KCTD12 AND ULK2 PARTNER TO REGULATE HABENULAR DENDRITOGENESIS

AND BEHAVIOR / SPINOPHILIN REGULATES DENDRITIC SPINE FORMATION AND

F-ACTIN DYNAMICS IN HIPPOCAMPAL NEURONS

By

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To my family for the gift of life and eternal support,

To my friends for guidance and encouragement,

And to my cats for companionship and amusement

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

Introduction

The Importance of Neuronal Development and Dendritogenesis

The brain is an enigmatic organ, containing billions of cells that work together to create thoughts, emotions, and movement. It is the central processing unit of the nervous system, coordinating information from specialized functional domains of the central nervous system (CNS) and allowing for swift response to environmental stimuli. The nervous system contains two basic types of cells: neurons, which form the circuitry of the brain and glia, which support neuronal development and function. My research focuses on the development of the neuron. A neuron is made up of three parts: an axon, to sends signals, a cell body containing the nucleus and cellular organelles, and one or more dendrites for receiving signals. Each of the neurons in the CNS communicates with one another through ion and peptide exchange at the synapse, the meeting site of an axon from one neuron and a dendrite from another. A post-mitotic neural progenitor cell undergoes extensive and intricate morphological changes to develop these structures and become a functional neuron that is capable of processing information from its inputs and sending information through its outputs.

Appropriate neuronal morphogenesis is essential for forming the distinct functional domains of each of the hundreds of types of neurons in the brain. Each subtype of neuron can form a unique dendritic arbor, first described by Ramón y Cajal in the late 1890s (Ramón y Cajal, 1891; Ramón y Cajal, 1899) (Figure 1). Generating the correct size and shape of dendrites is essential for a neuron to satisfactorily sample and process the signals that converge on its dendritic field. Abnormal formation of dendrites

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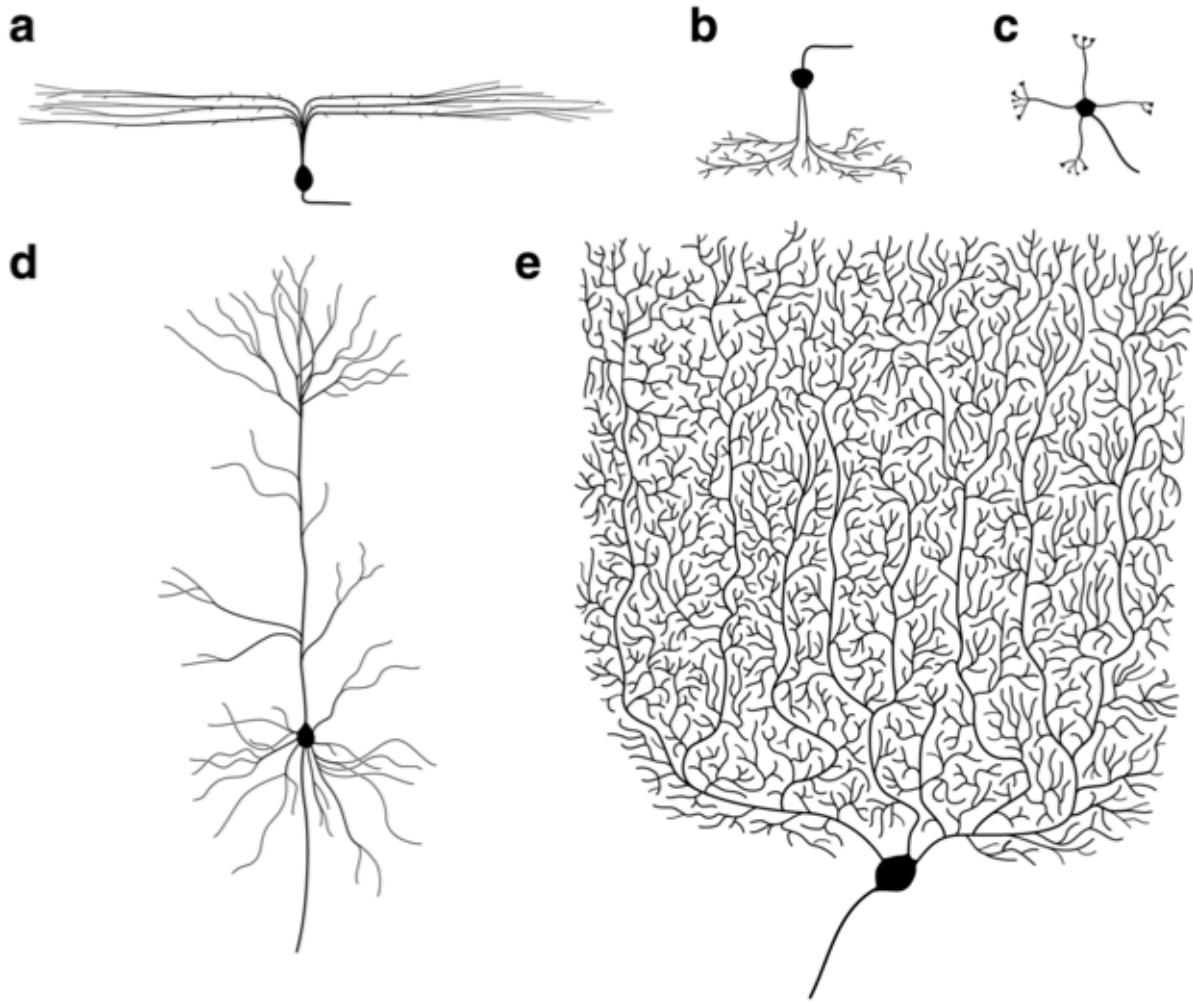
Figure 1. Dendritic arbor diversity in the nervous system. Dendritic arbors take on many intricate shapes. (**a**) Side view of a retinal ganglion cell. (**b**) Side view of an amacrine cell. (**c**) A cerebellum granule neuron. (**d**) A Purkinje cell, adapted from Ramón y Cajal 1995. (**e**) A cortical pyramidal neuron. Figure was published in Gao et al. 2007.

is observed in cortical neurons of patients with disorders including Rett's syndrome and fragile X syndrome, correlating with the emergence of behavioral symptoms (Emoto, 2011; Kaufmann and Moser, 2000). Dendrite morphogenesis is a multi-step process, which includes growth, branching, remodeling, and pruning of neuronal processes (Luo, 2002) (Figure 2). Understanding the control of neuronal circuit development is key to understanding normal and abnormal brain function and behavior.

The first part of this thesis addresses two components of neurodevelopmental biology: the cellular biology of dendrite formation and the behavioral consequences of altered dendritogenesis in an evolutionarily conserved brain nucleus. In order to understand the relationship between genes, cellular biology, and behavior, I used a wide variety of biochemical, molecular biology, and imaging techniques to understand the processes required to make a functioning brain. This introduction discusses the current knowledge of dendrite formation and sets the stage for my project.

<p align="center">**Neurite and Dendrite Formation**</p>

Axon and dendrite specification occurs in five stages in primary culture (Figure 3). First, a nonpolar cell extends lamellipodia, driven by F-actin rearrangements and polymerization (stage 1), followed by neurite extension (stage 2) (Flynn, 2014; Jan and Jan, 2003). Then one of the neurites is selected to form the axon (stage 3), and it extends a large growth cone, leaving the other neurites to form dendrites (stage 4). Finally, synaptogenesis occurs to complete development (stage 5). *In vivo*,
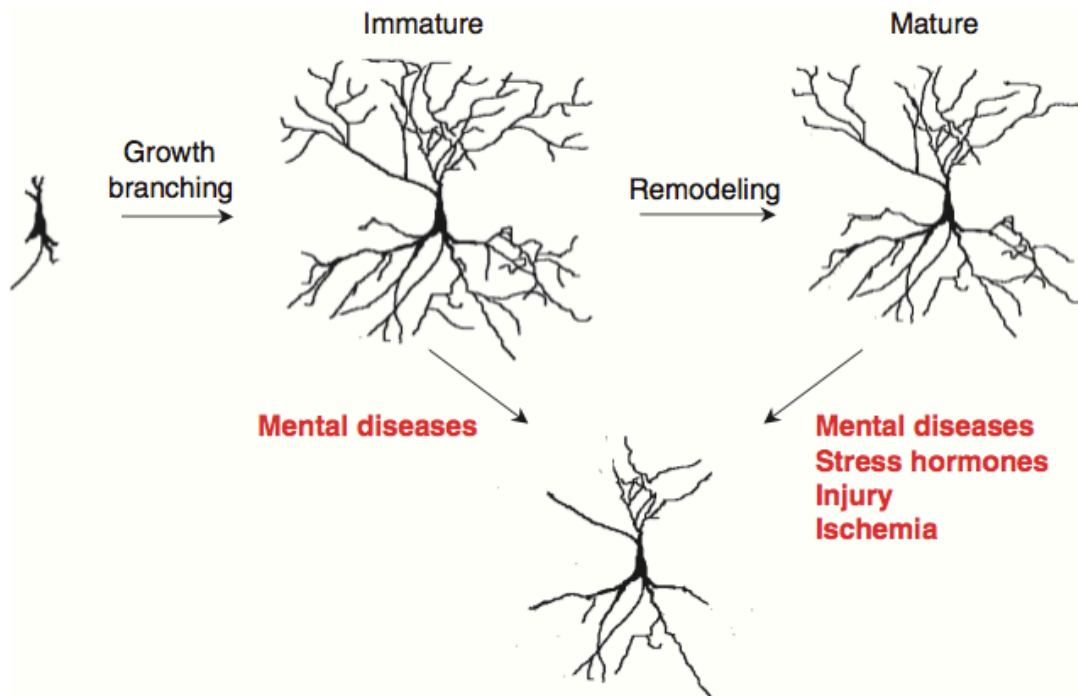
Figure 2. Dendrite remodeling in development and pathological conditions. In normal development, dendrites grow from morphologically unpolarized neurons and extend in a defined direction with formation of branches at defined intervals. Many neurons' dendrites stop growing at defined borders as they mature. At the same time, dendrites are remodeled by selective stabilization and elimination of particular processes to refine their connectivity. Some genetic disorders such as Down's syndrome (DS) and Rett syndrome (RS) appear to affect remodeling and ⁄ or maintenance of dendritic trees. Dendritic arbors of mature neurons in the adult brain can be abnormally remodeled in pathological conditions (injure, ischemia) and under stress conditions. Figure was published in Emoto et al. 2011.
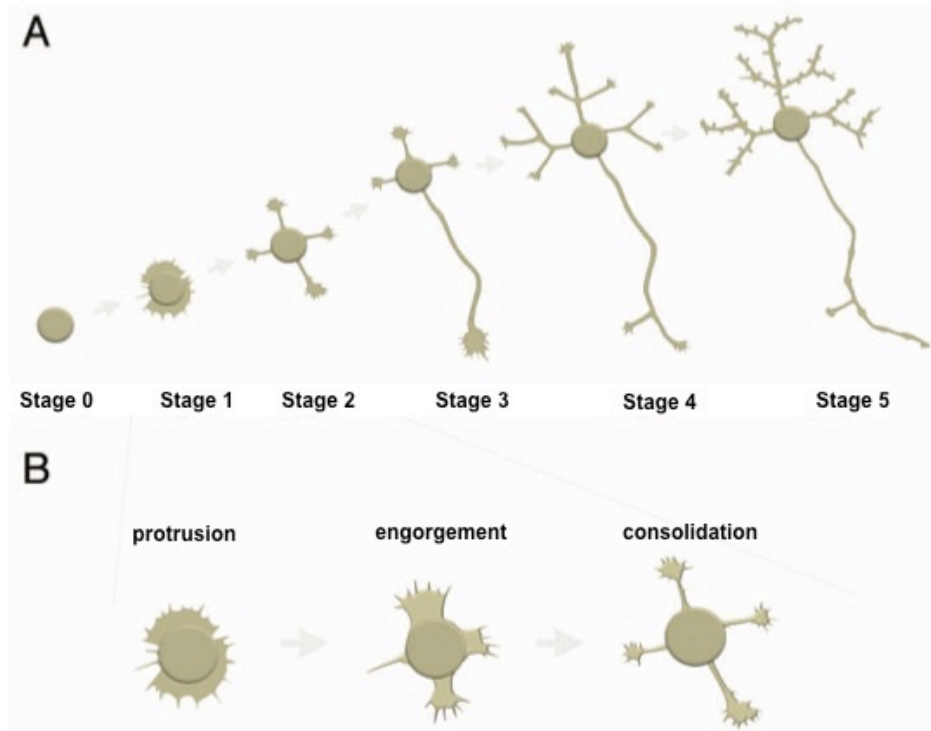
Figure 3. Neuronal development and neurite initiation. (A) Neuronal morphogenesis can be divided into a series of stages, initially characterized in culture (Dotti et al., 1988), but similarly occur in vivo. The stages provided here are broad generalizations and specific culture conditions determine the exact timing of the developmental milestones. Shortly after their birth (or after plating in culture), initially spherical neurons begin extending circumferential lamellipodia and filopodia (Stage 1). During the Stage 1–2 transition, the lamellipodia protrude forward and stable filopodia become engorged forming into neurites (neurite initiation or neuritogenesis). A Stage 2 neuron typically exhibits multiple minor neurites, all of which have the potential to become an axon. In the absence of graded external signals a stochastic process occurs in which one neurite with a large and dynamic growth cone begins elongating at a rapid rate and becomes the axon (Stage 3). In vivo, this typically occurs in a directed manner, as the presence of extrinsic cues guide axonal development. The axon continues to grow and differentiate while remaining processes then grow and arborize acquiring dendritic identities (Stage 4, 3–7 d). Neurons then begin to make synapses, develop dendritic spines, and form neuronal circuits (Stage 5). (B) Neurite initiation or neuritogenesis occurs during the stage 1–2 transition and can be subdivided into 3 phases. F-actin assembly drives membrane protrusion as either broad lamellipodia or finger-like filopodia. Then microtubules and other components move out into the lamellipodia and filopodia during the engorgement phase. Finally neurite formation at the base of the growth cone is consolidated as the membrane and underlying cytoskeleton reorganizes to form the cylindrical shaft of the neurite. Figure adapted from Flynn et al. 2014.

neurogenesis occurs slightly differently. Neurite extension also occurs simultaneously with migration, since the neurons are not confined to a coverslip (Barnes and Polleux, 2009). It appears that a nonpolar neuron sends out only one neurite to form the axon initially, later extending dendritic neurites to become multipolar (Gao et al., 1999; Myers et al., 1986; Westerfield et al., 1986). Despite evidence that neurons can form neurites and axons before migration, and that migration can occur without axon extension, it is still debated whether or not these initial neurites are in fact neurites or migratory protrusions (Hand and Polleux, 2011; Kwiatkowski et al., 2007; Lei et al., 2012; Noctor et al., 2004).

The structure of a neuron is maintained by its cytoskeletal microfilaments, which are made up of actin filaments, and intermediate filaments to bear tensile forces, and microtubules to resist compressive loads (Dennerll et al., 1988) (Figure 4). Concerning actin, neurite growth cones contain four actin-based structures: lamellipodia (flat actin sheets), filopodia (actin bundles), arcs (growth cone peripheral-central domain border), and intrapodia (β1-integrin-rich protrusions) (Dehmelt and Halpain, 2003; Flynn, 2014). Initial neuritogenesis occurs by two mechanisms. In the first, lamellipodia form nascent growth cones, followed by microtubule transport of vesicles and organelles to the leading edge. First, an actin meshwork will form a lamellipodium and undergo segmentation; the segmented lamellipodia will accumulate microtubules and migrate away from the cell body, elongating as the microtubules compress into a tighter bundle (Dehmelt et al., 2003; Tang and Goldberg, 2000; Yu and Bellamkonda, 2001). As the lamellipodium transitions into a neurite, marked by a microtubule-based shaft and an actin-rich growth cone, actin filaments will polymerize and extend via nucleation, cap its

6

ends to halt growth, bundle to form a filament, and interact with microtubules (Dehmelt and Halpain, 2003; Flynn, 2014; Sainath and Gallo, 2014). Concurrently microtubules will polymerize and bundle along actin filaments. Once a neurite has been invaded by microtubules, it can transport cargo such as mitochondria and vesicles throughout the protrusion to influence development (Dehmelt and Halpain, 2003). Finally the proximal cytoskeleton and membrane remodels to form a cylindrical neurite (Dehmelt et al., 2003; Flynn et al., 2012). Another model suggests a filopodium can form a neurite without lamellipodia by protruding, filling with microtubules and cytoplasm, and lastly forming a growth cone (Dent et al., 2007; Flynn et al., 2012; Smith, 1994). The site of primary dendrite formation in PCs is dictated by the Golgi Apparatus; mutation of atypical protein kinase C (aPKC) causes the Golgi to be more broadly spread throughout the cell and thus multiple primary dendrites form (Tanabe et al., 2010). aPKC also plays a role in determining axonal polarity and specification in mammalian neurons (Shi et al., 2003).

The clutch hypothesis is thought to be the molecular motor mechanism by which growth cones of developing neurites protrude. This process begins with the intracellular retrograde flow of actin monomers coupled with actin filament assembly at nucleation sites. Next, actin monomers are recycled at the proximal end of the growth cone while adhesion complex develop, resulting in forward protrusion of the growth cone (Dehmelt and Halpain, 2003; Mitchison and Kirschner, 1988). Retrograde flow and growth cone dynamics are controlled by myosin motor systems, including mysoin-1c, myosin II and myosin V (Bridgman et al., 2001; Brown and Bridgman, 2003; Diefenbach et al., 2002; Wang et al., 1996). The role of other cytoskeletal structures such as actin arcs and
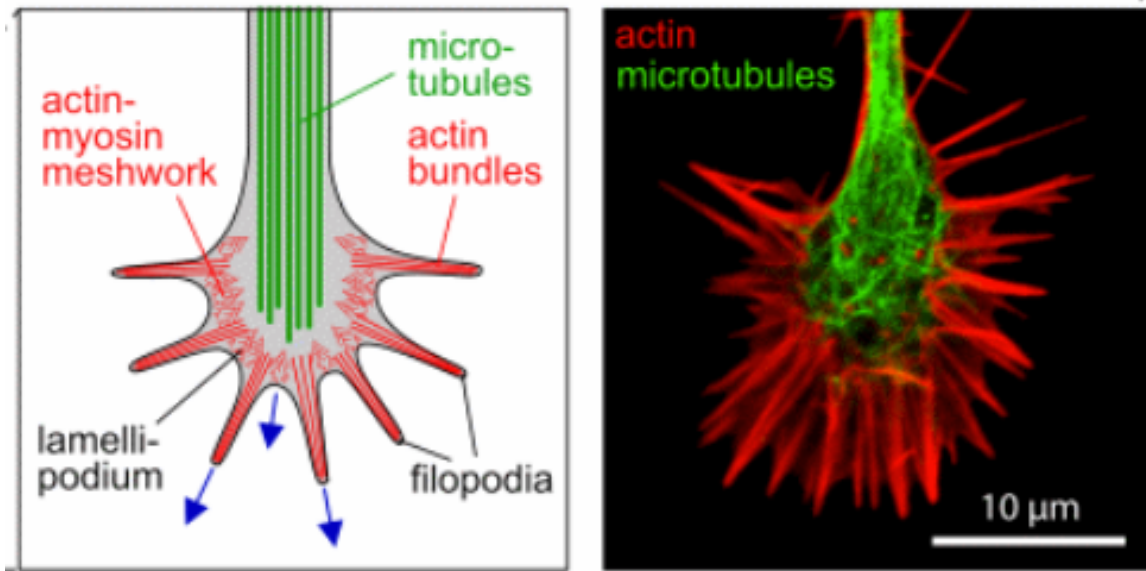
Figure 4. Schematic representation and image of a neuronal growth cone. The morphological structure of a developing neurite is regulated by the actin and microtubule cytoskeletons. The lamellipodium are made up of an actin meshwork, while the filopodia contain actin bundles. Microtubules innervate the neurite into the growth cone. The fluorescence image on the right shows an NG108 growth cone. Figure modified from diploma thesis of Daniel Koch, 2008 and Steve Pawlizak, 2009.

intrapodia are not well understood. Actin arcs result from the compaction of the actin network during retrograde flow, and organize microtubules in the central domain of the growth cone (Dent et al., 2007; Schaefer et al., 2002). Intrapodia are a poorly understood actin structure; however we know they are induced by microtubule polymerization and are potentially an important part of neurite initiation (Dehmelt and Halpain, 2003; Munnamalai and Suter, 2009; Rochlin et al., 1999).

Once a neurite has protruded, the next important step in dendritic development is growth and branch formation. Live imaging of developing *Drosophila* neurons has been valuable in understanding dendritic growth and branching. Dendrites can be seen forming new branches through interstitial sprouting of new branches from an existing branch, or from bifurcation of the growing tip of an existing branch (Gao et al., 1999; Grueber et al., 2003; Sugimura et al., 2003). These newly formed branches are highly dynamic and malleable and are susceptible to extrinsic and intrinsic factors. Extrinsically, Slit/Robo and Netrin/Frazzled act in both axonal and dendritic development (Furrer et al., 2003). *Robo* and *frazzled Drosophila* mutants have defects in motor neuron dendrite guidance that can be rescued by their respective proteins. This data was recapitulated in mammalian cell culture; Slit1 promoted dendritic growth and branching, whereas Robo proteins suppressed this phenotype (Whitford et al., 2002). Semaphorin 3A (mediated by Fyn, a tyrosine kinase, and cyclin-dependent kinase 5) plays a similar role in dendritogenesis by working as an apical dendrite attractant in mammalian neurons (Polleux et al., 2000; Sasaki et al., 2002). Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), Notch receptors, and bone morphogenic proteins (BMPs) all act extrinsically to regulate

9

dendritic morphology in vertebrates (Lein et al., 1995; McAllister et al., 1997; McAllister et al., 1995; Redmond and Ghosh, 2001). BMP7, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and interferonγ negatively regulate dendritogenesis (Drahushuk et al., 2002; Guo et al., 1999; Kim et al., 2002).

Neuronal activity is another key extrinsic aspect of dendritic development. Inhibition of neurotransmission causes complete dendritic retraction in cultured rat sympathetic neurons (Vaillant et al., 2002). Neurotransmitter-evoked Ca2+ signaling locally stabilizes dendritic arbors (Lohmann et al., 2002). Conversely, activity withdrawal activates the Rho/ROCK/LIMK/cofilin pathway to promote actin and dendritic retraction (Miller and Kaplan, 2003). Neuronal activity plays an important role in dendritic spine development, specialized structures along the dendritic shaft (Chen et al., 2014; Corty et al., 2009). This will be discussed in the second part of my thesis.

Intrinsically cytoskeletal regulators like Rho GTPases are important in dendrite formation (Luo, 2002; Redmond and Ghosh, 2001). Dendrite initiation is controlled by Rac1/PAK and Cdc42 in cortical neurons (Redmond and Ghosh, 2001; Threadgill et al., 1997). Microtubule transport is required for dendrite differentiation in cultured neurons; the absence of mitotic motor protein CHO1/MKLP1, dynein, or dynein-associated protein Lis1 alters dendritic maturation (Liu et al., 2000; Yu et al., 2000). Microtubule associated protein 2 (MAP2) knockout mice have reduced dendrite length and density (Harada et al., 2002). Finally, an actin-microtubule crosslinking protein Kakapo/Shortstop regulates dendritic branching; in mutants, sensory and motor neuron branching is dramatically reduced (Gao et al., 1999; Prokop et al., 1998).

10

Several signal transduction proteins have been identified in dendritic development. In *Xenopus* and mammalian systems, activity-dependent CaMKII stabilizes dendritic arbors and limits growth, while in rat neurons CaMKIV mediates Ca^{2+}-mediated growth via transcription factor cyclic-AMP responsive element binding protein (CREB) (Redmond et al., 2002; Vaillant et al., 2002; Wu et al., 2001; Wu et al., 1999). The MEK-ERK pathway plays an intertwining role in these signal transduction pathways; while both short and long-term ERK activation correlate with neurite extension, short-term activation is CamK-dependent and long-term activation is Ras-dependent (Wu et al., 2001).

Zebrafish as a Model for Dendritogenesis

The zebrafish is an excellent system for studying vertebrate dendrite development *in vivo*. The embryos are transparent, making them useful for imaging, and are easily manipulated genetically. There are a vast amount of genetic tools available, such as cell and tissue-specific labeling, the Gal4:UAS system, the Cre-loxP system, the CRISPR-Cas9 system, and more (Hocking et al., 2013). Relevance for understanding the human brain is high, as there is extensive conservation of neuroanatomical features and gene expression in many regions of the vertebrate brain (Aizawa et al., 2011). As discussed earlier, a neural progenitor cell must migrate and differentiate morphologically into a specialized neuron with elaborated dendritic arbors after it is fated to be a neuron by asymmetrical division (Hocking et al., 2013). *In vivo* imaging of zebrafish neurons have helped us to understand how a neural progenitor becomes a multipolar arbor. Live cell imaging of purkinje cells (PCs) in the zebrafish

11

cerebellum shows highly dynamic neurites during the first day of development, but a single stable primary dendrite and axon after 24 hours (Tanabe et al., 2010). A similar phenotype is seen in retinal ganglion cells (RGCs). The dendritic arbor is first highly dynamic, then becomes stabilized as basal branch formation slows (Choi et al., 2010). The next important step in development is targeting dendrites to their appropriate axonal counterparts to form synapses. Through live imaging studies, dendrites from individual RGCs have been observed elaborating within a specific lamina, or layer, to connect with laminated axons of interneurons (Mumm et al., 2006). This study countered the notion that pruning was the main mechanism of determining the dendritic arbor morphology in vertebrates. Despitre recent advances in live imaging, dendritic development still remains poorly understood compared with axonal development. The zebrafish model system is a valuable tool for scientists to use to study this process in real time within the context of an intact brain.

In addition to their use in cell biological studies, zebrafish are also an amenable model organism for studying how molecular and genetic effects on development ultimately change behavioral outputs (Cachat et al., 2011; Fonseka et al., 2016; Klee et al., 2011a; Klee et al., 2011b; Miklósi and Andrew, 2006). Around 70% of human genes have a zebrafish orthologue (Howe et al., 2013). Combined with genetic modifications discussed earlier, zebrafish can be used to study behaviors such as fear, anxiety, stress response, escape response, avoidance, sociality, response to novelty, memory, and more. For instance, zebrafish are a valuable model system for studying stress and anxiety-based behaviors. Scototaxis is a measure of anxiety based on preference for a dark vs. light environment (Maximino et al., 2010) (Figure 5). Treatment of larvae with

lorazapam correlates with an increase in time spent in their non-preferred environment during a scototaxis test (Chen et al., 2015). When put in a novel tank test, larvae that were more hesitant to enter the novel tank were slower to recover from stress than early emerges (Tudorache et al., 2015). Zebrafish can be trained in mazes, similar to rodents (Sison and Gerlai, 2010). One study found that acute inescapable stress impaired spacial memory during a maze test (Gaikwad et al., 2011). Behavioral phenotypes are an important aspect in understanding normal and abnormal dendritogenesis.

The Habenular Nuclei

In particular, the dorsal habenular nuclei are an advantageous region to study dendritogenesis in the central nervous system. The habenular nuclei have a superficial location in the zebrafish forebrain and have a stereotypical unipolar morphology, which simplifies analysis (Bianco et al., 2008). The nuclei act as a relay connecting forebrain regions to the dopaminergic and serotonergic networks in the brain (Aizawa et al., 2011; Hikosaka, 2010) (Figure 6). In zebrafish, the habenula receives sensory inputs from the pallium, eminentia thalami, and the posterior tuberculum, and sends efferent connections to downstream circuitry via the interpeduncular nucleus and raphe (Bianco and Wilson, 2009) (Figure 7). In mammals the habenula receive inputs through the septum, nucleus accumbens, hypothalamus, and globus pallidus, and projects to the rostromedial tegmental nucleus, substantia nigra, ventral tegmental area, raphe, and nucleus incertus (Figure 7). Although the formation of the habenular nuclei is poorly understood, the morphology and function of these nuclei are highly conserved throughout vertebrate evolution and coordinate cognitive processes including learning,

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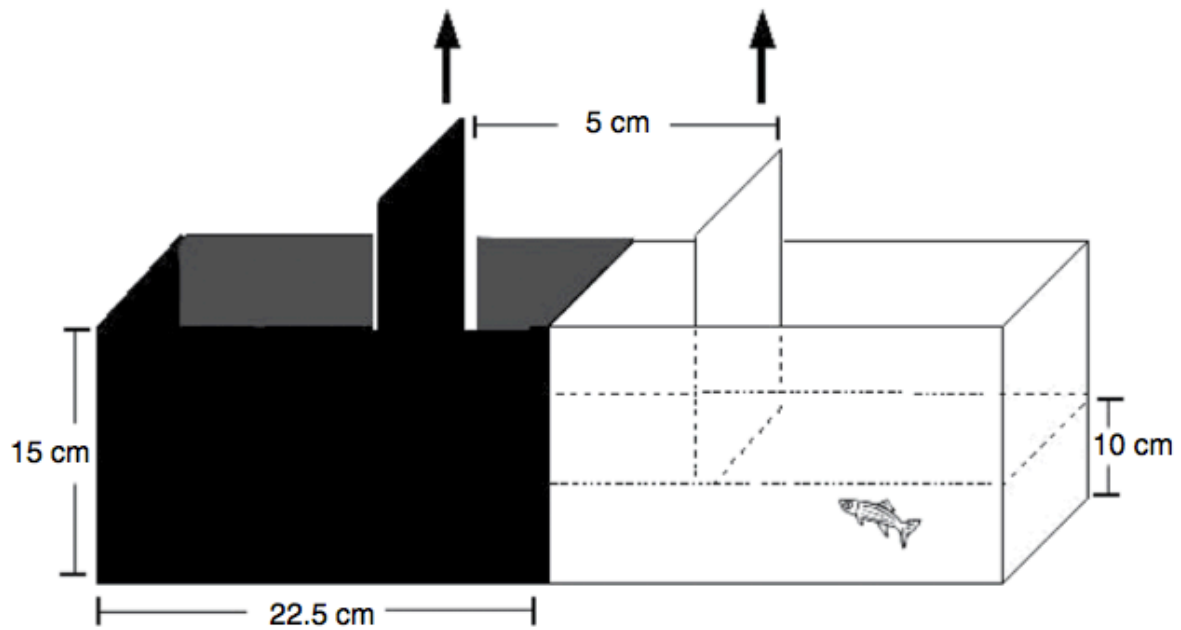
Figure 5. Scototaxis (light/dark) test apparatus. This apparatus is used to test for environmental preference of a zebrafish. It is made of matte acrylic to eliminate reflection. The size depicted is suitable for fish that have a maximum body length of 5 cm. Longer species require proportionally longer arenas, and larvae smaller arenas. Figure modified from Maximino et al. 2010.
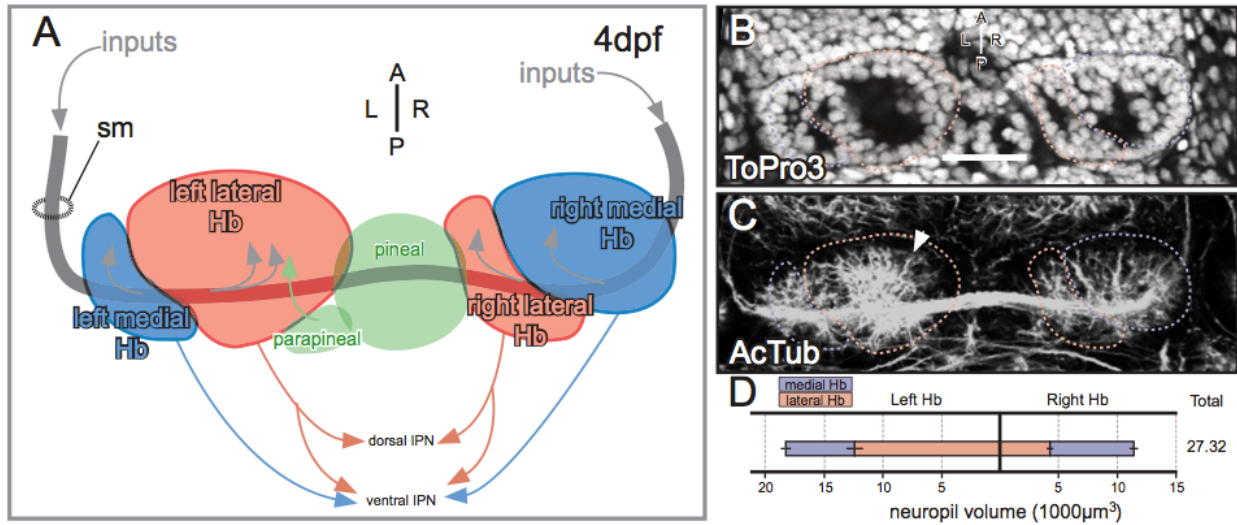
Figure 6. The structure and circuitry of the habenular nuclei. A, The paired Hb receive input via the stria medullaris (sm) and send efferents to the midbrain target, the interpeduncular nucleus (IPN). The asymmetric parapineal organ innervates the left lateral subnucleus (green arrow) and is instrumental in establishing Hb laterality. Each subnucleus is asymmetrically subdivided into medial (blue) and lateral (red) subnuclei, with the lateral subnucleus much larger on the left and the medial subnucleus larger on the right. The nomenclature distinguishing the medial from lateral subnuclei is based on their position in the adult brain, which is the opposite of their position in the 96 hpf larvae shown here. **B**, An optical slice through a ToPro3 (nuclear marker)-stained larvae demonstrates asymmetrical subnucleus organization. **C**, The soma-free regions inside each Hb subnucleus are filled with neuronal processes [acetylated tubulin (AcTub) immunofluorescence] including afferent axons and Hb dendrites. The greatest volume of neuropil is found in the large left lateral subnucleus (arrow). Figure modified from Taylor et al. 2011.

fear response, addiction, and anxiety (Aizawa et al., 2011; Hikosaka, 2010) (Figure 7).

Under normal, non-disease conditions, habenular activation is seen in humans when

receiving negative feedback from an external source, and in non-human primates when

experiencing an aversive stimulus (Matsumoto and Hikosaka, 2008; Ullsperger and

Cramon, 2003). In mice, the habenula is an important regulator of dopamine signaling

for reward-based learning and overconsumption of highly palatable foods (Stamatakis et

al., 2016; Tian and Uchida, 2015). Recently in zebrafish, the habenular nuclei were

shown to be important in social aggression and conflict resolution (Chou et al., 2016).

Overall, the function of the habenula is to process aversive or negative information from

the environment and coordinate an appropriate behavioral response.

Malfunction of the habenular circuitry is observed in schizophrenia and

depression (Morris et al., 1999; Ranft et al., 2009; Savitz et al., 2011; Shepard et al.,

2005). In schizophrenic patients, the habenula is not activated when negative feedback

is received from a short-term memory task, but it is activated in control subjects

(Shepard et al., 2005). This indicates a potential mechanism for the impairment of

negative feedback learning in schizophrenic patients. Additionally, an increased amount

of habenular calcification has been observed in patients with schizophrenia, although

the functional significance of this phenomenon is not well-studied (Caputo et al., 1998).

There is evidence in both animal models and in patients that the habenula is

involved in depression. Rats that are predisposed to developing learned helplessness

exhibit increased metabolic activity in the habenulo-interpeduncular circuit (Shumake

and Gonzalez-Lima, 2003). In patients with major depression, postmortem human

studies have shown decreased habenular volume in both the medial and lateral

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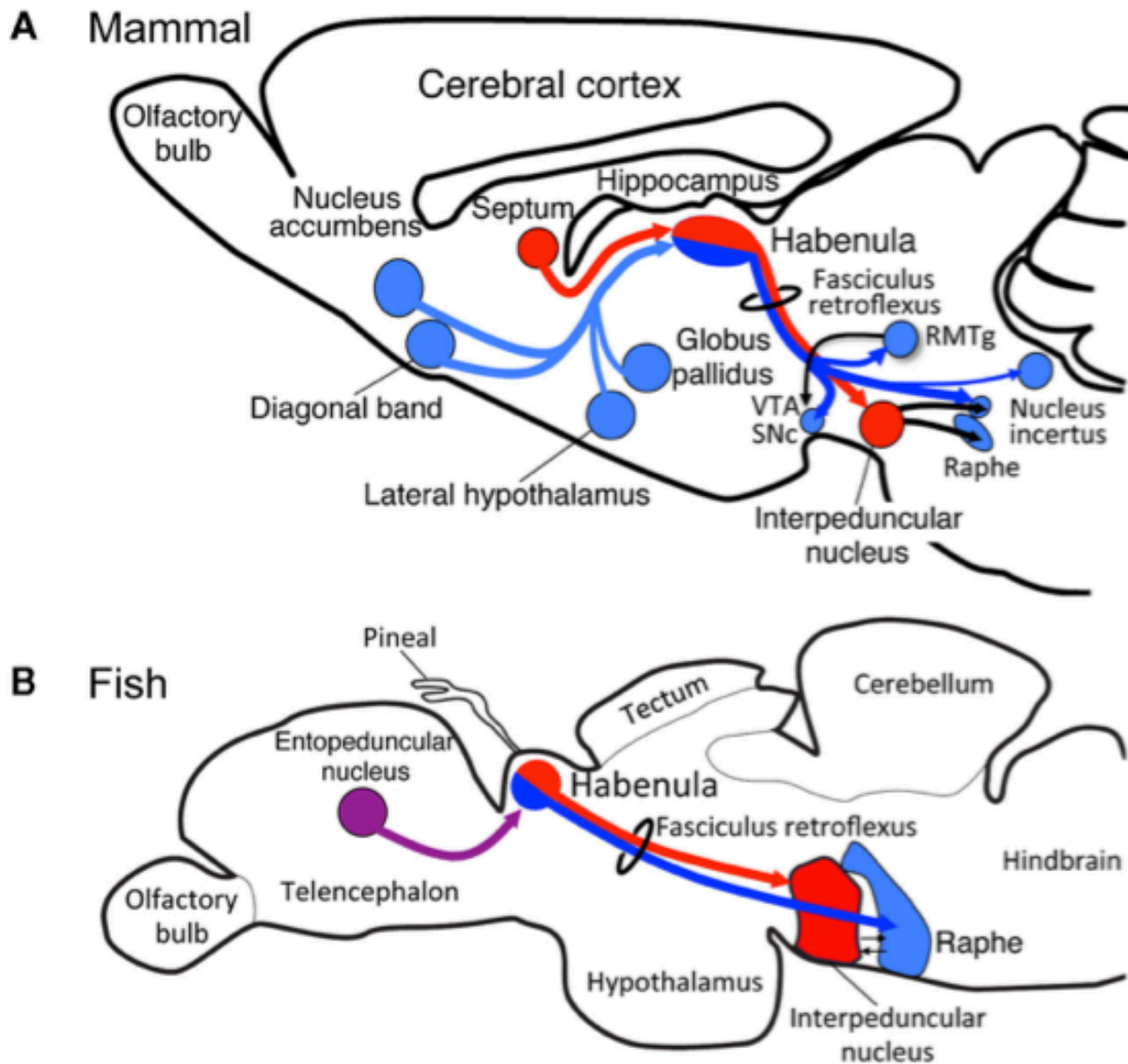
Figure 7. Evolutionary conservation of habenular pathways in vertebrates.
Schematic diagram of sagittal sections from rat **(A)** and zebrafish **(B)** showing homologs
for the medial (red) and lateral (blue) habenular circuitries. The entopeduncular nucleus
[purple in **(B)**] sends axons to the habenula in zebrafish, although the target of those
axons within the habenula remains unclear. RMTg, rostromedial tegmental nucleus;
SNc, substantia nigra, pars compacta; VTA, ventral tegmental area. Figure published in
Aizawa et al. 2011.

subnuclei (Ranft et al., 2009; Savitz et al., 2011). An increase in neuronal activity in the habenula is seen in depressive patients (Morris et al., 1999). Furthermore, deep brain stimulation in the habenula has successfully been used to treat a patient with major depression who was unresponsive to traditional pharmacological treatments (Sartorius et al., 2010).

Recent studies have implicated the habenular nuclei in fear and anxiety-like behaviors in zebrafish (Chen et al., 2015; Facchin et al., 2015). When treated with an estrogen receptor β agonist, WAY-200070, there is an increase in time spent in the non preferred environment in addition to an in *c-fos* expression in the habenula of larvae that have undergone a light-dark test (Chen et al., 2015). Reversal of habenular asymmetry in zebrafish larvae results in a thigmotaxis (edge preference) phenotype, an increase in the onset of swimming, and a decrease in distance swam, indicating an increase in anxiety (Facchin et al., 2015). My research focuses on understanding similar phenotypes in zebrafish larvae with defective dendritogenesis.

Kctd12 and Ulk2

Members of the K+ channel tetramerization domain-containing 12 (Kctd12) gene family are expressed in the habenular nuclei during zebrafish development (Gamse, 2003) (Figure 8A-C). Previous research has implicated Kctd12 genes in ubiquitin ligase adaptation, GABA$_B$ receptor modulation, and dendritogenesis (Bayón et al., 2008; Schwenk et al., 2010; Taylor et al., 2011). In the developing zebrafish, these genes negatively regulate habenular dendritogenesis; mutation of the Kctd12 genes results in increased neuropil volume, while overexpression of the Kctd12 genes results in

decreased neuropil volume (Taylor et al., 2011) (Figure 9-10). In zebrafish there are two orthologous genes that have distinct patterns of expression in the subnuclei of the habenula. Kctd12.1 is expressed in the lateral subnuclei, while Kctd12.2 is expressed in the medial subnuclei (Aizawa et al., 2011; Gamse, 2005; Hikosaka, 2010) (Figure 8). Kctd12 proteins are found in the cytoplasm of habenular neurons, including within the dendrites (Taylor et al., 2011). During dendritogenesis, Kctd12 regulates the activity of Unc-51 Like Autophagy Activating Kinase 2 (Ulk2), a serine/threonine kinase that promotes filopodial extension during neuronal process formation (Yan et al., 1999a; Zhou et al., 2007). Ulk2 has also been shown to suppress axonal branching through non-clathrin coated endocytosis in sensory growth cones (Zhou et al., 2007). Ulk2 is expressed throughout both habenular subnuclei, beginning at 48 hours post fertilization (hpf) until at least 96 hpf (Taylor et al., 2011). Previous work has shown that Kctd12.1 interacts with Ulk2. The presence of Kctd12 appears to inhibit Ulk2 activity, and as a result, dendritogenesis is reduced (Taylor et al., 2011). Expression of Kctd12 in the habenular nuclei is conserved throughout the vertebrate lineage, making the zebrafish system amenable to study Kctd12 function in behavior and disease (Gamse, 2005; Metz et al., 2011). However, the mechanisms by which Kctd12 inhibits Ulk2 activity to modulate habenular dendritogenesis are not known.

Discussion

In this study we focus on biochemical and genetic mechanisms of dendrite formation by Kctd12 and Ulk2 in the habenulae, and the functional repercussions on

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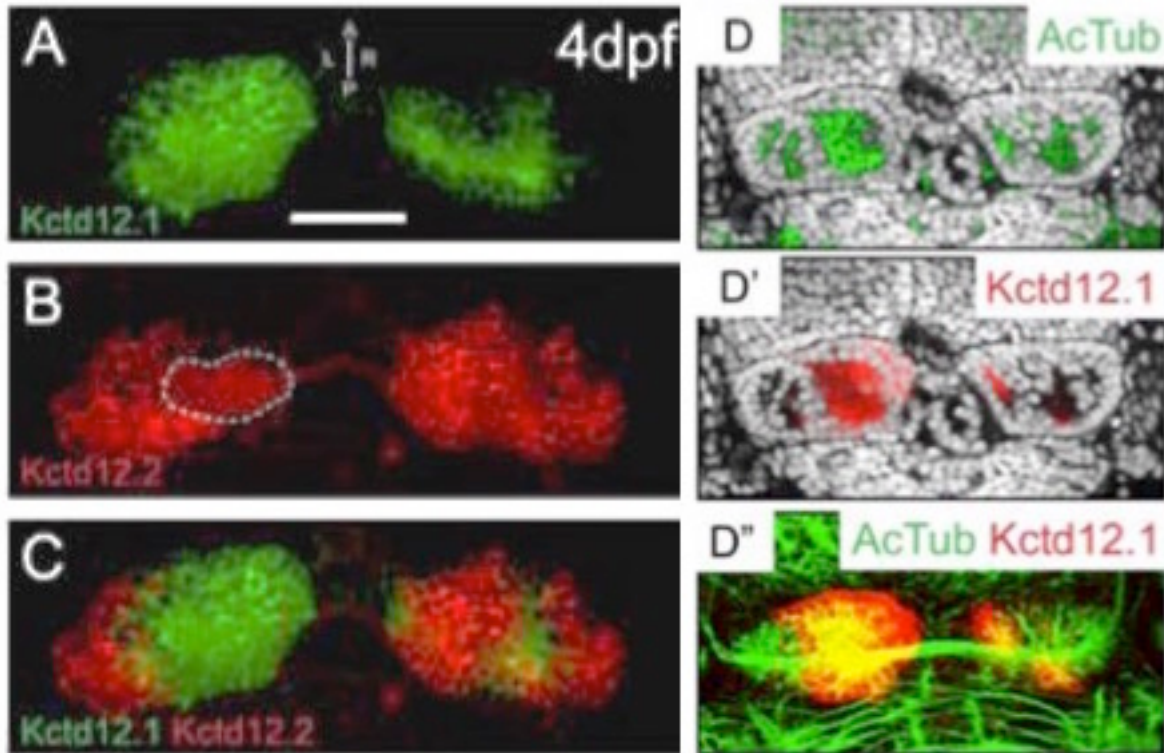
Figure 8. Kctd12 protein expression in the developing habenula. (A) Kctd12.1 protein (green) is primarily expressed in lateral Hb subnuclei. (B) Kctd12.2 (red) is expressed in most neurons of the medial Hb subnuclei, (white dashed line indicates signal from dendritic processes, not Kctd12.2-positive soma) (C) in a largely complementary pattern to Kctd12.1. (D) An optical slice through the 96 hpf Hb shows that Kctd12.1 expression in the left lateral subnucleus (red) is tightly correlated with the neuropil density (green). Figure modified from doctoral dissertation of Taylor 2011.

behavior when dendritogenesis is altered. The goal of this research was to better understand how dendrite development affects circuit function through behavioral outputs. By altering dendritogenesis genetically we are able to look at the relationship between genes and behavior. We expound upon the previously reported Kctd12-Ulk2 regulation of habenular dendritogenesis and show that Kctd12 and Ulk2 interact biochemically, regulate arborization of habenular dendrites, and ultimately affect behavior. Specifically, we show that Kctd12.1 interacts with a 26-amino acid segment of the PS domain of Ulk2. We then demonstrate that Ulk2 promotes branching and elaboration of developing dendrites, but has no effect on extension or retraction events. Finally, we show that increased habenular dendritogenesis decreases anxiety-like behavior. We conclude that Kctd12/Ulk2 regulates the development and ultimately the function of the habenular nuclei.
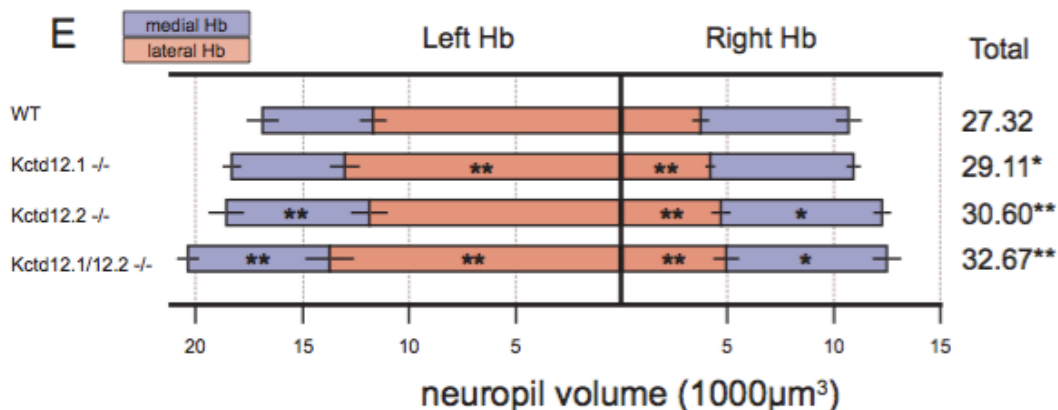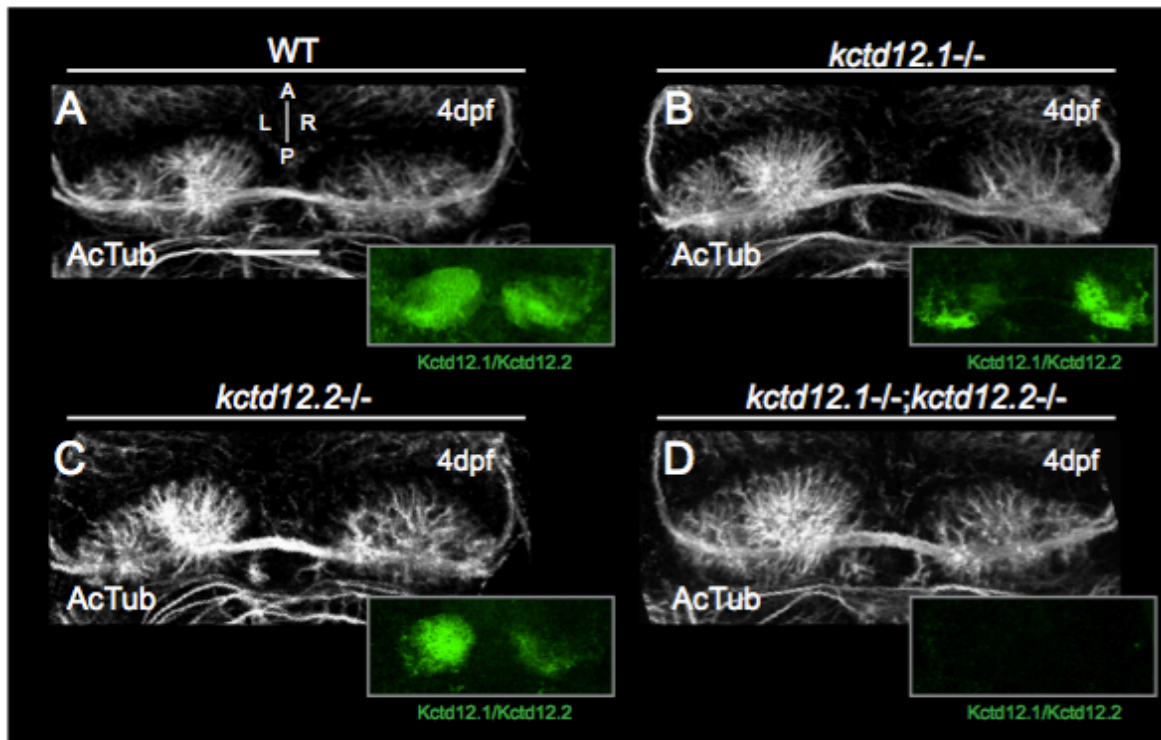
Figure 9. Mutation of Kctd12 proteins leads to excess Hb neuropil. A, At 4 dpf, WT larvae display elaborate extension of neuropil in both Hb and express both Kctd12.1 and 12.2 (green, inset). **B**, The *kctd12.1* coding sequence is disrupted by a large viral insertion in *kctd12.1* mutants (note lack of Kctd12.1 staining in lateral subnuclei in inset). Hb neuropil in Kctd12.1-negative larvae is slightly expanded in lateral subnuclei. **C**, An ENU-induced stop codon in the coding sequence of *kctd12.2* in *kctd12.2* mutants leads to loss of Kctd12.2 protein expression (note lack of Kctd12.2 staining in medial subnuclei in inset). Kctd12.2-negative larvae also display excess elaboration of Hb neuropil, particularly in the medial subnuclei. **D**, *kctd12.1; kctd12.2* double mutants are negative for both Kctd12 proteins (green, inset), and Hb neuropil is expanded in both the lateral and medial subnuclei. **E**, Volumetric quantification of neuropil expansion in *kctd12* mutants. Neuropil expansion is restricted to lateral subnuclei in Kctd12.1-negative larvae, consistent with the expression pattern of Kctd12.1. Neuropil expansion

22

in Kctd12.2-negative larvae affects medial subnuclei as well as the right lateral subnucleus. Double mutant larvae display neuropil expansion in all subnuclei (asterisks indicate statistical difference compared with WT). Scale bars, 50 um (*$p < 0.05$, **$p < 0.01$, two-tailed t-test). Figure published in Taylor et al. 2011.

Figure 10.1. Overexpression of Kctd12.1 inhibits elaboration of Hb neuropil. A, Kctd12.1 (green) is normally expressed only in Hb neurons of the lateral subnuclei. B, In Hb:Gal>Kctd12.1-MT larvae, Kctd12.1-MT fusion protein (red) is expressed at high levels in nearly all Hb neurons. C, D, WT larvae have an elaborate network of neuropil that segregates within each Hb subnucleus (C), but the presence of high levels of ectopic Kctd12.1-MT fusion inhibits the elaboration of Hb neuropil (D). E, Volumetric quantification of Hb neuropil reduction. Overexpression of Kctd12.1-MT causes significant reduction of total Hb neuropil volume compared with WT. All subnuclei are significantly affected with the exception of the right lateral subnucleus (asterisks indicate statistical difference compared with WT). Scale bars, 50 um (*p < 0.05, **p < 0.01, two-tailed t-test). Figure published in Taylor et al. 2011.

Figure 10.2. Overexpression of Kctd12.2 inhibits elaboration of Hb neuropil. A, Kctd12.2 (green) is normally expressed in neurons of the medial subnuclei. **B**, In Hb:Gal>Kctd12.2-MT larvae, expression of Kctd12.2-MT fusion protein is driven at high levels in nearly all Hb neurons. **C**, **D**, WT larvae have an elaborate network of neuropil that segregates within each Hb subnucleus (**C**), but the presence of high levels of ectopic Kctd12.2-MT fusion inhibits the elaboration of Hb neuropil (**D**). **E**, Volumetric quantification of Hb neuropil reduction. Overexpression of Kctd12.2-MT causes a significant reduction of total Hb neuropil volume compared with. All subnuclei are significantly affected (asterisks indicate statistical difference compared with WT). Scale bars, 50 um (*p < 0.05, **p < 0.01, two-tailed t-test). Figure published in Taylor et al. 2011.

CHAPTER II

Kctd12 and Ulk2 partner to regulate dendritogenesis and behavior in the habenular nuclei

Preface

Portions of this chapter have been published in *PLOS ONE* under the title "Kctd12 and Ulk2 partner to regulate dendritogenesis and behavior in the habenular nuclei," by Lee et al. 2014.

Abstract

The habenular nuclei of the limbic system regulate responses, such as anxiety, to aversive stimuli in the environment. The habenulae receive inputs from the telencephalon via elaborate dendrites that form in the center of the nuclei. The kinase Ulk2 positively regulates dendritogenesis on habenular neurons, and in turn is negatively regulated by the cytoplasmic protein Kctd12. Given that the habenulae are a nexus in the aversive response circuit, we suspected that incomplete habenular dendritogenesis would have profound implications for behavior. We find that Ulk2, which interacts with Kctd12 proteins via a small proline-serine rich domain, promotes branching and elaboration of dendrites. Loss of Kctd12 results in increased branching/elaboration and decreased anxiety. We conclude that fine-tuning of habenular dendritogenesis during development is essential for appropriate behavioral responses to negative stimuli.

Methods

Zebrafish Maintenance and Strains

Zebrafish (*Danio rerio*) embryos were obtained by natural spawning and raised at 28.5°C on a 14:10 light/dark cycle. Staging was by age (dpf; days post-fertilization). To prevent pigment formation, 0.003% phenylthiourea was added to embryo media during development. Zebrafish lines used: Tg[*cfos:gal4vp16*]^{s1019t} (Hb:Gal4 hereafter), *kctd12.1*^{vu442}, and *kctd12.2*^{fh312} (Scott et al., 2007; Taylor et al., 2011; Tomoda, 2004; Zhou et al., 2007). Zebrafish adults and embryos were euthanized with an overdose of Tricaine applied in the water. The Vanderbilt University Institutional Animal Care and Use Committee approved all animal work (protocol number C/07/024).

Cloning

The Ulk2 PS domain was divided into four fragments encoding 129-131 amino acids each and these fragments were cloned either individually or in combination into the pGBK bait vector. Fragments correspond to the following amino acids of Ulk2: PS-1 (272 - 401), PS-2 (401 - 531), PS-3 (531 - 661), PS-4 (661 - 790). These fragments were tested for interaction with Kctd12.1 in a yeast 2-hybrid assay (Matchmaker Gold Yeast 2-Hybrid System, Clontech). Subsequently, the first fragment (PS-1) was divided into five smaller fragments of 26 amino acids. Fragments correspond to the following amino acids of Ulk2: PS1.1 (272 - 297), PS1.2 (298 - 323), PS1.3 (324 - 349), PS1.4 (350 - 375), PS1.5 (376 - 401). Adjacent segments were cloned into the pGBK vector. These fragments were tested again by yeast 2-hybrid.

Yeast 2-hybrid

Kctd12.1 was cloned into an activation domain fusion protein plasmid (pGAD), and Ulk2 fragments into a DNA-binding plasmid (pGBK). Y2H Gold (Clontech) yeast were cotransformed according to the manufacturer's instructions with pGAD and pGBK fusion plasmids. Yeast were grown on synthetic media lacking leucine and tryptophan (-LEU, -TRP) to select for plasmid uptake. Single colonies were inoculated in liquid media lacking leucine and tryptophan, adjusted to and OD_{600} of 1, and five fold serial dilutions were prepared in sterile water. Five microliters of each dilution were spotted in parallel on –LEU –TRP plates and on plates additionally lacking adenine and histidine (-ADE, -HIS, -LEU, -TRP). Growth was monitored after 2-3 days incubation at 30°C.

Morpholino Knockdown of Ulk2

Morpholino antisense oligonucleotides (Gene Tools) were injected into the yolk underneath the blastomere(s) of 1-2 cell stage embryos (ulk2^{MO}). The splice site morpholino was injected at 2 ng/embryo, resulting in a ~50% knockdown of protein, as previously described (Taylor et al., 2011). Wild-type sibling controls were not injected with morpholino.

Transgenesis

Transient transgenic animals were generated using transgenes constructed with the Tol2kit (Kwan et al., 2007). Transgenic Hb:Gal4 embryos were injected between 2-8 cell stage with a Tol2 construct containing the upstream activating sequence (UAS) driving expression of GFP fused to a CaaX motif (UAS:CaaXGFP:pA). Embryos were

screened for cardiac GFP at 2 days post fertilization, and imaged between days 3-4 or

fixed at day 4.

Immunofluorescence

Embryos for whole mount immunohistochemistry were fixed at 4dpf in Prefer

fixative (Anatech) and processed as previously described (Taylor et al., 2011). Primary

antibodies were used at the following concentrations: mouse anti-acetylated tubulin

(Sigma-Aldrich) (1:500) and rabbit anti-GFP (Torrey Pines Biolabs) (1:500). Primary

antibodies were detected using donkey anti-rabbit or goat anti-mouse secondary

antibodies conjugated to Alexa 488 or Alexa 568 fluorophores (Invitrogen) (1:300).

Microscopy and Image Analysis

Embryos were anesthetized with Tricaine (Sigma), mounted in 0.6% agarose,

and imaged for one hour on a LSM510 META (Zeiss) confocal microscope with a

40X/1.30 Plan NEOFLUAR oil-immersion objective. Z-stacks of the habenula were

taken at 1 μm intervals for a total depth of 82 μm. Embryos were maintained at 28.5°C

using a forced air heating chamber. Whole mount embryos were imaged in the same

manner. Images were processed using Volocity (Improvision) software. Branches of the

single dendritic protrusion from habenular neurons [7] were counted manually. A

protrusion was counted as a branch (rather than a varicosity) if it was at least 0.70 μm

long. Dendrite length was calculated using Simple Neurite Tracer (ImageJ). A total of 38

sibling larvae were quantified, with 21 WT larvae and 17 morphant larvae measured.

Fifty-seven neurons were quantified in WT larvae, and forty-five in morphant larvae.

Individual dendrites during time-lapse imaging were quantified by on how many extension and retraction events occurred during the imaging period. Sixteen WT and sixteen morphant dendrites were quantified from 4 WT and 4 morphant larvae. Only dendrites that could be unambiguously tracked were selected for quantification. An extension was counted as the protrusion lengthening, and a retraction as the protrusion shortening.

Behavior

Behavioral assays were performed in a 6-well dish on 5 dpf larvae. Larvae were acclimated on a clear background for 2 minutes, and then placed on a half clear/half black background for the choice portion of the experiment. Images were taken every half second for 10 minutes. Images were processed using FIJI to determine the xy position of the fish in all movie frames (Schindelin et al., 2012). Position of the fish was converted to polar coordinates using the R software environment (r-project.org) and the well was divided into clear and dark background halves. Each half was then subdivided by the distance from the center of the well into the 60% of the area at the center and 40% at the edge. We observed highly variable preference for the center v. edge on the light half, while preference for the center for the edge was consistent between experiments. Total time in the arena does not always equal 1, as counts are normalized to total number of frames, not total number of observations.

Statistics

Statistical analysis consisted of one-way ANOVAs using web-based software at
http://vassarstats.net/anova1u.html

Results

Kctd12 interacts with Ulk2 via a 26-amino acid sequence in the PS domain

Previous work showed that Kctd12.1 and Ulk2 interact, which may be important
for the regulation of Ulk2 activity (Taylor et al., 2011). Ulk2 contains three distinct
domains: an N-terminal kinase domain, a carboxy-terminal domain, and a highly
disordered proline and serine-rich middle domain (PS domain) (Figure 11A). The exact
function of the PS domain is poorly understood; however, it is the only domain required
for interaction with Kctd12.1 (Taylor et al., 2011). The PS domain has been shown as a
site of autophosphorylation that is essential for Ulk2 activation (Yan et al., 1999a). We
sought to identify parts of the PS domain that are required for interaction with Kctd12.1
using the yeast 2-hybrid assay. The PS domain (519 amino acids) was divided into 4
subdomains (each containing 129-131 amino acids) and those regions were expressed
either individually or in combination as indicated (Figure 11A). The first subdomain (PS-
1) was sufficient for interaction with Kctd12.1 (Figure 11B,D). We further narrowed down
the potential interaction site by subdividing the PS-1 into five equal segments (each
containing 26 amino acids), and expressing constructs containing overlapping

Figure 11. Kctd12.1 interacts with a subset of amino acids in the PS domain of Ulk2. Transformants expressing a fragment of the PS domain of Ulk2 fused to the Gal4 DNA-binding domain were mated with transformants expressing Kctd12.1 fused to the Gal4 activation domain. **A.** Kctd12.1 contains two domains: an N-terminal domain (NTD) that promotes oligomerization, and a C-terminal domain (CTD) of undefined function. Ulk2 contains three domains: an N-terminal serine-threonine kinase domain (K), an internal proline-serine-rich region (PS rich), and a CTD involved in protein–protein interactions. Fragment 1.4 of the Ulk2 PS rich domain is the site of interaction with Kctd12. **B.** Region 1 of the Ulk2 PS domain is the site of interaction with Kctd12.1. **C.** PS domain fragments containing region 1.4 (PS1.3-1.4 and PS1.4-1.5) interact most strongly with Kctd12.1, suggesting the site of interaction is PS1.4. **D.** Summary of the yeast two-hybrid results. Fragment 1.4 of the Ulk2 PS rich domain is the site of interaction with Kctd12. Figure published in Lee et al. 2014.

combinations of these segments. This analysis showed that an interaction with Kctd12.1 only occurred when segment 1.4 was included in the assay (Figure 11C,D). We concluded that segment 1.4 within the PS domain of Ulk2 is required for interaction with Kctd12.1.

Ulk2 positively regulates dendritic branch formation

Previously we reported a decrease in neuropil volume in Ulk2 morphants, but the underlying mechanism of this phenomenon is not understood (Kwan et al., 2007; Taylor et al., 2011). Ulk2 could potentially alter neuropil volume by affecting dendrite extension, retraction, or branching. To test the specific function of Ulk2 on habenular dendritogenesis, we used an antisense morpholino oligonucleotide (MO) to interfere with Ulk2 function, and measured dendrite length and branch number at 4 dpf, after most habenular dendritogenesis has been completed. Individual habenular neurons were labeled with membrane-tethered GFP to visualize the dendrites (Figure 12A,B). When compared to controls, we found no apparent change in individual dendrite length (data not shown), but a significant decrease in the number of dendrite branches in Ulk2 morphants (Figure 12C).

Ulk2 positively regulates dendrite elaboration

Previous work in our lab showed a dramatic decrease in overall dendritic volume (Schindelin et al., 2012; Taylor et al., 2011) when Ulk2 is knocked down, but we had no explanation for the mechanism of this phenotype. While static images are convenient for
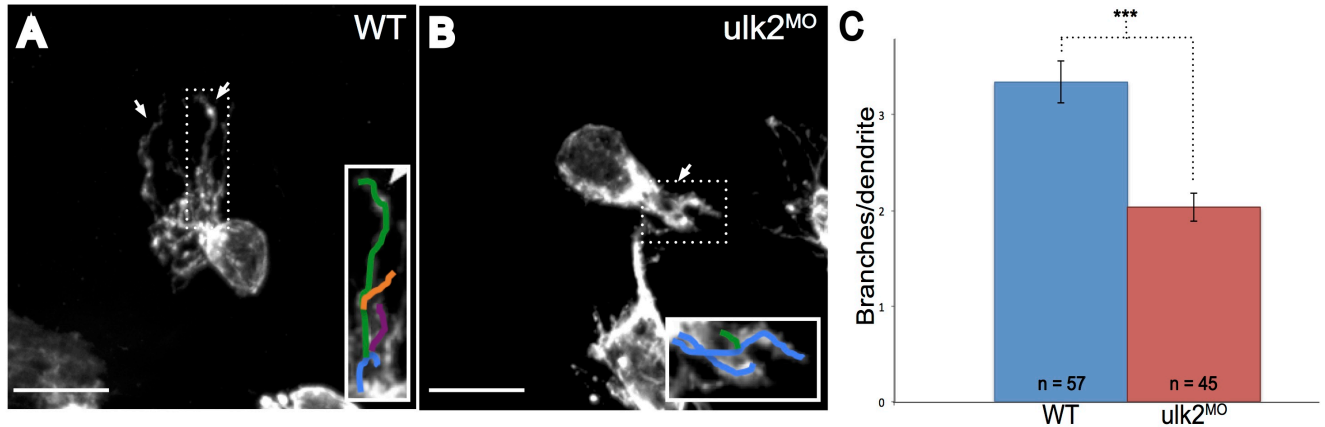
Figure 12. Ulk2 promotes dendritic branching. A and B. Habenular neurons were scatter labeled using Hb:Gal4 and UAS:CaaXGFP to label isolated neurons. Arrows point to dendrites on WT (highly branched) and morphant (reduced branched) neurons. Insets demonstrate how branches were quantified; each branch of a dendrite is drawn in a different color. Scale bar is 10 μm. C. Branches per dendrite were calculated by dividing the total number of branches by the number of dendrites per larva. Fifty-seven neurons were quantified in WT larvae and forty-five in morphant larvae. Ulk2 morphants had a decreased number of branches relative to WT (***$p < 0.0001$, ANOVA).

observing global phenotypic changes in dendrites, time-lapse imaging gave us insight

into the dynamics of dendritogenesis. From our time-lapse imaging, we concluded that

Ulk2 plays a role in dendrite elaboration. We quantified the number of extensions and

retractions of the GFP-labeled dendrites during a one-hour imaging period and

compared Ulk2 morphants and controls (Figure 13A,B). We found that there was no

difference in the number of extensions or retraction events (Figure 13C,D). However the

extension/retraction ratio was reduced in Ulk2 morphants (Figure 13E), which is

consistent with our results in fixed embryos and suggests that Ulk2 plays a role in

maintaining dendrite elaborations. We did not see a change in cell morphology or

polarity. This observation suggests that Ulk2 is required for dendrite elaboration, and

without it, dendrites are unable to be maintained and become stable.

**Kctd12 proteins negatively regulate thigmotaxic behavior in a non-preferred
environment**

The habenular nuclei function as an important regulator of various behaviors

such as learning, fear response, addiction, escape, and anxiety (Hikosaka, 2010; Taylor

et al., 2011). Recently, the zebrafish has become a useful model organism to study

genetic mechanisms of fear and anxiety related behaviors using paradigms such as

scototaxis (dark environmental preference) and thigmotaxis (edge preference)

(Maximino et al., 2010; Schnörr et al., 2012; Taylor et al., 2011). We investigated the

functions of Kctd12 proteins in scototaxic and thigmotactic behaviors. We observed no

scototactic phenotype, as all genotypes preferred the light, regardless of their

genotypes (Figure 14A). However, in the dark, *kctd12* mutants (*kctd12.1*, *kctd12.2*, or
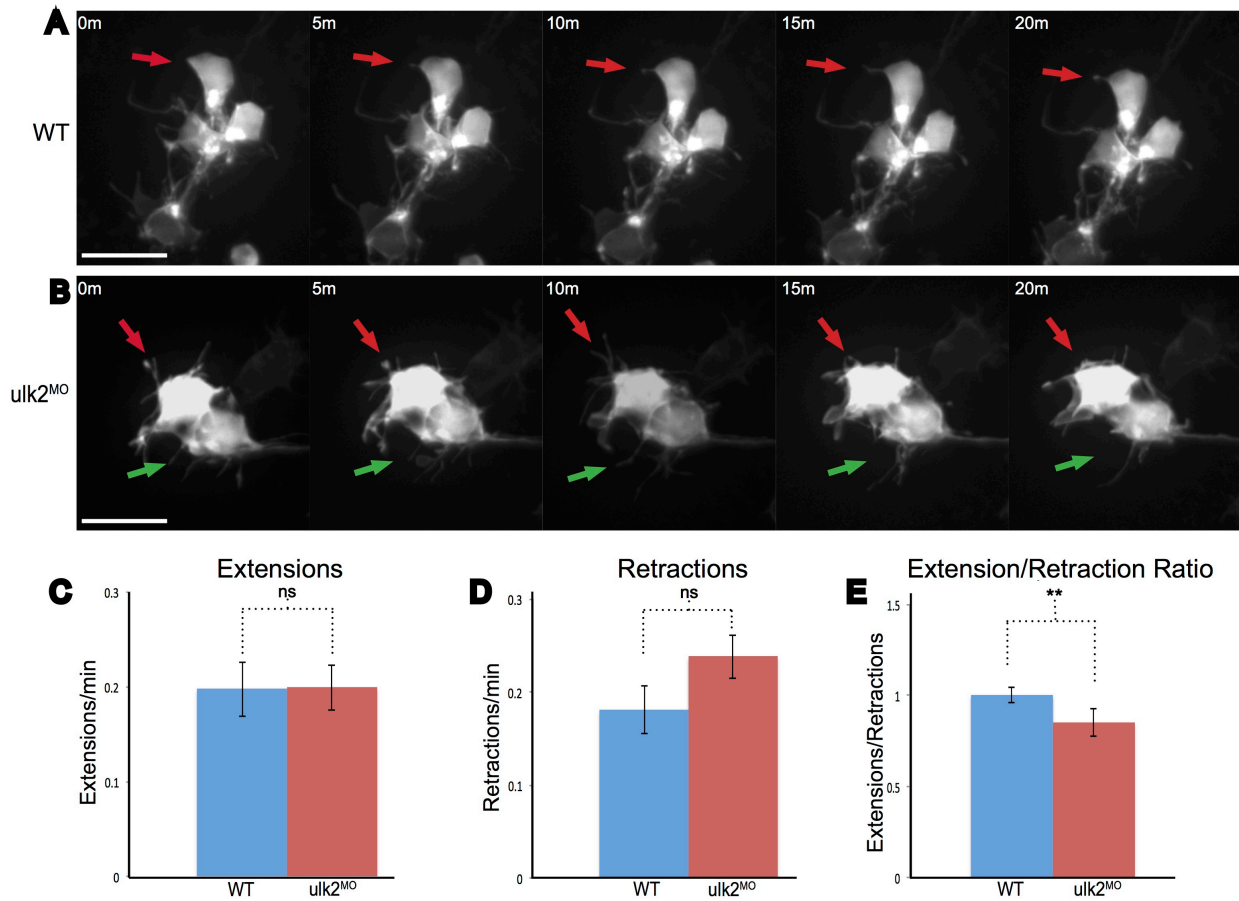
Figure 13. Ulk2 morphants have a decreased extension/retraction ratio. A. and B. Habenular neurons were scatter labeled and imaged for one hour. Red arrows point to dendrites being tracked over the 20-minute period displayed. Green arrows point to an additional dendrite tracked during the same time-lapse. Dendrites in the WT neurons are maintained for longer than in Ulk2 morphants. Scale bar is 10 μm. **C. and D.** The number of extension (p = 1) and retraction (p = 0.1095) events per dendrite per minute was similar between WT (n = 16) and Ulk2 morphants (n = 16), but **E.** the ratio of extension events to retraction events per dendrite was significantly reduced (**p = 0.00488, ANOVA) in Ulk2 morphants.

double mutants) exhibited an increased preference for the center of the arena, as opposed to the edge (Figure 14B). This phenotype indicates that overelaboration of habenular dendrites correlates with a decrease in anxiety-like behavior, as indicated by the increase in time spent by the mutant larvae in the center of the arena.

Discussion

Our analysis showed that an Ulk2 interaction with Kctd12.1 only occurred when segment 1.4 within the PS-1 domain was included in the assay (Figure 11C,D). This indicates that segment 1.4 is the likely area to which Kctd12 binds to inhibit Ulk2 from promoting dendritogenesis. Since Ulk2 autophosphorylation is required for its kinase activity, we hypothesize that Kctd12 binds to segment 1.4 of the PS domain to prevent autophosphorylation and thereby negatively regulate Ulk2 function (Yan et al., 1999a). Segment 1.4 contains 2 serines and 1 threonine. Future studies will indicate whether one or more of these residues are autophosphorylated, and whether or not Kctd12 can inhibit Ulk2 autophosphorylation in the absence of these key amino acids. It is also possible that other residues outside segment 1.4 are phosphorylated, and the inhibition of phosphorylation is indirect. It will also be interesting to see the phenotypic effect of a Kctd12-insensitive Ulk2 variant *in vivo*.

We found that Ulk2 promotes dendritic branching, while having no effect on dendrite length (Figure 12). Ulk2 has been previously reported to promote early endosome formation and axonal growth, and to suppress axon branching (Taylor et al., 2011; Tomoda, 2004; Zhou et al., 2007). In contrast, we found that Ulk2 promotes dendritic branching or branch stabilization. Knockdown of Ulk2 protein decreased

38
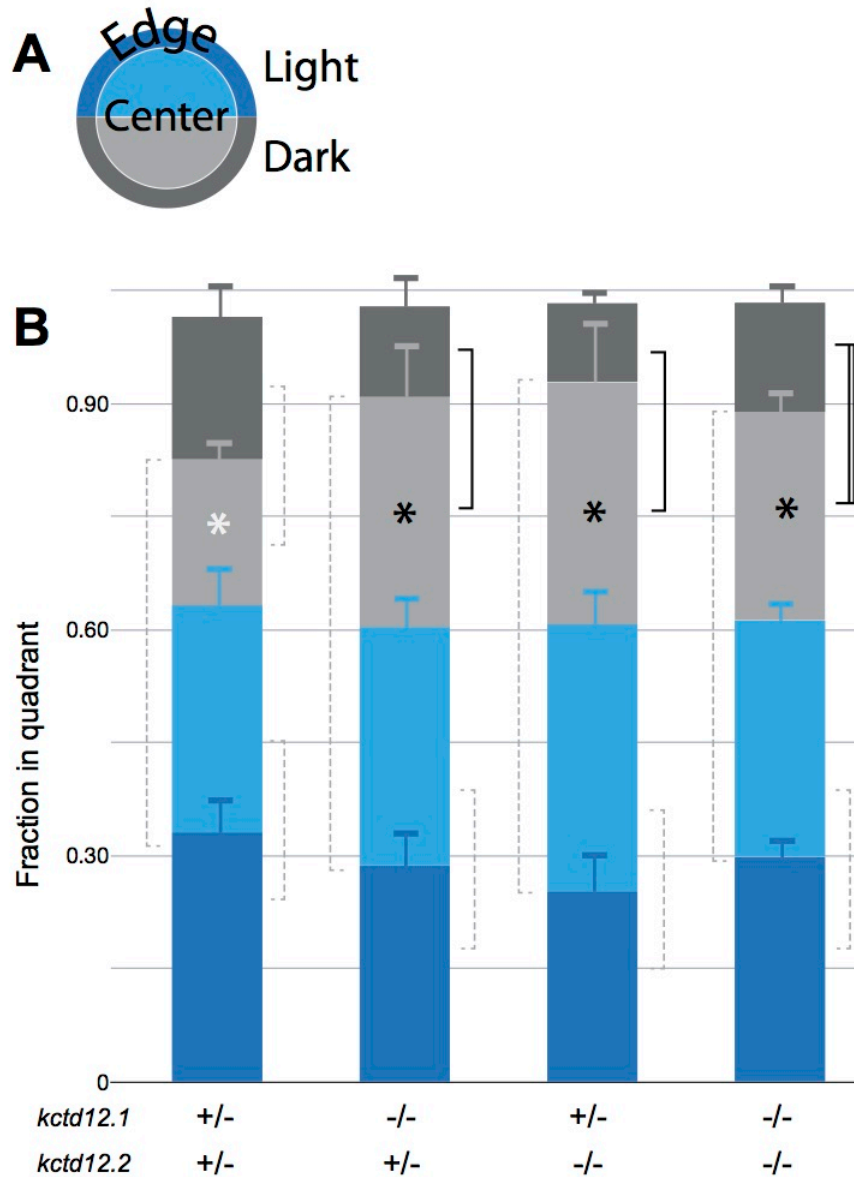
Figure 14: Kctd12 mutation causes a decrease in thigmotactic behavior. A. Each well of a 6-well plate is divided into halves with dark (gray) or clear (blue) bottoms. The well is further divided into center (light) and edge (dark). The position of the fish is recorded every half second to measure scototaxis or thigmotaxis. The center is defined as 60% of the area of the circle (inner) and the edge as the remaining 40% (outer). Double heterozygous larvae were used as controls **B.** Scototaxis. No change in scototaxis is detected between genotypes. All genotypes prefer the light. Dotted lines are ns. Thigmotaxis. Preference for the center increases in Kctd12 single and double mutants (solid line: $p < 0.05$, double line: $p < 0.01$, 2-tailed t-test). Pooling mutant animals for a comparison to double heterozygote controls showed a significant effect of genotype (white vs. black * $p < 0.01$). kctd12.1/2 +/- n=19; kctd12.1/2 -/- n=9; kctd12.1 -/-, kctd12.2 +/- n=11; kctd12.1 +/-, kctd12.2 -/- n=7.

branch number, without altering branch length. These results indicate that Ulk2 possibly has differing roles in axons and dendrites: in axons it promotes elongation and suppresses branching, while in dendrites it only promotes branching. This phenotype might also be due to cell-type or species differences. In addition, we found that Ulk2 regulates dendritic elaboration. Although Ulk2 knockdown did not change the number of extensions or retractions, the ratio of extensions to retractions decreased in Ulk2 morphants (Figure 13). This observation suggests that Ulk2 is required for dendrite elaboration, and without it, dendrites are unable to be maintained and become stable. It will be important to determine the effect of Kctd12 mutation on dendrite length and branching, as well as dendritic elaboration. Recently another habenular dendritogenesis factor was identified: Daam1, a formin family protein, mediates habenular dendritogenesis as well as axiogenesis (Colombo et al., 2013). Future studies should consider the possible interaction between Kctd12, Ulk2, and Daam1.

Finally, we found that overelaboration of habenular dendrites from Kctd12 mutation negatively regulate thigmotaxic behavior in a non-preferred environment (Figure 14). With these experiments, we cannot rule out that this behavior phenotype is not due to changes in non-habenular neurons where Kctd12 is expressed. Kctd12.1 is also expressed in the retina at 96 hpf (Gamse, 2005; Kwan et al., 2007; Thisse et al., 2001). Kctd12.2 is expressed in rhombomere 4 from 10-13 somites and in various small groups of neurons in the forebrain between 48-96 hpf (Choe et al., 2011; Gamse, 2005). Although Kctd12 expression is not restricted to the habenula, it is the only place where both Kctd12.1 and 12.2 are expressed together. Since the habenula and thigmotaxis are related to anxiety, and mutation of either Kctd12 reduces thigmotaxis, the most

40

parsimonious explanation is that altered Kctd12 function in the habenula is responsible (Hikosaka, 2010; Lee et al., 2010; Okamoto et al., 2012; Schnörr et al., 2012).

Our study is the first to implicate Kctd12 genes in the regulation of anxiety-like behavior. Mutation of Kctd12 causes an overelaboration of dendrites in the habenula, consistent with the negative regulation of Ulk2 by Kctd12 (Taylor et al., 2011). When Kctd12.1 or 12.2 are absent, the larvae appear less anxious. It is unclear how the overelaboration of dendrites causes a decrease in anxiety-like behavior. However, since this study there has been a publication showing that increased habenular activity corresponds to a decrease in anxiety-like behavior (Chen et al., 2015). This leaves the possibility that overelaboration of dendrites causes the same behavioral response as increased neuronal activity. Future studies to examine the relationship between dendrite volume and behavioral output will be important to parse out the relationship between genes, circuits, and behavior.

Acknowledgements

CHAPTER III

Future Directions

Proper habenular dendritogenesis is important for regulation of dopaminergic and serotonergic pathways and ultimately fear and anxiety-based behaviors in the vertebrate system. The zebrafish is an amenable system to study the relationship between genes, circuits, and behaviors, due to its genetic and imaging toolkits. In this study, I characterized a protein-protein interaction important for dendritic development, and showed how these proteins regulate dendrite morphology and behavior *in vivo*.

The Kctd12-Ulk2 interaction is potentially a novel Ulk2 regulatory system. Our data indicates that Kctd12.1 binds to the domain of Ulk2 required for autophosphorylation. I hypothesize that this physical interaction is how Kctd12 inhibits Ulk2 activity to suppress dendrite development. To test this hypothesis, I would use site directed mutagenesis to determine the minimum amino acids required for Kctd12.1-Ulk2 interaction. I would then construct a mutant Ulk2 that cannot interact with Kctd12.1, and quantify its ability to autophosphorylate in the presence of Kctd12.1 using an *in vitro* autophosphorylation assay. I would expect to see that wild type Ulk2 could not autophosphorylate in the presence of Kctd12, while mutant Ulk2 could. Next, I would express this mutant Ulk2 in zebrafish larvae, and quantify changes in neuropil density, branch number, and dendrite elaboration in the absence of a Kctd12-Ulk2 interaction *in vivo*. I predict that Ulk2 would promote overelaboration of dendrites, since Kctd12 would not be able to bind and suppress this phenotype. It would also be interesting to determine if Kctd12.2 interacts with Ulk2 in the same region as Kctd12.1

Towards the end of this study, we received an Ulk2 mutant generated by Cecilia Moen's laboratory. Unfortunately, I was not able to characterize the Ulk2 mutant; however, I would expect to see a similar if not more severe defect in dendrite branching and elaboration. Replicating these studies with the mutant would be important to determine if a complete absence of Ulk2 phenocopies an Ulk2 morphant.

Currently, we do not have a mechanism by which Ulk2 promotes dendritic branching and elaboration. There are several approaches we could use to identify targets in this pathway. First, we could use a candidate gene approach. Since Ulk2 plays a role in autophagy as well as endocytosis in axons, we could look at endocytic pathway components that Ulk2 could be using to promote branch formation and dendritic elaboration (Alers et al., 2012). Potential targets could include autophagy-related gene 13 (Atg13) or focal adhesion kinase family interacting protein of 20 kDa (FIP200), which are known Ulk2 interactors involved in vesicular trafficking. On the other hand, we could use next generation RNA sequencing techniques to identify alterations in gene expression levels in Ulk2 morphants/mutants versus wild type neurons. This would give us a more complete picture of the pathways Ulk2 uses to regulate dendritogenesis.

Another important study would be to determine how Kctd12.1 affects dendrite extension, retraction, and branching. I would repeat the Ulk2 morphant experiments with Kctd12 mutants, as well as with Kctd12 overexpressing larvae. I would expect that in opposition to Ulk2, Kctd12 would suppress dendrite branching and elaboration. Furthermore, I could corroborate our model by looking at dendritic dynamics in

43

Kctd12/Ulk2 mutant larva, where I would expect to see the Ulk2 mutant phenotype prevail, as Ulk2 appears to be epistatic to Kctd12 (Taylor et al., 2011).

Finally, I would repeat the scototaxis and thigmotaxis experiments with Ulk2 mutant larvae. Since Kctd12 mutation results in an overelaboration of habenular dendrites and a decrease in anxiety-like behavior, I would expect that Ulk2 mutants would have diminished habenular dendrite volume and increased anxiety-like behavior. This is another key experiment in validating the model that Kctd12-Ulk2 interaction regulates habenular dendritogenesis with consequence to behavioral outputs. This is a novel pathway in understanding how genes influence an organism's interaction with its environment.

On a different note, Kctd12 is also known to modulate $GABA_B$ receptor signaling (Schwenk et al., 2010). $GABA_B$ receptors are heterodimeric metabotropic G-protein coupled receptors consisting of one $GABA_{B1a}$ or $GABA_{B1b}$ subunit and one $GABA_{B2}$ subunit (Benarroch, 2012). I wanted to know if $GABA_B$ receptors were present in the developing zebrafish habenula. I found that only the $GABA_{B2}$ subunit was expressed during habenular development (Figure 27). There is data showing that $GABA_B$ homodimers can form functional receptors *in vitro*; however, this remains to be demonstrated *in vivo* (Kuner, 1999; Martin et al., 1999). It is possible that Kctd12s also modulate $GABA_B$ receptor signaling to further regulate habenular dendrite development.

CHAPTER IV

Introduction

Preface

Portions of this chapter have been published in *Cell Health and Cytoskeleton* under the title "Dendritic spine morphology and dynamics in health and disease," by Lee et al. 2015.

Introduction

The second part of this thesis takes a deeper look into dendritogenesis; specifically the formation of dendritic spines, which are protrusions emanating from the dendritic shaft that form excitatory synapses with incoming axons. The goal of this project was to understand how a cytoskeletal interacting protein influences the development of spines in cultured hippocampal neurons. Unlike habenunlar neurons, which are not spiny, hippocampal neurons have many spines and are important players in the learning and memory circuitry. This introduction discusses the current knowledge of dendritic spine formation and sets the stage for my project.

The Hippocampus, Memory, and Plasticity

A major attribute of the brain is its ability to store and retrieve information. Not only does the brain process information from the environment to the body (learning), it also encodes these experiences so that they may be stored and retrieved (memory). The neocortex and hippocampus are vital brain areas for learning and memory (Eichenbaum, 2004; Wiltgen et al., 2004). The neocortex plays a paramount role in

memory processing, encoding, and consolidation of memories. Over a century ago we began understand that the memories nearest in time to the onset of disease or trauma are more unstable than those from the distant past, indicating that memories can be reinforced over time (Ribot 1882, Burnham 1903, Barbizet 1970, Korsakoff 1887, Rose and Symonds 1960). More specifically the hippocampus's function first became apparent after the removal of a patient's medial temporal lobe resulted in an inability to form declarative memories and retrograde amnesia (Corkin et al., 1984; Scoville and Milner, 1957). More recent studies have demonstrated that hippocampal damage results in memory formation defects throughout mammalian systems (Anagnostaras et al., 1999; Victor and Agamanolis, 1990; Zola-Morgan and Squire, 1990). Currently the theory of memory processing and consolidation suggests that neocortical information is connected through the hippocampus to form a memory (Bontempi et al., 1999; Wiltgen et al., 2004) (Figure 15). At this point the memory is unstable and malleable (Duncan, 1949; Flexner et al., 1965; Gordon and Spear, 1973; McGaugh and Krivanek, 1970). During sleep a feedback mechanism consolidates the information back to the neocortex, and the memory is no longer associated with the hippocampus (Bontempi et al., 1999; Wiltgen et al., 2004) (Figure 15). There are several hypothesis as to how exactly memories are consolidated. One is that repetitive communication between the neocortex and hippocampus leads to strengthening of neocortical synapses (Takashima et al., 2009). Another suggests that related memories are stored in "schemas" by network modification (McClelland et al., 1995). Finally, the multiple trace theory suggests that context-independent memories, such as factual information, is stored in the neocortex, while context-specific information is stored in the hippocampus (Conway

46

et al., 1999; Dudai, 2012; Fink et al., 1996; Nadel and Moscovitch, 1997).
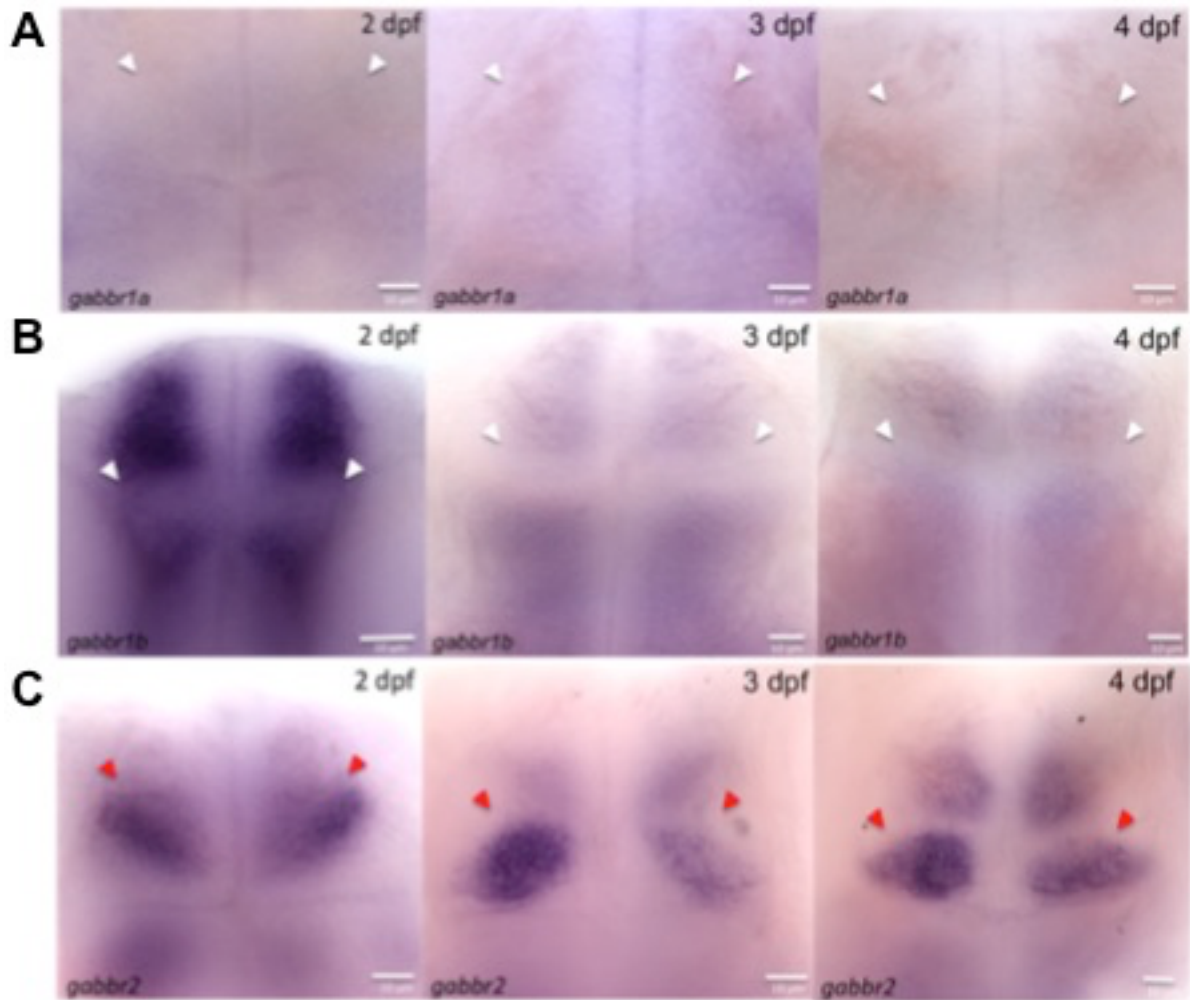
Figure 15. GABA$_{B2}$ receptors are present in the developing habenula. A-C. *In situ* hybridization of GABA$_{B1a}$ (**A**), GABA$_{B1b}$ (**B**), and GABA$_{B2}$ (**C**) receptors. GABA$_{B1a}$ receptors only show basal expression, while GABA$_{B1b}$ receptors are excluded from the habenula (white arrows). GABA$_{B2}$ receptors are highly concentrated in the habenula (red arrows) from day 2-4dpf.

Memory acquisition and consolidation also correlates with gene expression in the neocortex and hippocampus: retrieval of recent memories correlates with immediate early gene (IEG) expression in the hippocampus, but not neocortex, while retrieval of remote memories correlates with increased IEG expression in the neocortex but not hippocampus (Frankland et al., 2004; Maviel et al., 2004). Additionally synaptic remodeling occurs to accommodate these rapid changes (Chen et al., 2014; Cui et al., 2004; Nakazawa et al., 2004; Tsien et al., 1996). Alterations in spine number and maintenance, as well as activation of NMDARs and α–CaMKII activity and adrenergic signaling are seen with memory formation and consolidation (Frankland et al., 2004; Thomas, 2015; Wang et al., 2003; Xu et al., 2009; Yang et al., 2009).

Though seemingly permanent, memory consolidation and maintenance is a dynamic process. Stable memories can be adapted by reconsolidation (McKenzie and Eichenbaum, 2011; Misanin et al., 1968; Nader and Hardt, 2009; Nader et al., 2000). After retrieval a memory becomes labile, which must be stabilized by reconsolidation (Tronson and Taylor, 2007). By blocking protein synthesis after memory recollection, researchers have observed a disruption in long-term memory in rats (Nader et al., 2000). However, delaying treatment of the protein synthesis inhibitor negated the memory defects, suggesting there is a critical window of destabilization. Consolidation and reconsolidation are distinct molecular phenomena: consolidation is BDNF-dependent, while reconsolidation is dependent upon the transcription factor Zif268 (Lee et al., 2004). Reconsolidation is important for enhancing and updating memories, though the interrelated molecular mechanisms distinguishing storage and retrieval deficits remains unclear (Tronson and Taylor, 2007). Substantial advances have been

49
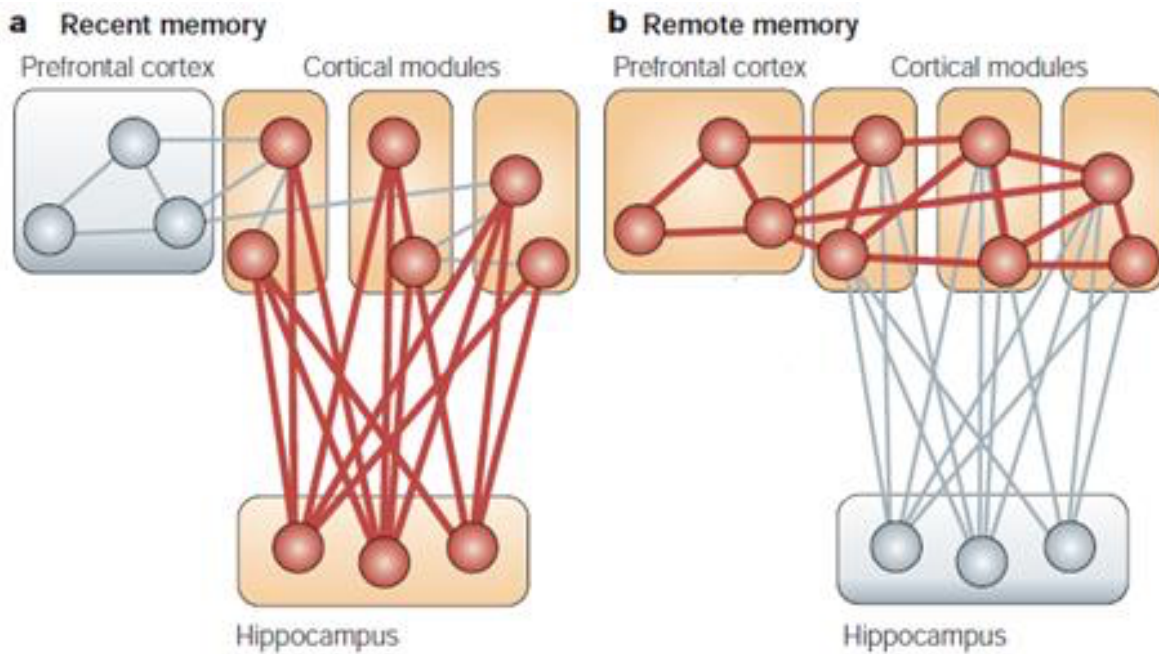
Figure 16. Memory Consolidation. A. Hippocampal-cortical networks are actively engaged during phases of memory acquisition. **B.** After consolidation, the role of the hippocampus is limited as memories become dependent on cortico-cortical networks Figure adapted from Frankland and Bontempi 2005.

made in understanding the molecular basis of learning and memory acquisition, while memory retrieval remains poorly understood (Thomas, 2015).

Dendritic Spines

Dendritic spines are actin-rich structures that form the postsynaptic terminals of excitatory synapses in the brain. Santiago Ramón y Cajal first described dendritic spines, using Golgi staining, near the end of the 19th century and proposed that these spines were sites of axonal and dendritic contact (Ramón y Cajal, 1891; Ramón y Cajal, 1899) (Figure 16). Decades later, with the advent of electron microscopy, these spines were indeed shown to be sites of excitatory synaptic contact between neurons, proving that Cajal's hypothesis was correct (Gray, 1959a; Gray, 1959b). These and subsequent studies highlight the importance of dendritic spines and pose interesting questions as to the specific functions of these structures (Koch and Zador, 1993; Yuste and Majewska, 2001). Dendritic spines most likely have functions other than to simply connect axons and dendrites. This is supported by the observation that many inhibitory synaptic inputs occur on dendritic shafts in the absence of spines; however, it should be noted that recent data indicate that some inhibitory neurons have functional spines, and inhibitory synaptic inputs can occur on spines of cortical pyramidal neurons (Chiu et al., 2013; Keck et al., 2011; Kubota et al., 2007; Scheuss and Bonhoeffer, 2014). A widely held theory is that spines serve as biochemical compartments in the cell (Koch and Zador, 1993; Müller and Connor, 1991). The unique morphology of spines, which consists of an enlarged head and a thin neck, makes them ideal structures to function as postsynaptic biochemical compartments that separate synaptic terminals from dendritic shafts (Müller

and Connor, 1991; Yuste et al., 2000). In addition, spines could serve as electrical compartments, which can maintain membrane potentials that are distinct from those of the parent dendrites (Harnett et al., 2012; Tsay and Yuste, 2004; Yuste, 2013). The electrical isolation of individual spines might provide a mechanism to allow neurons to integrate and independently regulate the strength of a large number of synaptic inputs (Yuste, 2013). Moreover, the compartmentalization of spines most likely contributes significantly to the efficiency of synaptic transmission and plasticity. Intriguingly, the spine neck width is reported to be an important factor in regulating compartmentalization (Tønnesen et al., 2014). Other roles for dendritic spines have been proposed, and the specific functions of spines are an active area of interest and debate that warrants continued research (Shepherd, 1996; Yuste and Majewska, 2001).

The functions of dendritic spines are governed, at least in part, by their morphology. They range in morphology from filopodia-like protrusions, which are thought to be spine precursors, to more mature stubby, thin, or mushroom-shaped structures (Peters and Kaiserman-Abramof, 1970). Stubby spines do not have a neck whereas thin and mushroom-shaped spines consist of long necks that are connected to small and large bulbous heads, respectively; filopodia-like protrusions are extensions from the dendrite that lack a bulbous head. Spine morphology is malleable, and their shape can change over time, even on a time scale of minutes or faster (Dunaevsky et al., 1999; Fischer et al., 1998; Lendvai et al., 2000) (Figure 17). In the case of dendritic filopodia, the dynamic, exploratory nature of these structures could be beneficial in forming connections with axons (Ziv and Smith, 1996). After an initial interaction between dendritic filopodia and axons, synapses can assemble on a relatively rapid
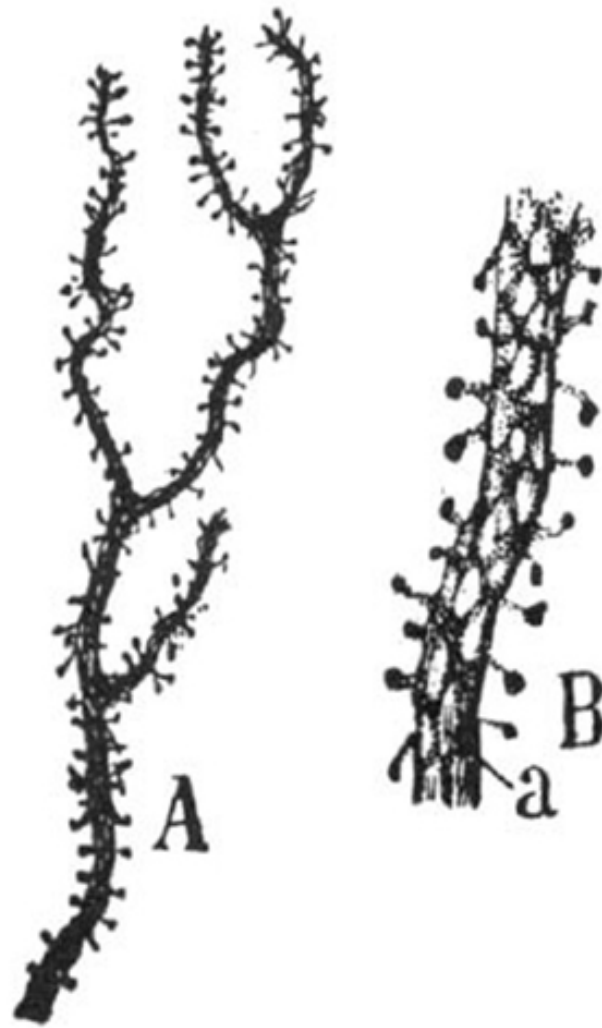
Figure 17. Original dendritic spine illustrations from Cajal. A. and B. Drawings depict dendritic spines (a marks the shaft) from a cerebellar Purkinje cell, as drawn from Golgi material. Figure modified from (Yuste, 2015) with permission from "Herederos de Santiago Ramón y Cajal."

time scale (hours) (Friedman et al., 2000; Li and Sheng, 2003). For most mammals, spine and synapse formation is widespread during early postnatal development and is followed by a pruning phase during adolescence that eliminates unnecessary or improper synaptic connections (Chen et al., 2014; Petanjek et al., 2011). In adults, dendritic spine formation and elimination are at an equilibrium with a fraction of spines being consistently added or removed (Chen et al., 2014). Morphological changes, which are usually activity-dependent, also occur in more mature spines and are associated with synaptic plasticity (Bliss and Lomo, 1973). Synaptic plasticity, which entails the strengthening or weakening of synapses over time as well as synapse formation and elimination (structural plasticity), is widely believed to be the cellular basis of learning and memory (Bliss and Lomo, 1973; Holtmaat et al., 2006; Ito and Kano, 1982). In vivo imaging in the cerebral cortex of mice has shown that spine dynamics/remodeling is associated with different forms of learning (Holtmaat et al., 2006; Lai et al., 2012; Xu et al., 2009; Yang et al., 2009). Spine head size is thought to correlate with memory formation, with smaller spine heads being associated with temporary memory storage and learning while large spine heads more permanent memories (Bourne and Harris, 2007). Synaptic plasticity is also thought to be necessary for the encoding and storage of memory. The foundation of this theory dates back to Donald Hebb, who postulated a link between alterations in synaptic activity and memory storage.

Experimental attempts to model Hebb's theory led to the discovery of long-term potentiation (LTP), which typically uses high frequency stimulation to increase synaptic transmission and spine head size (Bliss and Lomo, 1973) (Figure 18). In order to encode information efficiently, an increase in synaptic strength must be

counterbalanced by a weakening of synapses by a process termed long-term depression (LTD). LTD can be provoked experimentally with low frequency stimulation, causing a prolonged decrease in synaptic transmission and spine head size (Ito and Kano, 1982; Ito et al., 1982). These experimental models, LTP and LTD, have been invaluable in generating a wealth of data showing the essential function that synaptic plasticity has in learning and memory. Another line of evidence that supports this link is the well-documented association between abnormalities in dendritic spine/synapse formation and plasticity and numerous neurological disorders, including autism, Alzheimer's disease (AD), schizophrenia, and intellectual disability (Fiala et al., 2002; Penzes et al., 2011).

Cytoskeletal Interactions Governing Dendritic Spine Morphology and Development

Actin is the major cytoskeletal element in dendritic spines where filamentous actin (F-actin), which results from the polymerization of monomeric actin (G-actin), is found at high concentrations. Actin is highly dynamic in spines and is regulated by neuronal activity (Star et al., 2002) (Figure 19). Fluorescence recovery after photobleaching experiments with GFP-actin showed that 85% of the actin turned over in less than a 1 minute, and LTD stabilized a significant portion of the dynamic actin (Star et al., 2002). Spines have three distinct pools of F-actin (Honkura et al., 2008). A dynamic pool of actin at the spine tip with a high turnover rate and a more stable pool at the base where turnover is slow. A third pool of F-actin formed throughout the spine head following glutamate uncaging, which increases synaptic strength; this actin pool was relatively stable and mediated spine head enlargement, demonstrating that
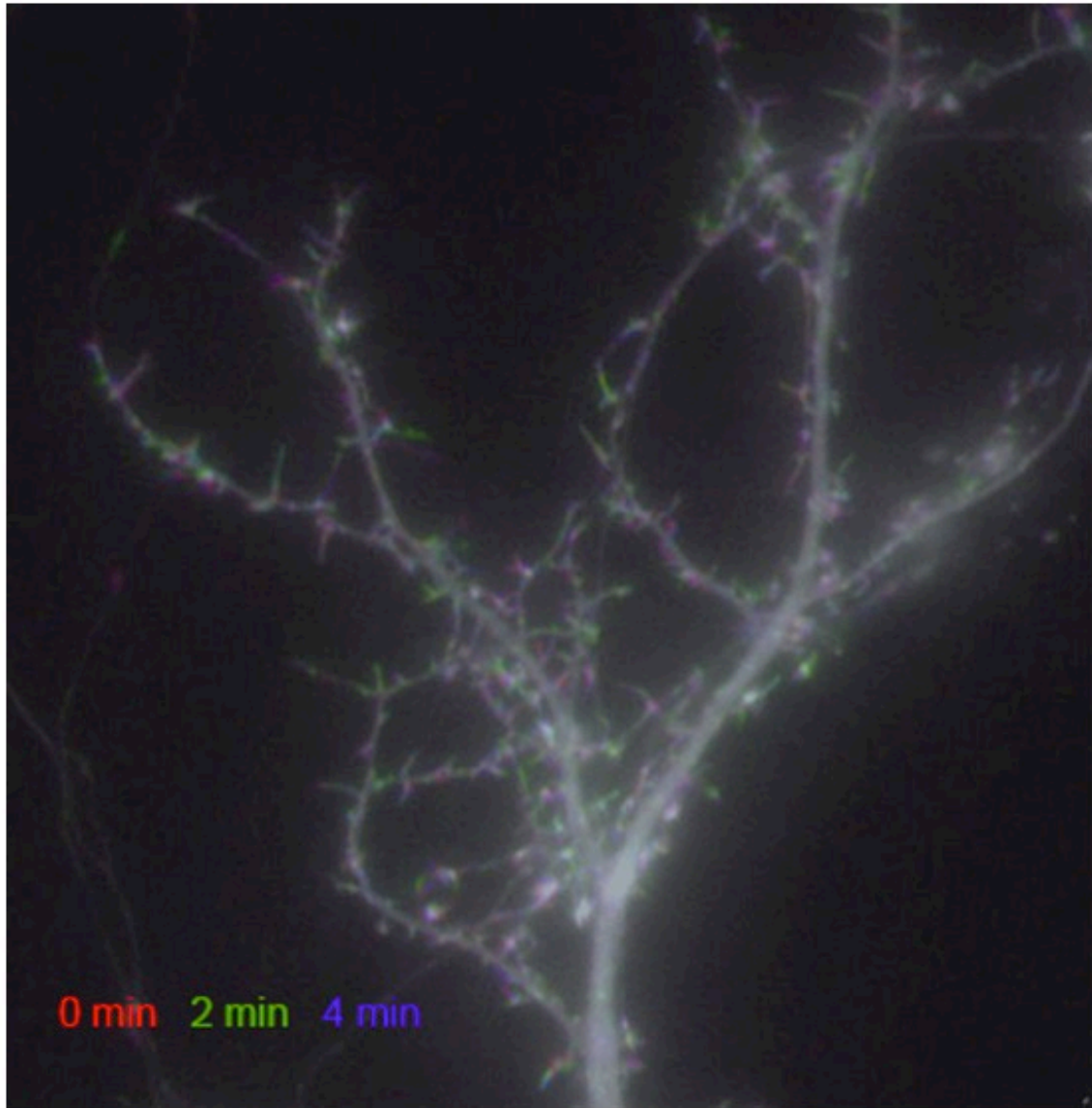
Figure 18. Dendritic spines are dynamic structures. A three-color temporal overlap of a hippocampal neuron expressing green fluorescent protein-tagged β-actin. In general, green and magenta indicate dynamic spines whereas white depicts more stable spines. Figure published in Lee et al. 2015.

neuronal activity modulates actin dynamics (Honkura et al., 2008). A dynamic actin cytoskeleton is critical for the morphological malleability of spines, which underlies the formation and plasticity of these structures.

Actin remodeling is regulated by the Rho family of small GTPases that includes Rho, Rac, and Cdc42. These small GTPases are molecular switches which exist in an active (GTP-bound) and an inactive (GDP-bound) state. The cycling of the GTPases between the active and inactive states is regulated by three types of proteins: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine dissociation inhibitors (GDIs). GEFs promote the exchange of GDP for GTP, activating the GTPase; GAPs increase intrinsic GTP hydrolysis, returning these proteins to an inactive state; and GDIs form soluble complexes with the GTPases and sequester them in an inactive state (Cherfils and Zeghouf, 2013). Rac and Cdc42 induce dendritic spine formation, whereas Rho promotes the retraction and loss of spines (Luo et al., 1996; Tashiro et al., 2000; Wegner et al., 2008b). Rac can promote spine formation through its downstream effector p21-activated kinase (PAK) (Zhang, 2005a). Cdc42 stimulates spineogenesis and enlargement of spine heads via activation of the actin-binding protein neural Wiskott-Aldrich syndrome protein (N-WASP) and the Arp2/3 complex, which mediates the formation of branched actin filaments (Wegner et al., 2008b). Moreover, loss of Cdc42 in mice results in deficits in synaptic plasticity and remote memory recall (Kim et al., 2014). Rho family GEFs and GAPs also have important roles in spine development and function. Mice lacking the Rac GEF karilin-7 exhibit defects in cortical spine density and in working memory (Cahill et al., 2009). The Rac GEF Tiam 1 is required for dendritic spine formation, and knockdown of Tiam1 causes a decrease in
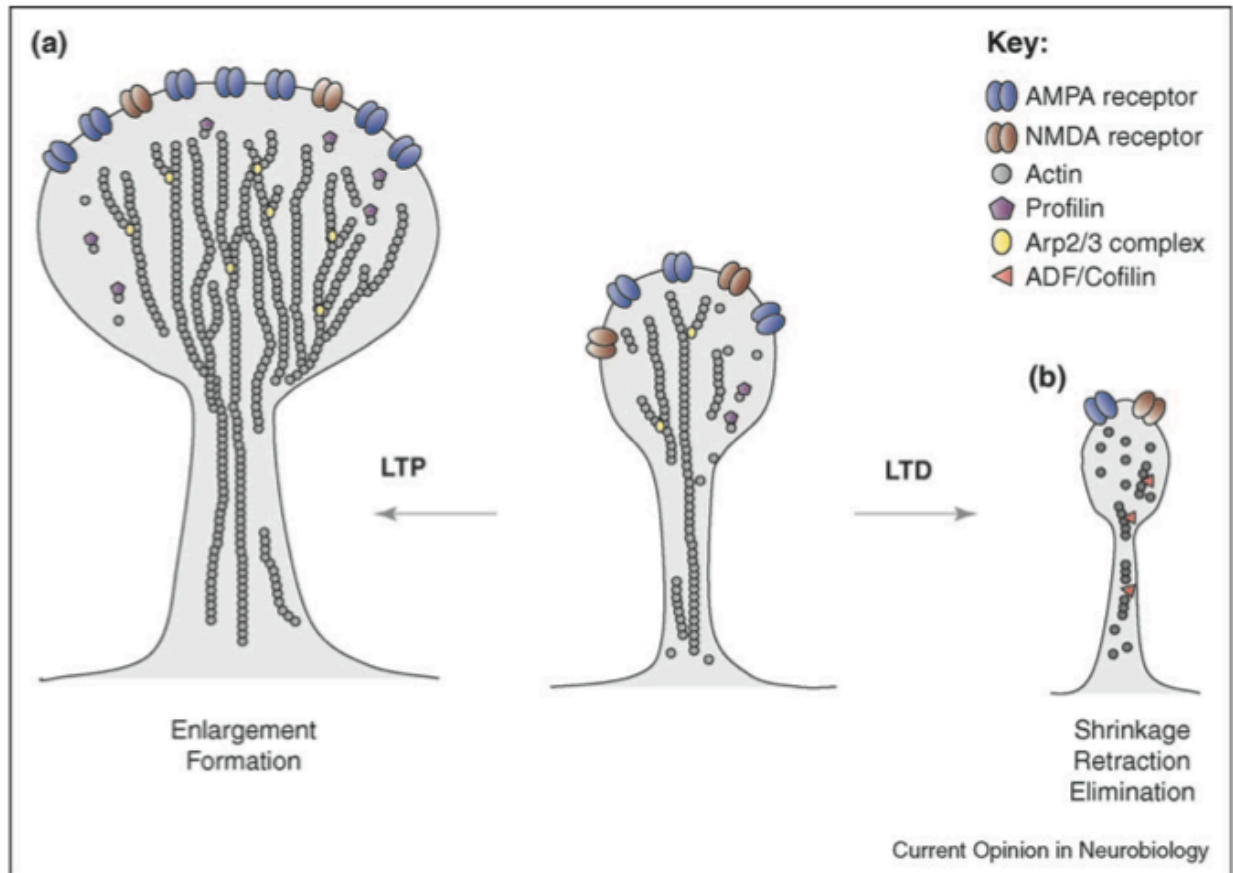
Figure 19. Changes in actin polymerization and spine morphology with LTP and LTD. (a) LTP is associated with a shift of actin equilibrium toward F-actin (F-actin is depicted as linear chains of monomeric G-actin [single circle]) in spines, enlargement of the spine head, and recruitment of more AMPA receptors to the postsynaptic membrane. Profilin promotes actin filament assembly by increasing the availability of actin-ATP for polymerization. The Arp2/3 complex stimulates nucleation of new actin filaments and formation of branches. (b) By contrast, LTD stimulation shifts the equilibrium toward actin depolymerization, resulting in shrinkage or loss of spines. The actin severing protein ADF/cofilin might be involved in spine shrinkage. Figure published in Tada et al. 2006.

spine and synapse density (Tolias et al., 2005; Zhang and Macara, 2006). β-PIX, another GEF, regulates spine formation through activation of Rac and subsequently PAK (Zhang, 2005b). GEF-H1, a Rho family GEF, inhibits spine formation and negatively regulates spine length through a Rho pathway (Kang et al., 2009). Rho family GAPs also contribute to the development of dendritic spines and synapses. Expression of the Rac GAP a1-chimaerin leads to a loss of spines by inhibiting new spine formation and by mediating the pruning of existing spines (Buttery et al., 2006; Van de Ven et al., 2005). Oligophrenin-1, a Rho-GAP, regulates the maturation and plasticity of excitatory synapses by inhibiting Rho activity (Nadif Kasri et al., 2009). Furthermore, p190 RhoGAP modulates spine morphogenesis by regulating Rho GTPase activity (Zhang and Macara, 2008). The function of GDIs in regulating spine development and plasticity is currently unknown and represents an exciting avenue for future investigation.

Actin-Binding Proteins in Spine Development

Actin binding proteins (ABPs) also play a large role in modulating actin dynamics. Therefore, a number of ABPs are known to regulate spine/synapse formation and plasticity via their ability to modulate actin. As already discussed, N-WASP, which promotes the polymerization of branched actin filaments through activation of the Arp2/3 complex, induces spine formation and enlargements of spine heads (Wegner et al., 2008b) (Figure 18). Knockout of ArpC3, a subunit of the Arp2/3 complex, in forebrain excitatory neurons in mice led to a loss of spines and defects in synaptic plasticity and episodic memory (Kim et al., 2013). WAVE1, another WASP family member and an effector of Rac, regulates spine morphology and density as well as synaptic plasticity,
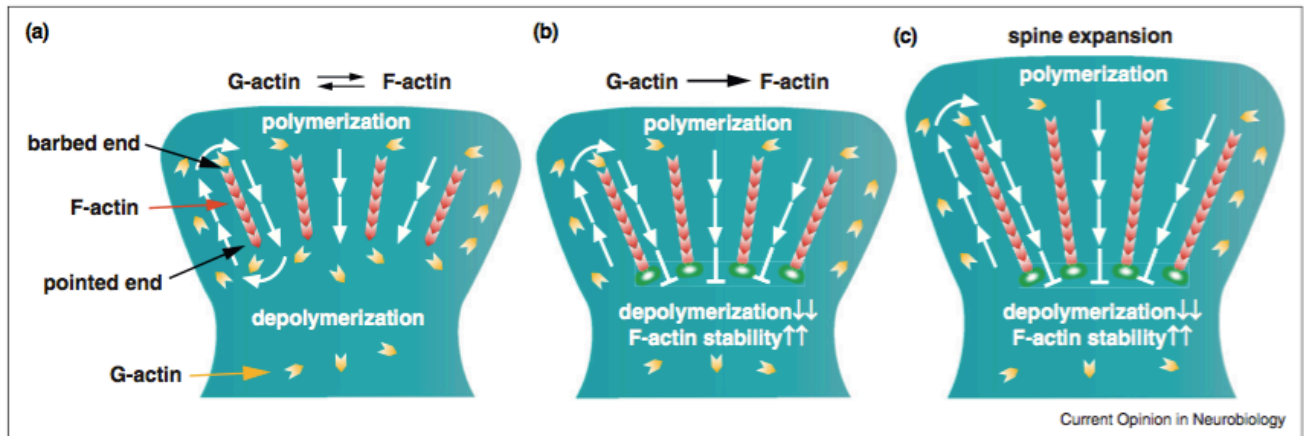
Figure 20. Proposed mechanisms for spine expansion. (a) In a naive spine, there is a constant treadmilling of actin from the periphery to the center of the dendritic spine, maintained by an equilibrated rate of F-actin polymerization/depolymerization. (b) LTP induction stabilizes the actin filaments and slows down the depolymerization at the pointed end of F-actin located at the core of dendritic spine. (c) Polymerization continues in the periphery of dendritic spine, thereby generating the driving force that expands the spine head. Figure published in Bosch et al. 2012.

and loss of this protein results in deficits in learning and memory (Kim et al., 2006; Soderling et al., 2007). Knockdown of cortactin, which also activates the Arp2/3 complex, similarly led to alterations in spine number and morphology (Hering and Sheng, 2003). Formins are another class of actin nucleators that are implicated in spine regulation. Formins can be activated by RhoGTPases, but unlike the Arp2/3 complex, formins produce unbranched actin filaments (Pruyne, 2002; Sagot et al., 2002). One study demonstrated that mammalian *diaphanous*-related formin2 (mDia2) promotes filopodia formation; however, future studies are needed to further explore the role of formins in spine formation and plasticity (Hotulainen et al., 2009). Proteins containing WASP homology 2 (WH2) actin binding domains are a third class of actin nucleators that were most recently identified (Quinlan et al., 2005). Mice lacking Spir-1, the founding member of the WH2 protein family, have reduced spine density on cortical neurons and exhibit increased fear memory (Pleiser et al., 2014).

Actin remodeling in dendritic spines is mediated by other proteins, such as profilin, cofilin, and gelsolin (Figure 18). Profilin promotes actin polymerization by acting as a nucleotide exchange factor, catalyzing ADP to ATP exchange on G-actin, and by binding G-actin and increasing its incorporation into actin filaments (Pollard and Cooper, 1984; Tilney et al., 1983). Activity-dependent targeting of profilin II, a brain-enriched isoform, is associated with the stabilization of spine morphology whereas blockade of profilin targeting to spines led to destabilization of spine structure (Ackermann and Matus, 2003). Interestingly, fear conditioning in rats resulted in profilin redistribution into spines in the lateral amygdala, which corresponded with an increase in size of their postsynaptic densities (Lamprecht et al., 2006). Mice deficient in profilin II unexpectedly

61

do not have defects in LTP/LTD or learning and memory (Pilo Boyl et al., 2007). Moreover, conditional knockout of profilin I in the forebrain of mice did not result in significant defects in excitatory synaptic transmission or in spine density or morphology (Görlich et al., 2012). Because profilin I and II could have overlapping functions, a double knockout will be necessary to decipher the functions of profilin in regulating spine/synapse development and plasticity. Cofilin is another key regulator of actin dynamics that binds to and severs actin filaments (Andrianantoandro and Pollard, 2006) (Figure 18). Cofilin-mediated actin turnover is important for controlling spine length and morphology (Hotulainen and Hoogenraad, 2010). Furthermore, suppression of cofilin activity is important for the stabilization of mature spines (Shi et al., 2009). Cofilin localization and activity in spines is modulated by synaptic plasticity (Chen et al., 2007; Pontrello et al., 2012). In addition, cofilin-mediated actin turnover regulates the size of spine heads during LTP and LTD, and loss of cofilin impairs synaptic plasticity and associative learning (Chen et al., 2007; Gu et al., 2010; Rust et al., 2010; Zhou et al., 2004) (Figure 18). The activity of gelsolin, which also severs actin filaments, is important for regulating actin turnover during LTD (Star et al., 2002).

The Neurabin Family of Actin-Binding Proteins

Neurabin I is an scaffolding F-actin binding protein first isolated from neural tissue (Nakanishi et al., 1997). It contains an F-actin binding domain, a PSD-95, D1gA, ZO-1-like (PDZ) domain, a PP1-binding domain, and a coiled-coil domain, and is concentrated at the synapse and growth cones of lamellipodia in developing neurons. This study also showed that antisense oligonucleotide treatment caused a decrease in

neurite formation in cultured rat hippocampal neurons. A 2007 study found that NrbI is required for GABAergic motor neuron synapse formation, neuronal polarity, and axonal/dendritic fate determination (Hung et al., 2007). Additionally, expression and knockdown of NrbI in cultured rat hippocampal neurons cause a decrease in neuronal complexity and neocortical migration (Causeret et al., 2007). NrbI targets PP1 to the synapse to promote synaptic depression; a PP1-defective mutant causes an increase in AMPAR phosphorylation a reduction in surface receptor expression (Hu et al., 2007). NrbI has been implicated in long-term potentiation (LTP) and fear-based memory as well (Wu et al., 2008). The NrbI knockout mice showed reduced LTP in hippocampal CA1 neurons, along with an increase in AMPAR-mediated synaptic transmission. When placed in a shock chamber and conditioned to learn that a sound would be accompanied by a shock, these mice exhibited less freezing behavior, indicating impaired contextual memory. NrbI knockout mice, as well as mice receiving an NrbI siRNA injection in the anterior cingulate cortex also show a decrease in anxiety-like behavior (Kim et al., 2011).

NrbII/spinophilin was first discovered as an F-actin and protein phosphatase 1 (PP1) binding protein with similar domain organization as neurabin (Allen et al., 1997; Satoh, 1998). The two proteins are 51% identical in sequence and 74% identical at the protein level (Sarrouilhe et al., 2006) (Figure 20). It contains an actin-binding domain (ABD), a receptor-binding domain, a PP1 binding domain, a PDZ domain, and a coiled-coil region. While NrbI expression is restricted to neural tissue, NrbII/spinophilin is ubiquitously expressed but specifically enriched in the cerebral cortex, caudatoputamen, hippocampus, and cerebellum (Ouimet et al., 2004; Satoh, 1998). Subcellularly, both of

these proteins are concentrated at the synapse as well as at cadherin-based cell-cell interaction sites (Satoh, 1998). While usually present in dendritic spines at excitatory synapses, spinophilin can also be found in dendritic shafts adjacent to inhibitory synapses of GABAergic interneurons in the hippocampus and ventral palladium (Ouimet et al., 2004). However spinophilin is not always associated with spines, as seen in the cerebral cortex. Spinophilin concentration varies in the spine microdomain (Muly et al., 2003). The PSD contains the highest amount of spinophilin, and the spine neck contains the lowest, with no spinophilin detected beyond 400 nm from the synapse. Spinophilin is also present in dendritic shafts, preterminal axons, and glia.

Spinophilin regulates F-actin bundling, depending on its phosphorylation status (Satoh, 1998). Protein kinase A (PKA) phosphorylates spinophilin in the ABD to modulate anchoring of the spinophilin-PP1 complex in dendritic spines (Hsieh-Wilson et al., 1999). PKA phosphorylation increases spinophilin affinity for actin filaments, but decreases stoichiometry of binding, indicating there are multiple actin binding sites at the N-terminus. Additional experimentation has shown that the actin-binding site extends beyond the first 155 amino acids, or that there is a second binding site between amino acids 151 and 282 (Barnes et al., 2004). This PKA phosphorylation also causes decreased binding to the PSD and cytosolic localization (Hsieh-Wilson et al., 2003). Ultimately phosphorylation of spinophilin by PKA might cause an increase in phosphorylation and activation of AMPA channels. In addition spinophilin is phosphorylated by CaMKII at Ser-100 and Ser-116 in the ABD1 domain, reducing its affinity for F-actin (Grossman et al., 2004). This alters the subcellular localization of
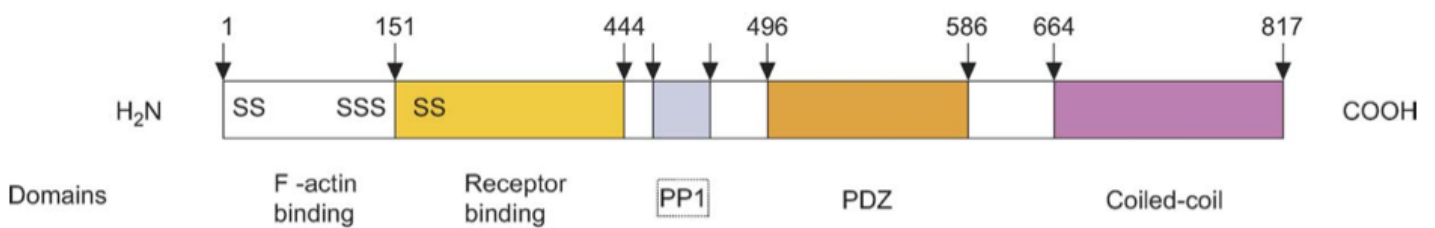
Figure 21. A schematic representation of the domain structure of full-length spinophilin. The protein phosphatase 1-binding domain is located within amino acids 447 and 451 (R/K-R/K-V/I-X-F). The main potential phosphorylation motifs are Ser-15 and Ser-205 (extracellular-signal regulated protein kinase 2), Ser-17 (cyclin-dependent kinase 5), Ser-94 and Ser-177 (protein kinase A), Ser-100 and Ser-116 (Ca^{2+}/calmodulin-dependent protein kinase II). Figure published in Sarrouilhe et al. 2006.

proteins within dendrites and spines. Spinophilin is also phosphorylated by ERK (Futter

et al., 2005). ERK phosphorylates ABD1 to reduce spinophilin's ability to bind and

bundle F-actin. ERK's role in spineogenesis is unclear. Conversely, Cdk5

phosphorylation of the spinophilin ABD1 increases filopodial density but has no affect

on spinophilin's actin affinity.

Spinophilin is known to interact with over 30 proteins, including itself. Both

spinophilin and neurabin can form homomers and can also interact in a heteromer

(spinophilin-neurabin-PP1) (MacMillan et al., 1999; Nakanishi et al., 1997; Satoh, 1998).

Spinophilin and PP1α or PP1γ1 interact via the R-K-I-H-F motif (Allen et al., 1997;

Carmody et al., 2004; Hsieh-Wilson et al., 1999). Spinophilin forms a complex with PP1

in an alternate site, leaving the active site available, suggesting spinophilin functions to

target PP1 to F-actin and the PSD (Hsieh-Wilson et al., 1999; Terry-Lorenzo, 2002).

Protein phosphatase inhibitors I2 and I4 are known spinophilin interactors(Terry-

Lorenzo, 2002). Spinophilin colocalizes with I2 in spines in hippocampal neurons,

suggesting spinophilin targets both PP1 and its inhibitor to the synapse. Spinophilin

interacts with doublecortin (DCX) via its coiled-coil domain (Tsukada et al., 2003;

Tsukada et al., 2005). DCX phosphorylation and spinophilin interaction enhances its

binding to F-actin (Tsukada et al., 2005). This interaction suggests that doublecortin-

spinophilin interaction is a connector between the actin and tubulin cytoskeleton.

Spinophilin also interacts with a similar protein, DCAMKL1, via the coiled-coil domain of

spinophilin (Tsukada et al., 2003). DCAMKL1 is homologous to DCX in its N-terminal

region, but contains a putative Ca^{2+}/calmodulin-dependent protein kinase. Spinophilin

and p70^{S6k}, an mRNA translation regulator and a Rac pathway protein, interact via its

PDZ domain (Burnett et al., 1998). Spinophilin promotes localization of Tiam1, a Rac-GEF, which in turn activates p70^{S6k} (Buchsbaum et al. 2003). Spinophilin also regulates actin dynamics by interacting with kalirin-7, another Rac1-GEF, via its C-terminal domain (possibly the PDZ domain) to target it to dendritic spines (Penzes et al., 2001). Finally spinophilin interacts with Rho-GEF Lfc, a microtubule-associated protein (Ryan et al., 2005). Following neuronal stimulation, Lfc translocates to spines and interacts with spinophilin, presumably to alter cytoskeletal dynamics.

Spinophilin regulates glutamatergic synapse activity. Yan et al. 1999 found that it colocalizes with AMPARs in neostriatal neurons and that constitutively active PP1 keeps AMPA dephosphorylated (low activity) (Yan et al., 1999b). Furthermore spinophilin KO mice have persistent AMPA signal, indicating a spinophilin-targeted protein like PP1 is not able to turn the receptors off (Feng et al., 2000). This phenotype is also observed with NMDARs. Spinophilin plays an important role in synaptic transmission and plasticity by targeting regulatory proteins to PSD.

Spinophilin knockout mice show transient increase in spine number during development (Feng et al., 2000). These mice also have decreased hippocampal size, reduced LTD, and reduced susceptibility to kainate-induced seizures and neuronal apoptosis. In another study, these mice displayed impaired conditioned taste aversion learning (Stafstrom-Davis et al., 2001). NrbI doesn't seem to compensate for this phenotype, indicating the two probably have different binding partners. Structural data has shown that the PDZ domains of NrbI and spinophilin have different peptide binding pockets (Kelker et al., 2007).

Spinophilin as a Tumor Suppressor and in Immunity

Spinophilin is also a tumor suppressor gene. Spinophilin is frequently associated with microsatellite instability and loss of heterozygosity in tumors (Carnero, 2012; Vilar and Tabernero, 2013). Reduction or loss of spinophilin correlates with a worse prognosis in breast, lung, kidney, head and neck cancer, colorectal cancer, and leukemia, and an increase in cancer stem cell population in breast cancer (Aigelsreiter et al., 2014; Carnero, 2012; Ferrer et al., 2015; Ress et al., 2014). In colorectal cancer cell lines, loss of spinophilin causes increased growth (Ress et al., 2014). Spinophilin knockout mice have a reduced lifespan, increased number of tumors, and increased tissue specific cellular proliferation (Ferrer et al., 2011).

Spinophilin also plays a role in antigen presentation at the immunological synapse, although this role is poorly understood (Seed and Xavier, 2008). Upon dendritic cell stimulus, spinophilin relocalizes from the cytoplasm to plasma membrane at contact sites (Bloom et al., 2008). Additionally, spinophilin is required for T cell activation. Spinophilin could potentially regulate synapse-cytoskeletal dynamics in both neurons and immunological cells.

Discussion

In this study we focus on the molecular mechanisms of spine development. Specifically we concentrate on understanding how the scaffolding protein spinophilin regulates spine number and F-actin dynamics in the spine head. Previous work has showed spinophilin's robust ability to interact with actin, as well as over 30 post-synaptic density proteins. We demonstrate that spinophilin expression promotes spineogenesis as well as F-actin accumulation in the spine head. It does this by a mechanism

68

independent of its C-terminal domain. We also report that spinophilin knockdown

drastically suppresses spine formation. Altogether we conclude that spinophilin's role in

spine development involves regulating the actin cytoskeleton locally in the spine head;

however the downstream mechanisms of this phenomenon need to be studied further.

CHAPTER V

Spinophilin Regulates Spine Formation and F-actin Dynamics in

Hippocampal Neurons

Abstract

Dendritic spines are protrusions emanating from the dendritic shaft that interact with axons to form excitatory synapses. Changes in spine number and morphology are seen in diseases such as autism, schizophrenia, and Alzheimer's disease; thus, identifying factors that regulate spine formation, maturation, and maintenance is critical for understanding normal and pathological brain function. Here, I show that spinophilin/neurabin II, a scaffolding protein that is highly expressed in dendritic spines, has an important role in dendritic spine and synapse formation in hippocampal neurons. Knockdown of endogenous spinophilin with a short hairpin RNA (shRNA) causes a significant decrease in synapse and spine density, as shown by immunostaining for the presynaptic marker synaptic vesicle protein 2 (SV2) and the postsynaptic marker postsynaptic density protein 95 (PSD95). On the other hand, expression of mCherry-spinophilin results in an increase in spine density. These results suggest that spinophilin is critical for dendritic spine and synapse formation. I hypothesized that spinophilin was promoting dendritic spine and synapse formation by regulating F-actin accumulation. Indeed, expression of GFP-spinophilin led to an increase in the amount of F-actin in spine heads. Furthermore, I wanted to determine if the C-terminal domain (CTD) of spinophilin was required to observe these phenotypes. I found that this domain was not required to promote spine formation or F-actin accumulation in the spine head.

Collectively, our data demonstrate an important function for spinophilin in modulating the formation of dendritic spines and synapses.

Methods

Reagents and Plasmids

SV2 monoclonal antibody was made by the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). PSD95 monoclonal antibody (clone 7E3-1B8 was purchased from EMD Millipore (Billerica, MA). Aqua-Poly/Mount was purchased from Polysciences, Inc. (Warrington, PA). The pTS2-mCherry-spinophilin vector, the C-terminal deletion mutant, as well as the spinophilin shRNA #1 were previously described (Evans et al., 2015).

Cell Culture and Transfection

Hippocampal neurons were isolated from day 19 rat embryos and were cultured at low density using a previously published protocol (Goslin et al. 1998). A calcium phosphate protocol was used to transfect the neurons at day *in vitro* (DIV) 6 (Wegner et al., 2008a).

Immunocytochemistry

At DIV 11-12 neurons were fixed with 4% paraformaldehyde (PFA), 4% sucrose in phosphate-buffered saline (PBS) for either 15 minutes at room temperature for SV2 and phalloidin labeling, or 5 minutes for PSD95 staining, followed by cold methanol for 10 minutes. Coverslips were washed 3 times with PBS, then permeabilized with 0.2%

Triton X-100 in PBS for 10 minutes, followed by another 3 PBS washes. Coverslips were then blocked with 20% goat serum in PBS for one hour. For immunolabeling, coverslips were incubated overnight at 4°C with primary antibodies diluted in 5% goat serum in PBS. The following day coverslips were washed 5 times with PBS for one hour, then incubated with secondary antibodies in 5% goat serum in PBS for 45 minutes at room temperature. Lastly the coverslips were washed 5 more times for one hour and mounted on glass slides with Aqua Poly/Mount for imaging.

Microscopy and Image Analysis

Images were collected using either a Retiga EXi CCD camera (Qimaging, Surrey, British Colombia) linked to an Olympus IX71 inverted microscope (Melville, NY) with a PlanApo 60X OTIRFM objective (NA 1.45) integrated with a Lambda 10-2 automated controller (Sutter Instruments, Novato, CA) or a Quorum WaveFX-X1 spinning disk confocal system containing a Yokogawa CSU-X1 spinning disk (Yokogawa Electric Corp., Newnan, GA) with Borealis upgrades and modifications (Guelph, Canada) connected to a Nikon Eclipse Ti microscope (Melville, NY) with an Apo TIRF 60X objective (NA 1.49) and EM-CCD camera (Hamamatsu, Hamamatsu City, Japan). Filter cubes on the Olympus microscope were an Endow GFP Bandpass filter cube (excitation HQ470/40, emission HQ525/50, Q495LP dichroic mirror) (Chroma, Brattleboro, VT), a TRITC/Cy3 cube (excitation HQ545/30, emission HQ610/75, Q570LP dichroic mirror), and a Cy5TM cube (excitation HQ620/60, emission HQ700/75, Q660LP dichroic mirror). For the Nikon microscope, mCerulean, GFP, mCherry, and Alexa Fluor 647 images were acquired by exciting laser lines at 441, 491, 561, and 642

nm, and emission filters were 470/24, 525/50, 593/40, and 700/75, respectively (Semrock, Rochester, NY). MetaMorph software (Molecular Devices, Sunnyvale, CA) was used to acquire the images. Fiji was used to analyze the images (Schindelin et al. 2012).

Dendritic spine densities were manually quantified along primary and secondary dendrites. Spines were defined as protrusions that co-localized with presynaptic terminals by SV2 staining, or that co-localized with the PSD95 staining. F-actin intensities were quantified in from background-subtracted images. Statistical analyses were performed using RStudio (Version 0.98.1091). Comparison of two means was performed using t-tests, and multiple means using one-way analysis of variance (ANOVA).

Results

Spinophilin expression promotes spineogenesis

Dendritic spines undergo F-actin modulated morphological changes during the processes of learning and memory (De Roo et al., 2008; Engert and Bonhoeffer, 1999; Frotscher, 2014; Leuner et al., 2003; Maletic-Savatic, 1999; Nägerl et al., 2004). Previous work has shown that spinophilin is an F-actin binding protein that localizes to dendritic spine heads in hippocampal neurons (Allen et al., 1997; Satoh, 1998). Therefore, I investigated the relationship between spinophilin and dendritic spine formation. First, I expressed mCherry-tagged spinophilin in neurons and examined its localization. mCherry-spinophilin localized in punctate structures along the dendrite (Fig. 21A-B). To show these structures were dendritic spines, I co-immunostained for the

73

presynaptic marker SV2 and the postsynaptic marker PSD95. mCherry-spinophilin co-localized with SV2 and PSD95 in synapses (Fig. 21A-B). Furthermore, expression of mCherry-spinophilin led to an increase in the number of dendritic spines and synapses compared to mCherry alone (Fig. 21C-D). These results suggest that spinophilin plays a role in dendritic spine and synapse formation.

Spinophilin knockdown suppresses spine formation

Next, I took an shRNA approach to knockdown expression of endogenous spinophilin. I transfected neurons with a spinophilin shRNA that we had previously shown to significantly knockdown endogenous expression of spinophilin in hippocampal neurons (Evans et al., 2015). Expression of spinophilin shRNA results in a significant decrease in the number of dendritic spines and synapses as compared to expression of empty pSUPER vector, suggesting that spinophilin regulates dendritic spine and synapse formation (Figure 22A-D).

Spinophilin regulates F-actin accumulation in the spine head

Because dendritic spines are comprised of a branched network of F-actin (Korobova and Svitkina, 2010; Star et al., 2002; Tatavarty et al., 2009) and spinophilin is an F-actin binding protein, I hypothesized that spinophilin regulates F-actin accumulation in spine heads (Satoh, 1998). To test this hypothesis, I expressed mCherry-spinophilin in neurons and quantified F-actin levels in dendritic spine heads by staining with fluorescently labeled phalloidin, which binds to F-actin (Allison et al., 1998; Drenckhahn et al., 1984; Wulf et al., 1979) Drenckhahn, Wulf, Allison). Expression of

mCherry-spinophilin increased the amount of F-actin in dendritic spine heads (Figure 23). In contrast, transfection of neurons with spinophilin shRNA resulted in a decrease in the level of F-actin in the heads of dendritic spines (Figure 24). Collectively, these results suggest that spinophilin regulates dendritic spine and synapse formation by modulating F-actin accumulation in these structures.

The CTD of spinophilin is not required to regulate spineogenesis or F-actin dynamics

Finally I wanted to determine if the CTD of spinophilin was required for the increase in spineogenesis and F-actin accumulation in spine heads. Using a mCherry-spinophilin construct with a deletion before the C-terminal domain (spinophilinΔCTD), I found that the CTD of spinophilin was not required to promote spine formation, as spine levels were similar between mCherry-spinophilin and mCherry-spinophilinΔCTD expressing neurons (Figure 25). Similarly, there was no change in F-actin accumulation in the spine heads of mCherry-spinophilinΔCTD expressing neurons, compared to mCherry-spinophilin expressing neurons (Figure 26). This indicates that the CTD of spinophilin is not required for spineogenesis or F-actin regulation.

Discussion

Previous research has shown spinophilin is a scaffolding protein that binds over 30 proteins, and over the years it has been used as a marker of mature spines due to its localization to the spine head (Sarrouilhe et al., 2006). Spinophilin's interaction with F-actin is carefully controlled by phosphorylation in its two actin-binding domains and is

vital for targeting from the shaft to the spine (Barnes et al., 2004; Grossman et al., 2004; Grossman et al., 2002; Hsieh-Wilson et al., 2003; Satoh, 1998). Furthermore, spinophilin can regulate AMPA and NMDA receptor activity by localizing PP1 to the synapse (Feng et al., 2000; Yan et al., 1999b). However, the function of spinophilin in dendritic spine and synapse formation is currently not well understood. Therefore, I investigated the function of spinophilin in the formation of dendritic spines and synapses.

Our data demonstrate that spinophilin expression increases the density of dendritic spines and synapses in hippocampal neurons, independently of the coiled-coiled domain found in the CTD. Conversely, knockdown of endogenous spinophilin via shRNA decreases the number of dendritic spines and synapses. These data suggest that spinophilin signaling is important for the assembly of dendritic spines and synapses. Because spinophilin has a number of binding partners outside of the coiled-coil domain, it probably regulates dendritic spines and synapses through its interaction with other proteins. Future studies will be needed to identify if these or other proteins are involved in spinophilin signaling in the regulation of dendritic spines and synapses.

Additionally, I observed a relationship between spinophilin levels and F-actin intensity in the spine head. Our previous studies have shown that Asef2 and spinophilin work together to promote spineogenesis via Rac signaling (Evans et al., 2015). Spinophilin has also been previously reported to interact with Rho GEFs kalirin-7 and Lfc (Penzes et al., 2001; Ryan et al., 2005). Because Rac and Rho signaling are critical for F-actin reorganization (Govek et al., 2005), it is possible that spinophilin regulates F-actin dynamics in dendritic spines via these signaling pathways. Alternatively,
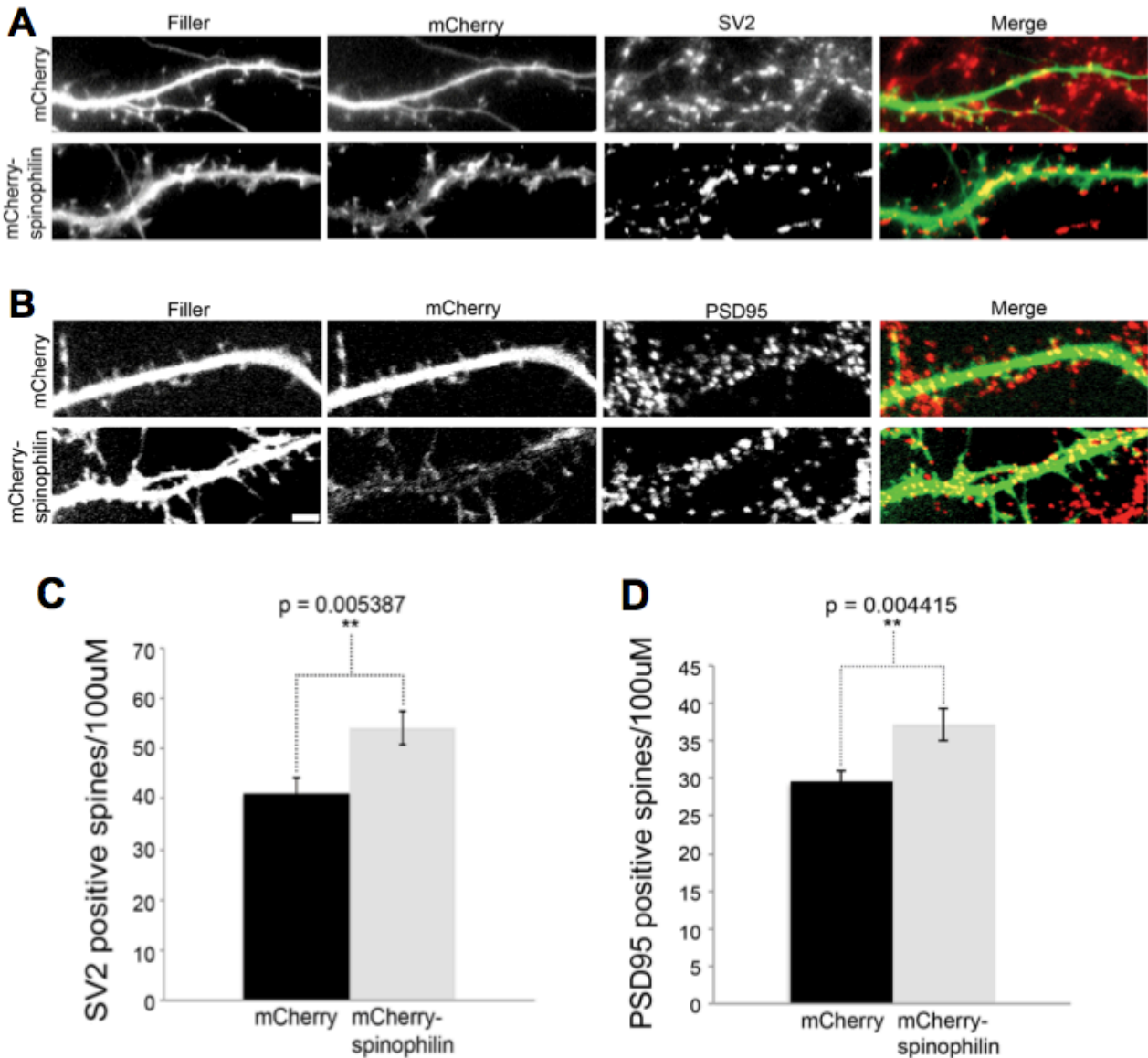
Figure 22. Spinophilin promotes dendritic spine formation. A-B. DIV5 neurons were transfected with mCherry or mCherry-spinophilin, then fixed and stained for SV2 (**A**) or PSD95 (**B**) at DIV11-12. **C.** Quantification of SV2-positive spine density in mCherry and mCherry-spinophilin expressing neurons (n = 44 control and 49 mCherry-spinophilin dendrites from 3 independent experiments). **D.** Quantification of PSD95-positive spine density in mCherry and mCherry-spinophilin expressing neurons (n = 47 control and 45 mCherry-spinophilin dendrites from 3 independent experiments). Scale bar = 10 um.

Figure 23. Spinophilin knockdown suppresses dendritic spine formation. A-B. DIV5 neurons were transfected with NT shRNA or spinophilin shRNA, then fixed and stained for SV2 (**A**) or PSD95 (**B**) at DIV11-12. **C.** Quantification of SV2-positive spine density in NT and spinophilin shRNA expressing neurons (n = 44 control and 42 spinophilin shRNA dendrites from 4 independent experiments). **D.** Quantification of PSD95-positive spine density in NT and spinophilin shRNA expressing neurons (n = 44 control and 40 spinophilin shRNA dendrites from 4 independent experiments). Scale bar = 10 um.

spinophilin could directly modulation F-actin reorganization, or it could exert its effect through other signaling proteins. Phosphorylation of spinophilin by PKA has been shown to decrease its ability to bind and crosslink F-actin (Hsieh-Wilson et al., 2003), which could affect F-actin organization in spines. Additionally, spinophilin targets PP1 to spines in order to modulate glutamatergic signaling, LTP and LTD, and spine maturation (Hu et al., 2007; Hu et al., 2006; Terry-Lorenzo et al., 2005).

Interestingly, the phenotype observed in the spinophilin knockout mice is increased spine formation in the caudatoputamen; however I hypothesize that this discrepancy is due to a tissue-specific and temporal difference in the experiments (Feng et al., 2000). The experiments were performed on two different brain areas, allowing the possibility that spinophilin has a differential, tissue-specific role in spineogenesis. In addition, the spinophilin knockout mouse data could be a spine maintenance phenotype, whereas the phenotype seen in our experiments is a spine developmental phenotype.

In conclusion, our data suggests that spinophilin is required for spineogenesis and F-actin accumulation in the spine head. Expression of spinophilin in hippocampal neurons promotes spineogenesis, even in the absence of the CTD, whereas knockdown suppresses spineogenesis. A similar phenotypic relationship is seen with F-actin accumulation in the spine head. Future studies will needed to assess the mechanism(s) by which spinophilin alters spines and the actin cytoskeleton.

Figure 24. Spinophilin promotes F-actin accumulation in the spine head. A-B.
DIV5 neurons were transfected with mCherry or mCherry-spinophilin, then fixed and stained for phalloidin at DIV11-12. Quantification of fluorescent phalloidin intensity in the spine heads of mCherry and mCherry-spinophilin expressing neurons (n = 53 control and 51 mCherry-spinophilin dendrites from 3 independent experiments). Scale bar = 10 um.
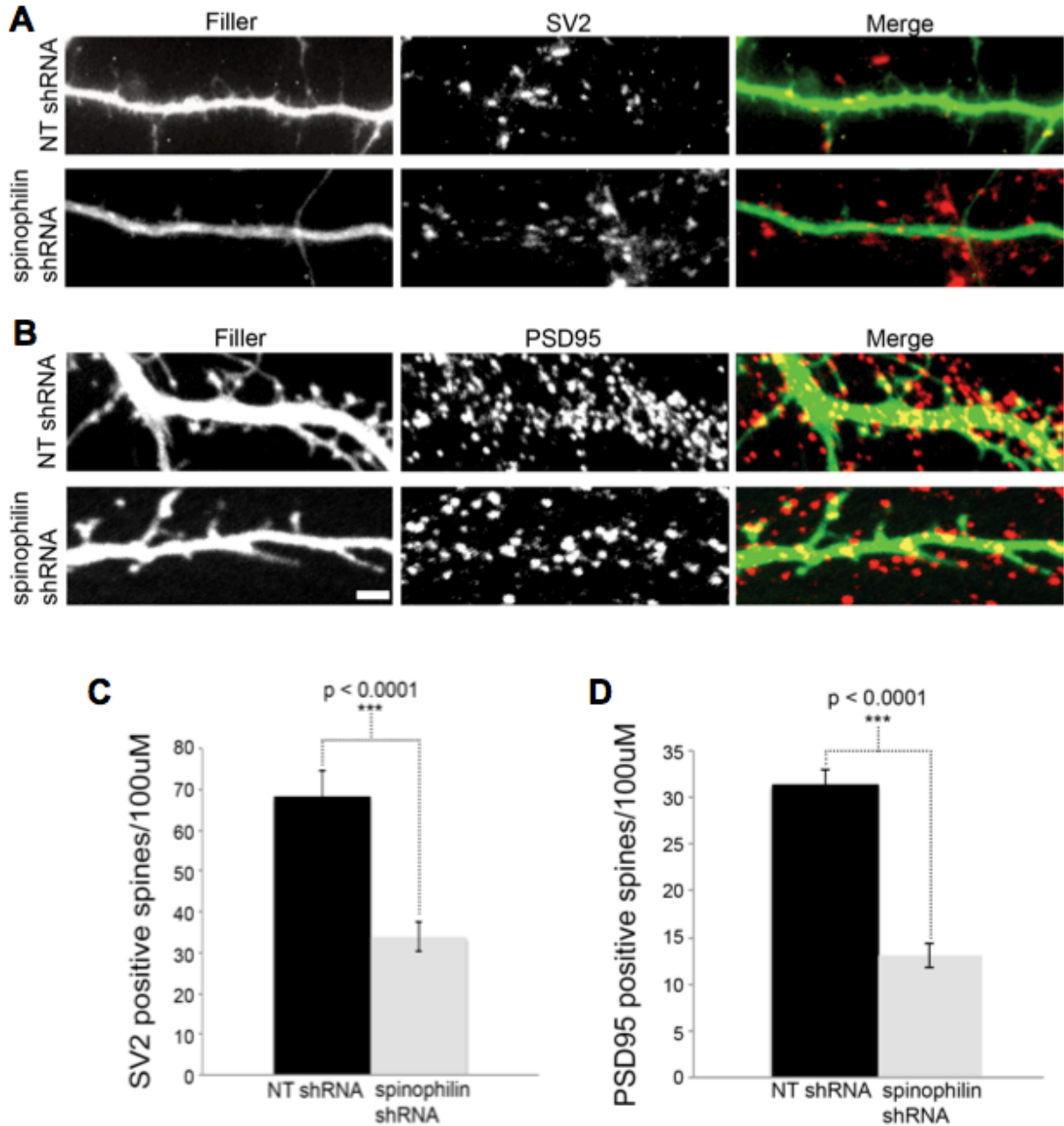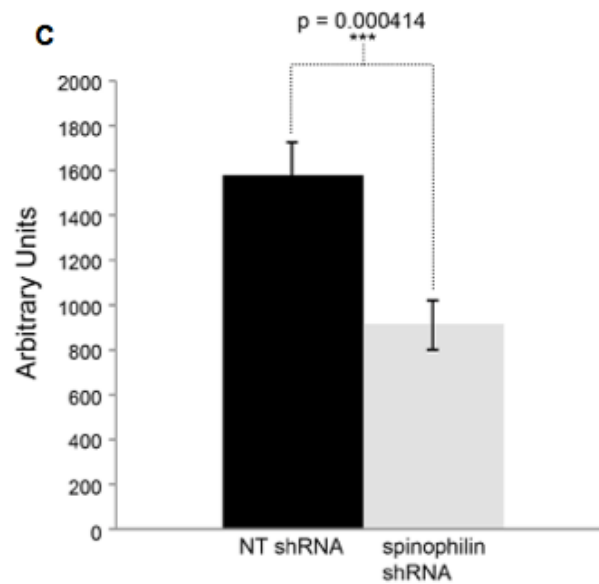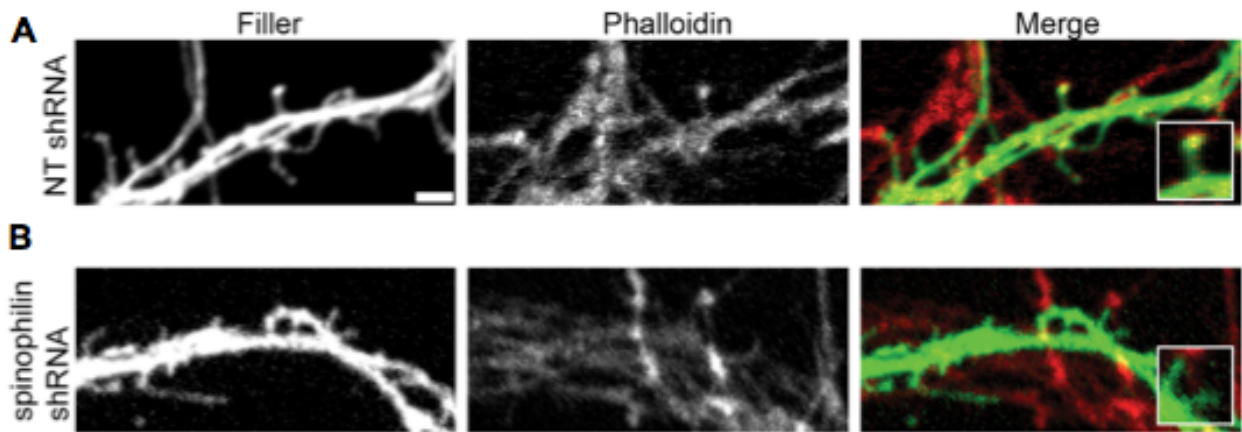
Figure 25. Spinophilin knockdown suppresses F-actin accumulation in the spine head. A-B. DIV5 neurons were transfected with NT shRNA or spinophilin shRNA, then fixed and stained for phalloidin at DIV11-12. Quantification of fluorescent phalloidin intensity in the spine heads of mCherry and mCherry-spinophilin expressing neurons (n = 52 control and 50 mCherry-spinophilin dendrites from 3 independent experiments). Scale bar = 10 um.
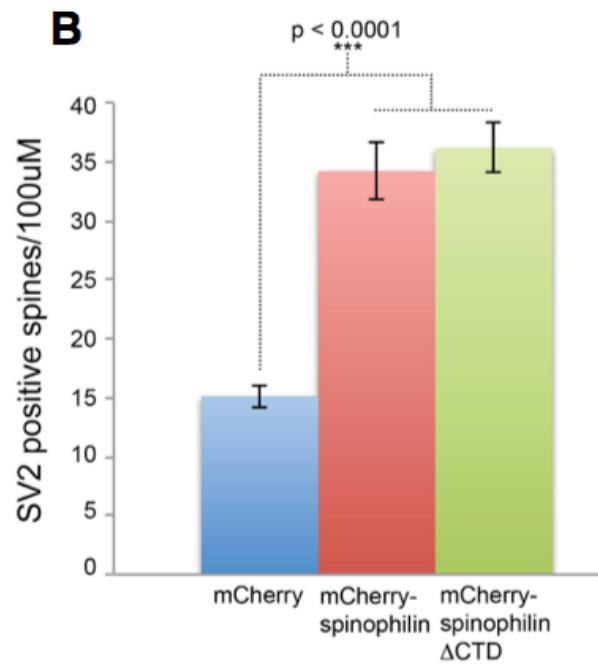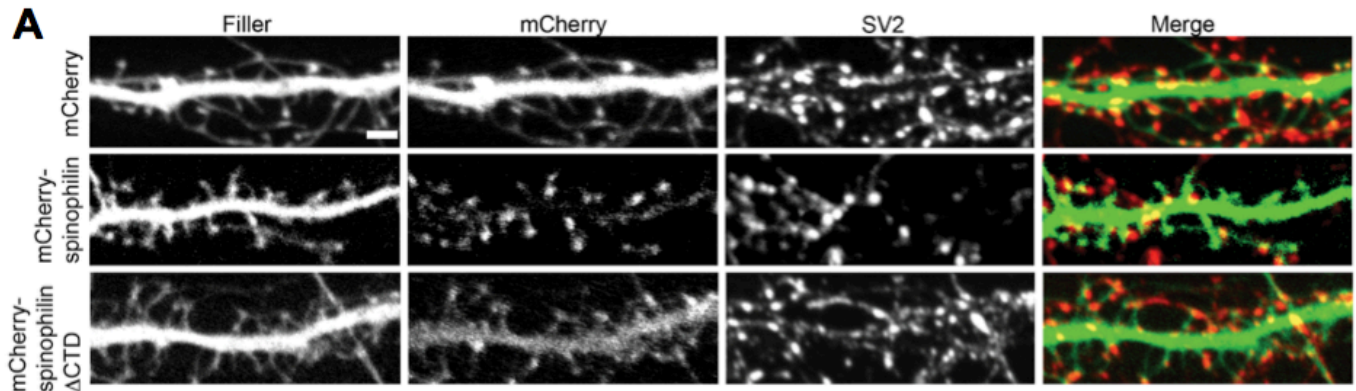
Figure 26.1. SpinophilinΔCTD promotes SV2-positive spine formation. A. DIV5 neurons were transfected with mCherry, mCherry-spinophilin, or mCherry-spinophilinΔCTD, then fixed and stained for SV2 at DIV11-12. **B.** Quantification of SV2-positive spine density in mCherry, mCherry-spinophilin, or mCherry-spinophilinΔCTD expressing neurons (n = 46 control, 40 mCherry-spinophilin, and 42 mCherry-spinophilinΔCTD dendrites from 4 independent experiments). Scale bar = 10 um.
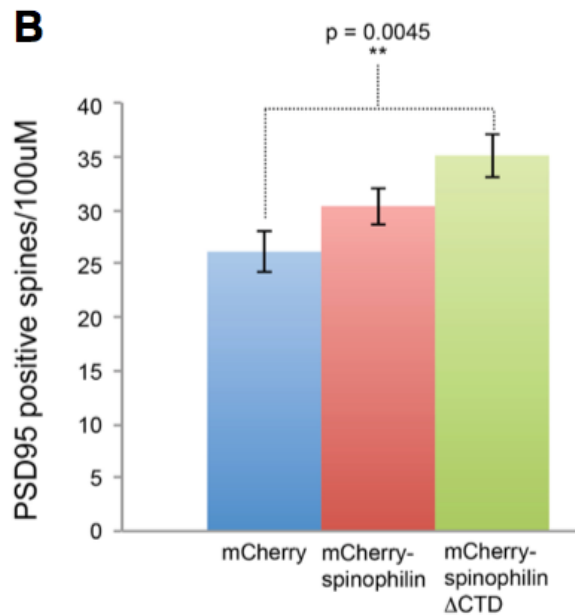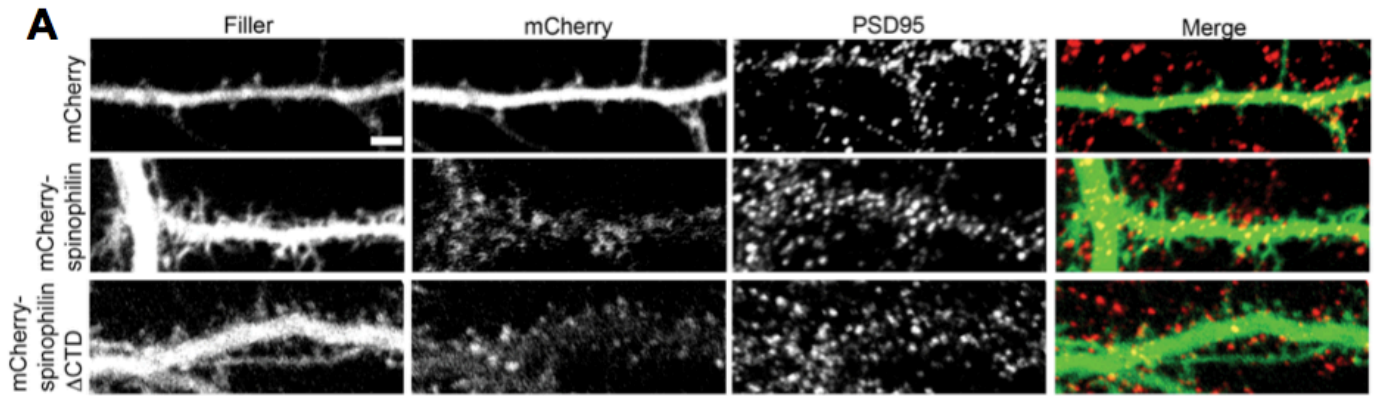
Figure 26.2. SpinophilinΔCTD promotes PSD95-positive spine formation. A. DIV5 neurons were transfected with mCherry, mCherry-spinophilin, or mCherry-spinophilinΔCTD, then fixed and stained for PSD95 at DIV11-12. **B.** Quantification of PSD95-positive spine density in mCherry, mCherry-spinophilin, and mCherry-spinophilinΔCTD expressing neurons (n = 41 control, 39 mCherry-spinophilin, and 41 mCherry- spinophilinΔCTD dendrites from 4 independent experiments). Scale bar = 10 um.
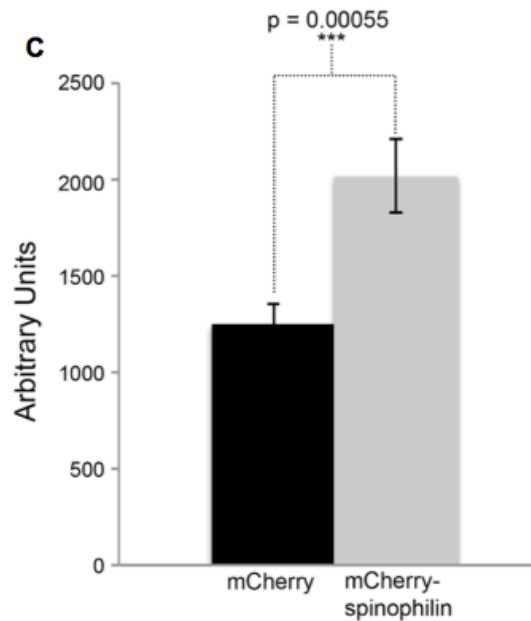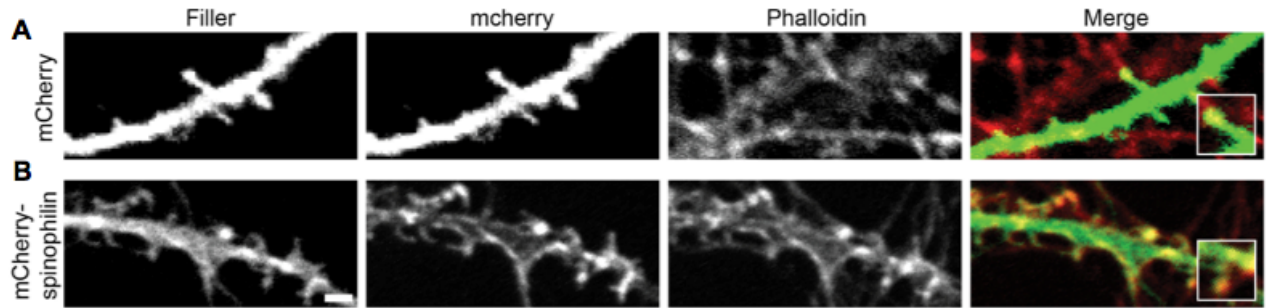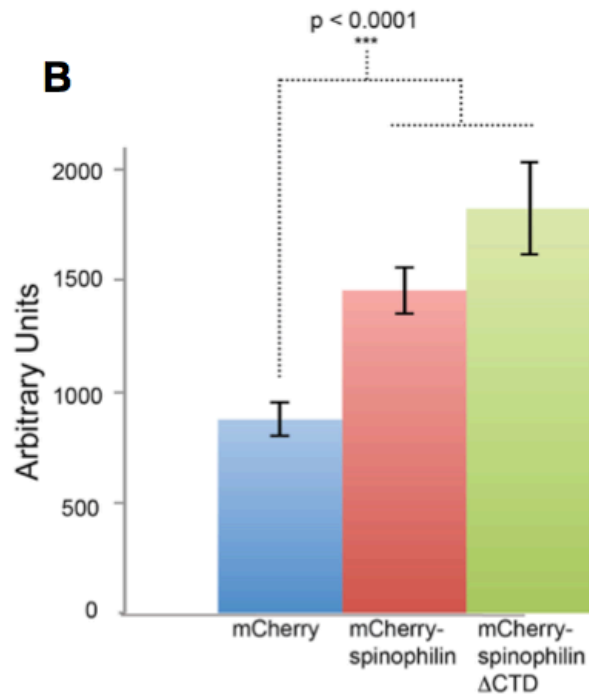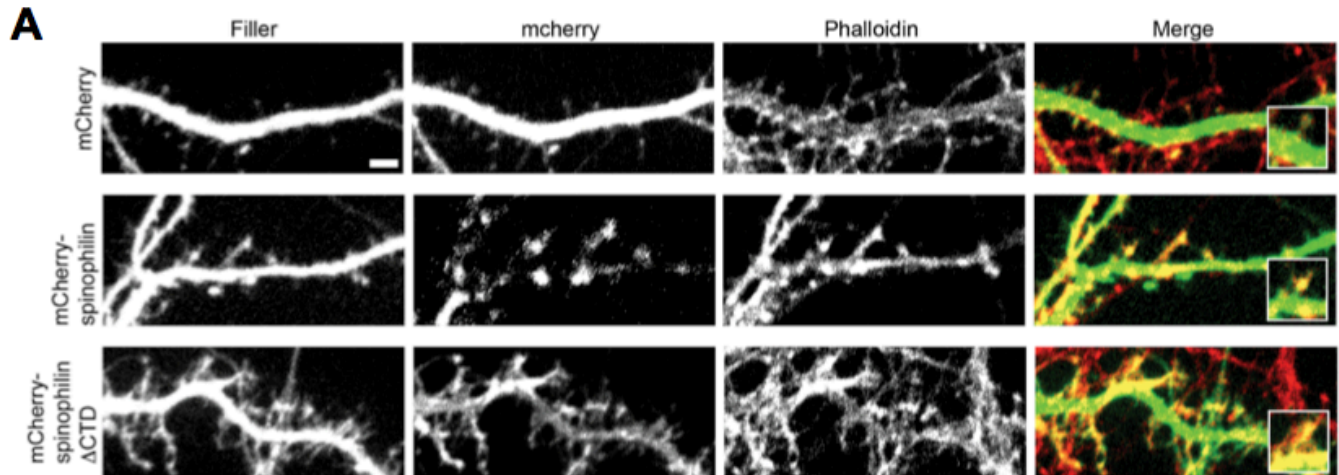
Figure 27. SpinophilinΔCTD promotes F-actin accumulation in the spine head. A. DIV5 neurons were transfected with mCherry, mCherry-spinophilin, or mCherry-spinophilinΔCTD, then fixed and stained for phalloidin at DIV11-12. **B.** Quantification of fluorescent phalloidin intensity in mCherry, mCherry-spinophilin, and mCherry-spinophilinΔCTD expressing neurons (n = 85 control, 84 mCherry-spinophilin, and 84 mCherry-spinophilinΔCTD spines from 4 independent experiments). Scale bar = 10 um.

Acknowledgements

CHAPTER VI

Future Directions

In this study, I have found that the scaffolding protein spinophilin promotes spineogenesis and regulates F-actin dynamics in the spine head, independently of its CTD. In rat hippocampal neurons, spinophilin promotes dendritic spine formation and increases F-actin accumulation in the spine head. Conversely, spinophilin knockdown suppresses spineogenesis and F-actin accumulation in the spine head. Furthermore, deletion of the CTD of spinophilin does not alter its ability to regulate spine development or F-actin dynamics. These results indicate that spinophilin plays a key role in mediating spine development through the cytoskeleton. An important next step is to characterize the relationship between spinophilin and spines using live-cell imaging. Visualizing real-time dynamics of spine formation will be important to determine how spinophilin affects filopodial extension, spine maturation, and spine maintenance. I hypothesize that spinophilin expression would promote spine extension and maintenance of spines, whereas spinophilin knockdown suppresses spine formation. These experiments would provide insight into the specific role spinophilin plays in spine development. These live-cell imaging experiments would be greatly enhanced by super resolution microscopy techniques to observe spine morphology with higher resolution compared to confocal microscopy (Tønnesen et al., 2014).

Because spinophilin is a scaffolding protein known to interact with over 30 proteins, it is likely that it serves as a mediator between synaptic proteins and the actin cytoskeleton (Sarrouilhe et al., 2006). Since proteins that interact with spinophilin's coiled-coil domain (neurabin, spinophilin, doublecortin, DCAMKL1, Rho GEF Lfc, and

86

TGN 38) do not appear to be required for these phenotypes, it is likely that proteins interacting with another domain, such as the PDZ or receptor-binding domain, are responsible (Sarrouilhe et al., 2006).

Two likely candidates are kalirin-7 and p70 S6 kinase (p70S6k), both of which are cytoskeletal regulators (Burnett et al., 1998; Penzes et al., 2001). Kalirin-7 is a brain-specific Rac1 GEF found in the PSD of dendritic spines that interacts with spinophilin (Penzes et al., 2001; Penzes et al., 2009). It has been shown to regulate spine development, maintenance, and plasticity in pyramidal neurons (Cahill et al., 2009; Clapcote et al., 2007; Millar et al., 2003; Sankoorikal et al., 2006; Vorhees and Williams, 2006). Additionally, kalirin-7 has been implicated in psychiatric disorders such as schizophrenia, attention deficit disorder (ADHD), and Alzheimer's disease, making its interaction with spinophilin potentially important in disease pathology (Chwang et al., 2007; Ma et al., 2008; Penzes and Jones, 2008; Sarnyai et al., 2000; Savonenko et al., 2008). Another potential target for spinophilin-mediated spineogenesis is p70^{S6k}, a mitogen-activated protein kinase that regulates mRNA translation and cyclic AMP (cAMP) response elements (Burnett et al., 1998). Spinophilin could interact with p70^{S6k} in order to alter local protein synthesis or to regulate cAMP signaling in the spine.

To determine if spinophilin is promoting spine development through PDZ-domain interactions, I would delete this domain, and express a mCherry-spinophilinΔPDZ in hippocampal neurons and quantify spine density and F-actin intensity in spine heads. If I saw reduced spineogenesis, similar to wild type neurons, I would then break down the mCherry-spinophilinΔPDZ into smaller deletion constructs to determine the site required for spineogenesis, then express the construct in neurons to confirm the abrogated

phenotype. Additionally, it would also be interesting to see what pathway(s) spinophilin is using to promote spine development with CRISPR-Cas9 technology. I would knock out PDZ-interacting proteins like kalirin-7 or p70^{S6k} and determine if spinophilin expression still promotes spineogenesis. A defect in spine formation in the absence of p70^{S6k} would potentially be an interesting new pathway showing local protein synthesis is required for spine development. I could also knockout Rac1 effectors such as PAK or MLK to determine if the Rac1 pathway is required for spinophilin-mediated regulation of F-actin dynamics in the spine head. All together the spinophilin-cytoskeleton interaction is an untapped area of study that has implication on proper spine development and potentially disease pathology.

Spinophilin's regulation of synaptic function is another area of study that should be pursued. In the knockout mouse, spinophilin interactor PP1 presumably isn't targeted to spines, causing a persistent AMPAR signal (Feng et al., 2000). This phenotype is corroborated in recordings from neostriatal cells expressing a peptide that blocks PP1 binding to spinophilin (Yan et al., 1999b). Feng et al. also demonstrated defective NMDAR signaling in the knockout mice; in the presence of a PP1 inhibitor, spinophilin knockout neurons do not display a spine in NMDAR transmission seen in the wild-type controls (Feng et al., 2000). Determining the role of the PDZ and coiled-coil domains of spinophilin in glutamatergic signaling in cultured neurons could be done using calcium sensing and FM464 dyes to observe and quantify synaptic activity. Transfected neurons expressing mutant forms of spinophilin could be characterized with these techniques to further describe spinophilin's role in synaptic activity.

Finally, it would be important to look at how spinophilin's phosphorylation status regulates its ability to influence spine and synapse development. Spinophilin's localization to the PSD is phosphorylation dependent; when phosphorylated by PKA, spinophilin localizes away from spines to the cytosol (Hsieh-Wilson et al., 2003). Additionally, spinophilin is phosphorylated by CaMKII, Cdk5, and Erk2 (Futter et al., 2005; Grossman et al., 2004; Hsieh-Wilson et al., 2003). I predict that spinophilin's phosphorylation status targets spinophilin to particular locations in dendritic spines. Using site-directed mutagenesis, I could mutate the residues phosphorylated by PKA, CaMKII, Cdk5, and Erk2. These experiments would allow better characterization of the biochemical interactions between spinophilin and its interactors and spinophilin's role in synaptic function.

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