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Regulation of Dendrite Development and Synapse Formation by Tropomodulin

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Regulation of Dendrite Development and Synapse Formation by Tropomodulin

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Abstract

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By Omotola F. Omotade

Neurons of the central nervous system elaborate highly branched dendritic arbors that host numerous dendritic spines, actin-rich protrusions that serve as the postsynaptic platform for excitatory synapses. The actin cytoskeleton plays an important role in dendrite development and controls the structure and molecular organization of spines during synapse formation. However, the molecules and mechanisms that regulate actin organization and remodeling during postsynaptic development are not fully understood. Tropomodulins (Tmods) are a multi-domain family of proteins that cap the pointed end of actin filaments, thereby regulating the stability, length, and architecture of complex actin networks in diverse cell types. Three members of the Tmod family, Tmod1, Tmod2, and Tmod3 are expressed in the vertebrate CNS, but their function in neuronal development is largely unknown. In this study, we present evidence that Tmod1 and Tmod2 are expressed in the hippocampus and play an important role in dendrite arborization and synapse formation. Loss-of-function analysis reveals that Tmod2, but not Tmod1, is required for dendritic branching during postsynaptic development. Both Tmod1 and Tmod2 localize to a unique sub-spine region, where they regulate F-actin stability in spines. Knockdown of either Tmod1 or Tmod2 disrupts spine morphogenesis and impairs synapse formation. Together, these findings establish an important role for Tmods in postsynaptic development. Regulation of F-actin stability by Tmod1 and Tmod2 represents a key mechanism underlying the cytoskeletal rearrangements required for spine morphogenesis and synapse development.

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Chapter I

Introduction

1.1 The Synapse: Gateway of Neuronal Communication

The human brain is comprised of over 100 billion neurons, making it the most complex organ in the human body. During neuronal development, these neurons establish an elaborate and exquisite circuitry – allowing communication between functionally distinct regions of the brain. All aspects of human consciousness – behavior, emotion, memory, and cognition – rely on rapid and precise communication between neurons. In the central nervous system, seamless communication is achieved through the *synapse*, a specialized junction that allows signals to be transmitted and integrated between cells. Over 100 trillion synapses exist in the human brain – allowing us to produce thoughts, evoke behavior, and recall memories in milliseconds. For more than a century, an overwhelming body of research has provided fundamental insight into the cellular pathways underlying the establishment, maintenance and modification of synapses. Despite over a century of fervent study, the synapse remains a mysterious frontier – revealing its secrets only when presented with the finest of human curiosity and ingenuity. Over the years, testable hypotheses and predications have morphed into concrete evidence, in turn producing well-established models of synapse development and function.

Here in this dissertation, I will focus the mechanisms and molecules responsible for the proper establishment of excitatory synapses during brain development. This general introduction is intended to serve as a guide through a series of published works that have provided fundamental insight into 1) the structure of the postsynaptic compartment (dendrites and dendritic spines) 2) the dynamics underlying its development and 3) the role of the actin cytoskeleton in the postsynaptic compartment, particularly during development. The introduction will culminate with a review of Tropomodulin molecules and their function in regulating diverse actin-based networks. The principles discussed in this introduction serve as a framework for the original research presented in Chapter 3 and Chapter 4. Finally, the discussion will offer thoughtful perspectives that contextualize the major research findings obtained during my doctoral studies.

1.2 Neuronal Morphology and Synaptic Transmission

Despite the morphological and functional diversity of neurons in the central nervous system, vertebrate neurons share conserved features (**Figure 1.1A**). Neurons are polarized and possess a single long axon (though collateral axon branches exist) and shorter dendritic branches. Both emanating from the cell body, these distinct processes are specialized for transmitting and receiving information, respectively (Bullock et al., 2005). A single axon emanates from the cell body and ends in a presynaptic terminal that transfers electrical or chemical information to a target cell. Dendrites possess many branches that contain the molecular machinery required to receive and integrate information from numerous inputs. Neurotransmission is achieved by pairing the presynaptic element of one neuron, which is equipped to deliver information, with the

postsynaptic element from a neighboring neuron, creating a synapse specialized for the transfer of information between nerve cells (Bullock, 1959).

Synaptic transmission in the vertebrate brain is largely achieved through the chemical synapse, a specialized junction comprised of paired pre- and post- synaptic structures (**Figure 1.1B**). These complimentary structures are exquisitely designed for neurotransmitter release and reception, respectively (Palay and Palade, 1955, Palay, 1956, Gray, 1959). The presynaptic terminal contains small, membrane-bound organelles, termed synaptic vesicles (Palay, 1956). These vesicles contain neurotransmitters, which, upon their release, bind to receptors on the postsynaptic membrane. In the vertebrate brain, small actin-rich membrane protrusions, referred to as dendritic spines, serve as the postsynaptic component of excitatory synapses (Hotulainen and Hoogenraad, 2010) (**Figure 1.1B**). Although only 1 μm^3 on average, dendritic spines contain the neurotransmitter receptors, scaffolding proteins, and signal transduction molecules needed to receive presynaptic input and transduce them into biochemical signals (Sorra and Harris, 2000, Bourne and Harris, 2008, Hotulainen and Hoogenraad, 2010). Together these molecules are clustered into a large, electron-dense organizing structure referred to as the postsynaptic density (PSD), which sits directly underneath the dendritic spine membrane (Palay and Palade, 1955, Gray, 1959). For optimal neurotransmission, the PSD is directly apposed to the presynaptic active zone (AZ), the site of neurotransmitter release (**Figure 1.1B**) (Palay and Palade, 1955, Gray, 1959, Sudhof, 2012).

Synaptic transmission in the excitatory glutamatergic nervous system is initiated when electrical signal, referred to as an action potential, is transmitted down the axon. Once the action potential reaches the presynaptic terminal, it triggers the opening of

voltage-gated Ca^{2+} channels on the presynaptic membrane, causing an influx of calcium ions into the axon terminal. Elevation of presynaptic Ca^{2+} concentration triggers the fusion and subsequent exocytosis of synaptic vesicles, which release stored neurotransmitters into the synaptic cleft, a small extracellular space of ~ 10-20 nm that separates the presynaptic terminal from the postsynaptic dendritic spine. Following exocytosis, neurotransmitters diffuse across the synaptic cleft and bind to receptors on the dendritic spine membrane, which in turn open their channels to allow an influx of ions. The resulting change in membrane potential activates a complex signal transduction cascade responsible for generating an action potential that travels to the presynaptic terminal, where it is again converted into a chemical signal. Glutamate is the principal excitatory neurotransmitter in the vertebrate central nervous system and its release from presynaptic terminals increases the probability an action potential will be produced. This tightly-regulated series of events constitutes the basis for neuronal communication between excitatory neurons in the vertebrate brain.

1.3 Postsynaptic Dendrite Development and Synapse Formation

The formation of synaptic connections requires the proper growth and extension of axonal and dendritic processes during development. During early development, growing axons must navigate through a complex environment in order to arrive at the correct destination and establish the appropriate synaptic contact. The directional steering of axons is achieved through the axonal growth cone, a broad, cytoskeletal-based projection at the tip of growing axons (Ramón y Cajal S, 1901). The growth cone serves as the primary sensory and motile structure of the axon – allowing the axon to sense and respond to diffusible chemotrophic cues (Kolodkin and Tessier-Lavigne, 2011). An

elaborate microtubule and actin-based cytoskeleton provides the structural support for the growth cone as well as powers its directional motility (Dent et al., 2011, Omotade et al., 2017). By translating extracellular signals into directional migration, growth cones steer axons towards the appropriate target in the developing brain – ensuring that intricate connectivity is established. The process of axon outgrowth and guidance, including the phenomenology and molecular events that drive these events, will not be the focus of this section but are detailed in several excellent reviews (Lowery and Van Vactor, 2009, Vitriol and Zheng, 2012). The following segment will focus on the development of postsynaptic structures: the establishment of intricate dendritic branches and the synapses that these such arbors support.

During development, dendrites do not merely serve as passive targets for axonal exploration; but rather, exhibit highly dynamic behavior – extending and retracting nascent branches (Dailey and Smith, 1996). The generation of synapses on these early arbors stabilize these branches, in turn causing the dendrite to grow in length, increase in complexity and subsequently establish further synaptic contacts (Ziv and Smith, 1996, Niell et al., 2004, Haas et al., 2006). Early indications that dendrite development is a dynamic process came nearly a century ago from observations of dendrites from fixed tissues. In the late nineteenth century, Cajal and Golgi found that dendrites isolated at different developmental stages showed increasing complex dendritic arbors as well as the progressive accumulation of spines. These observations would prove crucial to our understanding of the developing nervous system. Since then, researchers have examined dendrites from a diversity of organisms and experimental systems – ranging from *Xenopus* to Zebrafish to mammals. Each of these systems show a progressive increase in

dendrite branching, length, and number during development (Wong, 1990, Wu et al., 1999, Niell et al., 2004). Collectively, these studies have provided strong evidence that dendrites undergo a stereotypical sequence of outward growth and branching during development, which is concomitant with axon outgrowth and synapse formation.

Static images of dendrites were followed by a wave of studies employing time-lapse microscopy to unveil the spatiotemporal dynamics of postsynaptic development in live neurons. In particular, the use of two *in vitro* systems –primary dissociated neurons and organotypic brain slices – have been fundamental in shaping our understanding of synapse formation on developing dendrites. A major advantage of organotypic brain slice cultures is that this model system preserves the complex morphological and physiological features of brain tissue, allowing high-density synaptic connections to form in a manner that more closely recapitulates the circuitry established *in vivo*. In the early to mid 90's, researchers found that organotypic hippocampal slices derived from postnatal rodents undergo neurite outgrowth and synaptogenesis and can be maintained for weeks in culture (Stoppini et al., 1991, Muller et al., 1993, Stoppini et al., 1993, Robain et al., 1994). For these reasons, organotypic slice cultures have been widely used as a model system to study the processes of dendrite growth and synapse formation and plasticity. In addition to organotypic slice cultures, primary dissociated neurons have been widely used by neuroscientists to study neuronal development. Primary neuronal cultures allow researchers to examine the properties of neurons at the single-cell and single-synapse level, which has provided crucial mechanistic insight into the cellular and molecular mechanisms underlying postsynaptic development. As a complement to these *in vitro* systems, time-lapse two photon laser scanning microscopy has allowed researchers to

image the structural dynamics of postsynaptic development over periods of weeks to months *in vivo*. The deeper tissue penetration and reduced cellular phototoxicity allowed by two-photon microscopy has contributed to its popularity as a method for examining long-term postsynaptic development *in vivo* (Denk et al., 1990, Lendvai et al., 2000, Trachtenberg et al., 2002).

The aforementioned technological advances have contributed to our understanding of postsynaptic development, which can be divided into three general stages (**Figure 1.2**). In the first stage, primary dendrites containing dendritic growth cones at their distal tips, elongate from the soma. At this stage, axons are present in the vicinity of developing dendrites, though synapses have not yet formed. In the second stage, robust dendrite elongation and synapse formation occur concurrently, with synaptic contacts between dendritic filopodia and axons stabilizing nascent dendritic branches. As synaptic contacts are made, dynamic dendritic filopodia become stabilized and converted to mature spines, leading to a heterogeneous population of spines during period. As synaptic circuits stabilize, further addition and elimination of spines refines the circuitry. After postsynaptic development is complete, mature dendritic arbors support a high density of dendrite spines that make functional synapses with presynaptic boutons. The following segments will summarize the main events that occur during the stages of postsynaptic development.

1.3.1 Early postsynaptic development

During early postnatal development (P0-P7 in rats, *P:postnatal*), neurons extend highly dynamic dendritic arbors that exhibit periods of rapid outgrowth and retraction (Dailey and Smith, 1996, Niell et al., 2004). At this stage, branches are highly unstable

and many branches retract as well as fully resorb into the dendritic shaft (Dailey and Smith, 1996). Dendrites elongate through the advance of an actin-based growth cone structure present at dendrite tips (Ulfhake and Cullheim, 1988, Dailey and Smith, 1996, Fiala et al., 1998, Wu et al., 1999). In addition to growth cone structures at their proximal tips, dendrites at this stage are also covered in highly dynamic filopodia, long (~5-10 μm) protrusions, which lack a bulbous head (Purpura, 1974, Harris et al., 1992, Papa et al., 1995, Ziv and Smith, 1996, Fiala et al., 1998). These filopodia are extremely transient and exhibit rounds of rapid extension and retraction (Dailey and Smith, 1996, Ziv and Smith, 1996, Portera-Cailliau et al., 2003). Early in postnatal development, a high abundance of synapses can be detected on the dendritic shaft (Harris et al., 1992, Fiala et al., 1998). This period of early dendrite develop is therefore defined by the presence of dendritic growth cones and filopodia, which are required for dendrite extension.

1.3.2 A Coupled Process: Synapse Development and Dendrite stabilization

As postsynaptic development progresses (~P12-P23), secondary and tertiary branches extend from primary dendrites, creating an increasingly complex dendrite arbors. During this stage, synapse formation is initiated as dendritic filopodia establish contact with incoming axonal afferents. Axonal contact and subsequent synapse formation cause filopodia to undergo a reduction in motility, substantial shortening and rapid head expansion – generating a spine-like structure. Thus, in contrast to earlier time periods characterized by transient filopodia that extend and retract from the dendritic shaft, this period is characterized by a sharp increase in spine synapses (Dailey and Smith, 1996, Ziv and Smith, 1996, Marrs et al., 2001). Electron microscopy from motor sensory cortex in rat showed a sharp increase in synaptic density that commences in the

second postnatal week (Markus and Petit, 1987). During this period of robust synapse formation, a heterogonous population of spine-like protrusions can be detected on the dendritic shaft (Dailey and Smith, 1996, Fiala et al., 1998). 'Protospines', which embody features of both dynamic filopodia and stable spines, are also abundant during this time period (Dailey and Smith, 1996, Marrs et al., 2001). Like mature spines, protospines persist for longer periods of time, yet they exhibit rapid changes in length, which is characteristic of filopodia. The transient appearance of 'protospines' at an developmental stage preceded by filopodia and subsequently followed by the presence of mature spines, provided a key piece of evidence in support of the *'Filopodial Elongation Model'* of synapse formation (discussed in Section 1.3). According to this model, synapse formation is facilitated by dendritic filopodia, which serve as direct precursors to dendritic spines. After searching for and establishing the appropriate contact with an axon, synapse formation is initiated, thereby causing the filopodium to transition into a mature spine.

Guided by this hypothesis, a wave of studies using time-lapse and electron microscopy provided fundamental insight into the role of dendritic filopodia during synaptogenesis. Using confocal time lapse microscopy, Dailey *et al.* succeeded in directly visualizing the transition of a dynamic filopodia into a more stable, spine-like structures, suggesting that filopodia play an active role in establishing synaptic connection (Dailey and Smith, 1996). This study was closely followed by direct evidence that filopodia initiate physical contacts with nearby axons, subsequently leading to the formation of functional presynaptic boutons (Ziv and Smith, 1996, Fiala et al., 1998). Such evidence came in the midst of a highly contentious debate questioning the ability of filopodia to initiate synaptic contacts with axons and subsequently evolve into dendritic spines. From

these and other studies, a well-established sequence of synapse formation – in which filopodia play an active role –has been proposed (Purpura, 1974, Harris et al., 1992, Papa et al., 1995, Dailey and Smith, 1996, Ziv and Smith, 1996, Fiala et al., 1998, Maletic-Savatic et al., 1999, Okabe et al., 2001, Portera-Cailliau et al., 2003). Synapse formation is initiated when a dendritic filopodium probes the environment and establishes the appropriate axonal contact. Axo-dendritic contact stabilizes the filopodium, induces the formation of a presynaptic bouton and leads to the accumulation of postsynaptic components. This synaptic contact subsequently causes the filopodium to undergo the morphological changes required for conversion into a mature dendritic spine. This transient period of robust synaptogenesis is therefore characterized by a population of dendritic protrusions that are heterogeneous in both morphology and structural dynamics. Stable spines, dynamic filopodia, and persistent yet structurally dynamic 'protospines' cover the surface of the dendrite – reflecting a robust effort to establish the trillions of synapses require for proper brain function.

1.3.3 Synaptotrophic Hypothesis of Dendrite Development

The co-occurrence of dendrite development and synapse formation has long prompted researchers to question whether synaptic inputs are required for dendrite growth and stabilization (Vaughn, 1989). During development, only a small fraction of nascent branches that extend are maintained, while the majority rapidly retract (Niell et al., 2004). A leading hypothesis to explain the dynamic nature of new dendritic branches is that these transient branches 'sample' the environment for appropriate presynaptic contact, subsequently becoming stabilized by the formation and maturation of synapses. This so-called 'synaptotrophic hypothesis' was first articulated by Vaughn in the late 80's

and since then, convincing evidence suggests that dendrite development is indeed dictated by the nature and number of synaptic inputs (Wu et al., 1999, Niell et al., 2004, Haas et al., 2006). To observe the dynamic relationship between dendrite growth and synaptogenesis, Niell *et al.* used two-photon time-lapse microscopy to image dendrite development over multiple days in optic tectal neurons of living zebrafish. Synapses and dendrites were respectively labelled by expressing PSD-95-GFP, a scaffolding protein that localizes to the post synaptic density of excitatory. With this method, Niell *et al.* found that dendritic branches develop from stabilized filopodia that have established synaptic contact with an axon. Time-lapse imaging showed that a subset of filopodia that contact axons subsequently accumulate PSD-95, thereby becoming stabilized. Stabilized filopodia can then mature into dendritic branches, whereas as those that do not become stabilized are retracted. Successive iterations of selective stabilization leads to growth and branching of the arbor. A following study by Haas *et al.* demonstrated that AMPAR-mediated glutamatergic neurotransmission is required for synapse stabilization and dendrite growth (Haas et al., 2006). Collectively, these studies demonstrate that the formation and stabilization of synapses are required for robust dendrite growth and maturation.

1.3.4 Synaptic Refinement: Dendrite and Synapse Uncoupling

As synaptic circuits mature during adolescence and early adulthood, the structural plasticity of dendrites is greatly reduced. During this time, dendritic branches are mainly stabilized, with no major retraction or extension of branches (Wu et al., 1999, Trachtenberg et al., 2002, Holtmaat et al., 2005). Likewise, as neuronal development proceeds, the presence of filopodia is greatly reduced and the fraction of persistent spines

begins to increase, subsequently leading to a peak in spine and synaptic density (~ P26 - P30 in rat, depending on brain region) (Harris et al., 1992, Papa et al., 1995, Dailey and Smith, 1996, Portera-Cailliau et al., 2003, Holtmaat et al., 2005). As individuals transit adolescence and early adulthood, this peak in spine density is followed by a reduction in synaptic density, referred to as 'pruning' (Huttenlocher, 1979, Rakic et al., 1986, Markus and Petit, 1987, Huttenlocher, 1990, Bourgeois et al., 1994, Rakic et al., 1994). When dendritic spines of pyramidal neurons were imaged over days to months in the somatosensory cortex *in vivo*, Holtmaat *et al.* found that spine retractions exceeded spine additions in adolescent mice (P16- P25). Thus, the reduction in spine density that accompanies the late stages of synaptogenesis is not merely attributed to the loss of spines, but rather, reflects a dynamic remodeling of the synaptic circuitry – where synaptic connections are rapidly added and lost. In contrast to early development, in which stabilization of dendritic branches is he40avily dependent on synapse formation, this later stage of 'refinement' is characterized by an uncoupling of dendritic spine and dendritic branch stability. The uncoupling between dendrite and spine stability is critical for the long-term stability of synaptic circuits, as it allows mature neurons to fine-tine synaptic connections, while simultaneously maintaining the overall integrity of the dendritic arbor field. This gradual reduction and reorganization of synapses, referred to as *pruning*, can last throughout adolescence into early adulthood, depending on the brain region (Huttenlocher and Dabholkar, 1997). In the human prefrontal cortex, synaptic density increases between the age of 1–5 years, which is approximately 50% above the adult mean. By contrast, synaptic density sharply decreases between ages 2–16 (Huttenlocher, 1979, Huttenlocher and Dabholkar, 1997).

The overproduction of synapses as well as its gradual reduction during neuronal development, is highly dependent on experience and has important implications in learning, memory, and susceptibility to neuropsychiatric diseases (Molliver et al., 1973, Feinberg, 1982, Rakic et al., 1994). Pruning of synaptic circuits in the frontal cortex– a brain region associated with motivation, impulsivity, and addiction – is thought to underlie age-related behavior in adolescents. In support of this, experimentation with addictive drugs and onset of addictive disorders is primarily concentrated in adolescence and young adulthood (Chambers et al., 2003, Nixon and McClain, 2010). Age- related pruning of the frontal cortex is therefore believed to contribute to the increased risk-taking, susceptibility to drug addiction and vulnerability to neuropsychiatric diseases that characterizes the adolescence period (Feinberg, 1982, Rakic et al., 1994, Spear, 2000, Chambers et al., 2003). By contrast, the stabilization of synaptic circuits that is achieved during the transition from adolescence to adulthood is hypothesized to make individuals less likely to engage in impulsive decision making, addiction, and addictive-like behaviors (Spear, 2000, Chambers et al., 2003).

1.3.5 Adulthood: Stable Structures, Plastic Synapses

By adulthood, mature dendrites have achieved their final size and structure, extending many elaborate arbors. *In vivo* time-lapse imaging reveals that mature dendrites from adult rodents do not exhibit substantial elongation or retraction of existing branches or formation of new branches (Trachtenberg et al., 2002, Holtmaat et al., 2005). At this stage, synapse formation is complete and dendrites are uniformly covered in short (~1 um), stable spines that exhibit minimal changes in shape or length (Hosokawa et al., 1992, Papa et al., 1995, Dailey and Smith, 1996, Marrs et al., 2001). *In vivo* imaging

studies using adult mice reveal largely persistent spines in the multiple brain regions during this period (Grutzendler et al., 2002, Trachtenberg et al., 2002, Holtmaat et al., 2005, Zuo et al., 2005, Majewska et al., 2006). The majority of these spines support postsynaptic structures and are paired with presynaptic partner, demonstrating a mature synapse (Harris and Stevens, 1989, Harris et al., 1992, Ziv and Smith, 1996, Marrs et al., 2001). Although the majority of spines in the adult brain are stable, chronic time-lapse imaging of dendritic spines in the barrel cortex *in vivo* reveals that a portion of spines in the adult brain remain dynamic, with spines appearing and disappearing in response to novel sensory experience (Trachtenberg et al., 2002, Holtmaat and Svoboda, 2009). In addition, activity-induced modification of synaptic strength, referred to as *synaptic plasticity*, can alter both the number and size of dendritic spines (Hosokawa et al., 1995, Buchs and Muller, 1996, Engert and Bonhoeffer, 1999, Maletic-Savatic et al., 1999, Matus, 2000, Okamoto et al., 2004). Synaptic plasticity, and the resulting changes in spine structure, underlie many critical neurological processes, such as learning and memory (Lamprecht and LeDoux, 2004).

The sequence of events presented above form the basis for the development of postsynaptic dendrites and spines. Though dendrite development and synapse formation are intimately linked in early stages of development, the subsequent uncoupling of these processes is critical for the synaptic refinement and plasticity.

1.4 Models of Dendritic Spine Development

Though the existence of dendritic spines were documented as early as the late nineteenth century (Ramón y Cajal S, 1888), it would not be until nearly 100 years later before the first evidence-based theories of spine development began to coalesce into bona

fide models. Ultrastructural examination of fixed tissue as well as time-lapse imaging in cultured slices and dissociated neurons have provided a breadth of information about the mechanisms of spine development. From these studies, three main models of spine formation have emerged (**Figure 1.3**): 1) The Vaughn 'Filopodial' model 2) The Sotelo model and 3) The Miller/Peters 'Axonal Induction' model (Yuste and Bonhoeffer, 2004). The main discordance between these three models lies in the role ascribed to the presynaptic terminal during spine formation. In the Filopodial model, a dendritic filopodium searches for and captures an axon terminal, thereby causing the filopodium to transition into a mature spine. By contrast, the Sotelo model states that spines emerge independently from the axonal terminal. In the Miller/Peters model, spinogenesis is initiated when an axon terminal makes contact with the dendritic shaft, which then induces spine formation. More broadly, these theories debate the age-old question of cell-autonomy: are intrinsic signals sufficient for spine formation (e.g. is spine formation 'hard-wired') or are extracellular cues and signals required for the development of these structures?

Though dendritic spines possess conserved morphological and molecular features, it is important to note that a diversity of dendritic spines are present in the brain (even within the same neuron). The seemingly disparate observations between these models may therefore reflect physiologically relevant differences between brain regions, synapses, and spines. The following sections will summarize each model and detail the key pieces of evidence that has been instrumental in shaping each model.

1.4.1 The 'Filopodia Elongation' Model

Several early observations were instrumental in shaping the sequence of events that are proposed in the filopodial model of spine formation. Both *in vivo* (Purpura, 1974, Fiala et al., 1998) and *in vitro* (Papa et al., 1995, Dailey and Smith, 1996, Ziv and Smith, 1996), spine formation and synapse development are preceded by a period in which short-lived, dynamic structures transiently extend and retract from dendritic shaft (Dailey and Smith, 1996, Ziv and Smith, 1996). These dendritic filopodia are present in early development, but are absent from the mature brain, which is instead populated by stable, bulbous spines. As synapses form, filopodia numbers rapidly decline and appearance of stable-mature spines increase (Papa et al., 1995, Ziv and Smith, 1996, Fiala et al., 1998). Collectively, these findings suggest that filopodia play a developmental role as spine precursors.

Without directly observation filopodia motility, J.E. Vaughn proposed that dendritic filopodia function in 'short range exploration', sampling the environment in order to initiate synaptic contact with a nearby axon (Vaughn, 1989). Direct evidence of this hypothesis was provided by Dailey *et al.*, who showed that dendritic filopodia in cultured hippocampal slices exhibit rapid, protrusive motility, extending and retracting within 5-10 μm of the dendritic surface (Dailey and Smith, 1996). In addition, this study succeeded in directly visualizing the transition of a dynamic filopodium into a more stable, spine-like structure– thereby adding credence to the theory that filopodia can indeed be converted to spines. However, direct evidence that filopodia played an active role in synaptogenesis was not provided until Ziv. *et al.* directly visualized dendritic filopodia forming contact with nearby axons in dissociated hippocampal cultures. (Ziv

and Smith, 1996). Using time-lapse microscopy, the authors demonstrated that axonal contact 1) stabilized a dynamic filopodium and 2) was followed by the appearance (formation) of a presynaptic bouton that was precisely positioned at the sites where the filopodia contacted the nearby axon. These findings provided strong evidence that synapse formation between a dynamic filopodia and an axon is sufficient to stabilize a dynamic filopodia and promote its morphological conversion to a stable spine.

From this and subsequent studies (Fiala et al., 1998, Maletic-Savatic et al., 1999, Marrs et al., 2001, Portera-Cailliau et al., 2003), a widely-accepted sequence of events have been proposed (**Figure 1.3**). In the initial stages of dendritic spine formation, filopodia emerge from the dendritic surfaces in order to 'sample axons' and initiate a synaptic connection. Once a synaptic connection is established, filopodia undergo a 'filopodia to spine' transition, characterized by a decrease in motility, substantial shortening and rapid head expansion. These morphological changes give rise to mature, mushroom-shaped spine.

1.4.2 The Sotelo Model

Because the mammalian cerebellum is not vital for life, many genetic and natural mutations of the cerebellum have been exploited to study the mechanisms underlying spine formation (Yuste and Bonhoeffer, 2004). Most notably, *Weaver* and *Reeler* mutant mice have provided invaluable insight into the role of the axon terminal during dendritic spine formation (Landis and Reese, 1977). Through distinct mechanisms, both of these mice strains lack cerebellar granule cell fibers, which serve as the presynaptic component to the majority of spines in cerebellar Purkinje cells. In the absence of cerebellar granule cell fibers, Purkinje cells develop morphologically normal spines at roughly normal

densities (Landis and Reese, 1977, Sotelo, 1978). This observation suggests that the initial formation of spines does not require presynaptic axons. Indeed, many 'naked spines' (spines without presynaptic terminals) can be detected in early postnatal development (P0-P12) (Sotelo, 1975, 1978). Despite the absence of an axon terminal, *Weaver* and *Reeler* Purkinje cell spines possess electron dense postsynaptic 'particles' directly apposed to the membrane, suggesting that postsynaptic specialization, at least in part, is achieved by mechanisms independent of axonal innervation (Sotelo, 1975). These findings led Sotelo to propose a model in which spine formation (at least in portion of neurons) can be attributed to an intrinsic property of a neuron, rather than extracellular signals. In the case of the Purkinje cells, the neuron is 'hard-wired' to build spines as well as regulate spine density in the absence of granule cell fibers. In support of this hypothesis, using time-lapse microscopy, Dailey *et al.* observed the *de novo* appearance of stable spines that did not arise from the conversion of a dynamic filopodia or an intermediate 'protospine' structure (Dailey and Smith, 1996). Thus, at least in some systems, it appears that the *de novo* emergence of stable spines from the dendritic shaft is indeed a mechanism used to generate a population of spines.

1.4.3 The Millers/Peters Model

During early postnatal development, synapses formed directly on the dendritic shaft, referred to as shaft synapses, are present in the hippocampus (Fiala et al., 1998, Yuste and Bonhoeffer, 2004) and other brain regions (Miller and Peters, 1981). Like dendritic filopodia, the amount of shaft synapses is greatly reduced after the second postnatal week, during which dendritic spine begin to appear (Fiala et al., 1998). Based on observations in the rat visual cortex (Miller and Peters, 1981), Millers and Peters,

along with other researchers (Hamori, 1973, Mates and Lund, 1983), proposed an ‘axonal induction model’, in which the axon establishes contact with the dendritic shaft in order to induce spine formation (**Figure 1.3**). According to this model, an axon makes contact with the dendritic shaft, creating a shaft synapse that consequently gives rise to an immature, “stubby” spine. Subsequently, the presynaptic region and the dendritic spine become morphologically and functionally mature. Recently, a study showed that two-photon laser uncaging of the neurotransmitter glutamate can induce rapid, *de novo* formation of functional spines in cortical brain slices from young mice (P8-P12) within seconds (~6 seconds) (Kwon and Sabatini, 2011). This observation suggests that presynaptically-released glutamate may indeed trigger the formation of spines that bypass the ‘filopodial stage’. Rather than three isolated models, it is highly likely that multiple, non-mutually exclusive mechanisms are responsible for spine formation *in vivo*.

1.5 Dendritic Spine Structure

The primary function of dendritic spines is to compartmentalize the postsynaptic signaling molecules, scaffolding proteins, and receptors that are needed to respond to presynaptic stimuli. This biochemical compartmentalization necessitates that the spine cytoplasm be separated from the parent dendrite, a task that is largely mediated through the unique dendritic spine morphology. Dendritic spines are composed of three main compartments: 1) a base that is connected to the dendritic shaft 2) a constricted neck (~0.5 – 2 μm in length) and 3) a bulbous head (~1 μm in diameter) that directly apposes the presynaptic terminal (**Figure 1.4**). This unique structure allows spines to serve as independent, functional units of the excitatory nervous system. In addition, dendritic spines allow dendrites to establish synaptic connections with axons 1-2 μm beyond the

dendritic surface, thereby increasing synaptic density. Variability in spine dimensions regulates the degree of biochemical and electrical coupling to the parent dendrite, which in turn affects synaptic function (Wickens, 1988, Yuste and Denk, 1995, Hayashi and Majewska, 2005, Noguchi et al., 2005, Arellano et al., 2007, Biess et al., 2007, Araya et al., 2014).

Though spines possess conserved morphological features, they exhibit heterogeneity in shape (Jones and Powell, 1969, Harris et al., 1992). Studies using electron microscopy have identified three morphological classes of dendritic spines, each with distinct, measurable properties (**Figure 1.4B-1.4C**) (Harris et al., 1992). "Mushroom" spines contain a large bulbous head and a constricted neck, "stubby" spines are short without a well-defined neck, and "thin" spines have a long head and a small bulbous head. Dendritic filopodia, which are long protrusions with no distinct neck or head, are commonly accepted as the fourth category of dendritic protrusions. Though the functional significance of spine structure is not fully understood, an overwhelming body of evidence suggests that spine shape is correlated with synaptic efficacy (Arellano et al., 2007). For example, the volume of the spine head is correlated with the area of the postsynaptic density (PSD), the number of postsynaptic receptors on the membrane, and the size of the readily releasable pool of neurotransmitters (Freire, 1978, Harris and Stevens, 1989, Schikorski and Stevens, 1999), parameters which directly affect synapse function. In addition to having physiological consequences, modifications in spine structure are implicated in pathological conditions. For example, alterations in the size, shape and density of dendritic spines are hallmark features of many neuropathological conditions, such as schizophrenia, Fragile X syndrome and Alzheimer's disease (van

Spronsen and Hoogenraad, 2010, Kulkarni and Firestein, 2012). Aberrant spine morphology is also observed in addiction, cases of sensory deprivation, and chronic stress (van Spronsen and Hoogenraad, 2010, Kulkarni and Firestein, 2012). These alterations in spine morphology and density are accompanied by synapse loss and aberrant synaptic signaling, indicating that dendritic spine structure is tightly coupled to normal synapse function (Blanpied and Ehlers, 2004, van Spronsen and Hoogenraad, 2010, Penzes et al., 2011).

1.6 Ultrastructure of Dendrite Spines: The Actin Cytoskeleton and Post-Synaptic Density

Early EM studies examining the ultrastructure of dendritic spines revealed two main structural components: The F-actin cytoskeleton and an electron-dense assembly of postsynaptic proteins, referred to as the postsynaptic density (PSD). (Palade, Palay 1954) Collectively, these components act as a signal processing machine – allowing dendritic spines to dynamically regulate its structure and composition in response to synaptic activity. The following segment will summarize the key features of F-actin and the PSD in spines, in turn detailing their contribution to synaptic function.

1.6.1 The F- Actin Cytoskeleton in Dendritic Spines

Organization of F-actin in Spines

Actin is the major cytoskeletal component and structural determinant of dendritic spines (Fifkova and Delay, 1982, Landis and Reese, 1983) (Matus, 2000, Star et al., 2002, Okamoto et al., 2004). Early EM studies revealed the presence of both long filaments and a meshwork of short, branched actin filaments (Fifkova and Delay, 1982,

Landis and Reese, 1983) in the spine head. However, recent platinum EM images suggest that the organization of F-actin in spines is far more complex than originally appreciated (Korobova and Svitkina, 2010). Rather than a simplified model of branched actin in the spine head and linear actin in the spine neck, the actin cytoskeleton of the spine head, neck and base are all comprised of different proportions of branched and linear actin.

Similar to the organization of actin in the lamellipodia of motile cells, the spine head is mainly comprised of a short, cross-linked branched actin network (Korobova and Svitkina, 2010) (**Figure 1.5A**). Consistent with this organization, several actin binding proteins required for the formation of a dendritic branched network are enriched in dendritic spines. The Arp2/3 complex (Arp2/3), which binds to the sides of existing actin filaments to nucleate filaments, and multiple nucleation-promoting factors (NPFs) are critical for regulating spine structure. In addition, profilin, which transports ATP-actin monomers to polymerizing barbed ends, capping protein, which promotes Arp2/3-mediated polymerization, and ADF/cofilin, which depolymerizes pointed ends of actin to replenish the monomer pool, are all present in dendritic spines (Ackermann and Matus, 2003, Racz and Weinberg, 2006, 2008, Wegner et al., 2008, Korobova and Svitkina, 2010, Fan et al., 2011).

In the spine neck, linear actin filaments are organized into loosely arranged longitudinal actin bundles (Korobova and Svitkina, 2010) (**Figure 1.5A**). Interestingly, actin filament barbed ends, which are the site of polymerization *in vivo*, are frequently oriented towards the dendritic shaft (Frost et al., 2010, Korobova and Svitkina, 2010). The spine base is comprised of long actin filaments (and to a lesser extent, branched actin filaments) that converge with the microtubule network in the dendritic shaft.

Kinetic Pools of F-actin in Spines

Dendritic spines at rest contain two pools of F-actin: a dynamic pool and a stable pool (Star et al., 2002, Honkura et al., 2008) (**Figure 1.5B**). Using fluorescence recovery after photobleaching (FRAP), Star *et al.* found that 85% of actin in spines is highly dynamic, turning over in less than one minute. Subsequently, Honkura *et al.* found that the dynamic pool of F-actin undergoes retrograde flow from the spine apex to the base (Honkura et al., 2008). This finding is consistent with electron micrographs demonstrating that a significant fraction of actin filament barbed ends are oriented towards the spine membrane (Fifkova and Delay, 1982). Using a super-resolution microscopy technique called photactivated localization microscopy (PALM), Frost *et al.* found that, although spines possess a tip-to-base orientation of flow, actin polymerization could occur at subdomains throughout the spine. Moreover, the velocity and turnover of F-actin can be highly heterogeneous even within individual spines (Frost et al., 2010). In the last twenty years, advances in microscopy have provided a better understanding of the complex F-actin organization and dynamics within spines.

In contrast to the dynamic pool of F-actin, the stable pool of F-actin has a turnover of ~17 min (Honkura et al., 2008). This stable pool is located near the base of the spine head and is postulated to provide a scaffold by which the dynamic pool can generate an expansive force against the spine membrane, leading to structural modifications of spine shape and size(Honkura et al., 2008). Recently, Sidenstein *et al.* revealed the presence of a actin-βII/III spectrin periodic subcortical lattice in the base and neck dendritic spines (Sidenstein et al., 2016). This detergent resistant (Efimova et al., 2017) cytoskeletal network is discontinued at synaptic sites and does not reach the PSD

(Sidenstein et al., 2016). In non-neuronal cells, the actin-spectrin membrane skeleton forms a stable structure that is crucial for maintaining the structural integrity of cells (Yamashiro et al., 2012). The localization of this structure to a subspine region characterized by stable F-actin may account for the increased F-actin stability observed in the spine base.

Actin-based plasticity in spines

Two well-characterized models of synaptic plasticity, long term potentiation (LTP) and long term depression (LTD), enhance and decrease synaptic transmission respectively (Malenka and Bear, 2004). In response to LTP and LTD, dendritic spines undergo bidirectional spine remodeling, where LTP causes spine head enlargement and LTD causes spine shrinkage (Hosokawa et al., 1995, Buchs and Muller, 1996, Engert and Bonhoeffer, 1999, Maletic-Savatic et al., 1999, Matus, 2000, Okamoto et al., 2004). Dynamic rearrangements of F-actin drive the changes in spine morphology associated with activity-induced synaptic plasticity (Star et al., 2002, Okamoto et al., 2004, Fukazawa et al., 2003). Recently, Honkura *et al.* found that LTP-inducing stimuli causes the formation of a kinetically stable 'enlargement pool' of F-actin, which is required to mediate long-term expansion of the spine head (Honkura et al., 2008). The F-actin spine cytoskeleton is therefore dynamically regulated in response to synaptic activity.

1.6.2 The Postsynaptic Density

Approximately 60 years ago, EM images of synapses in the central nervous system revealed 'densities' directly underneath the presynaptic and postsynaptic plasma membranes (Palay and Palade, 1955, Palay, 1956). The presynaptic 'density' corresponds

to the active zone (AZ), a region of the axon terminal that contains the molecules required for the exocytosis of synaptic vesicles (Sudhof, 2012). On the postsynaptic side, the electron-dense 'thickening' underneath the dendritic spine membrane would later be revealed as a diverse assembly of proteins, referred to as the post-synaptic density (PSD) (**Figure 1.1-B**) (Okabe, 2007, Sheng and Hoogenraad, 2007). The PSD contains the glutamate receptors that detect presynaptically-released glutamate as well a myriad of signaling and cytoskeletal proteins that transduce glutamate into postsynaptic biochemical responses (Kennedy, 2000). Mass spectrometry has provided a global analysis of PSD constituents (Peng et al., 2004, Chen et al., 2005, Cheng et al., 2006), which is estimated to exceed over five-hundred proteins (Cheng et al., 2006, Dosemeci et al., 2007). However, the precise postsynaptic function of many identified PSD components remains to be determined.

For optimal synaptic transmission, the PSD is situated at the distal tip of the spine head, directly apposed from the presynaptic AZ (Palay, 1956). Though the dimensions of the PSD can be quite variable, most are approximately 200-500 nm in diameter and ~ 60 nm thick (Carlin et al., 1980, Harris et al., 1992, Fiala et al., 1998). The size of the PSD is correlated with spine head volume and the number of presynaptic vesicles (Harris and Stevens, 1989, Harris et al., 1992), which in turn affects synaptic function (Kennedy, 2000). Notably, the PSD is not a static structure, but has been shown to change shape and size during development and in response to synaptic activity (Marrs et al., 2001, Blanpied et al., 2008, MacGillavry et al., 2013). The physiological importance of the PSD has also been demonstrated through molecular disruption of PSD components, which causes

synaptic phenotypes associated with diseases (Gardoni et al., 2009, Bayes et al., 2011, Peca et al., 2011, Uchino and Waga, 2013).

Glutamate receptors

Excitatory synaptic transmission in the central nervous system relies on the release of glutamate from presynaptic terminals, which, after diffusing across the synaptic cleft, binds to postsynaptic glutamate receptors. Three principle subtypes of ionotropic glutamate receptors mediate excitatory transmission: the ligand-gated ionotrophic glutamate receptors, N-methyl-D-aspartate (NMDA)-type receptor and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type receptor as well as the kainate receptors (Dingledine et al., 1999, Traynelis et al., 2010). The number, composition, and location of receptors in neurons are critical determinants of synaptic transmission (Kennedy and Ehlers, 2006, Newpher and Ehlers, 2008, MacGillavry et al., 2011). The NMDAR family of glutamate receptors form a multi-subunit, nonselective ion channel that is permeable to Ca^{2+} as well as monovalent cations K^{+} and Na^{2+}. NMDAR receptors are highly enriched in the PSD fraction (Moon et al., 1994). Under basal conditions, when the dendritic spine membrane potential is at its resting state, NMDAR channels are blocked by a Mg^{2+} in the pore of the channel. Thus, NMDARs have little contribution to synaptic transmission at the resting potential (Higley and Sabatini, 2012). By contrast, AMPARs are permeable to ions at resting membrane potential and activation of these receptors is fast and transient (Malinow and Malenka, 2002). Depolarization of sufficient amplitude and duration removes the Mg^{2+} block from the NMDA pore, allowing the influx of Ca^{2+}, K^{+} and Na^{2+} into dendritic spines (Higley and Sabatini,

2012). NMDAR-dependent Ca^{2+} influx is heavily dependent on membrane potential and glutamate release.

In pyramidal neurons in the hippocampus, NMDARs are a predominant source of evoked calcium signals in the dendritic spine (Sabatini et al., 2001, Higley and Sabatini, 2012). Activation of NMDARs by glutamate produces a highly compartmentalized Ca^{2+} transient that is restricted to the spine head, allowing the synapse to be modulated in a spine by spine manner (Kennedy et al., 2005). Additional calcium entry through VGCC (voltage gated calcium channels) or release by intracellular calcium stores can contribute to postsynaptic depolymerization (Kennedy, 2000, Higley and Sabatini, 2012). In the spine, Ca^{2+} ions act as a principal signaling molecule, and together with calmodulin (CaM), Ca^{2+} regulates a wide variety of neuronal functions (Kennedy, 2000, Kennedy et al., 2005). In response to Ca^{2+} influx, many Ca^{2+} – dependent kinases and phosphatases activate and deactivate components of the postsynaptic signaling machinery, in turn regulating diverse processes such as protein trafficking and protein synthesis (Kennedy, 2000, Higley and Sabatini, 2012). This in turn has important functional consequences on synaptic development and plasticity (Newpher and Ehlers, 2008).

Scaffolding molecules

By interacting with both cytoplasmic and membrane bound proteins, scaffolding proteins provide the structural framework for the PSD (Kim and Sheng, 2004, Okabe, 2007). In order to mediate this interaction, scaffolding proteins contain multiple protein interaction domains, such as PDZ domains, SH3 domains, and polyproline domains (Sheng, 2001, Kim and Sheng, 2004). Both EM micrographs and advanced super-resolution microscopy show that the actin cytoskeleton is intimately intertwined with the

PSD, where it plays a crucial role in organizing receptors at the PSD and in the spine membrane (Fifkova and Delay, 1982, Allison et al., 1998, Frost et al., 2010). Interestingly, actin filaments near the PSD are very dynamic (Frost et al., 2010), suggesting that receptors are not statically anchored (Allison et al., 1998, Frost et al., 2010). The relationship between actin and PSD components are made even more complex due to the fact that different PSD components exhibit varying degrees of dependence on F-actin for PSD association (Allison et al., 1998, Allison et al., 2000). For example, clustering of the scaffolding protein PSD-95, a core member of the PSD that anchors NMDA receptors, is unaffected by actin depolymerization, whereas Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), is dependent on an intact actin cytoskeleton (Allison et al., 2000). Mass spectrometry has discovered an array of actin-binding proteins and actin cross linking proteins associated with the PSD (Cheng et al., 2006, Dosemeci et al., 2007) and many signaling pathways converge on the F-actin cytoskeleton, reflecting its importance in regulating postsynaptic biochemical responses to glutamate.

1.7 The actin cytoskeleton in dendritic spine morphogenesis

In addition to controlling the structure and molecular organization of postsynaptic spines, the actin cytoskeleton is crucial for spine development and synapse formation. During synapse formation, dendritic filopodia undergo distinct morphological changes to yield a mature spine. It is well-established that dynamic remodeling of the postsynaptic actin cytoskeleton drives spinogenesis, yet the precise mechanisms underlying filopodia initiation, elongation, and spine head formation are not fully understood.

At present, fundamental questions regarding actin regulation and remodeling during spine morphogenesis are still outstanding. What role does cytoskeleton play in the initiation of dendritic spines? How does the actin cytoskeleton of dendritic filopodia different from that in mature spines? What actin binding proteins are responsible for the dynamic cytoskeletal rearrangements underlying spine formation? Until recently, the precise organization of the actin filaments in dendritic filopodia was unknown, leading to an assortment of speculative models about the mechanisms by which actin is modified during spine morphogenesis.

Using platinum replica EM, Korobova *et al.* characterized the cytoskeleton of filopodia in DIV12 (*day in vitro*, 12) dissociated primary hippocampal cultures, a stage characterized by abundant dendritic filopodia (Korobova and Svitkina, 2010). Importantly, this study revealed fundamental differences between dendritic filopodia and conventional filopodia, a finding that provides crucial insight into the morphogenesis of spines. Unlike conventional filopodia, which consist of tight bundles of straight, cross-linked filaments, dendritic filopodia consist of loosely arranged actin filaments of varying lengths. Consistent with this arrangement, dendritic filopodia lack fascin, a conventional actin filament bundling protein. Interestingly, dendritic filopodia contain the Arp2/3 (Arp2/3) complex and capping protein (CP), consistent with the presence of branched actin (particularly in the filopodia tip). Consistent with notion that dendritic filopodia give rise to spines, the organization of F-actin in the base of filopodia is similar to that found in the base of mature spines. In the filopodia base, long actin filaments (and some branched filaments) converged with the dendrite, often extending into the microtubule network in the shaft. In contrast to the parallel, unipolar arrays of actin filaments that

predominate in conventional filopodia (Svitkina et al., 2003), dendritic filopodia contain a barbed ends that face the filopodia base (near dendritic shaft), rather than the tip. This mixed orientation of actin filaments is consistent with the reports stating that dendritic filopodia elongate from actin polymerization at both the filopodia tip and root (Hotulainen et al., 2009).

In addition to dendritic filopodia, dendrites of DIV10 primary dissociated neurons contain patches of highly branched actin, referred to as 'actin-patches'. Though filopodia were long appreciated to originate from the dendritic shaft (Harris and Stevens, 1989, Papa et al., 1995, Dailey and Smith, 1996) from pre-existing patches (Andersen et al., 2005), Korobova *et al.* showed that that filopodia originate from phase dense patches on the dendritic shaft. These phase dense filopodial precursors likely correspond to the patches of branched actin filaments that were observed in DIV10 dendrites using platinum replica EM.

Collectively, these findings provide a working model for cytoskeletal rearrangements that occur during spinogenesis (**Figure 1.6**). Spine formation begins with the formation of a patch of branched actin network on the dendritic shaft. This actin patch subsequently elongates into a dendritic filopodium. The small GTPase Rif and its downstream effector, mDia2 formin has been shown to be important for filopodia formation (Hotulainen and Hoogenraad, 2010). Time-lapse microscopy shows that filopodia-to-spine transition is accompanied by a 'swelling' in the tip of the filopodia (Marrs et al., 2001). The patches of branched actin at the filopodia tip (as revealed by EM) may therefore reflect an initiation of spine head enlargement by Arp2/3-mediated nucleation. In support of this, inactivation of Arp2/3 complex leads to defects in the

formation of bulbous spine heads from dendritic filopodia reduced spine density and altered spine morphogenesis (Kim et al., 2006, Wegner et al., 2008, Spence et al., 2016). Spine development may therefore involve a switch from mDia2-based nucleation, which promotes the formation of linear actin filaments, to Arp2-3 mediated nucleation, which is required for the formation of a branched actin network at the filopodial tip. In the non-neuronal cells, the formation of Arp2/3-mediated branched actin networks requires capping protein (CP). CP binds to the barbed ends of actin filaments, thereby inhibiting further elongation and promoting nucleation by the Arp2/3 complex (Pollard and Borisy, 2003, Akin and Mullins, 2008). Consistent with the presence of CP in dendritic filopodia, Fan *et al.* found that capping of actin filaments by capping protein is an essential step required to remodel the actin cytoskeleton during spine morphogenesis (Fan et al., 2011). Using cultured hippocampal neurons, Fan *et al.* found that depletion of capping protein leads to an increase in filopodia like protrusions and large, irregularly shaped spine heads. These results demonstrate the CP is required for restricting filament elongation and promoting the formation of branched actin via Arp2/3-mediated nucleation.

In addition to actin nucleation, ADF/cofilin-induced (cofilin-1) actin filament disassembly has been shown to be crucial for shaping spine head morphology during development. In neurons with reduced amounts of cofilin-1, actin filament turnover is substantially decreased and spines contain abnormal filopodial like protrusions and long spine necks (Hotulainen et al., 2009). Thus, in addition to replenishing the polymerization-competent pool of G-actin, actin filament depolymerization and severing by ADF/cofilin is necessary for restricting filaments length in the spine neck and head during spine formation. After spine head expansion, myosin II-dependent contractility

and cross-linking may further modulate the shape of the spine head and neck, generating a morphologically mature dendritic spine (Ryu et al., 2006).

Though the precise mechanisms underlying filopodia initiation, elongation, and spine head formation are not fully understood, many studies have been fundamental in elucidating the mechanisms of actin regulation underlying dendritic spine morphogenesis (**Table 1.1**). The sequence of events described above provides a conceptual framework for dendritic spine morphogenesis. Future studies characterizing the cytoskeletal organization and molecular composition of dendritic filopodia and spines will no doubt contribute to our understanding of how the actin cytoskeleton is dynamically remodeled during spine morphogenesis and synapse formation.

1.8 Modulation of Actin in Dendritic Spines by Capping Proteins

Actin in the cell exists in two forms: globular actin (G actin, 'actin monomers') and filamentous actin (F-actin). F-actin is polymerized from ATP-bound actin monomers, forming a polarized filament with a barbed (plus) end and pointed (minus) end that favors actin assembly and disassembly, respectively (**Figure 1.7A**) (Pollard and Borisy, 2003, Blanchoin et al., 2014). *In vivo*, actin filaments form diverse cytoskeletal structures, each contributing to distinct cellular functions. For example, in the leading edge of motile cells, actin forms a highly branched, dendritic network – an organization that is critical for membrane protrusion and subsequent cell migration. By contrast, actin filaments in the sarcomere of striated muscle are organized into straight filaments of uniform length, an orientation required for efficient muscle contraction. Formation and remodeling of distinct F-actin structures involves the concerted efforts of a diverse array of actin regulatory proteins (Pollard et al., 2000, dos Remedios et al., 2003). These so called

‘actin-binding proteins’ regulate actin dynamics, length, and higher order organization, consequently producing the diverse actin-based structures observed within and among cells. Of these actin regulatory proteins, those involved in filament nucleation, severing/disassembly, crosslinking, end capping, and monomer sequestering are believed to be crucial for the formation and dynamics of actin structures (Pollard et al., 2000, Pollard and Borisy, 2003).

In dendritic spines, a wide range of actin binding proteins exert spatiotemporal control over filament dynamics (Hotulainen and Hoogenraad, 2010), generating distinct structural and functional pools of F-actin (Honkura et al., 2008). Due to the extensive crosstalk between different actin regulatory molecules/pathways, delineating the specific functions of individual actin binding molecules during spine formation and remodeling remains a huge challenge. Strong evidence suggest that capping of actin filament minus ends represents an important mechanism for regulating F-actin at the synapse. The following segment will focus on the known role of F- actin capping proteins in regulating spine structure and function, particularly during synapse development.

Because filament polymerization and depolymerization occurs at filament ends, the regulation of free filament ends is dictates the dynamics and organization of F-actin. Capping proteins can specifically bind to either the barbed or pointed end of actin filaments, where they block the association and dissociation of G-actin monomers (**Figure 1.7A**). An assortment of actin plus end capping proteins are present *in vivo*, each with distinct properties. These include capping protein (CP), gelsolin, epidermal growth factor receptor pathway substrate 8 (Eps8), and adducin. Capping of the barbed end represents an important mechanisms for regulating filament length and promoting

Arp2/3-mediated nucleation. By contrast, the pointed ends of actin filaments are capped by the acumentin, Arp2/3 complex (Arp2/3) and Tropomodulin (Tmod) (Southwick and Hartwig, 1982, Weber et al., 1994, Amann and Pollard, 2001). Of these two, Arp2/3 and Tmod have been extensively studied, while acumentin has received considerably less attention. Unlike the Arp2/3 complex that caps pointed ends in order to nucleate daughter filaments, Tmod capping stabilizes preexisting filaments by inhibiting filament elongation or depolymerization.

Regulation of F-actin by plus end capping proteins is an essential step required for dynamic actin remodeling during spine morphogenesis. Capping protein (CP, also known as CapZ), is the best characterized barbed-end capping protein and is essential for actin-based motility (Pollard and Borisy, 2003, Akin and Mullins, 2008). In cultured hippocampal neurons, the loss of CP resulted in a substantial reduction in the number of mushroom-shaped dendritic spines with a concomitant increase in filopodia-like protrusions (Fan et al., 2011). The loss of CP also resulted in the emergence of aberrant filopodia-like protrusions from the spine head. These results suggest that barbed end capping regulates filament length and promotes Arp2/3-mediated growth of branched actin networks during spine head expansion. Epidermal growth factor receptor pathway substrate 8 (Eps8), is a multifunctional protein that can cap barbed ends, bundle actin filaments, and activate the Rac signaling pathway (Disanza et al., 2004, Hertzog et al., 2010). Genetic knockout of Eps8 in mice leads to the formation of immature spines and impaired cognitive function (Menna et al., 2013), phenotypes shown to be mediated by the capping function of Eps8. Spines lacking Eps8 also showed increased actin polymerization (Stamatakou et al., 2013), less stable PSD-95 dynamics (Menna et al.,

2013), and reduced synaptic strength (Menna et al., 2013). Together, these studies suggest that Eps8 regulates actin dynamics as well as the accumulation and/or clustering of PSD components during spine formation. It remains to be determined if Eps8 and CP function redundantly in spine development.

In addition to synapse formation, barbed end capping of actin filaments may represent a key event that enables the modification of the spine actin structure during synaptic plasticity. Consistent with this notion, both CP and Eps8 are recruited into spines during LTP-inducing stimuli (Kitanishi et al., 2010, Menna et al., 2013) and Eps8 capping activity is required for LTP-mediated spine formation (Stamatakou et al., 2013) and synaptic strengthening (Menna et al., 2013). However, the mechanism by which barbed end capping is regulated by synaptic activity is currently unclear. One possibility involves the actin severing protein gelsolin, which can be regulated by Ca^{2+} and PIP2 (Sun et al., 1999) and may function in activity-dependent barbed capping of actin filaments in spines without filament severing (Star et al., 2002). β-adducin is localized to dendritic spines and mice lacking β-adducin display impaired synaptic plasticity, motor coordination and learning deficits (Rabenstein et al., 2005, Porro et al., 2010, Bednarek and Caroni, 2011). Adducins may function during synaptic activity by capping barbed end of actin filaments in the actin-βII/III spectrin membrane lattice, which is present in the base and neck dendritic spines. This newly-identified cytoskeletal structure is necessary for the formation of a constricted dendritic spine neck and proper synaptic transmission (Bar et al., 2016, Sidenstein et al., 2016, Efimova et al., 2017). Modulation and specificity of capping activity amongst these various classes of barbed end capping proteins is likely specified by binding partners, differential affinity for barbed ends, other

actin-regulatory domains within the protein (such as bundling or severing) and post-translational modifications. Further studies will be needed to elucidate the mechanism by which barbed end capping proteins work in concert to regulate the actin cytoskeleton at the synapse.

Very little is currently known about pointed end capping and its function in dendritic spines. Tmods are the best known pointed end capping proteins and Tmod1, Tmod2 and Tmod3 are expressed in the nervous system (Sussman et al., 1994, Watakabe et al., 1996, Conley et al., 2001, Cox et al., 2003). The presence of unbranched actin filaments in the spine head and neck (Korobova and Svitkina, 2010) suggests that portion of pointed ends are regulated by Tmod capping, substantially impacting their stability. Altered expression of Tmod1 is observed in kainic-acid induced seizures and altered expression of Tmod2 is observed in Down's syndrome, epilepsy, prefrontal ischemia and methamphetamine exposure (Sussman et al., 1994, Iwazaki et al., 2006, Yang et al., 2006, Chen et al., 2007, Sun et al., 2011). These findings suggest that regulation of actin dynamics by Tmod is critical for brain function. In support of this, Tmod2 knockout mice exhibit synaptic and behavioral deficits, including altered learning and memory (Cox et al., 2003). However, the underlying cellular mechanisms responsible for this phenotype is not fully known. In a recent study using gain-of-function analysis of exogenously-expressed Tmods, Tmod1 and Tmod2 were found to play a role in dendritic branching and spine morphology in cultured rat hippocampal neurons (Gray et al., 2016). However, the precise mechanisms by which Tmod modifies F-actin dynamics in the postsynaptic compartment has not been investigated. At present, Tmod remains poorly characterized in the neurons.

1.9 Tropomodulin Family of F-actin Pointed End Capping Proteins

1.9.1 Tropomodulin isoforms

Tropomodulins (Tmod) are a conserved, multi-domain family of proteins that cap the pointed end of actin filaments (Yamashiro et al., 2012, Fowler and Dominguez, 2017) in metazoans (**Figure 1.7B**). By blocking monomer exchange at filament ends and inhibiting filament elongation or depolymerization, Tmod regulates the stability, length, and architecture of complex actin networks in diverse cell types (Yamashiro et al., 2012). At present, four vertebrate Tmod isoforms, each encoded by distinct genes, are expressed in a tissue specific and developmental manner (Yamashiro et al., 2012). Tmod1 is predominantly expressed in terminally differentiated, post-mitotic cells such as neurons, striated muscle, and red blood cells. Tmod2 is expressed predominately in neurons, Tmod3 is ubiquitously expressed, and Tmod4 expression is restricted to skeletal muscle (Yamashiro et al., 2012). Tmod1 was first discovered as a binding protein of tropomyosin (TM) in erythrocytes (Fowler, 1987), where it is associated with actin filaments in the erythrocyte membrane skeleton (Yamashiro et al., 2012). Tmod1 is by far the best studied of the Tmod family members and extensive studies examining the function of Tmod1 have provided fundamental knowledge about the biochemical properties and functions of Tmods *in vivo*.

1.9.2 Structural and Functional Domains of Tmods

Tmod1-4 are highly conserved and all four isoforms exhibit ~ 75% amino acid sequence similarity within the same species (Watakabe et al., 1996, Almenar-Queralt et al., 1999). All Tmods share two conserved domains: an unstructured N terminal ‘Tropomyosin (TM)/Pointed-End Capping’ (TMCap) domain and a compactly folded, C-

terminal Leucine-Rich Repeat/Pointed End Capping (LRRCap) domain (Kostyukova, 2008a, Yamashiro et al., 2012). Both the TMCap domain and the LRRCap domain are able to cap actin filament pointed ends, though the properties of these domains vary significantly. Tight binding of Tmod to F-actin pointed ends requires tropomyosin (TM), an α-helical coiled-coil protein that binds along the sides of F-actin filaments (**Figure 1.7B**) (Weber et al., 1999). The TM-Cap domain binds to both F-actin and TM, and caps actin pointed ends with high affinity (~ 0.2 nM K_D) (Weber et al., 1994, Weber et al., 1999, Kostyukova and Hitchcock-DeGregori, 2004). By contrast, the LRR-Cap domain does not require TM for F-actin binding and therefore caps the pointed ends of TM-free filaments very weakly (~ 0.1 μM K_D). Weak association of Tmod1 with TM-free actin filament pointed ends reduces, but does not completely block actin subunit exchange (Weber et al., 1994, Weber et al., 1999). Thus, by binding F-actin pointed ends in TM-dependent or independent manner, Tmods can function as both a 'tight cap' and a 'leaky cap' respectively.

1.9.3 Tropomyosins

Tropomyosins (TM) form head-to-tail polymers along the length of actin filaments to stabilize and regulate the access of actin binding proteins to the filaments (Martin and Gunning, 2008). In mammals, four tropomyosin genes (TPM1-4) produce over 40 protein isoforms (Guven et al., 2011). Several brain specific TMs have been identified, of which TMs from TPM3 and TPM4 genes have shown to localize to postsynaptic spines (Guven et al., 2011). However, the functions of TMs in neurons, especially in synapses, remain largely unknown. Importantly, Tmod isoforms have been shown to have different

affinities for TM isoforms (Kostyukova, 2008), thereby adding a layer of complexity to Tmod regulation in diverse tissues.

1.9.4 Proposed model of Tmod attachment to actin filament pointed ends

Deletion and mutagenesis studies, combined with a high-resolution crystal structure of Tmod1, have provided a model for Tmod docking at actin filament pointed ends (Kostyukova et al., 2001, Krieger et al., 2002, Kostyukova and Hitchcock-DeGregori, 2004, Lu et al., 2004, Kostyukova et al., 2005, Uversky et al., 2011).

Evidence suggests that both the LRR-Cap and TM-Cap domains contribute to the pointed end capping activity of Tmods. Because Tmods binds to TM directly, the two terminal TMs near the actin filament pointed end contribute binding sites for the TMCap, thereby enhancing its affinity for actin filament pointed ends. Moreover, the TM-Cap domain contains an F-actin-binding that contributes a third binding site at the pointed end (Kostyukova and Hitchcock-DeGregori, 2004, Kostyukova, 2008b). Partial crystal structures of Tmod1 suggests that The LRR-Cap domain provides an additional binding site for F-actin at the pointed end, leading to a total of four Tmod binding sites at the TM-coated actin filament pointed end (Krieger et al., 2002). Both TM-dependent and independent actin pointed end activity are required for maximum Tmod affinity to pointed ends (Kostyukova and Hitchcock-DeGregori, 2004, Kostyukova et al., 2005, Kostyukova, 2008b) as well as pointed end capping *in vivo* (Mudry et al., 2003, Tsukada et al., 2011). The model of Tmod docking presented above is predominantly based on studies of Tmod1, whose physical properties (including partial crystal structure) have been extensively characterized.

1.9.5 Other Actin Regulatory Functions of Tmods

Though pointed end capping is the most well-characterized function of Tmods, Tmod2 and Tmod3 can also sequester G- actin monomers in the absence of tropomyosin (Fischer et al., 2006, Yamashiro et al., 2014) *in vivo* and *in vitro,* and Tmods can also nucleate actin filaments weakly *in vitro* (Yamashiro et al., 2010). Unlike pointed end capping, these actin-regulatory functions have not been extensively studied in a cellular context.

1.9.6 Localization and Function in Diverse Cellular Structures

Tmods localize to diverse, actin-based structures, where they regulate actin dynamics, length, and organization. Genetic depletion of Tmod in a broad range of tissues have provided fundamental insight into the function of Tmod *in vivo*. In particular, loss-of-function studies in the 'membrane skeleton' of diverse cells have provided convincing evidence that Tmod is crucial for stabilizing cytoskeletal architecture and organizing membrane and cell morphology. The membrane skeleton is a highly-crosslinked, submembranous network comprised of spectrin, actin, and associated accessory proteins (Fowler, 2013). This cytoskeletal network is crucial for maintaining cell morphology, organizing membrane components, and establishing polarized membrane domains (Fowler, 2013). Short actin filaments (15-18 subunits long) are a feature of the membrane skeleton and are crucial for organizing spectrin molecules. This restricted filament length is achieved by capping filament barbed and pointed ends by adducin and Tmod, respectively, thereby inhibiting actin exchange at filament ends. In red blood cells (RBCs), the actin-spectrin membrane skeleton is a major determinant of cell shape, allowing the RBC to withstand circulatory stress. Depletion of Tmod1 from

RBCs depolymerizes actin filaments and disrupts the organization of the spectrin-actin lattice, in turn reducing the deformability and increased the fragility of RBCs (Yamashiro et al., 2012, Fowler, 2013). These results provide clear evidence that Tmods are important for restricting filament length and regulating actin dynamics.

The spectrin-based membrane skeleton in polarized epithelial cells is crucial for maintaining a tall, cuboidal morphology as well as the assembly of apical and basolateral membrane domains. Tmod3 is the sole isoform present in polarized intestinal, bronchiole, and kidney epithelial cells lines (Weber et al., 2007), where it is present in the membrane skeleton of these cells. SiRNA depletion of Tmod3 causes actin filament depolymerization, a reduction in TM levels, and a disorganized membrane skeleton, thereby causing shorter, collapsed cells. Similarly, loss of Tmod1 from lens fiber cells, which typically exhibit a near-perfect hexagonal geometry, leads to irregularly shaped cells with disordered packing (Gokhin et al., 2012, Nowak and Fowler, 2012). These phenotypes suggest that pointed end capping by Tmod are critical for maintaining membrane skeleton integrity and proper cell morphology.

Although Tmods preferentially localize to stable actin structures, such as the sarcomere of striated muscle (Yamashiro et al., 2012), Tmods are also found in dynamic actin structures. Tmod3 is associated with F-actin the leading edge of migrating endothelial cells (Fischer et al., 2003). Somewhat paradoxically, siRNA knockdown of Tmod3 increases migration rates, while overexpression of GFP-Tmod3 decreases migration. These effects may be due to the monomer sequestering activity of Tmod3, which would reduce the amount of G-actin available for barbed end polymerization, thus

slowing migration. To date, the mechanisms by which Tmods regulate dynamic actin networks are not well understood.

1.10 Summary

The development of dendritic arbors and dendritic spine are crucial for neuronal communication in the excitatory glutamatergic nervous system. A host of neuropsychiatric and neurodevelopmental disorders, ranging from schizophrenia to Alzheimer's disease, are characterized by defects in dendrite branching and changes in spine morphology and density. An understanding of how these postsynaptic structures are misregulated under pathological conditions will first require an understanding of the molecules and pathways responsible for their development under physiological conditions.

Dynamic remodeling of the actin cytoskeleton is crucial for the development of postsynaptic dendrites and spines. However, the molecules and mechanisms that regulate actin organization and remodeling during postsynaptic development are not fully understood. The Tropomodulin (Tmod) family of molecules cap the pointed end of actin filaments, thereby regulating the stability, length, and architecture of complex actin networks in diverse cell types. Tmods are highly expressed in the central nervous system, yet how these molecules function in the brain is largely unknown.

The overall goal of this dissertation is to characterize the expression profile, subcellular distribution, and function of Tmod in hippocampal neurons. We hypothesize that minus end capping of actin filaments by Tmod is essential for stabilizing the actin cytoskeleton during postsynaptic development. The research presented in this dissertation will address four essential questions: 1) What is the expression profile and subcellular

distribution of endogenous Tmod1 and Tmod2 in hippocampal neurons? 2) What is the role of Tmod during dendrite development? 3) What is the function of Tmod during dendritic spine development and synapse formation? and 4) How does Tmod regulate the F- actin cytoskeleton in dendritic spines? In Chapter 3, I present original work characterizing the expression of Tmod1 and Tmod2 in hippocampal neurons and examining their function in dendrite development. In Chapter 4, I present novel work assessing the function of Tmod1 and Tmod2 in dendritic spine morphogenesis and synapse formation. Collectively, the findings in this dissertation provide novel insight into the mechanisms by which F-actin is regulated during neuronal development and adds to the limited body of knowledge about Tmod function in neurons.

1.10 Figures

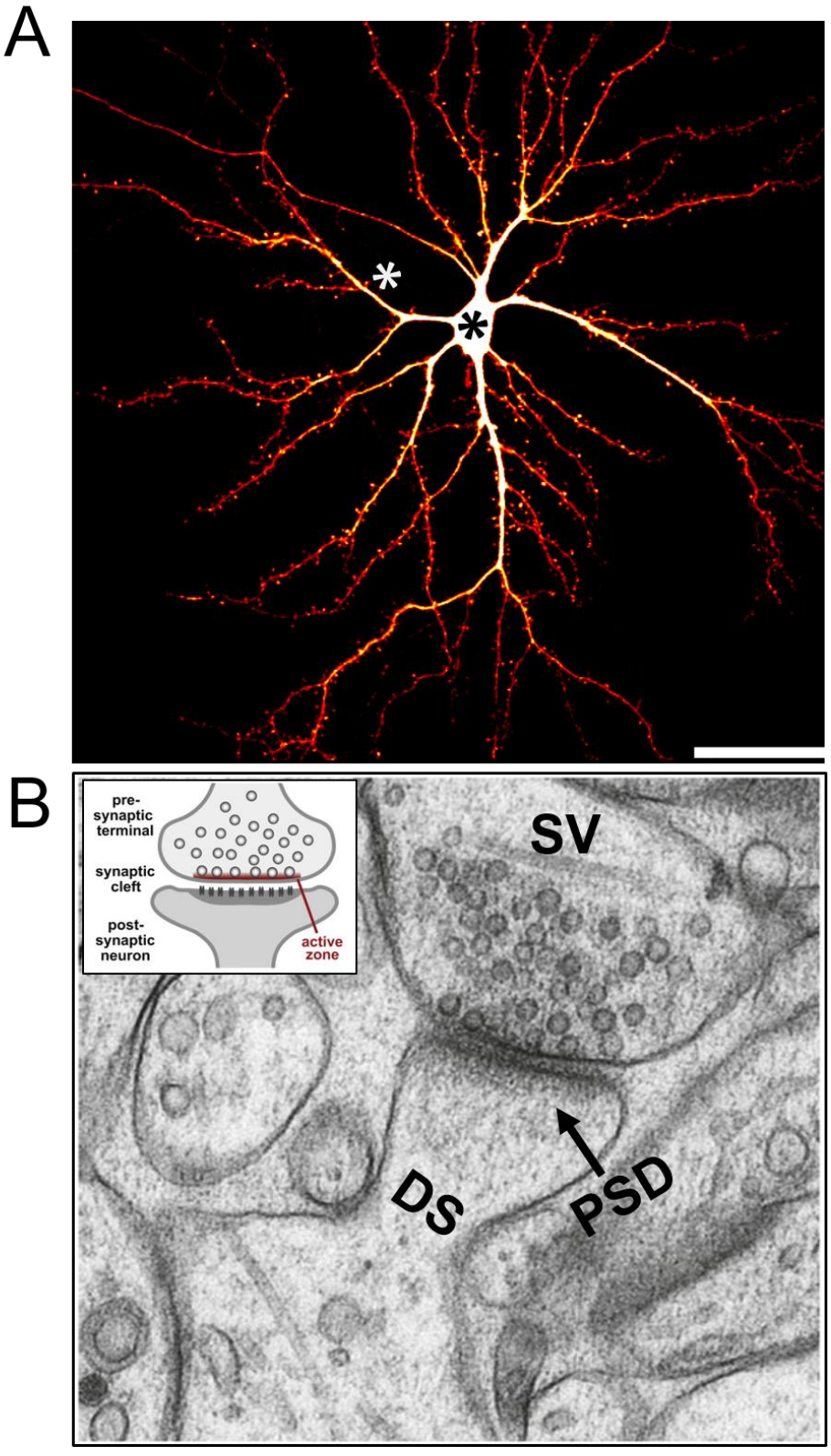

A
B
pre-
synaptic
terminal
synaptic
cleft
post-
synaptic
neuron
active
zone
SV
DS
PSD

Figure 1.1: Neuronal Morphology and Synapse Function.

A: Neuronal morphology. Confocal image of a mature neuron from dissociated hippocampal cultures transfected with green fluorescent protein (GFP). Dendritic branches (white asterisk) covered in dendritic spines extend from the cell body (black asterisk). Scalebar: 30 µm. **Image: Julia Omotade, Laboratory of James Zheng**. **B:** Inset: Schematic drawing of an excitatory chemical synapse with paired presynaptic (axon terminal) and postsynaptic (dendritic spine) structures. **Modified from (Sudhof, 2012). Reprinted with permission. C:** Electron micrograph of a synapse formed between neocortical neurons in an adult mouse. A dendritic spine (DS) forms a synapse with a presynaptic terminal filled with synaptic vesicles (SV). Some of these vesicles are docked in the active zone, which is directly apposed to the post-synaptic density (PSD). **Modified from (Korogod et al., 2015) . Reprinted with permission.**

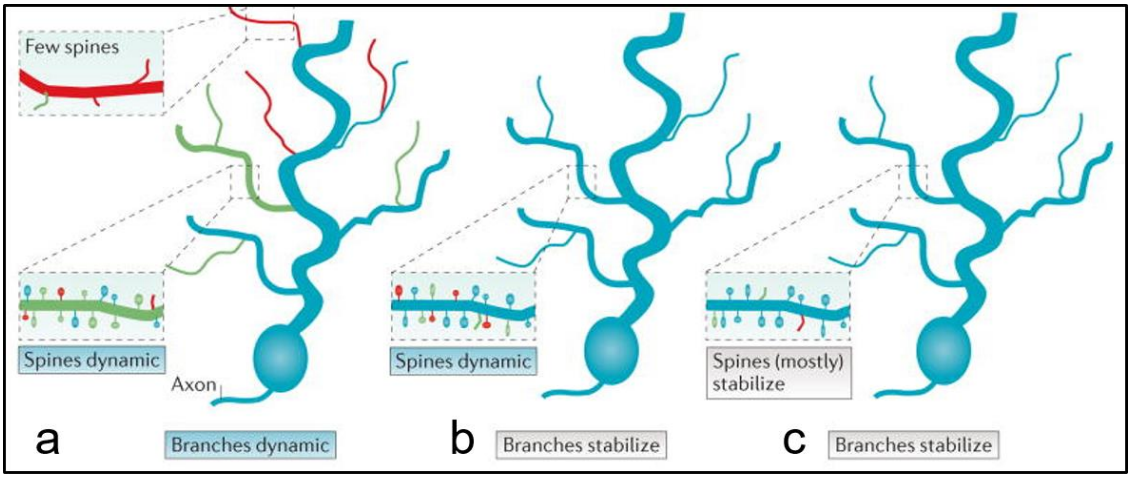


Figure 1.2: Dendrite and Dendritic Spine Dynamics during Development.

A: During early development, dendritic branches are highly dynamic, extending new branches (green) and retracting some existing branches (red). Failure to form productive synaptic contacts (inset, red dendrite segment) results in fewer spines and dendrite branch retraction. More stable branches (inset, green dendrite segment) contain a mix of stable spines, new spines and destabilizing spines**. B**: As animals enter and transit adolescence, some dendrite branches begin to stabilize. A peak in spine density during this period is subsequently followed by a net loss of spines. **C:** As animals enter adulthood, dendritic spine dynamics slow and most of the spines remain stable. © **(Koleske, 2013) Reprinted with permission.**

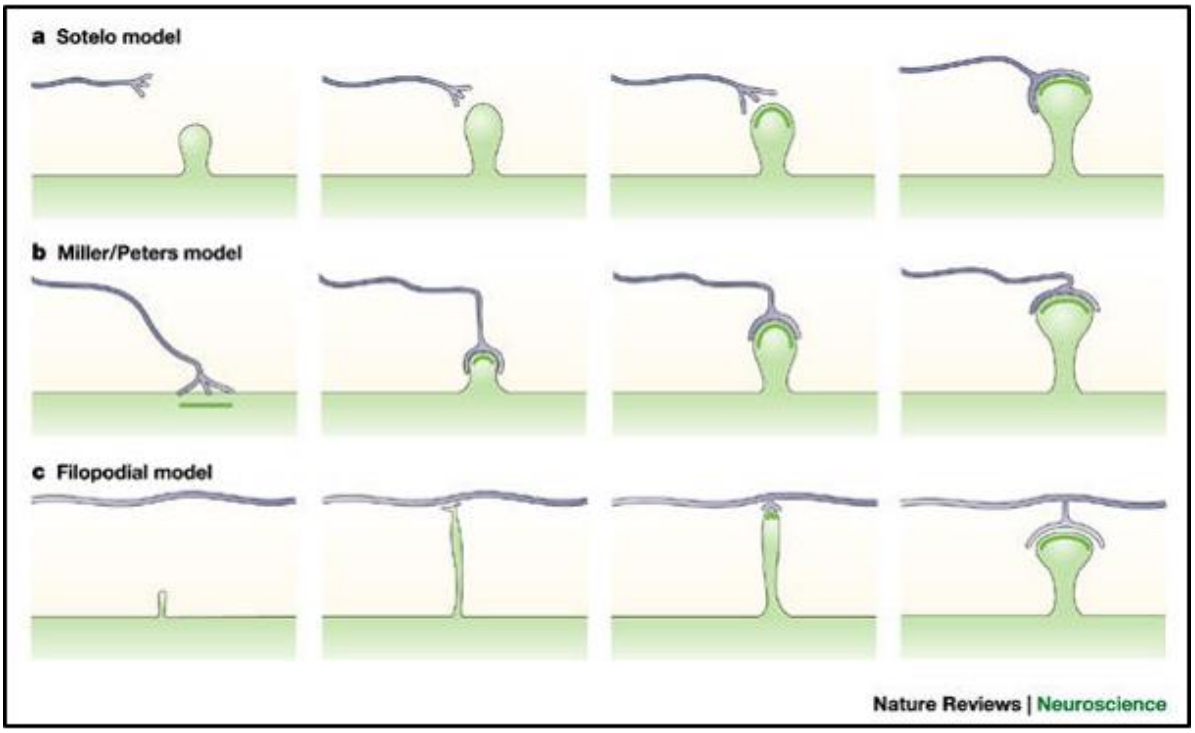


Figure 1.3 Three Models of Spinogenesis.

This diagram illustrates the essential features of the three models of spinogenesis. In the Sotelo model (a), spines emerge independently of the axonal terminal. In the Miller/Peters model (b), the axon terminal induces the formation of the spine. Finally, in the filopodial model (c), a dendritic filopodium captures an axonal terminal and becomes a spine. © **(Yuste and Bonhoeffer, 2004) Reprinted with permission.**

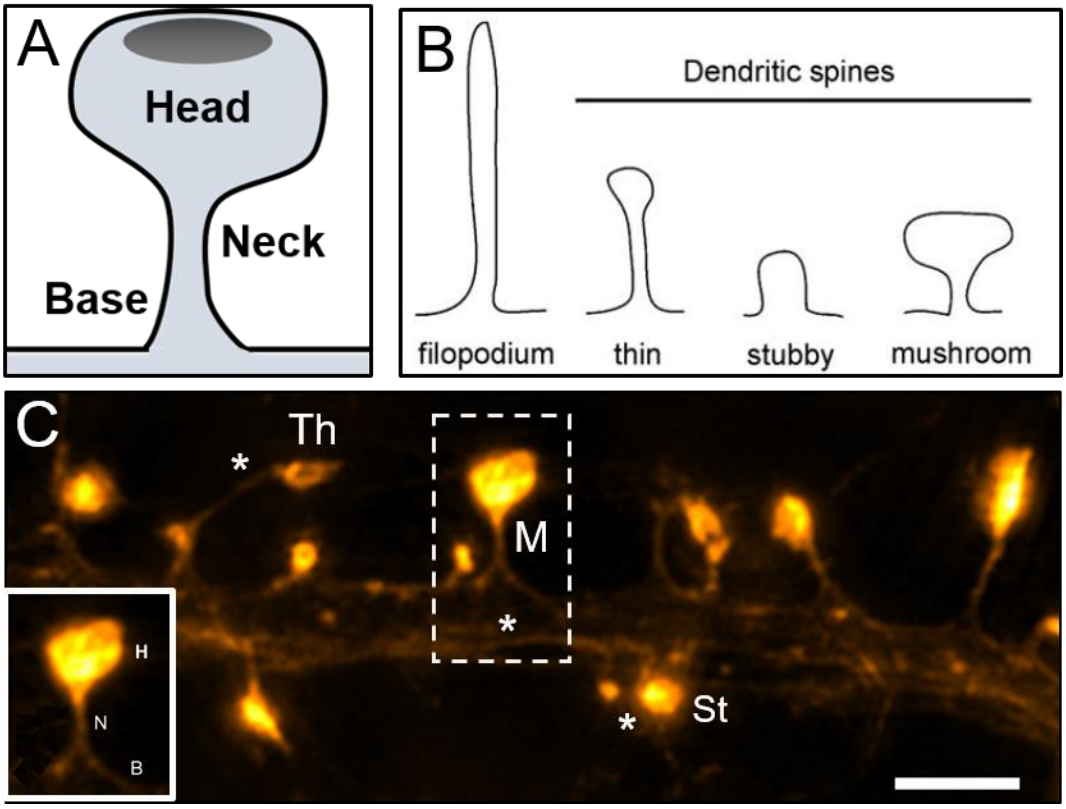


Figure 1.4: Dendritic Spine Structure and Morphology.

A: Schematic drawing illustrating dendritic spine structure. Dendritic spines are composed of three main compartments: a delta-shaped base that connected to the dendritic shaft, a constricted neck, and a bulbous head. **B:** Schematic representation of the morphological classes of dendritic spines. **© (Ethell and Pasquale, 2005) Reprinted with permission. C:** High magnification structured illumination microscopy (SIM) image of a select dendritic region from a hippocampal neuron in culture, demonstrating heterogeneity in dendritic spine morphology. Astericks indicate different morphological classes of dendritic spines: (**M:** mushroom, **St:** stubby, **Th:** thin) Scalebar: 4 µm. *Inset:* Spine compartments are indicated as follows **H:** head, **N:** neck, **B:** base. **Image: Julia Omotade, Laboratory of James Zheng**

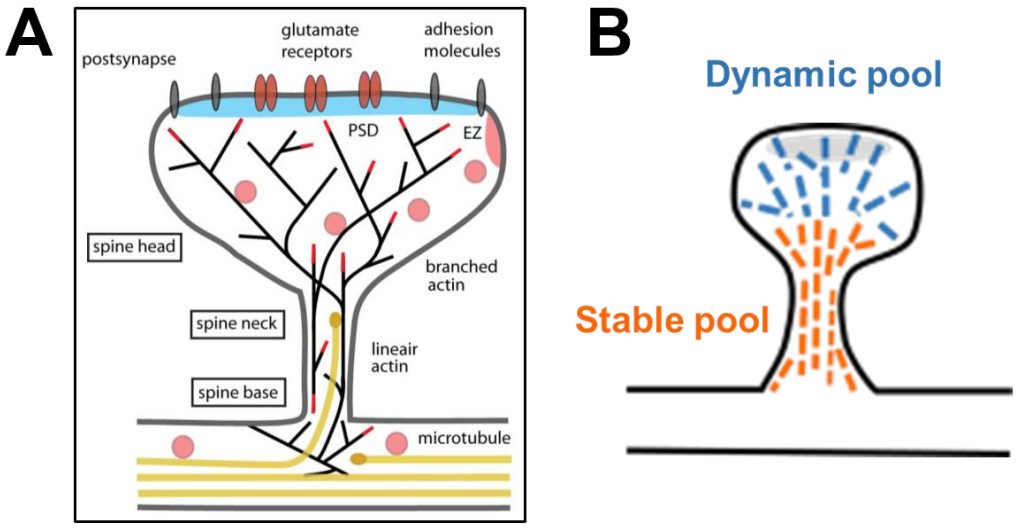


Figure 1.5: Organization of F-actin in Mature Spines.

A: Schematic diagram of a mature, mushroom-shaped spine showing the organization of the actin cytoskeleton (black) and the microtubules cytoskeleton (yellow). Dendritic spines exhibit a network of both branched and linear filaments, though branched filaments predominate in the spine head and linear filaments predominate in the spine neck. The postsynaptic density (blue) supports a myriad of scaffolding and signaling molecules, as well as adhesion molecules (gray), and glutamate receptors (reddish brown). The endocytic zone is located lateral to the PSD in the extrasynaptic regions. © **(Hotulainen and Hoogenraad, 2010) Reprinted with permission. B:** Schematic diagram showing the localization of the dynamic pool (spine head) and stable pool (spine base) in dendritic spines.

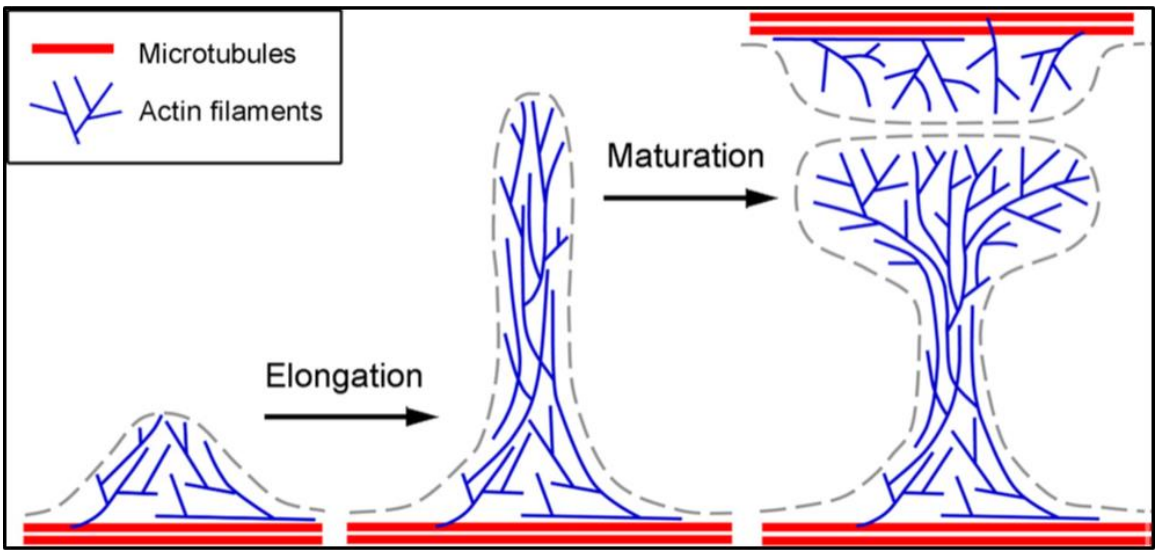


Figure 1.6: Model for actin cytoskeleton organization during spine morphogenesis.

The actin cytoskeleton in dendritic patches (left), dendritic filopodia (middle), and dendritic spines (right) has similar organization consisting of a mixed network of linear and branched actin filaments (blue) anchored to microtubules (red) or actin filaments in the dendritic shaft. The process of spine formation probably begins with the formation of a dendritic patch, which then elongates into a dendritic filopodium. On receiving of appropriate signals, the filopodium undergoes maturation into a spine. © (Korobova and Svitkina, 2010). **Reprinted with permission.**

1.11 Tables

Table I. **Actin-binding proteins and signaling pathways in dendritic spine morphogenesis**

Protein	Effect on actin cytoskeleton[a]	Function in dendritic spine morphogenesis	Signaling pathway[a]
Nucleating			
Arp2/3 complex	Nucleates branched actin filaments	Required for spine head growth (Wegner et al., 2008; Hotulainen et al., 2009)	Rac and Cdc42 signaling cascades activate Arp2/3 complex
DRF3/ mDia2	Induces elongation of straight actin filaments	Required for proper dendritic filopodia and spine neck formation (Hotulainen et al., 2009)	Rif activates DRF3/mDia2
Proteins regulating Arp2/3 complex			
Cortactin	Activates Arp2/3 complex	Regulates spine density; binds Shank (Hering and Sheng, 2003)	Src family kinases activate cortactin
N-WASP	Activates Arp2/3 complex	Regulates spine density (Wegner et al., 2008)	Cdc42, Rac, and PIP_2 activate N-WASP
WAVE-1	Activates Arp2/3 complex	Regulates spine density (Soderling et al., 2007)	Rac and PIP_3 binding activate WAVE-1
Abp1	Controls Arp2/3 via N-WASP	Abp1 expression increases mushroom spine; binds Shank (Haeckel et al., 2008)	
ADP/ATP exchanger			
Profilin	Enhances exchange of ADP to ATP and actin treadmilling rate	Stabilizes spine morphology and moves to spines upon activity (Ackermann and Matus, 2003)	
Depolymerizing			
ADF, Cofilin	Depolymerizes and severs actin filaments	Required for spine head morphology and stabilization during LTP formation (Chen et al., 2007; Hotulainen et al., 2009)	PAK3 phosphorylates LIM kinase, which inactivates ADF/cofilins
Capping			
Eps8	Caps plus-ends of actin filaments	Inhibits BDNF-induced neuronal filopodia formation (Menna et al., 2009)	MAPK phosphorylation inhibits Eps8
Cross-linking			
α-Actinin	Bundles actin filaments	Expression induces spine elongation and thinning (Hoe et al., 2009)	FAK reduces binding of α-actinin to actin
Calponin	Bundles and stabilizes actin filaments	Expression induces spine elongation and increase in density (Rami et al., 2006)	
CaMKIIβ	Bundles and stabilizes actin filaments	Required for spine maturation and LTP-induced stabilization (Okamoto et al., 2007)	CaMKIIβ autoinhibition is released by NMDA receptor activation
Neurabin I	Bundles actin filaments	Required for spine maturation (Terry-Lorenzo et al., 2005)	Cdk5 phosphorylation inhibits Neurabin I
Drebrin	Bundles and stabilizes actin filaments	Expression induces spine elongation (Ivanov et al., 2009)	
Motor proteins			
Myosin II	ATP-driven, actin-based motor	Required for proper spine head and neck morphology (Ryu et al., 2006)	RhoA activates myosin II
Myosin VI	ATP-driven, actin-based motor	Required for spine formation (Osterweil et al., 2005)	

[a]For review see Le Clainche and Carlier, 2008.

Table 1.1: Actin-binding proteins/signaling pathways in spine morphogenesis

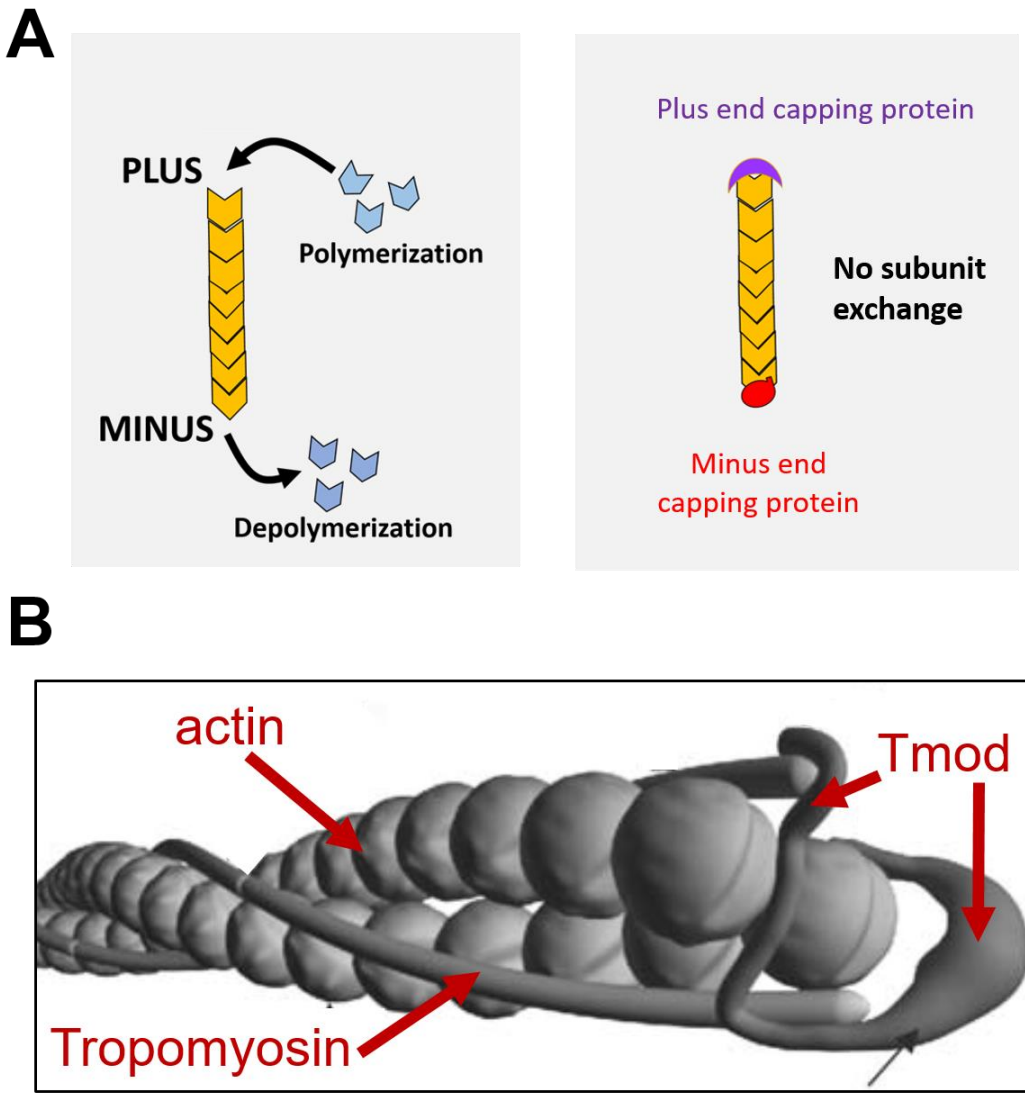


Figure 1.6: F-actin capping proteins at filament ends.

A: Schematic representation of a polarized actin filament. Actin filaments are polarized with a plus end and a minus end, which are the preferred site of filament polymerization and depolymerization, respectively. **Right:** Plus end capping proteins (purple) and minus end capping proteins (red) bind to filament ends and block the association and dissociation of G-actin monomers. **B:** Schematic representation of the slow growing pointed end of the actin filament capped by Tmod. **Modified from (Kostyukova, 2008).**

Chapter II

Methods

DNA constructs

DNA constructs of GFP-Tmod1 and GFP-Tmod2 were made by suncloning the full length rat sequences (Tmod1: NP_037176.2, Tmod2: NP_113801.1) in frame downstream of the full length eGFP sequence in an eGFP-C1 vector (Clonetech). For knockdown experiments, hairpins against rat Tmod1(shTmod1: 5'CACAGAAGTTCAGTCTGATAA 3', directed against 3' UTR. shTmod1-B: CCAGAACTTGAAGAGGTTAAT, directed against coding sequence) and Tmod2 (shTmod2: 5' CCAGTTGTTCTGGAACTTT 3', directed against coding sequence, shTmod2-B: 5' GTCAACCTCAACAACATTAAG 3', directed against coding sequence) were subcloned into a pSUPER backbone also encoding eGFP in order to allow visualization of transfected cells.

Immunostaining

For immunostaining of primary hippocampal cultures, neurons were fixed with 4% (w/v) paraformaldehyde in PBS for 15 minutes at room temperature. Fixed neurons were washed and permeabilized with 0.2% Triton X-100 (w/v) in PBS for 15 minutes. Neurons were blocked with PBS containing 4% BSA, 1% goat serum and 0.1% TX-100 for 1 hour and incubated with the following primary antibodies overnight at 4°C. Antibodies used are: anti-Tmod1 (affinity-purified custom rabbit polyclonal R1749bl3c,

Fowler et al. 1993. JCB 120:411-420), anti-Tmod2 (Abcam, ab67407) anti-PSD-95 (Thermo, MA1-046), anti-SV2 (DHSB). Cells were then washed and labelled with anti-rabbit or anti-mouse Alexa 488/546 antibody (Thermo Fisher) for 45 minutes at room temperature. Actin filaments were stained with phalloidin conjugated to Alexa fluor 488(A12379) or 568(A12380), purchased from Thermo Fisher.

Neuronal culture, transfection and imaging

Sprague Dawley timed-pregnant adult rats (8-10 weeks) were purchased from Charles River Laboratories. Primary hippocampal neurons were prepared from embryonic day 18 rat embryos and plated on 25-mm coverslips pretreated with 0.1 mg/ml poly-d-lysine (EMD Millipore) at a density of approximately 400,000 cells per dish. Neurons were plated and maintained in Neurobasal medium supplemented with B-27 and Glutamax (Invitrogen) and maintained at 37°C and 5% CO2. Cells were transfected using the calcium phosphate transfection kit (Clontech) at 5, 10 or 18 days in vitro (DIV18) and imaged between DIV21-23. Each experiment was replicated at least three times from independent batches of cultures.

Animal Care: All animals were treated in accordance with the Emory University Institutional Animal Care and Use Committee (IACUC) guidelines.

Microscopy and imaging

Cell imaging was performed using a Nikon C1 confocal system. Laser-scanning confocal images were collected on a Nikon C1 confocal system based on the Nikon Eclipse TE300 inverted microscope (Nikon Instruments, Melville, NY) equipped with a 60X/1.4 numerical aperture (NA) Plan Apo oil immersion objective. Typically, a 3-D

stack of images of a dendritic region was acquired and then projected into a 2D image (maximum intensity) for visualization and analysis. For multi-day live cell imaging of dendrite development, neurons cultured on glass-bottomed dishes were changed from normal culture medium to phenol-red free Neurobasal medium and sealed with Parafilm. The sealed dish was placed on the microscope stage and housed in a temperature controlled chamber (Warner Instruments, New Haven, CT) at ~35 °C. Cells were imaged using a Nikon C1 laser-scanning confocal system as mentioned above. To image all of the dendritic branches at different focusing planes of a dendritic segment, z-stacks of images of EGFP-expressing neurons were acquired and converted to 2-dimensional images by maximal intensity projection. After imaging, the dish was unsealed, changed back to the original neurobasal medium, and returned to the CO2 incubator for further culture and imaging at later time points.

STED Super-resolution Microscopy

All images were acquired using a commercially available multicolour STED microscope (Leica TCS SP8 STED 3X, Leica Microsystems GmbH, Wetzlar, Germany) equipped with a white light laser source operated in pulsed mode (78 MHz repetition rate) for fluorophore excitation and two STED lasers for fluorescence inhibition: one STED laser with a wavelength of 775 nm operated in pulsed mode with pulse trains synchronised to the white light laser pulses, and two continuous-wave STED laser with a wavelengths of 592 nm and 660 nm. Excitation wavelengths between 470 and 670 nm were selected from the white light laser emission spectrum via an acousto-optical beam splitter (AOBS). All laser beams were focused in the cell sample with a wavelength corrected, 1.40 numerical aperture oil objective (HC PL APO 100×/1.40 OIL STED

WHITE). The samples were scanned using the field-of-view beam scanner at uniform scanning rates and within specific scanning areas further specified below. The fluorescence from a given sample were detected by GaAsP hybrid detectors. The recorded fluorescence signal in the STED imaging mode was time-gated using the white light laser pulses as internal trigger signals.

For processing and visualization of acquired images. All acquired or reconstructed images were processed and visualized using ImageJ (imagej.nih.gov/ij/). For visualization of the images, the "fire" lookup tables (LUT) were used. Brightness and contrast were linearly adjusted for the entire images.

Structured Illumination Microscopy:

Three-dimensional structured illumination microscopy (3D-SIM) was performed on an inverted Nikon N-SIM Eclipse Ti-E microscope system equipped with Perfect Focus, 100u/1.49

NA oil immersion objective, and an EMCCD camera (DU-897, Andor Technology,

Belfast, UK). Images were reconstructed in Nikon elements. Images were analyzed using ImageJ software and NIS-Elements AR Analysis software.

Data analysis

STED microscopy: Quantitative analyses using the average interpeak distance generated by lines scans were used to determine periodicity. Using STED images immunolabelled for Tmod1 or Tmod2, ~100 µm regions that displayed more than two consecutive periodic structures were use for analysis. The distance between periodic structures was quantified using profile line scans to measure the distance between the

centers of two neighboring periodic structures. A 2 pixel wide line was used to generate line scans in Nikon elements AR. From the intensity profile line scans, 'the peak to peak' distance between consecutive peaks were measured. The full width at half maximum (FWHM) of the individual peaks that were used to generate the 'peak to peak distance' were calculated. The FWHM value was then subtracted from each 'peak to peak' value, generating the interpeak distance. The interpeak distance from an average of 222 peak and 233 peaks, for Tmod1 and Tmod2 respectively, were used to calculate the interpeak distance.

SIM analysis: To quantify subspine localization, dendritic spines were separated into three compartments: the spine neck, the distal most part of the spine head (area 1) and proximal-most part of the spine head (area 2: closest to spine neck). To equally divide the spine head into two equal compartments (area 1 and area 2), the height of the spine head was measured in Image J and a horizontal line corresponding to half the value of the measured height was placed through the spine head. Using the 'freehand selection' function in ImageJ, an ROI was drawn around circumference of the given region of the spine head, as demarcated by phalloidin staining. For each region (area 1, area 2, or spine neck) the raw integrated density value was measured for both the phalloidin channel and the Tmod channel. A ratio derived from the raw integrated density value of Tmod/phalloidin was generated for each area and the mean ratio values were used to generate graphs.

Spine analysis: The 3-D images of spines were reconstructed using Imaris 8.4 (Andor Technology). For spine analysis, filopodia were defined as thin protrusions without a distinguishable head, and spines were defined as protrusions with a length < 4

µm and an expanded, distinguishable head. Spine and filopodia numbers were counted manually to calculate the density (number per unit length of 100 µm of the parent dendrite). Spine head width was measured as spine diameter (the longest possible axis), and neck length was from the proximal edge of the spine head to the edge of the dendrite. For spines with no discernible necks, a minimum value of 0.2µm was used. To quantify synaptic density, the cluster number and area of DV2 and PSD-95 per unit neurite length were counted and measured using ImageJ.

Western blotting

For developmental immunoblots, hippocampal cultures at DIV4, DIV7, DIV14, and DIV21 were homogenized in lysis buffer containing (20 mM Tris HCl, pH8, 137 mM NaCl, 10% glycerol, 1% TX-100, 2 mM EDTA), supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and protein inhibitor cocktail (Sigma P2850). Hippocampi were collected from the brains of three independent littermates at E18, P8, P14, P21 and adult Sprague-Dawley rats of both sexes. Hippocampi were snap frozen in liquid nitrogen prior to homogenization in lysis buffer. Lysates were denatured in 1x Laemmeli sample buffer and boiled for 3 minutes. 15 µg of protein, as determined by Bradford assay, was loaded and fractioned by SDS-PAGE in a 12% Tris-glycine acrylamide gels (Invitrogen) and subsequently transferred to nitrocellulose membrane. Membranes were treated with 5% milk in PBS containing 0.05% Triton X-100 and then incubated with primary antibody overnight at 4°C: anti-Tmod1 (affinity-purified custom rabbit polyclonal R1749bl3c, Fowler et al. 1993. JCB 120:411-420), anti-Tmod2 (Abcam, ab67407). Bound antibodies were detected by HRP conjugated secondary

antibody (Jackson ImmunoResearch) and visualized by chemiluminescence using ECL (Pierce). Gels were quantified using the gel analysis function of ImageJ software (NIH).

Live cell extraction

Hippocampal neurons were extracted for 1 minute at room temperature in cytoskeleton buffer (10 mM MES, ph 6.1, 90 mM KCl, 2 mM EGTA, 3 mM $MgCl_2$, 0.16M sucrose containing 0.025% saponin, 0.1 mM ATP and 1 µM of unlabeled phalloidin). Neurons were immediately fixed in 4% PFA in PBS and stained with the phalloidin and Tmod1 or Tmod2 according to the protocol detailed above. Identical fluorescent labeling conditions and acquisition parameters were used in extracted and non-extracted neurons. In order to quantify the relative level of Tmod in dendrites and spines of extracted and non-extracted neurons, images of Tmod were merged with phalloidin-labelled F-actin in order to identify dendritic spines and corresponding dendrites. The fluorescence intensity of Tmod in spines within a dendritic region of approximately 100 µm was averaged and compared with the mean fluorescence intensity of the Tmod along the entire length of the corresponding dendritic region. 30 cells were examined from at least three independent batches of culture.

Fluorescence Recovery after Photobleaching (FRAP):

The FRAP assay was performed on a Nikon A1R laser scanning confocal microscope platform. The platform was equipped with the Perfect Focus System (PFS), multiple laser sources with AOTF control, motorized x-y stage, a modular incubation chamber with temperature and CO2 control, and a full line of photomultiplier tube detectors. Neurons grown on coverslips were mounted in a custom live cell chamber. A 60X PlanApo N TIRF oil immersion objective (1.49 NA) was used for all image

acquisitions. Time-lapse images were acquired through 4 stages. For stage one, 6 consecutive control images were acquired with 2 s interval between frames. For stage two, a single spine head within a preselected region of interest (ROI) was photobleached with 100% power of the 488 nm laser line from a 40 mW argon laser for 500 ms with the pixel dwell set at 3.9 µs. For stage three, a 5 s imaging sequence was acquired with no delay between frames immediately after photobleaching, yielding 19 frames in total. For stage four, a 5 min imaging sequence was taken with 2 s interval between frames, generating 151 frames in total. The following imaging settings were used for stage one, three and four: 2% 488 nm laser power, 1.2 µs pixel dwell, 0.07 µm/pixel resolution.

Experimental Design and Statistical Analysis

All the data from this study was collected from at least three replicates of independently prepared samples. Quantified data was statistically analyzed using a one or two tailed, unpaired Student's t-test for parametric data, and a Kruskal-Wallis one-way anova test, with a Dunn's multiple comparison for non-parametric data. Graphpad Prism v.7. was used for all statistically analysis. P-values are provided in the corresponding figure legends. Data are presented as the mean ± S.E.M., with in-text values stated. Astericks indicate a p value ≤ 0.05 and non-significant is denoted by "ns".

Chapter III

Tmod1 and Tmod2 in Dendrite Development

ABSTRACT

Neurons of the central nervous system elaborate highly branched dendritic arbors that host numerous dendritic spines, actin-rich protrusions that serve as the postsynaptic platform for excitatory synapses. The actin cytoskeleton is crucial for the formation of elaborate dendritic arbors during neuronal development, yet the molecules and mechanisms that regulate actin organization and remodeling during dendrite development are not fully understood. In metazoans, the Tropomodulin (Tmod) family of proteins cap actin filament minus ends and inhibit depolymerization, thereby regulating the stability, length and organization of diverse actin-based networks. Tmod1, Tmod2, and Tmod3 are expressed in the vertebrate CNS, but their function are not well understood. In this study, we present evidence that Tmod1 and Tmod2 are highly expressed in hippocampus and play an important role in dendritic arborization. Both Tmod1 and Tmod2 exhibit a unique nanoscale distribution in dendritic arbors. Loss-of-function analysis reveals that Tmod2, but not Tmod1, is required for dendritic branching. Together, these findings establish an important role for Tmods in postsynaptic development.

A portion of this chapter has been submitted to the Journal of Neuroscience and is under review:

Omotola F. Omotade, Wenliang Lei, Velia Fowler, James Q. Zheng. Regulation of Dendrite Development and Synapse Formation by Tropomodulins.

3.1. Introduction

Neurons of the central nervous system elaborate highly branched dendritic arbors that host numerous dendritic spines, actin-rich protrusions that serve as the postsynaptic platform for excitatory synapses. The formation of elaborate dendritic arbors is achieved through the dendritic growth cone, a motile, actin-based structure present at the tips of developing dendrites. Dendritic growth cones are crucial for dendrite extension as well as the formation of collateral dendritic branches. Despite fervent study, the molecules and mechanisms that regulate actin organization and remodeling during dendrite development are not fully understood.

Tropomodulins (Tmods) cap the pointed end of actin filaments, thereby regulating the stability, length, and architecture of actin networks in diverse cell types. Tmod1, Tmod2 and Tmod3 are expressed in the brain, though their exact functions remain unclear. Altered expression of Tmod1 is observed in kainic-acid induced seizures and altered expression of Tmod2 is observed in Down's syndrome, epilepsy, prefrontal ischemia and methamphetamine exposure (Sussman et al., 1994, Iwazaki et al., 2006, Yang et al., 2006, Chen et al., 2007, Sun et al., 2011). These findings suggest that regulation of actin dynamics by Tmod is critical for brain function. In support of this, Tmod2 knockout mice exhibit synaptic and behavioral deficits (Cox et al., 2003), though the underlying cellular mechanisms are unclear. Tmod1 and Tmod2 are present in growth cones of elongating neurites in cultured rat hippocampal neurons, and are suggested to play roles in neurite formation, based experiments in N2a cells (Fath et al., 2011) and PC12 cells (Moroz et al., 2013, Guillaud et al., 2014). A recent study (Gray et al., 2016) using GFP-Tmod1 and GFP-Tmod2 overexpression indicates that Tmod1 and Tmod2

regulate dendritic branching and spine morphology in cultured rat hippocampal neurons. However, fundamental knowledge about the endogenous distribution and role of Tmod during dendrite development remains largely unknown.

In this study, we investigated the expression profile, subcellular distribution and function of Tmod1 and Tmod2 in hippocampal neurons. We find that Tmod1 and Tmod2 are expressed in dendrites, where they exhibit a unique, nanoscale distribution. Tmod1 and Tmod2 are differentially expressed during dendrite development and loss-of-function analysis reveals that Tmod2, but not Tmod1, is required for dendritic branching during development. Together, these findings establish an important role for Tmods in postsynaptic development. Regulation of F-actin stability by Tmod1 and Tmod2 may represent a key mechanism underlying the cytoskeletal rearrangements required for dendrite development.

3.2. Results

Expression of Tmod1 and Tmod2 in hippocampal neurons

Previous studies using immunoblot analysis have shown the expression of Tmod1 (Sussman et al., 1994) and Tmod2 in rat brains (Watakabe et al., 1996). Tmod1 expression increases postnatally, while Tmod2 expression reaches adult levels during embryonic brain development. Here, we specifically examined the expression of Tmod1 and Tmod2 proteins in rat hippocampus at E18 (E: embryonic), P8 (P: postnatal), P12, P23 and adult, which correspond to the stages before, during and after synapse formation (Markus and Petit, 1987, Harris et al., 1992, Fiala et al., 1998). Immunoblot analysis shows that both Tmod1 and Tmod2 are expressed in rat hippocampus, but with different profiles **(Figure 3.1A)**. Tmod1 is undetectable at E18, with apparent expression

beginning around P8. Substantial Tmod1 expression is detected around P12 and continues to increase steadily until adulthood. By contrast, Tmod2 is expressed at E18 and its levels increase sharply from E18 to P8. After P8, expression of Tmod2 remains relatively steady until adulthood. The differential expression of Tmod1 and Tmod2 protein in hippocampal development parallels their mRNA expression during embryonic and postnatal development in brain (Watakabe, Kobayashi and Helfman. 1996. J. Cell Sci; Sussman, Sakhi et al 1994; Cox & Zoghbi Genomics 2000). The formation of spine synapses commences near the end of the second postnatal week (P14) and peaks during the end of the fourth postnatal week (P30) (Markus and Petit, 1987, Harris et al., 1992, Fiala et al., 1998). Therefore, both Tmod1 and Tmod2 are expressed during this critical window of spine development and synapse formation. On the other hand, robust dendrite development occurs during early postnatal development (P0-P7), during which Tmod2, but not Tmod1, is highly expressed. Since no Tmod3 protein was detected in hippocampal neurons (data not shown), we hypothesize that Tmod1 and Tmod2 may function in dendrite development and synapse formation.

We next examined the protein expression of both isoforms in primary rat hippocampal neurons during specific *days in vitro* (DIV) **(Figure 3.1B)**. Primary hippocampal neurons exhibit defined stages of postsynaptic development, thereby allowing us to align Tmod protein expression with the events during dendrite development and synapse formation. Hippocampal neurons in culture develop axon-dendrite polarity around DIV5, with substantial growth and arborization of dendrites occurring until DIV14 (Dotti et al., 1988). From DIV14 to DIV18, relatively stable dendritic arbors undergo continuous but small growth until maturation. In parallel with

dendrite development, synapse formation in cultured hippocampal neurons initiates on dendritic arbors around DIV7-9, peaks around DIV11-14, and is largely completely by DIV18 onward (Ziv and Smith, 1996, Friedman et al., 2000, Grabrucker et al., 2009). Consistent with the pattern of expression detected in hippocampal tissue, Tmod2 protein levels in cultured hippocampal neurons remain relatively steady before, during and after the processes of dendrite development and synapse formation **(Figure 3.1B)**. In contrast, Tmod1 expression is undetectable before DIV4 and remains low from DIV7-14, which corresponds to robust dendrite and synapse development. Robust expression of Tmod1 is not seen until DIV21, during which both dendritic arbors and spines are stable **(Figure 3.1B)**. Together, these immunoblot data indicate that both Tmod1 and Tmod2 are differentially expressed at distinct stages of development.

We also performed immunofluorescence staining to examine the expression and distribution of Tmod1 and Tmod2 in cultured primary hippocampal neurons **(Figure 3.1C)**. We found that both Tmod1 and Tmod2 are abundantly expressed in the somatodendritic compartment, as evidenced by co-localization with microtubule-associated protein 2 (MAP2), which is enriched in dendrites **(Figure 3.1C)**. Tmod signal is also detected in neuronal processes lacking MAP2, suggesting that Tmod is also present in in axons. Tmod1, but not Tmod2, is detected in the nucleus, consistent with previous reports of a nuclear localization signal/nuclear export signal within the Tmod1 molecule (Kong and Kedes, 2004). Together with the developmental expression profile, these immunofluorescence data support the notion that Tmod1 and Tmod2 may function in the postsynaptic compartment during development.

Membrane association of Tmods

While both Tmod1 and Tmod2 are present in the dendritic shaft of DIV21 hippocampal neurons in culture, a close examination revealed that Tmod2 appears to exhibit a membrane-enriched distribution in a portion of dendrites (**Figure 3.2,** bottom left panel **/Figure 3.3A**). This observation suggests that a fraction of Tmod2 molecules may be associated with the cortical actin cytoskeleton. To test if the Tmod1 and Tmod2 are associated with the F-actin cytoskeleton in dendrites, we performed live-cell extraction using the mild detergent saponin (Lee et al., 2013, Lei et al., 2017). Brief exposure of live neurons to saponin retains relatively stable structures such as the cytoskeleton and cytoskeleton-associated proteins (Lee et al., 2013, Lei et al., 2017). By comparing the fluorescence intensity of Tmod in extracted and non-extracted neurons, we were able to estimate the cytoskeleton-associated fraction of Tmod in dendrites. Identical fluorescent labeling conditions and acquisition parameters allowed us to directly compare the fluorescence intensity of Tmod in extracted vs. non-extracted neurons. When normalized to the fluorescence intensity value in spines (which are unaffected by saponin extraction), we find that live cell extraction eliminated ~30% of Tmod1 and Tmod2 immunoreactivity in the dendritic shaft, with 64.40 ± 26.12% (mean ± standard deviation, n = 180) and 67.20 ± 6.00% (mean ± standard deviation, n = 173) of shaft immunoreactivity remaining, for Tmod1 and Tmod2, respectively. Although Tmod1 is largely distributed throughout the dendritic shaft (**Figure 3.2,** top left panel/**Figure 3.3A**), Tmod1 immunoreactivity could be detected in a longitudinal, submembranous band in a subset of neurons exposed to saponin (**Figure 3.2**, top left panel). Although this cortical localization of Tmod1 is not apparent in dendrites of non-extracted neurons, our

live-cell extraction data reveal that both Tmod1 and Tmod2 are associated with the cortical actin cytoskeleton in dendrites.

Interestingly, the Tmod2 signal near the dendritic shaft membrane appears to exhibit a punctate distribution (**Figure 3.2,** bottom right panel). To better resolve the spatial pattern of Tmod2 associated with the cortical actin cytoskeleton, we utilized stimulated emission depletion (STED) microscopy, a super-resolution imaging method that provides an optimal lateral resolution of ~30-50 nm (Meyer et al., 2008). Our STED images further confirmed the punctate appearance of both Tmod1 and Tmod2 in dendrites, as well as the localization of Tmod2 in the subcortical region (**Figure 3.3B**). Interestingly, Tmod2 appear to display a periodic pattern within dendrites (**Figure 3.3C**). Although Tmod1 is distributed throughout the dendritic shaft, the Tmod1 signal along the membrane also exhibited a similar periodic pattern (**Figure3.3C**). Quantitative analysis indicated that the average interpeak spacing was 165 ± 64nm (n_{peak} =215 , $n_{dendrites}$ =16) and 155 ± 63nm (n_{peak} = 222, $n_{dendrites}$ = 22), for Tmod1 and Tmod2 respectively (**Figure 3.3D**). While first identified in axons (Xu et al., 2013), recent studies have also revealed the existence of a membrane-associated periodic skeleton (MPS) in dendrites (Xu et al., 2013, Zhong et al., 2014, D'Este et al., 2015, Han et al., 2017). The observed spacing between Tmod puncta is consistent with the observed lengths of associated molecular markers of the MPS structure, which has been reported to range from ~160 nm (D'Este et al., 2016) to 200 nm (Xu et al., 2013, He et al., 2016). These data suggest that Tmod1 and Tmod2 may be a component of the membrane cytoskeleton in dendrites.

Tmods in Dendrite Development

To investigate the roles of Tmod1 and Tmod2 in the postsynaptic compartment, we performed loss-of-function analysis in hippocampal neurons during dendrite development. Knockdown of endogenous Tmod1 and Tmod2 proteins levels were achieved using short hairpin RNA (shRNA) and knockdown efficiency was evaluated by measuring the level of endogenous Tmod1 and Tmod2 protein in Cath-A differentiated (CAD) neuroblastoma cells. Immunoblot analysis revealed a 51% and 58% reduction in Tmod1 and Tmod2 protein levels using the hairpins 'shTmod1' and 'shTmod2', respectively (**Figure 3.4A**). It was previously reported that neurons from Tmod2 knockout mice display an eight-fold upregulation of Tmod1 protein levels (Cox et al., 2003) and shRNA knockdown of Tmod2 in N2a cells results in a 2-fold increase in Tmod1 (Fath et al., 2011), making isoform-specific interpretation of Tmod function challenging. However, using 'shTmod1' and 'shTmod2', we did not detect significant compensatory up-regulation by either isoform (**Figure 3.4A**). The shRNA constructs 'shTmod1' and 'shTmod2' are therefore robust and specific and were used in all subsequent experiments.

To investigate the roles of Tmods in dendrite development, hippocampal cultures were transfected with shTmod1 or shTmod2 at DIV10, a developmental stage characterized by robust dendritic arborization and branching. Control neurons transfected at DIV10 and imaged at DIV21 displayed complex dendritic arbors that possessed many secondary and tertiary branches (**Figure 3.4B**). By contrast, neurons expressing shTmod2 displayed a dramatic reduction in dendritic branching (**Figure 3.4B –3.4C**), with a 55 ± 24.9 % reduction in branch number and a 54% ± 23.7 % reduction in dendritic length, as

compared to control (**Figure 3.4C**). Intriguingly, introduction of shTmod1 at DIV10 did not have any observable effect on dendrite development (**Figure 3.4B –3.4C**). These results indicate that Tmod2, but not Tmod1, is required for dendrite development.

To better understand how Tmod2 regulates dendritic growth and arborization, we tracked the growth of dendritic arbors during development in control vs shTmod2-expressing neurons. The dendrites of control shLucif-expressing neurons showed continuous, but small growth between DIV14 and DIV18 (**Figure 3.5A,** green arrows). However, hippocampal neurons expressing shTmod2 underwent progressive shrinkage of their dendritic branches over the same time period (**Figure 3.5A,** red arrows). To quantify the effects of Tmod2 depletion on dendritic growth, we performed Sholl analysis on neurons 1, 3 and 5 days after transfection. ShTmod2 expression significantly reduced the number of dendritic intersections between 20–100 μm radii, whereas shLucif had no effect (**Figure 3.5B**). To further quantify the effect of Tmod2 depletion on dendrite arborization, we summed the dendritic intersections within a radius of 10–200 μm. After 5 days of shTmod2 expression, the total number of dendritic intersections was significantly reduced (**Figure 3.5C**). Similarly, the total length of dendritic branches was reduced by shTmod2 expression, but not with shLucif alone (**Figure 3.5C**).

3.3 Discussion

The actin cytoskeleton is tightly regulated by a wide array of actin binding proteins that impart spatiotemporal control over its organization and dynamics (Pollard et al., 2000, Pollard and Borisy, 2003). Tropomodulin molecules are best known for controlling the length and stability of actin filaments in the sarcomere of striated muscle and the membrane skeleton of diverse cell types (Yamashiro et al., 2012). Tmod1,

Tmod2 and Tmod3 are expressed in the brain, though their exact functions remain unclear. To date, fundamental knowledge about the endogenous distribution and role of Tmod during dendrite development remains largely unknown. In this study, we performed a series of experiments to examine the expression profile, subcellular distribution, and functions of endogenous Tmod1 and Tmod2 in hippocampal neurons. Our findings show that both Tmod1 and Tmod2 molecules are present in dendrites, where they exhibit a unique subcellular distribution in dendritic arbors. We provide evidence that Tmod2 is expressed in the early stages of neuronal development and, importantly, required for dendritic arborization. This study provides fundamental insight into mechanisms underlying dendrite development and adds to the limited body of knowledge about Tmod function in neurons.

Our finding that Tmod2, but not Tmod1, is involved in dendrite development may not necessarily indicate an isoform-specific role for Tmod2 in dendritic arborization. Given that Tmod1 is minimally expressed during the early stages of neuronal development, the apparent “specific” role for Tmod2 in dendritic arborization may be attributed to its robust expression during this developmental stage. This notion is consistent with a recent study in which overexpression of exogenous Tmod1 or Tmod2 promoted dendrite branching (Gray et al., 2016). While we cannot rule out the possibility that inherent biochemical and structural differences between Tmod isoforms (Yamashiro et al., 2012) may contribute to the selective effects of Tmod2 on dendrite development, we propose that the specific expression profiles of Tmod1 and Tmod2 dictate their selected actions during distinct stages of neuronal development. In support of this, Tmod1 and Tmod2 are both expressed during synapse formation and appear to be equally

involved in regulating the spine F-actin stability and spine development (Chapter IV). Tmod1 is robustly expressed at DIV21 and may therefore be required for maintenance of mature dendritic arbors.

How does Tmod2 regulate dendrite development? There are several non-exclusive possibilities. First, Tmod2 may regulate F-actin in motile, actin-based, dendritic growth cones, which enable dendrite extension as well as the formation of collateral dendritic branches (Ulfhake and Cullheim, 1988, Fiala et al., 1998, Niell et al., 2004). In support of this, Tmod1 and Tmod2 are expressed in axonal growth cone of young primary hippocampal neurons (Fath et al., 2011). Moreover, Tmod1 and Tmod2 alter neurite formation in PC12 and N2A cells (Gray et al., 2017), which extend neurites through actin based growth cones. (Ulfhake and Cullheim, 1988, Fiala et al., 1998). Second, dendrite development is tightly coupled to synapse formation, whereby the amount and nature synaptic input determines the extent of dendritic branching (Koleske, 2013). It is plausible that Tmod2 regulation of dendritic arborization may be mediated by its effects on dendrite development and synapse formation (Chapter IV). The submembranous distribution of Tmod2 along with the observed periodicity in dendrites offers a third possibility for Tmod2 regulation of dendrite development. In dendrites, short actin filaments are organized into evenly spaced ‘actin rings’ that exhibit a periodicity of ~180-190 nm (Zhong et al., 2014, D'Este et al., 2015, D'Este et al., 2016, Han et al., 2017). These actin rings are connected by spectrin tetramers, forming a membrane-associated periodic skeleton (MPS) that wraps around the circumference of the dendrite (**Figure 3.6**). The dendritic MPS bears striking molecular similarity to the spectrin-actin ‘membrane skeleton’ found in diverse metazoan cells, of which Tmod

plays a crucial role. It is therefore plausible that Tmods may be a component of the MPS within dendrites, where it could cap the pointed ends of short actin filaments in periodic F-actin rings.

In addition to the MPS, our results show that Tmod2 may associate with the longitudinal F-actin cytoskeleton directly underneath the dendritic shaft membrane (lateral cytoskeleton) (**Figure 3.3B**). In the lateral membrane of polarized epithelial cells, Tmod3 caps the pointed end of F-actin filaments, generating a stable membrane skeleton required for maintaining cell height and cell shape (Weber et al., 2007). Though the dynamics and organization of F-actin in lateral dendritic cytoskeleton have not been carefully examined, the presence of Tmod2 (and to a lesser extent, Tmod1) suggests that this structure is relatively stable, likely providing structural and mechanical support to the dendrite. Though the contribution of the MPS and the lateral cytoskeleton to dendrite development is not known, Tmod2 may regulate dendrite development by stabilizing F-actin filaments in cortical F-actin structures during neuronal development. While Tmod1 has also been found to exhibit a similar periodic pattern in the dendrites of DIV21 hippocampal neurons, its late expression may exclude its participation in dendrite development.

Our finding that Tmod1 and Tmod2 are essential for dendrite development shed light on the mechanisms by which F-actin is regulated during brain development. Although future studies are needed to reveal the precise mechanisms by which Tmod2 regulates dendrite branching during postsynaptic development, this work adds to the limited body of knowledge about Tmod function in neurons.

3.4 Figures

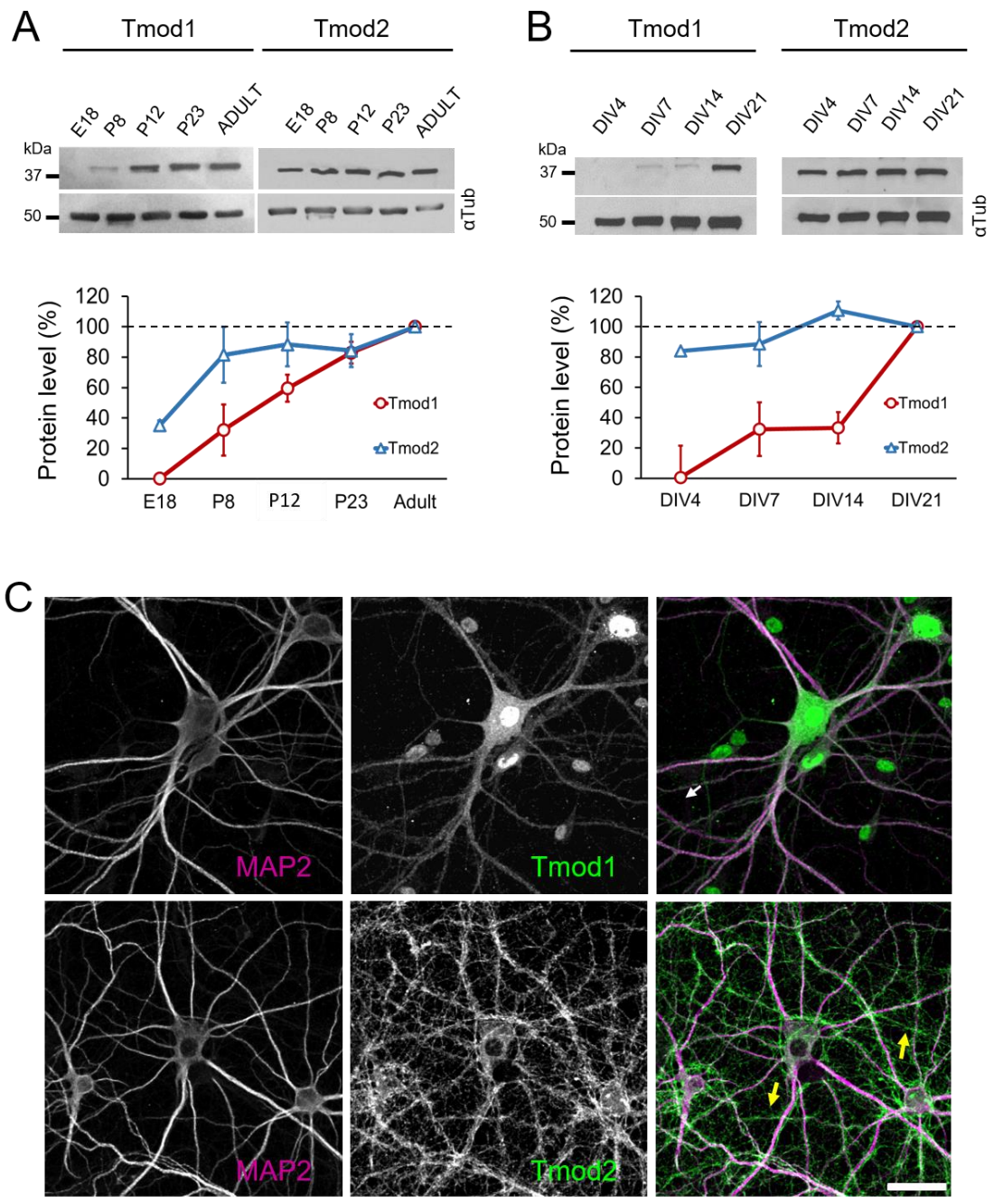


Figure 3.1: Expression profile and subcellular distribution of Tmod1 and Tmod2

Representative western blots of Tmod1 and Tmod2 in rat hippocampal tissue **(A)** and primary hippocampal neurons **(B)** depict the changes in Tmod expression over time. All bands in gel are cropped for clarity. Quantification from three separate replicates is

shown in the corresponding line graphs (N=3 for each timepoint). The mean value for each timepoint was normalized to the corresponding tubulin loading control, and then to the ‘Adult’ (A) or ‘DIV21’(B) value. **C:** Immunostaining of endogenous Tmod1 and Tmod2 (green), together MAP2 (magenta) in cultured hippocampal neurons. Arrows indicate axons. Scalebar = 30 µm.

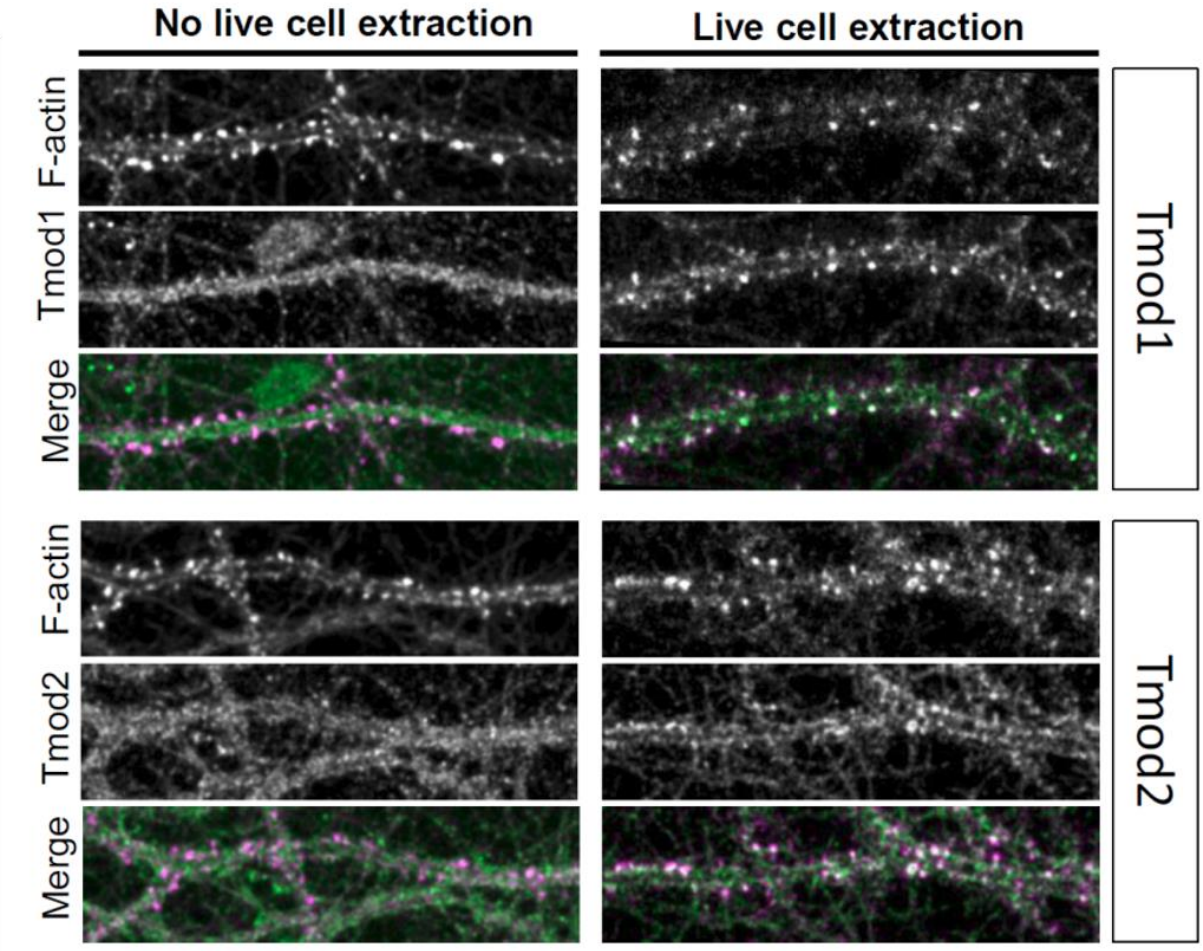


Figure 3.2. Membrane association of Tmod1 and Tmod2

Representative immunofluorescence images of dendritic regions stained for Tmod1 (top) or Tmod2 (bottom) in extracted and non-extracted hippocampal neurons. Live cell extraction retains a significant portion of Tmod1 and Tmod2 in subcortical regions of the dendritic shaft (right panels).

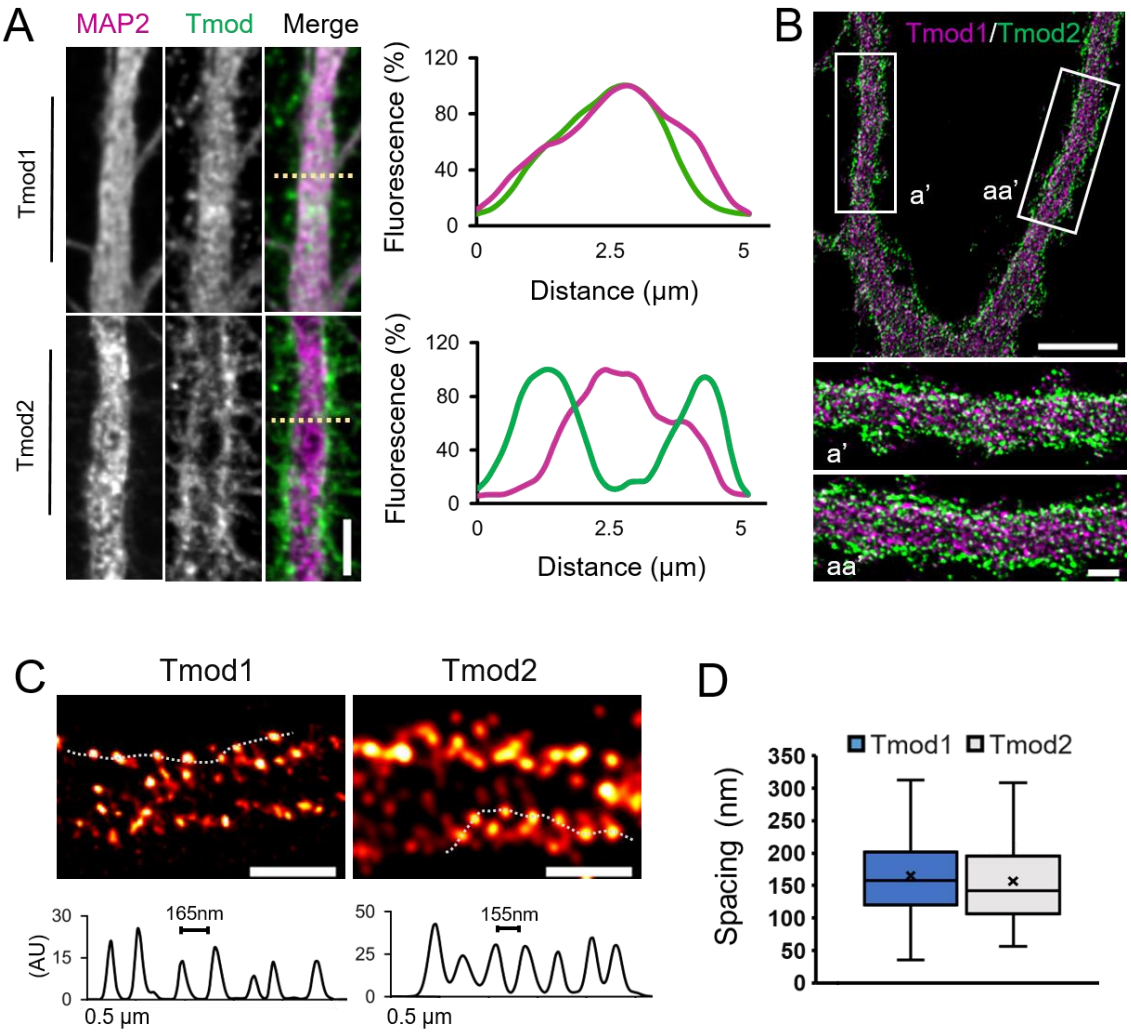


Figure 3.3 Distribution of Tmod1 and Tmod2 in Dendrites

A: Representative confocal immunofluorescence images of dendritic regions in non-extracted neurons stained for Tmod1/Tmod2 and MAP2. Scalebar: 4.5 μm. **B:** Representative STED images of dendrites from DIV21 hippocampal neurons co-stained with Tmod1 and Tmod2. Scalebar: top panel, 5 μm. Bottom panel: 1 μm. **C:** Representative STED images showing periodic distribution of Tmod1 and Tmod2 in DIV21 hippocampal neurons. Images represent individual Z-slices, rather than a composite Z-stack. Scale bar for both images:1 μm. Bottom: Representative line profiles

from corresponding regions above (dashed line). **D:** Box plot of Tmod1 and Tmod2 interpeak spacing in primary rat hippocampal neurons. ‘X’ within blox plots indicates average interpeak spacing for Tmod1 (n_{peak} =215 , $n_{dendrites}$ =16) and Tmod2 (n_{peak} = 222, $n_{dendrites}$ = 22).

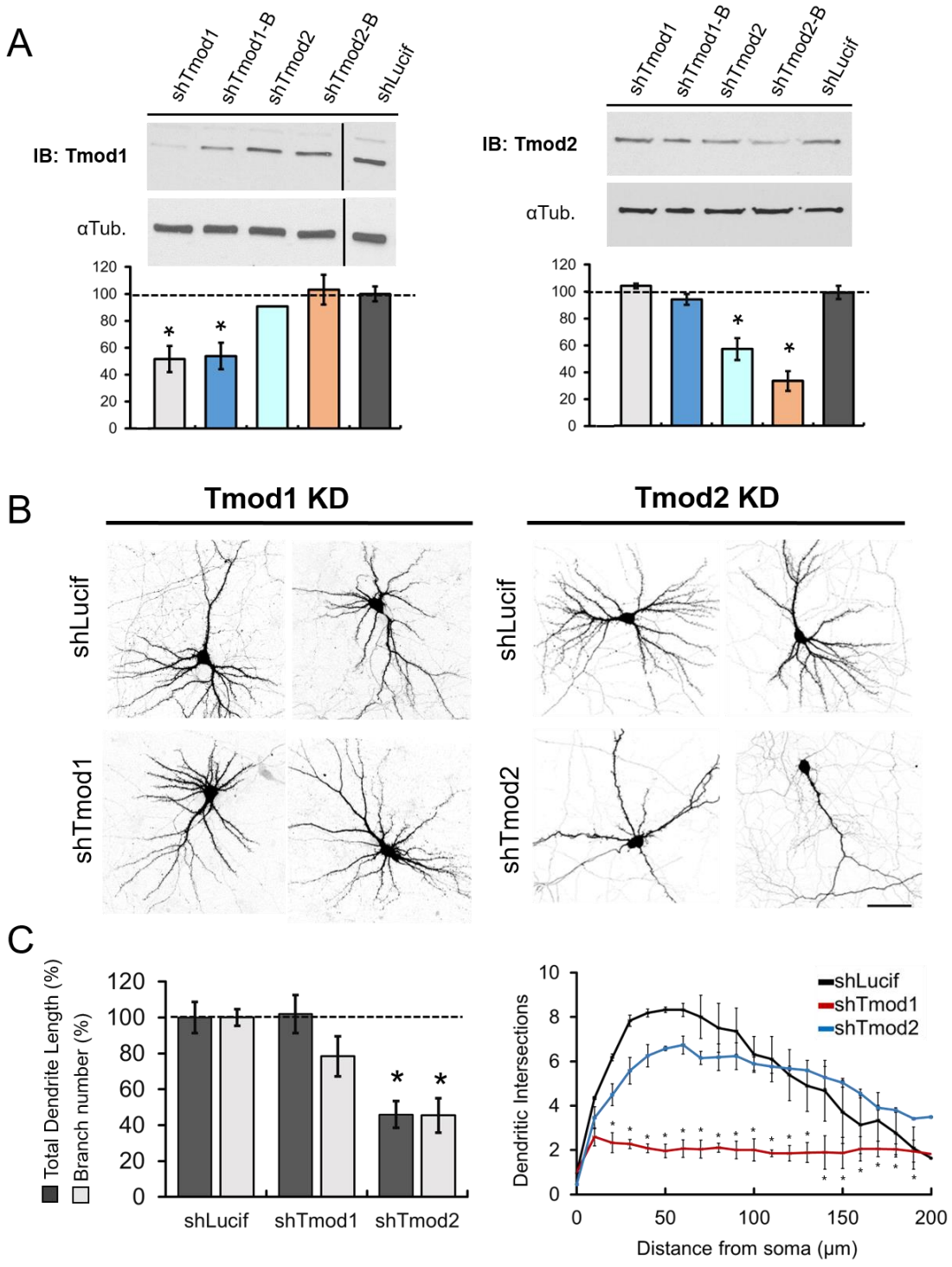


Figure 3.4. Tmod1 and Tmod2 in Dendrite Development

A: Representative immunoblot images reveal robust and specific reduction of Tmod1 and Tmod2 protein levels in Cath-A differentiated (CAD) neuroblastoma cells expressing

knockdown constructs (48 hours). Black vertical bar in Tmod1 blot (left panel) delineates the boundary between two non-adjacent lanes from the same gel (processed identically). All bands in gel are cropped for clarity. Bar graphs depict mean Tmod1 (left) and Tmod2 (right) knockdown levels in CAD cells from three independent replicates. Data are normalized to Tmod protein levels in shLucif-expressing cells. Statistical analysis was performed using Student's t-test. Error bars represent standard error of the mean (S.E.M). *$p < 0.05$, n.s.= not significant. $p^{shTmod1} = 0.001$, $p^{shTmod1\text{-}B} = 0.0001$, $p^{shTmod2} = 0.028$, $p^{shTmod2\text{-}B} = 0.023$. **B:** Representative confocal images of neurons expressing shLucif, shTmod1, or shTmod2. Images were inverted in grayscale for presentation. **C:** Bar graphs show changes in total dendritic length and branch number as labeled. For each condition, values are normalized to shLucif. Statistical analysis was performed a Student's t-test. Error bars represent standard error of the mean (S.E.M).*$p < 0.001$. Right: Line graph depicts the results of sholl analysis from control, shTmod1-, and shTmod2-expressing neurons. Statistical analysis was performed using a two-way anova test, with a Dunn's multiple comparison test to determine statistical differences. Error bars represent standard error of the mean (S.E.M).*$p < 0.05$, compared to shLucif.

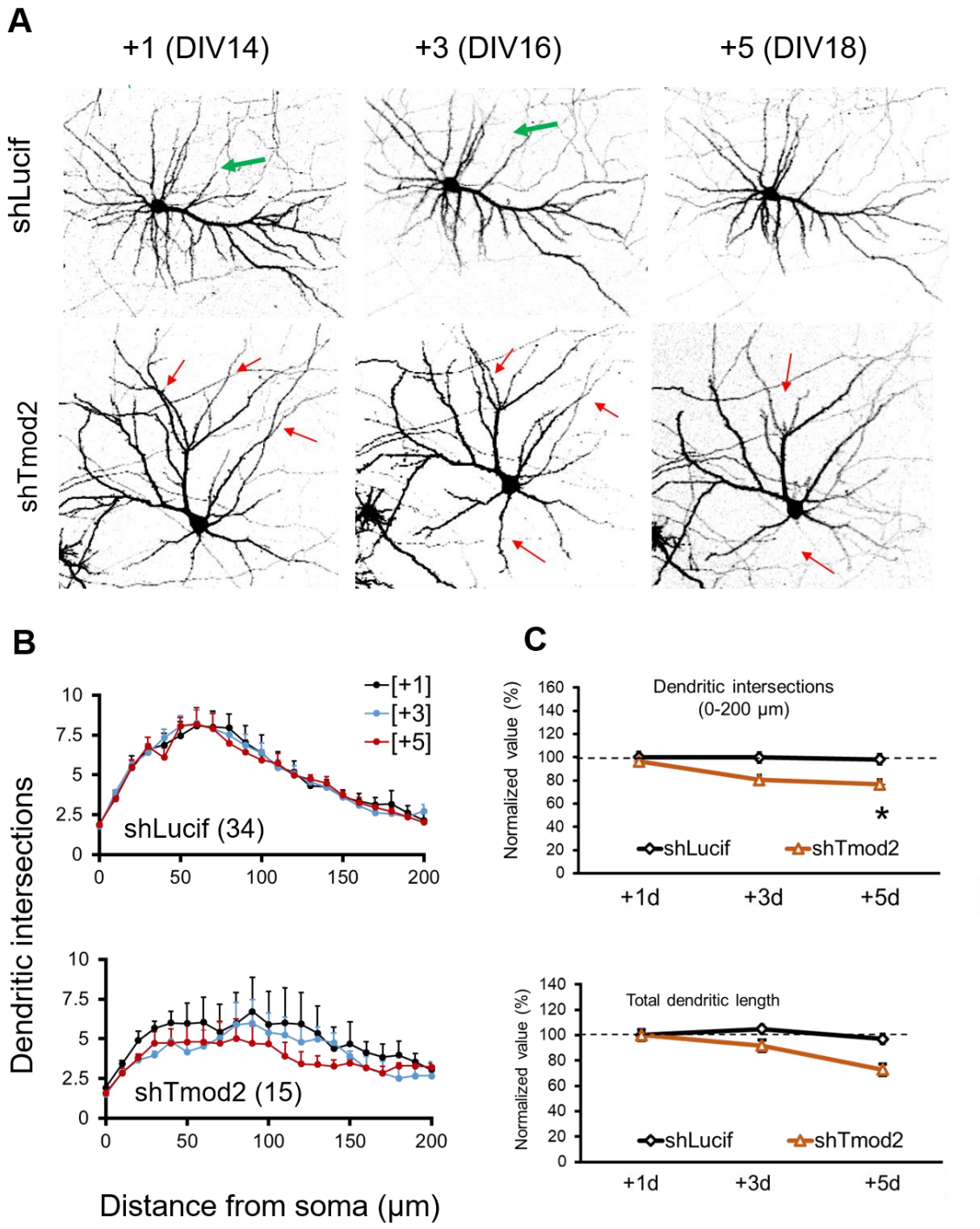


Figure 3.5: Regulation of dendritic development by Tmod2

A: Representative confocal images of hippocampal neurons expressing shLucif and shTmod2 at 1, 3, and 5 days after transfection. Images were inverted in grayscale for presentation. Red and green arrows indicate the shrinkage and growth of dendritic

branches, respectively. **B:** The line graphs (right) depict the results of sholl analysis. Numbers in brackets indicate the total number of cells examined (from at least three independent batches of culture). Error bars represent standard error of the mean (S.E.M). **C:** Line graphs depict the changes in dendritic intersections from the Sholl analysis and the total dendritic length at different days post transfection. *p <0.05; (two-way, repeated measures anova test, posthoc: Dunn's multiple comparison test), comparing to the corresponding point of the shLucif group. For each condition, the data are normalized to the +1day. Data is derived from at least three independent cultures. Error bars represent the standard error of the mean (SEM).

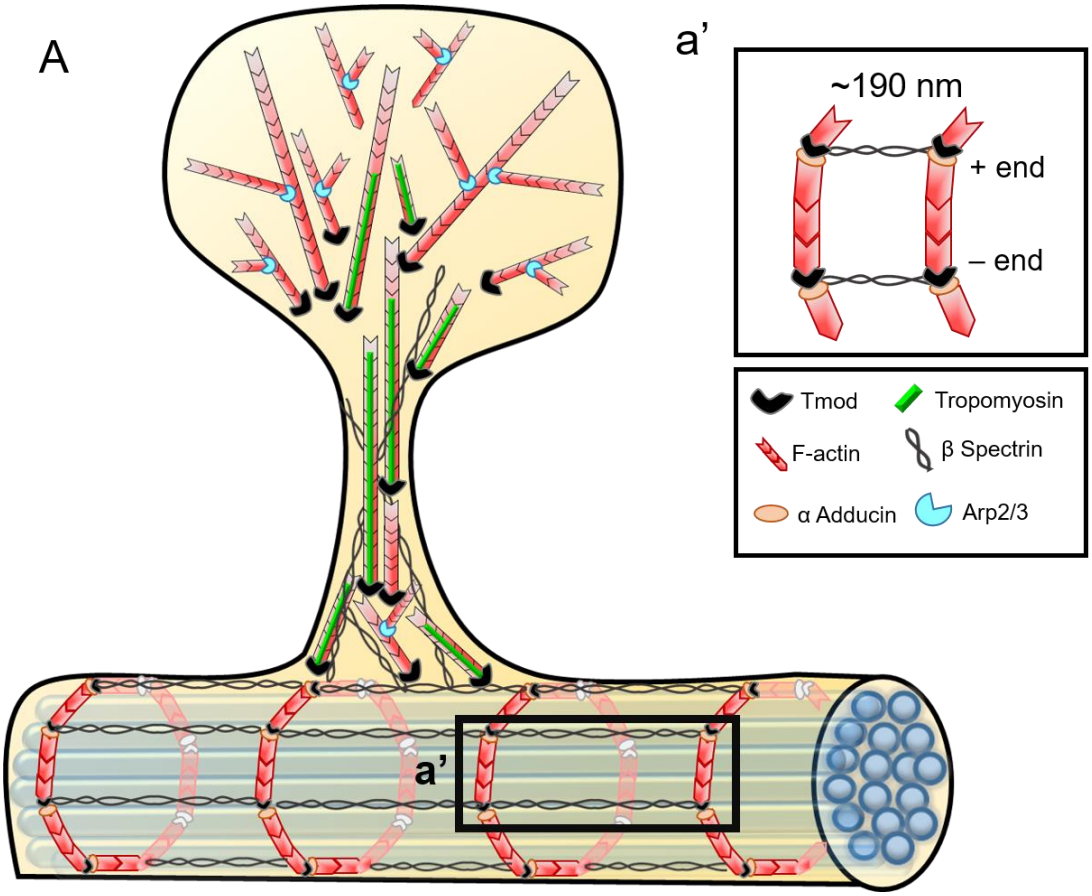


Figure 3.6. Cytoskeletal organization in dendrites

A: In dendrites, evenly-spaced 'F-actin rings' are connected to spectrin tetramers, forming a membrane-associated periodic skeleton (MPS) that is similar to the 'membrane skeleton' of erythrocytes. The F-actin rings in the dendritic MPS are comprised of short actin filaments that are capped at their plus ends by adducin **(a')**. The observed periodicity of Tmod1 and Tmod2 in dendrites may indicate their presence in this structure **(a')**. Capping of F-actin minus ends by Tmod and plus ends by adducin would restrict filament length and impart filament stability, contributing to the generation of ring-like structures.

Chapter IV

Regulation of Dendritic Spine Development and Synapse Formation by Tropomodulin

ABSTRACT

The majority of excitatory synapses in the vertebrate brain are formed on dendritic spines – tiny, actin-rich protrusions on the dendritic surface that provide a platform for presynaptic input. The actin cytoskeleton controls the structure and molecular organization of spines and its dynamic remodeling underlies spine development and synapse formation. Despite intense study, the molecules and mechanisms that regulate actin organization and remodeling during postsynaptic development are not fully understood. In metazoans, the Tropomodulin family (Tmod) of proteins cap the pointed end of actin filaments, thereby regulating actin filament stability and organization. Tmod1, Tmod2, and Tmod3 are expressed in the vertebrate CNS, but if and how these molecules function during synapse formation is not known. In this study, we present evidence that Tmod1 and Tmod2 localize to dendritic spines, where they play an important role in dendritic spine development. Loss of Tmod1 or Tmod2 increases F-actin dynamics in dendritic spines and disrupts spine morphogenesis and synapse formation. These findings suggest that regulation of F-actin stability by Tmod1 and Tmod2 is essential for the cytoskeletal rearrangements underlying synaptogenesis.

A portion of this chapter has been submitted to the Journal of Neuroscience and is under review:

Omotola F. Omotade, Wenliang Lei, Velia Fowler, James Q. Zheng. Regulation of Dendrite Development and Synapse Formation by Tropomodulins

4.1 Introduction

Actin is the major cytoskeletal component in dendritic spines (Fifkova and Delay 1982), where it provides structural support and spatially organizes postsynaptic components (Hotulainen and Hoogenraad 2010). During synapse development, highly motile filopodia are converted into stable, mushroom-shaped spines that contain the synaptic components needed to receive presynaptic input (Yuste and Bonhoeffer 2004). This morphological transition is characterized by a conversion from longitudinal, F-actin filaments into a highly branched, dendritic network that predominates in the spine head (Hotulainen and Hoogenraad 2010, Korobova and Svitkina 2010). Despite fervent study, the molecules and mechanisms that regulate actin organization and remodeling during dendrite development and spine morphogenesis are not fully understood.

In metazoans, the Tropomodulin (Tmod) family of proteins cap actin filament minus ends and inhibit depolymerization, thereby regulating the stability, length and organization of diverse actin-based networks. Tmod2 knockout mice exhibit synaptic and behavioral deficits, including altered learning and memory (Cox, Fowler et al. 2003), though the cellular mechanisms that account for these phenotypes are unclear. A recent study (Gray, Suchowerska et al. 2016) using GFP-Tmod1 and GFP-Tmod2 overexpression indicates that Tmod1 and Tmod2 regulate dendritic branching and spine morphology in cultured rat hippocampal neurons. However, fundamental information such as the subcellular distribution, expression profile, and mechanisms by which Tmod modifies F-actin dynamics in dendritic spines has not been investigated. Therefore, if and how Tmods regulate F-actin synapse formation remain unknown.

In this study, we investigated the expression profile, subcellular distribution and function of Tmod1 and Tmod2 in hippocampal neurons. We find that Tmod1 and Tmod2 are expressed during synapse formation and are present in postsynaptic spines, where they exhibit a nanoscale, subspine distribution. Loss of either Tmod1 or Tmod2 increases F-actin dynamics in dendritic spines, perturbs spine morphology and disrupts synapse formation. Our results demonstrate that Tmod1 and Tmod2 are essential for regulating the actin cytoskeleton during dendritic spine morphogenesis and synapse formation, likely by promoting F-actin stability.

4.2 Results

To understand the function of Tmod in neurons, we examined the expression of Tmod1 and Tmod2 in rat hippocampus at E18 (E: embryonic), P8 (P: postnatal), P12, P23 and adult, which correspond to the stages before, during and after synapse formation (Markus and Petit 1987, Harris, Jensen et al. 1992, Fiala, Feinberg et al. 1998). Immunoblot analysis shows that both Tmod1 and Tmod2 are expressed in rat hippocampus, but with different profiles (**Figure 4.1A**). Tmod1 is undetectable at E18, with apparent expression beginning around P8. Substantial Tmod1 expression is detected around P12 and continues to increase steadily until adulthood. By contrast, Tmod2 is expressed at E18 and its levels increase sharply from E18 to P8. After P8, expression of Tmod2 remains relatively steady until adulthood. Formation of spine synapses commences near the end of the second postnatal week (P14) and peaks during the end of the fourth postnatal week (P30) (Markus and Petit 1987, Harris, Jensen et al. 1992, Fiala, Feinberg et al. 1998). Therefore both Tmod1 and Tmod2 are expressed in this critical window of synaptogenesis.

We next examined the protein expression of both isoforms in primary rat hippocampal neurons during specific *days in vitro* (DIV). Synapse formation in cultured hippocampal neurons initiates on dendritic arbors around DIV7-9, peaks around DIV11-14, and is largely completely by DIV18 onward (Ziv and Smith 1996, Friedman, Bresler et al. 2000, Grabrucker, Vaida et al. 2009). Consistent with the pattern of expression detected in hippocampal tissue, Tmod2 protein levels in cultured hippocampal neurons remain relatively steady before, during and after the processes of synapse formation and dendrite development (**Figure 4.1A**). In contrast, Tmod1 expression is undetectable until DIV7, with robust expression appearing around DIV21.Together, these immunoblot data indicate that both Tmod1 and Tmod2 may function in dendritic spine development and synapse formation.

To determine if either Tmod1 or Tmod2 is present in dendritic spines, we fluorescently labeled dendritic spines with a low concentration of fluorescent phalloidin, a phallotoxin that binds F-actin with high affinity (Gu, Firestein et al. 2008). Both Tmod1 and Tmod2 are expressed in dendritic spines containing post-synaptic density protein 95 (PSD-95), a marker of excitatory synapses (**Figure 4.2A**). When comparing the fluorescence intensity of Tmod in dendritic spines and the corresponding shaft region (spine/shaft ratio), we find that Tmods are slightly enriched in dendritic spines, with a spine/shaft ratio of 132.24 ± 16.68% (mean ± standard deviation) and 119.59 ± 5.96% (mean ± standard deviation) for Tmod1 and Tmod2, respectively. These results show that Tmod1 and Tmod2 are slightly enriched in postsynaptic spines.

The high level of Tmod signal in the dendritic shaft suggests the presence of a diffusible pool of Tmod in the postsynaptic compartment. To test if Tmod1 and Tmod2 in

spines are associated with the spine F-actin cytoskeleton, we performed live-cell extraction using the mild detergent saponin (Lee, Vitriol et al. 2013, Lei, Myers et al. 2017). Brief exposure of live neurons to saponin retains relatively stable structures such as the cytoskeleton and cytoskeleton-associated proteins (Lee, Vitriol et al. 2013, Lei, Myers et al. 2017). By comparing the fluorescence intensity of Tmod in extracted and non-extracted neurons, we were able to estimate the cytoskeleton-associated fraction of Tmod in distinct postsynaptic regions. Identical fluorescent labeling conditions and acquisition parameters allowed us to directly compare the fluorescence intensity of Tmod in extracted vs. non-extracted neurons. We find that the levels of endogenous Tmod1 and Tmod2 detected in dendritic spines of extracted and non-extracted neurons are nearly identical, with 98.24 ± 24.27% (mean ± standard deviation, n = 196), and 96.97 ± 29.86% (mean ± standard deviation, n = 233) of immunoreactivity remaining in spines after extraction, for Tmod1 and Tmod2, respectively (**Figure 4.2B-4.2D**). These data indicate that the majority of Tmod molecules in spines are associated with the actin cytoskeleton and/or PSD. Collectively, these results provide evidence that Tmod1 and Tmod2 exhibit tight cytoskeletal association in the dendritic spines, likely through F-actin binding.

It should be noted that the Tmod signal in dendritic spines do not appear to completely overlap with phalloidin-labeled F-actin. Rather, Tmod1 and Tmod2 appear to be restricted to the spine neck and center of the spine head (**Figure 4.2A-4.2B**). To obtain a more detailed analysis of the spatial organization of Tmod in dendritic spines, we turned to structured illumination microscopy (SIM), which utilizes overlapping moiré patters to give a resolution of ~115 nm (Gustafsson 2000). SIM images revealed that

Tmod1 and Tmod2 are largely absent from the distal-most and lateral regions of the spine head, instead localizing to the center most region of the spine head and the spine neck (**Figure 4.3A**). To quantify the apparent sub-spine localization of Tmod1 and Tmod2, dendritic spines were separated into three compartments: the spine neck, the distal most part of the spine head (area 1) and proximal-most part of the spine head (area 2: closest to spine neck). For each region (area 1, area 2, or spine neck) the raw integrated density value was measured for both the phalloidin channel and the Tmod channel and a subsequent Tmod/phalloidin ratio was generated. Quantitative analysis reveals that Tmod1 and Tmod2 are indeed enriched in the spine neck and lower part of the spine head (area 2) (**Figure 4.3B**). Further analysis using STED microscopy confirmed the subspine distribution of Tmod as well as revealed a punctate distribution in spine necks (**Figure 4.3C**). Interestingly, the distribution of Tmod1 and Tmod2 in the dendritic spine neck and head are largely non-overlapping (**Figure 4.3D**). Our results indicate that Tmod1 and Tmod2 exhibit a distinct sub-spine localization, concentrating in the spine neck and the base of the spine head.

The unique localization of Tmod to the spine neck and a subspine region of spine head prompted us to investigate the functional role of Tmod in spines. Studies using sophisticated imaging techniques have shown that dendritic spines contain different pools of F-actin, namely a dynamic and stable pool (Star, Kwiatkowski et al. 2002, Honkura, Matsuzaki et al. 2008, Frost, Shroff et al. 2010). Importantly, the dynamic pool of F-actin appears to be at the distal tip and lateral edges of the dendritic spine head and the stable pool of F-actin is located at the base of the spine head (Honkura, Matsuzaki et al. 2008). We therefore hypothesized that the localization of Tmod in the neck and base of the spine

head, a region enriched in stable F-actin, may be a mechanism used to regulate the stability of F-actin in dendritic spines. To test this hypothesis, we used fluorescence recovery after photobleaching (FRAP) to study the turnover of actin in dendritic spines from neurons depleted of Tmod1 or Tmod2. In control cells expressing shLucif, EGFP-actin fluorescence after photobleaching reached 50% of pre-bleaching levels in ~ 9 sec, consistent with our previous findings (Lei, Myers et al. 2017) (**Figure 4.4B-4.4C**). In contrast, actin filaments in spines of shTmod1 and shTmod2-expressing neurons recovered significantly quicker, achieving 50% recovery in ~7 seconds and ~ 6 seconds, for Tmod1 and Tmod2 respectively **(Figure 4.4B-4.4C)**. In control neurons, the plateau of EGFP-actin at 300 sec was ~87%, indicating that the proportion of stable F-actin that had not yet recovered was ~13%. By contrast, the proportion of stable actin was significantly decreased in shTmod1 and shTmod2-expressing neurons, with only ~ 8% and ~ 3% of GFP-actin left unrecovered by 300 sec, for Tmod1 and Tmod2, respectively (**Figure 4.4C**). Collectively, these results provide strong evidence that Tmod 1) stabilizes actin filaments in dendritic spines and 2) maintains the size of the stable F-actin pool.

To examine the function of Tmod during dendritic spine development and synapse formation, we introduced shTmod1 and shTmod2 into hippocampal neurons at DIV10, a developmental stage characterized by abundant dendritic filopodia that have not yet converted to mushroom-shaped spines. In control neurons transfected with shLucif, the majority of dendritic protrusions had developed into mushroom-shaped spines with a distinct head and neck. By contrast, knockdown of Tmod1 and Tmod2 caused a significant reduction in spine density and a modest increase in filopodia-like protrusions (**Figure 4.5A-4.5B**). Interestingly, we find that knockdown of Tmod2, but not Tmod1,

causes a significant increase in aberrantly-shaped spine heads, leading to an increase in spine head width (**Figure4.5B, 4.5E**). The abnormalities in spine density and morphology caused by Tmod knockdown can be better appreciated by 3D reconstruction of select dendritic protrusions (**Figure 4.5B**, bottom panel). To ensure that these phenotypes are due to Tmod depletion, rather than off-target effects, we generated a second set of hairpins directed against non-overlapping regions of Tmod1 and Tmod2, referred to as shTmod1-B and shTmod2-B, respectively. Similar to shTmod1 and shTmod2-expressing neurons, neurons expressing shTmod1-B and shTmod2-B exhibited defects in spine morphology and density (**Figure 4.5C, 4.5E**), indicating that depletion of Tmod is responsible for the observed spine defects. These results demonstrate that Tmod1 and Tmod2 are essential for the morphological development of dendritic spines.

We next used immunofluorescence to examine if Tmod1 or Tmod2 knockdown affects the formation of functional synapses. Synapses were defined as paired pre- and post synaptic markers using SV2 (presynaptic marker) and PSD-95 (postsynaptic marker). Our analysis revealed that the majority of dendritic spines from control neurons were apposed by SV2 puncta and contained PSD-95 signal (**Figure 4.6A**), consistent with spines representing the postsynaptic component of functional synapses. By contrast, in cells expressing shTmod1 and shTmod2, the density of PSD-95 and SV2 signals associated with protrusions was greatly reduced, with the majority of filopodia-like protrusions lacking PSD-95 signal and an opposing SV2 signal (**Figure 4.6A-46B**). Though PSD-95 and SV2 density was significantly reduced, the majority of spines that were present in shTmod-expressing neurons, contained PSD-95 signal and were directly

apposed by SV2 signal. Collectively, our data support a role for Tmod in dendritic spine development and synapse function.

4.3 Discussion

Dynamic remodeling of the actin cytoskeleton underlies dendritic spine development and synapse formation. Despite intense study, the molecules and mechanisms that regulate actin organization and remodeling during postsynaptic development are not fully understood. Tropomodulin molecules are best known for controlling the length and stability of actin filaments in the sarcomere of striated muscle and the membrane skeleton of diverse cell types (Yamashiro et al., 2012). To date, fundamental knowledge about the endogenous distribution and role of Tmods during synapse formation remains unknown. In this study, we performed a series of experiments to examine the expression profile, subcellular distribution, and function of endogenous Tmod1 and Tmod2 in hippocampal neurons. We find that Tmod1 and Tmod2 localize to a unique sub-spine region, where they regulate F-actin stability in spines. Together with our loss-of-function analysis, our data indicate that Tmod molecules regulate the actin cytoskeleton during dendritic spine morphogenesis and synapse formation. This study provides fundamental insight into mechanisms underlying neuronal development and adds to the limited body of knowledge about Tmod function in neurons.

A significant observation of this study is the sub-spine localization of Tmod1 and Tmod2 (**Figure 4.6-C**). Tropomodulin molecules are best known for capping filament minus ends and inhibiting depolymerization, thereby stabilizing actin filaments. Previous studies have shown that the peripheral region of spine heads contain branched F-actin structures that undergo dynamic turnover (Fifkova and Delay 1982, Landis and Reese

1983, Korobova and Svitkina 2010) (Star, Kwiatkowski et al. 2002, Honkura, Matsuzaki et al. 2008). By contrast, the core of spine head and spine neck contains relatively stable F-actin. The localization of Tmod1 and Tmod2 to a subspine region characterized by stable filaments is consistent with the classic function of Tmods in stabilizing diverse actin networks. The existence of two distinct, kinetic pools of F-actin may allow the spine to maintain a stable core for structural integrity, while simultaneously undergoing polymerization-driven structural rearrangements that underlie synapse development and plasticity. For example, during LTP and LTD, the spine head increases and decreases in size respectively (Star, Kwiatkowski et al. 2002, Honkura, Matsuzaki et al. 2008), yet the overall mushroom shape is preserved. Consistent with a role for Tmods in plasticity, loss of Tmod2 leads to altered LTP (Cox, Fowler et al. 2003) and LTD (Hu, Yu et al. 2014) – though the precise role of Tmod during synaptic plasticity remains to be explored.

What other mechanisms besides pointed end capping could account for the stability of F-actin in spines? Tight binding of Tmod to F-actin pointed ends requires tropomyosin (TM), an α-helical coiled-coil proteins that binds along the sides of F-actin filaments (Weber, Pennise et al. 1999). The subspine localization of Tmod is therefore indicative of TM-coated filaments in this region. The presence of TM along the length of F-actin blocks cofilin binding, thereby inhibiting cofilin-mediated severing and disassembly of TM-coated filaments (Bernstein and Bamburg 1982, Kuhn and Bamburg 2008). Spatiotemporal exclusion of cofilin in the spine ‘core’ may contribute to the generation or maintenance of relatively stable filaments, consistent with kinetic studies. In support of this, EM studies have shown that cofilin preferentially localizes just beneath the plasma membrane in dendritic spines (Racz and Weinberg 2006). In addition to an

antagonistic relationship with actin-binding proteins, Tmod may promote F-actin stability in spines by capping pointed ends in the actin-βII/III spectrin membrane lattice, which is present in the base and neck dendritic spines (Sidenstein, D'Este et al. 2016). Intriguingly, this detergent resistant (Efimova, Korobova et al. 2017) membrane lattice is discontinued at synaptic sites, entering the spine head but not reaching the PSD (Sidenstein, D'Este et al. 2016). Strikingly, we find that Tmod distribution in spines is 1) enriched in the neck and base of the spine head 2) exhibits a non-overlapping distribution with the PSD and 3) is overwhelmingly resistant to detergent extraction. These data, coupled with the well-established presence of Tmod in diverse actin/spectrin-membrane skeletons, suggests that Tmod may be a component of subcortical lattice in dendritic spines. Because this lattice is not detected in the region of the spine characterized by dynamic actin, its presence may contribute to the observed increase in F-actin stability in the spine center.

Despite the observed functional overlap of Tmod1 and Tmod2 in regulating spine morphology, the developmental expression profile of Tmod1 and Tmod2 suggests that they might have distinct roles during synapse formation and stabilization. The onset and peak of Tmod1 expression corresponds to a time period characterized by robust synaptogenesis and synapse maturation. By contrast, Tmod2 expression is relatively robust before, during and after synapse formation. These data suggest that Tmod1 may function primarily in the late stages of synaptogenesis, such as spine stabilization and maturation. Divergence in Tmod1 and Tmod2 function is likely mediated through differential binding to TPM3 and TPM4, two TM isoforms expressed in the postsynaptic compartment (Had, Faivre-Sarrailh et al. 1994, Guven, Gunning et al. 2011). Tmod1 and Tmod2 have differential affinities for TM isoforms (Kostyukova 2008, Uversky, Shah et

al. 2011, Colpan, Moroz et al. 2016), which are known to alter the spectrum of actin-regulatory proteins that can bind to F-actin (Gunning, O'Neill et al. 2008, Gunning, Hardeman et al. 2015). The spatiotemporal association of Tmod1 or Tmod2 with distinct Tm-coated filaments may enable Tmod1 and Tmod2 to adopt distinct roles during the various stages of dendritic spine development and synapse formation. In support of this, we show that Tmod1 and Tmod2 have largely non-overlapping distributions in spines, where they differentially regulate F-actin dynamics at the synapse. The inherent biochemical and structural differences between Tmod isoforms (Yamashiro, Gokhin et al. 2012), coupled with differential affinity for TM-coated actin filaments, are likely mechanisms used to fine-tune the isoform-specific regulation of F-actin by Tmod1 and Tmod2.

How might Tmod1 and Tmod2 regulate the actin cytoskeleton during spine and synapse development? The localization of Tmod1 and Tmod2 to a subspine region characterized by stable F-actin provides important insight into this question. Synapse formation is accompanied by a significant increase in the size of stable pool of F-actin in dendritic spines (Koskinen, Bertling et al. 2014), consistent with the conversion of highly motile filopodia into stable spines. We propose that capping of filament pointed ends by Tmod1 and Tmod2 inhibits depolymerization, thereby leading to filament stabilization and/or net actin polymerization during spine formation and maintenance. Consistent with this model, introduction of shTmod1 or shTmod2 at DIV10, during the filopodia-to-spine transition (FST), causes a significant decrease in stable spines and functional synapses. These studies shed light on the mechanisms by which F-actin is regulated in neurons and provide a platform for future studies to uncover the precise mechanisms by which Tmod

modifies the F-actin cytoskeleton during dendritic spine development and synapse formation.

4.4 Figures

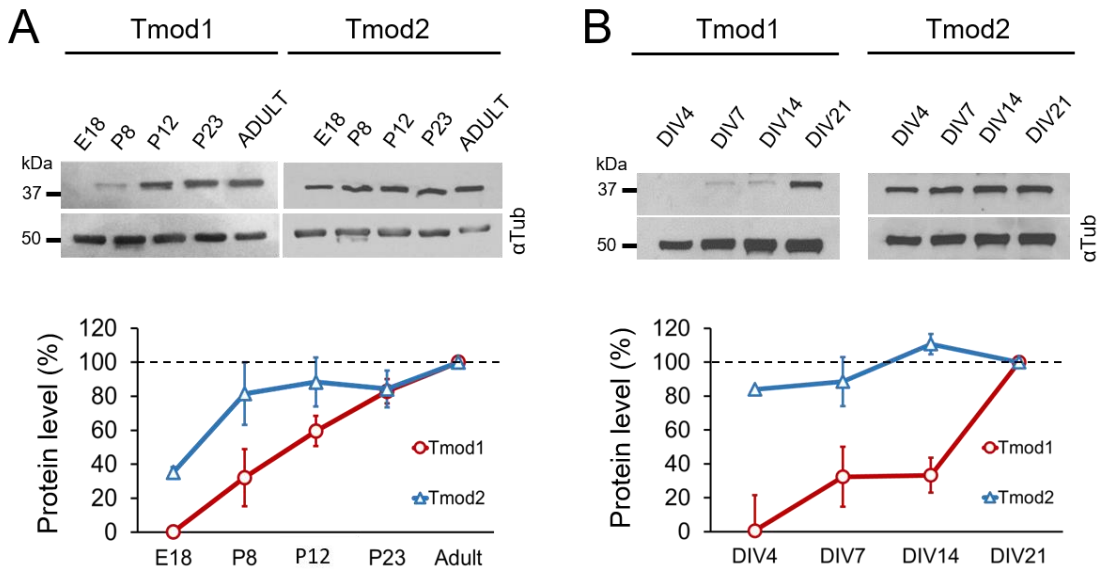
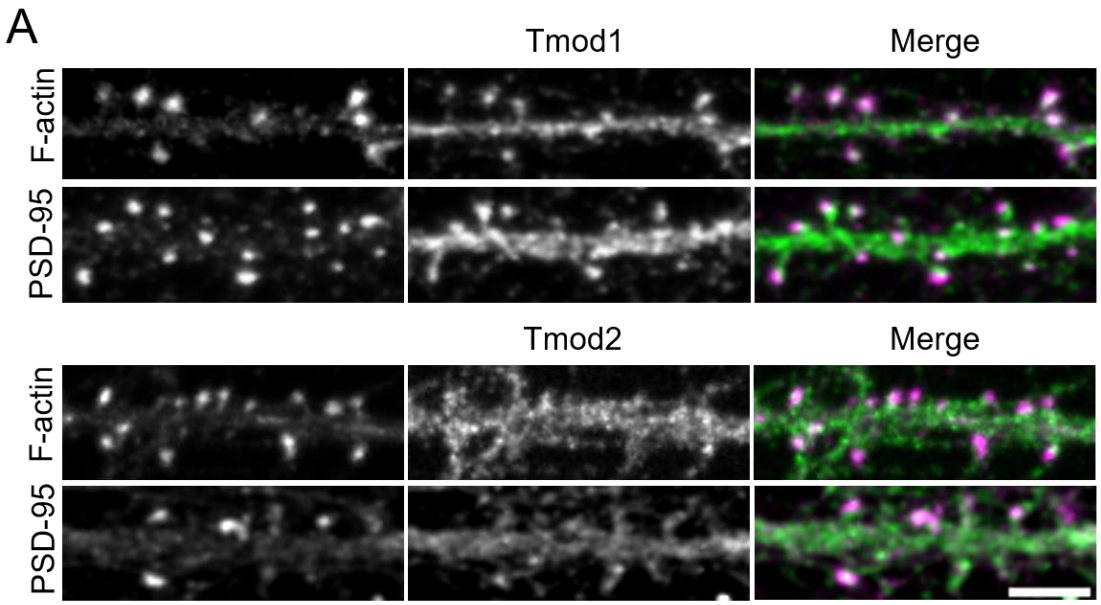
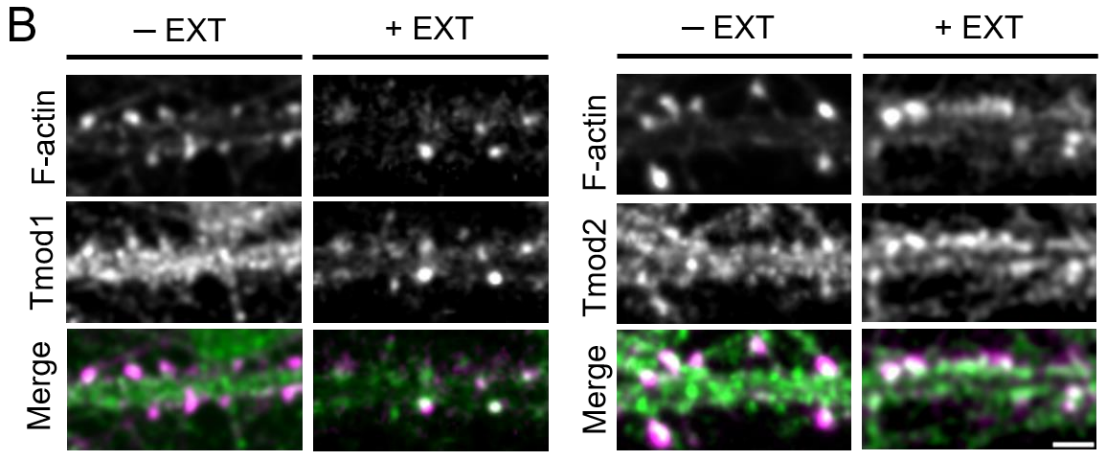


Figure 4.1: Expression profile of Tmod1 and Tmod2 in hippocampal neurons

Representative western blots of Tmod1 and Tmod2 in rat hippocampal tissue **(A)** and primary hippocampal neurons **(B)** depict the changes in Tmod expression over time. All bands in gel are cropped for clarity. Quantification from three separate replicates is shown in the corresponding line graphs (N=3 for each timepoint). The mean value for each timepoint was normalized to the corresponding tubulin loading control, and then to the 'Adult' (A) or 'DIV21'(B) value.


A
Tmod1
Merge
F-actin
PSD-95
Tmod2
Merge
F-actin
PSD-95


B
– EXT
+ EXT
– EXT
+ EXT
F-actin
Tmod1
Merge
F-actin
Tmod2
Merge


C
NO EXT.
EXT.
Spine value
Fluorescence Intensity (%)
120
100
80
60
40
20
0
Tmod1
Tmod2

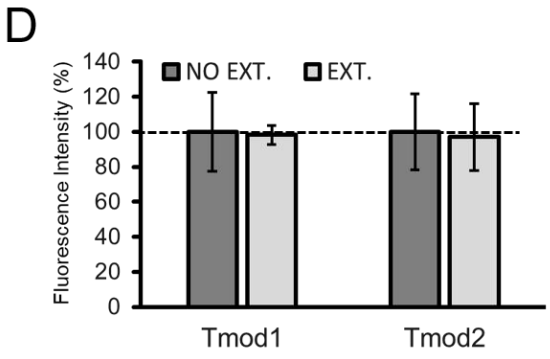

D
NO EXT.
EXT.
Fluorescence Intensity (%)
140
120
100
80
60
40
20
0
Tmod1
Tmod2

Figure 4.2. Subcellular distribution of Tmods in hippocampal neurons.

A: Immunostaining of endogenous Tmod1 and Tmod2 (green), together with phalloidin-labelled dendritic spines and PSD-95 (magenta). Scalebar = 5 µm **B:** Representative immunofluorescence images of Tmod1 and Tmod2 in select dendritic regions of extracted and non-extracted hippocampal neurons. Scalebar = 2.5 µm. **C:** Bar graph depict the mean fluorescence intensity of Tmod1 and Tmod2 in the dendritic shaft of control and extracted neurons. The fluorescence intensity values of Tmod1 and Tmod2 in the shaft are normalized to the mean corresponding value in the spine. **D:** Bar graphs depict the mean fluorescence intensity of Tmod1 and Tmod2 in dendritic spines of control and extracted neurons. 'Extracted' values are normalized to the 'non-extracted' values of Tmod1 and Tmod2, respectively. (C and D): Quantification from three separate replicates is shown in each corresponding bar graph. N_{spines} =196 and 233, for Tmod1 and Tmod2 respectively. N_{shaft} =180 and 173, for Tmod1 and Tmod2 respectively. Error bars: standard error of the mean (S.E.M). Mann-Whitney test was used to determine significance. The values for Tmod1 and Tmod2 in the shaft (C) and spine (D) are non-significant (extraction vs. non- extraction).

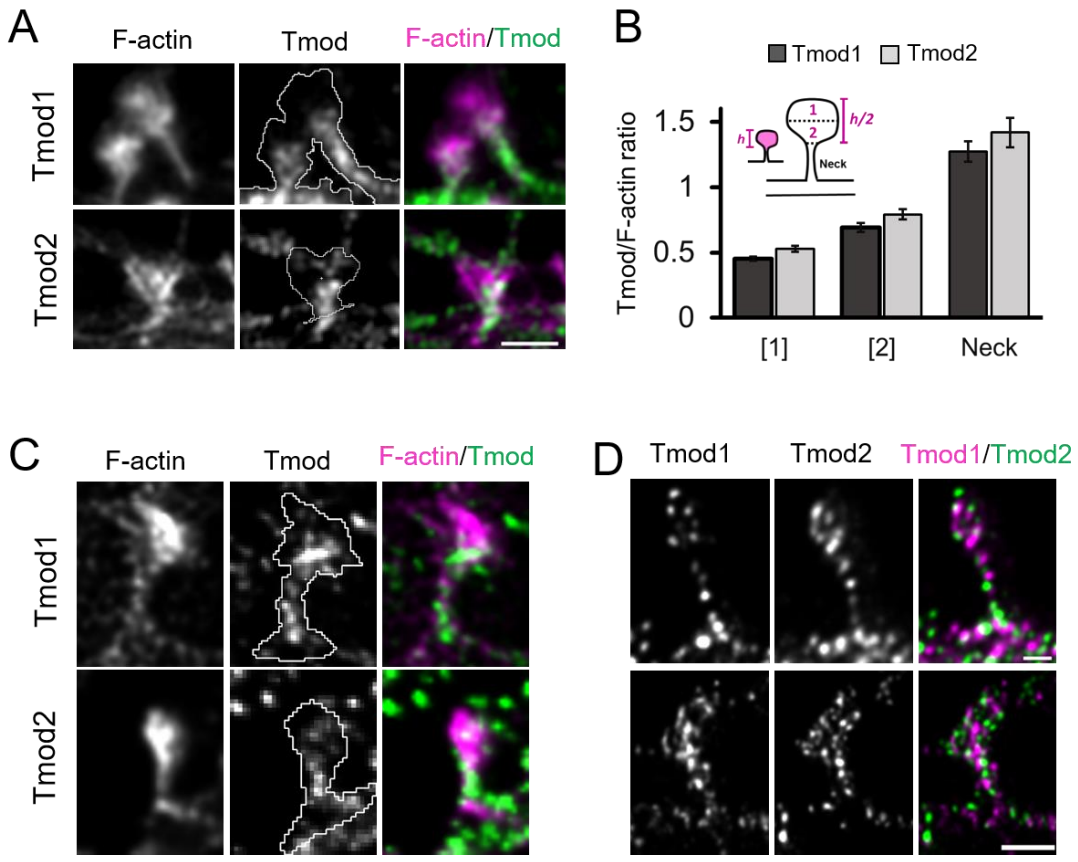


Figure 4.3 Subspine localization of Tmod1 and Tmod2.

A: 3D-SIM images of Tmod1 or Tmod2 (green) and F-actin (magenta) in dendritic spines reveal a subspine localization in the neck and base of spine head. **B:** Bar graphs represent the mean raw integrated density Tmod/F-actin ratios in the proximal and distal region of the spine head, as well as the spine neck **C:** STED image showing the distribution of Tmod1 or Tmod2 (green) and F-actin (magenta) in spines. **D:** STED image showing the distribution of Tmod1 (green) and Tmod2 (magenta) in the same dendritic protrusion.

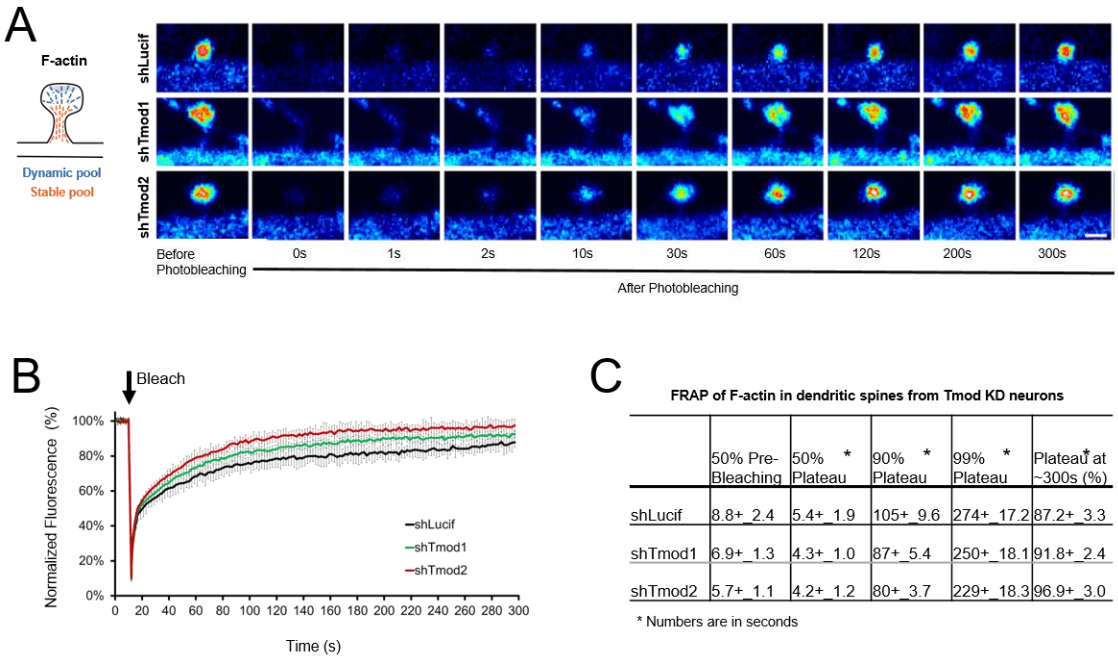


Figure 4.4. FRAP analysis of actin in Tmod1 or Tmod2 KD neurons.

A: Representative image sequences showing the fluorescence recovery of EGFP-actin after single-spine photobleaching in neurons expressing shLucif, shTmod1 or shTmod2. Scale bar = 1 μm. **B:** FRAP curves for control or shTmod-expressing neurons. The fluorescent signals in spines are normalized to the prebleaching mean and corrected for acquisition-based bleaching using the signals from the adjacent shaft regions. N = 15, 24 and 20 for shLucif, shTmod1 or shTmod2 groups respectively, and error bars represent the ± SEM. **C:** Quantification table showing the EGFP-actin recovery time for 50% prebleaching levels, 50% plateau levels, 90% and 99% plateau levels, and the final recovery percentages in dendritic spines. All numbers are presented as mean ± SEM.

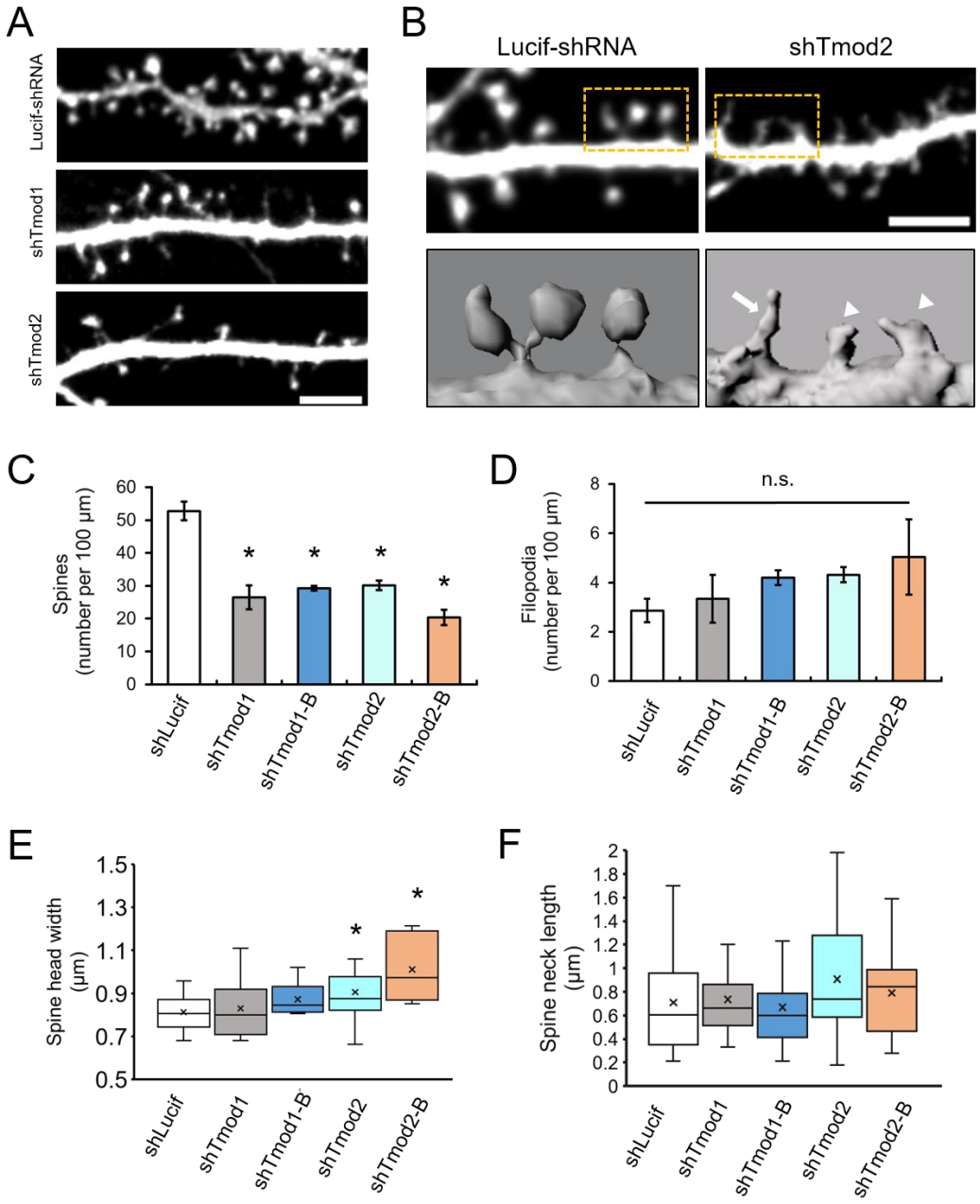


Figure 4.5. Tmod1 or Tmod2 knockdown impairs spine formation. A: Select dendritic regions in control vs shTmod expressing cells demonstrate reduced spine density in shTmod1 or shTmod2 neurons. Scalebar = 5 µm **B:** Representative images of dendritic protrusions in primary hippocampal neurons expressing shLucif, shTmod1 or shTmod2. 3D reconstruction of protrusions shown in the bottom panel. Arrows: thin

filopodia, arrowheads: aberrantly-shaped spines. **C-F:** Quantitative analysis of spine and filopodia densities and other morphological features in control vs. knockdown neurons. Error bars represent standard error of the mean (S.E.M). Statistical analysis was performed a Student's t-test, Asterisks: $p < 0.05$. p value $^{\text{spine density}}$: shTmod1 = 0.002 , shTmod1-B = 0.003 , shTmod2 = 0.0002, shTmod2-B = 0.001. p value $^{\text{spine head witdth}}$: shTmod2 = 0.016 , shTmod2-B = 0.001.

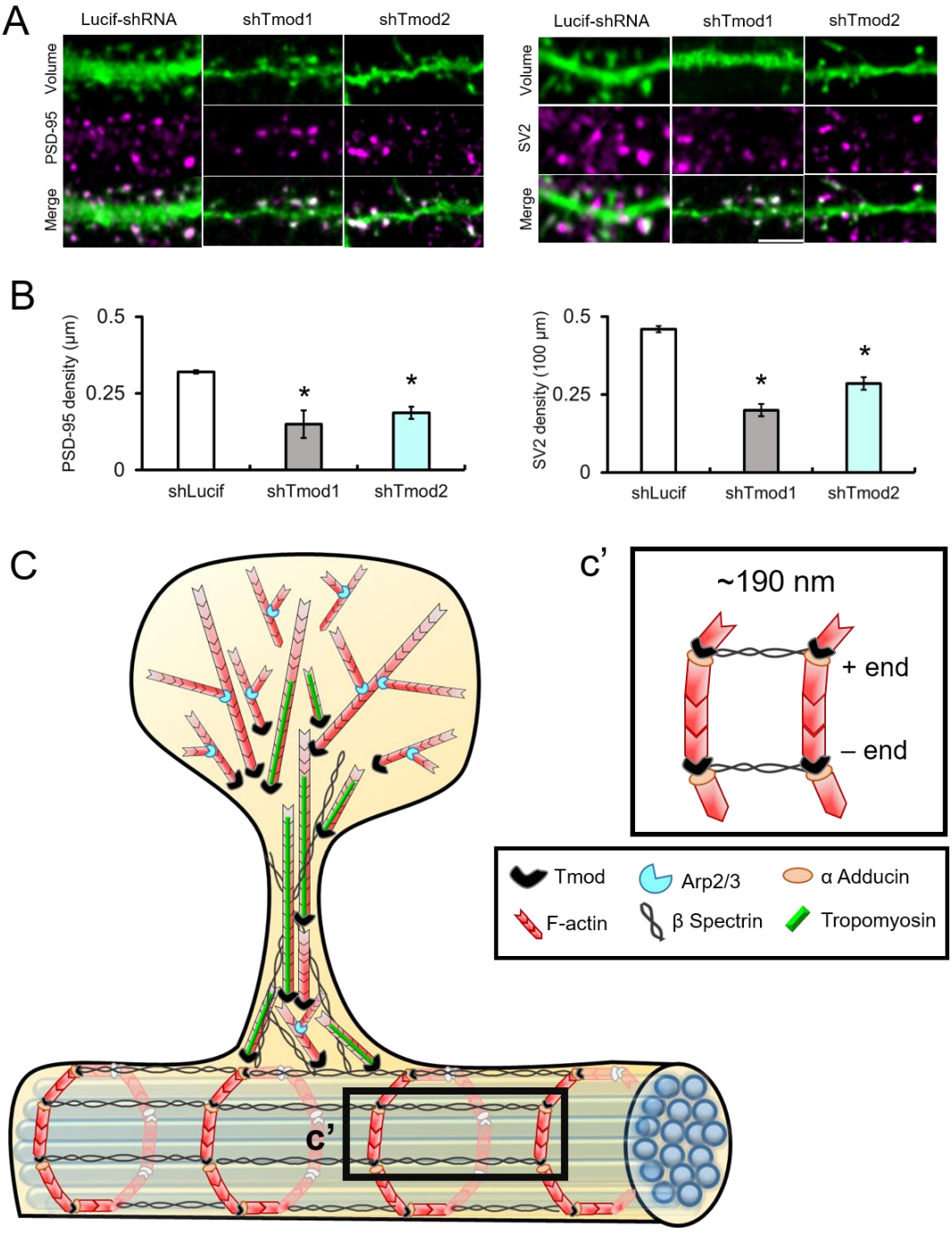


Figure 4.6. Tmod loss impairs synapse formation.

A: shLucif, shTmod1- or shTmod2-expressing neurons were stained for PSD-95 (left panel) and SV2 (right panel). Scale bar = 5µm **B:** The density of PSD-95 and SV2 are

quantified in the groups below. Asterisks: $p < 0.05$, Student's t-test. p value $^{\text{PSD density}}$: shTmod1 = 0.016 , shTmod2 = 0.003. p value $^{\text{SV2 density}}$: shTmod1 = 0.007 , shTmod2 = 0.017. Quantification from three separate replicates is shown in the corresponding bar graphs. **C: Schematic representation of Tmod localization and function in postsynaptic compartment.** Actin filaments in the spine head are oriented with their barbed ends towards the membrane and their pointed ends towards the spine center, or core. Tmod molecules are enriched in the spine neck and core of the spine head, where they cap the pointed ends of actin filaments. By contrast, Tmod molecules are rarely detected in the peripheral region of the spine head, which is characterized by newly nucleated filaments whose pointed ends are associated with the Arp2/3 complex, leading to a high number of branched filaments. βII/βIII spectrin tetramers, together with short actin filaments, form a membrane-associated periodic skeleton (MPS) in dendrites, the spine neck, and the base of the spine head. Like βII/βIII–spectrin, Tmod immunofluorescence is detected in the spine head, but does not overlap with the postsynaptic density (PSD). The presence of Tmod in this subspine region is highly indicative of Tropomyosin (TM) expression, though which TM isoform coats actin filaments in the stable core is unknown. In dendrites, short actin filaments capped by adducin at the barbed end (and possibly Tmod at the pointed end) are organized into evenly-spaced rings that are connected by spectrin tetramers.

Chapter V

Discussion: Tropomodulins in the Postsynaptic Compartment

Broadening our Understanding of F-Actin Organization and Dynamics at the Synapse

For over a century, the mechanisms underlying synapse development, maintenance, and plasticity have captured the curiosity of scientists. Strong evidence now suggests that synapse dysfunction, such as synapse loss, aberrant synaptic signaling and aberrant synaptic plasticity, is a feature of many neurological diseases. An understanding of how synapses are misregulated during pathological conditions will first require an understanding of the molecules and mechanisms that regulate synapse function under physiological conditions. Despite intense study, the mechanisms underlying the establishment, maintenance and modification of excitatory synapses are not fully understood.

The actin cytoskeleton is crucial for the development, maintenance and plasticity of dendritic spines. To date, an impressive body of work has uncovered the many molecules that exert spatiotemporal control over actin dynamics during development. Despite these efforts, our understanding of how actin is modified at the synapse is far from complete. Given the importance of actin in dendritic spine function, it is no surprise that misregulation of actin remodeling pathways are associated with a sleuth of

psychiatric, neurodevelopmental, and neurodegenerative diseases. Understanding the actin regulatory mechanisms responsible for proper neuronal function will no doubt shed light on the cellular processes that are disrupted during pathological conditions. The work presented in this dissertation examines the mechanisms by which Tropomodulin molecules regulate F-actin during dendrite development and synapse formation. The novel observations presented in this dissertation extend our understanding of how F-actin regulation contributes to the establishment of functional excitatory synapses.

5.1 Characterizing Tmods at the synapse

For the past thirty years, an impressive body of work has characterized the properties and function of Tmod in diverse actin networks. Despite a wealth of information about Tmod in non-neuronal tissues, the localization of Tmod in mature neurons has, until now, been unknown. The work in Chapter 3 and Chapter 4 examines the distribution, expression and function of Tmod1 and Tmod2 in hippocampal neurons. We find that both Tmod1 and Tmod2 localize to dendrites and dendritic spines, where they exhibit tight cytoskeletal association. Our in-depth, nanoscale investigation of Tmod distribution reveals that Tmods are enriched in a subspine region that contains stable F-actin (**Figure4.6-C**). From this key observation, we hypothesized that the selective localization of Tmod to the spine core, and its apparent exclusion from a region characterized by dynamic filaments, may be a mechanism used to promote F-stability in spines. Indeed, our FRAP results show that a loss of Tmod molecules increases F-actin dynamics in dendritic spines. Tropomodulin molecules are best known for capping pointed ends and stabilizing actin filaments in the sarcomere of striated muscle and the

membrane skeleton of diverse cell types. Our findings suggest that, like other diverse actin networks, pointed end capping by Tmod is a mechanism used to generate stable F-actin at the synapse. To our knowledge, this is the first work to demonstrate the endogenous localization of Tmods in mature neurons as well as provide insight into the mechanisms by which Tmod regulates F-actin dynamics in spines.

5.2 How does the subspine localization of Tmod extend our understanding of the spine cytoskeleton?

The spatiotemporal localization of actin binding proteins can provide crucial insight into the organization and dynamics of F-actin in a given cellular structure. For example, the spatiotemporal accumulation of the Arp2/3 complex within a given cellular region oftentimes accompanies a branched actin network. Likewise, the Formin family of actin nucleators are responsible for generating linear filaments, and are thus located in the proximity of non-branched actin networks. What migh the localization of Tmod suggest about the organization and function of F-actin in spines? Unlike the Arp2/3 complex that caps pointed ends in order to nucleate daughter filaments, Tmod capping stabilizes preexisting filaments by inhibiting depolymerization. The confinement of Tmod to a relatively small region of the spine head suggests that pointed ends near the periphery of the spine head are either 1) uncapped or 2) capped by the Arp2/3 complex. This model is consistent with studies showing a high degree of branched (Fifkova and Delay, 1982, Landis and Reese, 1983, Korobova and Svitkina, 2010), highly dynamic (Star et al., 2002, Honkura et al., 2008), actin filaments in the spine head.

A major outstanding question is if the subspine localization of Tmod is required for its apparent stabilization of F-actin in spines. Future studies disrupting the targeting of Tmod to the spine core will be crucial to determine if Tmod capping of F-actin filaments at any region in the spine proper promotes F-actin stability. Alternatively, capping of F-actin pointed ends specifically in the spine core (e.g. through the actin-spectrin periodic membrane skeleton) may be required to generate a stable F-actin pool. At present, our work does not distinguish between these possibilities, but future experiments will be critical in providing insight into the function of the stable pool of F-actin in spines. It should be noted that although we do not directly test the role of pointed end capping in this study, our data indicating that approximately 97% of Tmod in spines is resistant to detergent extraction suggests a role for pointed end capping of F-actin, rather than other actin regulatory functions of Tmods.

5.3 How is the stable pool of F-actin generated?

Pointed end capping of filaments is a well-studied mechanism used to inhibit depolymerization and promote stability; however, it is likely that mechanisms other than filament end capping are required to generate and maintain the stable pool of F-actin in spines. In addition to pointed-end capping of filaments, how might the localization of Tmod to a subspine region promote F-actin stability? A strong body of evidence suggests that Tmod has a synergistic and antagonistic relationship with actin binding proteins (ABPs) that regulate on F-actin stability. These spatiotemporal, epistatic interactions between Tmods and other actin regulators likely extend into the synapse –regulating the precise balance of dynamic and stable actin in the postsynaptic compartment. Tight binding of Tmod to F-actin pointed ends requires tropomyosin (TM), suggesting that

TMs are also present in the spine core (though it is unknown which isoform). The presence of TM along the length of F-actin blocks binding of the Arp2/3 complex (Blanchoin et al., 2001, DesMarais et al., 2002) as well as cofilin severing (Bernstein and Bamburg, 1982, Kuhn and Bamburg, 2008), inhibiting both nucleation and cofilin-mediated disassembly of TM-coated filaments. Though the distribution of Arp2/3 and cofilin in spines has not yet been investigated using nanoscale fluorescence microscopy, EM studies have shown that Arp2/3 concentrates in region between the spine plasma membrane and the center of the spine (Racz and Weinberg, 2008), whereas cofilin preferentially localizes just beneath the plasma membrane (Racz and Weinberg, 2006). Spatiotemporal exclusion of Arp2/3 and cofilin in the spine 'core' would thereby generate relatively stable, linear filaments, consistent with ultrastructural and kinetic studies (Hotulainen et al., 2009) .

Conversely, the exclusion of Tmod/TM complex from perisynaptic sites may be a mechanism to ensure that filaments near the periphery are competent for Arp2/3 binding and cofilin severing. An enrichment of these molecules near the membrane would be essential for replenishing the G-actin pool and generating the force required for rapid polymerization and subsequent head expansion (Lei et al., 2016). In addition, the segregation of Tmod-capped linear filaments in the neck and Arp2/3-capped branched filaments in the head would enable the spine to undergo dynamic rearrangements during development and plasticity, while maintaining a stable neck to preserve overall spine morphology. Indeed, loss of Tmod2 leads to altered LTP (Cox et al., 2003) and LTD (Hu et al., 2014) – though the precise role of Tmod during synaptic plasticity remains to be explored. A complex interplay between TM isoforms, Tmod1 and Tmod2, and other

actin-binding proteins is likely responsible for dictating the structural and kinetic properties of F-actin in spines during development and plasticity. Future experiments examining the epistatic relationship between Tmods and other actin binding proteins will be crucial in determining the mechanisms regulating F-actin stability in spines.

5.4 The role of Tmod in dendritic spine development and synapse formation

We find that knockdown of Tmod1 and Tmod2 during synaptogenesis significantly impairs spine development and synapse formation. How might Tmod specifically regulate the actin cytoskeleton during spine and synapse development? Synapse formation is accompanied by a significant increase in the size of stable pool of F-actin in dendritic spines (Koskinen et al., 2014), consistent with the conversion of highly motile filopodia into stable spines. It is likely that capping of filament minus ends inhibits depolymerization, thereby leading to filament stabilization and/or net actin polymerization. This would in turn account for the increase in stable actin observed during spinogenesis. Consistent with this model, introduction of shTmod1 shTmod2 at DIV10, during the filopodia-to-spine transition (FST), causes a significant increase in dynamic filopodia and a concomitant decrease in stable spines and functional synapses. We find that depletion of Tmod also reduces spine density, suggesting that Tmod-mediated stabilization of the cytoskeleton may be required for nascent spines to stabilize. During the FST, Tmod may therefore be required to generate a stable cytoskeletal structure that serves as platform for Arp2/3- mediated expansion (Hotulainen et al., 2009). The aberrantly shaped spine heads that are characteristic of shTmod2-expressing

neurons may suggest that Tmod2 is needed not only for the conversion of filopodia to spines, but to stabilize the cytoskeleton of mature spines. This finding is consistent with our FRAP data suggesting that Tmod2 has a more prominent role than Tmod1 in maintaining F-actin stability in spines. If and how the membrane skeleton contributes to spine development is unknown, but it is plausible that Tmod capping of minus ends in this structure may promote dendritic spine stability during development.

5.5 Tmods and plasticity

Because synapse activity affects the proportion of stable to dynamic actin (Star et al., 2002, Honkura et al., 2008) it is likely that Tmods have a specific role during synaptic plasticity. Under stimuli that led to LTD, Star *et al*. found that the amount of stable actin in spines increased from 5% to 41%, over 8 times the amount in spines at rest. LTD is often associated with a loss of F-actin and spine shrinkage (Fukazawa et al., 2003, Okamoto et al., 2004), and an increase in the stable pool of F-actin upon LTD is seemingly paradoxical. However, it is possible that while the overall amount of F-actin in spines decreases during LTD due depolymerization of actin, an increase in the amount of stable F-actin in the spine core may be required to maintain spine shape. This would suggest that during LTD, molecules that promote F-actin stability would need to be activated or recruited to spines. Capping of minus ends by Tmod would effectively inhibit depolymerization, which, when coupled with plus end capping proteins, could increase the proportion of stable filaments in spines. Consistent with this model, neurons expressing a hairpin against Tmod2 exhibit impaired LTD (Hu et al., 2014) and are unable to undergo spine shrinkage associated with LTD. In a somewhat conflicting report, mice lacking Tmod2 exhibit enhanced LTP. This may be due to an 8-fold

compensatory upregulation by Tmod1 in Tmod2 knockout mice. In addition, LTP-inducing stimuli causes the formation of a kinetically stable ‘enlargement pool’ of F-actin, which is required to mediate long-term expansion of the spine head (Honkura et al., 2008). Thus, in addition to LTD, Tmods may play a role in increasing filament stability during structural rearrangements underlying LTP. Inhibition or depletion of Tmod during LTP and LTD will be essential in understanding the possible role for Tmods in synaptic plasticity.

5.6 Summary

The actin cytoskeleton plays an important role in dendrite development and, importantly, controls the structure and molecular organization of the spines during synapse formation. Understanding the molecules and mechanisms that regulate actin organization and remodeling during postsynaptic development is of utmost importance. The questions addressed in this dissertation were: 1) What is the expression profile and subcellular distribution of endogenous Tmod1 and Tmod2 in hippocampal neurons? 2) What is the role of Tmod during dendrite development? 3) What is the function of Tmod during dendritic spine development and synapse formation? and 4) How does Tmod regulate the F- actin cytoskeleton in dendritic spines? Through the work presented in this thesis, I have uncovered a novel role for Tmod1 and Tmod2 in regulating the actin cytoskeleton during dendrite development and synapse formation, likely by promoting F-actin stability. Collectively, the findings in this dissertation provide novel insight into the mechanisms by which F-actin is regulated during neuronal development and adds to the limited body of knowledge about Tmod function in neurons.

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