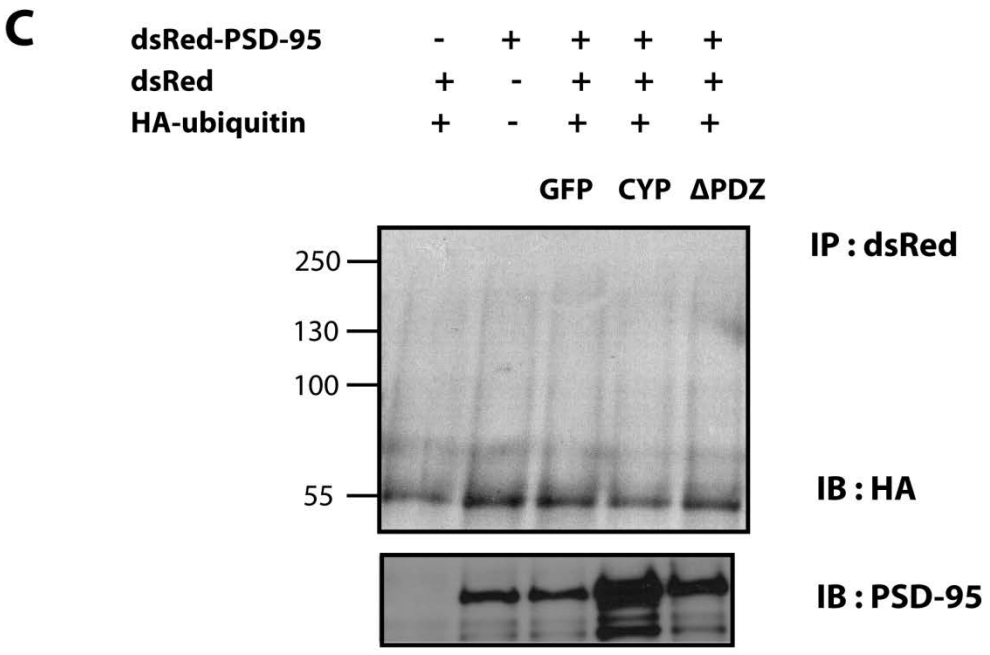

C
dsRed-PSD-95 - + + + +
dsRed + - + + +
HA-ubiquitin + - + + +
GFP CYP ΔPDZ
250
130
100
55
IP : dsRed
IB : HA
IB : PSD-95

Figure 5-5. Overexpression of cypin does not affect PSD-95 ubiquitination.

A, Cultured hippocampal neurons or ***B***, cortical neurons were transfected with plasmids encoding the indicated proteins, and cell lysates were subject to immunoprecipitation with an anti-PSD-95 antibody. Polyubiquitinated PSD-95 was examined by probing the blots with an anti-ubiquitin antibody. ***C***, COS-7 cells were co-transfected with pDsRed2-N1-PSD-95 and pCMV-HA-ubiquitin along with plasmids for expression of GFP-tagged proteins as indicated, and cell lysates were subject to immunoprecipitation with an anti-dsRed antibody. Polyubiquitinated PSD-95 was examined by probing the blots with anti-HA antibody. Little or no polyubiquitinated PSD-95 was detected, and overexpression of either cypin or cypinΔPDZ did not have any effect on PSD-95 ubiquitination. n=2 experiments.

DISCUSSION

The dynamic rearrangement and remodeling of the postsynaptic density is fundamental mechanism for neuronal synapse formation and plasticity in the brain. In response to activity, postsynaptic proteins change their composition at synapses by altering protein levels. It has been reported that many PSD proteins undergo increased transcription and translation (Steward and Schuman, 2001; West et al., 2001), or turnover by the ubiquitin-proteasome dependent pathway (DiAntonio et al., 2001; Hegde and DiAntonio, 2002; Murphey and Godenschwege, 2002; Ehlers, 2003).

PSD-95 is one of the most crucial components of the postsynaptic density, which serves as a molecular scaffold for the assembly of signaling complexes, receptors, and adhesion molecules. Dynamic changes in PSD-95 protein levels occur at synaptic sites, contributing to the determination of synaptic strength (Stein et al., 2003; Ehrlich and Malinow, 2004). This process may occur by either incorporation of new proteins or degradation of proteins.

In this chapter, we have shown that cypin increases total cellular PSD-95 protein levels in neurons when cypin is overexpressed. Cypin regulates dendrite morphology by promoting microtubule assembly (Akum et al., 2004), and its binding partner PSD-95 can act in an antagonistic manner to regulate dendrite branching (Charych et al., 2006). Accordingly, cypin also reduces the number of clusters of PSD-95 at synaptic sites ((Firestein et al., 1999), Figure 5-2)). Here, we showed that cypin increases total PSD-95 levels, suggesting that cypin plays role in the redistribution of PSD-95 at synaptic sites by increasing cellular levels of PSD-95. Moreover, cypin interacts with the proteasome (Figure 5-4), and we have found that cypin increases PSD-95 protein levels via a

proteasome-dependent pathway. Taken together, our data suggest that cypin might act to inhibit proteasomal degradation of PSD-95. In line with this idea, MG132 treatment resulted in a slight decrease in the synaptic staining of PSD-95 (Ehlers, 2003). In addition, cypin may act as an anti-ubiquitination agent when interacting PSD-95. This possibility was tested by detection of polyubiquitinated PSD-95 in neurons or COS-7 cells where is cypin overexpressed (Figure 5-5). Surprisingly, we did not see any changes in ubiquitinated PSD-95. Although a report exists that PSD-95 is targeted for ubiquitination when the NMDA receptor is stimulated and this contributes to AMPA receptor endocytosis (Colledge et al., 2003), others groups have been unable to detect ubiquitination of PSD-95 (Ehlers, 2003; Bingol and Schuman, 2004). PSD-95 has a PEST motif, which is rich in proline(P), glutamic acid (E), serine (S), and threonine (T) and usually acts as a signal peptide for protein degradation (Rogers et al., 1986); thus it is more likely that PSD-95 is targeted for rapid degradation by ubiquitination. However, small ubiquitin-related modifier (SUMO) can also target PEST sequences, and SUMO can act as an antagonist of ubiquitin in protein degradation, suggesting that this PEST motif can also serve as an anti-degradation signal.

The results shown in this chapter suggest that cypin-mediated changes in PSD-95 protein levels might be a chronic event since we transfected neurons and allowed cypin proteins to be expressed for 17 days *in vitro* due to the fact that we see a higher transfection efficiency with nucleofection. Changes in synaptic PSD-95 clustering were seen in shorter time periods of overexpression, showing that cypin can act during shorter windows of time. Since PSD proteins often show rapid redistribution and remodeling in response of activity, further investigation of the effect of cypin on PSD-95 protein levels

during short periods of time must also be addressed. Additional studies will focus on the mechanism underlying cypin action on PSD-95 re-localization and the involvement of the proteasome in this process.

AKNOWLEDGEMENTS

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Chapter 6. Summary and Future Directions

Proper development of dendritic arbors and spines is important for normal brain function. Defects in dendritogenesis and synaptogenesis are found in many neurodegenerative diseases, such as Rett syndrome, autism, and Alzheimer's disease (Zoghbi, 2003). Many proteins have been reported to regulate dendrite development, including BDNF, cypin and PSD-95. Our laboratory previously reported that cypin increases dendrite branching in hippocampal cultured neurons when overexpressed, and this regulation is accomplished by promoting microtubule assembly (Akum et al., 2004). Recently, we reported that PSD-95 decreases dendrite numbers by altering microtubule organization (Sweet et al., 2011). Since crosstalk between extracellular signals from other neurons or supporting cells and intracellular molecules is required for proper signaling, it is much of importance to understand how they coordinate with each other.

It has been reported there are cypin isoforms that are produced by alternative splicing; however, these isoforms are all C-terminal variants. Here we reported the existence of a new isoform of cypin, which has a shorter protein sequence and lacks N-terminal region of cypin. This short isoform does not have guanine deaminase activity, and its function needs to be elucidated. Since the N-terminus of cypin is required for zinc binding, it is possible that this short form is unable to bind to zinc and exhibit guanine deaminase activity although essential residues for zinc binding remain intact. Our data also suggest that this novel isoform may function in development differently than full length cypin since the two isoforms differ in their distribution in different organs. Further functional studies of this short isoform are needed to understand the role of cypin and its isoform during development. We also determined cypin expression patterns in

developing rat brains. Interestingly, cypin is expressed in a pattern in developing brain, especially in cerebellum, that is different from its expression in adult. This raises interest in the roles of cypin in the regulation of dendrite branching in numerous cell types. Most of the work performed to elucidate the function of cypin in dendritic arborization were done in cultured hippocampal neurons, and cypin is highly expressed in hippocampus in both developing and adult brains. Purkinje cells are one of the cell types that have the most branched dendrite morphology, and the fact that cypin is expressed in this cell layer in developing brain, but not in adult brain, suggests a role for cypin in the development of the cerebellum. Furthermore, it is known that cypin regulates the synaptic clustering of PSD-95, suggesting that cypin might play distinct roles in different developmental stages. (Chapter 2)

In Chapter 3 and 4, we investigated the global and local effects of BDNF on dendrite branching. BDNF binds to its receptors and signals through the MAPK signaling pathway to activate the transcription factor called CREB. Activated CREB, which is phosphorylated, increases gene expression, which can mediate a number of functions in a cell. Cypin has a CRE cis-acting element in its promoter, and CREB binds to this element to increase expression of cypin. Elevated mRNA and subsequent protein levels of cypin increase the number of dendrites proximal to the cell body. This BDNF-promoted increase of proximal dendrites is seen only when BDNF is administrated globally. Local application, on the other hand, shows a very different effect on dendrite morphology. Local BDNF administration using microbeads coated with BDNF shows increased dendrite branching not only proximal to the soma but also at distal regions in the arbor. The characterization of how exactly this local source of BDNF regulates the dendritic

arbor at the site of stimulation is needed to understand spatial control of the neuronal network. The proteins involved in this process can be uncovered by further studies.

Cypin was originally described as a cytosolic interactor of PSD-95, and it reduces synaptic clustering of PSD-95. How cypin regulates PSD-95 synaptic clustering is largely unknown. In Chapter 5, we suggested the possibility that changes in PSD-95 protein levels might contribute to this regulation. It has been suggested that involvement of the proteasome is one of the mechanisms for changes in synaptic localization of PSD-95 (Colledge et al., 2003; Ehlers, 2003; Bingol and Schuman, 2004), and there is some controversy between the groups studying ubiquitin-dependent proteasomal degradation of PSD-95. We suggest that cypin acts as a regulator for proteasome-dependent changes in PSD-95 protein levels. Further studies must be done to understand the mechanism underlying this cypin action on PSD-95 levels.

The establishment of neuronal networks is based on multiple processes, and proper spatial and temporal regulation of dendrite growth and branching and synaptic protein targeting are required for normal brain function. Most studies by our laboratory and others were performed *in vitro,* usually cultured hippocampal or cortical neurons. Verifying the mechanism *in vivo* is necessary for the development of drugs to target many neurological diseases. In fact, *in vivo* overexpression and knockdown studies of proteins involved in dendrite development we discussed in this thesis are currently being performed in our laboratory, and we are in the process of generating conditional knockout mice of cypin. These studies with *in vivo* animal models can aid us in fully understanding the mechanisms underlying changes in dendrite and spine morphology during development and while learning and retrieving memories.

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Curriculum Vitae

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Publications

1) **Kwon M**, Fernández JR, Zegarek GF, Lo SB, Firestein BL. BDNF-promoted increases in proximal dendrites occurs via CREB-dependent transcriptional regulation of cypin. *Journal of Neuroscience*, 2011 Jun 29; 31(26):9735-45

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Teaching Experience

Mentoring and Supervising undergraduate students and summer students (3) over the past 5 years