Analysis of BDNF-Induced Dendritic Growth in Hippocampal Neurons

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I have read Beth's thesis and I approve of it for her submission.

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Via email to Peggy Perkins, 03/25/13

Précis

Proper growth and development of the central nervous system is critical for healthy brain function. Dendrites, branch-like extensions off of the cell body of a neuron, and dendritic spines, short protrusions from the sides of dendrites, allow neurons to transmit signals and communicate with one another. There is a growing body of literature exploring the molecules involved in regulating the development and extension of dendrites and spines, collectively referred to as the dendritic arbor, but many of these mechanisms are not yet well understood. Brain derived neurotrophic factor (BDNF) is a protein known to stimulate dendrites to increase in length and complexity; cAMP response element binding protein (CREB) is a transcription factor shown to do the same. Because BDNF is a well recognized activator of CREB dependent transcription, we hypothesized that binding of BDNF to its receptors starts a signaling cascade that stimulates dendritic growth via CREB-dependent transcription. Two genes we have identified that are essential for dendritic growth are Par6C and Rnd3. The expression of both genes is stimulated by BDNF and regulated by CREB.

Previous work has shown that activity of the protein RhoA decreases the growth of dendrites and spines. Its activity is inhibited by another protein, called p190GAP, and both Par6C and Rnd3 are known activators of p190GAP. We hypothesized that BDNF-induced dendritic outgrowth would activate CREB-dependent transcription; induce Par6C and Rnd3 expression, which would then activate p190GAP to inhibit RhoA, thus allowing for dendritic growth. Our results showed that BDNF-induced dendritic outgrowth required inhibition of RhoA, and that BDNF-induced growth required Par6C and Rnd3 activity.

A wide range of disorders result from abnormal growth and function of the dendritic arbor; some of these include memory impairments, genetic disorders such as Down's syndrome and Fragile X syndrome, mental illnesses such as schizophrenia and neurodegenerative disorders such as Alzheimer's disease. By understanding the precise mechanisms that direct growth and development of dendrites and dendritic spines, it is possible to better able to identify the abnormalities present in these disorders, which may provide valuable insight in developing new therapies that more accurately target the problem. There is still much to be discovered in this field before we can start producing dugs, but we hope that each study brings us one step closer to the next medical breakthrough in treating neurocognitive disorders.

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Figures

Introduction

The dendritic arbor is a series of projections emanating from the cell body of a neuron that receives synaptic signals from other neurons. Proper development and orientation of the dendritic arbor is required for healthy brain function (Negishi and Katoh 2005). The size and shape of the dendritic arbor is specific to neuronal cell type, and closely tied to the function of the neuron (Parrish et al 2007). Development of neural circuitry involves both extrinsic factors, such as neurotrophins and extracellular ligands, and intrinsic factors, such as enhanced neuronal excitability and the regulation of gene expression (Parrish et al 2007). A thorough understanding of the processes involved in dendritic arborization is critical to understanding the diseases caused by errors in this process. A large number of neurological disorders, including schizophrenia, Alzheimer's Disease, Down's Syndrome, Rett's Syndrome, Fragile X syndrome, and autism spectrum disorders, are all associated with abnormalities in the dendritic arbor (Kauffman and Moser 2000; Pickett and London 2005; Penzes and Remmers 2012). A clear picture of the molecules and mechanisms involved in the growth of dendrites and dendritic spines will allow for specific targeting of the irregularities that cause disease.

Synaptic activity has an important influence on dendritic development, and has been shown to produce rapid outgrowth in neurons involved in learning and memory (Maletic-Savatic et al 1999). While excitatory input can stimulate growth and stabilize existing dendrites and spines, the absence of synaptic activity can reduce local dendritic branching (Rajan and Cline 1998). The mechanism by which synaptic activity leads to dendritic growth is not entirely understood; however, it is clear that calcium plays an important role (Wong and Gosh 2002). Calcium can affect changes in the postsynaptic cell through NMDA receptors and neuronal nicotinic cholinergic receptors (nAChRs), the opening of voltage gated calcium channels

(VGCCs), and via intracellular stores of calcium, and different neuronal cell types may use different combinations of these mechanisms to bring about local structural changes (Wong and Ghosh 2002).

NMDA receptors act as ligand and voltage-gated ion channels, and when activated, increase membrane permeability to calcium (Kandel et al 2000). Activation of NMDA receptors has been shown to induce rapid, local dendritic outgrowth and to stabilize existing branches (Rajan and Cline 1998). Both NMDA receptors and AMPA receptors are activated by glutamate, but AMPA seems to work only in more complex neurons and does not seem to have a significant impact on the initial development of the dendritic arbor (Rajan and Cline 1998). Calcium influx primarily activates calcium/calmodulin-dependent protein kinases (CaM kinase II and CaM kinase IV), mitogen-activated protein kinase (MAPK), and protein kinase A (PKA) (Redmond et al 2002). These molecules are important parts of signaling cascades that influence dendritic structure (Wong and Ghosh 2002). One proposed mechanism for calcium-mediated dendritic growth is through CaM kinase IV activation of CREB-dependent transcription (Redmond et al 2002). CaM kinase II has been shown to stabilize dendrites in mature neurons, preventing both outgrowth and retraction of branches (Wu and Cline 1998). Activation of the membrane-anchored CaM kinase CLICK-III/CaMKIγ can stimulate activity of STEF, a RacGEF, leading to activation of Rac1 and turning on local dendrite formation (Takemoto-Kimura et al 2007). Intracellular calcium stores can also influence dendritic outgrowth. The ryanodine receptor controls release of intracellular calcium from the endoplasmic reticulum; stimulation of ryanodine activity has been shown to be sufficient for eliciting dendritic outgrowth (Wayman et al 2012).

Extracellular signals, such as neurotrophic factors, also play an important role in the development and regulation of the dendritic arbor and their influence on growth, differentiation, and survival has been thoroughly investigated. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and NT4/5 are all neurotrophins shown to play a role in regulating dendritic growth (Scott and Luo 2001). Neurotrophins bind to the family of tyrosine kinase (Trk) receptors with high affinity, and it is thought that Trk receptor expression is required for neurotrophin-induced dendritic growth (Bergami et al 2008; Scott and Luo 2001). When BDNF is released locally, it requires the presence of the TrkB receptor on the recipient cell to stimulate dendritic growth (Horch and Katz 2002). Activation of the TrkB receptor activates multiple signaling pathways including the Phospholipase C/calcium pathway as well as the MEK/ERK signaling cascade. Downstream of the MEK/ERK signaling pathway is the eventual phosphorylation and activation of CREB. This phosphorylation of CREB at Ser-133 is mediated by the mitogen- and stress-activated protein kinase 1 (MSK1) and initiates CREB transcriptional activation (Finkbeiner et al 1997; Arthur et al 2004; Lang et al 2007; Bergami et al 2008).

CREB (cAMP response element binding protein) is a transcription factor that has been shown to play a role in learning and memory, addiction, circadian rhythms, and cell survival. It is part of the bZIP superfamily of transcription factors and is closely related to CREM (cAMP element modulator) and ATF-1 (activating transcription factor 1). A basic domain on the C-terminal of these proteins allows for efficient DNA binding, and another region allows for easy dimerization with co-activators. All proteins in the CREB family bind to the cAMP response element (CRE), which includes the consensus sequence TGACGTCA. Additionally, the CREB protein also contains a kinase inducible domain (KID) at Ser-133. When this residue is

phosphorylated, the KID domain becomes a target for the CREB binding protein (CBP). CBP works as a CREB co-activator, and the interaction between CBP and CREB allows for recruitment of the other cofactors and transcriptional machinery including RNA Polymerase II which then initiates gene expression (Lonze and Ginty 2002).

CREB-dependent transcription has been shown to be necessary and sufficient for activity regulated dendritic growth (Wayman et al 2006, 2008). Although inhibition of CREB activity does not affect basal dendritic development, it does eliminate activity-induced outgrowth, indicating that CREB is an essential mediator of activity-dependent dendritic plasticity (Wayman et al 2006). Inhibition of CREB also prevents activity-dependent growth of dendritic spines (Impey et al 2010). One proposed mechanism for activity and CREB-induced development is through NMDA receptors, which then initiate a signaling cascade that sequentially involves CaM kinase kinase (CaMKK)/CaM kinase I (CaMKI), MEK, ERK, CREB, and Wnt2 (Wayman et al 2006).

Although a variety of different cellular conditions can induce Ser133 CREB phosphorylation, many are initiated by an extrinsic stimulus, such as neuronal activity or neurotrophic factors, which have both been shown to activate CREB (Finkbeiner et al 1997; Lonze and Ginty 2002; Redmond et al 2002). CREB, neuronal activity, and neurotrophin expression have all been shown to be involved in growth of the dendritic arbor, and there is significant evidence that synaptic activity and neurotrophins such as BDNF work through CREB-regulated genes to induce plasticity in dendrites (Finkbeiner et al 1997; Redmond et al 2002; Wayman et al 2006; Suzuki et al 2011).

The formation of the dendritic arbor requires precise regulation of the neuronal cytoskeleton. The cytoskeleton provides structural support for dendritic projections, and is made

up of two essential main elements: actin and microtubules (Scott and Luo 2001; Luo 2000). Actin filaments are long, thin, and highly dynamic, while microtubules are larger, hollow tubes that are more stable than actin filaments (Scott and Luo 2001). Actin filaments can undergo cycles of rapid polymerization and depolymerization, which allows the growth cone to be highly mobile and enables the neuron to extend and retract processes quickly (Kandel et al 2000). Both actin filaments and microtubules require binding proteins for stabilization (Kandel et al 2000). In microtubules, these microtubule-associated proteins (MAPs) are phosphorylated in response to depolarization, which facilitates binding to and stabilization of microtubules (Chen and Ghosh 2004). MAP2 is a high affinity microtubule binding protein that is found exclusively in dendrites and expression of green fluorescent protein (GFP)-tagged Map2B protein is a useful tool for selectively visualizing dendrites (Scott and Luo 2001).

There are five basic steps in the development of the dendritic arbor: initiation, extension, branching, spine formation, and restriction of growth (Scott and Luo 2001). Neurons begin as unpolarized cells that are acted on by a variety of signaling events, such as increased synaptic activity and growth factor signaling that can promote dendrite formation, branching, and extension (Scott and Luo 2001). One mechanism for determining the final shape of a developing neurite is through regulation by chemoattractants and chemorepellants. For example, the protein semaphorin 3A (Sema3A) is an important chemoattractant that stimulates the extension of dendrites in cortical pyramidal neurons. When deprived of Sema3A treatment, cortical pyramidal neurons grow abnormally and lack proper orientation (Polleux et al 2000). Appropriate extracellular signaling is an important step in correctly orienting the dendritic arbor.

Dendritic branching can occur either through the splitting of the growth cone, or the appearance of a new branch from the side of a pre-existing dendritic shaft (Scott and Luo 2001).

Destabilization of the cytoskeleton allows actin filaments to extend in new directions as filopodia, small, finger-like protrusions containing long bundles of actin, which must be invaded by microtubules in order to become stable dendrites (Van Aelst and D'Souza-Schorey 1997; Scott and Luo 2001). These branches are stabilized by microtubules and MAPs and become part of the dendritic arbor.

Dendritic spines are small protrusions off of dendritic branches that mark the primary locations of excitatory synaptic input (Zhang and Macara 2008). Like branches, spines can begin as filopodia, although only a very small percentage of filopodia mature into spines, and most filopodia simply disappear (Alvarez and Sabatini 2007). Limitation of dendritic growth is regulated by a variety of genes and depends in part on the neuron's location in the nervous system. One important protein involved in restriction of dendritic growth is the small Rho GTPase, RhoA (Scott and Luo 2001).

Small Rho GTPases play an essential role in regulating the actin cytoskeleton in dendritic development through interaction with downstream effectors that manipulate actin dynamics (Negishi and Katoh 2005). RhoGTPases act as molecular switches, cycling between the active, GTP-bound state, and the inactive, GDP-bound state (Wennerberg et al 2003). This transition between active and inactive states is regulated by enzymes called GAPs, GTPase Activating Proteins, and GEFs, Guanine Exchange Factors (Wennerberg et al 2003). GEFs work to activate G proteins by exchanging GDP for GTP, while GAPs stimulate the RhoGTPase to hydrolyze GTP, halting G protein activity (Wennerberg et al 2003). The activity of some members of the small RhoGTPase family is thought to stimulate dendritic outgrowth, like Rac1 and Cdc42, while other members such as RhoA inhibit outgrowth (Luo 2000; Negishi and Katoh 2005).

Activity of Rac1 has been shown to stimulate dendritic outgrowth (Luo 2000; Impey et al 2010). One likely mechanism that leads to Rac1 activation is through microRNA132 (miR132) regulation. MicroRNAs are small RNA that bind to target mRNA and prevent translation, effectively silencing gene expression. Initiation of CREB-dependent transcription, through a variety of signals including neuronal activity, results in the expression of miR132 (Wayman et al 2008; Impey et al 2010). miR132 regulates Rac1 activity by inhibiting the translation of p250GAP, a GAP protein that inactivates Rac. Downregulation of p250GAP is essential for cytoskeletal growth and synaptogenesis (Wayman et al 2008). In order for Rac1 to be activated, and thus regulate the cytoskeleton, it must be activated by a Rac-GEF. Kalirin-7 is a Rac-GEF shown to be one protein responsible for activating Rac1 following miR132-induced downregulation of p250GAP (Impey et al 2010). Neuronal activity leads to the activation of CREB-dependent transcription, which increases miR132 expression, leading to the inhibition of p250GAP activity, while allowing for Kalirin-7 to activate Rac1, which modulates the actin cytoskeleton and induces synaptogenesis (Wayman et al 2008).

Activation of another RhoGTPase, RhoA, is thought to have the opposite effect on dendritic outgrowth and synaptogenesis as Rac1. For example, in optic tectal neurons, RhoA activation inhibits activity-dependent dendritic growth, while activation of Rac1 and Cdc42 stimulate dendritic branching dynamics. Visual stimulation inhibits RhoA activity and allows for arbor growth, demonstrating that RhoA is regulated by light-induced activity (Sin et al 2002). Expression of dominant-negative forms of Rac1 and Cdc42, or constitutively active RhoA, prevents a light-induced increase in branching, indicating that activation of Rac1 and Cdc42 in conjunction with inhibition of RhoA are necessary for light-induced dendritic outgrowth (Sin et al 2002).

Because CREB-dependent transcription is believed to be an important element in activity-dependent dendritic outgrowth (Wayman et al 2006), it is likely that some CREB-regulated genes are involved in cytoskeletal modification in some capacity. To identify genes regulated by CREB-dependent transcription, our lab conducted a Chromatin Immunoprecipitation Sequencing (ChIP-Seq) experiment. After crosslinking CREB to the promoter of its target genes, the DNA was fragmented, then using an antibody for CREB, the CREB-bound DNA fragments were immunoprecipitated and isolated. CREB was de-crosslinked from the DNA, and the previously CREB-bound DNA fragment promoter regions were sequenced and mapped to the genome. This process identified a library of genes with CREB-bound promoter regions, which are thus likely regulated by CREB (Lesiak et al 2013). Additionally, an RNA sequencing screen (RNA-Seq) was conducted wherein all mRNA produced in control conditions and following BDNF stimulation was collected and sequenced, producing a library of genes which identified genes that were either up or down regulated by BDNF treatment. These two libraries identified thousands of genes, and by cross-referencing the CREB ChIP-Seq library with the BDNF RNA-Seq library, numerous BDNF-induced and CREB-regulated genes were identified. From this subset of genes, Par6C and Rnd3, two known RhoA inhibitors, were identified as likely BDNF-induced CREB-regulated genes that are likely involved in dendritic growth and synaptogenesis (Lesiak et al 2013).

Par6C has been shown to function as a RhoA inhibitor, and acts as a scaffolding protein that suppresses RhoA by co-localizing the RhoA-GAP p190GAP with RhoA. It is believed to be necessary for dendritic spine formation and maintenance, but its effects on dendritic outgrowth have yet to be determined (Zhang and Macara 2008). Likewise, the small RhoGTPase, Rnd3 (RhoE), has previously been shown to inhibit RhoA activity through activating p190GAP,

inhibiting the RhoA effector ROCK I, and potentially inhibiting the RhoA-GEF Syx (Wennerberg et al 2003; Riento et al 2003; Goh and Manser 2010). Unlike most Rho family proteins, Rnd3 lacks a functional GTPase domain and is thus constitutively active, and therefore unaffected by GAP and GEF regulation (Riento et al 2005). Recent evidence suggests that overexpression of Rnd3 leads to spinogenesis via inhibition of the RhoA/ROCK I signaling pathway (Lesiak et al 2013).

Due to the role of Rnd3 and Par6C in BDNF-induced spinogenesis, we chose to examine the effects of these proteins in BDNF-induced dendritic outgrowth. Both Rnd3 and Par6C are known to modify the actin cytoskeleton in dendritic spines, and RhoA, another modulator of the actin cytoskeleton, is inhibited by CREB-dependent transcription. We believe that BDNF can trigger CREB transcription via the MEK/ERK/MSK I pathway to inhibit RhoA activity, and we sought to establish whether this pathway was sufficient to regulate dendritic growth.

Thesis Activity

This project investigates whether the RhoA inhibitors, Rnd3 and Par6C, are necessary for BDNF-induced, CREB-regulated dendritic outgrowth.

Methodology

Cell culture

Animal use for hippocampal cultures was carried out in compliance with Washington State University IACUC approved protocols ASAF 0317-011 and ASAF 04020-003. Hippocampal neurons ($3.0X10^4$ cells per square centimeter) were cultured from P1–2 Sprague–Dawley rats on plates coated with poly-L-lysine (Sigma; molecular weight 300,000) as described previously (Lesiak et al 2013). Hippocampal neurons were maintained in Neurobasal A (NBA)

media (Invitrogen) supplemented with B27 (Invitrogen), 0.5 mM L-glutamine, and 5 μM cytosine-D-arabinofuranoside (Sigma; added at DIV2). Hippocampal neurons were treated on DIV6 as written in text and figure legends. For BDNF treatment, reagent was first diluted prior to being added to native in-well media to reach the final in-well target concentration. Hippocampal neurons used for dendrite analysis were transfected with various constructs on DIV6 and then treated on DIV7 ± BDNF in media to a final in-well concentration of 50ng/mL BDNF. Cells treated without BDNF received the same amount of media at this time. On DIV9, cells were fixed.

RNA isolation, reverse transcription and quantitative real-time PCR

Total or nuclear RNA was isolated using the Trizol (Invitrogen) method following manufacturer's protocol, and reverse-transcribed using MMLV (Invitrogen). Real time PCR was conducted as previously described (Lesiak et al 2013). All RT-PCR data utilized standard curve real-time PCR.

Western blotting

To analyze Par6C and Rnd3 expression, cultures were treated as specified in figure legends, and each well was lysed in 50μL RIPA buffer (Upstate) with Phosphatase Inhibitor II and III (Sigma) and Protease Inhibitor (Roche). Samples were then dounce homogenized and spun in a microcentrifuge to pellet debris, and frozen at -80˚C for storage. Samples were prepared using NuPage LDS Sample Buffer (Invitrogen) with 0.5M DTT and heated at 70˚C for 10 minutes before equal volumes were loaded into NuPage 4-12% Bis-Tris gels (Invitrogen) and run using NuPage Mops Buffer. Protein was transferred to PVDF using Tris-Glycine

buffer. Blots were blocked using Aquablock (East Coast Biotech) for 1 hour, and then probed with anti-Par6C (Sigma, c-terminal), anti-Rnd3 (Upstate), and anti-ERK ½ (Loading control)(Santa Cruz) diluted in Aquablock overnight. Blots were then washed with PBS before application of Rockland IR secondary antibodies (anti-RB 700 and anti-MS 800) for 2 hours. Finally, blots were washed with PBS again before they were scanned using Li-COR infra-red Odyssey Scanner to image blots. Band intensities were measured using ImageJ and normalized to ERK2 band intensities (Lesiak et al 2013).

Transfection

Primary hippocampal neurons were transfected with LipofectAMINE 2000 (Invitrogen). For transfection of 24 wells of a 24 well plate, 50µL of Lipofectamine 2000 (L2K) was added to 2.5mL of NBA and incubated for 5 minutes. DNA plasmids for each of the treatments were mixed in separate tubes, with enough total plasmid DNA for 1µg/well. Native media in wells were collected and kept warm, and 500µL of warm growth media (GM) was placed in the well. NBA/L2K mix was added to DNA mixtures for 20 minutes, before 100uL/well of DNA/NBA/L2K mix was added to each well for 30 minutes. After 30 minutes, media was aspirated, and 500µL of warm native media was returned to well. In each experiment, we optimized DNA amounts to minimize toxicity and maximize transfection efficiency. Lipofectamine 2000 transfection efficiency was 0.5–5%.

Quantification of morphology

High-density hippocampal neurons were transfected with GFP-MAP2B±test plasmids or (sh-RNAs/stealth RNA) oligoprimers. Expression of fluorescently tagged MAP2B allows visualization of the dendritic arbor because MAP2B associates with microtubules exclusively in the soma and dendrite while being excluded from the axon. Expression of low levels of MAP2B

has been shown to have no effect on manipulating dendritic architecture. Neurons were transfected on DIV 6 then fixed (4% paraformaldehyde, 3% sucrose, 60mMPIPES, 25mMHEPES, 5mMEGTA, 1mMMgCl2, pH7.4) on DIV 9 for 20 minutes at room temperature. Fluorescent images were acquired using a cooled CCD camera (Hamamatsu Photonics) attached to a Zeiss Axioplan2 (Carl Zeiss). Morphometric measurements were performed using Image J and Neuron J software (NIH.gov). Total dendritic length was measured on each of approximately 20 neurons in at least two replicate experiments.

Results

BDNF Induced Dendritic Growth Requires RhoA Inhibition

BDNF treatment induces a 25% increases in total dendritic length and dendritic branches (Figure 1). To examine whether BDNF-induced increases in dendritic growth and branching requires inactivation of RhoA, we used two methods to activate RhoA: targeted knockdown of the RhoA inhibitor, p190GAP using a small hairpin RNA (sh-p190GAP), and overexpression of a constitutively active RhoA mutant (ca-RhoA) to activate RhoA. sh-p190GAP expression prevented BDNF-induced dendritic outgrowth and branching (Figure 1). Transfection with constitutively active RhoA (caRhoA), a RhoA mutant that cannot be inhibited, also blocked BDNF-induced increases in dendritic length and branching (Figure 1). This data suggests that BDNF requires RhoA inhibition to stimulate dendritic outgrowth and branching.

Figure 1: BDNF Induced Dendritic Growth Requires RhoA Inhibition

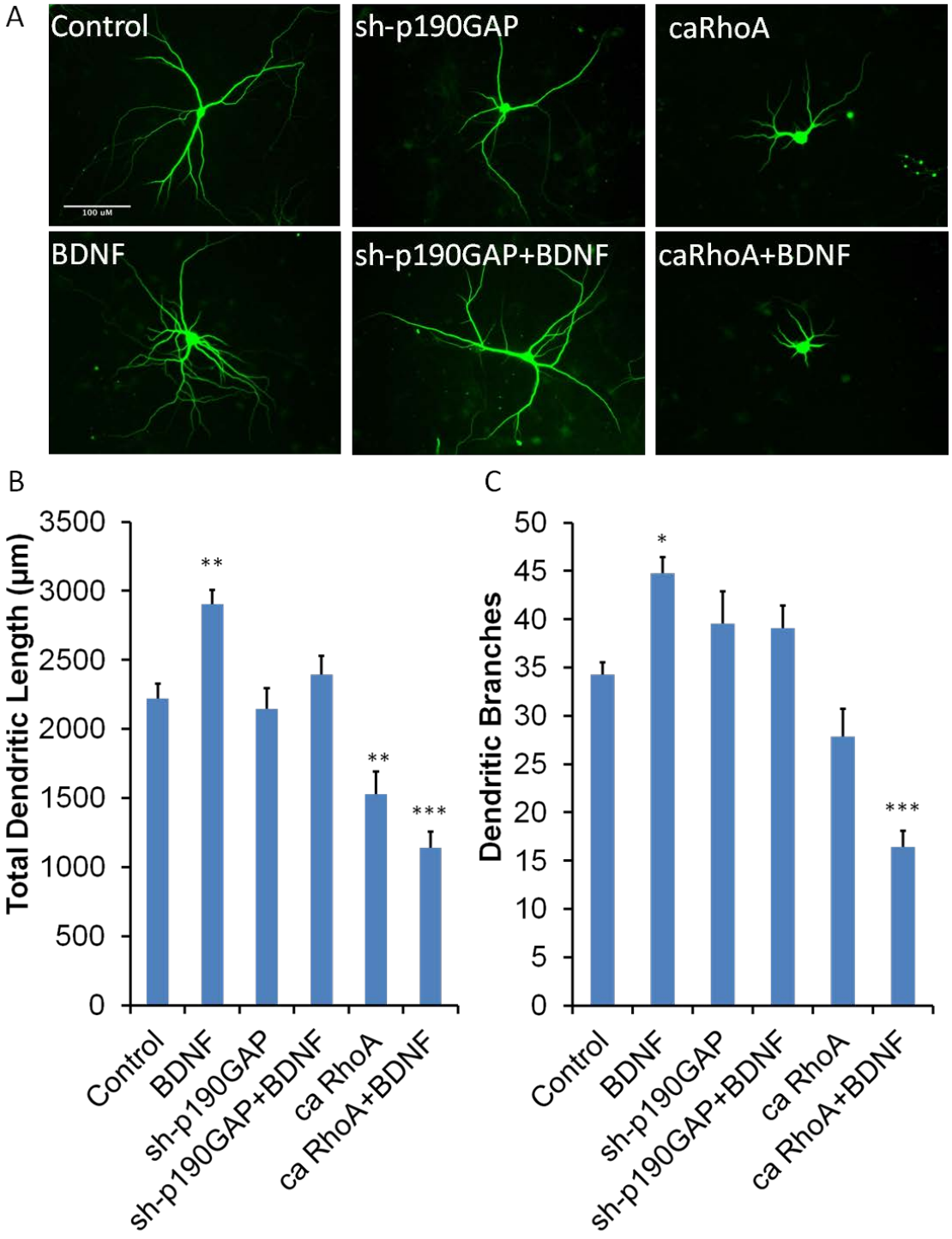


Figure 1: DIV6 cultured hippocampal neurons were transfected with MAP2B ± empty vector (Control and BDNF), ± sh-p190GAP, ± ca RhoA, w/ or w/o 50ng/mL BDNF stimulation on DIV7 until fixed on DIV9. A) Representative images. B) Average Total Dendritic Length. C) Average Dendritic Branches. Dendrites were imaged and analyzed using NeuronJ Dendrite tracing program. (± SEM, *p<0.05, **p<0.01, ***p<0.001 compared to control).

BDNF Induces Par6C and Rnd3 Expression

Stimulation of cultured hippocampal neurons with BDNF resulted in a significant increase in Par6C and Rnd3 mRNA levels (Figure 2A,C). To inhibit CREB-dependent transcription, we transfected ACREB, a potent dominant interfering mutant of CREB. Transfection of the neurons with ACREB resulted in a significant decrease in Par6C and Rnd3 mRNA expression in the presence of BDNF stimulation (Figure 2A,C). If mRNA levels increase, it would be expected that protein levels might also increase, and BDNF treatment increased the expression of both Par6C and Rnd3 protein by approximately 50%. This increase was diminished by pretreatment with the MEK inhibitor U0126 (Figure 2B,D). These results demonstrate that activation of the BDNF-MEK/ERK-CREB pathway leads to the increased expression of Par6C and Rnd3. (Experiments featured in Figure 2 originally carried out in Lesiak et al 2013).

Figure 2: BDNF induces expression of Par6C and Rnd3

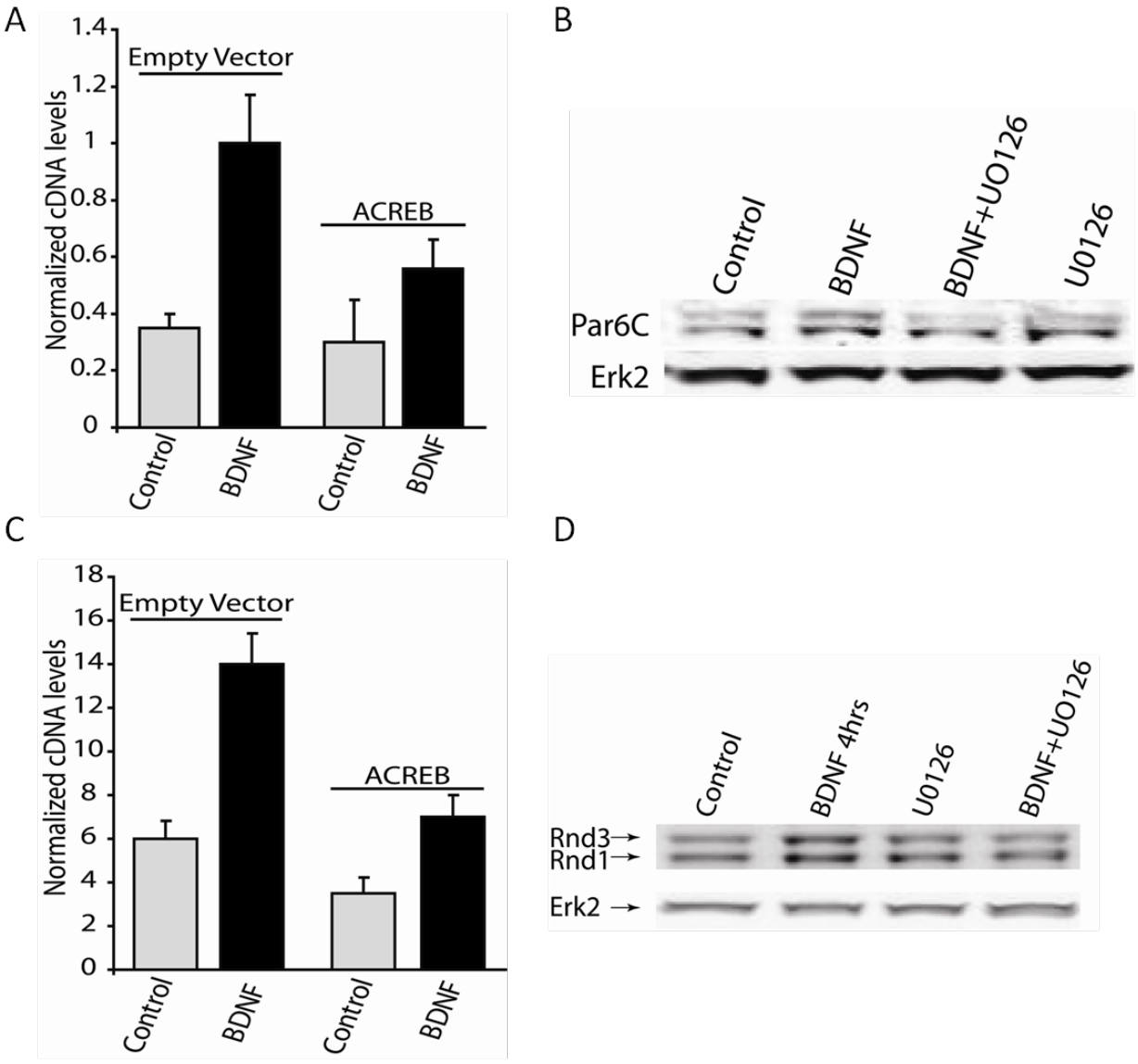


Figure 2: DIV6 cultured hippocampal neuron were transfected with either empty vector or ACREB expression construct and treated with or without 50ng/mL BDNF for 2 hours. Total RNA was isolated and reverse transcribed into cDNA using OligoDT primers, and levels of Par6A (Par6C) RNA were determined using RT-PCR (normalized to GAPDH). A) Par6C mRNA and C) Rnd3 mRNA. DIV6 cultured hippocampal neurons were treated with or without 50ng/mL BDNF, with or without pre-treatment of 20μM U0-126, lysed and used for Western blot, using primary antibodies against Par6C, Rnd3, and ERK2 (loading control). Representative blots B) Par6C and D) Rnd3.

Par6C is Required for BDNF Induced Outgrowth

Transfection and overexpression of Pa6C, like BDNF, increased both total dendritic length and the number of branches (Figure 3). Conversely, sh-RNA-mediated repression of Par6C expression effectively blocked BDNF-induced increases in total dendritic length and branching (Figure 3). This supports the hypothesis that BDNF requires Par6C expression to stimulate an increase in dendritic growth. It is known that Par6C suppresses RhoA activity under some circumstances, and to determine whether the effects of Par6C overexpression are mediated by RhoA inhibition, we used two methods to activate RhoA in conjunction with Par6C overexpression. Sh-RNA-mediated repression of p190GAP, with simultaneous expression of Par6C, did not increase growth above control levels (Figure 3). Likewise, co-expression of constitutively active RhoA (caRhoA) with Par6C also blocked Par6C-induced increases in dendritic outgrowth and branching, and inhibited dendritic outgrowth to a level significantly below control neurons (Figure 3). This data suggests that Par6C is critical to BDNF-induced dendritic outgrowth, and likely requires the inhibition of RhoA by p190GAP activity.

Figure 3: Par6C is Required for BDNF Induced Dendritic Outgrowth

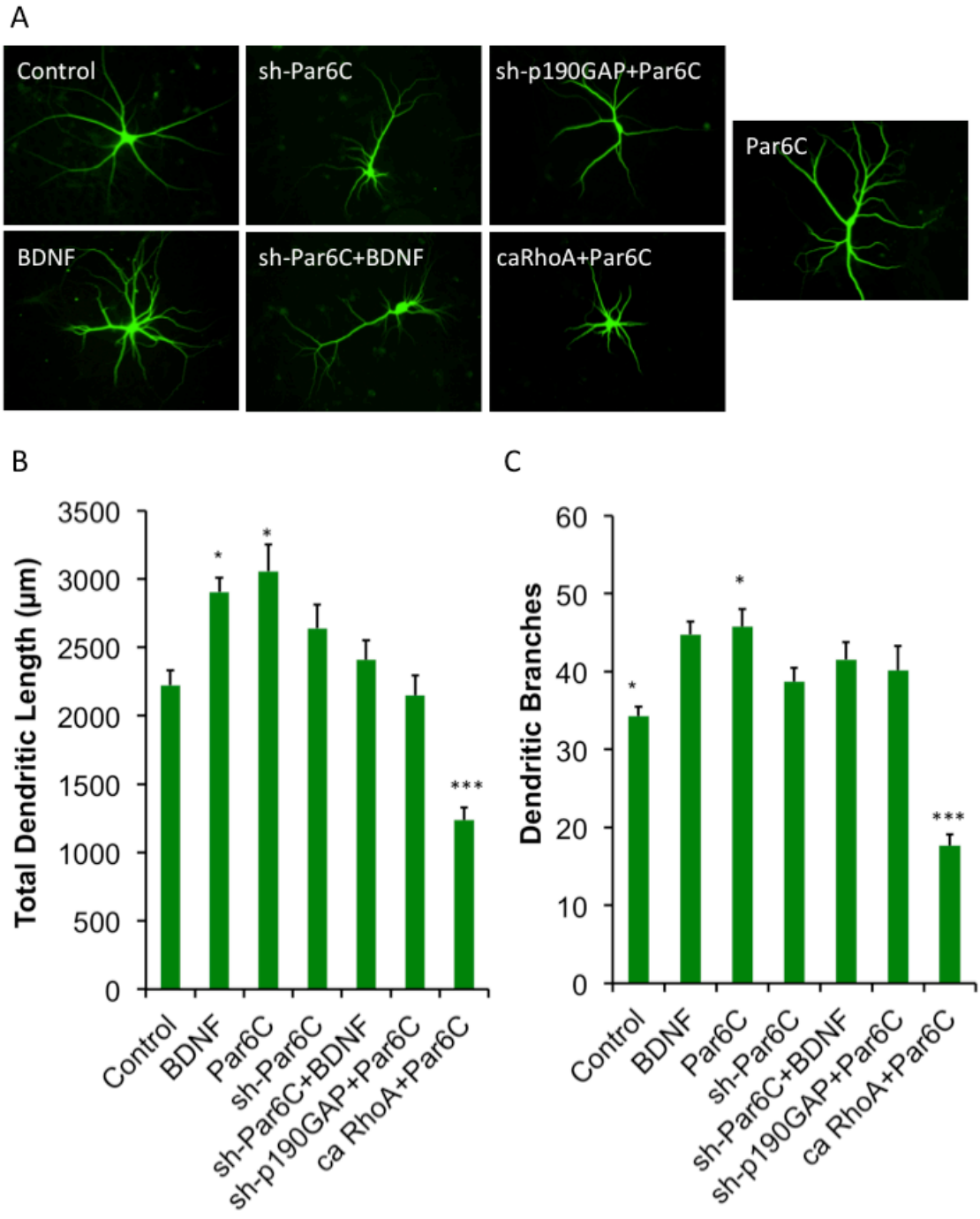


Figure 1: DIV6 cultured hippocampal neurons were transfected with MAP2B ± empty vector (Control and BDNF), ±Par6C, ± sh-Par6C, ± sh-p190GAP, ± ca RhoA, w/ or w/o 50ng/mL BDNF stimulation on DIV7 until fixed on DIV9. A) Representative images. B) Average Total Dendritic Length. C) Average Dendritic Branches. Dendrites were imaged and analyzed using NeuronJ Dendrite tracing program. (± SEM, *p<0.05, **p<0.01, ***p<0.001

Rnd3 is Required for BDNF Induced Outgrowth

Like Par6C, overexpression of Rnd3 significantly increases total dendritic length and number of branches, similar to increases seen following BDNF treatment (Figure 4). siRNA-mediated repression of Rnd3 protein expression blocked BDNF-induced increases in dendritic length and branching (Figure 4). As with Par6C, sh-p190GAP and caRhoA were used to determine the role of RhoA inhibition in Rnd3-induced dendritic growth. Both co-transfection of sh-p190GAP with Rnd3 and caRhoA with Rnd3 prevented an Rnd3-induced increase in growth (Figure 4). These results indicate that Rnd3 plays a critical role in BDNF-induced dendritic outgrowth and that it likely requires the inhibition of RhoA by p190GAP activity.

Figure 4: Rnd3 is Required for BDNF Induced Dendritic Outgrowth

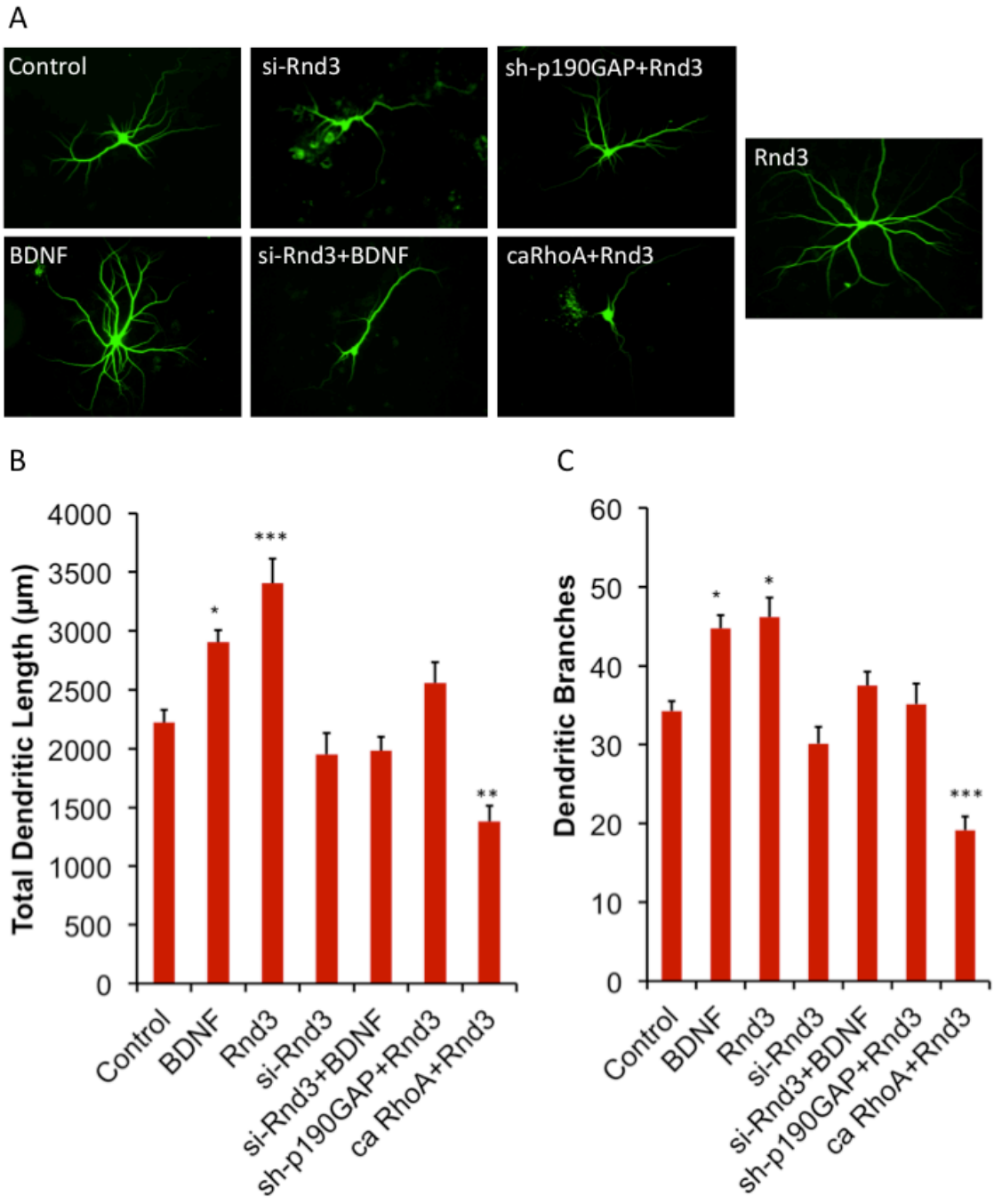


Figure 1: DIV6 cultured hippocampal neurons were transfected with MAP2B ± empty vector (Control and BDNF), ±Rnd3, ± si-Rnd3, ± sh-p190GAP, ± ca RhoA, w/ or w/o 50ng/mL BDNF stimulation on DIV7 until fixed on DIV9. A) Representative images. B) Average Total Dendritic Length. C) Average Dendritic Branches. Dendrites were imaged and analyzed using NeuronJ Dendrite tracing program. (± SEM, *p<0.05, **p<0.01, ***p<0.001

Discussion

BDNF-induced Dendritic Outgrowth Requires Par6C and Rnd3

Many neurological disorders are associated with abnormal dendritic morphology, and understanding the precise regulation of dendritic growth and patterning could provide useful insight to aid the development of new and better therapies for these conditions. Lasting changes in the morphology of the dendritic arbor require transcriptional activity, and the transcription factor CREB has been shown to play an essential role in the growth of dendrites (Wayman et al 2006). Importantly, neurotrophins, such as BDNF, have been shown to stimulate dendritic growth in part through a CREB-dependent transcriptional mechanism (Redmond et al 2002; Arthur et al 2004).

Although it is well established that CREB-dependent transcription and BDNF stimulation both can both lead to expansion of the dendritic arbor, the exact mechanism by which they regulate growth remains unclear. We hypothesized that CREB-regulated genes that inhibit RhoA would be involved in BDNF- and CREB-mediated dendritic outgrowth. We show that prevention of RhoA inhibition by suppressing expression of the RhoA-GAP p190GAP or by expressing a constitutively active RhoA mutant prevents BDNF-induced dendritic outgrowth. This data indicates that BDNF-induced dendritic outgrowth requires the inhibition of RhoA.

We found that Par6C and Rnd3 expression increased with BDNF stimulation in a CREB-dependent manner, and that overexpression of Par6C or Rnd3 is sufficient to stimulate significant increases in dendritic length and branching in cultured hippocampal neurons. Interference with RhoA inhibition using sh-p190GAP and caRhoA prevented dendritic outgrowth brought on by BDNF, Par6C or Rnd3. We propose a mechanism where BDNF binds to TrkB, initiates the MEK-ERK-MSKI signaling cascade, stimulating CREB-dependent

transcription of Par6C and Rnd3. This increase in Par6C and Rnd3 expression leads to the activation of p190GAP, which inhibits RhoA and allows for dendritic outgrowth (Figure 5). This chain of events illuminates a novel mechanism for regulating dendritic growth and branching in hippocampal neurons, and is an important step in understanding the pathways that become abnormal in neurocognitive disorders.

Future Directions

Further studies are needed to confirm the role of CREB in BDNF-induced dendritic outgrowth and verify the actions of other molecules in this pathway. Because BDNF has been shown to initiate a signaling cascade involving MEK to induce growth, we would expect that activation of MEK would be sufficient to induce growth, while inhibition of MEK would block BDNF-induced dendritic outgrowth. MEK has been shown to play an essential role in activity-induced dendritic outgrowth and to be sufficient to induce dendritic growth, so we would expect to see a similar outcome in BDNF-induced dendritic growth (Wayman et al 2006). Another study could investigate whether overexpression p190GAP alone is sufficient to induce growth, and if it is able to do so when its activators, Par6C and Rnd3, are inhibited. Because p190GAP activity inhibits RhoA, it is likely that overexpression of p190GAP would induce dendritic outgrowth, even when Rnd3 and Par6C are repressed. Finally, the consequence of suppressing RhoA is not well established, although the results of this study suggest RhoA inhibition will lead to dendritic outgrowth.

Experimental Limitations

One drawback in our experimental model that limited the completion of additional experiments is that the variability in neuronal health from culture to culture can be extreme. Due to the extensive evidence suggesting that BDNF induces an increase in dendritic outgrowth, we

used BDNF-induced increases as a positive control indicating a healthy culture and a reliable experiment. When BDNF treatment did not lead to an increase in dendritic outgrowth, this caused significant issues in the execution of experiments and prevented the collection of data repeating previously published experiments looking at the role of the MEK/ERK pathway and CREB function in BDNF-induced dendritic outgrowth. Additionally, the varied health of neuronal cultures prevented further elucidation of the precise role of p190GAP in dendritic outgrowth; however, failure to collect these data does not detract from the essential role Par6C and Rnd3 play in BDNF-induced dendritic outgrowth.

Abnormal Dendritic Morphology in Disease

Structure and function are closely tied in the dendritic arbor, and abnormalities in structure are highly correlated with functional deficits. Learning and memory, neurocognitive disorders such as depression, schizophrenia, Alzheimer's disease, and autism spectrum disorders (ASD), and genetic disorders such as Down's syndrome, Rett syndrome, and Fragile X syndrome all show atypical dendrite and spine morphologies as part of their pathologies (Kauffman and Moser 2000; Sheline et al 2003; Pickett and London 2005; Penzes et al 2011; Wong et al 2013).

Several proteins discussed previously that play important roles in the development of the dendritic arbor have also been shown to be involved in aberrant neurocognitive function. For example, transgenic mice overexpressing miR132, a CREB-regulated product, has been shown to impair novel object recognition memory (Hansen et al 2010). This study also found that overexpression of miR132 increased spine density, so it is interesting to note that the memory deficit was correlated with an increased number of spines in the hippocampus (Hansen et al 2010). Other studies have found that upregulation of CREB improved memory function along

with increased BDNF expression (Suzuki et al 2011). These results indicate that a precise regulation of CREB-dependent gene expression may be critical for normal brain function.

Another protein involved in dendritic arborization and linked to psychiatric and neurocognitive disorders is the Rac/Rho-GEF kalirin-7; altered kalirin signaling seems to play an important role in schizophrenia (Penzes and Remmers 2012). DISC I (disrupted in schizophrenia) is a protein product of a primary schizophrenia susceptibility gene. DISC 1 acts as a scaffolding protein that increases kalirin's GEF activity in regulating dendritic spines; mutations in DISC 1 found in schizophrenic patients may affect its scaffolding properties, preventing DISC 1 from augmenting kalirin's GEF activit and thus negatively affect spine formation (Penzes and Remmers 2012).

In addition to schizophrenia, other diseases such as Austism spectrum disorder (ASD) are also associated with abnormal dendritic morphology. ASD is characterized by impaired social interaction and communication, sensory hyper- and hyporeactivity, and stereotyped, repetitive behaviors (Pickett and London 2005). In patients with ASD, an increase in spine density along the middle section of apical dendrites was seen in individuals with the lowest cognitive function (Pickett and London 2005). A similar phenomenon is seen in Fragile X syndrome individuals, with an overabundance of thin, elongated spines (Penzes et al 2011). Recent evidence points to a hypothesis of neurons that are over-connected in local circuits, but lack adequate connectivity between brain regions (Penzes et al 2011). Individuals with ASD have also been found to have altered serum levels of BDNF, which could play a role in the atypical synaptic morphology and connectivity of ASD (Chapleau et al 2009).

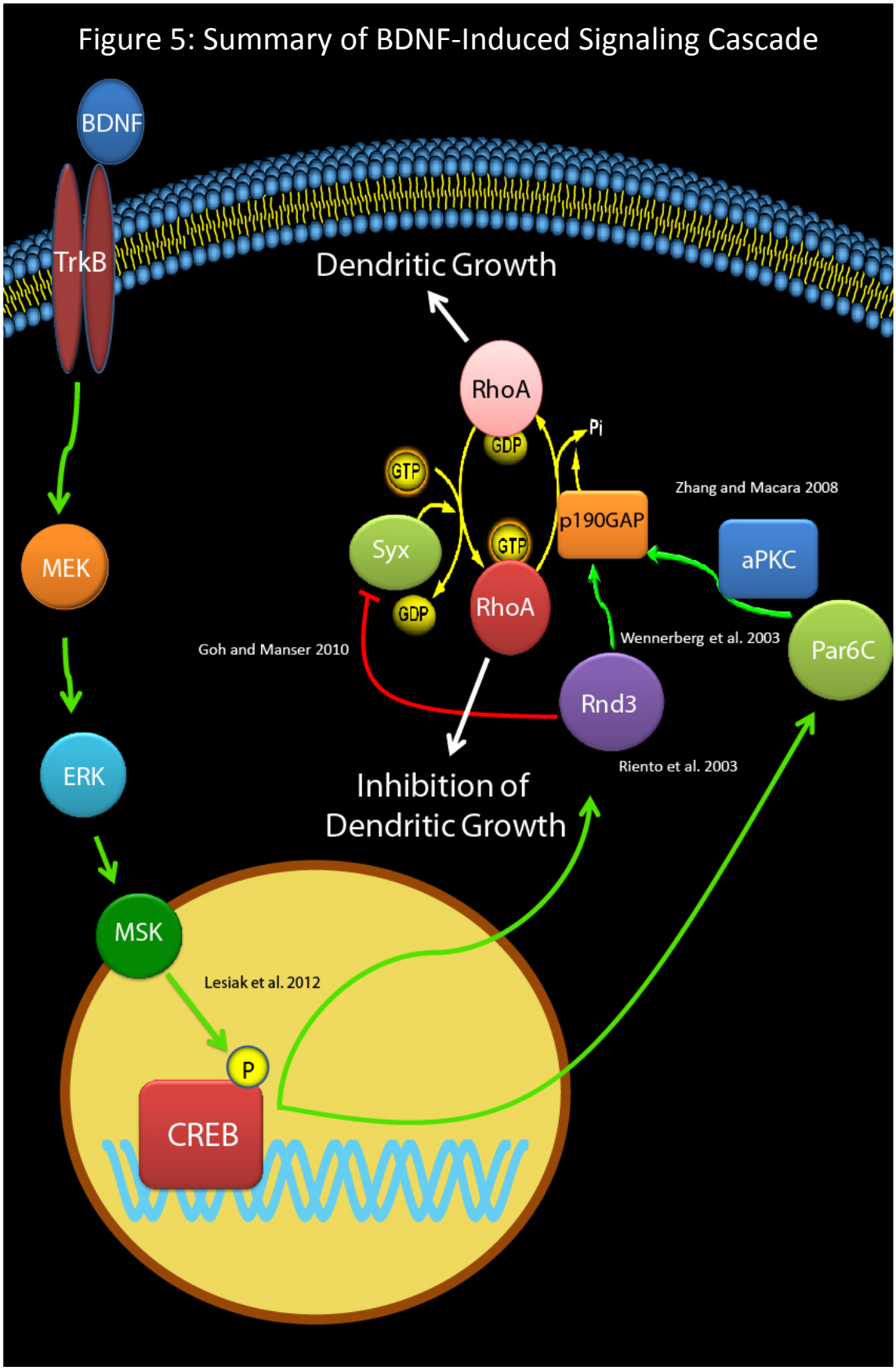
In Alzheimer's disease, postmordem tissue samples reveal dendritic spine loss in the hippocampus and throughout the cortex (Penzes et al 2011). Synapse loss is thought to be an

early consequence of Alzheimer's disease that gets increasingly worse as the disease progresses (Penzes et al 2011). Familial Alzheimer's disease is associated with mutations in three genes crucial for beta amyloid (A**β**) production; A**β** oligomers have been shown to induce spine dysgenesis and reduce spine density (Penzes et al 2011). In non-familial, late-onset Alzheimer's disease (LOAD), the primary risk factor is the ApoE gene, which has been found to influence dendrite and spine morphology but the mechanisms for this have yet to be uncovered (Penzes et al 2011).

Conclusion

This study showed that BDNF-induced dendritic outgrowth requires the CREB-dependent expression of Par6C and Rnd3 to inhibit RhoA. Small yet novel, this contribution to the greater body of knowledge regarding the regulation of the dendritic arbor is significant. Mutations and abnormalities in these pathways can quickly lead to disease and impairment of cognitive function, and these disorders not only disrupt daily life for millions of individuals, but also pose a tremendous cost to society. By understanding the mechanisms underlying dendritic outgrowth, our hope is that we, and others, will be better able to produce accurate, localized medications and therapies to treat those affected by neurocognitive disorders.

Figure 5: Summary of BDNF-Induced Signaling Cascade



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