Neuronal morphology is crucial for normal communication and health and is altered in many disease states. Microtubules are key regulators of neuronal morphology and are key components in transport of molecular cargo. Therefore, regulation of microtubule behavior is very important for establishing proper neuronal morphology and function. Thus, we examined the role of two proteins, PSD-95 and cypin, on microtubule dynamics and structure. First we show that PSD-95, a well-known synaptic protein, negatively regulates dendrite branching through a novel interaction with EB3, a plus-end microtubule-binding protein. Second, we show that cypin, a protein known to positively regulate dendrite branching, alters microtubule behavior and binds to assembled microtubules as well as tubulin heterodimers. We also show that PSD-95 and cypin have opposite but complimentary roles in changing microtubule stability, with cypin increasing and PSD-95 decreasing the stability of microtubules. Finally, we examine the
ability of small molecules compounds to modify the cellular functions of cypin. We show that cypin’s guanine deaminase activity and interaction with PSD-95 can be altered by these compounds, affecting cellular behavior.
I want to dedicate this thesis to my wife for supporting and encouraging me though this process. Without her I never would have made it through the tough times and the good times wouldn’t have been nearly as enjoyable.

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Chapter 1 – Introduction

Neurons

Neurons are highly organized cells that are used by the body for communication and information storage. In order to communicate, neurons send and receive electrical signals through very complex extensions, called the axon and the dendrites. Axons are used to transport signals from the neuron to a target, while dendrites are used to receive the incoming signal. During neurogenesis, axons and dendrites form extensive networks of connections with other neurons and cells to facilitate this communication. Neurogenesis occurs during very early stages of development, but new neurons can also be formed through adult life, and these neurons also need to make connections\textsuperscript{1,2}. Malfunctions in the development of the neuronal network during development can lead to disrupted communication that results in disease, so understanding how neurons develop is crucial to treating diseases in the brain.

Dendrites

Since Ramon y Cajal first illuminated that the dendrites of neurons form unique and highly regulated shapes, referred to as the dendritic field, there has been great interest in how neurons can form and regulate such unusual and complex morphologies.

Dendrite branching depends on shape and alterations in shape result in disease

The shape of the dendritic field is of particular interest to researchers because precise dendritic patterning is of crucial importance for signal processing by neurons. Normal communication between neurons is dependent on the shape and size of the
dendritic field. It is extremely important to understand the process by which dendrites are formed because when that process is disturbed, there are serious consequences for the brain that can result in disease. Diseases such as Alzheimer’s disease, epilepsy, Rett syndrome, and some other forms of cognitive developmental disorders (reviewed in) show changes in the dendritic field structure in at least a subset of neurons that are associated with the disease phenotype.

Cell culture system

Dissociated cell culture is one of the most common methods used to study the regulation of dendrite branching. This type of system is well-characterized, and neurons advance through 5 stages of neurite growth and development. Time in this system is measured by the number of days the cells have grown in culture after plating. This is referred to as “days in vitro” (DIV). During the first phase, undifferentiated lamellipodia extend from the cell body. This stage occurs immediately after the cells are plated. In stage 2, the lamellipodia become recognizable neurites. This stage occurs quickly, and these stages 1 and 2 together last for approximately 1 day. Then, one of the neurites differentiates into the axon in stage 3 at approximately DIV 2-3. During this period, dendrites grow directly from the cell body and are referred to as primary dendrites. The axon follows a different progression from the dendrites and will not be reviewed here. During stage 4, the extension of the dendritic field occurs. Primary dendrites branch into more complex structures, consisting of secondary branches that extend directly off of primary branches, and tertiary branches extend off of secondary branches or beyond. This occurs from approximately DIV 7 to DIV 12. Once in stage 5, the dendritic field is
consolidated, the neuron prunes unneeded branches, and spines proliferate to form the mature field. This process occurs throughout the life of the neuron but begins in earnest at approximately DIV 15. The stages are well-established, but the time frame in which each stage occurs is variable and highly dependent on culture conditions and model system used.

**Regulation of dendrite development**

By using cell culture and numerous model systems, much has been learned about the control of dendrite branching. Many signaling molecules, such as BDNF, NGF, neurotrophins, reelin, and Wnt, have been identified as factors that can alter dendrite morphology (reviewed in [16]). Internal regulators of dendrite branching, such as cytosolic PSD-95 interacting protein (cypin), postsynaptic density-95 (PSD-95), snapin, and LRRK2 [17–20], have also been identified. Recently, the cytoskeleton has been an area of intense study because of its newly emerging role in the active regulation of neuronal morphology [21]. This thesis will focus on microtubules and several proteins that regulate them in an effort to further explore the role of microtubules in the formation of the dendritic field.

**Microtubules**

Microtubules are key components of how neurons achieve their unique morphology. Microtubules are long polymers constructed with heterodimers of alpha and beta tubulin. The dimers assemble and disassemble in a highly dynamic fashion with
tubulin heterodimers, assembling mainly at the plus-end of the microtubule (for review see 22). The dynamic assembly of GTP bound tubulin on the plus-end of the microtubule and GDP bound tubulin falls off of the minus-end of the microtubule is referred to as tread-milling. This means that the microtubule is constantly being recycled, incorporating new tubulin molecules. This dynamic microtubule can undergo periods of growth, in which the microtubule grows longer, and periods of pausing or collapse, in which the microtubule stops growing or shortens. The collapsing of the microtubule is referred to a catastrophe and is a natural process but can also be altered by outside molecules. The molecules pertinent to this thesis are reviewed in more detail in Chapter two but they can include: plus and minus end binding proteins, microtubule severing proteins, microtubule polymerization proteins, microtubule stabilizing proteins and MAPs. By using these proteins to alter the microtubule cytoskeleton any cell can regulate its shape during processes, such as cytokinesis and cell motility, but neurons take shape changing to an extreme when forming the dendritic field.

**Microtubules in axons and dendrites**

The differentiation of axons and dendrites is one of the most distinctive characteristics of neurons. Microtubules behave and are regulated differentially in axons and dendrites. One of the first differences between these two neurite types seen was the separation of MAP2 and tau, microtubule associated proteins (MAPs) that bind to microtubules, into the dendrite and axon respectively 23-26. MAP2 and tau also play a role in differentially spacing the microtubules from each other 27. The orientation of the microtubules is one of the other major distinctions. The axon contains almost entirely
uni-polar microtubules that are oriented with their plus-ends, site of the majority of tubulin assembly, towards the growth cone, while the dendritic microtubules are oriented with some of their plus-ends toward the growth cone and some towards the cell body. It is generally accepted that approximately fifty percent of the microtubules in the dendrite are oriented with the plus-end toward the nucleus, but there are recent examples where this idea has been challenged. In flies, the microtubules in axons are oriented one way and dendrites the other, and modern live cell imaging techniques suggest a microtubule orientation of 80% toward the tip of the branch and 20% toward the cell body in adult mouse brains. However, neither of these examples argues that the microtubules in axons and dendrites are the same. How this mixed orientation is achieved is not yet completely understood, but it is thought to be caused by the transport of small sections of already assembled microtubules to the desired orientation by motor proteins rather than by new microtubules being made to face in a specific direction. This mixed orientation is also thought to be important for selective transport of molecules to the axon or dendrite.

Microtubules in neurite branching

The transporting of small parts of assembled microtubules is also considered one of the main mechanisms by which neurites branch. Microtubules at the site of the nascent neurite will debundle and invade the new neurite. The invasion is made of small portions of assembled microtubules created by microtubule severing molecules, such as spastin and katanin. These small lengths of microtubules are then encouraged to grow by molecules, such as collapsin response mediator protein 2 (CRMP2), that increase
microtubule polymerization \(^{41}\). This invasion and stabilization of microtubules in the newly forming neurite is crucial for maintaining the neurite and allowing it to grow into a mature extension \(^{36,37}\). Microtubules also encourage the growth and stabilization of neuronal spines \(^{42,43}\).

Through their ability to control both dendrite branching and transport of cellular cargo, microtubules are key players in the normal function of neurons. This thesis will explore the role that two proteins, cypin and its binding partner PSD-95, play in regulating microtubule behavior, and the consequences of that regulation in dendrite arborization. Cypin is a well-known positive regulator of dendrite branching and microtubule polymerization, while PSD-95 has been shown to decrease dendrite branching. The opposing roles of these two proteins will elucidate how the cell can regulate the microtubule cytoskeleton to bias itself towards a state of dendrite branching and growth or towards a state of dendrite maturity and communication with other neurons. The first section will focus on PSD-95 and explore the mechanisms by which PSD-95 acts to downregulate dendrite branching \(^{19,44,45}\). The second section will dig deeper into the understanding that we have of the role of cypin in the regulation of dendrite branching. Finally, the third section will begin to examine how we can alter the activity or interaction of these two proteins using small molecules to potentially aid us in alleviating disease or expand our ability to learn about neuronal development.
References

Chapter 2 – PSD-95 and microtubules

Introduction

Postsynaptic density protein-95 (PSD-95/SAP-90) belongs to a protein family of membrane-associated guanylate kinases (MAGUKs). The MAGUK proteins are scaffolding proteins and have complex binding domains that allow them to interact with many different proteins. Their most well-known function is to assemble synaptic signaling complexes, such as AMPA and NDMA receptors, at synapses. Post-translation modifications, such as palmitoylation, play important roles in the localization of PSD-95. The PSD-95 domain structure consists of three postsynaptic density protein-95, discs large, zona occludens-1 (PDZ) domains, a src homology 3 (SH3) domain, and a guanylate kinase (GK) homology domain. PSD-95 has two conformations: closed, in which the SH3 and GK domains overlap and limit exposure, and open, in which the SH3 and GK domains are exposed and can interact their binding partners. The SH3 domain is also required for formation of a stable lattice of PSD-95 at the synapse but it is not required for localization to the synapse. To make the situation even more complex, PSD-95 forms oligomers through N terminal interactions to create even more possibilities for protein interaction.

In addition to this very well-studied synaptic function, it has been recently discovered that PSD-95 plays a role non-synaptically. PSD-95 negatively affects dendritogenesis, resulting in fewer dendrites in neurons that overexpress PSD-95. This overexpression even overcomes any increases that are promoted by cytosolic PSD-95 interactor (cypin). Our work on cypin strongly suggests that local regulation of microtubule polymerization influences dendrite arborization. While much is known
about how microtubules are regulated in the axon by motors and severing, stabilizing, and growth-promoting proteins 18-21, less is known about the regulation of microtubule dynamics in determining dendritic arbor shape. PSD-95 associates with microtubule-associated proteins, such as microtubule-associated protein 1A (MAP1A), CRIP1, adenomatous polyposis coli (APC), and cypin 16,22-25; however, it remains unclear as to how PSD-95 affects microtubules.
Illustration 2.i

Illustration 2.i. **PSD-95 is a scaffolding protein that has interactions with many proteins.** Although all of the domains play many roles, the effects can be generalized. The three PDZ domains in PSD-95 bind to proteins that control synaptic content. The SH3 and GK domain can interact with each other and often interact with the surrounding environment to control PSD-95 localization and PSD content.
Current research focuses on the proteins that bind to the plus-ends of microtubules. The plus-end is where new tubulin is added during microtubule assembly. The plus-end accumulates a diverse group of microtubule-associated proteins, collectively referred to as the plus-end-tracking proteins (+TIPs). +TIPs encompass a large number of unrelated proteins that can be grouped into several major types of proteins: end-binding proteins (EBs), cap-gly proteins, microtubule motors, basic/ser motif proteins, and CLASP proteins (for review see 26-29). One +TIP binding protein family is the end-binding protein (EB) family. The EB proteins were initially discovered as interactors of APC 30. Like other +TIPs, EB proteins mediate interactions between the plus-ends of microtubules, organelles, and protein complexes as well as altering microtubule stability 26,29,31,32. Of the three EB family members, EB3 is preferentially expressed in the central nervous system and is used to track microtubule dynamics 26,28,33,34.

Here, we show that PSD-95 alters microtubule organization in neurons, resulting in decreased dendrite branching. PSD-95 performs this function via direct interaction with EB3 through the src homology 3 (SH3) domain on PSD-95. Deletion of the SH3 region (PSD-95ΔSH3) blocks the effects of PSD-95 on dendrite branching and microtubules. Overexpression of PSD-95 in cultured neurons results in decreased lifetime of EB3 comets in dendrites, suggesting that decreased microtubule stability and alteration of microtubule organization decrease dendrite branching. Mutation of the polyproline region of EB3 eliminates binding of EB3 to PSD-95 and blocks the effect of PSD-95 on
comet lifetime. Electron microscopic analysis of microtubules in neurons that overexpress PSD-95 confirms that PSD-95 alters microtubule organization, resulting in more microtubules crossing between the shafts at dendrite branch points. Based on these data, we present a novel mechanism by which PSD-95 can shape the dendritic arbor and cytoskeleton in developing hippocampal neurons via an interaction with EB3.
Materials and methods

Statistics and graphs

All statistics were calculated using the Prism 4.0 software from GraphPad (La Jolla, CA).

Antibodies

Mouse monoclonal antibodies recognizing PSD-95 (MA1-045, MA1-046) were purchased from Affinity Bioreagents (Golden, CO). Rabbit anti-PSD-95 was previously characterized. Rabbit anti-EB3 was generously provided by Dr. Anna Akhmanova (Rotterdam, The Netherlands). Both monoclonal (MA1-046) and rabbit anti-PSD-95 are specific for PSD-95. Mouse anti-MAP2 and rabbit anti-DsRed were purchased from BD-Pharmingen (San Diego, CA). Rat anti-GFP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

Neuronal culture, immunocytochemistry, and transfection

Hippocampal cultures were prepared from rat embryos at 18 days gestation as previously described. For transfection, the neurons were grown for 10 days in culture (DIV) and transfected with the appropriate constructs using Effectene from Qiagen (Valencia, CA) or by the calcium phosphate method. For immunocytochemical studies, neurons were fixed at DIV 12 or DIV 17 with ice cold methanol for 15 minutes and immunostained with the following primary antibodies: mouse anti-PSD95 (1:200), rat anti-GFP (1:1000), rabbit anti-EB3 (1:3000). Staining was visualized using secondary antibodies conjugated to Cy2 or Cy3 from Jackson ImmunoResearch Laboratories (West
Grove, PA). Cells were then rinsed briefly with phosphate-buffered saline and mounted on glass slides using Fluoromount G from Southern Biotech (Birmingham, AL).

Co-immunoprecipitation from brain detergent extracts

Rat forebrain (1 gram wet weight) was homogenized in 5 ml TEE (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 7.4) and incubated with an equal volume of 2X RIPA buffer (100 mM Tris-HCl, pH 7.4, 300mM NaCl, 1% deoxycholate, 2% NP-40 and 0.2% SDS, 2mM EDTA, pH 7.4) containing 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at 4°C for 1 hour. Detergent-insoluble material was pelleted by centrifugation at 15,000 x g for 10-15 min at 4°C. Antibody (10 µl) was added to the extract and incubated overnight at 4°C, followed by the addition of 25-50 µl protein A sepharose (GE Healthcare, Piscataway, NJ). After a 1-2 hour incubation at 4°C, washed beads were incubated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.01 M Tris-HCl pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 2.3% SDS, 0.005% bromophenol blue) for 20 min at room temperature (RT) followed by centrifugation. The supernatant was boiled and subjected to SDS-PAGE and Western blotting using the indicated antibodies.

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) supplemented with 7.5% fetal bovine serum in a 5% CO₂ atmosphere. Cells were transfected with 1.5 µg of the indicated plasmid DNA
encoding the indicated proteins using LipofectAMINE 2000 from Invitrogen (Carlsbad, CA) following the manufacturer's instructions. COS-7 cells were transfected with cDNAs encoding wildtype EB3-mRFP and either wildtype PSD-95-GFP, PSD-95△SH3-GFP, or GFP using Lipofectamine 2000 from Invitrogen (Carlsbad, CA) as per the manufacturer’s protocol. Cells were lysed in TEE and solubilized using Triton X-100 at a final concentration of 1%. Insoluble material was pelleted at 15,000 x g, and lysate was incubated with anti-PSD-95 antibody overnight at 4°C and then with protein A sepharose for 1 hour. Beads were isolated and bound proteins were eluted in SDS-PAGE sample buffer. Samples were boiled and subjected to SDS-PAGE and Western blotting using the indicated antibodies.

**Microtubule immunoprecipitation**

Immunoprecipitation of microtubules was performed as described elsewhere. Briefly, COS-7 cells were incubated and proteins were extracted for 15 min at RT with 0.5% NP-40 in microtubule stabilization buffer (20 mM Tris [pH 6.9], 0.5% (v/v) NP-40, 2 M glycerol, 10% (v/v) DMSO, 1 mM MgCl2, 2 mM EGTA, 200 M sodium orthovanadate, 1 mM PMSF, and the aforementioned protease inhibitor cocktail). Detergent-insoluble material was pelleted by centrifugation at 15,000 x g, and soluble extracts were used for immunoprecipitation with a mouse anti-acetylated tubulin antibody. Immunoprecipitation procedure was done as described above except that all steps were carried out at room temperature.

**Microtubule polymerization assay**
The assay was performed using the Microtubule Polymerization Kit BK004 from Cytoskeleton Inc. (Denver, CO). Briefly, 97% pure tubulin (30 µM) was mixed with purified GST fusion proteins (4 µM) in PEM buffer containing 10% glycerol and 1 mM GTP on ice to prevent polymerization. The mixture was then incubated at 37°C, and tubulin polymerization was detected by measuring the absorbance at 355 nm once per minute for one hour in a clear 96 well plate.

Filter overlay assay

Bacterial expression and purification of GST fusion proteins were performed as previously reported. For overlay assay, lysates from COS-7 cells expressing EB3-GFP were subjected to SDS-PAGE and transferred to PVDF membrane. Membrane strips containing immobilized EB3-GFP were washed 3 times with TBST (10 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.2% Tween-20) for five minutes each and blocked with TBST containing 5% non-fat dried milk (NFDM) for two hours. Strips were incubated with 0.25 µM of the indicated GST fusion protein for 15 hours, followed by three washes with TBST for 5 minutes each. All of the above steps were done at RT. The strips were incubated with TBST containing 5% NFDM for 30 minutes at RT followed by incubation with anti-GST antibody (Upstate Biotechnologies; Lake Placid, NY) diluted 1:2000 in TBST with agitation at 4°C overnight.

Molecular Modeling

The active structures of the PSD-95 fragment containing the SH3, HOOK, and guanylate kinase (GK) domains (pdb ID = 1JXO) and the solution structure of EB3
containing the calponin homology (CH) domain (pdb ID = 1WYO) were chosen as the models for the protein binding simulation. The structures were retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb) \(^{42}\). All of the molecular depictions were studied and visually analyzed using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco \(^{43}\).

**Molecular Docking.**

Chain A of the PSD-95 structure (pdb ID = 1JXO) and a heptapeptide from the EB3 structure (APPPNPNG) (pdb ID = 1WYO) were extracted and manually modified using Sybyl v7.2. Prior to ligand docking, the PSD-95 structure was energy-minimized using the Tripos Force Field \(^{44}\) and Powell method using 1000 iterations after addition of H-bond hydrogens to the structures. In addition, the heptapeptide from the EB3 structure was energy-minimized with the Tripos Force Field and a Conjugated Gradient method with 1000 iterations procedure after addition of hydrogens and Gasteiger-Hückel charges assignation \(^{45}\). The GOLD (Genetic Optimization of Ligand Docking) docking program \(^{46}\) was used to dock the heptapeptide into the proline-rich peptide binding groove in the SH3 domain of PSD-95 as previously described \(^{41}\). The default algorithm speed was selected during GOLD docking, and the number of poses for each inhibitor was set to 10. The peptide binding site was defined as SH3-PSD-95 residues within a 30 Å radius of the center of Trp470. The best conformation solution result was used to analyze the protein-peptide binding interface.
**X-gal staining and electron microscopy**

Hippocampal cultures were grown \(^47\) for 10 days in culture on Thermanox® Coverslips (Electron Microscopy Sciences; Hatfield, PA). The neurons were co-transfected with cDNA encoding β-galactosidase and GFP or PSD-95-GFP using the calcium phosphate transfection method. Neurons were fixed at DIV12 with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperature for 10 min, washed at room temperature in wash buffer (0.1 M Tris pH7.3, 2 mM MgCl\(_2\)) and staining buffer (5 mM K\(_4\)[Fe(CN)\(_6\)]·3H\(_2\)O, 5 mM K\(_3\)[Fe (CN)\(_6\)], 2 mM MgCl\(_2\), 20 mM Tris pH 7.3, 0.1% Triton X-100). Staining for β-galactosidase was performed overnight at 37º C with 1 mg/ml X-gal (Omega Bio-Tek Inc.). The cultures were post-fixed in 1% OsO\(_4\) in wash buffer for 1 h. Post-fixed cultures were rinsed with water, dehydrated in ethanol, and embedded using EMbed 812 kit (Electron Microscopy Sciences). The embedded cultures were cut parallel to the culture surface and imaged with a JEOL 100 CX transmission electron microscope (Rutgers University Electron Imaging Facility, Division of Life Sciences). Microtubule crossings at branch points were counted along a linear axis that was 0.5 µM long, beginning at the branch point and extending into the neuron perpendicular from membrane edge using Image J (NIH, Bethesda MD). Spacing between microtubules in the shaft was measured in a similar manner using ImageJ. A line (0.5 µM long) was drawn perpendicular to the microtubules in the shaft. An intensity map along the line was generated in ImageJ, and measurements were taken using the distance between the peaks representing microtubules. See Figure A.1 in the appendix for an example of how we have taken these measurements.
Time-lapse video microscopy of EB3-GFP comets in hippocampal neurons

Hippocampal cultures were prepared as described above and plated at a density of 700 cell/cm$^2$ on glass-bottom 35 mm culture dishes (WPI, Sarasota, FL). Neurons were transfected with the appropriate constructs at plating using AMAXA electroporation (Lonza Biologics; Basel, Switzerland). GFP-tagged EB3 was used in all experiments for consistency and to achieve the lowest amount of photobleaching. Transfection was performed according to the manufacturer’s protocol using ‘Alternative rat neuron protocol O-23.’ After electroporation, a 5 minute recovery period in RPMI was performed. After plating, a ½ media change after 2-4 hours and full media change after 18 hours were performed. Neurons were then grown as usual. Time-lapse fluorescence imaging was performed 12-18 hours after transfection. For live cell imaging, culture medium was replaced with Ringer's solution (150 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM Glucose, 10 mM HEPES, pH 7.4) 12-18 hours after transfection, and images were acquired on an Olympus Optical IX50 microscope (Tokyo, Japan) equipped with a 60x oil-immersion objective, heated stage, Cooke Sensicam CCD cooled camera, fluorescence imaging system and ImagePro software (MediaCybernetics; Silver Spring, MD). Images were taken 1 per second for 100 seconds. Movies were adjusted for straightness, brightness, contrast, and gamma correction and then cropped to create time-lapse representations. Comet velocity and lifetime were analyzed using Image J (NIH; Bethesda, MD) Comet movement was measured from the tip of each comet in the frame in which it initially appears to the tip of the comet in the frame previous to when it disappears by drawing a box which connected beginning tip to end tip. Velocity was quantified as length traveled (µm) vs. time (s). Comet lifetime was quantified by
measuring the length of time that comets are visible in the dendrite. Images were prepared for presentation with Adobe Photoshop to adjust cropping, levels, and contrast, and all images were subjected to the same adjustments.

**Dendrite analysis and imaging**

Neurons were imaged in the GFP channel at 200x using an Olympus Optical IX50 microscope (Tokyo, Japan) with a Cooke Sensicam CCD cooled camera, fluorescence imaging system and ImagePro software (MediaCybernetics; Silver Spring, MD). Dendrite morphology was digitized in three stages based on these initial images. In the first stage, the semi-automated tools available through the NeuronJ plugin to ImageJ (NIH; Bethesda, MD) were used to define coordinates of all dendrites in the x-y plane. In the second stage, NeuronStudio was used to define the pattern of connectivity between dendrites. These two steps fully determine the structure of each cell’s dendritic arbor and encode it in a digital format. Custom scripts written in MATLAB (MathWorks; Natick, MA) were used to transfer the data from NeuronJ to NeuronStudio. Using these digitized dendritic arbors, a second set of MATLAB scripts were then used to analyze data and perform Sholl analysis with a 9.3 µm ring interval. The data were transferred to Excel to facilitate statistical analysis. The experimenter was blinded to conditions during all data analysis. Dendrites less than 3 µm in length were not counted.

\[15,48\]
Results

**EB3 is in a protein complex with PSD-95**

We have previously shown that PSD-95 regulates dendrite branching in cultured hippocampal neurons and alters microtubule organization in COS-7 cells\(^{15}\). Because PSD-95 family members have been shown to indirectly bind to microtubules\(^{22,23}\), we hypothesized that PSD-95 may regulate dendritic arborization by interacting with the cytoskeleton. To test this hypothesis, we characterized the time course of co-localization of PSD-95 and EB3, a neuronal form of the plus-tip binding protein that is associated with growing microtubules\(^{33,49}\), in cultured hippocampal neurons (Figure 2.1A and B). We found that the percentage of co-localization was highest at DIV12 (Figure 2.1B), which is the peak of dendrite branching\(^{16,50}\). We then hypothesized that PSD-95 and EB3 may reside in a protein complex, and to test whether such an interaction exists, we performed co-immunoprecipitation experiments using rat brain extracts. We found that EB3 binds to PSD-95 (Figure 2.1C) but not to SAP-102 or SAP-97 (Figure 2.1C). These data indicate that the interaction between EB3 and PSD-95 is specific.
Figure 2.1. PSD-95 interacts with EB3

A

DIV 8

DIV 12

DIV 16

PSD-95  EB3  Merge

B

C

IB: αEB3

IB: αPSD-95  IB: αMAGUK
Figure 2.1. PSD-95 interacts with EB3.

A. Co-localization of EB3 comets with PSD-95 puncta. Primary cultures of hippocampal neurons were fixed with ice-cold methanol followed by 4% paraformaldehyde. Double-label immunocytochemistry with antibodies to PSD-95 (left column) and EB3 (middle column) was performed. Images are overlaid for direct comparison (right column). *Inset*, High magnification indicating co-localization of PSD-95 and EB3 puncta (arrows). B. Co-localization of EB3 and PSD-95 peaks at DIV12. Co-localization was quantified by calculating the proportion of total EB3 comets that overlay PSD-95 puncta. ***p < 0.001 by ANOVA followed by Bonferroni multiple comparisons test. C. Co-immunoprecipitation of EB3 with PSD-95 from adult rat brain. EB3 co-immunoprecipitates with PSD-95 but it does not co-immunoprecipitate with SAP-97 or SAP-103. Detergent extracts from adult rat brain were subjected to immunoprecipitation with the indicated antibodies.
**EB3 interacts with PSD-95 via a direct interaction between a polyproline region in EB3 and the SH3 domain of PSD-95**

We then asked whether the interaction between PSD-95 and EB3 is direct. Overlay assays indicated that filter-bound EB3-GFP binds to an overlaid portion of the PSD-95 polypeptide that contains the SH3 domain as well as that containing both the SH3 and guanylate kinase domains but not to those portions of PSD-95 containing PDZ domains 1-3 or the guanylate kinase domain alone (Figure 2.2A). Interestingly EB3-GFP did not interact with the full-length PSD-95 in this assay, suggesting that this interaction cannot occur when the full-length PSD-95 is not properly folded. To test this possibility, we co-incubated equimolar amounts of purified GST-EB3 and GST-PSD95 followed by immunoprecipitation with an antibody to PSD-95 or with mouse IgG. As shown in Figure 2.2B, an antibody to PSD-95 but not mouse IgG co-immunoprecipitated EB3 in this assay, suggesting that both full-length PSD-95 and EB3 must assume specific three-dimensional structures to bind. Notably, the SH3 and GK domains of PSD-95 can bind intra- and inter-molecularly, altering the availability of the SH3 domain for EB3 binding.

We then performed sequence analysis of the EB proteins to identify a potential binding site in EB3 for the SH3 domain of PSD-95. This analysis and in silico molecular docking analysis indicated that the SH3 domain of PSD-95 interacts with a proline-rich hexapeptide (APPPNP), corresponding to amino acids 136-141 on the EB3 polypeptide (Figure 2.2C and 2.2D). These results suggest that the SH3 domain of PSD-95 interacts directly with EB3, possibly via a proline-rich region in EB3.

While EB2 contains a similar proline-region, we did not use it as a focus of our
studies because EB3 is preferentially expressed in the central nervous system\textsuperscript{33}. EB2 is also not as significant a contributor to suppression of microtubule catastrophe\textsuperscript{32} and does not form dimers with either EB1 or EB3\textsuperscript{52}. EB2 does not localize to the plus-end of the microtubule in distinct comets\textsuperscript{53}. Thus, our studies focused on the interaction between PSD-95 and EB3.
Figure 2.2. The SH3 domain of PSD-95 directly binds to a polyproline-rich region of EB3.
Figure 2.2 The SH3 domain of PSD-95 directly binds to a polyproline-rich region of EB3.

A. EB3 directly interacts with the SH3 domain of PSD-95. Lysates from COS-7 cells expressing EB3-GFP were resolved by SDS-PAGE and transferred to PVDF membrane. Membrane strips were overlayed with the indicated GST fusion proteins of PSD-95 domains followed by immunoblotting with an antibody to GST. B. Purified EB3 and PSD-95 interact directly. Equimolar amounts of purified, bacterially expressed GST-EB3 and GST-PSD-95 were incubated at 4°C overnight, followed by immunoprecipitation with an antibody to PSD-95. Precipitates were subjected to immunoblotting with an antibody to EB3. C. Sequence alignment of the RP/EB microtubule-associated protein family members. EB3 contains a polyproline region. Conserved residues are highlighted in yellow and boxed. The gaps are indicated with (-). An EB3-specific proline-rich residue motif is highlighted in red from amino acids 136 to 141. D. Display of the best EB3 proline-rich peptide conformation and its accommodation in the SH3-binding groove of PSD-95 after docking using GOLD. The peptide was rendered in element-coded sticks (oxygen=red; nitrogen=blue; hydrogen=white; carbon=gray) and the N- and C- termini are indicated. The PSD-95 structure is shown using a gray-coded Solvent Accessible Surface (SAS) and residues in the SH3 domain in PSD-95 involved in the peptide binding are shown in cyan.
The interaction between PSD-95 and EB3 regulates the dendritic arbor

PSD-95 stops dendrite branching\textsuperscript{15}, and the interaction between PSD-95 and EB3 may play a role in this process, perhaps by sequestering EB3, thereby inhibiting microtubule growth and organization. To test this hypothesis, we asked whether direct binding of PSD-95 to EB3 is essential for proper dendritogenesis. We created a mutant of amino-terminal GFP-tagged PSD-95, which lacks the SH3 domain (PSD-95\text{\textregistered}SH3. As seen in Figure 2.3, overexpression of wildtype PSD-95-GFP significantly decreased dendritic complexity while overexpression of GFP-PSD-95\text{\textregistered}SH3 had no effect on dendrite number, adding further support to the importance of the interaction between PSD-95 and EB3 in shaping the dendritic arbor.
Figure 2.3. Deletion of the SH3 binding region in PSD-95 rescues PSD-95-induced branching deficits.
Figure 2.3. Deletion of the SH3 binding region in PSD-95 rescues PSD-95-induced branching deficits.

A. Representative inverted GFP images of hippocampal neurons transfected with the cDNA constructs for the indicated proteins. Neurons were transfected on DIV7 and analyzed for dendrite number at DIV12. Scale bars = 50 µm. B. Sholl analysis of neurons transfected with cDNA constructs encoding PSD-95-GFP, GFP-PSD-95ΔSH3, or GFP. Neurons expressing PSD-95 ΔSH3-GFP do not show PSD-95-promoted decreases in dendritic arborization but instead show similar levels of branching to neurons expressing GFP up to a distance of 100 µm from the cell body. ***p<0.001 by ANOVA followed by Bonferroni multiple comparisons test. n = 42 neurons for each condition.
 Binding of PSD-95 to EB3 decreases EB3 binding to the +TIPs of microtubules and slows microtubule assembly in a cell-free system

We reasoned that by binding to EB3, PSD-95 prevents EB3 from accessing microtubules. Thus, we asked whether the interaction of EB3 and PSD-95 affects the binding of EB3 to the plus-ends of microtubules. To address this question, we used COS-7 cells since overexpression of PSD-95 in COS-7 cells results in disorganized microtubules. Extracts from COS-7 cells transfected with cDNA encoding EB3 fused to GFP, prepared using a microtubule stabilization buffer, were subjected to immunoprecipitation using an antibody to acetylated tubulin in the presence or absence of purified GST, GST-PSD-95, or PSD-95ΔSH3 (Figure 2.4A). As shown in Figure 2.4, the association of EB3 with immunoprecipitated microtubules was reduced in the presence of GST-PSD-95 but not GST-PSD-95ΔSH3. These results suggest that the binding of EB3 to PSD-95 destabilizes the binding of EB3 to the plus-ends of microtubules in these cultures.

How does the binding of PSD-95 to EB3 alter microtubule growth? We used an in vitro cell-free microtubule polymerization assay to assess the kinetics of tubulin polymerization in the presence of purified PSD-95. Surprisingly, these tubulin preparations contain detectable amounts of EB3, as shown by Western blotting with an antibody to EB3 (Figure 2.4B). Our results show that GST-PSD-95, but not GST-PSD-95ΔSH3, decreases the total amount of tubulin polymerization, as determined by light scattering at OD_{355} (Figure 2.4B and C). These results are consistent with the idea that PSD-95 acts to inhibit the binding of EB3 to the plus-ends of microtubules, contributing to the destabilization of microtubule polymerization in this assay.
Figure 2.4. PSD-95 decreases the binding of EB3 to microtubules in heterologous cells and inhibits microtubule assembly in a cell-free system.
Figure 2.4. **PSD-95 decreases the binding of EB3 to microtubules in heterologous cells and inhibits microtubule assembly in a cell-free system.**

A. Microtubules were immunoprecipitated from lysates of COS-7 cells expressing EB3-GFP and exogenously added GST-PSD-95 or GST-PSD-95ΔSH3. Immunoprecipitates were subjected to SDS-PAGE and Western blotting for the presence of EB3. B. Light scattering of microtubules at OD\textsubscript{355} in the presence of 1 mM GTP was used to detect the effect of PSD-95 on microtubule polymerization. Addition of 4 M GST-PSD-95, but not GST-PSD-95ΔSH3, slows the polymerization of purified tubulin compared to the addition of equimolar concentrations of GST alone. *Inset*, Western blots of purified tubulin fractions (right lane), used in the above polymerization assay, show detectable amounts of EB3. The immunoreactive protein band co-migrates with that from a rat brain homogenate (left lane). C. Quantification of total tubulin polymerization shows that GST-PSD-95, but not GST-PSD-95ΔSH3, lowers the total amount of polymerization that occurred as measured by V\text{max} of the curve. *p<0.05 by Two-way RM ANOVA followed by Bonferroni multiple comparisons test to compare each point on the curve. n = 3.*
**Overexpression of PSD-95 results in altered microtubule organization**

Because EB3 binds to the plus-ends of microtubules and rescues them from catastrophe \(^{32}\) and our results suggest that binding of PSD-95 to EB3 negatively affects EB3 binding to microtubules and subsequent assembly (Figure 2.4), we hypothesized that the interaction between PSD-95 and EB3 may cause a change in microtubules in neurons. To assess whether PSD-95 alters microtubule structure in neurons, we performed electron microscopic analysis on transfected cultured neurons. As shown in Figure 2.5A, B, and D, overexpression of PSD-95-GFP results in more microtubules that cross at dendrite branch points (see Figure 5E for diagram). This change in microtubule architecture was not seen in neurons overexpressing GFP-PSD-95\(\Delta\)SH3 (Figure 2.5A, C, and D). We did not observe a change in the total number of microtubules observed in the plane of sectioning in neurons overexpressing GFP or PSD-95-GFP (Figure 2.5E). We also observed a change when the space between microtubules was measured in the shaft of the neuron (Figure 2.6). Overexpression of PSD-95 significantly increases the space between microtubules when compared to over expression of GFP while GFP-PSD-95\(\Delta\)SH3 only partially rescues this effect (Figure 2.6D).
Figure 2.5. PSD-95 increases microtubule crossing at dendrite branch points

A

B

C

D

E

PSD-95 increases microtubule crossing at dendrite branch points.
Figure 2.5. PSD-95 increases microtubule crossing at dendrite branch points.

A-C. EM images of microtubules of hippocampal neurons transfected at DIV10 and fixed on DIV12. Scale bar = 0.5 µM. * = branch point from a dendrite. A. Control neurons that express GFP have organized microtubules at dendrite branch points. B. Neurons that overexpress PSD-95-GFP show disorganized microtubules at that cross at dendrite branch points when compared to control GFP expressing neurons. C. Neurons that express GFP-PSD-95∆SH3 show organized microtubules at dendrite branch points, similar to control neurons expressing GFP. D. Quantitation of the number of microtubules that cross at dendrite branch points in transfected hippocampal neurons represented in panel. Microtubules cross dendrite branch points more often in neurons that overexpress PSD-95-GFP when compared to microtubules of neurons that overexpress GFP or GFP-PSD-95∆SH3. **p<0.01 by Kruskal-Wallis test followed by Dunn’s multiple comparison test. n = 5 neurons for each condition. E. Quantitation of the total number of microtubules present at dendrite branch points. There is no difference between control neurons that express GFP or neurons that express PSD-95-GFP.
Figure 2.6. PSD-95 causes an increase in microtubule spacing along the shaft of the dendrite.
Figure 2.6. PSD-95 causes an increase in microtubule spacing along the shaft of the dendrite.

A. Representative images of control hippocampal neurons that express GFP. B. Representative images of hippocampal neurons that overexpress PSD-95-GFP. C. Representative images of hippocampal neurons that overexpress GFP-PSD-95∆SH3 D. Quantification of the spacing between microtubules. Neurons that overexpress PSD-95-GFP have a significantly increased inter-microtubule distance. ***p<0.001 compared to neurons that express GFP. Neurons that overexpress GFP-PSD-95∆SH3 have a microtubule spacing pattern that is in between neurons that overexpress PSD-95-GFP and control neurons that express GFP. *p<0.05 compared to neurons that express GFP. All statistics analyzed by Kruskal-Wallis test followed by Dunn’s multiple comparison test. n > 20 neurons for each condition. Scale bar = 0.5 µM.
Interaction of PSD-95 and EB3 reduces EB3 comet lifetime

We hypothesized that this change in microtubule structure may be due, at least in part, to an effect of PSD-95 on EB3 comet dynamics. To assess this, we co-expressed EB3-GFP or EB3-THR-GFP, a mutant in which the prolines have been mutated to threonines, rendering the EB3 unable to bind PSD-95 (Figure 2.7A and B) in hippocampal neurons. We measured the movements of these GFP-tagged comments in the growing dendrites of young hippocampal neurons (Figure 2.7C). We found that the threonine mutation of EB3 lowers the time that EB3 comets spend on the plus-tips of the microtubules, referred to as the comet lifetime, but that the mutation does not alter the velocity of the comets (Figure 2.7D and F). We also found that co-expression of PSD-95 with EB3-GFP lowered the lifetime of the wildtype comets, but this co-expression did not affect the lifetime of the EB3-THR comets by decreasing it further (Figure 2.7D and E). Co-expression of PSD-95 lowered the velocity of both wildtype and mutant comets, suggesting that an interaction of PSD-95 with other +TIP proteins at other domains in PSD-95 may regulate comet velocity (Figure 2.7D and F). We also found that when EB3-GFP was co-expressed with PSD-95ΔSH3, comet lifetime did not change; however, comet velocity was significantly decreased, similar to the effect of co-expressing wildtype PSD-95. Our data strongly suggest that PSD-95 influences microtubule dynamics in neurons via direct interaction of EB3 by specifically affecting how long EB3 comets associate with the microtubule plus-end and via interaction with other proteins to affect EB3 comet velocity.
Figure 2.7. PSD-95 decreases the lifetime of EB3 comets in hippocampal neurons.

A) Sequence comparison of EB3 and EB3-THR

B) Western Blot analysis of PSD-95 and EB3

C) Immunofluorescence images

D) Comet lifetime analysis

E) Comet lifetime graph

F) Comet velocity graph
Figure 2.7. PSD-95 decreases the lifetime of EB3 comets in hippocampal neurons.

A. Diagram of a mutant form of EB3 with key amino acids of the proline-rich region changed to threonines. B. Wildtype, but not EB3-THR, co-immunoprecipitates with PSD-95 from extracts of COS-7 cells co-expressing the indicated proteins. The anti-EB3 antibody used for Western blotting recognizes a nonspecific (ns) ~50 kDa protein band in COS-7 cell lysates. C. Low magnification images of GFP-tagged EB3 comets in a growing neurite. Asterisks indicate representative comets. D. Representative time series images of GFP-tagged EB3 and EB3-THR comets in the dendrites of hippocampal neurons and GFP-tagged EB3 and EB3-THR comets in hippocampal neurons also expressing PSD-95-mRFP or PSD-95ΔSH3-mRFP. Images were taken at 18-24 hours post-transfection, which was performed at the time of plating. Scale bars = 1 µm. Arrows indicate first and last appearance of comet. Asterisks track with tip of comet. E. Quantitation of the effect of PSD-95-mRFP overexpression on EB3-GFP and EB3-THR-GFP comet lifetime. Overexpression of PSD-95-mRFP decreases the lifetime of EB3-GFP comets but does not decrease the lifetime of EB3-THR-GFP comets. *p<0.05 and **p<0.01 by Kruskal-Wallis test followed by Dunn’s multiple comparison test compared to EB3-GFP. n ≥ 15 comets for each condition. E. Quantitation of the effect of PSD-95 overexpression on EB3-GFP and EB3-THR-GFP comet velocity. Overexpression of PSD-95-GFP decreases the velocity of both EB3-GFP and EB3-THR-GFP comets, suggesting that this effect is not specific to the direct interaction between PSD-95 and EB3. *p<0.05 and **p<0.01 by Kruskal-Wallis test followed by Dunn’s multiple comparison test compared to EB3-GFP. n ≥ 15 comets for each condition.
Reduced EB3 comet lifetime correlates with decreased dendritic arborization

To further explore the physiological significance of PSD-95-mediated reduction in EB3 comet lifetime, we examined the effect of overexpression of PSD-95 and EB3 on dendritic arborization. We found that overexpression of EB3-THR-GFP, but not wildtype EB3-GFP, reduced dendritic complexity (Figure 2.8A and B). We also observed that overexpression of PSD-95-mRFP reduced dendrite number whether expressed alone or co-expressed with either EB3-GFP or EB3-THR-GFP (Figure 2.8A and C). These results are consistent with the effects observed on comet lifetime when these proteins are overexpressed. This indicates that comet lifetime may play a role in the process of dendrite arborization. It is important to note that the effects of PSD-95 on comet velocity may also contribute to decreases seen in dendrite number; however, based on our data, it appears that the direct binding of PSD-95 to EB3 may play a major role in regulating dendritogenesis.
Figure 2.8. Overexpression of PSD-95 decreases branching even in the presence of a non-binding mutant of EB3
Figure 2.8. Overexpression of PSD-95 decreases branching even in the presence of a non-binding mutant of EB3.

A. Representative inverted GFP images of hippocampal neurons transfected with the indicated cDNA constructs. Neurons were transfected on DIV 7 and analyzed for dendrite number at DIV12. Scale bar = 50 µm. B. Sholl analysis of neurons transfected with cDNA constructs for the indicated proteins. B. Analysis of neurons represented in panel A shows that overexpression of EB3-THR-GFP results in decreased dendritic complexity when compared to dendrite numbers of control neurons expressing GFP or neurons expressing EB3-GFP. ***p<0.01 by Two-way RM ANOVA followed by Bonferroni multiple comparisons test to compare each point on the curve. n = 24 neurons for each condition. C. Sholl analysis of neurons expressing EB3-GFP or EB3-THR-GFP alone or with PSD-95-mRFP. Neurons expressing EB3-THR-GFP show a decreased dendritic complexity when compared to control neurons. C. Expression of PSD-95-mRFP decreases dendrite number. Co-expression of either EB3-GFP or EB3-THR-GFP with PSD-95-mRFP results in a decrease in dendritic complexity that is equal to the decrease mediated by PSD-95 alone. ***p<0.01 by Two-way RM ANOVA followed by Bonferroni multiple comparisons test to compare each point on the curve. n = 24 neurons for each condition.
A mutation of SH3-binding motif in EB3, constructed with charged amino acid substitutions for prolines, acts in a manner opposite to the EB3-THR mutant.

We constructed two mutants of EB3 to examine the interaction of EB3 with PSD-95. One, the EB3-THR mutant, which contains substitutions of threonine in place of prolines required for binding to PSD-95, has been discussed above. Another mutation was made with arginines in place of prolines (EB3-ARG; see Figure 2.9A for diagram of this change). Unlike EB3-THR, EB3-ARG was not competed off of microtubules by exogenous PSD-95 (Figure 2.9). Neurons overexpressing EB3-ARG-GFP have increased dendrite complexity when compared to neurons overexpressing wildtype EB3-GFP or GFP (Figure 2.10). Thus, substituting prolines with arginines in this region of EB3 may serve as a dominant mutation that can be used to explore the relationship of PSD-95 and EB3 in microtubule dynamics.
A mutant EB3 with arginines in place of crucial prolines in the SH3 region of EB3 was created. Microtubules were immunoprecipitated from lysates of COS-7 cells expressing EB3-ARG-GFP and exogenously added GST-PSD-95 or GST. Immunoprecipitates were subjected to SDS-PAGE and Western blotting for the presence of EB3. A. Diagram of a mutant form of EB3 with key amino acids of the proline-rich region changed to arginines. B. Representative images of Western blots from immunoprecipitations. C. Quantitation of Western blot. No significant changes were found when PSD-95 was added to microtubule preparation. Statistics were performed using ANOVA followed by Bonferroni multiple comparisons test using GraphPad Prism. n=3.
Figure 2.10. Overexpression of EB3-ARG mutant increases dendrite branching.

Sholl analysis of neurons transfected with cDNA constructs encoding the indicated proteins. Neurons that overexpress EB3-ARG-GFP show increased branching when compared to control neurons overexpressing GFP and neurons overexpressing wildtype EB3-GFP. **p<0.01 by two-way RM ANOVA followed by Bonferroni multiple comparisons test to compare each point on the curve. n = 48 neurons for each condition.
Discussion

Since dendrite branching involves reorganization of the neuronal cytoskeleton, \(^{55,56}\), and because the effects of PSD-95 on dendrite branching may involve the alteration of cytoskeletal dynamics \(^{15}\), we hypothesized that PSD-95 achieves these morphological effects through an interaction with microtubule-associated proteins. Our results show that PSD-95 directly interacts with the microtubule plus-end binding protein EB3, a member of the +TIP family of microtubule-associated proteins, by a direct interaction of the PSD-95 SH3 domain with a proline-rich region within the EB3 polypeptide. When the interaction between PSD-95 and EB3 was disturbed using functional mutational analysis, we observed an elimination of the effect of PSD-95 on dendrite number and branching and EB3 comet behavior. It is important to note that while the SH3 domain of PSD-95 interacts with the GK domain and is required for establishing a stable lattice in the postsynaptic density, it is not required for localization to the synapse \(^{9-12}\). Thus, the lack of effect of the PSD-95ΔSH3 mutant on dendrite number and comet velocity is not due to improper localization.

This is the first time it has been shown that PSD-95 alters EB3 comet dynamics, and hence microtubule dynamics, in developing neurons. Time-lapse studies of EB3-GFP comets showed a reduced lifetime of only wildtype comets in the presence of PSD-95. The reduction of the time that EB3 spends at the tip of the microtubule in the presence of excess PSD-95 may be due to displacement of EB3 from microtubules, as evidenced by our cell-free studies, or it may be due to a reduction of the available pool for the rapidly cycling EB3 molecule \(^{57}\). Our observed effect of PSD-95 overexpression on comet lifetime matches the results from our studies on the effects of PSD-95 and EB3.
on dendritic arborization. Overexpression of EB3-THR alone or PSD-95 in the presence of EB3 or EB3-THR results in decreased dendritic complexity. These data suggest that decreased EB3 comet lifetime may play a role in regulating dendritic arborization. This role of comet lifetime is also supported by the fact that expression of a mutant PSD-95 containing a deletion of the SH3 domain from PSD-95 results in no effect on dendrite branching or comet lifetime but does decrease comet velocity. Our new data, summarized by the model shown in Illustration 2.ii, provide a potential mechanism for the function by which PSD-95 acts during the course of neuronal development.
Illustration 2.ii. Model of the roles of PSD-95 and EB3 in regulating dendritogenesis

A. EB3 cycles on and off microtubules over time.

B. Lowered comet lifetime decreases dendritogenesis.

C. Normal dendritogenesis.

D. Increased PSD-95 decreases dendritogenesis.

Microtubules are highly organized at branch points.

Disruption of the organization decreases dendritogenesis.
Illustration 2.ii. Model of the roles of PSD-95 and EB3 in regulating dendritogenesis.

A. Under control conditions, EB3 comets have a lifetime of ~10.5 seconds. This time is influenced by the cycling of EB3 on and off of the plus-end of the microtubule and the cycles of microtubule catastrophe and rescue. Endogenous PSD-95 interacts with EB3.

B. When PSD-95 or EB3-THR is overexpressed, EB3 comet lifetime decreases, leading to reduced dendritic complexity. EB3-THR comets remain on microtubules for a shorter amount of time than do wildtype comets, and increased PSD-95 expression may sequester EB3, reducing its lifetime at the plus-end of microtubules. C. In control neurons, microtubules do not cross at branch points frequently. D. When PSD-95 is overexpressed, the frequency of microtubules that cross between dendritic branches increases. Scale bar in example images of neurons = 50 µM.
It is also of interest that the velocity of both wildtype and mutant EB3 comets were both reduced by PSD-95. This result suggests that PSD-95 may be acting in an indirect manner to control this characteristic of EB3 comets and that other molecules may play a role in this interaction. The regulation of the dynamic nature of the plus-ends of microtubules is very complex and involves many protein interactions. For example, the PDZ domains of PSD-95 can bind to APC, and EB3 interacts with APC to regulate microtubule dynamics and targeting. CLIP-170, another +TIP protein, can bind to microtubules and EB1 through separate domains, providing a potential model for interaction at the plus-end of the microtubule. Although APC and CLIP-170 represent two possible interactors for PSD-95 action at the plus-end, an indirect interaction of another +TIP protein binding to a different domain of PSD-95 may act to regulate EB3 comet velocity, while the direct interaction between PSD-95 and EB3 regulates comet lifetime.

The EB3-ARG mutant may prove to be a useful tool to further examine this complex relationship. EB3-ARG is not competed from microtubules by PSD-95 and increases branching, while EB3-THR is competed from microtubules and decreases branching. This may be due to the fact that arginine is a charged amino acid, and the substitutions were made near the domain that is important for EB dimerization. This mutation may cause EB3 to form more stable dimers with itself or EB1. Since expression of EB3-ARG acts in an opposite manner to expression of EB3-THR, this mutant may be useful for exploring which effects of the EB-PSD-95 interaction are due to PSD-95 competition and which are due to changes in EB3 binding behavior.

What is the physiological significance of the regulation of EB3 comet lifetime by
PSD-95? There has been some debate in the field about the affect of EB proteins on microtubule dynamics. Studies done in a number of cell lines and conditions have attributed a very complex, sometimes conflicting, set of behaviors to the EB proteins \textsuperscript{32,62-64}. What all of the studies agree on is that disrupting comet appearance on the \textit{+TIP} alters microtubule dynamics. Our data indicate that the effects of PSD-95 on EB3 comet lifetime include changes in microtubule dynamics and that these changes correlate to altered microtubule organization that may influence dendrite morphology. We confirmed these effects of PSD-95 using electron microscopy and observed that microtubules of neurons that overexpress PSD-95 cross more at branch points and have more space between them in the shaft of the dendrite than do microtubules of neurons expressing GFP or PSD-95\textcopyright{SH3}. This organizational change could influence dendrite morphology by inhibiting the formation of new branches or stabilizing existing ones. The microtubules that cross at the branches may be oriented with their plus-ends toward the nucleus, which would have implications in transport of dendritic cargo \textsuperscript{65}. One confounding factor may be the role of the actin cytoskeleton. It has been shown that EB3 acts to bring actin remodeling molecules to the cell membrane, and disrupting this interaction may prevent microtubules from interacting with F-actin, which is important for neurite extension \textsuperscript{66,67}.

The present research sheds light on the newly discovered interaction between EB3 and PSD-95 at the \textit{+TIP}. We previously reported that PSD-95 alters dendrite branching in cultured hippocampal neurons and that this effect might result from the alteration of microtubule dynamics \textsuperscript{15}. We now have shown that the direct interaction of PSD-95, an integral part of the post-synaptic apparatus, and EB3, an essential regulator of
microtubule dynamics, correlates to changes in the microtubule network, which shapes dendritic structure. The study of this interaction provides additional insight into the complex organization of molecules at the plus end of microtubules and into the mechanism of dendritogenesis.
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Data from this chapter has appeared in 68 and 69.

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Chapter 3 – Cypin and microtubules

Introduction

Cytosolic PSD-95 interactor (cypin) is a 50 kDa protein that is a well-known regulator of dendritogenesis. Cypin has several important domains that give it functionality (Illustration 3.i). Initially, Dr. Firestein found that cypin interacts with PSD-95 through a C-terminal, PDZ-binding motif and decreases the clustering of PSD-95 at the postsynaptic density \(^1\). It is, thus far, the only protein capable of this effect on PSD-95. Cypin acts as a guanine deaminase (GDA) and is thus a part of the purine metabolic cycle. It functions to deaminate guanine to xanthine and is the major guanine deaminase in the brain \(^1,2\). Zinc binding to several crucial amino acids in the N-terminus of the protein is important for functional GDA activity \(^2,3\). Finally, through the collapsin response mediator protein homology (CRMPH) domain, cypin binds tubulin heterodimers and increases microtubule polymerization \(^2\). Two of these functions, GDA activity and microtubule assembly, are required for cypin to increase dendritogenesis \(^2\). The PDZ-binding motif is not required to increase dendritogenesis, but it is required for the stabilization of the mature dendrite arbor \(^2,4\).

In addition to the internal protein motifs that mediate cypin function, there are several protein interactions and signaling pathways that are important for cypin-promoted increases in dendrite branching to occur. Two other proteins have been found to interact with cypin, and both act as negative regulators. Overexpression of PSD-95 can negate any effect of cypin on dendritogenesis \(^4\), and the protein snapin, a SNAP-25 interacting protein, binds to the CRMPH domain and eliminates any increases in cypin-promoted dendritogenesis by competing tubulin binding to cypin \(^5\). Our laboratory has also recently
identified the signaling pathways which regulate cypin function. BDNF signaling acts through CREB-dependent transcriptional regulation of cypin \(^6\). Interestingly, cypin is required for BDNF-mediated increases in dendrite branching. Negative regulation of cypin occurs via activated RhoA, which decreases cypin protein levels through translational regulation \(^7\).

Although our laboratory has studied cypin for over a decade, there are still some gaps in our knowledge of cypin function. We still do not yet know how cypin associates with microtubules and how this association changes microtubule dynamics in the cell. As seen in Chapter 1, microtubule dynamics in the cell play a powerful role in determining the fate of the dendritic arbor. We do know, however, that cypin associates with tubulin and increases microtubule polymerization in a cell-free system. However, cypin-promoted changes in microtubule dynamics have not yet been observed in the cell. This chapter will address this and other gaps in our knowledge of cypin action on microtubules and tie them to physiologic consequences in the cell.
Illustration 3.i Diagram of cypin domains and protein interactions.

Cypin binds to zinc via amino acids 76-84, 240, and 330. These amino acids are required for guanine deaminase activity, and cypin binds to tubulin heterodimers with a CRMP homology domain located at amino acids 350-403. This domain is required to increase microtubule polymerization and is negatively regulated by snapin. A PDZ-binding motif is located at the C-terminal end of cypin and is required for the interaction of cypin and PSD-95.
Materials and methods

Statistics and graphs

All statistics were calculated using the Prism 4.0 software from GraphPad (La Jolla, CA).

Microtubule binding

This assay is performed according the protocol given in the BK029 microtubule binding protein spin-down assay kit from Cytoskeleton Inc. (Denver, CO) with the following exceptions. Microtubules were prepared fresh for each assay using 5mg/ml in a 50% glycerol solution. Microtubules were then stabilized using taxol and kept at RT. A control using an equivalent amount of tubulin without polymerization and kept on ice was prepared at the same time. A second control of GST alone was used to control for the effect of the GST tag on the assay. GST purified protein (25 µM) was incubated with the microtubules at RT and 4 ºC, respectively, for 30min. The mixtures were then spun through a 50% glycerol cushion buffer at 18,000 g at RT or 4 ºC for 25 min. Soluble fractions were collected. Pellet fractions were washed 2 times with 80 mM PIPES pH 7 buffer and resuspended in an equal volume of GTB (80 mM PIPES pH 7, 1 mM MgCl₂, 1 mM EGTA). Samples were run using SDS-PAGE, analyzed by Western blot, and probed with mouse anti-GST.

Microtubule End binding

The procedure was performed similarly to the microtubule-binding assay above except for microtubule preparation. The principle behind this assay is that a solution of 5
mg/ml of tubulin in a 20% glycerol GPEM (80mM PIPES, pH 6.9, 0.5mM EGTA, 1mM MgCl₂, 1mM GTP) buffer will produce microtubules with an average length of 2 µm, whereas a solution of 3 mg/ml tubulin in a 5% glycerol GPEM buffer produces microtubules with an average length of 16.5 µm. This produces two populations of microtubules. The population of microtubules made with 5 mg/ml tubulin has many short microtubules that contain more microtubule ends for the protein to bind to. The population made with 3 mg/ml tubulin population has fewer microtubules with more length along the shaft of the microtubule. The different length microtubules were then incubated with exogenously produced GST-tagged proteins, and the assay was performed as in the microtubule-binding assay above.

**Microtubule polymerization assay**

The assay was performed using Microtubule polymerization kit BK004 from Cytoskeleton Inc. (Denver, CO). Briefly, 97% pure tubulin (30 µM) was mixed with purified GST fusion proteins (4 µM) in PEM buffer containing 10% glycerol and 1 mM GTP, on ice to prevent polymerization. The mixture was then incubated at 37 °C, and tubulin polymerization was detected by measuring the absorbance at 355 nm once per minute for one hour in a clear 96 well plate.

**Neuronal culture**

Hippocampal cultures were prepared from rat embryos at 18 days gestation as previously described. Cells were plated on glass coated with 0.2 mg/ml poly-D-lysine (PDL). Neurobasal containing B27 supplement, Glutamax, and penicillin/streptomycin
was used for culture growth. Plating density, transfection method, and other deviations are listed with each method.

**X-gal staining and electron microscopy**

Hippocampal cultures were grown for 10 days in culture on Thermanox® Coverslips (Electron Microscopy Sciences; Hatfield, PA). The neurons were co-transfected with cDNA encoding β-galactosidase and GFP or cypin-GFP using the calcium phosphate transfection method. Neurons were fixed at DIV12 with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperature for 10 min, washed at room temperature in wash buffer (0.1 M Tris pH7.3, 2 mM MgCl₂) and staining buffer (5 mM K₄[Fe(CN)₆]·3H₂O, 5 mM K₃[Fe(CN)₆], 2 mM MgCl₂, 20 mM Tris pH 7.3, 0.1% Triton X-100). Staining for β–galactosidase was performed overnight at 37 °C with 1 mg/ml X-gal (Omega Bio-Tek Inc.). The cultures were post-fixed in 1% OsO₄ in wash buffer for 1 h. Post-fixed cultures were rinsed with water, dehydrated in ethanol, and embedded using EMbed 812 kit (Electron Microscopy Sciences). The embedded cultures were cut parallel to the culture surface and imaged with a JEOL 100 CX transmission electron microscope (Rutgers University Electron Imaging Facility, Division of Life Sciences). Microtubule crossings at branch points were counted along a linear axis that was 0.5 μM long, beginning at the branch point and extending into the neuron perpendicular from membrane edge using ImageJ (NIH, Bethesda MD). Space between microtubules in the shaft was measured in a similar manner using ImageJ. A line 0.5 μM long was drawn perpendicular to the microtubules in the shaft. An intensity map along the line was generated in ImageJ and measurements were taken using the
distance between the peaks representing microtubules. See figure A.1 in the appendix for
an example of how to take these measurements. In addition, the number of microtubules
along this line was also calculated.

Dendrite analysis and imaging

Neurons were grown as above but plated on glass coverslips at a density of 100K
cells/cm². For transfection, the neurons were grown for 10 days in culture (DIV) and
transfected with the appropriate constructs using the calcium phosphate method. Fixation occurred at DIV 12 with 4% PFA, 4% sucrose in PBS for 15 min at 37 ºC.
Neurons were imaged in the GFP channel at 200x using an Olympus Optical IX50
microscope (Tokyo, Japan) with a Cooke Sensicam CCD cooled camera, fluorescence
imaging system and ImagePro software (MediaCybernetics; Silver Spring, MD).
Dendrite morphology was digitized in three stages based on these initial images. In the
first stage, the semi-automated tools available through the NeuronJ plugin to ImageJ
(NIH; Bethesda, MD) were used to define coordinates of all dendrites in the x-y plane. In
the second stage, NeuronStudio was used to define the pattern of connectivity between
dendrites. These two steps fully determine the structure of each cell’s dendritic arbor and
encode it in a digital format. Custom scripts written in MATLAB (MathWorks; Natick,
MA) were used to transfer the data from NeuronJ to NeuronStudio. Using these digitized
dendritic arbors, a second set of MATLAB scripts was then used to analyze data and
perform Sholl analysis with a 9.3 μm ring interval. The data were transferred to Excel to
facilitate statistical analysis. The experimenter was blinded to conditions during all data
analysis. Dendrites less than 3 μm in length were not counted.
**Time-lapse video microscopy of EB3-GFP comets in hippocampal neurons**

Hippocampal cultures were prepared as described above and plated at a density of 700 cell/cm² on glass-bottom 35 mm culture dishes (WPI, Sarasota, FL). Neurons were transfected with the appropriate constructs at plating using the Amaxa electroporation (Lonza Biologics; Basel, Switzerland). mRFP tagged EB3 was used in all measures. This transfection was done according to the manufacturer protocol using ”Alternative rat neuron protocol O-23.” After electroporation a 5-minute recovery period in RPMI was performed by pipetting the cells out of the electroporation cuvette and into a 1.5 ml microcentrifuge tube containing 500 µl of equilibrated and warm RPMI media. Cells were then plated in Neurobasal media containing B27 and glutamax. After plating, a ½ media change after 2-4 hours and full media change after 18 hours were performed using equilibrated and warmed Neurobasal media containing B27, Glutamax and Pen-Strep. Neurons were then grown as normal. Time-lapse fluorescence imaging was performed 12-18 hours after transfection. For live-cell imaging, culture medium was replaced with Ringer's solution (150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl₂, 10 mM Glucose, 10 mM HEPES, pH 7.4) 12-18 hours after transfection, and images were acquired on a Olympus Optical IX50 microscope (Tokyo, Japan) equipped with a 60x oil-immersion objective, heated stage, Cooke Sensicam CCD cooled camera, fluorescence imaging system and ImagePro software (MediaCybernetics; Silver Spring, MD). Images were taken 1 per second for 100 seconds. Movies were adjusted for straightness, brightness, contrast, and gamma correction and then cropped to created time-lapse representations. Comet velocity and lifetime were analyzed using ImageJ (NIH;
Bethesda, MD). Comet movement was measured from the tip of each comet in the frame in which it initially appears to the tip of the comet in the frame previous to when it disappears by drawing a box connecting beginning tip to end tip. Velocity was quantified as length traveled (µm) vs. time (s). Comet lifetime was quantified by measuring the length of time that comets are visible in the dendrite. Images were prepared for presentation with Adobe Photoshop to adjust cropping, levels and contrast, and all images were subjected to the same adjustments.

**Measurement of EB3 comet size**

DIV 10 neurons were transfected using the calcium phosphate transfection method. Neurons were fixed at DIV 12 with 4% PFA and sucrose for 15 min at 37 ºC. For immunocytochemical studies, neurons were fixed at DIV 12 with ice cold methanol for 15 minutes and then 4% PFA at 37 ºC for 15min. Cells were then immunostained with the following primary antibodies: mouse anti-MAP2 (1:200) and rabbit anti-EB3 (1:3000). Staining was visualized using secondary antibodies conjugated to Cy2 or Cy3 from Jackson ImmunoResearch Laboratories (West Grove, PA). Cells were then rinsed briefly with phosphate-buffered saline and mounted on glass slides using Fluoromount G from Southern Biotech (Birmingham, AL).
Results

Cypin binds to assembled microtubules as well as tubulin heterodimers

In order to understand more fully the way that cypin interacts with microtubules, we wanted to determine if cypin binds to assembled microtubules as well as tubulin heterodimers. There are several ways for a protein to interact with tubulin and microtubules. CRMP2, a microtubule polymerizing protein, whose function is axon branching and extension, binds to both tubulin heterodimers and assembled microtubules. This is in contrast to the other microtubule-associated proteins (MAPs), such as MAP1A and tau, that bind only to assembled microtubules and stabilize their structure. We hypothesized that cypin, like CRMP2, would bind to assembled microtubules as well as tubulin heterodimers. To test this, we used a glycerol cushion spin-through assay to isolate assembled microtubules from tubulin heterodimers in the presence of cypin. We found that full-length cypin and all constructs containing the first 220 amino acids of cypin bind to microtubules. Constructs not containing this region did not significantly bind to microtubules. (Figure 3.1A).
Figure 3.1. Cypin binds to assembled microtubules using a region located between amino acids 120 and 220.

Purified exogenous cypin, full length or mutants containing the indicated amino acid, and purified tubulin were used in a cell-free assay to examine microtubule binding. Taxol indicates the assembled microtubule condition and 4 °C indicates the unpolymerized tubulin control. A. Representative Western blots from spin-down assay. B. Quantitation of densitometry from pellets (microtubule-associated fraction) in spin-down assay. Only full-length cypin and amino acids 1-220 of cypin bind significantly to assembled microtubules when compared to the GST control. All other comparisons are not statistically significant. *p<0.05 using 2 way RM ANOVA with Tukey’s multiple comparisons post test.
Cypin does not preferentially bind to the ends of assembled microtubules.

Since cypin binds to assembled microtubules we wanted to determine if it preferentially associated with ends of microtubules or along the shaft of the microtubule. We tested this by creating two microtubule populations in a cell-free system: one population is composed of long microtubules with few ends and long shafts, and one population is composed of short microtubules with many ends and short shafts. We repeated the same microtubule spin-down procedure that was used in Figure 3.1 using these different populations of microtubules. We found that cypin pelleted with microtubules at a slightly higher ratio in the microtubule population with more shaft than in the population with more ends (Figure 3.2). This assay suggests that cypin does not bind preferentially to microtubule shafts or ends because there is a higher amount of binding surface in the shaft condition. This would be expected since both condition have microtubule shafts. Interestingly cypin pelleted through the glycerol cushion in all conditions to some degree, even the 4 °C condition, in which there should be no microtubules present. This pelleted cypin may be a result of cypin-mediated microtubule polymerization, even in these adverse conditions. If the tubulin control is excluded for this reason, and only the condition with formed microtubules is examined, there is no significant difference in binding between the two populations (data not shown).
Figure 3.2. Cypin does not bind preferentially to the end of the microtubule

Purified, exogenous cypin was incubated with two populations of microtubules, one population with many microtubule ends and one with few ends and longer shafts. Microtubules were spun through a glycerol cushion, and associated proteins remained with microtubules in the pellet (P) while non-associated proteins remained in the supernatant (S). A. Representative images of Western blots probed for cypin. B. Quantitation of Western blots. Ratio of cypin incubated with formed microtubules vs. cypin incubated with free tubulin was calculated. Statistics were analyzed using GraphPad Prism. No significant difference was found using Student’s t-test.
Cypin increases the rate and amount of microtubule polymerization in a cell-free system, but it does not increase nucleation

While our laboratory previously established that cypin can increase microtubule polymerization in a cell-free system\(^2\), we did not determine the mechanism by which cypin acts to increase microtubule assembly. We repeated the microtubule polymerization assay using a tubulin preparation that contains the necessary proteins for polymerization in the absence of any exogenously added proteins (Figure 3.3A). We reconfirmed that cypin does increase the Vmax of the polymerization reaction. This means that total microtubule polymerization in the reaction increased (Figure 3.3B). We also determined that cypin increases the reaction velocity of the polymerization reaction. This means that the rate of microtubule polymerization increased (Figure 3.3D). We compared initial portion of the polymerization curves (Figure 3.3A and C) and found that cypin does not increase microtubule nucleation as there is not a lag in the start of polymerization when compared to the GST control. New nucleation of microtubules would result in a longer period of time before the start of polymerization.
Figure 3.3. Cypin increases the polymerization rate and total amount of microtubule polymerization.
Figure 3.3. Cypin increases the polymerization rate and total amount of microtubule polymerization

A cell-free microtubule polymerization assay was performed using purified, exogenous cypin. A. Microtubule polymerization curves generated by cell-free microtubule polymerization assay. Box encloses region of interest when looking for delay of polymerization caused by nucleation. B. Quantitation of Vmax (total amount of microtubule polymerization). Cypin increases Vmax (total microtubule polymerization). *p<0.05 by two-way, paired t-test using Mann-Whitney post-test. C. Linear portion of curve from box in 3.3A. Black lines are best-fit linear values of reaction velocity. Best-fit values were generated using GraphPad Prism 4.0. D. Quantitation of reaction velocity from best-fit values. Cypin increases reaction velocity of the microtubule polymerization reaction. ***p<0.001 by comparing best-fit linear slopes generated using Student t-test in GraphPad Prism.
Cypin alters microtubule behavior in the cell by decreasing inter-microtubule distance and increasing microtubule number and EB3 comet velocity

After examining how cypin affects microtubules in a cell-free system, we examined how cypin alters microtubules in culture neurons. First, we examined changes caused to the static arrangement of microtubules. We performed EM imaging of neurons overexpressing GFP-cypin or GFP. We found that overexpression of cypin decreased the spacing between microtubules when compared to control neurons (Figure 3.4A and B). We also found that cypin did not change the number of microtubules that cross between dendritic branches (Figure 3.4C and D). However, cypin did increase the number of microtubules in the area measured (Figure 3.4E). This behavior is in contrast to the effects of PSD-95 on microtubule arrangement as seen in Chapter 2. To assess the effect of cypin on microtubule dynamics in the cell, we used live-cell imaging techniques to capture the movement of EB3 comets. These comets are only found on the plus-ends of growing microtubules, so their behavior can be used to track microtubule behavior. We found that overexpression of cypin increased the velocity of EB3 comets when compared to comets in control neurons (Figure 3.5). The increase in EB3 comet velocity indicates that cypin increases microtubule polymerization in the neuron as well as in a cell-free system. The fact that EB3 comet size does not change suggests that the increase in velocity is due to cypin-mediated increases in microtubule polymerization rate and is not due to a direct effect of cypin on EB3 dynamics. Again, this is in contrast to the comet behavior seen in neurons overexpressing PSD-95, where the comets show a decreased comet velocity and lifetime.
Figure 3.4. Cypin decreases the spacing between microtubules but does not change microtubules crossing at branch points.
Figure 3.4. Cypin decreases the spacing between microtubules but does not change microtubules crossing at branch points

EM images taken from DIV 12 neurons were used to quantify microtubule structure. A. Representative images along the shaft of neurites transfected with the cDNA for the indicated protein. Scale bar = 0.5 µm. B. Quantitation of the spacing between microtubules. Cypin decreases the spacing between microtubules. *p<0.05 using paired, two-tailed t-test with Mann-Whitney post-test for significance. C. Representative images of branch points from dendrites transfected with the cDNA for the indicated protein. Scale bar = 0.5 µm. D. Quantitation of the number of microtubules crossing between branch points. There is no significant difference between neurons expressing GFP or neurons expressing GFP-cypin. E. Quantitation of the number of microtubules in a given unit area. Cypin increases the number of microtubules in a given area. *p<0.05 using paired, two-tailed t-test with Mann-Whitney post-test.
Neurons overexpressing cypin were examined for EB3 comet appearance and behavior. A. Quantification of EB3 comet velocity in DIV 2 neurons. Cypin increases EB3 comet velocity. **p<0.01 by unpaired student t-test. B. Quantification of EB3 comet area in DIV 12 neurons. No significant differences were found using Kruskal-Wallis ANOVA and Dunn’s multiple comparisons test.
Discussion

We previously showed that cypin can bind to tubulin heterodimers and increase microtubule polymerization in a cell-free system, and thus, we wanted to further examine how cypin can affect microtubule behavior in both a cell-free system and in the neuron. We found that cypin binds to assembled microtubules (Figure 3.1). We also determined that it does not preferentially bind to the ends or to the shaft of microtubules (Figure 3.2). We also identified a novel microtubule-binding region between amino acids 120 and 220 of cypin (Figure 3.1). This region is of interest because it does not lie in the same location as any other known domain of cypin. This implies that it may have a separate function from tubulin heterodimer-binding, which occurs between amino acids 350-403 of cypin.

We propose that cypin binds to microtubules and tubulin using separate domains. The functionality of the microtubule-binding domain needs to be further explored, and the role it plays in other known functions of cypin established.

The experiments performed in this thesis have shown that cypin increases the total amount and rate of microtubule polymerization in a cell-free system. We also have shown that it does this, not by nucleating new microtubules, but by acting on microtubules that are already forming (Figure 3.3). This is of interest because it places cypin in contrast to proteins, such as spastin and katanin, that increase branching in the axon by severing microtubules to create new sites of nucleation. This could mean that cypin plays a role in stabilizing microtubules along newly formed dendritic branches rather than by creating new branches. Interestingly we found that cypin increased the number of microtubules in the dendrite (Figure 3.4E). This may indicate that cypin nucleates new microtubule
growth; however, our biochemical data suggest that cypin increases the stability of microtubules that would normally fail to grow.

To examine how cypin affects microtubules in the neuron, we used two techniques: Electron microscopy to examine the static microtubule structure and live-cell imaging of EB3 comets to observe the dynamic nature of the microtubules. We found that cypin decreased the spacing between microtubules in neurons and increased the velocity of microtubule polymerization, as measured by EB3 comet velocity (Figure 3.5). These effects are consistent with the effect of cypin on microtubules in cell-free and cell culture systems (Chapter 3 and 2). They are also consistent with reports that microtubules enter into nascent neurites and stabilize them

The decrease in microtubule spacing and discovery of microtubule-binding domains place cypin in a unique position in the world of microtubule associated proteins (MAPs). Typically, MAPs interact with microtubules in one of three ways. Molecules, such a MAP2 and tau, bind to microtubules and alter the bundling of microtubules in the cell 12-14. Molecules, such a CRMP2, bind to tubulin heterodimers and encourage microtubule polymerization 11. End-binding proteins bind to and stabilize growing microtubules 19. Cypin may be unique in that it performs both microtubule polymerization and microtubule bundling/stability. Further experiments must be performed to confirm these preliminary studies, but the presence of a tubulin-binding domain in, microtubule-binding domain in, and a change in microtubule arrangement by cypin strongly suggest multiple functions for cypin.
The results presented here have continued to expand upon the knowledge of how cypin can regulate microtubule behavior in the neuron. They confirm that cypin is an important regulator of both microtubule dynamics and structural arrangement and present potentially new functions for cypin that can be explored. Although cypin was discovered almost 12 years ago, study of its basic functions continues to provide insight into the behavior of microtubules in the neuron.
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Chapter 4 – Identification of small molecule compounds that alter cypin function

Introduction

The study of biochemical and physiological properties of proteins provides an excellent opportunity to expand our knowledge of neuronal biological functions. Yet to truly benefit society, this research must be taken one step further and be used to alleviate human disease. The first step in using research for this purpose is to identify a biological target that alters cellular function in a meaningful way. Since defects in synaptogenesis and neuronal growth are underlying causes of many neurological diseases, they are an ideal starting place. In this context, cypin is an attractive drug target because of its direct influence on neuronal shape, its interaction with PSD-95, and its role in purine metabolism.

First, cypin plays a direct role in the development of neuronal shape by promoting microtubule polymerization. Since it has been shown that dendrite shape can directly alter the communication behavior of neurons, this is a relevant parameter to target for manipulation. Diseases, such as Alzheimer’s disease, epilepsy, Rett syndrome, and other forms of cognitive developmental disorders (reviewed in) show changes in dendrite arbor structure in at least a subset of neurons associated with the disease phenotype. While cypin has not been associated with causality in these diseases, it may prove valuable in manipulating neuronal structure in a therapeutic fashion to alleviate the symptoms.

Second, cypin interacts with and decreases PSD-95 protein levels and localization to the synapse. PSD-95, as discussed in Chapter 2, plays many roles at the synapse,
including assembling synaptic signaling complexes. PSD-95 stabilizes dendritic spines, which play a crucial role in maintaining proper neuronal communication. In particular, autism spectrum disorders (ASD) have a disturbed balance of excitatory and inhibitory synapses. Since cypin can mediate decreases in expression and localization of PSD-95, it may play a role in maintaining this balance. It has also recently been shown that certain mutational variants of PSD-95 associate with mouse models of autistic behavior.

Finally, cypin is the main guanine deaminase in humans and functions in purine metabolism to deaminate guanine to xanthine, which can then be further metabolized into uric acid. Several diseases are marked by an increase in uric acid. For example, Lesch-Nyhan syndrome occurs when an excess of guanine and xanthine build up and cause an excess secretion of uric acid. Interestingly, it has also been shown that there are changes in dendrite morphology in mouse models of Lesch-Nyhan syndrome. Hyperuricemia, a type of gout, is a ubiquitous increase in levels of uric acid. Individuals with autism have increased levels of purines and succinyl purines in cerebral spinal fluid (CSF) and plasma. It has also been shown that a subset of patients with autism have excess uric acid secretions. Conversely, there are scenarios and diseases in which uric may be beneficial, as it is a powerful anti-oxidant. In Parkinson’s disease, uric acid levels in the CSF and plasma may be diagnostic of disease protection, and it has been suggested that these high levels may be protective and slow disease progression. Also, uric acid may be protective in cases of spinal cord injury. Since cypin plays a critical role in the salvage pathway for purine metabolism, a pathway altered in these diseases, it is important to understand how cypin regulates this metabolism.
For all of these reasons, cypin is an excellent target for therapeutics for treating disease. The next step in using a biological target to study disease is to find a way to manipulate the biology. Typically, this means finding a small molecule compound which can interact with the protein and alter its function. Once a small molecule compound is found, it can be used as a therapy or tool to further research about cypin’s function without having to directly change proteins in the cell. To this end, our laboratory has collaborated with other members of the scientific community to find small molecules that can change the biochemical properties and interactions of cypin. We have two sets of compounds: one set, which may interfere with the guanine deaminase (GDA) activity of cypin, and one set, which may disrupt the binding of cypin to PDZ2 of PSD-95. This chapter will explore how these molecules can interact with cypin, what their potential is as tools, and how we can manipulate the GDA activity of cypin and cypin’s association with PSD-95.
Materials and methods

Statistics and graphs

All statistics were calculated using Prism 4.0 software from GraphPad (La Jolla, CA).

COS-7 cell culture and transfection

COS-7 cells were plated at 70-80% confluence and maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen), supplemented with 7.5% fetal bovine serum in a 5% CO₂ atmosphere. At 60% confluence, cells were transfected with 5 μg DNA using 7 μL Lipofectamine (Invitrogen) and OptiMEM (Invitrogen) using the manufacturer’s protocol. Cells were left in OptiMEM for 12 hours. After 12 hours, a full media change in DMEM, penicillin/streptomycin and FBS (7.5%) was performed for each cell culture plate, and cells were allowed to grow for 24 hours before protein was harvested for assay.

Guanine deaminase assay

The manufacturer’s protocol for the Amplex Red Xanthine/Xanthine Oxidase Assay Kit (Invitrogen) was used to screen for presence of H₂O₂, a downstream product of cytin’s guanine deaminase activity. Guanine (4 μl suspended at a concentration of 6.25 mM in 0.1 M NaOH was added to the reaction mix (prepared as per manufacturer’s instructions but for 2.5 ml total volume) to serve as the substrate for cytin. The product of this reaction, xanthine, can be measured readily by exciting the reactions at 530nm and recording at 590 nm once/min at room temperature for 60 min in a 96 well plate format.
A maximum of 48 wells were assayed at once to eliminate variability due to pipetting time. Cypin and control protein concentrations were measured by Bradford assay and normalized before each assay. GST proteins were aliquoted and stored at -80 °C while COS-7 cell-produced proteins were isolated fresh from culture the day of the assay.

Guanine was suspended at 6.25 mM in 0.1 M NaOH and stored at -20 °C. Higher concentrations of guanine did go into solution, and if they did, they precipitated out of solution when added to the assay, and thus were spun out of solution before plate reading occurred.

**Definition of 1 U of cypin in GDA assay**

It was also observed that there can be variability between batches of purified GST protein, so specific activity was determined and 1 unit (U) defined as the amount of cypin needed to create an absorbance change of 250 A/min in the presence of excess substrate. For all experiments performed with GST protein, 1 U is equal to 22.5 pmol of GST-cypin. This can be measured by observing the change in absorbance caused by cypin in the presence of 1000X greater concentration of guanine.

**Preparation of proteins for use in GDA assay**

**COS-7 cell lysate**

Twenty four hours after COS-7 cell transfection with GFP-Cypin or GFP cDNA, cells were washed twice with phosphate-buffered saline (PBS) and scraped into 1 ml of GDA lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.4 and 1 mM PMSF). Lysate was homogenized by passing it through a 25-gauge needle five times and was then
centrifuged at 15,000 x g at 4 °C for 10 min. Concentration of cytosolic proteins in the supernatant was measured using Bradford assay.

GST-tagged cypin assay for enzyme kinetics

Aliquots of GST-tagged proteins were thawed on ice and diluted to the proper concentration in assay buffer (0.15 M Tris-HCl) and stored on ice during assay preparation.

Assay variations for different experiments

COS-7 cell lysate experiments

COS-7 cell lysate was diluted to 0.5 µg/µl of total protein. 25 µl of lysate was used in each well of the assay to provide cypin.

Cypin concentration varied

Guanine concentration was held steady at 25 µM while cypin concentration was varied between 25 and 7.5 nM

Guanine concentration varied

Cypin amount was held steady at 1 U while guanine concentration was varied from 25 µM to 0.034 µM in ½ log increments.

Compounds added to guanine concentration varied

Cypin amount was held steady at 1 U while guanine concentration was varied from 25 µM to 0.034 µM in ½ log increments. Compounds were tested at 40 µM.
**Primary hippocampal neuron cultures**

Embryos for dissection were obtained from a wildtype male crossed with transgenic female rat expressing GFP under universal promoter. Hippocampal cultures were prepared from rat embryos at 18 days gestation as previously described in \(^1,10,25\) with the following exceptions: coverslips were coated with 0.2 mg/ml poly-D-lysine (PDL), cells were treated with 0.25% trypsin for 5 min at 37 °C followed by 3 washes with Hanks buffered saline solution (HBSS)+HEPES buffer, GFP-expressing cells were added to non-GFP-expressing cells at a ratio of 1:49 to ensure that GFP-expressing neurons would be sufficiently spread out for branching analysis.

**Treatment of neurons with compound**

Neurons were grown for 10 days *in vitro* (DIV) in Neurobasal (Invitrogen) media containing B27, Glutamax, and penicillin/streptomycin (Full Neurobasal). On DIV 10, cells were treated with compound or an equivalent volume of DMSO as a control. Treatment was performed by mixing the compound or control in conditioned media in a 1:1 ratio with pre-warmed Full Neurobasal and media removed from the culture. The remaining culture media was aspirated, and the treatment-containing media was immediately returned to the culture. The culture was allowed to grow until DIV 12 before fixation. The remaining culture media was immediately aspirated and the treatment-containing conditioned media added back to the well.
Fixation and immunocytochemistry of neuronal cultures

Neurons were fixed on DIV 12 with 4%PFA/4% sucrose for 10 min at 37 ºC. Cultures were then immunostained with chicken anti-GFP (1:500; Rockland Immunochemicals, Gilbertsville, PA), mouse anti-MAP2 (1:500; BD Pharmingen, San Diego, CA) and 1 μM Hoechst stain for nuclei. Staining was visualized using secondary antibodies conjugated to Cy2 or Cy5 from Jackson ImmunoResearch Laboratories (West Grove PA). Cells were then rinsed briefly with PBS and mounted on glass slides using Fluoromount G from Southern Biotech (Birmingham, AL).

Dendrite analysis and imaging

Neurons were imaged in the GFP channel at 200x using an Olympus Optical IX50 microscope (Tokyo, Japan) with a Cooke Sensicam CCD cooled camera, fluorescence imaging system, and ImagePro software (MediaCybernetics, Silver Spring, MD). Dendrite morphology was digitized in three stages based on these initial images. In the first stage, the semi-automated tools available through the NeuronJ plugin to ImageJ (NIH, Bethesda MD) were used to define coordinates of all dendrites in the x-y plane. In the second stage, NeuronStudio was used to define the pattern of connectivity between dendrites. These two steps fully determine the structure of each cell’s dendritic arbor and encode it in a digital format. Custom scripts written in MATLAB (MathWorks, Natick, MA) were used to transfer the data from NeuronJ to NeuronStudio. Using these digitized dendritic arbors, a second set of MATLAB scripts was then used to analyze data and perform Sholl analysis with a 9.3 μm ring interval. The data were transferred to Excel to facilitate statistical analysis. The experimenter was blinded to conditions during all data
analysis. Dendrites less than 3 µm in length were not counted \(^{26,27}\).

**Co-immunoprecipitation from whole rat brain**

Rat forebrain was homogenized in 10 ml TEE (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 7.4) and incubated with an equal volume of 2X RIPA buffer (100 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1% deoxycholate, 2% NP-40 and 0.2% SDS, 2 mM EDTA, pH 7.4) containing 1 mM phenylmethanesulfonylfluoride (PMSF) and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at 4 °C for 1 hour. Detergent-insoluble material was pelleted by centrifugation at 15,000 x g for 10 min at 4 °C. Brain lysate was then diluted to 20 µg/µl using RIPA buffer, and rabbit anti-cypin antibody (5 µl or 5 µg of IgG) was added to the 450 µl of diluted extract and incubated overnight at 4 °C, followed by the addition of 25-50 µl protein A sepharose (GE Healthcare, Piscataway, NJ). After a 1-2 hour incubation at 4 °C, washed beads were incubated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.01 M Tris-HCl pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 2.3% SDS, 0.005% bromophenol blue) for 20 min at RT followed by centrifugation. The supernatant was boiled and subjected to SDS-PAGE and Western blotting using mouse anti-PSD-95 (Millipore, Billerica, MA) as primary antibody and HRP-tagged donkey anti-mouse secondary (Jackson ImmunoResearch Laboratories, West Grove, PA). Blots were quantified using ImageJ to create an ROI around the desired bands with all quantitation normalized to the IgG control lane.
Microtubule polymerization assay

Polymerization assay was performed using Microtubule Polymerization Kit BK004 from Cytoskeleton Inc. (Denver, CO). Briefly, 97% pure tubulin (30 μM) was mixed with purified GST fusion proteins (4 μM) in PEM buffer containing 10% glycerol and 1 mM GTP on ice to prevent polymerization. The mixture was then incubated at 37 °C, and tubulin polymerization was detected by measuring the absorbance at 355 nm once per minute for one hour in a clear 96 well plate. Guanine or guanine analogs were used at 100 mM.
Results

Cypin

Alterations in cypin GDA activity can alter the microtubule polymerization activity of cypin

The Firestein laboratory previously showed that a subset of guanine-like molecules can bind to cypin with higher affinity than guanine and that these molecules can alter GDA activity. To determine whether these compounds change other aspects of cypin activity, we performed microtubule polymerization assays in the presence of these compounds using guanine as a control. We found that a subset of the compounds altered microtubule polymerization in the presence of cypin. Xanthine and 7-methylguanine attenuate cypin-mediated increases in microtubule polymerization (Figure 4.1). These data suggest that compounds that alter GDA activity also alter the ability of cypin to increase microtubule polymerization.
Figure 4.1. Guanine-like molecules alter cypin-mediated increases in microtubule polymerization

Cell-free microtubule polymerization was performed in the presence of GST or GST-cypin at 4µM with the indicated compound at 100 mM. 7-methylguanine and guanine inhibit cypin-mediated microtubule polymerization when compared to GST-cypin alone and are statistically similar to microtubule polymerization in the presence of GST alone. Diamino-purine and xanthine do not alter cypin-mediated microtubule polymerization.
A subset of compounds alter the GDA activity of cypin purified from COS-7 cell lysate

In collaboration with Fox Chase Cancer Center we obtained 288 compounds to screen for their effects on cypin’s GDA activity. In order to more closely replicate cellular conditions, we expressed GFP-cypin in COS-7 cells and used the lysate in the GDA assay as performed previously in the laboratory \(^{28,29}\). We used this COS-7 cell lysate to screen the 96 compounds for their effects on GDA activity. We normalized the activity levels of all compounds so that average activity was 1. We found several positive and negative regulators (Figure 4.2 A). A subset of the compounds had a significant effect on cypin’s GDA activity that was either 1 (inhibitors) or 2 (activators and inhibitors) standard deviations away from the normalized average. These were selected for further testing. (Figure 4.2B)
Figure 4.2. A subset of compounds alter the GDA activity of cylin purified from COS-7 cell lysate

COS-7 cell lysate was used to assay GFP-cylin and GFP protein and screen the effect of small molecule compounds on cylin’s GDA activity. A. Normalized slopes from the screening of 96 compounds. Red bars are two standard deviations above the normalized average. Orange bars are one standard deviation about the normalized average. Blue bars are within one standard deviation above or below the normalized average. Purple bars are one standard deviation below the normalized average. Black bars are two standard deviations below the normalized average. B. Compounds that were two standard deviations away from the normalized average were selected for further testing. B9 and B10 were only one standard deviation away from the normalized average but were selected as the second lowest group of inhibitors.
**Fox Chase compounds can alter branching activity**

To further study these small molecule compounds, we tested their ability to alter dendrite branching in hippocampal neurons. In order to simplify this procedure and allow high through-put of cell treatment, we used cells from a rat expressing GFP under a universal promoter to obtain GFP-positive neurons. We mixed these with wildtype neurons at a ratio of 1:50 to obtain a culture that is appropriate for branching analysis (Figure A.2). In an initial study, we treated neuronal cultures with compound for two days and found that these compounds alter branching, with the change correlating to the effect on GDA activity (Figure 4.3). General Sholl analysis revealed that two compounds with inhibitory effects on GDA activity, B9 and G5, significantly decrease dendrite complexity (Figure 4.3A). To identify branch orders that had been altered, we performed Sholl analysis on each order of dendrite branch and found that B9 significantly decreased the complexity of secondary branches and that E9 decreased the complexity of tertiary branches (Figure 4.3B). Interestingly, G5 did not change any specific branch orders, and the changes that occurred in neurons treated with E9 were not revealed until this specific analysis was used. To further characterize the changes that occur in neurons treated with these small molecules, we quantified changes in dendrite number and length (Figure 4.3C). There was no significant change found in dendrite length (data not shown). The only change discovered in dendrite number was that H9, an excitatory compound, increased the number of secondary branches. To determine where these branches were located in the dendritic field, we used a type of analysis that categorizes branches as: a root branch, any branch extending from the cell body; a tip branch, branches that terminate without branching; or intermediate, any branches between root and tip. We
found that H9 increased the number of intermediate and tip branches (Figure 4.3D). This indicates that changes caused by compound H9 increases the branching that occurs further out in the dendritic field, not causing existing branches to grow longer.
Figure 4.3. Small molecule compounds alter branching in hippocampal neurons

A

B

Primary

Secondary

Tertiary

C

Primary #

D

RIT Root #

Secondary #

RIT Int #

Tertiary #

RIT Tip #
Figure 4.3. Small molecule compounds alter branching in hippocampal neurons

Branching analysis of GFP-expressing neurons treated with small molecule compounds or DMSO control. A. Sholl analysis of GFP-expressing neurons. Neurons treated with compounds B9 and G5 significantly decrease dendritic field complexity when compared to control neurons treated with DMSO. **p<0.01 for G5 and B9 when compared to DMSO treated control using two way ANOVA with Bonferroni multiple comparisons test. B. Sholl analysis of specific branch orders in the dendritic field. No compound treatment resulted in a significant change in primary dendrite complexity. B9 decreased the complexity of secondary branches. **p<0.01 for B9 when compared to DMSO-treated control using two way ANOVA with Bonferroni multiple comparisons test. C. Quantification of the number of branch orders. H9 increases the number of tertiary branches when compared to DMSO-treated control cells. D. Quantification of root, intermediate, and tip branch types. H9 significantly increases the number of intermediate and tip branches. C and D **p<0.01 using two way RM ANOVA with Bonferroni multiple comparisons test. n = ~10 neurons for each condition.
Establishment of GDA Assay using purified proteins

To more fully define the effect of the compounds on cypin’s GDA activity, we designed an assay that uses a more defined source of cypin. We cannot properly calculate enzymatic variables or compound effectiveness unless we know the quantities of enzyme being added to the assay. We also needed to screen larger amounts of compounds in a more efficient manner. To achieve this, we optimized the GDA activity assay for a 96 well format and use of purified GST-tagged cypin protein. First, we confirmed that GST-cypin behaves in a linear manner when cypin concentration is increased in the presence of excess guanine (Figure 4.4A). We then quantified the enzymatic variables for cypin alone. We found that 1 U of cypin, defined in materials and methods, has a K of 8.629 µM, a Vmax of 247.8 A/min, and a cooperativity constant of 1.924 (Figure 4.4B, D, and E). This cooperativity constant shows that cypin cooperates with itself during guanine deamination. This is not an unreasonable idea since most guanine deaminase is purified from tissue as a dimer 31-33. We then examined the effects of several inhibitors on cypin behavior in the purified GST protein assay. We found that no change in cypin behavior occurred even in the presence of the same amount of compound used in the GDA assays from COS-7 cells (Figure 4.4C, D and E).
Figure 4.4. Purified cypin assay

A

B

C

D

E

Figure 4.4. Purified cypin assay

A

B

C

D

E
Figure 4.4. Purified cypin assay

Purified GST-cypin was used in a 96 well format for guanine deaminase assay to determine enzyme kinetics for cypin and types of inhibition. A. Slopes of GDA velocity with different amounts of cypin and guanine held steady at 25 nM for all conditions. Reaction velocity is linear in response to increases in concentration. B. Slopes of reaction velocity with cypin held at 1 U while guanine concentration was varied by ½ log increments to observe Vmax of 247.8 A/min with a 95% CI of 238.0 to 257.6, a Km of 8.629 µM with a 95% CI of 6.353 to 10.90 and a cooperativity constant of 1.924 with a 95% CI of 1.543 to 2.305. Dotted line is curve fit using linear regression. C. Slopes of reaction velocity for 1 U of cypin in the presence of compounds B9 and H9. DMSO was used as a control. Dotted lines are curves fit using linear regression. D. Km measured using 1 U of cypin in the presence of compounds or DMSO. No significant differences were found. E. Vmax of 1 U of cypin in the presence of compounds or DMSO. No significant differences were found. All statistics were generated using GraphPad Prism 4.0 using non-linear regression with the equation Y=Vmax*X^h/(Kprime+X^h). n=3 with each reaction run in duplicate.
**PSD-95**

**CN2097 competitor can partially displace cypin from interacting with PSD-95**

We asked what the effects would be if cypin is displaced from PSD-95 using small molecules. This would allow us to examine the effect on the cells without having to introduce variables such as transfection, overexpression, or mutation. To do this, we collaborated with Dr. Mark Spaller. He synthesized compounds that bind to specific PDZ domains of the MAGUK proteins and created membrane-permeable small molecules that bind with very high affinity to PDZ 1 and 3 of PSD-95\(^{34-36}\). These compounds have already been used in a study on thermal hyperalgesia, and should therefore, have a high probability of being effective in our hands\(^37\). The active compound, referred to as CN2097, should at least partially displace cypin from PSD-95, since cypin binds to PDZ 1 and 2 of PSD-95\(^10\). The control compound has the active residues in CN2097 replaced with inactive glycine residues (CN5125) and alanine residues (CN5135). First, we examined the compounds’ ability to disrupt the interaction between cypin and PSD-95 using rat whole brain extract. We performed co-immunoprecipitation using cypin antibody and found that the compound CN2097 disrupted cypin binding to PSD-95 (Figure 4.5). These data are consistent with the idea that cypin binds to PDZ 1 and 2 of PSD-95.
Figure 4.5. PDZ 1 and 3 competitor CN2097 inhibits cypin binding to PSD-95 in rat brain extract

Whole brain extract was incubated with CN2097, and co-immunoprecipitation was performed using anti-cypin. Control is immunoprecipitation in the presence of control compound CN5135. A. Representative Western blots. B. Quantitation performed by densitometry of Western blot. *p<0.05 by two tailed t-test followed by Mann Whitney post-test. n=4.


**CN2097 decreases dendrite arborization but has no effect on PSD-95 clustering**

Since the compounds proved to be effective in displacing cypin from PSD-95, we next wanted to see if these compounds have any effect in the cell. We tested CN2097 in two ways. First, we asked whether CN2097 alleviates decreased PSD-95 expression and localization caused by cypin\(^{10}\). We grew neurons in culture and treated them with 50 µM CN2097 or control compound CN5135 from DIV 10 until DIV 12. We found that there was no significant change in either number or size of PSD-95 puncta when comparing cells treated with CN2097 vs. control compound, CN5135 (Figure 4.6). We also assessed dendrite branching in the presence of the compounds to determine if they would have any effect on dendrite development. We used the same mixed culture of neurons from a rat with GFP expressed under and ubiquituous promoter and wildtype neurons used to examine the compounds previously (Figure 4.7). Neurons treated with CN2097 for two days and fixed on DIV 12 show significant decreases in dendrite field complexity when compared to neurons treated with both CN5135 and CN5125 controls. (Figure 4.7A). This decrease in complexity corresponds to a decrease in secondary and tertiary dendrite number and length (Figure 4.7B and C). This negative effect on dendrite morphology corresponds to the behavior of dendrites in cells that overexpress PSD-95 in the presence of cypin or cells that overexpress a mutant of cypin lacking the PDZ-binding motif\(^{27}\). This indicates that the compounds have a similar effect on the neuron as altering the cypin/PSD-95 interaction using traditional manipulation methods but does not fully alleviate all of the consequences of this interaction.
Figure 4.6. Treatment of cells with CN2097 causes no significant change in PSD-95 clusters

![Graphs showing no significant change in PSD-95 clusters](image)

PSD-95 clusters were quantified after treatment with CN5135 (control) or CN2097 (active) from DIV 10 to DIV 12. Number and area of PSD-95 clusters were measured using ImagePro. A. Quantification of PSD-95 cluster number. There was no significant change when neurons were treated with CN2097. B. Quantification of PSD-95 cluster area. n=1 experiment of 25 neurons.
Figure 4.7. Treatment of neurons with CN2097 causes decrease in dendrite branching.
Figure 4.7. Treatment of neurons with CN2097 causes decrease in dendrite branching

Branching analysis of GFP-positive neurons treated with the indicated compounds. A. Sholl analysis of GFP-expressing cells. Neurons treated with CN2097 show a significant decrease in dendritic field complexity. B. Quantification of dendrite number. Neurons treated with CN2097 show decreased number of secondary and tertiary dendrites. C. Quantification of dendrite length. Neurons treated with CN2097 showed decreased dendrite length in secondary and tertiary dendrites. **p<0.01, *p<0.05 using two way ANOVA with Bonferroni multiple comparisons. n>14 for all conditions.
Discussion

Through the first part of this chapter and previous work done in the Firestein laboratory, it had been shown that small molecules can regulate the GDA activity of cypin and that these molecules can change other biochemical behaviors of cypin through such regulation \(^{28}\) and Figure 4.1. This thesis shows that the GDA activity assay is a meaningful assay for compounds that may have an effect on cellular morphology. In initial tests, inhibitory compounds of cypin’s GDA activity decreased dendritic field complexity and excitatory compounds increased dendritic field complexity (Figure 4.3). Highly specialized analysis software that our laboratory has developed \(^{30}\) uncovers effects of these small molecules that would not be detected by conventional Sholl analysis. Conventional Sholl analysis did not reveal that H9 has any effect on the dendritic field; however, when using branch order analysis, we observed that tertiary branches change in number in response to treatment with compound H9. By using root, intermediate, and tip analysis, we found that H9 increases branches that terminate and do not branch as well as in tertiary dendrites that branch. This conclusion is supported by the general appearance of extensions that extend off of dendrites in neurons treated with H9 that are MAP2-positive, indicating they contain microtubules and are true dendritic branches (Figure 4.8). As shown in Langhammer et. al. 2010, these changes can occur without changing the curve generated by Sholl analysis and are only elucidated using more specialized analysis methods. The subtlety of the changes caused by the compounds needs to be more fully explored beyond the initial tests performed here. It may indicate that small molecule inhibition is a good way to examine the effects of cypin in a more sensitive manner. It may also indicate that to achieve a larger effect on neuronal morphology, compounds
with a larger affinity for cypin may need to be identified. It is also possible that the modulation of cypin’s GDA activity does not have a great effect on neuronal morphology and would not be useful for the treatment of diseases in which morphology is the primary deficit. This seems unlikely for all morphologically linked diseases because, as an example, the deficits seen in a subset of Rett syndrome patients are not with primary dendrites but with the formation of the extended dendritic field, consisting of secondary and tertiary branches where the compounds seem to have their effect.\(^8\).
Figure 4.8. Neurons treated with H9 show growth of MAP2-positive tertiary extensions

![Image of Neuron with MAP2-positive tertiary extensions]

Figure 4.8. Neurons treated with H9 show growth of MAP2-positive tertiary extensions

Images from fluorescence microscopy of a GFP-positive neuron treated with compound H9 and exhibiting a characteristic branching pattern. A. GFP channel only image with dendrites highlighted by red circle. B. Pseudo-colored neuron with fine extensions highlighted by white circle. Green=GFP and red=MAP2. MAP2 is present in the fine extensions, indicating they are dendrite branches and not filopodia.
Importantly, for these studies, a 96 well format assay for the study of cypin’s GDA activity was developed, allowing a more efficient study of cypin. This assay has been developed using cypin protein from COS-7 cell lysate and purified GST-tagged cypin protein. These initial studies allowed us to determine kinetic data for cypin, showing that cypin has a 0.8629 µM Km for guanine as substrate (Figure 4.4). While this Km does not exactly match previously described Km values of guanine deaminases which run from ~ 9.5 to 15 µM \cite{31-33}, our assay was carried out under different conditions than most assays. We were limited and could only carry out the assay at 25 °C, which can negatively impact the efficiency of enzymatic reactions greatly. The high through-put nature of the 96 well format also allowed us to screen 96 compounds of interest and select for compounds that have the greatest effect on cypin’s GDA activity.

The inability to accurately measure the amount of cypin protein in the COS-7 lysate makes it unsuitable for truly quantitative and high through-put screen of compounds. Unfortunately, the compounds H9 and B9 did not change cypin activity in any manner in the assay using GST-tagged proteins (Figure 4.3C, D and E). It is possible that H9 and B9 function by altering the dimerization of untagged cypin in the GDA assay with COS-7 cell lysate. The self-dimerization characteristics of GST protein may negate any effects the compounds have on cypin dimerization. To address this, cypin protein should be purified in a manner, such as HIS tag purification or cleavage of the GST tag, that does not leave a large purification tag on the protein. It is also possible that the compounds altered a different protein interaction with cypin that is present in the COS-7 cell lysate but not in the purified GST-tagged protein.
Using the membrane-permeable compounds produced by Dr. Spaller, we showed that compounds specifically designed to interfere with PDZ 1 and 3 of PSD-95 can significantly disrupt the cypin/PSD-95 interaction. This is notable in several respects. First it shows that disruption of only one of the potential places where cypin binds to PSD-95 is sufficient to disrupt a large amount of the interaction between the two molecules. This means that this interaction is a viable target for manipulation. The cellular effects of the compounds are also significant. Treatment with the compounds is sufficient to alter dendrite branching, and this disruption is very similar to that seen when PSD-95 is overexpressed in neurons or the PDZ-binding motif is deleted from cypin. This indicates that the changes in branches may be regulated by the interaction between cypin and PSD-95.

Interestingly, these compounds were not able to alter cypin-mediated changes in endogenous PSD-95 levels in neurons at DIV 12. It would have been expected that neurons treated these compounds would show an increase in the levels of PSD-95 localized to clusters by relieving the negative regulation of PSD-95 by cypin. It is possible that the effect of cypin on PSD-95 localization does not play a role until later in development or that the level of negative regulation by cypin earlier in development is minimal since a majority of PSD-95 is not synaptically localized during the peak of branching.

These experiments have shown that the biochemical behavior and interactions of cypin can be manipulated using small molecules. They have established new assays that will allow future screening of the effect of small molecules on cypin behavior and have shown that the use of GDA activity is a valid screen for the future effectiveness of
compounds. Manipulation of cypin may one way to treat patients with diseases, such as autism, hyperuricemia, and cognitive developmental delay. The discovery of compounds that both increase and decrease cypin’s GDA activity is especially important as diseases, such as autism, result in increased levels of uric acid and may benefit from a decrease in cypin activity \(^{18,39}\). In contrast, there are indications that high levels of uric acid may play a protective role in diseases, like Parkinson’s disease, and these cases may benefit from an upregulation of cypin’s GDA activity\(^{21-23}\). Finding small molecules to manipulate cypin in a non-invasive manner is the first step to utilizing cypin as a tool to treat patients with neurological and cognitive disorders.
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I would also like to thank several undergraduates who worked very closely with me to produce these results. Rachel Swanson and Emilie Transue worked on the cypin guanine deaminase experiments. Vibhu Chandrashekhar worked on the effects of the cell-permeable compounds that interact with PDZ 1 and 3 of PSD-95 on the cypin/PSD-95 interaction. All three of these students worked very hard, added thoughtful insights, and will make excellent future scientists.

Figure 4.1 has been previously published in 28.
References


Chapter 5 – Microtubule Stability and Conclusions

Microtubule Stability

Introduction

An emerging area of interest is in the post-translational modifications (PTMs) that occur to microtubules in the neuron. To advance the study of cypin and PSD-95 and examine the implications of the changes they cause this is a good place to start looking. Microtubules can undergo many modifications, but a few of them are better-studied than others (reviewed in 1). Three of the main PTMs are detyrosination, acetylation, and polyglutamylation. Detyrosination is the removal of a tyrosine on the C-terminus of alpha tubulin. This occurs to assembled microtubules that have been present for a short period of time, but are stable. Acetylation is the addition of acetyl groups to alpha tubulin. Acetylation only occurs on very stable microtubules, but the modification does not confer stability. The third well-studied PTM is glutamylation, in which glutamate residues are attached to the C-terminus of tubulin. A distinct modification, polyglutamylation, in which long chains of glutamates are attached, is present in many neuronal microtubules.

These modifications make up a new layer of regulation that is now being referred to as the “tubulin code,” in reference to the histone code, because these modifications of tubulin influence how microtubule associated proteins (MAPs) associate with the microtubule and regulate their function. Each modification seems to play a distinct role in cargo transport, with each altering the behavior of different proteins. Tyrosination/detyrosination can influence end-binding protein association, kinesin-1 motor localization, and the effect of depolymerizing motor proteins on microtubules 2-5. Glutamylation plays a role in KIF1A targeting, dynein activity and synaptic activity-
dependent transport\textsuperscript{6-8}. Acetylation can localize KIF5A to microtubules and increase transport speed, and increased levels of acetylation have been associated with the rescue of some transport deficits seen in Huntington’s disease\textsuperscript{9-12}. All of these PTMs are important and play a function in normal neuronal transport. By localizing motor proteins and MAPs to microtubules, PTMs may play a key regulatory role in the transport of many molecules, including mitochondria, through KIF5A\textsuperscript{13}, and even microtubules, through dynein\textsuperscript{14}.

The fact that cypin can increase microtubule polymerization and dendrite branching while PSD-95 decreases microtubule polymerization and dendrite branching led us to ask whether these proteins can affect microtubule stability by examining microtubule PTMs.

**Methods**

Hippocampal neurons were plated at 20K cells/cm\textsuperscript{2} on 35 mm dishes with a 20 mm glass insert. Cells were transfected using AMAXA transfection using CMV promoters for protein expression. Fixation was performed by washing briefly with PBS and then fixing with ice-cold methanol for 10 min at -20 °C. The methanol was then aspirated and coverslips were allowed to air dry. Ice-cold acetone was then added, and cells were incubated for another 10 min at -20 °C. Cells were rinsed twice with PBS and stored at 4 °C for no longer then 5 days before staining. Cells were stained with antibodies against: GFP, to show transfected cells; one microtubule PTM (acetylation or detyrosination), to show areas of modification; βIII tubulin, as an indicator of total tubulin amount and neuronal lineage; and Hoechst dye to indicate nuclei. Imaging was performed by adjusting the time of acquisition for each channel based on the neurons expressing GFP.
to achieve a neutral intensity level that could increase or decrease without loss of data. Times were held constant for each trial. ROIs were selected on the dendrites of each neuron and mean intensity calculated using ImageJ. The mean intensity of each PTM was normalized to the total amount of tubulin using the βIII tubulin immunostaining intensity. The normalized ratio of PTM to total tubulin was used to calculate the ratio of detyrosination to acetylation.

**Results/Discussion**

To determine the effects of cypin and PSD-95 on neuronal microtubules, we transfected cells with the cDNA encoding GFP, GFP-cypin, or PSD-95-GFP and examined the ratio of different types of post-translational modifications on tubulin in the cell. Detyrosinated tubulin is a characteristic of newly formed microtubules while acetylation only occurs in microtubules that have been present for a longer period of time. While we did not find any significant results, the data trends indicate a scenario in which cypin and PSD-95 continue to play complementary roles in neuronal morphology. We found that cypin shows a trend towards a decreased ratio of detyrosinated to acetylated tubulin (Figure 5.1). This means that overexpression of cypin biases the neuron towards a more stable population of microtubules. PSD-95 shows a trend towards an increased ratio of detyrosinated to acetylated tubulin (Figure 5.1). This means that overexpression of PSD-95 biases the microtubules towards a less stable microtubule population. This change in microtubule stability, as discussed in Chapter 1, could lead to a change in the ability of microtubule motor proteins and plus-end molecules to associate with the microtubule. A change in acetylation and detyrosination could directly affect
transport speed of dynein motors and depolymerizing molecules to act upon microtubules 2-59-12.
Figure 5.1 – Cypin increases microtubule stability and PSD-95 decreases microtubule stability

Neurons overexpressing indicated proteins and immunostained for the indicated microtubule PTM. A. Representative neurons showing the unmodified intensity of each PTM. Images were selected to represent the normalized ratio of detyrosinated to acetylated tubulin and the general trend of intensities for each PTM. B. Quantitation of detyrosinated to acetylated tubulin. No significant differences were detected, although the data trend with cypin lowering the detyrosinated/acetylated tubulin ratio and PSD-95 increasing the detyrosinated/acetylated tubulin ratio. Statistics were calculated using a one-way ANOVA with Bonferroni post-test for significance. N= 2 trials of 10-15 neurons each.
Future Directions

Cypin/PSD-95 interaction

The data and conclusions presented in this thesis are excellent starting points for future exploration of the consequences of microtubule regulation in neurite behavior. The complementary roles of PSD-95 and cypin should be further explored. The changes that occur in microtubule post-translational modifications (PTMs) when cypin and PSD-95 are present are very interesting. Do any other microtubule PTMs change with cypin and PSD-95 expression? What domains of PSD-95 and cypin cause these changes to occur? Is the same domain in PSD-95 that is responsible for EB3 binding also responsible for this, or is it the more complex behavior that is seen in the EB3 comet and MT spacing behavior that alters microtubule PTMs? Is it necessary for cypin to bind assembled microtubules to change the stability of the microtubule, or is it the CRMPH region that facilitates this effect? What changes occur in microtubule stability and PTMs when the PDZ domain of cypin is deleted? It is possible that PSD-95 will assume regulatory control like it does for dendrite branching?

Given the changes that occur to the microtubule PTMs and arrangement, experiments should focus on the effects of altering cypin and PSD-95 levels on microtubule motor behavior. It is possible to use live-imaging techniques to visualize the movement of these motors along the microtubules in cell culture, and determine any changes in behavior. Speed and amount of transport should be examined, but direction of travel should also be observed. These experiments would also address the question of polarity changes caused by the alteration of microtubule arrangement and dynamics. The increase in microtubules seen crossing between branches with increased PSD-95
expression could mean that branches can communicate with each other, in addition to the cell body, and this too could be addressed by examining microtubule motor behavior. The changes in microtubule spacing seen with changes in cypin and PSD-95 expression could play a role, even if no changes are found with the microtubule PTMs. Transport may be inhibited by changes in the inter-microtubule distance as is seen in mitochondrial transport in the axon.\textsuperscript{16}

The compounds from Dr. Spaller used in Chapter 4 also show great promise in exploring the interplay between cypin and PSD-95. The initial studies show that these compounds are effective at disrupting this interaction and that this disruption can alter cellular behavior, but a more detailed characterization is needed before more complex experimentation, such as \textit{in vivo} work, can be performed. The EC50 of these compounds should be calculated by using co-immunoprecipitation experiments. The compounds can be titrated and used to aid further experiments. Cell morphology should be further explored using mature neurons to examine how disrupting the cypin/PSD-95 interaction affects dendritic pruning and synapse development. The complexities of blocking PDZ1 and 3 must also be explored. Co-immunoprecipitation of PSD-95 and cypin can be performed to determine what other molecular interactions are disrupted, and these interactions then probed for cellular effect. Once these other effects are known, the compounds can be applied to more complex models of cellular behavior in a consistent and thoughtful manner.

\textbf{Cypin biochemistry}

As seen in Chapter 3, Cypin has been shown to have four domains/activities that are important for its function: GDA activity, microtubule-binding, tubulin-binding, and
interaction with PSD-95. The exact mechanism by which cypin regulates microtubule dynamics has been further elucidated but remains unclear in the light of the microtubule-binding domain that it possesses. What roles do these domains play in regulating microtubules? A series of biochemical experiments designed to test the microtubule-binding domain and assess the role that it plays in microtubule polymerization, microtubule stability, dendrite branching, cellular microtubule dynamics, and cellular microtubule architecture will address such questions. The interplay between the tubulin- and microtubule-binding domains may allow cypin to regulate microtubule dynamics by stabilizing microtubules, such as MAPs, and also to increase microtubule polymerization directly as does CRMP2. Further experiments should address the combination of these domains to determine the cooperativity, if any, between them. Many of these experiments have been established in the Firestein laboratory, and the work performed in this thesis lays the groundwork for these experiments.

**Small molecules**

This thesis has established assays that can be used to screen small molecule compounds for their effect on cypin’s GDA activity. Work should continue on refining these assays by finding a viable way to produce exogenous cypin protein for use in the 96 well format. Our laboratory possesses an additional 192 compounds to screen, which should be performed using these assays when they are established. Compounds that have a similar structure to already active compounds can be selected in an effort to improve the success rate of screening. A further effort can be made to design and select compounds that are purposefully membrane-permeable to improve cellular effect. During and after the discovery period, compounds with promise should be screened in more
depth, i.e. in dendrite branching assays and in cellular GDA assays as described in \(^{17}\). Since changes in GDA have been associated with changes in dendrite number, the compounds should also be screened for their effect on cell-free and cellular microtubule dynamic assays. This can be performed using biochemical methods and live-cell imaging of EB3 comets as demonstrated in Chapter 2. Microtubule stability should also be determined if mutational analysis of cypin reveals a role for GDA or microtubule polymerization in the increase of MT stability seen with cypin overexpression.
Conclusions

In this thesis, we examined the role of cytoarchitecture and PSD-95 in regulating the microtubule cytoskeleton. Our published and unpublished data (Tseng and Firestein, submitted) suggest that PSD-95 and cytoarchitecture moderate whether a cell will be biased towards active dendritic branching or towards pruning the arbor and forming dendritic spines. We propose that the acts of creating a dendritic branch and a spine are complimentary. This means that the neuron can be in two states: In one state, the neuron is growing and forming dendritic branches, and in the other, it is growing dendritic spines for communication. This, in itself, is not novel, but we propose that the decision is made, at least in part, depending on the state of the microtubule skeleton and that this regulation occurs through the interaction of PSD-95 and cytoarchitecture. Based on the data presented in Chapters 2 and 3, we suggest that young neurons expressing cytoarchitecture have a microtubule structure that makes them more prone to grow and branch, but that as the neuron matures, increasing levels of PSD-95 push the dendrites to adopt a microtubule structure that makes them capable of forming more spines than branches.

Cytoarchitecture causes microtubules to polymerize faster and makes them more stable and more tightly packed. This could be a state that is preferential for dendrite branching. As previously stated, microtubule stabilization is an important part of forming a branch that can mature into a stable dendrite \(^{18,19}\). As neurons mature and the dendritic field stabilizes, the number of synapse increases. Cytoarchitecture negatively affects this maturation by decreasing PSD-95 expression and localization to the synapse \(^{20}\). In addition, growing microtubules are capable of disrupting the postsynaptic density \(^{21}\). The cytoarchitecture-mediated
increases in microtubule growth and stability may cause more microtubules to enter the spine or filopodia and disrupt the PSD.

PSD-95 causes the opposite change in the microtubule architecture. The resulting increased spacing between the microtubules, increase in number of microtubules that cross between branches, and decreased stability of microtubules from reduced EB3 binding could bias a neuron towards being ready to make smaller protrusions that require different cytoskeletal dynamics than those forming a dendritic tree. Importantly, an increase in PSD-95 can abolish the effect of cypin on the dendritic arbor, indicating that as a neuron matures and produces more PSD-95, microtubule cytoskeleton may move away from cypin-generated dendrites and into PSD-95 generated spines. It is also important to remember that these events occur in a balance and are not mutually exclusive. This balance is important, as illustrated by the events that occur during the disruption of the interaction between PSD-95 and cypin. When this interaction is interrupted, by deletion of the PDZ-binding motif on cypin or by small molecule inhibition (Figure 4.7), secondary branching mediated by cypin is inhibited. This indicates that PSD-95 can act as a negative regulator that overrides cypin-promoted changes in neuronal morphology.

This change in microtubule architecture and neuronal morphology is one that needs to be further researched, and as seen in Chapter 4, we are establishing less invasive ways to examine the behavior of cypin in the cell. Using small molecules, we will be able to modulate cypin-mediated increases in microtubule stability, polymerization, and dendrite growth, or to allow PSD-95 to exert its effects and alter microtubules to allow for growth of spines instead of dendrites. Using these small molecules and the assays
described in Chapters 2, 3, and 4, we can also begin to explore the role of guanine metabolism in dendrite morphology and disease. This opens new avenues of research for cypin action in neuronal function.

**Final conclusion**

Study of the mechanisms behind the interaction between cypin and PSD-95 has led to the conclusion that cypin and PSD-95 play opposite roles in determining neuronal morphology. PSD-95 acts to decrease branching through its effect on microtubule behavior, and cypin acts to increase branching through its effect on microtubule behavior. This relationship is ripe for further research because of the many potential implications for dendritic cellular transport, morphology, and the effects in disease. In addition to providing scientific insight into the relationship of PSD-95 and cypin, this thesis has established methodologies in the laboratory that will be useful for future research and has further expanded upon the idea of cypin as a molecular drug target worthy of examination.
References


Appendix

Making EM measurements

Figure A.1 How to count microtubules in EM images

Figure A.1. How to count microtubules in EM images. A. Example of how microtubule crossings were counted. A 0.5 μM line (black box) was drawn and any microtubules crossing that line were counted (arrows). Scale bar = 0.5 μM. B Example of how microtubule spacing was counted. A 0.5 μM line (yellow line) was drawn. C. Using ImageJ an intensity profile was drawn along the line and peaks that correspond to microtubules are visually marked. The distance between the center line of each peak is measured and recorded. The number of measurement recorded + 1 is the number of microtubules in the measured area. This can also be used in data analysis.
Neuronal cultures from GFP expressing rats

In order use cells from a rat expressing GFP under a ubiquitous promoter the conditions needed to plate cells must be established. Cells from a pregnant wild type female crossed with GFP homozygous male were harvested at E18. Typically, Mendelian genetics are true and ½ of the embryos are GFP positive and and ½ are wildtype. They were trypsinized and plated at 100,000 cells/cm². Cells were grown for 12 days and fixed using 4 % PFA and 4% sucrose. The ratios of GFP to wt were plated as indicated. We found that the numbers of neurons were similar regardless of plating. While the number of neurons that are GFP positive appears to be too low when the ratio goes from 1:9 to 1:19 the visualization of the neurons is much better because the neurons at dilutions 1:3 and 1:9 are not spread widely enough apart to separate processes from one neuron to the other. 1:49 is the best spread of neurons for visualization of all of the processes (Figure A.2)
Figure A.2. 1:49 is an appropriate mixture of GFP rat cells to WT cells to get an even spread of GFP cells for branching analysis at 100K/cm².
Figure A.2. 11:49 is an appropriate mixture of GFP rat cells to WT cells to get an even spread of GFP cells for branching analysis at 100K/cm²

A. Representative neurons from plating of GFP rats cells mixed with wild type cells at the indicated ratios. B Quantitation of neuronal populations.
Microtubule stability fixation tests

The microtubule stability fixation conditions were taken from\(^{23}\). Cells were fixed on DIV 12 with the indicated treatment. Blocking was done with 5% BSA, 1% triton X-100 and 0.02% sodium azide for 1 hr at RT. Immunostaining was performed with antibodies to GFP, one microtubule PTM, BIII tubulin and Hoechst stain for nuclei. See table T.X for antibody information and dilutions. Secondaries are from JacksonImmuno and used at 1:250 dilution.

The conditions below were tested to determine their effect on the microtubule PTMs and background staining. It was found that the methanol and acetone provided good staining on the PTMs and the best signal to noise ratio on the background. The GFP must be immunostained for since the cold MeOH quenches the fluorescence. The saponin the prefixation condition leached all the GFP from the cells during the pre-fixation treatment. The PHEM fixation seemed that the signal to noise ratio was too high.
Figure A.3. MT stabilization buffer fixation results

**MeOH**

- Acetylated Tubulin Stain
- Detyrosinated Tubulin Stain
- Polyglutamylated Tubulin Stain

- GFP, Acetylated
- GFP, Detyrosinated
- GFP, Polyglutamylated

- Bill, Hoechst

**MeOH + Acetone**

- Acetylated Tubulin Stain
- Detyrosinated Tubulin Stain
- Polyglutamylated Tubulin Stain

- GFP, Acetylated
- GFP, Detyrosinated
- GFP, Polyglutamylated

- Bill, Hoechst

**PHEM**

- Acetylated Tubulin Stain
- Detyrosinated Tubulin Stain
- Polyglutamylated Tubulin Stain

- GFP, Acetylated
- GFP, Detyrosinated
- GFP, Polyglutamylated

- Bill, Hoechst

**Saponin prefix - no GFP found on most cells**

- Acetylated Tubulin Stain
- Detyrosinated Tubulin Stain
- Polyglutamylated Tubulin Stain

- GFP, Acetylated
- GFP, Detyrosinated

- Bill, Hoechst
Figure A.3. MT stabilization buffer fixation results

Representative images of neurons treated with the indicated conditions and stained for the indicated antibodies.

Table T.1. MT stability antibody dilutions

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Company</th>
<th>Cat #</th>
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<tbody>
<tr>
<td>Gt-GFP</td>
<td>1:250</td>
<td>Rockland</td>
<td>600-101-215</td>
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<tr>
<td>Ck-BIII</td>
<td>1:250</td>
<td>Millipore</td>
<td>AB3201</td>
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<td>Ms-Ace tubulin</td>
<td>1:1000</td>
<td>Sigma</td>
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<td>1:250</td>
<td>Thermo</td>
<td>MA1-25062</td>
</tr>
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Curriculum Vitae

Education

Rutgers, The State University of New Jersey, Piscataway, NJ, PhD, Cell Biology and Neuroscience, October 2011

Virginia Polytechnic Institute and State University, Blacksburg VA
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Publications in Press

Sweet, E.S., Tseng, C.Y., Firestein, B.L. To branch or not to branch: How PSD-95 regulates dendrites and spines. Bioarchitecture (2011) vol. 1 (2) online


