STRUCTURAL CHARACTERIZATION AND TRANSCRIPTIONAL REGULATION OF THE CYTOSOLIC PSD-95 INTERACTING PROTEIN (CYPIN) AND ITS ROLE IN NEURONAL DENDRITE BRANCHING

by

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Dendrite morphology regulates how a postsynaptic neuron receives information from presynaptic neurons. The specific patterning of dendrite branches is promoted by extrinsic and intrinsic factors that trigger the activation of functional signaling pathways. However, only a handful of the regulatory factors and biochemical mechanisms involved in determining dendrite morphology are known. The Firestein laboratory previously reported that cypin (cytosolic PSD-95 interactor), the mammalian guanine deaminase (GDA), plays an active role in regulating dendrite branching in hippocampal neurons. Cypin-promoted increases in dendrite number are dependent on binding of zinc ions to cypin and cypin’s guanine deaminase activity.

This work focuses on the identification of specific structural properties of cypin that lead to the multifunctional roles in guanine metabolism and dendrite
development. We first employed phylogenetic analysis and computational structure modeling techniques to construct a three dimensional structural model of cypin. Inspection of our structural model confirmed that specific predicted residues coordinate with a zinc ion to play a role in enzymatic activity and promotion of dendrite branching in developing neurons. In addition, we used a combination of protein structure analysis, experimental kinetic studies, and cell culture tests to uncover novel potential ligands for cypin. We obtained a list of compounds that demonstrate higher binding affinity to GDA than does guanine. Our results provide evidence that an in silico drug discovery strategy coupled with in vitro verification can be successfully implemented to discover compounds that may have therapeutic value for the treatment of diseases and disorders where GDA activity is abnormal.

Since the regulation of dendrite branching function by cypin is dependent on intracellular levels of cypin protein, identification of transcriptional regulators of cypin gene expression may elucidate how cypin regulates neurite development. Therefore, we also investigated how extracellular factors can regulate cypin expression. Our data show that in developing neurons, BDNF increases cypin protein via activation of the MEK pathway, and consequently, CREB transcription factor-dependent cypin gene expression. The discovery of intrinsic regulators of cypin expression aids in our understanding of molecular mechanisms underlying dendritic patterning, and hence, synaptic plasticity, learning and memory.
Preface

A significant extent of the material in this dissertation is published or is in the process of publication in peer-reviewed scientific journals. The Introduction was previously published, with substantial modification, in the journal *Central Nervous System Agents in Medicinal Chemistry* (2008) 8(2), 100-06. Chapter 1, “Phylogenetic analysis and molecular evolution of guanine deaminases: from guanine to dendrites,” has been submitted for publication and is currently under review. Chapter 2, “Structural characterization of the zinc binding domain in cytosolic PSD-95 interactor (Cypin): role of zinc binding in guanine deamination and dendrite branching,” was published in *Proteins: Structure, Function and Bioinformatics* (2008) 70, 873-81. Chapter 3, “Characterization of novel substrates for guanine deaminase: lessons from computer-aided drug design,” and Chapter 4, “Transcriptional regulation of cypin and its role in dendrite branching,” are in preparation for submission.
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INTRODUCTION:

NEURONAL DENDRITE BRANCHING AND CYPIN, THE MAMMALIAN GUANINE DEAMINASE
Neuronal circuitry is established when neurons receive their inputs from other cells. The specific place where this occurs is on highly branched cellular extensions called dendrites. Thus, dendrite patterning and development are central to neuronal function and are responsible for determining how input signals are processed in the central nervous system (CNS). The degree of dendritic branching can regulate the cellular electrical properties of a neuron (Miller and Jacobs, 1984). Vast experimental evidence supports the idea that dendritic development is regulated by intrinsic and extrinsic factors (Landgraf and Evers, 2005; Libersat, 2005). In addition, cellular signaling pathways regulate transcriptional activity and expression of developmental regulators during neuronal growth (Goldberg, 2004). Early studies of dendritic shape and development using *Drosophila* as a model led to the identification of several genes involved in dendritic development (Gao et al., 1999; Tassetto and Gao, 2006). Specific transcription factors, including *sequoia*, *flamingo*, *hamlet*, and *cut* are critical determinants for the expression of genes that regulate dendrite morphology (Grueber et al., 2002 and 2003). In vertebrates, signaling proteins, such as β-catenin, adenomatous polyposis coli (APC), Numb, Numblike, and Notch, show a polarized cellular localization in neuronal precursor cells, suggesting an asymmetric activation of signaling pathways in developing neurons (Nishimura et al., 2006; Redmond and Gosh, 2001).

Interestingly, the number and pattern of dendrites are affected in patients suffering from neurodegenerative diseases, including autism and Rett Syndrome (Zoghbi, 2003). In addition, neurons isolated from Down Syndrome patients show
dendritic atrophy and a marked decrease in dendritic branching and length compared to control subjects (Takashima et al., 1989 and 1994; Ferrer and Gullotta, 1990). The abnormal cellular morphology in these individuals may be related to loss of neuronal communication, leading to mental retardation. Therefore, there is an emerging need to reveal the major molecules that regulate dendritic development, and consequently, to identify a series of pharmacological agents that target these molecules for the treatment of patients with neurodegenerative disorders.

**Modulators of dendrite branching**

Current research shows that extrinsic factors can modulate specific patterns of dendritic growth and branching by activating intrinsic cues (Whitford et al., 2002). These regulators activate precise signaling cascades that directly affect the cytoskeleton or the transcriptional regulation of gene expression. In fact, a number of secreted molecules have been identified that regulate dendritic development in neuronal cultures (Libersat and Duch, 2004). For example, chemoattractive signals, such as Semaphorin 3A, serve as regulators of specific developmental growth of axons versus dendrites by selectively targeting proteins to apical dendrites (Polleux, et al., 2000). Additionally, specific receptors in cellular membranes regulate the trafficking of these secreted molecules, and hence, the activation of dendritic signaling pathways.

Over the past two decades, it has been reported that neurotrophic factors play important roles in regulating neurite growth and branching in neurons of the CNS. The major neurotrophic factors are called neurotrophins, which consist of
nerve growth factor (NGF) (Levi-Montalcini, 1987; Shooter, 2001), brain-derived neurotrophic factor (BDNF; Barde et al., 1982), and neurotrophin-3 (NT-3; Maisonpierre et al., 1990). These factors are secreted from neurons and glia and exert their effects through the tyrosine receptor kinase family (Trk) of receptors in response to neuronal activity (Chao, 1992; Levine et al., 1995; Muller et al., 1995). There is some degree of receptor specificity with NGF binding to TrkA, BDNF and NT-4 binding to TrkB, and NT-3 preferentially binding to TrkC, all with high affinity (Reichardt, 2006). Characterization of these receptors has immensely advanced the study of signaling pathways that are triggered by these important factors to control neurite development, especially dendrite growth and branching. Inspection of experimental data provides clear evidence that neurotrophins increase the dendritic complexity of pyramidal neurons in the CNS by increasing total number and length of dendritic branches (McAllister, 2000). While there has been rapid and significant progress in understanding the mechanisms of action by these factors, more detailed clinical studies on the application of neurotrophins are needed. Therefore, there has been an increasing interest over the past years in the development of novel drug delivery strategies for the treatment of neurodegenerative diseases that show cellular morphology changes, and consequently, failure in proper neuronal activity (Haller and Saltzman, 1998; Tuszynski, 2007).

Since one of the main disadvantages of protein-based drugs is poor pharmacokinetic behavior, these drugs have met without success in clinical trials. Consequently, the aspiration to develop small molecule based drugs, which can
contain a neurotrophin mimetic by effectively activating the Trk family of receptors, has also been investigated (Lee and Chao, 2001; Pollack and Harper, 2002). In addition, natural compounds initially tested as anti-cancer agents, including staurosporine and K-252b, alter neurite development (Pollack et al., 1999). Structural analogues of staurosporine and K-252b can bind directly to Trk receptors and increase neurotrophin affinity by a putative allosteric effect (Pollack et al., 1999). For example, compounds that contain only the bis-indole carbazole subunit of staurosporine-like molecules but not the sugar subunit lose activity on Trk receptors. Active compounds contain the bis-indole carbazole subunit with no significant chemical substitutions, suggesting that this subunit is required for protein/receptor interaction. In contrast, any chemical substitution in the sugar subunit affects the specific activity effect on modulating Trk activity and neurotrophin affinity.

Unfortunately, most of the tested compounds lack concrete specificity in \textit{in vivo} models. This limitation has been particularly restrictive for compounds designed to directly interact with Trk family receptors. However, compounds that can be designed to contain a combinatorial function of interacting with Trk receptors and activating neuronal signaling pathways downstream of these receptors can provide an enhanced potential for the future use of neurotrophin-like drugs for the treatment of neurodegenerative diseases.

For more than four decades, it has been shown that zinc levels in the CNS play an important role in regulating neuronal morphology and synaptic transmission (Haug, 1967). Hippocampal zinc secretion occurs in excitatory
neurons of the dentate gyrus that contain unmyelinated axon bundles, called mossy fibers, which project to the CA3 region. These zinc-containing neurons pump zinc ions into vesicles emerging from the Golgi apparatus and act as presynaptic cells to alter the sensitivity of excitatory and inhibitory amino acid receptors in postsynaptic excitatory neurons (Frederickson, 1989; Smart et al., 1994; Frederickson et al., 2000). The postsynaptic neurons contain specific transmembrane channels that admit zinc ions into the cytosol to further initiate intracellular signaling pathways (Yin et al., 1998; Jia et al., 2002).

Although the specific functional significance of the modulatory role of zinc is not clear, proper dendritic arborization is disrupted in developing animals consuming a zinc-deficient diet (Dvergsten et al., 1983 and 1984). Morphological and functional studies of rodents given a zinc-reduced diet showed that neurons were reduced in size and had fewer branches and that their cytoplasmic maturation was interrupted (Dvergsten et al., 1983). In addition, zinc inactivity has been linked to Alzheimer’s disease (AD; Dvergsten et al., 1984). The cognitive functions of AD patients are significantly preserved when the patients are given zinc supplementation (Van Rhijn et al., 1990). On the other hand, non-regulated synaptically-released zinc in these patients results in increased Aβ amyloidosis (Bush, 2002). While the specific function of zinc-induced precipitation of Aβ into plaques needs to be determined, it is known that zinc homeostasis in neurons is a key modulator of neuronal excitability and development.
Dendritic reorganization, at the level of dendritic growth and branching, occurs in response to synaptic activity (McAllister et al., 1996). These changes are mediated by calcium signaling. Intracellular calcium levels are directly regulated by voltage-sensitive calcium channels (VSCC), N-methyl D-aspartate (NMDA) glutamate receptors, and ligand-gated calcium channels. Calcium levels can trigger two major signaling pathways mediated by a family of calcium/calmodulin-dependent protein kinases (CaMKs) and mitogen-activated kinases (MAPK). CaMKs and MAPKs are central mediators of calcium-dependent dendritic growth and branching since both families can alter the neuronal cytoskeleton by activation of microtubule polymerization and actin filament formation. For example, the CaMKII isoform CaMKIIβ can promote filopodial extension and dendrite development by interaction with actin (Fink et al., 2003). On the other hand, MAPKs can modulate dendrite formation by phosphorylation-mediated activation of microtubule-associated proteins (e.g. MAP2; Vaillant et al., 2002). The above agents that control dendrite growth and branching are shown in Figure I-1.

The cytosolic PSD-95 interactor (cypin): a linker between extrinsic and intrinsic regulation of dendrite branching

Cytosolic PSD-95 interactor (cypin) is a 454 amino acid protein, which acts as a guanine deaminase in mammalian cells. Cypin was initially isolated as a detergent-soluble PSD-95-interacting protein by affinity chromatography using brain extracts (Firestein et al., 1999). Cypin contains several domains, including a conserved nine residue Zn$^{2+}$-binding motif, a collapsin response mediated
protein homology (CRMPH) domain, and a PDZ-binding sequence (~SSSV) at its carboxyl terminus. The PDZ-binding sequence is responsible for cypin’s interaction with PDZ domains 1 and 2 of PSD-95 (Firestein et al., 1999). Cypin is expressed at high levels in neurons and intestinal epithelial cells. Functional studies demonstrated that overexpression of cypin in cultured hippocampal neurons disrupts the synaptic clustering of PSD-95 at the PSD, suggesting that cypin plays a role in the maturation of postsynaptic neurons (Firestein et al., 1999). Further work also demonstrated that overexpression of cypin increases dendrite number and branching (Akum et al., 2004).

Interestingly, cypin’s guanine deaminase activity has been linked to cypin-promoted increases in dendrite branching since overexpression of guanine deaminase activity–deficient mutants of cypin results in decreased branching in cultured neurons (Akum et al., 2004). The unexpected multi-functional properties of this particular protein may establish a key connection between neuronal morphology and purine metabolism. Cypin binds directly to tubulin heterodimers via its CRMPH domain to promote microtubule assembly. Since microtubule assembly is important for dendrite outgrowth and branching, these studies uncovered a mechanism by which cypin acts to control dendritic arborization. In addition, increases in electrical activity result in increases in cypin protein (Akum et al., 2004). Furthermore, activation of the small-GTPase RhoA, which decreases dendritic arborization, decreases cypin protein expression via a translation-dependent mechanism (Chen and Firestein, 2007). Similarly, dendrite numbers increase when an inactive form of RhoA is overexpressed in cultured
hippocampal neurons. Therefore, extracellular agents and subsequent signaling pathways that can regulate cypin protein levels and/or increase the enzymatic activity of cypin in neurons might be of pharmacological interest for targeting purine metabolic and cognitive disorders.

Since the enzymatic activity of cypin connects guanine metabolism and neuronal morphology, it is of great interest to use this protein as a novel drug target. De novo drug design can be significantly enhanced by the availability of a high-resolution cypin or guanine deaminase crystal structure model.

**Structural characterization of cypin to modulate its enzymatic activity and effects on dendrite branching**

The expression of cypin, the mammalian GDA, is tissue-specific (Paletzki, 2002; Firestein et al., 1999) and in brain, plays a role in the development of neuronal morphology by regulating dendritic arborization (Akum et al., 2004; Chen et al., 2005; Charych et al., 2006; Chen et al., 2007). Interestingly, promotion of dendrite formation by GDA in neurons is dependent on the breakdown of guanine during development (Akum et al., 2004; Fernández et al., 2008). Despite the central role played by cypin in neuronal development, many unanswered questions remain pertaining to its three-dimensional structure, the actual amino acids that coordinate zinc ion binding to cypin, the effect of zinc binding on dendrite branching, and the analysis of the protein-ligand biointerface to develop rational drug design.

In order to understand the molecular and structural properties that determine cypin’s dual function in purine metabolism and neurite development, a
phylogenetic analysis of the molecular evolution of guanine deaminases was performed. In Chapter One, we performed a complete evolutionary analysis of the full-length sequences and the principal domains in guanine deaminases. We reconstructed the molecular phylogeny of guanine deaminases to serve the purpose of understanding the molecular relationship between the functions of purine metabolism and dendrite branching. Furthermore, this study revealed domain acquisition and specific evolutionary positions of accumulative mutations and transferences between organisms that express other GDA protein forms.

A structural characterization of the cypin is described in Chapter Two. To identify the specific structural role of zinc binding in cypin-mediated dendrite branching and guanine deaminase activity, we employed computational homology modeling techniques to construct a three-dimensional structural model of cypin. Analysis of the protein-ion sequestration scaffold of this model identified several histidines and one aspartic acid residue responsible for zinc binding. Single substitution mutations in these specific sites completely disrupted the guanine deaminase enzymatic activity and rendered cypin unable to promote dendrite branching in rat hippocampal neurons. Furthermore, we showed that promotion of dendrite branching by cypin is zinc-dependent.

In addition to its role in the maturing brain, GDA is thought to play a role in proper liver function since abnormal levels of GDA activity have been correlated with liver disease and transplant rejection. Although mammalian GDA is an attractive and potential drug target for treatment of liver diseases and cognitive disorders, prospective novel inhibitors and/or activators of this enzyme have not
been actively pursued. In Chapter Three, we used the combination of protein structure analysis and experimental kinetic studies to test novel potential substrates for human GDA. We obtained a list compounds that demonstrate a higher binding affinity to GDA than does guanine. The results in this study provide evidence that an *in silico* drug discovery strategy coupled with *in vitro* verification can be successfully implemented to discover compounds that may have therapeutic value for the treatment of diseases and disorders where GDA activity is abnormal.

Since the cypin-promoted increases in dendrite branching correlate with cypin protein levels, the identification of transcriptional regulators of cypin gene expression may elucidate the mechanism by which cypin regulates neurite development. Therefore, in Chapter Four, we investigated how extracellular factors can regulate cypin gene and protein expression. Our data show that in developing neurons, BDNF increases cypin protein by the MEK pathway and that the cAMP responsive element binding protein (CREB) transcription factor regulates cypin gene transcription. The discovery of intrinsic regulators of cypin expression aids in our understanding of the molecular mechanisms underlying dendritic patterning, and hence, synaptic plasticity, learning, and memory.

Since aberrant dendritic arborization occurs in neurodegenerative diseases, including autism, Rett Syndrome, and mental retardation (Zoghbi, 2003), suitable and rational pharmacological drug targeting studies in this work will help in the discovery of additional agents for the control of dendritic branching and differentiation during CNS development and in disease states.
A

Neuronal Differentiation

B

Dendrite growth and branching
Figure I-1. Molecular agents and their corresponding targets that control dendrite growth and branching. (A) Schematic of the morphological changes in neuronal precursor cells after neuronal differentiation (B) Schematic of the agents that control dendrite growth and branching.
**Figure I-2. Cypin protein domains and interacting partners.** The rodent and human cypin are 454 amino acid proteins. Colored boxes indicate protein domains. The interacting partners are indicated with arrows and referenced.
CHAPTER ONE:

PHYLOGENETIC ANALYSIS AND MOLECULAR EVOLUTION OF GUANINE DEAMINASES
Summary

Guanine deaminase (GDA; guanase), is a ubiquitous enzyme that catalyzes the first step of purine metabolism by hydrolytic deamination of guanine, resulting in production of xanthine. This hydrolase subfamily member plays an essential role maintaining homeostasis of cellular triphosphate nucleotides for energy, signal transduction pathways, and nitrogen sources. In mammals, GDA protein levels can regulate neuronal development by regulating dendritic arborization. We previously demonstrated that the most abundant alternative splice form of GDA in mammals, termed Cypin (cytosolic PSD-95 interactor), interacts with postsynaptic density proteins, regulates microtubule polymerization, and increases dendrite number. Since purine metabolism and dendrite development were previously thought to be independent cellular processes, this multi-functional protein serves as a new target for the treatment of cognitive disorders characterized by aberrant neuronal morphology and purine metabolism.

Although the enzymatic activity of GDA has been conserved during evolution from prokaryotes to higher eukaryotes, a detailed evolutionary assessment of the principal domains in GDA proteins has not yet been put forward. In this Chapter, I perform a complete evolutionary analysis of the full-length sequences and the principal domains in guanine deaminases. Furthermore, we reconstructed the molecular phylogeny of guanine deaminases with neighbor-joining, maximum-likelihood, and UPGMA methods of phylogenetic inference. This study can act as a model whereby a universal housekeeping
enzyme may be adapted to also act as a key regulator of a developmental process.
Results

Guanine deaminases are multifunctional proteins

Guanine deaminase (GDA) plays an important role in purine metabolism since it mediates the first step of catabolism by converting guanine to xanthine and ammonia. GDA also plays a key role in the purine salvage pathway, where it mediates a molecular switch between the uses of purines as either a nitrogen or carbon source and nucleotide synthesis (Fig. 1A). In higher eukaryotes, GDA plays an important role in the regulation of dendrite development. As we have previously reported (Firestein et al., 1999; Akum et al., 2004; Chen et al., 2005; Charych et al., 2006; Chen and Firestein, 2007; Fernández et al., 2008), overexpression of cypin, the mammalian GDA, increases primary and secondary dendrites in cultured primary rat hippocampal neurons (Fig. 1B-C). This increase in dendrites is dependent on GDA activity (Akum et al., 2004; Fernandez et al., 2008). Therefore, we believe that cypin is a molecular and functional linker between purine metabolism and neurite development. Therefore, this multi-functional protein represents a new branch drug target for the treatment of cognitive disorders that manifest aberrant by neuronal morphology and purine metabolism.

Evolutionary pathways of guanine deaminases

In order to perform a comprehensive evolutionary analysis of GDA and to understand the possible molecular pathways which determine the acquisition of specific protein motifs to regulate both guanine deamination and dendrite
development, we analyzed prokaryotic and eukaryotic GDA sequences. We found 219 guanine deaminase sequences from the NCBI non-redundant (nr) protein database. Sequences from the Reference Sequence (RefSeq) protein database collection contain 143 prokaryotic, 10 archaeal, and 66 eukaryotic GDA sequences based on annotation as guanine deaminase, suggesting that guanine deaminase has been conserved during evolution from prokaryotes to higher eukaryotes. Phylogenetic analysis of these sequences revealed that there are several related clusters of GDA sequences. Although GDA sequences seem to be a fine model to evolutionally cluster prokaryotes, eukaryotes, and archaea organisms, specific GDA sequences from prokaryotes and archaea clustered into relatively independent clades as represented in the NJ tree in Fig 1-2.

To further analyze the specific origins of guanine deaminases, we performed an evolutionary assessment of the clusters founded in the initial phylogenetic analysis. As shown in Fig. 1-3, we found that 18 prokaryotic sequences cluster and form a specific clade in the UPGMA tree. Interestingly, analysis of these sequences revealed that all of them form part of the cytidine deaminase-like protein family. The members of this protein family contain 149-188 residues and are metallo-dependent nucleoside deaminases that contain a zinc ion in the active site. One of the sequences from this clade, the *Bacillus subtilis* guanine deaminase, has been crystallized and its structure has been solved (Liaw et al., 2004). Structural analysis of this protein revealed that this homodimeric protein contains a swapped C-terminal motif, which forms an intersubunit active site and dimerization domain. A zinc ion is found in the active
site and is coordinated by a water molecule, a histidine, and two cysteine residues. In contrast, the remaining and vast majority of the GDA sequences analyzed in our study are part of the amidohydrolase family. The members of this protein family contain a TIM barrel tertiary structural fold, which contains the active site and a divalent cation. Furthermore, this family is distinguished by having a nine-residue motif that is identified as a cation-binding motif. Distinct from the cytidine deaminase-like family, the amidohydrolase family contains a different cation coordination system formed only by histidines and an aspartic acid residue (Fernández et al., 2008; PDB ID=2I9U).

**Analysis of GDA protein domains**

Since the amidohydrolase family of proteins comprises the vast majority of the guanine deaminase sequences analyzed, we performed position-specific evolution analysis of the conserved domains, specifically zinc- and PDZ-binding. Within the GDA sequences, we found that all (100%) of the archaea and eukaryotic sequences analyzed contain the consensus domain pattern of PGX[VI]DXH[TVI]H. In contrast, only 7% of the prokaryotic sequences contain this domain pattern, suggesting that the acquisition of this domain in eukaryotes may arrive from early prokaryotes. As shown in Fig. 1-4A, the third and sixth variable positions in the nine-residue zinc-binding motif are not randomly occupied. Hydrophobic residues, typically phenylalanine and leucine, usually occupy the third position. In the case of the sixth position, polar amino acids,
including threonine and cysteine, occupy this location, suggesting that these positions are not randomly occupied.

We also studied the molecular identity and evolution of the PDZ-binding domains in guanine deaminase sequences. PDZ-binding domains are of three different types based on the last four residues of the sequences (Sheng and Sala, 2001). The PDZ domain is a structural protein domain found in signaling proteins, ranging from bacteria, yeast, and animals (Ponting et al., 1997). PDZ domains help to physically cluster membrane-anchoring proteins, cytoskeletal elements, and signaling proteins (Sheng and Sala, 2001). As such, Table I provides a list of the PDZ-binding domain present in all of the guanine deaminase sequences analyzed in this study and classified as class I, II, or III, based on the last four amino acids of the protein sequences (Sheng and Sala, 2001). Our results show that class II PDZ-binding domains are more prominent in guanine deaminases than class I, but class III PDZ-binding domains are not present in any of the analyzed sequences. Interestingly, we found that a wide range of vertebrates, ranging from birds to mammals, contain class I domains. This phenomenon can be correlated with the expression of PDZ-containing proteins in the same organisms.

In order to evaluate the hypothesis that the consensus sequences of proteins containing PDZ and PDZ-binding domains determine an evolutionary node in multifunctional proteins, including signaling enzymes, we further analyzed the conservation of zinc- and PDZ-binding domains between eukaryotes and prokaryotes. Figures 1-5 and 1-6 show a position occupation
analysis in these domains within eukaryotes and prokaryotes, respectively. In eukaryotes, as shown in Fig. 1-5A, the third and sixth variable positions in the nine-residue zinc-binding motif are not randomly occupied. The third position is usually occupied by hydrophobic residues and the sixth position by polar amino acids. Eukaryotic proteins with class I PDZ-binding domains contain a consensus sequence of –SSSV as their last residues (Fig. 1-5B). Conversely, proteins containing class II PDZ-binding domains show more diversity in the first and last position of the domain (Fig. 1-5C). In prokaryotes, the third and sixth variable positions in the nine-residue zinc-binding motif are also not randomly occupied, as shown in Fig. 1-6A. The third position is usually occupied by hydrophobic residues, and the sixth position is occupied by polar amino acids. Prokaryotic proteins containing class II PDZ-binding domains show more diversity in the first and third position of the domain (Fig. 1-6B).
Discussion

Intracellular levels of purine derivates are regulated also by GDA with a reversible guanine conversion in the purine salvage pathway (Nygaard et al., 2000; Saint-Marc and Daignan-Fornier, 2004). The function of guanine deamination is well conserved from prokaryotes (Liaw et al., 2004; Maynes et al., 2000) to higher eukaryotes (Kumar et al., 1965; Prodanov and Jerev, 1971; Fogle and Bieber, 1975; Rossi et al., 1978; Galilea et al., 1981; Yuan et al., 1999; Paletzki, 2002). In prokaryotes and lower eukaryotes, guanine deaminase plays an exclusive role in the purine salvage pathway where it regulates intracellular guanylic derivative pools for nucleotide anabolism. In these organisms, the balance of synthesis and degradation activities rigorously regulates the homeostasis of purine nucleotides. On the otherhand, in higher eukaryotes, the expression of GDA is tissue specific (Paletzki, 2002; Firestein et al., 1999), and can also play a role in the development of neuronal morphology by regulating dendritic arborization (Akum et al., 2004; Chen et al., 2005; Chen et al., 2007). Interestingly, promotion of dendrite formation by GDA in neurons is dependent on the breakdown of guanine during development (Akum et al., 2004; Fernández et al., 2008). Therefore, the molecular evolution of guanine deaminases must reveal domain acquisition and specific evolutionary positions of accumulative mutations and transferences between organisms that express other GDA protein forms.

Guanine deaminases are members of the aminohydrolase subfamily of proteins, which are dependent of divalent cations for the catalytic deamination mechanism (Liaw et al., 2004; Yao et al., 2007). Zinc is the predominant ion
founded in most of the structures currently deposited in the Protein Data Bank (Berman et al., 2000; PDB ID = 1WKQ, 2OOD, 2PAJ, 2UZ9), although ferrous ions can also be present in the ion-protein coordination (PDB ID=2I9U). The aminohydrolase family members share a nine-residue motif containing a PGX[VI]DXH[TVI]H ProSite database pattern (http://ca.expasy.org/prosite), where X can be any of the twenty standard aminoacids. In high eukaryotic sequences, the last four aminoacids of the carboxy termini form a PDZ-binding domain, an important protein-protein interaction motif suggested to be involved in neuronal development function (Firestein et al., 2003; Akum et al., 2004). Although the conservation and integrity of these motifs have not been studied across species, the evolution of the positions on these domains may reveal the specific molecular lineage and acquisition of the multiple functions in guanine deaminases.

In conclusion, we performed a phylogenetic and molecular evolution analysis of guanine deaminases from low prokaryotes to high eukaryotes. Following our results, we proposed a evolution model for the molecular evolution of guanine deaminases from early small cytidine deaminase-like peptides to a TIM barrel protein containing a consensus zinc-binding domain, a PDZ-binding domain and thus the ability to function in both purine metabolism and dendrite branching in neurons. In our model, three events are proposed to have led to the mammalian GDA. An ancestral cytidine deaminase-like protein evolved possibly by gene duplication to a larger protein sequence with a common TIM barrel 3-dimensional fold. In a second step, acquisition of a PDZ-binding domain at C-
terminus in the last four residues is introduced in eukaryotes from archaea and high GC% content prokaryotes. In a last step, a specific nine-residue divalent cation-binding domain is acquired in mammals from lower eukaryotes.

In general, our studies provide a molecular evolution analysis of the positions on the zinc- and PDZ-binding domains and reveal the specific molecular lineage and acquisition of the multiple functions in guanine deaminases. In this work, we performed a phylogenetic analysis and evolutionary study of guanine deaminases from prokaryotic to higher eukaryotic sequences. This work will help to understand how a simple enzyme in lower prokaryotes get to evolved into a multifunctional protein and associate purine metabolism and dendrite development in complex eukaryotes.
Figure 1-1. Biochemical and cellular functions of guanine deaminase (Cypin). (A) A schematic representation of the deamination reaction mediated by guanine deaminase. In both prokaryotic and eukaryotic proteins, GDA mediates the conversion from guanine (left) to xanthine (right) and ammonia by a hydrolysis mechanism. (B-C) In eukaryotes, GDA regulates dendrite growth and branching in neurons. As shown previously (Akum et al., 2004), in contrast to control neurons expressing GFP (B), rat hippocampal neurons overexpressing Cypin-GFP (C) have a greater number of primary and secondary dendrites.
Figure 1-2. Phylogenetic analysis of guanine deaminases. The evolutionary history tree was inferred using the Neighbor-Joining method (Saitou and Nei, 1987), and the bootstrap consensus tree shown here was inferred from 500 replicates. This tree represents the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The eukaryotic and prokaryotic protein sequences are shown as red and blue clades, respectively, and the archaeal sequences are represented as green clades. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in units of number of amino acid substitutions per site.
Figure 1-3. Evolutionary tree of guanine deaminases. The evolutionary tree was inferred using the UPGMA method (Sneath and Sokal, 1973). A bootstrap consensus tree was built by inferring 500 replicates (Felsenstein, 1985). The tree is drawn to scale, with branch lengths shown above and branches in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Members of the metallo-dependent hydrolases and cytidine deaminase-like protein families are indicated and shown with straight and line branches, respectively. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).
Figure 1-4. Comparison of conserved motifs in guanine deaminases.

Results of analysis of **(A)** the nine-residue zinc-binding and **(B)** C-terminal PDZ-binding domain present in all retrieved sequences are represented by a graphical representation with the overall height of the stack indicating the sequence conservation at that position. The height of the letter symbols (one-letter symbol amino acid) within the stack indicates the relative frequency of each amino acid at that specific position. Green (G, T, C, S, Y); Black (A, V, L, I, P, W, F, M); Red (D, E); Magenta (N, Q); Blue (H, R, K).
Figure 1-5. Amino acid position conservation from motifs in eukaryotic guanine deaminases. Results of (A) the nine-residue zinc-binding, (B) C-terminal PDZ-binding (class I-III), and (C) C-terminal PDZ-binding (class II) domain present in 66 eukaryotic sequences. Green (G, T, C, S, Y); Black (A, V, L, I, P, W, F, M); Red (D, E); Magenta (N, Q); Blue (H, R, K).
Figure 1-6. Amino acid position conservation from motifs in prokaryotic guanine deaminases. Results of (A) the nine-residue zinc-binding and (B) C-terminal PDZ-binding (class II) domain present in 153 eukaryotic sequences. Green (G, T, C, S, Y); Black (A, V, L, I, P, W, F, M); Red (D, E); Magenta (N, Q); Blue (H, R, K).
Figure 1-7. General model for the molecular evolution of guanine deaminases.
CHAPTER TWO:

STRUCTURAL CHARACTERIZATION OF THE CYTOSOLIC PSD-95 INTERACTOR (CYPIN)
Summary

Dendrite morphology regulates how a postsynaptic neuron receives information from presynaptic neurons. The specific patterning of dendrite branches is promoted by extrinsic and intrinsic factors that trigger the activation of functional signaling pathways. However, most of the regulating factors and the biochemical mechanisms involved regulating dendrite branching are unknown. Our laboratory previously reported that cypin (cytosolic PSD-95 interactor) plays an active role in regulating dendrite branching in hippocampal neurons. Cypin-promoted increases in dendrite number are dependent on guanine deaminase activity.

In this Chapter, we employed computational homology modeling techniques to construct a three dimensional structural model of cypin in order to identify the specific structural role of zinc binding in cypin-mediated dendrite branching and guanine deaminase activity. Analysis of the protein-ion sequestration scaffold of this model identified several histidines and an aspartic acid residue responsible for zinc binding. Single substitution mutations in these specific sites completely disrupted the guanine deaminase enzymatic activity and rendered cypin unable to promote dendrite branching in rat hippocampal neurons. The specific zinc ion-binding function of each residue in the protein scaffold was also confirmed by Inductively Coupled Plasma–Optic Emission Spectrometry. Inspection of our structural model confirmed that His82 and His84 coordinate with the zinc ion together with His240, His279, and Asp330 residues.
that until now were unknown to play a role in this regard. Furthermore, promotion of dendrite branching by cypin is zinc-dependent.
Results

Structure of cypin by homology modeling

The lack of an available three-dimensional structure of cypin, a guanine deaminase that has been shown to regulate dendrite patterning in neuronal development (Akum, et al., 2004), motivated us to develop an in silico model of the protein structure using homology modeling. The determination of a structural model facilitates the understanding of mechanisms that may contribute to the enzymatic activity of this protein. We identified the metal-dependent guanine deaminase from C. acetobutylicum with bound guanine in the active site [PDB ID = 2I9U] as the most suitable structural template for cypin, both being members of the metal-dependent hydrolases with a 56% of primary sequence similarity. The multiple sequence alignments of guanine deaminase between human, rat, and C. acetobutylicum as templates are shown in Figure 2-1 and were conducted using the ClustalW1.8 routine with a Protein Gap Open penalty of 10.0, Gap Extension penalty of 0.2, END GAP of -1, GAP DIST of 4 with the Gonnet Matrix (Castagnetto et al., 2002). This template contains conserved functional secondary structural motifs as verified by secondary structure predictions. Secondary structure prediction of the rat cypin (guanine deaminase) using three methods was performed, and the consensus α-helix and β-strand structures in the primary sequence of the protein are shown in Figure 2-2A. A WHATIF check of the 1P1M template structure was performed prior to the model assembly, adjusting for slight structural modifications. To identify structurally and functionally conserved regions across the family of the metal-dependent
hydrolases, multiple sequence alignment was performed between these sequences and the sequence of rat cypin. The multiple alignments were followed by a pairwise sequence alignment between cypin and the template 2I9U, and as expected, functionally conserved motifs were found, allowing us to determine a three-dimensional homology model of cypin as shown in Figure 2-2B.

**Identification of novel amino acid residues involved in zinc ion binding to cypin**

In order to determine the specific zinc-binding residues of the rat cypin protein structure, the Fe\(^{3+}\) ion was manually extracted from the guanine deaminase template structure and replaced with a Zn\(^{2+}\) ion positioned into the preliminary cypin model. Further de novo loop generations, along with energy minimization, were assessed in order to confirm zinc metal ion stabilization. It was previously reported that histidines 82 and 84 (His82, His84) are involved in zinc binding to cypin (Yuan et al., 1999); however, our model also reveals for the first time that His240, His279, and Asp330 are likely to be coordinating residues by virtue of the orientation of their side chains and their close proximity (<5.0 Å) to the zinc ion. The specific residues His82 and His84, previously predicted to bind zinc by Snyder and colleagues (Yuan et al., 1999), and His279 and Asp330 were identified within a close intramolecular distance. As shown in Figure 2-3, a specific tetracoordinate system was observed to be consistent with metal ion stabilization with an additional amino acid, His240, in high proximity, indicating a possible folding stabilization of the zinc domain scaffold. The novel zinc ion-
binding motif in cypin differs when compared to the only available structure of a guanine deaminase protein from *Bacillus subtilis* (Liaw et al., 2004; Chang et al., 2004), which has two cysteines, one histidine, and a water molecule as its zinc binding coordination.

**Guanine deaminase activity is absent in zinc-binding residue mutants**

The binding of zinc to cypin is thought to play an essential role in mediating cypin’s guanine deaminase activity (Akum et al., 2004; Yuan et al., 1999). To confirm our cypin structural homology model, and specifically the zinc-binding scaffold, we mutated the specific residues found to be responsible for the coordination of the zinc ion in the protein structural model and other residues as controls. Using site-directed mutagenesis, we created single alanine substitutions for each residue (His82, His84, His240, His279, and Asp330) using constructs that encode amino terminally GFP-tagged mutants. His71 and I83 were also mutated to alanines to serve as negative controls for eliminating zinc binding and to assure that lack of zinc binding to other mutants is not solely due to a disruption in the overall three-dimensional structure of cypin. COS-7 cells overexpressing wild type or mutant cypin proteins were lysed, and the lysates were subjected to a colorimetric guanine deaminase activity assay. Single substitution mutations of the predicted residues that bind to the zinc ion caused a drastic attenuation of wild type guanine deaminase activity measured over time (Figure 2-4). As expected, mutation of His71 and I83 had no effect on cypin’s guanine deaminase activity, suggesting that these mutants are not disturbing the
required three-dimensional structure of the protein, and therefore, the wild type enzymatic activity remains unaffected (Figure 2-4). These results indicate that the lack of wild type deaminase activity in the zinc-binding mutants is specific to those amino acids predicted to bind zinc. These data indicate that His82, His84, His240, His279, and Asp330, which are predicted to be essential for zinc binding, are also necessary for cypin’s enzymatic activity (Akum et al., 2004; Yuan et al., 1999).

**Zinc-binding residue mutations eliminate cypin-promoted increases in dendrite branching**

Given that cypin has been shown to increase dendrite branching when overexpressed in primary cultures of hippocampal neurons and that the guanine deaminase activity is necessary for this increase in dendrites (Akum et al., 2004), we tested whether mutating amino acids that bind zinc affects cypin-promoted increases in primary and secondary dendrite number. We transfected embryonic rat hippocampal neurons at 10 days *in vitro* (d.i.v.) with the same cDNAs encoding cypin-GFP constructs that we used in the guanine deaminase assay (Figure 2-3) and assessed primary and secondary dendrite number. As expected, overexpression of wild type cypin resulted in a significant increase in both primary and secondary dendrites when measured at 12 d.i.v. (Figure 2-5A-C) when compared to GFP-expressing neurons. Cells overexpressing the single alanine substitution mutants of cypin that are predicted to lack zinc binding also lack the ability to increase both primary and secondary dendrites when compared
to control GFP-expressing neurons (Figure 2-5A-C). In contrast, when residues, which are predicted not to be involved in zinc binding (His71 or Ile83) are mutated, cypin’s ability to increase dendrite number above control (GFP) conditions is not eliminated (Figure 2-5A-C). It does appear, however, that there is a small attenuation in the number of cypin-promoted dendrites, possibly due to reasons not related to zinc binding (Figure 2-5B). These data suggest that the predicted structural zinc-binding amino acids are necessary for promoting dendrite branching.

**Inductively Coupled Plasma-Optical Emission Spectrometry demonstrates that His82, His84, His240, His279, and Asp330 are essential residues for zinc binding to cypin**

To quantitatively demonstrate whether the predicted amino acids in our homology structure model of cypin are essential for zinc binding, we measured the amount of zinc bound to purified GST-cypin fusion proteins expressed in transformed bacteria. Equal concentrations of GST-cypin or GST-cypin mutants (Figure 2-6A) were subjected to Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES), and the radiation emission intensity at 213 nm was measured and compared to the control GST protein. We found that the levels of zinc ions bound to the single alanine mutants were significantly less than the level of zinc bound to wild type cypin (Figure 2-6B), indicating that our predicted zinc binding residues indeed help to stabilize zinc binding in the secondary and
tertiary structures of cypin. Mutation of His71 or Ile83 had no effect on zinc binding, as expected (Figure 2-6B).

Discussion

In this study, we used homology modeling to predict the three-dimensional structure of the multifunctional protein cypin. Although the *B. subtilis* guanine deaminase protein structure is available (pdb ID: 1WKQ; 7), the mammalian cypin proteins do not share any sequence similarity even though they share the same cellular substrate and enzymatic function. The rat, human, and mouse guanine deaminase orthologues contain 454 amino acids with a nine-residue metal-binding motif and a four-residue PDZ binding motif at the C-terminus, which is responsible for interaction with PSD-95 at the postsynaptic density in neurons (Firestein et al., 1999). On the other hand, the *C. acetobutylicum* guanine deaminase structure contains 428 residues, which align with the mammalian orthologue sequences. This guanine deaminase protein also contains the putative nine-residue metal binding motif and the predicted residues that bind to zinc in the rat and human guanine deaminase sequence, and these amino acids are conserved (Figure 2-1).

Cypin has been shown to be involved in purine metabolism by catalyzing the break down of guanine to xanthine and ammonia (Yuan et al., 1999). In addition, cypin has been reported to act as a negative regulator of PSD-95 clustering in neurons (Firestein et al., 1999) and as an important intrinsic factor that regulates dendrite branching in hippocampal neurons (Akum et al., 2004;
Chen et al., 2005). How these three functions of cypin are related has been a focus of study for our laboratory.

The primary finding of our current work is that, in addition to two previously known amino acids, we have identified three novel amino acids that play an important role in zinc binding to cypin. Furthermore, we have now definitively shown that zinc binding to cypin regulates guanine deaminase activity, which has been hypothesized but never demonstrated. Our current mutational analysis suggests that single substitutions of histidines 82, 84, 240, and 279 and aspartate 330 to alanine, a small non polar side-chain that does not allow for coordination to zinc, lowers the affinity of cypin for Zn$^{2+}$. This result suggests that our predicted residues play an important role in the stabilization of the native fold of cypin and ensure optimal residue orientation of key residues (His82, 84, 240, 279 and Asp330) within the binding site. Furthermore, cypin variants, which contain mutations in the zinc-binding region, lack the ability to increase both primary and secondary dendrites in rat hippocampal neurons when compared to control neurons. These same residues also disrupt the guanine deaminase activity of the wild type protein, suggesting that the predicted zinc-binding scaffold is necessary for both activities and that a functionally active enzyme is crucial for dendrite formation. This is consistent with previous work using deletion mutations of a nine-residue motif (amino acids 76-84) in cypin, resulting in attenuation of guanine deaminase activity and cypin-promoted increases in dendrite number (Akum et al., 2004). However, since disruption in cypin protein folding due to this large deletion could possibly disrupt the overall three-
dimensional structure of cypin, our mutation of single amino acid residues offers stronger evidence supporting a role for zinc binding, and hence guanine deaminase activity and dendrite formation.

What are the sources of zinc that can influence cypin’s guanine deaminase activity? In our cell-free zinc-binding assay, the source of zinc that was incorporated into the proteins during bacterial culture growth was obtained from yeast extract used in the LB media (0.2 mM; Outten and O'Halloran, 2001). This is a much higher concentration of that found in the media used to grow COS-7 cells for guanine deaminase assays (5 µM from fetal bovine serum; MacDonald et al., 1998; Paski SC, Xu, 2001). The concentration range of cellular zinc that is needed for cypin’s effect on dendrite branching is currently unknown. Interestingly, it was previously reported that zinc is released from the perinuclear area, including the endoplasmic reticulum, in mast cells in response to stimulation and can act as a second messenger (Yamasaki et al., 2007); however, it is unknown whether this occurs in neurons and whether this pool of zinc can activate cypin and promote dendrite branching.

Since the global three-dimensional structure of a protein determines the accessible motifs and residues necessary for its enzymatic activity, the lack of available solved structures for cypin and/or related proteins has hampered our understanding of specific mechanisms of protein action leading to cellular processes, such as purine metabolism and neuronal development. In the absence of a high-resolution crystal structure, rational predictions of protein structure using bioinformatics tools can provide valuable insight into protein
structure-function relationships when combined with experimental validation. Using the guanine deaminase from *C. acetobutylicum* as template to predict cypin structure by homology, we determined that the mammalian guanine deaminase contains a TIM barrel tertiary structure and a novel zinc ion-binding domain. Analysis of the *Bacillus subtilis* guanine deaminase crystal structure (Liaw et al., 2004; Chang et al., 2004) suggests that the residue-ion binding components diverge, where this bacterial enzyme contains a cysteine-histidine coordination to bind zinc, and the mammalian guanine deaminase/cypin utilizes four histidines (at positions 82, 84, 240, 279) and an aspartate residue (at position 330) to bind zinc. A high percentage of crystal structures of zinc-containing proteins contain at least three histidines involved in zinc binding, suggesting that our predicted interaction is an ion-protein interface by analysis using the Metalloprotein Database (Castagnetto et al., 2002).

The validation of the specific binding of zinc to cypin was established using ICP-OES, and our results confirmed that the protein structural model positively contains the zinc-binding scaffold interface of the mammalian guanine deaminase protein. Our data suggest that the zinc-binding stability of cypin is crucial for the guanine deaminase activity, possibly participating in the deamination reaction mechanism. This ion-protein biointerface may alter the overall three-dimensional conformation of cypin, thus possibly regulating its interaction with additional intrinsic molecules that may facilitate or promote dendrite branching. For example, cypin binds tubulin heterodimers and promotes microtubule polymerization, leading to increased dendrite number in growing
hippocampal neurons (Akum et al., 2004). As of yet, there are few available
templates for modeling the interaction between cypin and tubulin. Interestingly,
the collapsin response mediator protein (CRMP) homology domain of cypin
mediates this interaction, and a crystal structure for CRMP has just been
published (Deo et al., 2004). Furthermore, CRMP also binds zinc, and it too uses
a three-histidine motif (Deo et al., 2004). Thus, our ongoing studies are focused
on identifying critical residues for cypin’s interaction with tubulin and, together
with our current study, will aid in our understanding of cypin’s structure and
function in neurons.
Figure 2-1. Sequence alignment of guanine deaminases. Conserved residues are shaded and boxed, while class specific residues are boxed within the sequence alignment. The gaps are indicated with (−). The nine-residue motif thought to be responsible for the zinc ion binding is highlighted with a bar.
Figure 2-2. Structural model of cypin by homology modeling. (A) Secondary structure prediction. The α-helices are represented as blue boxes and β-sheets as red arrows. (B) Homology model of rat cypin. The Zn$^{2+}$ ion is colored magenta and the secondary structures colored as in (A). Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).
Figure 2-3. Zn$^{2+}$ scaffold coordination in cypin, the mammalian guanine deaminase. The protein residues are displayed as sticks and colored by atom-type (oxygen = red; nitrogen = blue). The Zn$^{2+}$ ion is colored magenta. The ion coordination system is indicated by solid green lines.
Figure 2-4. Guanine deaminase activity is absent in cypin zinc binding mutants. Lysates (50 µg) from COS-7 cells expressing GFP, GFP-cypin, GFP-cypin H71A, GFP-cypin H82A, GFP-cypin I83A, GFP-cypin H84A, GFP-cypin H240A, GFP-cypin H279A and GFP-cypin D330A were examined using the Amplex Red dye in a colorimetric assay (n = 3). Cypin H71A and I83A were not predicted to be involved in zinc binding and were used as controls. p< 0.01 for wild type, H71A, and I83A cypin as determined by ANOVA followed by Dunnett's multiple comparisons test compared with GFP as control. SEM values are showed for each datapoint; note that some values are smaller than the data symbols.
Figure 2-5. Zinc binding mutations eliminate cypin-promoted increases in dendrite number. (A) Immunostaining for GFP shows dendrite morphology changes in primary hippocampal neurons overexpressing GFP-cypin single substitution mutations. (B) Average number of primary dendrites in hippocampal neurons overexpressing GFP, GFP-cypin, GFP-cypin H71A, GFP-cypin H82A, GFP-cypin I83A, GFP-cypin H84A, GFP-cypin H240A, GFP-cypin H279A or GFP-cypin D330A (n = 35). ***p< 0.001 and **p< 0.01 by ANOVA followed by Dunnett's multiple comparison test compared with GFP as control. (C) Average number of secondary dendrites as in (A).
Figure 2-6. ICP-OES demonstrates that His82, His84, His240, His279, and Asp330 are crucial residues for zinc binding to cypin. (A) Coomassie Blue-stained 10% SDS-PAGE gel of 2 µg of each purified GST fusion protein. Traces of GST are present in GST-cypin and GST-cypin mutant preparations, due to a small amount of proteolysis often seen with our purification protocol. (B) Bar graph summarizing the average (n = 3) radiation intensity ratio for GST-cypin, GST-cypin H82A, GST-cypin H84A, GST-cypin H240A, GST-cypin H279A and GST-cypin D330A detected at 213 nm. ***p< 0.001 by ANOVA followed by Student-Newman-Keuls multiple comparison test compared with GST as control.
CHAPTER THREE:

CHARACTERIZATION OF NOVEL SUBSTRATES OF GUANINE

DEAMINASE/CYPIN
Summary

Guanine deaminase (GDA) is a metalloenzyme, which processes the first step in purine catabolism, converting guanine to xanthine by hydrolytic deamination. In higher eukaryotes, GDA plays an important role in the development of neuronal morphology by regulating dendritic arborization. In addition to its role in the maturing brain, GDA is thought to play a role in proper liver function since abnormal levels of GDA activity have been correlated with liver disease and transplant rejection. Although mammalian GDA is an attractive and potential drug target for treatment of liver diseases and cognitive disorders, prospective novel inhibitors and/or activators of this enzyme have not been actively pursued.

In this chapter, we use the combination of protein structure analysis and experimental kinetic studies to test novel potential ligands for human GDA. We obtained a list compounds from our molecular modeling studies, and these compounds demonstrate a higher binding affinity to GDA than does guanine. In fact, one of the compounds identified as a potential inhibitor is caffeine, which is easily synthesized and is an attractive candidate for drug discovery due to the fact that it is safely consumed. The results from this study provide evidence that an *in silico* drug discovery strategy coupled with *in vitro* verification can be successfully implemented to discover compounds that may have therapeutic value for the treatment of diseases and disorders where GDA activity is abnormal.
Results

Ion coordination analysis

The crystal structure of the *Bacillus subtilis* and human guanine deaminase proteins have been recently solved with a bound Zn$^{2+}$ ion at 1.17-Å and 2.30-Å resolution, respectively (Liaw et al., 2004; Chang et al., 2004; Moche et al., 2008). Although zinc binding is conserved for GDA from bacteria to higher eukaryotes, the specific ion coordination diverges between these homologs. As shown in Figure 3-1A-B, the Zn$^{2+}$ ion coordination in human GDA (hGDA) is comprised of an imidazole environment with three histidines and an aspartate residue (Fernández et al., 2008). In contrast, the Zn$^{2+}$ ion coordination in the bacterial protein is maintained by two cysteines, one histidine, and a water molecule for conservation of tetrahedral coordination. Since the substrate binding site of the enzyme is directly adjacent to the Zn$^{2+}$ ion, and the ion itself has been proposed to play a role during the mechanism of deamination (Liaw et al., 2004; Chang et al., 2004), this coordination difference influences the stability of substrate binding and product release during the enzymatic reaction.

Substrate binding characterization

The hGDA crystal structure was solved with a xanthine molecule bound to the specific substrate-binding site (Moche et al., 2008). In order to develop new substrates that show high affinity and specificity for hGDA, we performed a detailed characterization of the substrate-binding site. Analysis of the intramolecular interactions between the substrate or product molecules and the
residues in hGDA within this site revealed that hydrophobic and van der Waals forces control the heterocyclic aromatic ring interactions in the guanine molecule (Figure 3-2A). The carboxyl groups in the purine ring can form hydrogen bonds with two nitrogen atoms in residues Arg213 and Gln87. Moreover, the amine group in the guanine molecule forms two novel hydrogen bonds with the imidazole ring in His279 and the carboxylic acid side chain in Glu243. These essential interactions between the substrate (guanine) and residues in hGDA must be considered during substrate modification so as to increase enzyme-compound specificity.

**Energy Minimization and Molecular Dynamics simulations**

To refine the crystal structure coordinates and analyze the structure stability in a realistic water solvated system, we performed Energy Minimization (EM) to diminish possible interatomic clashes during the molecular ligand docking experiment. We used the AMBER9 package and force field (Case et al., 2005) to assess if the experimentally retrieved structure parameters preserve the 3D structure of hGDA. As shown in Figure 3-3A, we observed an EM from a high-energy unstable structure to a low-energy stable structure. Molecular dynamics (MD) simulations on the hGDA structure raised confidence in structure stabilization, as evidenced by the rapid convergence of the root mean squared deviation (RMSD) of the \( \alpha \)-carbon backbone in the structure inside of the water solvated system (Figure 3-3B).
The eukaryotic GDA has been implicated in diverse cellular signaling pathways, including its ability to bind and regulate neuronal protein trafficking to the postsynaptic density (PSD; Firestein et al., 1999; Akum et al., 2004). GDA binds to PSD-95, a PDZ-containing protein that associates with receptors and cytoskeletal elements at synapses. GDA binds to the first and second PDZ domains of PSD-95 via its PDZ-binding tail, consisting of the last four amino acids at the carboxyl terminus (-SSSV; Firestein et al., 1999). In order to investigate the dynamics of these four carboxyl terminal residues, we determined the Debye-Waller factor (DWF) or B-factor of the hGDA crystal structure in each residue position in the protein primary sequence after the MD simulation. As shown in Figure 3-3C, only the first and last amino acids demonstrated a fluctuation in atom-angle divergence about their average positions, suggesting that these residues are very dynamic compared to the other residue positions in the protein.

**Molecular Docking of guanine analogues**

An *in silico* molecular docking approach was conducted to assess the suitability of the hGDA structure for structure-based drug design. To investigate potential novel ligands for hGDA, we used the docking and scoring algorithm implemented in GOLD (Jones et al., 1997). In our experiments, we used a list of 188 guanine analogues, including previously studied GDA inhibitors (Rajappan and Hosmane, 1998; Ujjinamatada et al., 2006; Liaw et al., 2004) and several novel compounds. The chemical properties and docking scores of all of the
compounds tested are described in Table 3-1. A total of 30 compounds (15.95%) show better fitness scores than the typical substrate (guanine). Among these compounds, we observed that five of them contain different alkane group numbers at the same position. When we compared the \((\text{CH}_3)_n\) group number against their GOLD Score in hGDA, there was a strong linear correlation with the homologous series of alkane side chains \((r^2 = 0.78)\), suggesting that extension of the alkane group can increase the binding affinity of the ligand, possibly by increasing hydrophobic interactions with hGDA (Figure 3-4).

**Kinetic Analysis of guanine analogues**

To determine whether the novel small molecules that we identified can modify the enzymatic activity of mammalian GDA, we performed kinetic experiments using GDA isolated from rabbit, due to the fact that it is commercially available, and determined the experimental binding affinity constants for the compounds. Lineweaver–Burk Plots were used to calculate inhibition constants \((K_i)\). Results for the tested compounds are shown in Table 3-2. Our biochemical results demonstrate that guanine has a \(K_m\) of \(11.21 \pm 0.11 \times 10^{-6}\) M, consistent with previously reported values (Lewis and Glantz, 1974; Yuan et al., 1999; Ujjinamatada et al., 2006). Interestingly, most of the tested compounds significantly and competitively inhibited guanine binding to GDA, with higher \(K_i\) values than guanine, with the exception of caffeine, which has a lower \(K_i\) value than guanine. These results suggest that the compounds tested may be
potential substrates or inhibitors of the human guanine deaminase and may potentially act \textit{in vivo} with a high affinity to alter GDA activity.

\textbf{Guanine deaminase activity is disrupted by substrate analogues}

Competitive binding of substrates to cypin’s active site during deamination may affect the enzymatic activity. Thus, we tested the identified small molecules in a guanine deaminase assay using GFP-tagged rat cypin and purified rabbit guanine deaminase. Lysates from COS-7 cells overexpressing GFP or GFP-tagged wildtype cypin were subjected to a colorimetric guanine deaminase activity assay as previously described (Akum et al., 2004; Fernández et al., 2008; Chapter 2). Purified rabbit guanine deaminase was also used to test guanine analogues. Compounds that resemble the chemical structure of guanine and contain an amine group caused a drastic attenuation of wild type guanine deaminase activity measured over time (Figure 3-6). Among the tested compounds, we found that 6-thioguanine and 2,6-diaminopurine significantly attenuated cypin’s guanine deaminase activity, suggesting that chemical substitutions in the 2- and 6-position in the guanine molecule can inhibit the wildtype enzymatic activity. In contrast, 2-dimethylamino-6-hydroxypurine promotes higher enzymatic activity when added with guanine. This may be due to the deamination of 2-dimethylamino-6-hydroxypurine, resulting in release of dimethylamine molecule. These results indicate that only substrates that contain an amino group at the 2- site can compete with guanine for binding to the active
site or that compounds with substitutions in the 2-position of the purine ring may be a substrate for deamination.

**Cypin-mediated microtubule assembly is regulated by substrate analogues**

Conformational changes during substrate binding to cypin may also affect cypin-promoted microtubule assembly. Thus, we tested the effect of our guanine analogues on cypin-mediated microtubule assembly. We used a cell-free system, which permits the assessment of direct regulation by these analogues on cypin (Akum et al., 2004). Purified tubulin was polymerized, and the reaction path was monitored by absorbance over time (Figure 3-7). Among the tested compounds, we found that guanine, 6-thioguanine, and 2,6-diaminopurine significantly regulate cypin-promoted microtubule assembly. 6-thioguanine completely diminished the lag phase compared to control, suggesting that the presence of this molecule can cause microtubule catastrophe. In contrast, 2,6-diaminopurine increases cypin-promoted microtubule assembly as it increases the maximum velocity of reaction, suggesting that this molecule might cause a favorable conformational change to mediate microtubule polymerization. These results indicate that cypin substrates can regulate cypin-mediated microtubule polymerization, possibly by a structural modification of cypin’s tubulin-binding domain.
Discussion

Guanine deaminase plays an essential role in diverse cellular signaling pathways, including purine salvage and dendritic arborization in neurons (Akum et al., 2004; Chen et al., 2005; Chen and Firestein, 2007). Mammalian GDA is a potential drug target for treatment of purine metabolism deficiency and cognitive disorders by virtue of the fact that its enzymatic activity is necessary for normal purine salvage and brain development and function. Previous kinetic studies using purified rabbit GDA were performed using guanine analogues selected simply for their structural similarity to guanine (Baker and Santi, 1967; Baker and Wood, 1967; Baker, 1967). Although potential substrates can be revealed using this deductive method, more efficient and effective strategies are available to accelerate and streamline the process of finding potential inhibitors. The current availability of a high-resolution human GDA crystal structure affords the use of methods in rational, computer-aided, structure-based drug design for identifying effective inhibitors and/or activators of the mammalian GDA. In this study, we employed rational drug design approaches using the crystal structure to study the ion and substrate coordination of human GDA. A major finding of our current work is the identification of specific residues that play a major role in the deamination mechanism by cypin. This identification allowed us to perform rational implemented drug design using the described pharmacophore, rather than using structure similarity selection, to obtain novel substrate candidates for the enzyme.
Guanine deaminase orthologues are found in prokaryotes and lower eukaryotes, suggesting that purine metabolism is a critical cellular process. Although these orthologues share the same enzymatic activity, during evolution, the mammalian GDA proteins acquired an additional function in regulating neuronal development. A four-residue PDZ binding motif at the C-terminus is responsible for the interaction of and regulation of protein levels by GDA at the postsynaptic density (Firestein et al., 1999; Akum et al., 2004). These multi-functional activities of the mammalian GDA are conferred by the specific three-dimensional amino acid arrangements of the internal ion and substrate binding sites.

Although a direct conclusive link between purine metabolism and cognitive function remains elusive, several studies revealed that neurobehavioral and cognitive impairment syndromes, including Lesch-Nyhan disease, are linked to changes in purine homeostasis resulting from purine salvage failure (Schretlen et al., 2001; Jinnah and Friedmann, 2000). Abnormally high levels of guanine and derivatives have been shown to accumulate in media from Lesch-Nyhan disease cell line models (Shirley et al., 2007). A specific reduction in guanine to adenine nucleotide levels occurs in these cell lines (Shirley et al., 2007). In addition, other studies revealed that elevated serum levels of uric acid, another guanine metabolite, are directly related to cerebral ischemia in adults (Schretlen et al., 2007). These results suggest that enzymes involved in purine metabolism, like guanine deaminase, may play an essential role in regulating proper neuronal activity and communication.
Elevated GDA activity has been correlated with several liver diseases, including hepatitis and liver transplantation rejection (Shiota et al., 1989; Nishikawa et al., 1989; Crary et al., 1989). As such, liver and serum GDA activities are currently used as molecular markers for liver disease diagnosis. Anomalous purine metabolism is reflected in clinical problems associated with nucleotide salvage during cellular and DNA repair, ranging from mild to severe and fatal disorders. Regulation of purine metabolism controls the cellular nucleotide pool, and the nucleotide levels available directly affect DNA synthesis during cellular proliferation and differentiation. However, the major clinical manifestations of atypical purine catabolism arise from uric acid insolubility and its degradation, leading to inflammatory diseases like gout (reviewed in Kutzing and Firestein, 2008). Since anti-metabolic compounds have been used to treat purine metabolism-related diseases, our findings suggest a new array of molecules, which may inhibit overactivity of guanine catabolism seen in disease states.

Our results suggest that the combination of computer-aided protein structure analysis and experimental kinetic studies can be effectively implemented, facilitating rational drug design. We have used this method to discover compounds, which can be potential substrates or inhibitors of the human guanine deaminase for potential use in purine metabolism disorders, liver disease, and/or cognitive dysfunction. Thus, our future studies will focus on identifying the specific activity of these newly identified compounds in models of
neuronal and liver disease in order to screen candidates for therapies for these diseases.
Table 3-1. Chemical properties and docking scores of all of the compounds used in the molecular docking simulations.

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<th>IUPAC Name</th>
<th>Molecular Formula</th>
<th>PubChem Compound ID</th>
<th>Formula Weight (g/mol)</th>
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<th>Chem Score</th>
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<td>C5H14N10ORu</td>
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<td>1,3,7-trimethylpurine-2,6-dione</td>
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<td>11.98</td>
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<tr>
<td>4,5,6,7-tetrahydro-6-hydroxy-3H-imidazo-(4,5e)(1,4)diazepin-8-one</td>
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<td>Azepinomycin analogue 1</td>
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<td>BakerXIII</td>
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<td>N/A</td>
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<td>261.042</td>
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<tr>
<td>BakerXV</td>
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<tr>
<td>5-aminoimidazole-4-carboxamide</td>
<td>C9H10O3</td>
<td>8434</td>
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<tr>
<td>(ethyl 4-hydroxybenzoate)9-(p-carbetoxyphenyl)</td>
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<td>9679</td>
<td>50.80</td>
<td>15.25</td>
<td></td>
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<tr>
<td>(ethoxycarbonyl ethyl carbonate)Diethylpyrocarbonate</td>
<td>C9H10O3</td>
<td>8434</td>
<td>53.98</td>
<td>21.16</td>
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Table 3-2. Properties of experimentally tested compounds as mammalian guanine deaminase substrates.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
<th>Docking Score</th>
<th>$K_m$ or $K_i$ ($\times 10^{-6}$ M)</th>
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<td><img src="image1.png" alt="Structure" /></td>
<td>Guanine</td>
<td>60.65</td>
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<tr>
<td><img src="image2.png" alt="Structure" /></td>
<td>Caffeine</td>
<td>47.13</td>
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<tr>
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<td>5-amino-4-imidazole carboxamide</td>
<td>50.80</td>
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<tr>
<td><img src="image4.png" alt="Structure" /></td>
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<td>3.44 ± 0.18</td>
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<td>2-dimethylamino-6-hydroxypurine</td>
<td>71.14</td>
<td>1.09 ± 0.11</td>
</tr>
</tbody>
</table>
2,6-diaminopurine 49.65  1.88 ± 0.13
Xanthine 48.04  1.96 ± 0.07
7-methylguanine 59.61  5.55 ± 0.14
Uric acid 49.27  4.34 ± 0.21
Figure 3-1. Zinc ion coordination in guanine deaminases. (A) Structure of the human guanine deaminase protein (PDB ID = 2UZ9). The α-helices are colored in light blue and the β-sheets in green. The side chains of residues that interact with the zinc ion are displayed as green sticks. (B) Zinc ion coordination in human GDA. Protein residues are displayed as sticks and colored by atom-type (oxygen = red; nitrogen = blue). The Zn$^{2+}$ ion is colored violet. The ion coordination system is indicated by dashed green lines. (C) Zinc ion coordination in *Bacillus subtilis* GDA. Protein residues and Zn$^{2+}$ ion are colored as in (B). The water molecule is displayed as a red sphere. Molecular graphic images were produced using the UCSF Chimera package from the Resource for Biocomputing (Pettersen et al., 2004), Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).
Figure 3-2. Protein-ligand interactions in the substrate-binding site of human GDA. (A) Guanine-hGDA interactions. (B) Xanthine-hGDA interactions. Two-dimensional representation of ligand–protein interactions were analyzed using Ligplot (Wallace et al., 1995). Hydrogen bonds formed between protein residues and ligands are indicated by dashed green lines and the corresponding distance (Å) and residues engaged in hydrophobic interactions are represented in red.
Figure 3-3. Energy Minimization and Molecular Dynamics studies of human GDA. (A) Total structure energy plot against the minimization cycle number. (B) The α-carbon backbone RMSD of hGDA determined from its starting conformation obtained from a 300ps simulation. (C) B factor plot against the primary sequence in hGDA.
Figure 3-4. Alkane group substitutions in the guanine catalytic amine group increase binding affinity to hGDA. Molecular Docking results using the GOLD score are plotted against number of alkane groups added to the R1 position in the guanine molecule (inset). A linear correlation is noted ($r^2 = 0.78$).
Figure 3-5. Kinetics of novel guanine deaminase substrates. Lineweaver-Burk plots were used to calculate kinetic constants and substrate type analysis using a competitive substrate assay with guanine. Seven guanine concentrations in the µM range were combined with 0, 8, or 20 µM of each inhibitor. (A) Caffeine (B) 5-amino-4-imidazole carboxamide (C) N-2-acetyl-guanine (D) 2-dimethylamino-6-hydroxy-purine (E) 2,6-diamino-purine (F) xanthine (G) 7-methylguanine (H) uric acid.
Figure 3-6. Guanine deaminase activity is altered by substrate analogues.  

(A) Schematic of the guanine metabolism pathway used in the colorimetric assay. Guanine is hydrolyzed by guanine deaminase (GDA; cypin), releasing xanthine and ammonia. Xanthine is oxidized by xanthine oxidase (XO), releasing uric acid and peroxide. When Amplex Red reagent is present, it gets oxidized by horseradish peroxidase (HRP) with peroxide to produce the colored resofurin (shown in magenta), which can be detected from optical absorbance. (B) Lysates (50 µg) from COS-7 cells expressing GFP and GFP-cypin incubated with guanine analogues (10 µM) in DMSO were examined using the Amplex Red dye in a colorimetric assay (n = 3). Lysate from cells expressing GFP does not show guanine deaminase activity and were used as a negative control. (C) Purified rabbit guanine deaminase (8x10^{-4} U) incubated with guanine analogues (10 µM) in DMSO was subjected to a guanine deaminase assay as in (A). Standard error (SEM) is shown for each data point. Note that some SEM values are smaller than the data symbols.
Figure 3-7. Cypin-mediated microtubule polymerization is affected by substrate analogues. A tubulin polymerization assay was performed using tubulin (>97% pure; 3 mg/mL) in a 96-well plate at 37°C. Absorbances at 340 nm were measured every 1 min for 70 min. GST or GST-Cypin (0.1 nmoles) were incubated with or without guanine analogues (0.1 mM).
CHAPTER FOUR:

TRANSCRIPTIONAL REGULATION OF CYPIN IN HIPPOCAMPAL NEURONS
Summary

Alterations in dendrite branching and morphology are present in many neurodegenerative diseases, including Rett Syndrome and Alzheimer’s Disease. These variations disrupt postsynaptic transmission by providing an alteration of cell-cell interface in neuronal communication. Therefore, specific regulators of neuronal morphology have been intensely studied during the past few decades. Recently, our laboratory showed that cypin, a mammalian guanine deaminase, increases dendrite number when overexpressed in cultured hippocampal neurons (Akum et al., 2004). Since the intracellular protein levels of cypin correlate with an increase in dendrite number, we investigated how cypin expression is regulated in hippocampal neurons.

In this chapter, we study the regulation of cypin mRNA and protein expression by neurotrophic factors. We used a rational approach using a combination of bioinformatic analysis and pharmacological experiments in rat hippocampal neurons to identify the mechanism by which neurotrophic factors act. Here, we report that the cypin promoter region contains putative conserved cyclic adenosine 3',5'-monophosphate (cAMP) response element (CRE) regions, which we suggest can be recognized and activated by cAMP response element-binding protein (CREB). A pharmacological assessment in cultured hippocampal neurons with trophic factors revealed that brain-derived neurotrophic factor (BDNF) increases cypin levels via a tyrosine-receptor kinase B- (TrkB) and mitogen-activated protein kinase kinase (MEK)-dependent pathway. Quantitative PCR and Western blot analysis demonstrated that BDNF is the only neurotrophin
family member that can increase cypin mRNA and protein levels in hippocampal neurons. In addition, treatment with cAMP increases primary and secondary dendrites in neurons and promotes expression of a potential gene/protein variant as demonstrated by Western blot analysis. Taken together, these studies suggest that neuronal cypin expression can be regulated by a specific trophic factor pathway and also raise the possibility of a cypin gene variant expressed in hippocampal neurons. The BDNF-induced cypin promoter activity appears to be regulated by CREB in hippocampal neurons, thus, providing a molecular framework for the etiology of cypin-promoted dendrite development.
Results

**BDNF increase cypin protein levels in hippocampal neurons**

In order to investigate the possibility that neurotrophic factors may modulate cypin expression to promote increases in dendrite number, we treated cultures of primary hippocampal neurons with the three major factors: neuronal growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). These trophic factors have been widely studied as being secreted from neurons and glia to exert their effects through the tyrosine receptor kinase family (Trk) of receptors in response to neuronal activity (Chao, 1992; Levine et al., 1995; Muller et al., 1995). This receptor family has been classified by the interaction of specificity with NGF binding to TrkA, BDNF and NT-4 binding to TrkB, and NT-3 preferentially binding to TrkC (Reichardt, 2006). The detailed characterization of these receptors has advanced the study of signaling pathways that control neurite development, especially dendrite growth and branching. In addition, it has been reported that neurotrophins increase the dendritic complexity of pyramidal neurons in the CNS by increasing total number and length of dendritic branches (McAllister, 2000). Therefore, we performed a pharmacological assay using NGF, BDNF, and NT-3 at concentrations that should significantly exert an effect on the Trk-mediated pathway (Reichardt, 2006). We investigated the role of these neurotrophins in changes in cypin gene expression by examining mRNA levels by quantitative PCR (qPCR) and protein expression Western blotting. Figure 4-1 shows that only exposure to BDNF significantly increases cypin mRNA and protein levels after 72 hours of
treatment. This increase is dose-dependent and is due to TrkB activation since at the concentrations tested, no apparent cross-stimulation of TrkA, TrkC, or p75 should occur.

**Cypin gene promoter contains putative cAMP responsive elements (CRE)**

To identify possible cis-acting regulators of the cypin gene promoter, we performed a bioinformatics-based analysis to predict regulatory binding regions. Since the minimal promoter region of the rodent or human promoter region of cypin has not yet been characterized, we analyzed a vast region upstream of the first exon start region (10Kb). We compiled a list of predicted transcription factors, including AP-1, Sp-1, E2F, NF-kappaB, and CREB, that may bind to regulatory region of the cypin gene (Figure 4-2). Both NF-kappaB and CREB obtained the higher binding probability than the other factors, and thus, raised the question of whether there is conservation of these regions between rodent and human genes. CRE regions were conserved between the mouse, rat, and human cypin genes, as shown in Figure 4-3, although the positions of these regions differed within the gene. In addition, distinct TATA box domains were also found in this region and in proximity to the CRE elements, suggesting that together, these domains may represent an important regulatory region of the cypin gene.
**BDNF treatment increases cypin expression via a transcription dependent-mechanism**

Since both experimental and genomic data analyses suggest that BDNF may increase cypin expression via activation of CREB binding to consensus CRE regions within the gene promoter, we further investigated the specific downstream pathway that is activated. We first tested the hypothesis that extracellular BDNF promotes cypin gene expression via a transcription-dependent mechanism by pharmacological treatment with actinomycin D, which inhibits transcription by binding DNA at the transcription initiation complex and preventing elongation of transcription by the RNA polymerase (Sobell, 1985). As shown in Figure 4-4, actinomycin D treatment inhibited both BDNF-induced cypin mRNA and protein expression. This significant decrease in expression suggests that, in fact, BDNF increases cypin gene expression via a transcription-dependent mechanism.

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

We further investigated the downstream pathway of BDNF-activated cypin gene expression. CREB activation leads to changes in phosphorylation states of downstream proteins by activating several cytosolic kinases. A number of different pathways have been described for CREB activation, including signals
that elevate intracellular calcium levels, that elevate cAMP, than enhance growth, and that stimulate anti-apoptotic pathways (Shaywitz and Greenberg, 1999). However, the increasing intracellular cAMP levels are the usual secondary messenger pathways that lead to CREB translocation via cAMP-dependent protein kinase A (PKA). Upon binding of cAMP, regulatory subunits of PKA dissociate and release active catalytic subunits, which are capable of phosphorylating target proteins, including CREB family members (Taylor et al., 1990). Therefore, we tested the possibility that BDNF-mediated increases in cypin expression are mediated by a cAMP/PKA pathway, leading to CREB activation. To test this possibility, we treated cultures of hippocampal neurons with a membrane permeable cAMP analogue and assayed for cypin mRNA and protein changes. Figure 4-5 shows that this treatment increases the number of primary and secondary dendrites in hippocampal neurons, while in contrast, cypin mRNA and protein expression is not altered. On the other hand, our Western blot analysis revealed cAMP-promoted expression of a lower molecular weight band in the cypin blot, suggesting a possible splice variant. In addition, we treated cultures with a PKA inhibitor peptide to test the role of PKA activation in BDNF action on cypin expression. Figure 4-6 shows that inhibition of PKA does not block BDNF-promoted increases in cypin mRNA or protein. These results suggest that the activation of PKA by BDNF is not required for increased cypin expression. However, the appearance of a lower molecular weight band in our Western blot analysis suggests that cAMP signaling pathway may alter expression of a cypin variant.
The BDNF/MEK/CREB pathway is responsible for increased cypin expression in hippocampal neurons

Given that activation of CREB by the canonical cAMP/PKA pathway appears not to be involved in BDNF-promoted expression of cypin in neurons, we further tested other pharmacological agents, which specifically target other kinases that have been shown to increase CREB-mediated transcription. The phosphoinositide 3-kinase (PI3K) and the mammalian target of rapamycin are additional kinases that can be simultaneously stimulated by BDNF and growth factors (Du and Montminy, 1998; Peltier et al., 2007). PI3K kinase activates the Akt/PKB/mTOR pathway, and consequently, CREB can be activated by target phosphorylation of Ser119/Ser133 by Akt/PKB (Du and Montminy, 1998). In addition, activation of this pathway increases primary dendrite formation by upregulating gene expression in developing neurons (Dijkhuizen and Ghosh, 2005). Therefore, we tested the possibility that BDNF-mediated increases in cypin expression occur via the PI3K/Akt and mTOR pathway. We treated cultures of hippocampal neurons with wortmannin and rapamycin, potent and specific PI3K kinase and mTOR inhibitors, respectively. We then assayed for cypin mRNA and protein expression. Figure 4-7 and Figure 4-8 demonstrates that inhibition of the PI3K/Akt/mTOR pathway does not alter BDNF-mediated increases in cypin mRNA or protein expression, suggesting that the activation of PI3K/Akt and mTOR by BDNF is not required for increases in cypin expression.
Next, we used the highly selective mitogen-activated protein kinase kinase- (MAPK/MEK) specific inhibitor U0126 (Favata et al., 1998) to test whether the MEK kinase pathway is involved in BDNF promotion of cypin expression. Figure 4-9 shows that U0126 inhibited BDNF-promoted increases in cypin mRNA and protein overexpression. These results suggest that BDNF specifically increases cypin protein expression in hippocampal neurons via activation of MAPK/MEK.

Overall, our results suggest that extracellular BDNF increases cypin gene expression via a MAPK/MEK-dependent pathway, and consequently, this pathway may be responsible for regulating cypin-mediated dendrite morphology in developing hippocampal neurons.

Discussion

Although an increasing number of extracellular factors have been found to regulate neuronal dendrite morphology and branching (reviewed McAllister et al., 1996 and 2000), intracellular transducers that control gene expression during development have not been extensively studied. Elucidating the signaling mechanisms that regulate dendrite morphology and branching will enhance our understanding of how the developing brain regulates neuronal communication by neurite guidance, as well as lead to potential exploration of therapies for treatment of neurodegenerative disorders.

A number of signaling molecules have been identified as regulators of
dendrite morphology (Redmond and Ghosh, 2001; Vaillant et al., 2002; Konur and Ghosh, 2005). Importantly, cypin regulates dendrite formation by promoting microtubule assembly, and negatively regulates trafficking of PSD-95, which is associated with signaling networks at excitatory synapses and decreases dendrite branching (Firestein et al., 1999; Akum et al., 2004, Charych et al., 2006). Our results are the first to demonstrate that extracellular factors, specifically by BDNF, can regulate cypin gene expression.

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

MEK phosphorylates extracellular signal-regulated kinases (ERKs), and these activated ERKs phosphorylate CREB (Bonni et al., 1999). Subsequently, CREB phosphorylation and activation has been linked to neuroprotection in experimental animal models of stroke (Walton et al., 1996). Particularly, CREB is activated by the MEK signaling pathway during N-acetyl-O-methyldopamine (NAMDA) protection in CA1 neurons after transient forebrain ischemia (Park et al., 2004). Similar to our results, these effects were reversed by U0126, the specific MEK kinase inhibitor. The NAMDA-induced neuroprotection was
abolished by U0126, suggesting that the MEK-CREB signaling pathway might be an effective therapeutic strategy to treat stroke or other neurological syndromes. In contrast, other reports have suggested that high K(+) -induced depolarization, promotes neuronal survival by activating MAP kinase and increasing CREB-dependent transcription of specific genes that promote neuronal survival (Zhong et al., 2004).

CREB activation is associated with acquisition of learning and memory, as demonstrated to be crucial for hippocampal function (Sherrington, et al., 1995). Among other types, pyramidal neurons in the hippocampus display rich numbers of dendritic branches, and their synapses contain an abundance of TrkB receptors (Ip et al., 1993; Beck et al., 1993). The activation of TrkB receptors leads to CREB phosphorylation and other signaling cascades, which are critical for synaptic transmission maintenance. This interaction can activate kinases, such as MEK and ERK, which function to phosphorylate CREB, resulting in increased cypin expression.

We have shown by pharmacological and cell biological methods that the activation of the BDNF-CREB pathway regulates cypin expression. Since cypin has been associated with dendrite branching by modulating microtubule assembly and trafficking of proteins at the post-synaptic density, we suggest that synaptic plasticity of hippocampal neurons might be associated with the activation of the BDNF-CREB pathway to regulate synaptic plasticity and long-term memory. Further work should also focus on downstream targets of MEK to promote dendrite branching during development, particularly transcriptional
regulation of other neurite modulatory genes in neurogenesis *in vivo*. 
Figure 4-1. Endogenous cypin expression increases in response to BDNF.

(A) Schematic representation of neurotrophins and their specific Tyrosine kinase receptors (TrK). NGF bind to TrkA, BDNF binds to TrkB, and NT-3 binds primarily to TrkC. (B) Cells were treated with the indicated concentrations of neurotrophins beginning on DIV 7 for 72 hours. Extracts from untreated and treated cultures of hippocampal neurons were analyzed by SDS-PAGE and Western blotting using antibodies that recognize cypin or actin. (C) Densitometry analysis of cypin normalized to actin expression. (D) Quantitative RT-PCR assay for the rat cypin gene (GDA) after indicated treatments of cultures of hippocampal neurons. **p<0.01 by ANOVA followed Student-Newman-Keuls multiple comparison test compared with untreated as control. Cypin mRNA and protein levels are upregulated by increasing doses of BDNF, potentially activating TrkB.
Figure 4-2. Prediction of transcription factor binding sites in the promoter region of rat cypin. The 10Kb upstream sequence of the first exon of cypin was used to predict consensus-binding sequences using the TFSearch algorithm and TRANSFAC database (Heinemeyer et al., 1998). Each position is represented by the transcription factor and the binding probability (expressed as percentage of identity) is indicated as predicted by TFSearch-specific matrices.
Figure 4-3. Conserved cAMP responsive elements (CRE) regions are present in rodent and human cypin gene promoters. Genomic sequences from mouse, rat, and human were retrieved from the NCBI database up to 2Kb upstream from the first exon of cypin. These sequences were analyzed for the occurrence of conserved CRE and TATA-Box regions. Relative nucleotide distances from the first exon codon (ATG) are shown.
Figure 4-4. BDNF promotes cypin expression via a transcription-dependent mechanism. (A) Schematic of the BDNF/TrkB signaling pathway, which can promote increases in gene expression. Actinomycin D is a potent inhibitor of transcription, which can bind to the transcription initiation complex and impede gene expression. (B) Hippocampal neurons were incubated with DMSO or actinomycin D (5 µM) in the absence or presence of 25ng/mL BDNF, beginning on DIV 7 for 72 hours. Extracts from treated cultures were then analyzed by quantitative RT-PCR using specific rat cypin/GDA primers. (C) Hippocampal neurons were treated as in (B) and cytoplasmic protein extracts were resolved by SDS-PAGE and Western blotting using antibodies that recognize cypin or actin. (D) Densitometry analysis of cypin protein expression normalized to actin protein expression. Cypin mRNA and protein levels are upregulated by BDNF treatment, but this increase is inhibited by actinomycin D. **p< 0.01 by ANOVA followed Student-Newman-Keuls multiple comparison test compared with DMSO as control.
Figure 4-5. Treatment with membrane-permeable cAMP analogue primary and secondary dendrites and promotes expression of a cypin variant. (A) and (B) Average number of primary and secondary dendrites in hippocampal neurons treated with membrane-permeable cAMP (n = 20). ***p<< 0.001 and **p< 0.01 by ANOVA followed Student-Newman-Keuls multiple comparison test compared with untreated as control. (C) Cultures of hippocampal neurons were incubated with the indicated concentrations of membrane-permeable cAMP beginning on DIV 7 for 72 hours. Extracts from treated cultures were then analyzed by SDS-PAGE and Western blotting using antibodies that recognize cypin.
Figure 4-6. The cAMP/PKA pathway is not involved in BDNF-mediated increases in cypin expression. (A) Schematic of the possible BDNF/TrkB signaling pathway which could increase cAMP and activate PKA to increase gene expression. PKA inhibitory peptide (PKAi; Myr-N-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH₂; Pahan et al., 1998) is a specific PKA inhibitor which can bind to the kinase catalytic activity, and thus, inhibit the PKA/CREB pathway. (B) Hippocampal neurons were treated with DMSO or 25ng/mL PKAi and in the absence or presence of 25ng/mL BDNF beginning on DIV 7 for 72 hours. Extracts from treated cultures were then analyzed by quantitative RT-PCR using specific rat cypin/GDA primers. (C) Hippocampal neurons were treated as in (B) and cytoplasmic protein extracts were resolved by SDS-PAGE and Western blotting using antibodies that recognize cypin or actin. (D) Densitometry analysis of cypin normalized to actin protein expression. Cypin mRNA and protein levels are upregulated by BDNF treatment, and treatment with a PKA inhibitor is not sufficient to inhibit this mechanism. **p< 0.01 by ANOVA followed Student-Newman-Keuls multiple comparison test compared with DMSO as vehicle control.
Figure 4-7. The PI3K/Akt/PKB pathway is not involved in BDNF-promoted increases in cypin expression. (A) Schematic of the possible BDNF/TrkB signaling pathway which could activate phosphoinositide 3-kinase PI3K to then activate Akt/PKB to increase gene expression. Wortmannin is a PI3K inhibitor, which can specifically inhibit the PI3K/Akt/CREB pathway. (B) Hippocampal neurons were treated with DMSO or wortmannin (100nM) in the absence or presence of 25ng/mL BDNF beginning on DIV 7 for 72 hours. Extracts from treated cultures were then analyzed by quantitative RT-PCR using specific rat cypin/GDA primers. (C) Hippocampal neurons were treated as in (B) and cytoplasmic protein extracts were resolved by SDS-PAGE and Western blotting using antibodies that recognize cypin or actin. (D) Densitometry analysis of cypin normalized to actin protein expression. Cypin mRNA and protein levels are upregulated with BDNF treatment, and treatment with a PI3K inhibitor is not sufficient to block this increase. **p< 0.01 by ANOVA followed Student-Newman-Keuls multiple comparison test compared with DMSO as vehicle control.
Figure 4-8. The PI3K/mTOR pathway is not involved in BDNF-promoted increases in cypin expression. (A) Schematic of the possible BDNF/TrkB signaling pathway which could activate mTOR to increase protein translation. Rapamycin is a specific mTOR inhibitor which can bind to the mTOR complex 1 (mTORC1) and inhibit the PI3K/mTOR pathway. (B) Hippocampal neurons were treated with DMSO or 25ng/mL rapamycin in the absence or presence of 25ng/mL BDNF beginning on DIV 7 for 72 hours. Extracts from treated cultures were then analyzed by quantitative RT-PCR using specific rat cypin primers. (C) Hippocampal neurons were treated as in (B) and cytoplasmic protein extracts were resolved by SDS-PAGE and Western blotting using antibodies that recognize cypin or actin. (D) Densitometry analysis of cypin normalized to actin protein expression. Cypin mRNA and protein levels are upregulated with BDNF treatment, and an mTOR inhibitor is not sufficient to block this increases. **p<0.01 by ANOVA followed Student-Newman-Keuls multiple comparison test compared with DMSO as vehicle control.
**Figure 4-9.** BDNF increases cypin expression by the MEK signaling pathway. (A) Schematic of the BDNF/TrkB signaling pathway which could activate the Ras/Raf/MEK to increase gene expression. U0126 is a specific MEK inhibitor (Favata et al., 1998), which can inhibit the BDNF/TrkB/MEK/CREB pathway. (B) Hippocampal neurons were treated with DMSO or 10nM U0126 in the absence or presence of 25ng/mL BDNF beginning on DIV 7 for 72 hours. Extracts from treated cultures were then analyzed by quantitative RT-PCR using specific rat cypin primers. (C) Hippocampal neurons were treated as in (B) and cytoplasmic protein extracts were resolved by SDS-PAGE and Western blotting using antibodies that recognize cypin or actin. (D) Densitometry analysis of cypin normalized to actin protein expression. Cypin mRNA and protein levels are upregulated with BDNF treatment, and inhibition of MAPK/MEK by U0126 is sufficient to block this increase. **p< 0.01 by ANOVA followed Student-Newman-Keuls multiple comparison test compared with DMSO as vehicle control.
EXPERIMENTAL PROCEDURES

Computational Modeling

All calculations were conducted on 3.2 GHz RedHat Linux workstations. Development of the computer-aided inhibitor analysis on the guanine deaminase (GDA; cypin) structure proceeded in three steps: 1) sequence analysis, 2) energy minimization (EM) and molecular dynamics (MD) simulations, and 3) inhibitor docking and scoring.

Protein Sequence Retrieval

Two hundred nineteen guanine deaminase sequences were retrieved from the National Center for Biotechnology Information protein database (NCBI, http://www.ncbi.nlm.nih.gov/) based on annotation as guanine deaminase. Only non-redundant (nr) sequences from the Reference Sequence (RefSeq) protein database collection were selected for all of the analyses. All prokaryotic and eukaryotic sequences were analyzed to determine the evolutionary relationships of guanine deaminases.

Phylogenetic Analysis

All two hundred nineteen guanine deaminase sequences were aligned using the ClustalW v.2 routine with default parameters (Larkin et al. 2007). The full-length protein sequences were used in the multiple alignments. A Neighbor-Joining (NJ) cluster (Saitou and Nei, 1987) phylogenetic analysis method of the protein alignments was conducted using the PHYLIP package v3.65
(Felsenstein, 1985). The phylogenetic trees were constructed and edited with
MEGA4 (Tamura et al., 2007) with 500 bootstrap replicates and a cut-off value of
70%, and ambiguous alignments in highly variable regions were excluded from
the phylogenetic analysis.

**Position Domain Analysis**

Conservation of the protein domains of guanine deaminases (zinc- and
PDZ-binding) were performed by analyzing 143 prokaryotic, 10 archaeal, and 66
eukaryotic sequences. The zinc-binding domain was examined in all sequences
using the consensus domain pattern of PGX[VI]DXH[TVI]H. For PDZ-binding
domain analysis, the last four residues of all sequences were retrieved and
selected using the patterns X[ST]-X-V, X[ST]-X-L, X-φ-X-φ and X-D-X-V. Each
sequence was classified as one of the three classes according to Sheng and
Sala (2001). The domains were further segregated and multiply aligned using the
Weblogo tool with default parameters (http://www.weblogo.berkeley.edu/logo.cgi).

**Homology Modeling**

The crystal structure of the guanine deaminase from *C. acetobutylicum* with
bound guanine in the active site (PDB ID = 2I9U; Kumaran et al., to be published),
was chosen as the template for the homology modeling of rat cypin with NCBI
Accesion #NP_113964. The primary cypin sequence was submitted to the Swiss-
Modeller homology modeling tool [http://swissmodel.expasy.org//SWISS-
MODEL.html], and the resulting structure was submitted to the WHATIF server for
structural verification (http://www.cmbi.kun.nl/gv/servers/WIWWWI/; Vriend, 1990). The initial homology model was also constructed using the InsightII 2000 software package (http://www.accelrys.com; Insight©, 2000). 2I9U and rat cypin structurally conserved regions (SCRs) were assessed by secondary structure predictions using three different programs: nnpredict (Kneller et al., 1990), PHD (Rost and Sander, 1993) and JPRED (Cuff et al., 1998) and were identified based on sequence and functional similarity. The three dimensional coordinates within SCRs were copied from the template of 2I9U to cypin. De novo loop generation was employed to yield the coordinates for variable regions (VRs), which consisted of loop regions not contained within the SCRs. In the existing 2I9U structure, an iron cation (Fe$^{3+}$), a glycerol, and a guanine molecule are present in the structure, but only the cation has been observed to aid in the coordination between guanine and binding site residues. Previous initial work using purified recombinant guanine deaminases was found to contain an atom of zinc per protein monomer (Yuan et al., 1999). Therefore, the Fe$^{3+}$ ion was manually extracted from the template structure and replaced with a Zn$^{2+}$ ion positioned into the preliminary cypin model. After subsequent resolution of atomic clashes, the protein complex was submitted to Energy Minimization to refine the cypin structural model.

**Energy minimization (EM) and molecular dynamics (MD) simulations**

Energy minimization and molecular dynamics calculations were performed for refinement of the hGDA structure using the AMBER9 force field (http://amber.scripps.edu; Case et al., 2005). To study the preservation and
integrity of the three-dimensional structure of hGDA, MD simulations were first run on the modified hGDA crystal structure (2UZ9). The structure was checked for missing atoms according to residue templates. The AMBER9 package added 3531 lone-pair hydrogen atoms, and 6 extra atoms were manually extracted (LPD1 and LPD2 from Met1 and Met6; LPG1 and LPG2 from Cys2) from the raw structure using Sybyl v.7.2 to pass the AMBER9 parameters to neutralize the unit structure 16 Na\(^+\) ions in the AMBER9 package. The xanthine molecule, Zn\(^{2+}\) ion, and water molecules associated with the retrieved structure were extracted, and the unit was solvated by a 9 Å radius shell of TIP3 water molecules (Ryckaert et al., 1977). Subsequently, the solvated system was energy-minimized in two phases: first, the atoms in the protein structure were restrained using a 500 kcal/mol/rad force for 1000 iterations of constrained steepest descent (SD), but the water and ion molecules were free to move in order to eliminate bad contacts; second, the entire system was energy-minimized for 1000 iterations of SD and 2500 iterations of conjugate gradient (CG). Furthermore, MD simulations were then run on the system for electrostatic and van der Waals (vdW) interactions, using the standard force-field parameter set parm99 in AMBER9 with dielectric constant \( \varepsilon = 1 \) and cutoff distance = 12.0 Å. During the MD simulation, the solvated system was coupled to a Berendsen bath at 300 K by using a coupling constant \( \tau_T = 2 \) ps (Berendsen et al., 1984), and the temperature was gradually increased from 0 to 300 K over 10 fs of simulation time with the volume held constant (ensemble NVT). The hGDA structures derived from the final 300 ps MD simulation were averaged and energy-minimized using 1000
iterations of SD and 4000 iterations of CG. The average structure was used for further molecular docking experiments.

**Inhibitor Molecular Docking**

Guanine and a list of 188 guanine analogues, including previously studied GDA inhibitors (Rajappan and Hosmane, 1998; Ujjinamatada et al., 2006; Liaw et al., 2004) and novel compounds, were docked to the hGDA structure using GOLD (Genetic Optimization of Ligand Docking; Jones et al., 1997). The IUPAC names and chemical properties of all of the compounds tested are described in the Supplemental material. The majority of the small molecules were retrieved from the NCBI PubChem Compound database, and others were built with Sybyl v7.2 ([http://www.tripos.com](http://www.tripos.com)). All molecules were optimized with the MMFF94 force field and Gasteiger-Hückel atomic charges. The number of poses for each compound was set to 10, and the default algorithm speed was selected during the docking procedure. The hGDA residues within a 10 Å radius of the centroid of the guanine/xanthine binding site were designated as the hGDA substrate/ligand pocket. During molecular docking, early termination was allowed if the RMSD of the top five bound conformations of the ligand were within 1.5 Å. If the compound was previously assigned an experimental inhibition constant, the top-ranked conformation of this compound was selected and the corresponding GOLD score was then correlated with the known inhibition constant. The binding conformations of inhibitors in the ATP-binding site were displayed using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and
Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081; Ryckaert et al., 1977).

**CREB cDNA subcloning**

The CREB cDNA sequence was subcloned into the pe-GFP-C1 plasmid using the following protocol. Cultured rat hippocampal neurons were grown for 10 days in vitro (DIV10) and total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) following manufacturer’s instructions. Total cDNA was then generated using the high-capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) using 1µg of total RNA and following manufacturer protocol. Then, the CREB cDNA was amplified using 50ng of total cDNA and the following primers containing XhoI and EcoRI restriction sites: forward, 5′- GCA CTC CGA GCT ATG ACC ATG GAC TCT GGA GCA GAC -3′ and reverse, 5′- GCA CGA ATT CGA TTA ATC TGA CTT GTG GCA GTA AAG GTC C -3′. The PCR product was ligated into the pe-GFP-C1 plasmid with a T4 ligase (Invitrogen, Carlsbad, CA). Plasmid DNA from selected clones was isolated and confirmed by sequencing to be the rat CREB cDNA isoform A sequence and compared with NCBI Accession #NM_114443.1.

**Site-Directed Mutagenesis**

The cypin zinc-binding alanine substitution mutants, CREB constitutive active (CA) and CREB dominant negative (DN) were prepared using the QuickChange II site-directed mutagenesis kit (Stratagene). All of the substitution
mutations were performed using the pEGFP-C1 and pGEX4-T1 vectors containing the *Rattus norvegicus* wild type full-length sequence of cypin or CREB, using a cloned *Pfu* polymerase and *DpnI* endonuclease from Stratagene (La Jolla, CA). The following forward primers were used for cypin mutants: H71A, 5'- GAT CAG AGA GCT GAG CCA CGC TGA GTT CTT CAT GCC AG -3'; H82A, 5'- GGC CTT GTT GAT ACA GCC ATC CAT GCC CCT CAG TAT GCC -3'; I83A, 5'- GCC TTG TTG ATA CAC ACG CCC ATG CCC CTC AGT ATG CC -3'; H84A, 5'- CTT GTT GAT ACA CAC ATC GCT GCC CCT CAG TAT GCC -3'; H240A, 5'- GTA CAT CCA GAG CGC TAT AAG TGA AAA TCG TG -3'; D330A, 5'- GAT AGG GCT TGG GAC AGC TGT GGC TGG TGG TTA C -3'. The following forward primers were used for CREB mutants: S119D, 5'- CTT TCA AGG AGG CCT GAC TAC AAG AAA ATC TTG AAT GAC TTA TC and S119A, 5'- CCT TTC AAG GAG GCC TGC CTA CAA GAA AAT CTT GAA TGA CTT ATC-3'. For the generation of individual mutants, the following were mixed in a PCR-tube and placed in a thermal cycler: 10 ng plasmid containing a *Rattus norvegicus* wild type sequence of cypin and CREB, 5 ng of each forward and reverse primers, 10X *Pfu* polymerase buffer, dNTPs, sterile distilled H₂O and *Pfu* DNA polymerase to a volume of 50 µl. The following thermal cycle protocol was followed: (1) denaturing temperature 95°C 30 s, cycled once, (2) denaturing temperature 95°C 30 s, (3) annealing temperature 55°C 60 s, (4) extension temperature 68°C 530 s (1 minute per kb) and (5) steps 2 to 4 were cycled 16 times. Then, *DpnI* restriction enzyme (10 U) was added when the protocol was
complete, and incubated at 37°C for 1 h to completely digest the methylated (wild type template) plasmid. Finally, the cypin zinc-binding mutant sequences carrying the desired mutations were transformed into E. coli DH5α supercompetent cells, and plasmid DNA from selected clones was isolated and confirmed by sequencing.

**Antibodies**

Mouse anti-MAP2 and anti-actin were purchased from Sigma-Aldrich, and Cy2- and Cy3-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA). Rabbit anti-Cypin (RTG-55) was generated from animals immunogenized with GST-cypin fusion protein. Rat anti-GFP was a gift from Dr. Shu-Chan Hsu (Rutgers University, Piscataway, NJ).

**Neuronal Culture, Transfection, and Dendrite Branching Number Analysis**

Neuronal cultures were prepared from hippocampi of rat embryos at 18 days of gestation as previously described (Firestein et al., 1999; Akum et al., 2004; Chen et al., 2005). After 10 days in vitro (d.i.v.), neurons were transfected using the Effectene reagent (QIAGEN, Valencia, CA) as is done routinely in our laboratory (Firestein et al., 1999; Akum et al., 2004; Charych et al., 2006). Cultures were allowed to express the transfected proteins for 48 hours. Images of transfected hippocampal neurons were taken as described previously (Akum et al., 2004), and neurons were confirmed by MAP2 immunostaining. Primary
and secondary dendrites were counted as previously described (Firestein et al., 1999; Akum et al., 2004; Charych et al., 2006). In order to acquire unbiased dendrite number counts, the analyzing person was blinded to the transfection condition.

**Immunohistochemistry**

Hippocampal neurons were plated on coverslips and were fixed and blocked as previously described (Akum et al., 2004). Rat anti-GFP and mouse anti-MAP2 antibodies (both at 1:1000 dilutions) were added at room temperature for 2 hours. Coverslips were washed with PBS and incubated at room temperature with Cy2-conjugated donkey anti-rabbit IgG and Cy3-conjugated donkey anti-mouse IgG secondary antibodies (1:250 dilutions) (Akum et al., 2004). Coverslips were mounted onto frosted glass microscope slides using Fluormount G (Southern Biotechnology). Labeled cells were visualized by immunofluorescence using an Olympus IX50 microscope with a Cooke Sensicam charge-coupled device cooled camera, fluorescence, imaging system, and Image Pro software.

**GST Fusion Protein Purification**

GST, GST-cypin (wild-type) and GST-cypin mutants were expressed in *Escherichia coli* cultures grown in Luria-Bertani (LB) media and purified using glutathione-Sepharose beads (Amersham Biosciences, Piscataway, NJ). Transformed cells were grown overnight and seeded in a 100 mL LB media
culture at 37°C until reaching an OD$_{600}$ of 0.8. In order to obtain GST fusion protein products that have lower solubility, we induced cells with a low IPTG concentration (1 mM) and then grown overnight at room temperature. After centrifugation, cell pellets were homogenized in 10 volumes of ice-cold MTBSE buffer (1X PBS + 2 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated two times for 20 seconds each. Triton X-100 was added to 1%, and proteins were extracted for 1 hr at 4°C. Extract/beads were then loaded into the appropriately sized columns and were washed with 100 column volumes of MTBSE buffer, 1% Triton X-100, and 1 mM PMSF. Proteins were eluted consecutively with 5 column volumes of MTBSE containing 1% Triton X-100 and 2.5 ml of elution buffer (1X PBS + 1% SDS + 100 mM NaCl + 20 mM glutathione, pH 8.0). Finally, eluates were dialyzed extensively against PBS to remove glutathione and salt excess.

Zinc Binding Assay

GST fusion proteins were purified from E. coli as stated above using glutathione sepharose and eluted from the beads with excess glutathione. Zinc ions incorporated into the fusion proteins were from uptake by the bacteria, and the source was the yeast extract included in the LB medium in which the bacteria cultures were grown. Eluates were dialyzed extensively against PBS to remove the glutathione. Equal concentrations of protein samples (determined by Bradford protein assay and confirmed by SDS-PAGE) were subjected to Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) using a Varian Vista-
PRO Model CCD Simultaneous ICP-OES spectrometer (VISTA, Palo Alto, CA), and the absorbance intensity at 213 nm (specific for incinerated zinc) was compared with a standard curve of known ZnCl$_2$ concentrations (10 µM, 100 µM, 1 mM, 5 mM, and 10 mM in elution buffer) to calibrate the instrument. Radiation intensities were measured at 213 nm and the intensity ratios of samples to GST were plotted.

**Sequence Analysis**

All sequence and structure data were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Evaluation of the primary sequence in the active crystal structure of human guanine deaminase (hGDA; cypin; PDB ID =2UZ9; Moche at al., 2008) was conducted using the accession number of the hGDA protein sequence NP_004284. Pairwise sequence alignment between NP_004284 and hGDA was conducted using the ClustalW1.83 routine with default parameters (Thompson et al., 1994; Chenna et al., 2003). This alignment revealed a 22-residue (MHHHHHHSSGVDLGTENLYFQS) insertion in the N-terminus of the hGDA structure. In order to resemble the Reference Sequence (RefSeq) from the NCBI human protein database, the inserted peptide was manually extracted from the hGDA structure using Sybyl v7.2 (http://www.tripos.com).

**GDA Inhibitor Kinetic Assay**
GDA kinetic assays were performed as previously described (Rajappan and Hosmane, 1998; Ujjinamatada et al., 2006; Lewis and Glantz, 1974). Briefly, purified guanine deaminase from rabbit liver (purchased from MP Biomedicals) was used for biochemical studies. Commercially available purine and guanine analogues were used in our assays. With the exception of N2-Acetylguanine (Toronto Research Chemicals, Ontario, Canada), all tested compounds were purchased from Sigma-Aldrich. The colorimetric assays were performed at 25 °C and pH 7.4 in Tris buffered saline buffer (TBS; 25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl; Fisher Scientific). Guanine hydrolysis rates were measured by optical density changes at 245 nm. An enzyme unit was defined as the amount of enzyme that deaminates 1.0 μmole of guanine to xanthine per minute at pH 7.4.0 and 25 °C. In the assay, a fixed concentration of $8 \times 10^{-3}$ U guanine deaminase was incubated with seven guanine concentrations ([S] = 2, 5, 7, 10, 12, 15, 17 and 20 μM) and each inhibitor concentration (8 or 20 μM). Lineweaver–Burk plots ($1/V$ vs $1/S$) were used to calculate kinetic constants.

**Guanine Deaminase Assay**

To measure guanine deaminase enzyme activity, COS-7 cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) and 2.5 μg of DNA per 100 mm culture dish of pEGFP-C1 vector alone, containing the *Rattus norvegicus* wild type full length or mutant sequences of cypin. After 48 hours of expression, cells were washed twice with phosphate-buffered saline (PBS) and scraped into GDA lysis buffer (150 mM NaCl, 25 mM
Tris-HCl, pH 7.4 and 1 mM PMSF). Lysates were homogenized by passing them through a 25-gauge needle five times and centrifuged at 10,000 x rpm at 4°C for 10 min. Concentration of cytosolic proteins in the supernatant was measured using the Bradford Assay. Protein samples were resolved by SDS-PAGE for equal cypin expression verification. Fifty micrograms of lysate was then used for each guanine deaminase sample assay in GDA lysis buffer containing 0.025 U/ml xanthine oxidase (Sigma-Aldrich), 0.002 U/ml peroxidase (Sigma-Aldrich), Amplex Red reagent (Molecular Probes, Eugene, OR) and 125 mM guanine (Sigma-Aldrich, St. Louis, MO). For the guanine analogues assay, we used 10µM of each compound (Chapter 3). Negative controls were performed using protein lysates in assay solution with no guanine added. Negative control and samples were incubated at 37°C, and absorbance at 512 nm using a single beam Genesys 10 UV/Vis Spectrophotometer (Spectronic, Garforth, UK) was measured during the indicated time intervals after samples were centrifuged at 10,000 x rpm for 1 minute to remove insoluble guanine. Experiments were performed in triplicate and results were normalized by subtracting nonspecific background absorbance from the negative controls.

**Quantitative RT-PCR**

Primary embryonic (E18) rat hippocampal neurons were seeded at 1x10^6 cells per plate on 35 mm PDL-coated culture dishes (Corning, Corning, NY) in complete Neurobasal media supplemented with B-27 (Invitrogen, Carlsbad, CA). After seven days in vitro (DIV7), medium was replaced with treatment media
containing indicated concentrations of neurotrophins, kinase inhibitors, cell-permeable cAMP or DMSO vehicle. On day ten (DIV10) RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) following manufacturer’s instructions. Total cDNA was then generated using the high-capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) using 1µg of total RNA and following manufacturer protocol. We used a Stratagene Mx3000P QPCR system (Stratagene, La Jolla, CA) to perform multiplex assays using 50ng of total cDNA for cypin/GDA and GAPDH as an internal control. The TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA), containing primers and probes were used in our experiments. The cypin/GDA probe contained the FAM490 fluorophore and the GAPDH probe contained the HEX fluorophore both with the MGB quencher. Results were analyzed following the $2^{-\Delta\Delta Ct}$ method using GAPDH as an internal control and non-treated or DMSO as control.

**Tubulin polymerization and competition Assay**

The tubulin polymerization assay kit was purchased from Cytoskeleton (Denver, CO). Tubulin (>97% pure) was mixed with general tubulin buffer (GTB, 80 mM PIPES pH 6.9, 2 mM MgCl2, 0.5 mM EGTA, and 1 mM GTP) in a 96-well plate at 37°C. Absorbances at 340nm were measured every 1 min for 70 min by a VICTOR X3 Multilabel Plate Reader with Wallac 1420 v.3.0 software (PerkinElmer, Waltham, MA). GST or GST-Cypin (0.1 nmoles) was incubated with or without guanine analogues (0.1mM). Then, tubulin (3-4mg/mL) was added in the GTB buffer at 37°C for 5 min. The analysis was performed with the
Statistical Analysis

Dendrite counts were performed using n≥35 per condition, and statistical significance was determined by ANOVA followed by a Dunnett multiple comparisons test using p<0.05 as a significant difference. For the zinc-binding assay, protein samples were assayed in triplicate. For ICP-OES measurements, band densitometry and quantitative RT-PCR samples were compared using ANOVA followed by the Student-Neuman-Keuls comparison test also using p <0.05 as a significant difference.
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