ROLE OF PKMZ IN MORPHOLOGICAL AND SYNAPTIC DEVELOPMENT OF OPTIC TECTAL NEURONS IN *XENOPUS LAEVIS* TADPOLES *IN VIVO*

by

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B.Sc., Peking University, 2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

September 2010

ABSTRACT

PKMz (Protein Kinase M zeta) is a recently identified isoform of Protein Kinase C. It is persistently active upon synthesis because its sequence resembles the catalytic domain of PKC zeta but lacks the auto-inhibitory regulatory domain. Previous studies found that PKMz is critical for LTP maintenance, as well as learning and memory in the adult rat brain. However, it is not known whether and how it functions in developing neural systems. I have identified endogenous PKMz in *Xenopus laevis* tadpoles brain and found that its expression pattern is temporally and spatially correlated with synaptogenesis and dendritogenesis within tadpole retino-tectal system. By *in vivo* rapid time-lapse imaging and three-dimensional analysis of dynamic dendritic growth, I find that exogenous expression of PKMz within single neurons stabilizes dendritic filopodia by increasing dendritic filopodial lifetimes and decreasing filopodial additions, eliminations, and motility, whereas long-term *in vivo* imaging demonstrates restricted expansion of the dendritic arbor. Alternatively, blocking endogenous PKMz activity in individual growing tectal neurons with ZIP (zeta-inhibitory peptide) destabilizes dendritic filopodia and over long periods promotes excessive arbor expansion. Consistent with its established roles in regulating adult glutamatergic synaptic transmission, I also examined role of PKMz in regulating developing synapses, using both immunohistochemistry and *in vivo* patch clamp recording. Specifically, I find that knocking down endogenous PKMz using a morpholino impairs both transmission and maturation of glutamatergic synapses, and consistently induces promoted dendritic expansion as seen in ZIP treated neurons. The model that PKMz regulates dendritogenesis by regulating glutamatergic synaptic transmission was further investigated using a novel seizure model based on Xenopus tadpoles. I find that PTZ induced seizure activity increases normalized expression level of brain PKMz, which is required for over-stabilization of dendritic filopodia dynamics induced by seizure activity. Based on these findings, together with previous results from other related studies, I have constructed a discreet and stochastic computational model to simulate synaptotropic dendritic growth mechanism. I show that as formation of nascent synapses promotes dendritic expansion into region of synaptic partners by promoting maintenance of dendritic filopodia, synapse maturation drives further dendritic refinement and stabilization of appropriate dendritic structure.

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ACKNOWLEDGEMENTS

I would like to thank my committee members – including Dr. Shernaz Bamji, Dr. Catherine Rankin, and Dr. Yutian Wang – for their invaluable supports and insights to guide me through my PhD study.

Special thanks go to my supervisor, Dr. Kurt Haas, for providing supervision and assistance in every aspect of my study and work here. Working in a new lab is challenging, especially as an international student. Mistakes and failures are commonly seen. It's Kurt's patience and enthusiasm that help me work through this process and gain a more independent mind and hand of scientific research.

I would also like to thank my colleagues in Haas Lab for devoting their time and energy to give help and advice on my work, especially through those day-long lab meetings.

Finally, I would like to thank my girl friend Fan Yang for sharing my joys and sorrows in the past six years.

DEDICATION

To my beloved parents,

who always stand behind my exploration of life.

CO-AUTHORSHIP STATEMENT

Chapter 2

The experiments in Chapter 2 were conceived of and designed by me. I conducted all of the experimental work and data analysis, prepared the figures, and wrote the manuscript. Parisa Karimi Tari assisted with immunohistochemistry. Dr. Kurt Haas provided advice on the overall experimental design and helped edit the manuscript.

Chapter 3

The experiments in Chapter 3 were conceived of and designed by me. I conducted all of the experimental work and data analysis, prepared the figures, and wrote the manuscript. Dr. Kurt Haas provided advice on the overall experimental design and helped edit the manuscript.

Chapter 4

The experiments in Chapter 4 were conceived of and designed by Sesath Hewapathirane and Me. We conducted all of the experimental work and data analysis, prepared the figures, and wrote the manuscript. I was responsible for the PKMz related part and electrophysiology. Simon Chen, Wesley Yen and Shay Neufeld provided assistance in the software-based manual tracking and measurement of dendritic filopodia, and Parisa Karimi Tari assisted with immunohistochemistry and immunoblotting. Dr. Kurt Haas provided advice on the overall experimental design and helped edit the manuscript.

Chapter 5

The experiments in Chapter 5 were conceived of and designed by me. I conducted all of the experimental work and data analysis, prepared the figures, and wrote the manuscript. Dr. Kurt Haas provided advice on the overall experimental design and helped edit the manuscript**.**

Appendix A:

The experiments in Appendix A were conceived of and designed by me. I conducted all of the experimental work and data analysis, prepared the figures, and wrote the manuscript. Dr. Kurt Haas provided advice on the overall experimental design and helped edit the manuscript.

1 GENERAL INTRODUCTION

1.1 Dendritogenesis

1.1.1 Dendritogenesis in early development: a overview

Neurons are ‘polarized’ cells, with distinct elaborate dendritic and axonal arbor membranous protrusions extending from the soma. The flow of neural signals propagates from dendrites to soma to axons. Synaptic signals from upstream neurons are received primarily on dendrites, integrated in the dendritic arbor and soma to influence action potential firing which is transmitted though axons to send signals to dendrites of downstream neurons. Axons and dendrites can be distinguished by distinctive morphological differences, restricted protein expression and subcellular composition. For example, growth associated protein 43 (GAP-43) and tau protein are only expressed in axons, while microtubule-associated protein 2 (MAP2) exclusively localizes in dendrites (Kosik and Finch, 1987; Goslin et al., 1988). Asymmetric localization was also found on cell organelles such as Golgi outposts, which are only present at dendrites (Horton et al., 2005). Dendritic arbors of different neuronal sub-types exhibit enormous variety and complexity in morphology. The conservation of dendritic arbor morphologies within each sub-type supports an important relationship between arbor structure and function.

The primary roles of dendrites are to increase cell surface area for synaptic contacts and to reach regions of axonal input. Therefore, the size and shape of dendritic arbors determines the number and type of synapses formed on a neuron. The contribution of dendritic processes to

increasing cell surface area is evident when comparing typical neurons to spherical cells. For example, dendrites of mammalian motor neurons can have a surface area of approximately 370, 000 μm^2, yet only occupy a volume of 300,000 μm^3. In contrast, a spherical cell with the same surface area would be more than 60 times larger in volume. The finding that 80% of the surface area of motor neuron dendrites is dedicated to synapses demonstrates that dendritic arbor size is critically associated with number of synapses (Kellerth et al., 1979). In addition, the dendritic arbor morphology also decides a neuron's connectivity since its extent of branching and extension into specific spatial regions determines the distinct populations of axons that can be contacted (Harvey and Napper, 1991).

Dendritic morphology also plays an important role in the electrical integration and processing of synaptic transmission. Dendritic branches have been modeled as passive cables in which postsynaptic currents from multiple synaptic sites propagate to sum at the soma to trigger an action potential when a specific transmembrane potential threshold is reached. The dendritic arbor morphology therefore affects the extent of current decay, as well as temporal summation at the soma. This model has been elaborated to incorporate recent findings on active electrical properties of dendrites. Expression of voltage-gated ion channels throughout the dendritic membrane allows local excitatory synaptic inputs to integrate and trigger local regenerative responses. Dendritic spike can propagate to the soma to modulate action potential generation. In addition, the voltage-dependent dendritic response can be dynamically modulated. In rat hippocampal CA1 pyramidal neurons, propagation of local Na+ spike in individual dendritic branches can be enhanced by repetitive stimulation applied on that branch

through down-regulation of A-type potassium channel function. In this manner, recent experience could be stored in individual branches by compartmentalized dendritic plasticity. These findings indicate a complex and dynamically changing role for dendritic arbor processing and integration of synaptic input (Makara et al., 2009).

Given that the morphological structure of a neuron's dendritic arbor determines the number and types of synapses available and how synaptic signals are processed, proper dendritic arborization during early brain development is therefore critical for normal function of both the neuron itself and its contribution to the local circuit function. The majority of studies on dendritic development have utilized dissociated neurons grown in low-density cell culture because this system allows direct imaging with standard light microscopy. The morphogenesis of cultured neurons begins with extension of undifferentiated 'neurites', a term denoting processes that may be precursors of either dendrites or axons. After one neurite dedicates to an axonal phenotype, the remaining neurites differentiate into dendrites and elaborate into the dendritic arbor (Craig and Banker, 1994). The rapid progress of *in vivo* labeling and imaging techniques has made it possible to directly observe the dynamic dendrite growth of developing neurons within their natural environments. Such *in vivo* imaging studies of brain neuron growth have shown that, at least within intact *Xenopus laevis* tadpoles and zebrafish, neuronal polarity appears to be predetermined as the first projected process is the axon, from which dendritic branches emerge (Myers et al., 1986; Wu et al., 1999). Time-lapse imaging reveals that growing dendritic arbors are composed of unstable dendritic filopodia and more stable dendritic branches. Filopodia are short, typically less than 10 μm, and thin, ~1μm, protrusions that are actin-rich and highly motile.

During arbor growth, these filopodia are precursors to longer branches. Although the vast majority of filopodia retract within minutes or hours of emerging, a small fraction of these filopodia stabilize and elongate to create new branches (Niell et al., 2004). Dendritic branches are typically longer than 10 μm, contain microtubules, and exhibit growth cones at their leading tips when growing. While branches are relatively stable, during growth, even large branches may be retracted in a dynamic process of morphological refinement.

Long-interval *in vivo* time-lapse imaging of dendritic arbor growth has found that patterns of dendritic arborization can be generally divided into three phases (Wu et al., 1999; Wong and Wong, 2000). The earliest phase, Stage 1, occurs during initial periods following neuronal differentiation when dendritic processes first emerge and arbor sizes are small. During Stage 1, high process turnover occurs, yet with little or no net increase in arbor size. In Stage 2, dendritic arbor size increases dramatically due to stabilization and elongation of both filopodia and branches. Arbor morphology in Stage 2 is exceptionally plastic with continuous remodeling involving extension and retraction of filopodia and branches. In the final phase, Stage 3, the dendritic arbor becomes stabilized without further large-scale remodeling. The branch dynamics measured by addition and retraction rates of Stage 3 neuron are half of Stage 2 neurons. Branch extension ceases and the mature dendritic arbor is formed and is believed to remain largely stable throughout life.

1.1.2 Intrinsic factors directs cell-type specific morphological phenotypes

Initial theories of dendrite development suggested solely intrinsic control of morphologic patterning, supported by shared general morphologies between neurons within distinct cell types, and the conservation of these neuron-type specific arbor patterns when neurons are grown in dissociated cultures (Bray, 1973; Banker and Cowan, 1979). Maintenance of stereotyped arbor shapes has been attributed to regulation of patterning by genetic programs. *Drosophila* has been proven to be a useful model organism for identifying proteins regulating arborization, including transcription factors (Parrish et al., 2006; Tassetto and Gao, 2006). One example is the transcription factor *hamlet*. Hamlet is transiently expressed during development of 'external sensory' (ES) neurons in the *Drosophila* peripheral nervous system. ES neurons have a simple dendritic arbor, while 'multiple dendritic' (MD) neurons, another type of peripheral sensory neuron express arbors with bifurcating dendrites. Loss-of-function mutations of Hamlet have been found to transform ES neurons into MD-like morphologies (Moore et al., 2002). Another transcription factor *cut* has been found to determine specific morphological subtypes of MD neurons (Grueber et al., 2003). Overexpression of Cut in type I MD neuron leads to its transformation into the more complex dendritic morphologies typical of type III or IV neurons. Given the complexity of the dendritic morphogenesis of these neurons it is likely that these transcription factors regulate patterning by controlling expression of numerous other proteins. The role of transcription factors in regulating dendritic morphology has also been found in other animals. For example, knock-out mice lacking the transcription factor *calcium responsive*

transactivator (CREST) showed abnormal dendritic morphology of neurons within intact hippocampus and cortex, with less elaborate basal and apical dendrites compared to control mice (Aizawa et al., 2004).

1.1.3 Extrinsic factors directing dendritic arbor morphogenesis

However, mounting evidence from recent studies indicates that although basic arbor patterns, such as number of primary branches, may be intrinsic, external signals play pivotal roles in shaping the fine details of the dendrite arbor including branch length and total number of higher order branches. Subsequent studies have identified a wide range of extrinsic factors that can influence the complexity and growth of dendritic arbors. Therefore, it is now believed that while internal genetic programs may dictate the general morphological phenotype of a neuron's dendritic arbor, cues from the local environment help to sculpt the mature and functional structure (McAllister, 2000; Jan and Jan, 2003).

1.1.3.1 Guidance cues

Guidance cues have been identified and well characterized for their roles in directing growth of axons. The axonal growth cone is a well-studied organelle with specific guidance cue receptors mediating attractive and repulsive guidance to specific soluble and membrane-bound ligands encountered in the extracellular space allowing long distance axonal navigation through convoluted 3D tissues. Whether guidance cues similarly direct dendrite growth is a poorly addressed question. Few studies have recognized the existence of growth cones at the tips of

growing dendritic branches or characterized guidance cue effects on dendrite growth. One example is Semaphorin3A (Sema3A), which has been shown to direct general orientation of dendritic arborization in cortical pyramidal neurons. Sema3A was originally identified as an axonal guidance molecule based on its repellent effects on axon growth. Interestingly, the gradient expression of Sema3A in cortex was also found critical for orientating the growth of apical dendrite towards the pial surface as a chemoattractant. Interfering with Sema3A signaling using function-blocking antibodies to the semaphoring receptor Neuropilin1 leads to disorientated apical dendrites (Polleux et al., 2000). The divergent effects of Sema3A on axons (repellent) and dendrites (attractant) has been attributed to asymmetric expression of the intracellular downstream effecter of semaphorin receptors, soluble guanylate cyclase (sGC) in axons and dendrites (Polleux et al., 1998). Immunostaining of cortical neurons found low expression of sGC in axons and high levels in apical dendrites. Furthermore, application of an inhibitor of sGC to cortical slice cultures leads to disorientation of dendrites (Polleux et al., 2000).

1.1.3.2 Neurotrophic factors

Previous studies have found that neurotrophic factors could modulate dendrite growth, while the exact effects depend on the types of neurotrophic factors and neurons examined. Neurotrophic factors, such as nerve growth factors (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 and 4, exert their effects mainly through Trk family of receptor tyrosine kinases. In the peripheral nervous system, systematic application of NGF in rats was

found to cause dramatic expansion of dendritic arbors of sympathetic ganglion cells, while dendritic arbor growth was blocked by NGF antiserum (Snider, 1988; Ruit et al., 1990). In developing *Xenopus laevis* tadpole retina, application BDNF significantly decreased retinal ganglion cell (RGC) dendritic arbor complexity, whereas neutralizing endogenous BDNF levels with function-blocking antibodies significantly increased dendritic arbor complexity (Lom and Cohen-Cory, 1999). Extracellular application of neurotrophic factors to slice cultures of developing ferret visual cortex generally promotes dendrite growth of pyramidal neurons by increasing dendritic branch length and number. However, neurons in each cortical layer respond to different subset of neurotrophins, and within a layer, each neurotrophin elicits different effects on dendritic morphology. Furthermore, basal dendrites of neurons in each cortical layer respond strongly to a single neurotrophin, while apical dendrites are responsive to a range of neurotrophins (McAllister et al., 1995). Conversely, blocking endogenous neurophic factors function by Trk antibodies leads to layer specific effects on dendrites (McAllister et al., 1997). Interestingly, it was found that the growth promotion effect by neurotrophic factors requires neural activity, as inhibition of spontaneous activity, synaptic transmission or VGCCs was found to block enhanced growth elicited by BDNF(McAllister et al., 1996).

1.1.4 Neural activity regulates dendritic arbor elaboration

In addition to intrinsic genetic programming and extrinsic cues, a growing body of evidence supports a role for neural activity in regulating patterning of growing dendritic arbors. The term ‘neural activity’, however, encompasses a number of distinct phenomenon including action

potential firing, spontaneous synaptic transmission, and postsynaptic receptor activation.

1.1.4.1 Neuronal firing

One manipulation commonly employed to increase neural activity is the increase in extracellular potassium to induce membrane depolarization and increase neuronal firing. Elevated potassium increases total dendritic branch length and tip number in cultured growing neurons (Jin et al., 2003; Yu and Malenka, 2003). On the other hand, application of tetrodotoxin (TTX), which blocks action potential generation, was found to have variable effects on dendrite growth. Intracranial mini-pump infusion of TTX did not affect the arborization of neurons in embryonic cat lateral geniculate nucleus (Dalva et al., 1994). Similarly, TTX failed to affect dendritic arborization of tadpole tectal neurons *in vivo* (Rajan and Cline, 1998). In hippocampal co-cultures, afferent-induced dendritic branching is insensitive to chronic application of TTX (Kossel et al., 1997). In ferret cortical slices, neurotrophin-induced promotion of dendrite growth of pyramidal neurons was found to be blocked by TTX (McAllister et al., 1996). However, this requirement was absent in non-pyramidal neurons. Blocking action potentials with TTX did not prevent the BDNF-induced dendrite growth in cultured neocortical interneurons (Jin et al., 2003). The variability of TTX's effects may arise from the fact that TTX can only block action potential-induced neural transmission, but spares spontaneous neurotransmitter release that may mediate other aspects of activity dependent dendrite growth.

1.1.4.2 Excitatory glutamatergic synaptic transmission

Glutamatergic synaptic transmission, the major excitatory inter-neuronal chemical signaling in the central nervous system (CNS), has been shown to play a clear role in regulating dendritogenesis. Blocking transmission mediated by the NMDA and AMPA subtypes of glutamate receptors in acute cortical brain slices of neonatal rats using specific antagonists reduces the turnover and density of dendritic shaft filopodia, while focal glutamate application increases length of shaft filopodia (Portera-Cailliau et al., 2003). Glutamate receptor blockade also reduces filopodial length in RGCs in retina explant from chick embryos (Wong et al., 2000). Similarly, *In vivo* time-lapse imaging in *Xenopus* tadpoles demonstrates that blocking or reducing synaptic transmission mediated by AMPA and NMDA receptors impairs dendrite growth, characterized by destabilization and retraction of growing dendritic processes (Rajan and Cline, 1998; Haas et al., 2006). Interestingly, newly differentiated neurons with small dendritic arbors were found to be less sensitive to AMPA transmission blockade, which is consistent with delayed developmental expression of AMPA receptors at glutamatergic synapses (Rajan and Cline, 1998).

1.1.4.3 Sensory stimulation

A role for brain neural network activity driven by sensory stimulation on dendrite development was initially supported by the findings that visual cortical neurons of animals reared in stimulation-rich environment have more complex dendritic arbors than those from animals raised in sparse environments(Volkmar and Greenough, 1972). Further, occluding

visual input by dark rearing leads to abnormal dendrite growth in layer 4 stellate cells of visual cortex (Borges and Berry, 1976). However, these effects appear to be cell specific, since no change on dendritic arbor growth of layer 3 pyramidal cells was found in dark reared animals (Tieman et al., 1995).

Dramatic effects on dendritic development are found by introducing asymmetric right eye/left eye stimulation in animals with binocular vision. Monocular deprivation leads to atrophy of dendrites innervated by the deprived eye in the LGN (Wiesel and Hubel, 1963). Similarly, unilateral ear plugging disrupts the symmetric development of dendritic arbors of nucleus laminaris neurons innervated binaurally (Smith et al., 1983).

In vivo imaging of neurons in the optic tectum of *Xenopus* tadpoles provides direct evidence for regulation of dendritic arborization by sensory stimulation. Visual stimulation was found to influence dendrite growth of tectal neurons, which receive direct glutamatergic innervation from RGC axons. These studies found that exposing freely swimming tadpoles to darkness for 4 hours decreased dendrite growth, while growth rates increased by a subsequent 4 hours of moving bar visual stimulation (Sin et al., 2002). Furthermore, it was found that brief exposure to light stimulation could provide continuous promotion to dendrite growth even after the tadpoles were returned to darkness, suggesting a long-lasting structural plasticity mechanism was recruited. This visual input-induced enhancement in dendrite growth was blocked by application of antagonists of either NMDA or AMPA receptors (Sin et al., 2002).

1.1.5 Synaptotropic model of dendritogenesis

1.1.5.1 Description of the model

A theoretical model addressing the correlation between synaptogenesis and dendrite arborization was first proposed by Vaughn in the 1980s based on electron microscopic investigations of synaptogenesis and dendritic arborization in fixed rat spinal tissue. Vaughn found that dendrites of motor neurons predominantly grew into regions of the developing mouse spinal cord enriched with afferents and synapses (Vaughn, 1989). The original model posited that nascent dendritic processes are morphologically stabilized by synapses formation, while processes that fail to form synapses retract and are eliminated. Furthermore, new process could protrude from stabilized processes to probe the local environment for additional new synaptic contacts. In this manner, dendritic arbors can grow by the iterative extension and stabilization of processes leading to formation of dendritic arbor structures that are directed by synaptic connections. Assuming that synapse formation requires 'correct' functional pre- and postsynaptic activity, synaptotropic dendritogenesis would promote formation of dendritic morphologies optimized to process the activity encountered during development. Direct evidence supporting synaptotropic dendritogenesis comes from *in vivo* time-lapse imaging of zebrafish tectal neurons expressing the fluorescently-tagged postsynaptic marker PSD-95. In the developing tectum, PSD-95 puncta develop on nascent dendrite filopodia, and new filopodia branch from sites of PSD-95 puncta. The morphological stability of dendritic filopodia, which are precursors of longer branches, was found to correlate with the presence of PSD-95 puncta.

Most nascent dendritic filopodia lacking PSD-95 puncta rapidly retracted and were eliminated. In filopodia expressing puncta, retraction only occurred distal to the PSD-95 puncta, with complete filopodial elimination only observed once the PSD-95 puncta first dissolved (Niell et al., 2004).

1.1.5.2 Synaptogenesis and dendritic stabilization

Synapse formation is a dynamic process requiring interaction between pre- and post-synaptic partners. Synapse formation initiates when growing axonal and dendritic processes first contact with each other. Cell adhesion molecules (CAMs) on nascent pre- and postsynaptic elements, including Cadherin, Neuroligin/Neurexin, and SynCaM, confer specificity for correct pairing (Dalva et al., 2007). CAM-mediated adhesive interactions provide physical stability linking pre- and post- synaptic membranes and direct intracellular synaptic specialization. For example, neuroligin expressed by non-neuronal cells is sufficient to promote the formation of functional presynaptic structures in co-cultured neurons (Scheiffele et al., 2000). It is believed that assembling presynaptic machinery involves membrane integration of pre-packaged vesicles, while postsynaptic differentiation is mediated through gradual and sequential accumulation of proteins. Postsynaptic neuroligin is thought to bind and recruit the scaffolding proteins SAP90/PSD95 and NMDA receptors to postsynaptic site. Such complexes then mediate clustering of other postsynaptic molecules, including CaMKII, AMPA receptors, cytoskeleton proteins and other scaffolding proteins, either by direct binding or by glutamatergic transmission mediated mechanisms, and carry on cascades for recruiting more postsynaptic proteins implicated with formation of the postsynaptic density (PSD) matrix(Ziv, 2001; Ziv and

Garner, 2001).

Scaffolding proteins, including SAP90/PSD95, PSD93, SAP102, Homer/Shank, stargazing and others, are essential components of PSD assembly by binding multiple proteins including glutamate receptors, cytoskeleton proteins, kinases and other scaffolding proteins to form functional and stable postsynaptic matrix (Ziv, 2001; Ziv and Garner, 2001; Garner et al., 2002; Sheng and Hoogenraad, 2007). Findings that deleting single scaffolding proteins do not cause severe perturbation of the postsynaptic assembly suggests that these synaptic molecules are interconnected and provide functional redundancy, which confers sufficient stabilization of postsynaptic structure(McGee et al., 2001). Also, synapses may confer stabilization of dendritic filopodia by acting as a nucleation sites for the cytoskeleton network, and mediates the linkage between the trans-synaptic adhesive interactions with intracellular cytoskeleton network.

While actin is the major cytoskeleton component of dendritic filopodia, microtubules form the stable core of dendritic branches, and invasion of microtubules mediates stabilization of newly formed branches and consolidation of the dendritic tree (Whitford et al., 2002). Since most synapses are present at branching points of dendritic arbors (Sanchez et al., 2006), it is possible that synapses may act as anchor points for microtubules to stabilize dendritic segments proximal to the synaptic site. In addition, signaling cascades recruited in the postsynaptic matrix may also regulate the dynamics of microtubules by interacting with microtubule associated proteins (Quinlan and Halpain, 1996).

1.1.5.3 Synapse maturation

The vast majority of our knowledge of synapse formation, function, and maturation comes from studies of glutamatergic synapses. Developmental studies reveal a maturational progression in receptor and receptor subunit expression. Nascent glutamatergic synapses express NMDARs, yet lack AMPARs, and are therefore termed ‘silent’ since NMDAR channels are blocked by magnesium ions at resting membrane potentials (Kerchner and Nicoll, 2008). Without co-activation of AMPARs by presynaptically released glutamate, the magnesium ion blockade of NMDA receptors would not be removed and no glutamatergic postsynaptic current would be evoked. With maturation, AMPA receptors are incorporated into synapses which render these synapses ‘functional’ since AMPA receptor transmission provides the local depolarization required to unblock NMDA receptors. AMPAR incorporation into developing synapses is regulated by neural activity, and is thought to share some signaling mechanisms with synapse potentiation in mature neural systems, including activation of NMDA receptors and CaMKII (Constantine-Paton and Cline, 1998; Zhao et al., 2006).

CaMKII is highly enriched in central glutamatergic synapses, constituting approximately 2-5% of the protein in the postsynaptic density (Lisman et al., 2002). In mature brain, CaMKII activity is found to be critical for induction but not maintenance of long-term synaptic potentiation (LTP). Upon activation by calcium influx, CaMKII directly phosphorylates GluR1 subunits of AMPARs, increasing the channel conductance. Activated CaMKII is also found to translocate to the PSD where it binds to NMDARs, acting as an anchor protein to promote synaptic integration of AMPARs and other synaptic proteins (Soderling and Derkach, 2000; Lisman et al., 2002).

Intracellular infusion of persistently activated CaMKII is sufficient to induce synaptic potentiation in hippocampal neurons (Pettit et al., 1994). In developing *Xenopus* tectum, endogenous expression of CaMKII is correlated with the maturation stage of tectal neurons. CaMKII is absent in newly differentiated neurons but highly expressed in mature neurons. Exogenous expression of persistently active CaMKII in immature tectal neurons is found to promote maturation of retino-tectal synapses, electrophysiologically characterized as a decrease in the ratio of "silent" synapses expressing only NMDARs to "functional" synapses expressing both NMDARs and AMPARs (Wu et al., 1996).

Besides enhancement of AMPAR mediated synaptic responses, synapse maturation is also associated with changes in subunit composition of glutamate receptors. AMPARs in immature synapses are calcium-permeable because they lack the GluR2 subunit. With maturation, calcium-permeable GluR4 subunits are replaced by calcium-impermeable GluR2 subunits (Aizenman et al., 2002; Migues et al., 2007). A maturational shift is also seen in NMDARs, in which immature receptors contain NR2B subunits, which have slower decay kinetics and therefore larger calcium conductance, are exchanged for NR2A subunits that have rapid decay kinetics (Williams et al., 1993; Monyer et al., 1994). While the initial addition of AMPARs to silent synapses enhances the synaptic transmission efficacy, the subsequent changes in subunit composition confer more restricted calcium influx.

In addition to functional changes, synapse maturation also involves structural changes in postsynaptic assembly. Synaptic trafficking of glutamate receptors is mediated through interaction between receptors with various scaffolding and cytoskeleton proteins. Addition of

receptors to synapses is therefore accompanied by enhancement of both size and stability of the postsynaptic density matrix. Time-lapse imaging of neurons expressing fluorescently tagged PSD-95 shows that the PSD-95 puncta increase in intensity with time as synapses mature (Niell et al., 2004). Treatments affecting functional maturation of glutamatergic synapses are found to induce correlated changes in size of PSD-95 puncta (Haas et al., 2006; Yoshii and Constantine-Paton, 2007). Conversely, overexpressing scaffolding proteins like PSD-95 or Homer enhances the maturation of glutamatergic synapses (El-Husseini et al., 2000; Van Keuren-Jensen and Cline, 2008).

Collectively, structural maturation of synapses may stabilize local cytoskeleton networks underlying nascent dendritic processes, and the functional maturation of synapses may regulate synaptic transmission dependent dendritic arborization.

1.1.5.4 Calcium signaling mediates synaptic activity-dependent dendritic stabilization

Intracellular elevation of calcium levels evoked by synaptic activation may be an important intracellular signaling cascade regulating activity-dependent dendritic stabilization (Lohmann et al., 2002; Lohmann et al., 2005). Upon synaptic stimulation, intracellular calcium levels increase by calcium influx through NMDARs and voltage-gated calcium channels (VGCCs). In addition, infusion of calcium from extracellular sources can then initiate release of further calcium from intracellular stores, termed ‘calcium-induced calcium release’ (CICR). Local calcium transients restricted around synaptic sites have been shown to influence local dendritic arborization by

regulating filopodial stabilization (Lohmann et al., 2002; Lohmann et al., 2005). Selective blockade of local calcium transients leads to destabilization and retraction of dendritic filopodia (Lohmann et al., 2002). This effect of local calcium transients on dendritic filopodia dynamics is spatially and temporally specific. When filopodia first emerge from the dendritic shaft, low calcium levels are detected. Elevated calcium levels are observed and become frequent as filopodial growth accelerates, and peak levels are associated with filopodial stabilization (Lohmann et al., 2005). These observations suggest that intracellular calcium levels may affect filopodia motility by directly activating molecules regulating the cytoskeleton. For example, the calcium-activated kinase CaMKIIβ was found to regulate actin filament stability by directly binding F-actin (Sanabria et al., 2009).

1.1.5.5 Rho GTPases as downstream regulators of activity-dependent dendrite arborization

Synaptic activity may also regulate dendritic stability through regulation of small Rho GTPases, given their established roles in regulating the actin cytoskeleton (Etienne-Manneville and Hall, 2002). RhoA, Rac1 and Cdc42 are the three Rho GTPases most intensively studied for their roles in dendrite morphogenesis (Threadgill et al., 1997; Ruchhoeft et al., 1999; Li et al., 2000). Previous studies have shown that RhoA deters filopodia outgrowth by destabilizing the cytoskeleton while Rac1 and Cdc42 facilitate branching by stabilizing filopodia (Yin et al., 1981; Li et al., 2002). Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) through catalyzing the exchange of GDP for GTP, and are deactivated by GTPase-activating

proteins (GAPs). GEFs have been localized at postsynaptic sites, indicating Rho GTPases may act as convergent effectors downstream of intracellular signals implicated with cytoskeleton modulation (Tolias et al., 2005; Ma et al., 2008). Previous studies have shown that elevation of local calcium transients regulates activity of Rho GTPases in various cell types (Fleming et al., 1999; Price et al., 2003), and a recent study has found that extracellular application of a low concentration of ryanodine to cerebellar neurons triggers CICR, up-regulates Cdc42/Rac and down-regulates RhoA. Ryanodine treatment also leads to morphological changes in cultured *Xenopus* spinal cord neurons, which requires Cdc42 activation (Jin et al., 2005).

In vivo studies of neuron growth in *Xenopus* tadpoles also find that electrical stimulation of the optic nerve increases Rac activity and decreases RhoA activity in tectal neurons, which is dependent of AMPAR and NMDAR transmission (Li et al., 2002). This differential activation pattern supports crosstalk between small Rho GTPases. Furthermore, Rac/Cdc42 and RhoA show interdependent effects on dendritic arborization of tectal neurons. Enhanced Rac and Cdc42 activity selectively increased dendritic branch dynamics, which is blocked by RhoA activation. Conversely, branch elaboration induced by RhoA inhibition requires Rac activation (Li et al., 2000). In addition, visual stimulus-induced dendritic arbor growth requires decreased RhoA activity and increased Rac and Cdc42 activity (Sin et al., 2002). These results collectively suggest that Rho GTPases are important downstream regulators of synaptic activity-dependent dendritic arborization.

1.1.5.6 Activity-dependent protein synthesis in dendrite growth

Excitatory synaptic transmission may also regulate local dendrite arborization by affecting protein synthesis in dendrites. Both mRNAs and translation machinery have been identified in dendrites. Using cDNA microarray technology, over one hundred dendritic mRNA candidates have been identified from hippocampal pyramidal neurons (Zhong et al., 2006). Furthermore, strong evidence for activity-dependent dendritic protein translation comes from studies treating freshly severed dendritic arbors with glutamate that found increased protein translation, evident from elevated ^{35}S-methionine incorporation (Leski and Steward, 1996). In addition, direct synaptic stimulation that induces late-phase LTP in hippocampal neurons was found to increase expression of elongation factor 1A (eEF1A) in dendrites that had been severed from their cell bodies before stimulation (Tsokas et al., 2005). Further direct evidence supporting a role for activity-dependent dendritic translation in dendritic arborization in *Xenopus* tectal neurons comes from studies on cytoplasmic polyadenylation element binding protein (CPEB). CPEB is an mRNA binding protein regulating dendritic protein synthesis through dendritic delivery of mRNA and activity-dependent polyadenylation of target mRNAs (Ule and Darnell, 2006). CPEB was found to be required for dendrite arbor growth, and exogenous expression of an inactive mutant of CPEB specifically impairs visual stimulation-induced dendrite growth and development of glutamatergic synapses (Bestman and Cline, 2008).

Previous studies have identified CaMKIV as an important kinase mediating activity-induced gene transcription and dendrite outgrowth. CaMKIV is expressed in the nucleus and is activated by high calcium influx through NMDARs or VGCCs (Redmond and Ghosh, 2005). Exogenous

expression of a constitutively-active form of CaMKIV promotes dendritic growth in cortical neurons, which mimics the effects induced by calcium influx (Redmond et al., 2002). cAMP response element-binding protein (CREB), a transcription factor that is critically associated with activity-dependent dendrite growth, and its co-activtor CREB binding protein (CPB) are also required for CaMKIV's effects on dendrite growth(Hu et al., 1999; Impey et al., 2002). Recently, another transcription factor named calcium responsive transactivator (CREST) has been identified as a specific activity-dependent transcription factor (Aizawa et al., 2004; Pradhan and Liu, 2005). CREST is constitutively expressed at the nucleus at developmental periods of high dendrite growth. It interacts with CBP and is activated by calcium. Interestingly, neurons cultured from CREST knock-out mice have similar dendrite arborization patterns compared to controls, but they are less responsive to enhanced growth induced by calcium influx, and the impairment can be rescued by transient CREST expression (Aizawa et al., 2004).

1.1.5.7 Elaboration of synaptotropic theory

The synaptotropic model suggests that synaptogenesis in developing neurons direct dendritic arborization towards regions with high levels of appropriated presynaptic partners. This model is supported by various theoretical analyses (Niell, 2006; Tsigankov and Koulakov, 2009). Furthermore, given a positive feedback between synapse formation and filopodia maintenance, the model also predicts an exponential increase in dendritic arbor growth rate, which is observed in the 'Stage 2' of dendritic arbor growth. However, one important caveat of this conventional model is that it does not explain the gradual stabilization of dendritic arbor structure as neurons

mature. A self-restraining mechanism is necessary to prevent continued and excessive growth.

The mechanisms underlying maturation-associated loss of dendritic growth plasticity are unclear. However, this morphological shift occurs concomitantly with maturation of glutamatergic synapses (Wu et al., 1996). Therefore, one possibility is that graded levels of synapse maturation confer graded amounts of morphological stabilization. Synapse strength correlates with the intensity of local calcium transients produced by synaptic activation. While calcium transients have been shown to prevent filopodia retraction, large calcium transients at strong and mature synapses may halt filopodia extension (Segal et al., 2000; Wong and Wong, 2000). Alternatively, since synapse formation involves nucleation of cytoskeletal-associated proteins, it is possible that while immature synapses cluster sufficient cytoskeletal–associated proteins to prevent filopodial retraction, accumulation of greater amounts of these proteins at mature, stronger synapses prevents further growth plasticity.

Support for a modified synaptotropic model comes from studies altering levels and activity of CaMKII. CaMKII promotes maturation of glutamatergic synapses, and its endogenous expression increases as neurons mature. If synapse maturation is purely associated with dendritic growth, as predicted from a simple interpretation of the synaptotropic model, one would expect that expression of constitutive-active CaMKII would results in larger arbors. However, exogenous expression of CaMKII in actively growing neurons transforms the dendritic arborization from a dynamic stage to a stabilized one, evident from decreased branch addition and retraction rate (Wu and Cline, 1998). Thus, precocious neuron-wide maturation of glutamatergic synapses results in restricted growth and may mimic morphological effects of

synapse maturation in mature neurons. Conversely, inhibition of endogenous CaMKII activity either pharmacologically or by expressing an inhibitory peptide maintains neurons in a state of dynamic growth, leading to increased arbor expansion (Wu and Cline, 1998).

Such an elaborated model is able to explain the characteristic growth patterns of tectal dendrite observed *in vivo*. During Stage 1, limited synaptogenesis restricts maintenance of motile filopodia and branches, resulting in high process turnover and minimal process elongation. In Stage 2, the positive feedback between synapse formation and filopodia maintenance promote rapid expansion of dendritic arbor. A large proportion of synapses formed at this stage are immature, primarily expressing NMDARs (Wu et al., 1996). These immature synapses are sufficient to prevent filopodial retraction, but permissive for further elongation. Delayed expression of CaMKII in newly differentiated neurons may underlie the switch from Stage 1 to Stage 2. Without CaMKII during Stage 1, glutamatergic synaptic transmission may not be able to induce activity-dependent synaptogenesis and synapse maturation required for morphologic stabilization. Subsequent synaptic plasticity mechanism would then gradually promote synapse maturation leading to further stabilization to produce the loss of plasticity seen in Stage 3. In Stage 3, tectal neurons exhibit a high proportion of mature synapses with high AMPA/NMDA ratios and relatively few silent NMDA synapses, express high levels of CaMKII, and have mature and stabilized dendritic arbors. (Wu et al., 1996; Wu et al., 1999)

1.2 Protein Kinase M zeta, an activity-regulated constitutively-active Protein Kinase C isoform

1.2.1 Overview and classification

Protein kinase C (PKC) is a family of protein kinases regulating phosphorylation of serine or threonine residues on substrate proteins. The PKC family is comprised of about ten isoenzymes, which share conserved protein structure, including a catalytic domain and a regulatory domain that auto-inhibits the kinase in the resting state. Depending on different activation mechanisms, PKC isoenzymes can be categorized into three groups: conventional, novel and atypical. Conventional PKCs, including α, βI, βII and γ isoforms, require calcium and diacylglycerol (DAG) binding for initial activation. Novel PKCs, including δ,ε,θ and μ isoforms, do not require calcium binding. Atypical PKCs (aPKC), including ζ and ι/λ isoforms, are not responsive to DAG or calcium, but are activated by different secondary messengers (Mellor and Parker, 1998). Binding of phosphatidylinositol-3,4,5-(PO4)3 (PIP3), a lipid product by phosphoinositide 3-kinase (PI3K), initiates a conformational change of aPKCs, which releases their auto-inhibition and facilitates targeting of the kinase to the membrane. Then 3'-phosphoinositide-dependent kinase-1 (PDK1), another downstream target of PIP3, directs phosphorylation of a threonine residue (Thr 410) on the catalytic domain of aPKC, which is required for aPKC activation. Full activation is achieved by a further phosphorylation on T560, and *in vitro* studies suggest that this may be mediated by autophosphorylation (Hirai and Chida, 2003; Suzuki et al., 2003).

Protein Kinase M zeta (PKMz) is a recently identified variant of PKCζ (or PKCz). PKMz has

been found to be critically associated with neural plasticity and higher brain functions. PKMz possesses the catalytic domain of a full length PKCz, yet lacks the auto-inhibitory regulatory domain, and therefore is believed to be a constitutively active form of PKCz (Ling et al., 2002).

1.2.2 Distribution and expression of PKMz

PKMz has been found to be highly expressed in rat brain, and specifically enriched in hippocampus, frontal and occipital cortex, striatum, and hypothalamus (Naik et al., 2000; Hernandez et al., 2003). PKMz is phylogenetically conserved, with nervous system expression in other animals including *Drosophila*, Aplysia and *Xenopus* (Drier et al., 2002; Liu et al., 2009; Villareal et al., 2009). In studies of hippocampal LTP, levels of PKMz expression were found to rapidly increase upon tetanic stimulation. The NMDAR-activation-dependent increase is first detectable 10 minutes following stimulation, and lasts for more than 2 hours (Osten et al., 1996). Initially, it was believed that PKMz represented the product of calpain digestion of PKCz. *In vitro* studies found that activated PKCz was susceptible to limited cleavage at its hinge, producing independent catalytic segment, or PKM (Kishimoto et al., 1983), and it was hypothesized that calpain activated by calcium influx through NMDARs initiated this proteolysis (Vanderklish et al., 1995). In Aplysia, application of a calpain inhibitor blocks serotonin-induced enhancement of sensorimotor synapses, which was considered PKMz dependent (Villareal et al., 2009). However, this mechanism has not been found in rats or mice. Instead, increasing evidence from recent studies indicate that PKMz may be a product of *de novo* protein synthesis. Up-regulation of PKMz during LTP could be blocked by protein synthesis inhibitors (Osten et al., 1996). In

addition, western blotting using an antibody specific to the catalytic domain of PKCz, which could detect both PKMz and PKCz, detected only PKMz in hippocampus. Using the same antibody, it was also found that knock-out mice with the coding region of PKCz regulatory domain disrupted failed to express full length PKCz, but PKMz expression was not affected. Further investigation identified a unique PKMz mRNA from an alternating splicing variant of the PKCz gene. The PKMz mRNA is transcribed from an internal promoter within the PKCz gene, and therefore creates a unique 5' untranslated region (UTR) sequence, but has the same 3' UTR as PKCz mRNA. The open reading frame (ORF) of this mRNA contains sequences from the hinge to the end of PKCz. *In vitro* translation of the PKMz mRNA produces a protein with the same molecular weight as endogenous PKMz, but larger than the product of calpain cleavage of PKCz (Hernandez et al., 2003). Upon transcription in the nucleus, PKMz mRNA is rapidly transported and localized to synapto-dendritic sites. Two cis-acting dendritic targeting elements (DTEs) contained in the 3'-UTR and ORF of PKMz mRNA are critical for its precise localization. Specifically, DTE1 is responsible for its dendritic export, while DTE2 is required for its delivery to distal dendritic sites. Translation of dendritic PKMz mRNA trigged by intense synaptic stimulation was found to be mediated by activation of a comprehensive kinase network consisting of PI3-kinase, CaMKII, MAPK, protein kinase A (PKA), mammalian target of rapamycin (mTOR). Interestingly, activity of pre-existing PKMz was also found to be required for PKMz synthesis, suggesting a positive feedback between PKMz activity and expression that might underlie persistent upregulation of PKMz (Kelly et al., 2007a). Furthermore, actin polymerization, which is critically associated with LTP consolidation and rapid spine

morphological changes (Lin et al., 2005; Kramar et al., 2006), is also required for PKMz synthesis (Kelly et al., 2007b). In a recent study, a peptidyl-prolyl isomerase Pin1, which is present at dendritic spines as a synaptic protein synthesis suppressor, was identified as an additional factor regulating PKMz synthesis. Pin1 knockout mice showed dramatically elevated brain expression of PKMz and enhanced late phase LTP. Interestingly, PKMz activity was found to inhibit Pin1 and promote protein synthesis. The interaction between PKMz and Pin1 may provide a feed-forward mechanism underlying late LTP maintenance (Westmark et al.).

1.2.3 PKMz in long-term synaptic plasticity in mature brain

Studies on adult rat hippocampal slices have shown that PKMz is both necessary and sufficient for the maintenance of long-term synaptic potentiation (LTP). One hour after hippocampal LTP is induced by tetanus stimulation, bath application of either 1 μM chelerythrine, a PKC inhibitor that was found to preferentially inhibit PKMz, or a specific cell-permeable peptide inhibitor, myristoylated - Zeta pseudo-substrate inhibitor peptide (myr-ZIP), was found to reverse this synaptic potentiation (Ling et al., 2002). Furthermore, intracellular application of a low concentration (1-3 nM) of PKMz through a patch clamp recording electrode increased the amplitude of AMPAR-mediated EPSCs by 98.7% within 10 minutes following infusion. The potentiation by PKMz infusion was persistent for the entire recording period, and completely occluded further LTP induction by pairing tetanus stimulation with postsynaptic depolarization, indicating the PKMz-induced potentiation recruits the same signaling mechanism as tetanic LTP (Sacktor et al., 1993).

LTP consists of two stages: induction or short term potentiation, and maintenance (Malenka and Bear, 2004). The maintenance stage can be further divided into early and late phases. Early phase LTP is protein synthesis independent, and lasts for approximately 1 hour, while late phase LTP requires new protein synthesis and can last for many hours. Synaptic potentiation can be achieved through both pre- and postsynaptic mechanisms. Presynaptic potentiation is mediated through enhanced transmitter release, and may primarily contribute to induction of LTP. Postsynaptic potentiation is mainly mediated through enhanced AMPAR transmission, and is triggered by NMDAR activation upon strong synaptic stimulation. Strong activation of AMPARs induces sufficient membrane depolarization to release Mg2+ blockade of NMDARs. NMDAR activation leads to high calcium influx, which triggers activation of kinases, including PKC, PKA and CaMKII. Direct phosphorylation of synaptic AMPARs by these kinases enhances transmission efficacy. Upon activation, CaMKII translocates to synaptic sites and undergoes auto-phosphorylation to achieve a persistently active state in the absence of sustained elevated calcium. The persistent CaMKII kinase activity at synapses is considered critical for the transition of synaptic potentiation from induction to early LTP, maintenance phases. CaMKII has been hypothesized to play a role in regulating synaptic integration of AMPARs by anchoring and activating AMPAR interacting proteins (Soderling and Derkach, 2000; Boehm and Malinow, 2005). Trafficking of AMPARs to synapses is believed to be required for LTP maintenance (Malenka, 2003; Collingridge et al., 2004).

In order to elucidate the roles of PKMz in signaling cascades underlying LTP, AMPAR mediated miniature excitatory postsynaptic currents (mEPSCs) were examined from

hippocampal neurons infused with PKMz. These studies found that mean AMPAR mEPSC amplitude was doubled by PKMz infusion, but no change was found in either the frequency or the kinetics of the mEPSC responses, indicating that PKMz is involved in potentiating functional glutamatergic synapses but not the unsilencing of NMDAR-only synapses. Non-stationary noise analysis of AMPAR mEPSCs found that PKMz infusion into hippocampal neurons increases the number, but not the single channel conductance of AMPARs, indicating that PKMz may direct synaptic potentiation by driving synaptic integration of AMPARs (Ling et al., 2006). Further studies have found that PKMz activity regulates the interaction between N-ethylmaleimide -sensitive factor (NSF) and the GluR2 subunit of AMPARs, and promotes AMPAR trafficking through an exocytosis-independent pathway. In this model, PKMz promotes NSF to interact with C-kinase 1 (PICK1) for GluR2-containing receptors, to release receptors from reserve pools into extrasynaptic membrane. Receptors then move laterally into synaptic sites. Inhibiting PICK1-GluR2 interactions by intracellular infusion of an inhibitor peptide mimics synaptic potentiation and occludes the effect by PKMz infusion. Upregulation of GluR2/GluR3 expression was found in synaptosomes prepared from tetanized hippocampal slices, and the upregulation, as well as the synaptic potentiation, could be blocked by either myr-ZIP or a peptide inhibiting NSF-GluR2 interaction (Yao et al., 2008). As further evidence of this signaling mechanism, it was also found that myr-ZIP induced LTP reversal could be prevented by blocking the GluR2-dependent endocytosis of postsynaptic AMPARs with a synthetic peptide derived from the GluR2 carboxy tail ($GluR_{23Y}$)(Migues et al.).

Given the persistent kinase activity of PKMz, the synthesis of PKMz upon LTP stimulation

may act as a mechanism to transition of synaptic potentiation from early to late phases after CaMKII is deactivated by dephosphorylation. In this model, signaling cascades activated during early phase LTP, including CaMKII and other kinases, promote PKMz translation from its dendritic mRNA. PKMz activity mediates synaptic potentiation by promoting AMPAR delivery to synapses during the late phase, as well as sustained PKMz translation through a positive-feedback mechanism. This sustained PKMz synthesis may underlie the persistent potentiation lasting for hours to days.

Consistent with this model, PKMz's critical role in LTP was found to be specific for the late-phase, protein synthesis-dependent LTP. When PKMz activity is continuously blocked by bath application of myr-ZIP or protein synthesis inhibitors such as anisomycin and emetine, tetanus stimulation can still induce short-term potentiation that returns to baseline within two hours. Conversely, application of myr-ZIP at different time points after tetanization reversed the potentiation in a dose-dependent manner. Infusion of 5μM myr-ZIP one hour after tetanization completely reversed potentiation to baseline in 2 hours, while less than 50% reversal was achieved in 2 hours if 0.25 μM myr-ZIP was applied. No effect was found for 0.1 μM myr-ZIP (Serrano et al., 2005). In an *in vivo* study, persistent potentiation of fEPSP was induced by delivering high frequency stimulation through electrodes embedded in the perforant path of rats. Strikingly, intra-hippocampal infusion of a small amount of myr-ZIP at 22 hours after tetanization reversed the potentiation (Pastalkova et al., 2006).

Previous studies found that weak stimulation, which normally only induces early phase LTP, can induce long lasting late phase LTP if applied shortly before or after a strong stimulation

is applied to other inputs to the same neuron (Frey and Morris, 1997). A hypothetical synaptic tagging process was proposed to explain this phenomenon. In this model, both weak and strong stimulation initiate the creation of short lasting 'synaptic tags' at activated synapses. Strong stimulation also promotes expression of plasticity related proteins (PRP) in dendrites or soma, which are essential for late phase LTP. When weak and strong stimulations are applied within short intervals of each other, synaptic tags on weak synapses can direct sequestrate PRPs to these previously activated synapses to produce late phase LTP (Sajikumar et al., 2005; Serrano et al., 2005). PKMz was found to be involved in this process as a potential PRP. Application of myr-ZIP was found to block late phase LTP at synapse receiving strong titanic stimulation, as well as at weakly stimulated synapse. Interestingly, PKMz was not involved in “cross-tagging”, a phenomenon in which a strong LTP stimulation could transform an early phase LTD elsewhere into a late phase long-lasting LTD. Myr-ZIP could not block the late phase LTD produced by cross-tagging after strong LTP stimulation to another synapse(Sajikumar et al., 2005). These findings demonstrate that PKMz specifically mediates synaptic potentiation, but is not a general mechanism underlying long lasting synaptic changes.

1.2.4 PKMz in long-term memory

LTP has been hypothesized as a promising candidate mechanism underlying memory. Analogous to the induction and early and late maintenance phases of LTP, memory has formation and storage phases. Furthermore, many molecules implicated with LTP are also found to have important roles in memory (Sanes and Lichtman, 1999). As a candidate memory

molecule, roles of PKMz in learning and memory have been examined in various animal models. The implication of PKMz with memory was first tested in Drosophila. Using a Pavlovian olfactory learning task, it was found that transient induction of a PKMz transgene within 30 minutes after massed training enhanced memory tested 24h after training. Expression of the PKMz transgene also corrected memory defects induced by the *radish* mutant. Conversely, blocking PKMz activity either by chelerythrine or expressing a dominant negative form of PKMz impairs long-term memory after massed training. However, learning was not affected, indicating specific effects of PKMz on long-term memory (Drier et al., 2002).

To further test the model that PKMz mediated synaptic potentiation is an underlying mechanism of memory, the role of PKMz in long-term spatial memory was examined in rats. Infusion of myr-ZIP into rat hippocampus was found to reverse LTP induced *in vivo*. Spatial memory, which is dependent on hippocampus, was induced using an active avoidance paradigm. This paradigm consists of a slowing rotating platform that brings the animal into a zone with electric shock. Therefore, the animal has to remember the shock zone in order to avoid further shocks. Memory for the shock zone can be rapidly induced and can last for up to a month. One day after training, hippocampal infusion of myr-ZIP was found to rapidly and irreversibly erase the spatial memory. Rats failed to actively avoid the shock zone when tested 2 hours after myr-ZIP treatment. Animals were also tested 1 week after myr-ZIP infusion to rule out the possibility that myr-ZIP may impair memory retrieval. Treated rats failed to remember place avoidance. A further test found that 1-month-old memory can be eliminated by myr-ZIP. In addition, rats previously treated with myr-ZIP can rapidly form new long-term memories induced

by a second exposure to active avoidance training, indicating the memory elimination by myr-ZIP was not due to permanent damage to memory function. Collectively, these results indicate that maintenance of both *in vivo* LTP and long-term spatial memory require persistent PKMz activity (Pastalkova et al., 2006).

Although the precise mechanisms are unclear, the hippocampus plays an important role in new memory acquisition, while the neocortex is required for long-term storage. To examine whether PKMz is specifically critical for hippocampal memory or essential for general memory storage, the role of PKMz in neocortex was tested. A conditioned taste aversion (CTA) memory task was used to elicit neocortex-dependent single-trial long-term memory. Strikingly, memory was eliminated by injection of myr-ZIP into the insular neocortex up to 28 days following training. Furthermore, memories were insensitive to myr-ZIP during or immediately after training, indicating PKMz is not involved in short-term CTA memory, but is critical for formation of long term memories in neocortex (Shema et al., 2007).

Further studies also examined the roles of PKMz activity in other forms of memory. Using myr-ZIP, the effects of PKMz inhibition in dorsal hippocampus (DH) and basolateral amygdala (BLA) on retention of 1-day-old memory acquired in the radial arm maze, water maze, inhibitory avoidance, and contextual and cued fear conditioning paradigms were examined. Myr-ZIP infusion in the DH was found to selectively disrupt reference memory in the radial arm maze test without affecting working memory. In the water maze test, although myr-ZIP treated rats concentrated their swim time in the correct quadrant of the pool, they crossed the platform location fewer times than control rats, indicating that accuracy of spatial memory was impaired

by myr-ZIP. Collectively, these results suggest that PKMz is required for retention of accurate spatial, but not procedural and contextual information (Serrano et al., 2008). Furthermore, PKMz inhibition in the hippocampus did not affect contextual fear memory, but BLA infusion of myr-ZIP impaired classical conditioned memory for both contextual and auditory fear. Importantly, post-shock freezing was not affect by BLA infusion of myr-ZIP at either 5 minutes or 2 hours before the test, indicating PKMz activity in BLA mediates fear memory but not the fear expression (Serrano et al., 2008).

Recent studies have implicated synaptic trafficking of GluR2-containing AMPAR in PKMz mediated memory storage both in amygdale and hippocampus. It was found that fear memory impaired by PKMz inhibition was mediated through synaptic removal of GluR2-containing AMPARs. Blocking GluR2-dependent AMPAR endocytosis by the $GluR_{23Y}$ peptide abolished both behavioral impairment and associated synaptic removal of GluR2 caused by PKMz inhibition. Similarly, $GluR_{23Y}$ treatment also prevents the impairment of object location memory induced by PKMz inactivation in the dorsal hippocampus. Together, these findings indicate that PKMz maintains long-term memory by regulating the trafficking of GluR2-containing AMPA receptors (Migues et al.)

The involvement of PKMz in brain diseases like Alzheimer's disease (AD) has also been reported. It was found that PKMz, but not conventional and novel PKC isoforms accumulate in neurofibrillary tangles (NFTs) of limbic and medial temporal lobe structures, areas implicated in memory loss in AD. Interestingly, PKMz was not identified in any NFTs in control brains derived from elderly individuals without known cognitive impairment, suggesting a linkage between

PKMz-mediated synaptic plasticity and memory impairment in AD (Crary et al., 2006).

1.3 The *Xenopus laevis* tadpole as an animal model of neural development

Xenopus laevis, also known as African claw frog, has a long history being used as an important model organism in developmental biology research, mainly because of its large and easily accessible embryo. Using *Xenopus laevis* tadpoles as a model for neural development originates from a series of experiments conducted by Sperry, which provides critical evidence for the chemoaffinity model of axon guidance during visual system development (Sperry, 1963). Following work from other groups, which demonstrate more evidence supporting shared cellular and molecular mechanisms underlying neuronal development and circuit formation between Xenopus and other mammalian system, further establishes Xenopus tadpoles as an elegant model for studying early development of brain circuits (Cline, 1991; Debski and Cline, 2002; Cline, 2001; Zhou and Poo, 2004).

Those studies have mainly focused on the development of Xenopus retinotectal system. Similar as the mammalian system, axons from Xenopus retinal ganglion cells terminate in conserved positions and synapse with neurons inside the contralateral optic tectum, forming a retinotopic map as a result of combined influence of both activity dependent and independent mechanisms(Sperry, 1963; Cline 1991; Ruthazer et al., 2003). The development of this map initiates in tadpoles at stage as early as 30, when axons from retinal ganglion cell start to project into the tectum (Grant et al., 1980). Activity independent mechanisms, including diffusible and cell surface cues are thought to guide axons into conserved regions inside tectum and form a

coarse map, which is then refined by neural activity through a competition-based mechanism towards formation of exact spatial mapping between retinal surface and neuropil in tectum (Ruthazer et al., 2003). The refinement, which is functionally represented by decreased receptive field size and increased visual evoked response of individual tectal neuron, is heightened in retinotectal system of tadpoles of older stages (Stage 48-49) (Dong et al.,2009).

Optic tectum, which equals to superior colliculus in mammals, is the primary visual area in frog and fish (Dong et al.,2009). The continuous addition of new neurons from proliferate zone leads to an age gradient of cell body alignment, as well as a dynamically expanding neuron circuit at the tectum region, making tectum particularly useful for studying neuronal morphological growth and circuit formation. Furthermore, by using transparent tadpoles from natural albino mutants of Xenopus, direct visualization of developing events occurring at the intact tectum region can be easily achieved. Two recently developed techniques, single-cell electroporation and two-photon microscopy time-lapse imaging, have further made this system as a leading *in vivo* animal model for studying neuronal morphological development. Single-cell electroporation is a method used for targeted delivery of electrically charged macromolecules, including plasmids, dyes or peptide drugs into individual neuron inside the intact brain, thereby allowing study of cell autonomous effects on a treated neuron within unaltered environment (Haas et al., 2001). Due to deeper tissue penetration, more efficient light detection and reduced phototoxicity compared to conventional confocal microscopy, two-photon microscopy is especially useful for such *in vivo* preparations to capture accurate, 3D structure of dendritic arbor of a labeled neuron. Recently, our lab has developed novel protocols for imaging rapid

dynamic dendritic growth behavior over timescales of seconds to minutes of neurons within the unanesthetized tadpoles. The power of this model system comes from the ability to observe neurons growth within native environments exhibiting normal circuit activity. This model is well-suited to test the synaptotropic model of dendritogenesis since the pertinent early stages of dendrite growth can be directly imaged. Rapid imaging of growth *in vivo* finds highly dynamic dendritic filopodia on which new synapses are formed (Niell et al., 2004). By imaging dendritic filopodia at high temporal and spatial resolution using rapid time-lapse two-photon microscopy, we are able to study the dynamic interaction between synapse formation and dendritogenesis.

Neurons within the optic tectum receive direct innervation from retinal ganglion cells (RGCs) from the contralateral eye. Therefore, afferent glutamatergic synaptic responses can be readily induced on tectal neurons by delivering visual stimulation to the eye, or by electrically stimulating the optic nerve. This well-controlled afferent input pathway allows easy comparison of electrophysiological measures of synapse maturation to morphological development. In addition, the permeability of the tadpole to drugs applied to the bath allows easy access to pharmacological manipulations within an intact *in vivo* system.

Together, these natural features of the Xenopus tadpoles and technologies our lab and others have developed provide a powerful vertebrate model system for investigating molecular mechanisms underlying structural and functional plasticity of developing brain neurons and circuits.

1.4 Research aims and hypotheses

Previous studies have found critical roles of PKMz in long-term synaptic plasticity and

memory maintenance in the mature brain. It remains unknown whether PKMz is also involved in plasticity of developing synapses. In addition, synaptotropic mechanism associates dendritic arborization of immature neurons with synaptogenesis, and many molecular signaling pathways associated with mature synaptic transmission and plasticity have been found to regulate dendritic growth. It remains unknown if PKMz is involved in regulating synaptotropic dendritic growth. In my Ph.D. thesis research, I proposed to test the involvement of PKMz in the development of synapses and dendritic arbors within the intact developing brain of the albino *Xenopus laevis* tadpole. This powerful *in vivo* model system allows study of dendrite growth of developing neurons in their natural environment. Targeted, single-cell electroporation techniques have been specifically developed for this system to control expression of multiple genes in individual newly formed brain neurons for subsequent *in vivo* imaging of morphology. The transparency of the albino tadpole allows direct, *in vivo* time-lapse imaging of neuronal growth within the intact brain over intervals ranging from seconds to days.

First, I tested if **PKMz is endogenously expressed in tadpole brain, and whether the expression profile is consistent with stages of synaptic and dendritic development**. RT-PCR was used to clone *Xenopus laevis* PKMz. Using immunoblotting, I identified the developmental expression patterns of endogenous PKMz in tadpole brain. Using immunohistochemistry, I characterized the localization pattern of PKMz in the tadpoles retino-tectal system.

Second, I tested whether **PKMz activity regulates dendritic arborization of growing optic tectal neurons.** Using single cell electroporation to increase or inhibit PKMz activity in

individual tectal neurons, I studied the cell-autonomous effects of altered PKMz activity on dendritogenesis. I used short interval time-lapse two-photon imaging to capture the rapid growth dynamics of dendritic filopodia, and used long interval imaging to study the accumulated effect on dendritic arbor elaboration over multiple days.

Third, I tested whether **PKMz activity regulates synapse maturation in the developing brain.** PKMz's critical roles in adult synaptic plasticity suggest it may affect developing retino-tectal synapses. I used immunostaining of pre- and postsynaptic markers to examine roles of endogenous PKMz activity in synapse maintenance. I also used *in vivo* patch clamp electrophysiology recording to directly study the function of PKMz activity in maturation of tadpole retino-tectal synapses.

Fourth, I tested whether **excessive neural activity during seizures regulates PKMz expression, and whether PKMz is involved in seizure-associated abnormal dendritogenesis.** Studies in adult neural system find PKMz expression could be transiently elevated upon tetanus stimulation. Using a seizure model based on *Xenopus* tadpoles, I studied if abnormal high neural activity during seizure regulates PKMz expression. By manipulating PKMz expression in individual tectal neurons, I examined if the effect of seizure on dendritogenesis are mediated through PKMz activity.

Fifth, I **performed a theoretical analysis of synaptotropic model of dendritogenesis incorporating new information achieved through my studies of the effects of altered PKMz activity on synapse maturation and dendritogenesis.** Using computational simulation of dendritic growth, I examined if the results from the above studies of PKMz's effect on dendrite

growth are consistent with a synaptotropic mechanism. I also examined if such mechanisms could direct dendritic arbor to grow toward appropriate synaptic partners and stabilize in an optimal structure.

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2 PKMZ STABILIZES DENDRITIC ARBORIZATION OF OPTIC TECTAL NEURONS IN XENOPUS LAVIES TADPOLES [1]

2.1 Introduction

The creation of functional neural networks during early brain development requires appropriate axonal and dendritic structural growth and formation of precise synaptic connections. While neuronal transmission and correlated inter-neuronal activity are associated with synaptic plasticity underlying learning and memory in the mature brain, these factors likely influence neural circuit morphological growth during embryogenesis by directing synapse formation and maturation. The “synaptotropic model” of dendrite growth suggests that activity-dependent synaptogenesis directs growth by conferring morphological stabilization to otherwise labile processes, optimizing dendritic arborization and network connections to process the specific afferent activity innervating the maturing circuit. Dendritic arbor structural growth is critical to circuit function since dendritic surface area and length limits the number and type of synaptic contacts that can be formed, and arbor morphology influences the biophysical integration of synaptic potentials. Evidence for activity-dependent synaptotropic regulation of dendritogenesis comes from *in vivo* rapid time-lapse imaging of neuronal growth within the developing brain,

[1]A version of this chapter has been published: **Liu XF**, Tari P, Haas K (2009) PKM zeta restricts dendritic arbor growth by filopodial and branch stabilization within the intact and awake developing brain. *J.Neurosci 29(39):12229–12235* [Article content reproduced here with permission from Society for Neuroscience, Copyright, (2009)]

which reveals that glutamatergic transmission and synapse formation influences stability of highly dynamic filopodia and branches (Wu et al., 1999; Sin et al., 2002; Niell et al., 2004).

The molecular mechanisms underlying synaptotropic dendritic arborization within developing brain circuits are poorly understood, however, mounting evidence implicates shared pathways with those underlying synaptic plasticity in the mature brain. Glutamatergic transmission and activation of CaMKII, critical components of both initiation and the early-phase of long-term potentiation (LTP) at mature synapses, have been shown to regulate dendritic arborization in developing central neurons *in vivo* (Rajan and Cline, 1998; Wu and Cline, 1998; Rajan et al., 1999; Haas et al., 2006). Here, we investigate whether mechanisms underlying late phase LTP in the mature brain are also involved in developmental morphologic plasticity. PKM zeta (PKMz), a recently discovered constitutively active isoform of Protein Kinase C (PKC), has been implicated in late phase hippocampal LTP, as well as learning and memory in *Drosophila* and rodents (Jiang et al., 1994; Drier et al., 2002; Ling et al., 2002; Pastalkova et al., 2006). PKMz has high homology to the catalytic domain of PKC zeta (PKCz), yet lacks the PKC auto inhibitory domain, and thereby exhibits persistent kinase activity (Sacktor et al., 1993). Intracellular introduction of PKMz to hippocampal pyramidal neurons in mature brain slices potentiates synaptic transmission and occludes LTP induction. Inhibiting endogenous PKMz activity with the specific ZIP peptide inhibitor abolishes late phase LTP maintenance, but not LTP induction or short term potentiation (Hrabetova and Sacktor, 1996; Osten et al., 1996; Ling et al., 2002). Here, we test whether PKMz mediates morphological plasticity in early brain development by controlling expression and activity of PKMz in single neurons within the

retinotectal system of the intact albino *Xenopus laevis* tadpole brain. We use rapid and long-interval *in vivo* two-photon time-lapse imaging combined with comprehensive 3D quantification involving tracking of all dendritic filopodia and branches. Results demonstrate bidirectional regulation of dendritic stabilization by PKMz activity resulting in altered arbor expansion during periods of dynamic dendritogenesis.

2.2 Methods

2.2.1 Animals

Freely swimming stage 47 albino *Xenopus laevis* tadpoles (Nieuwkoop and Faber, 1994) were reared in 10% Steinberg's solution (1X Steinberg's: 10 mM HEPES, 60 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, pH 7.4), and housed at 22 ^{o}C on a 12hour light/dark cycle. Experiments were conducted at room temperature (22 ^{o}C). All experimental procedures were conducted according to the guidelines of the Canadian Council on Animal Care, and approved by the Animal Care Committee of the University of British Columbia's Faculty of Medicine.

2.2.2 DNA constructs

Xenopus PKMz was cloned from brain cDNA using RT-PCR and amplified using primers (upstream: 5'-GGCAAGGCTACAAGTGTATCAACTGC-3', downstream: 5'-GCTATAGAGAGATCAGCTTATACGGTCTCC-3') based on the sequences flanking the coding region of the Xenopus laevis PKMz mRNA (GI: 1220553). The PCR product was inserted into a

pCDNA expression vector. GFP fusions of Xenopus and mouse forms of PKMz were constructed and used for over-expression studies (mouse PKMz was a gift from Dr. Jerry Yin). Mouse and *Xenopus laevis* sequences share 87% homology and have conserved ATP and substrate binding motifs. The kinase activity of fusion proteins was verified by using a PKCz specific kinase assay. No significant differences were found between the effects of expression of the *Xenopus* or mouse PKMz on dendrite arborization, so data from these groups were combined.

2.2.3 Single-cell electroporation for transfection of individual neurons in vivo

Individual immature neurons within the optic tectum of the intact tadpole brain were fluorescently labeled using single-cell electroporation (SCE) (Haas et al., 2001; Hewapathirane et al., 2008). Tadpoles were briefly anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222). Under visual guidance using an upright stereomicroscope, a sharp glass pipette (tip diameter ~0.6μm) loaded with a solution of plasmid DNA (1-2μg/μl) was inserted into the optic tectum with the tip opposed to newly differentiated neurons in the tectal proliferative zone. An Axoporator 800A (Molecular Devices, Sunnyvale, CA) was used to deliver electric pulses. Stimulus parameters were: pulse intensity = 1.5 μA; pulse duration = 1 msec; pulse frequency = 300Hz; train duration 300 msec.

2.2.4 PKMz inhibitor peptide application

Tadpoles were anesthetized with 0.02% MS222, and cell-permeable myristoylated-PKMz inhibitor peptide (mZIP, QCB, Hopkinton, MA) dissolved in PBS at 0.5 nmol/μL and a total volume of 100-200 nL was slowly perfused into tadpole tectum using fine glass pipette attached to a Picospritzer (General Valve Corp, Fairfield, NJ). PBS was used as vehicle control. Alternatively, targeted inhibition of PKMz solely in individual neurons being imaged was achieved by SCE of ZIP peptide along with Alexa Fluor 488, 3000MW (Invitrogen, Eugene, OR) to visualize neuron morphology. An intra-pipette concentration of ZIP peptide of 5 nmol/μL and 3 mM for Alexa Fluor 488 was used. Delivery stimulus parameters for SCE of peptide and dye were: pulse intensity = 2 μA; pulse duration = 700 μsec; pulse frequency = 700Hz; train duration = 20 msec. In some experiments, TAMRA-dye labeled ZIP peptide (Pepmetric, Vancouver, BC) was used to determine duration of peptide retention within neurons over days. Loading of TAMRA labeled peptide was confirmed by fluorescence and confocal microscopy, and co-electroporation with Alexa Fluor 488 yielded 100% co-loading. No significant differences were found between the effects of labeled or non-labeled ZIP on dendrite arborization, so data from these groups were combined.

2.2.5 Immunoblotting

Tadpoles were anesthetized with 0.02% MS222, sacrificed, and brains were dissected and homogenized in lysis buffer (50 mM Tris-HCl, 1X Roche protease inhibitor cocktail, 1% Triton x-100, pH 7.4). Following centrifuge separation at 15000g for 10 min at 4^{o}C, the supernatant

was denatured, separated on a 4-12% Bis-Tris acrylamide gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. The blots were then probed with a PKMz antibody (gift from Dr. T. C. Sacktor).

2.2.6 Immunohistochemistry

For immunostaining of brain sections, tadpoles were fixed in 4% paraformaldehyde in PBS for two hours at room temperature followed by cryoprotection in a solution of 30% sucrose at 4 ^{o}C overnight, and sliced at 20 µm (horizontal cryosections). The sections were blocked in 5% normal goat serum for 1h and incubated in primary antibodies at 4^{o}C overnight. After washing with PBS buffer, sections were incubated in Alexa Fluor conjugated secondary antibodies (Invitrogen, Eugene, OR) for 1 hr at room temperature, mounted and imaged with an Olympus FV-1000 confocal microscope. The primary antibodies and dilutions used were: PKMz antibody (rabbit polyclonal, 1:100), SNAP-25 (rabbit polyclonal, 1:400, Stressgen, Ann Arbor, MI), and PSD-95 (mouse IgG, 1:200, Millipore, Billerica, MA). For secondary antibodies, we used Alexa 568 conjugated goat anti-rabbit antibody, and Alexa 633 conjugated goat anti-mouse antibody (1:200). For measurements of immunostained PSD-95 and SNAP-25 puncta density, all puncta in the range of 0.2-3.0 µm^2 were included. The cutoff size was selected based on the size distribution of “false-positive” puncta on images from brain sections incubated with only secondary antibodies. The density of total PSD-95 and SNAP-25 puncta, as well as co-localized puncta was determined using NIH ImageJ software. Colocalization probability was calculated by multiplying the percentage of colocalized puncta in each channel. Results from mZIP sections

were normalized to control sections on the same staining slide, and tested for significance with unpaired Student's t-tests. Colocalization was dramatically decreased by rotating either PSD-95 or SNAP-25 images by 90 degrees to each other (43.2±2.6% of original value), indicating the colocalization was not solely due to randomness.

2.2.7 *In vivo* time-lapse two-photon imaging of dendritogenesis

Images of neurons were captured using a custom-built two-photon microscope consisting of a modified Olympus Fluoview 300V confocal coupled to a Chameleon Ti:Sapphire laser (Coherent, Santa Clara, CA). An Olympus LUMPlanFl_IR 60X, water-immersion objective (1.1 NA) was used, with a z-axis step size of 1.5 µm. In order to capture rapid dendritic filopodial dynamics, un-anesthetized tadpoles were immobilized for imaging using the reversible paralytic panacurarium dibromide (PCD, 3 mM, Tocris), and embedded under a thin layer of agarose (0.8%) in a chamber continuously perfused with oxygenated 10% Steinberg's solution. 3D stacks of images through entire dendritic arbors were captured every 5 minutes over 1h. Long-term dendrite growth was imaged with 24h intervals. Single stacks of images through the entire dendritic arbor were captured by briefly anaesthetizing tadpoles with 0.02% MS222. Following imaging, tadpoles were returned to rearing solution where they rapidly recovered from anesthesia.

2.2.8 Morphometric analysis of dendritic growth

Three-dimensional skeletonized reconstructions of entire dendritic arbors were constructed

using Neurolucida software (MicroBrightfield, Williston, VT) and used to analyze total dendritic branch length (TDBL), branch tip number (BTN), and 3D Sholl analysis. All morphometric data analysis, except for Sholl analysis, was tested for significance with unpaired Student's t-tests. Results from Sholl analysis were tested with one-way ANOVA tests, followed by Turkey post-hoc analysis. Rapid dendritic filopodial and branch growth dynamics were analyzed using custom-written IGOR-based software (based on a program created by Dr. Jamie Boyd, UBC). All filopodia and branches were identified and tracked across multiple time-point image stacks.

2.3 Results

2.3.1 Endogenous expression of PKMz in Xenopus tadpole brain

A PKMz mRNA previously identified in *Xenopus laevis* (GI: 1220553) was used as the basis for PCR primers to clone PKMz and confirm expression in tadpole brain. Putative translation of this mRNA generates a protein containing 401 amino acids with 87% homology to rat PKMz (Hernandez et al., 2003) and conserved ATP and substrate binding motifs (Fig. 2.1A). Using an antibody raised against the catalytic domain of rat PKCz and immunoblots, we detected PKMz in tadpole brain lysates as a 50 KD protein, consistent with its putative translation product. Western blot analysis found developmental regulation of PKMz expression in tadpole brain, with levels increasing significantly from stage 35 to stage 50 (Fig. 2.1B). This period spans initial innervation and subsequent activity-dependent refinement of afferent axonal input from retinal ganglion cells (RGCs), and tectal neuron dendritogenesis (Grant et al., 1980). Immunohistochemistry staining of tadpole brain cyrosections found PKMz immunoreactivity

enriched in tectal neuropil where innervating RGC axons synapse with tectal neuron dendrites (Fig. 2.1C). Therefore, the endogenous expression of PKMz in *Xenopus* tadpole brain demonstrates a spatial-temporal pattern consistent with a role in synaptic and morphologic plasticity.

2.3.2 PKMz over-expression stabilizes dendritic filopodial growth dynamics

In developing neurons, dendritic filopodia are highly dynamic actin-rich protrusions typically less than 10 µm in length that emerge from the dendritic shaft and are precursors of longer branches (Portera-Cailliau et al., 2003; Niell et al., 2004). The majority of these processes retract within tens of minutes following initial extension, but a small percentage become stabilized and elongate to form new persistent dendritic branches. In order to comprehensively characterize filopodial growth dynamics, we employed rapid *in vivo* time-lapse imaging followed by tracking and measuring all processes at 5 min intervals for 1h. Measures allowed precise quantification of dendritic filopodia and branch motility, addition and elimination rates, and lifetimes. Results from rapid time-lapse imaging and analysis of growing tectal neurons transfected to express exogenous PKMz found a marked increase in filopodial stabilization compared to controls (Fig. 2.2A, B). PKMz over-expression induced a significant reduction in rates of filopodial addition and elimination (Fig. 2.2C), resulting in a decrease in overall turnover rate. Accordingly, the percentage of stable filopodia existing over the entire 1h imaging period, and average filopodial lifetime were significantly higher in PKMz neurons than controls (Fig.

2.2D, E). Furthermore, PKMz over-expression reduced both the absolute filopodial motility, represented by average length change per 5 minutes, and the filopodial dynamic range, the maximal change in length during the imaging period (Fig. 2.2F, G).

2.3.3 PKMz overexpression restricts dendritic arbor expansion

Effects of altered PKMz activity on long-term dendrite arborization were examined by imaging transfected developing tectal neurons at 24h intervals over several days. Dendrite morphologies of neurons over-expressing PKMz did not differ from controls at the initial imaging time point 12h following electroporation, but their growth patterns diverged in subsequent days (Fig. 2.2). Over four days of imaging, dendritic arbors of control neurons increased in size and complexity, while neurons expressing exogenous PKMz exhibited restricted arbor expansion. Increased PKMz activity produced a reduction in daily addition of total dendrite arbor length (Fig. 2.2H, I) and reduced arbor complexity (Fig. 2.2K), without significantly altering dendrite branch tip number (data not shown) or total length or number of filopodia (Fig.2.2J). Results demonstrate a persistent restriction of branch extension.

2.3.4 Inhibition of endogenous PKMz destabilizes dendritic filopodia

To examine the role of endogenous PKMz activity in regulating dynamic dendritic growth, we imaged rapid growth behavior during a 1h baseline, followed by intra-tectal infusion of the selective PKMz inhibitor mZIP. Following 4h incubation, a second 1h rapid imaging session was conducted. Within-cell comparisons found significant destabilization of dendritic filopodia

induced by PKMz inhibition (Fig. 2.3 A, B). Intra-tectal mZIP infusion destabilized dendritic filopodia, clear from a significant increased turnover rates of dendritic filopodia due to increased rates of both filopodial addition and elimination (Fig. 2.3C), reduction in the maintenance of pre-existing filopodia (Fig. 2.3D), and average filopodial lifetime (Fig. 2.3E). Furthermore, the absolute motility and dynamic range of filopodial growth were significantly increased (Fig. 2.3F, G).

2.3.5 Long-term inhibition of endogenous PKMz promotes dendritic arbor expansion

The role of endogenous PKMz activity in the long-term growth patterns of dendritic arbors was examined by delivering the ZIP PKMz inhibitor peptide along with Alexa Fuor dye into individual developing tectal neurons using SCE. Labeled neurons were imaged 6h after electroporation to capture initial baseline images prior to significant effects of ZIP on growth, and to match control and ZIP neurons based on initial arbor size. Daily dendritic arbor growth was imaged over 3 days while fluorescently tagged peptide (TAMRA dye labeled ZIP) could be reliably detected within neurons following SCE delivery during this period. Inhibition of PKMz by intracellular application of ZIP promoted increased dendrite outgrowth compared to dye labeled controls, represented by significantly larger total dendritic branch length (Fig. 2.3H). In contrast, dendritic branch tip number was not significantly affected by ZIP application (data not shown). 3D Sholl analysis found that PKMz inhibition increases arbor expansion as well as complexity (Fig. 2.3K). The primary effect of inhibition of endogenous PKMz by ZIP was an enhanced

extension of branches, with no effects on the total filopodial length or number (Fig. 2.3I, J).

2.3.6 PKMz inhibition impairs synapse maintenance *in vivo*

Given the role of PKMz in synapse plasticity in the adult brain and the importance of synaptogenesis in filopodial stabilization during dendritogenesis, we examined the effects of PKMz on synapse density in tadpole tectum. We employed immunostaining of tadpole brain cryosections for the pre- and postsynaptic markers SNAP-25 and PSD-95. PKMz inhibition by intra-tectal infusion of mZIP significantly reduced tectal synapse density after 4h, evident from decreased density of PSD-95 puncta juxtaposed to SNAP-25 puncta, and colocalization probability. No significant changes were found in the densities of either puncta (Fig. 2.4). These results demonstrate that inhibition of PKMz during active periods of developmental synaptogenesis impairs maintenance of excitatory synapses.

2.4 Discussion

The synaptotropic model of dendritogenesis postulates that synapses stabilize growing dendritic processes, thereby preventing retraction, yet allowing further growth and branching from synaptic sites (Vaughn, 1989; Niell et al., 2004; Cline and Haas, 2008). However, this model does not explain the progressive decline in dendritic growth and structural plasticity as neurons mature, characterized by reduced growth, motility, and additions of dendritic filopodia and branches (Wu et al., 1999). A possible explanation is that synaptotropic dendritogenesis is influenced by the maturational state of constituent glutamatergic synapses, thereby linking growth with the functional maturation of neurons (Wu et al., 1996). In this framework, nascent

immature synapses forming on new filopodia act to prevent retraction while permitting further growth. Mature synapses, in contrast, would restrict continued growth by conferring further structural stabilization. Here, we identify PKMz, a naturally expressed constitutively active kinase mediating late-phase synaptic LTP in the mature brain (Ling et al., 2002), as a potential regulator of synapse maturation state-dependent synaptotropic dendrite growth.

Increasing or decreasing levels of PKMz activity in individual actively growing brain neurons produces a similar shift in dendritic growth plasticity. Increasing PKMz activity in immature neurons dramatically reduces dendritic morphological plasticity, resulting in stunted arbors demonstrating precocious stabilization similar to mature neurons. PKMz's stabilizing effects on growing dendrites are likely mediated through synapse maturation rather than a direct effect on the cytoskeleton, since we found no effects on the motility of new filopodia within the first 30 minutes of extension, a period prior to detectable synapse formation (Niell et al., 2004; data not shown). Inhibiting endogenous PKMz activity with ZIP, shown to prevent late phase synaptic potentiation in the mature brain (Ling et al., 2002), and here to reduce synapse density in the *Xenopus* tectum, destabilizes growing dendrites with increased dendritic motility and turnover.

The identification of a role for PKMz in dendritogenesis adds to a growing list of molecular pathways shared between developmental neuronal morphogenesis and synaptic plasticity in the mature brain. Glutamatergic transmission, critical to initiation of mature synaptic plasticity, promotes dendrite growth (Sin et al., 2002), while inhibition of NMDA or AMPA receptor transmission destabilizes filopodia and inhibits growth (Rajan and Cline, 1998; Sin et al., 2002; Haas et al., 2006). At mature synapses, calcium entry through NMDA receptors activates

CaMKII a critical component of early-phase LTP. Exogenous expression of a constitutively active form of CaMKII in immature *Xenopus* tectal neurons both promotes maturation of retinotectal glutamatergic synapses (Wu et al., 1996), and restricts dendritic arbor plasticity similar to increasing PKMz activity (Wu and Cline, 1998). Inhibition of endogenous CaMKII activity destabilizes growing dendrites. Since PKMz expression is regulated by activity of CaMKII during induction of LTP in the mature hippocampus (Kelly et al., 2007), these kinases may act in concert during development to confer early and late-phase synaptic maturation and associated morphologic stabilization. These findings suggests that synapse maturation during brain circuit development which underlies morphological stabilization involves molecular mechanisms common to synaptic plasticity associated with learning and memory in the mature brain.

Manipulations predicted to shift synaptic plasticity in growing neurons *in vivo*, however, do not produce arbor morphologies predicted from a simple interpretation of the synaptotropic model, where enhancing synapse formation and maturation would be expected to promote growth, while the inhibition of synaptic strengthening would limit growth. Rather, enhanced PKMz or CaMKII activity, expected to lower a neuron's threshold for synapse maturation, results in stunted, non-motile arbors, while inhibiting these plasticity-associated kinases produces unrestricted arbor elongation. Thus, synapse maturation during dendritogenesis appears to function not only to stabilize nascent processes to prevent retraction, but also to restrict excessive arborization and may underlie the loss of dendrite morphological plasticity with neuronal maturation.

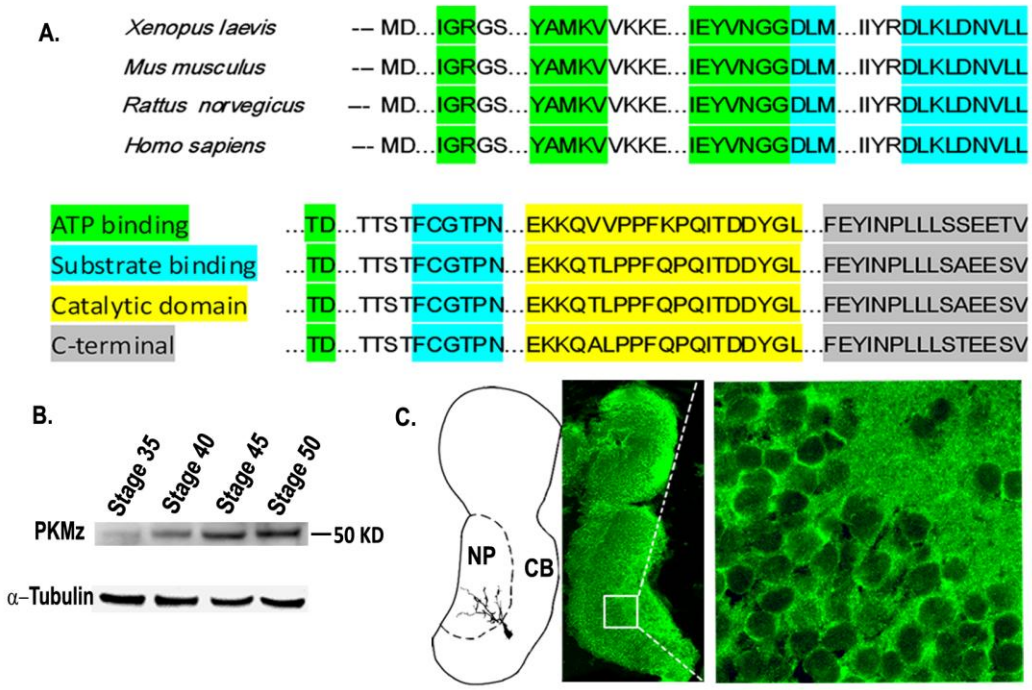

Figure 2.1 | Characterization of *Xenopus* PKMz. (A) Alignment of predicted PKMz amino acid sequences from *Xenopus laevis* and other species. Regions corresponding to postulated major motifs are highlighted. (**B**) Western blot using an antibody raised against the catalytic domain of rat PKMz identifies a 50 KD protein from *Xenopus* brain extracts, demonstrates a progressive developmental increase in expression. (**C**) Schematic drawing of tadpole brain (left) and horizontal section stained with PKMz antibody demonstrating enriched immunoreactivity in the neuropil region (NP) and the membrane portion of cell body (CB) region of the tectum.

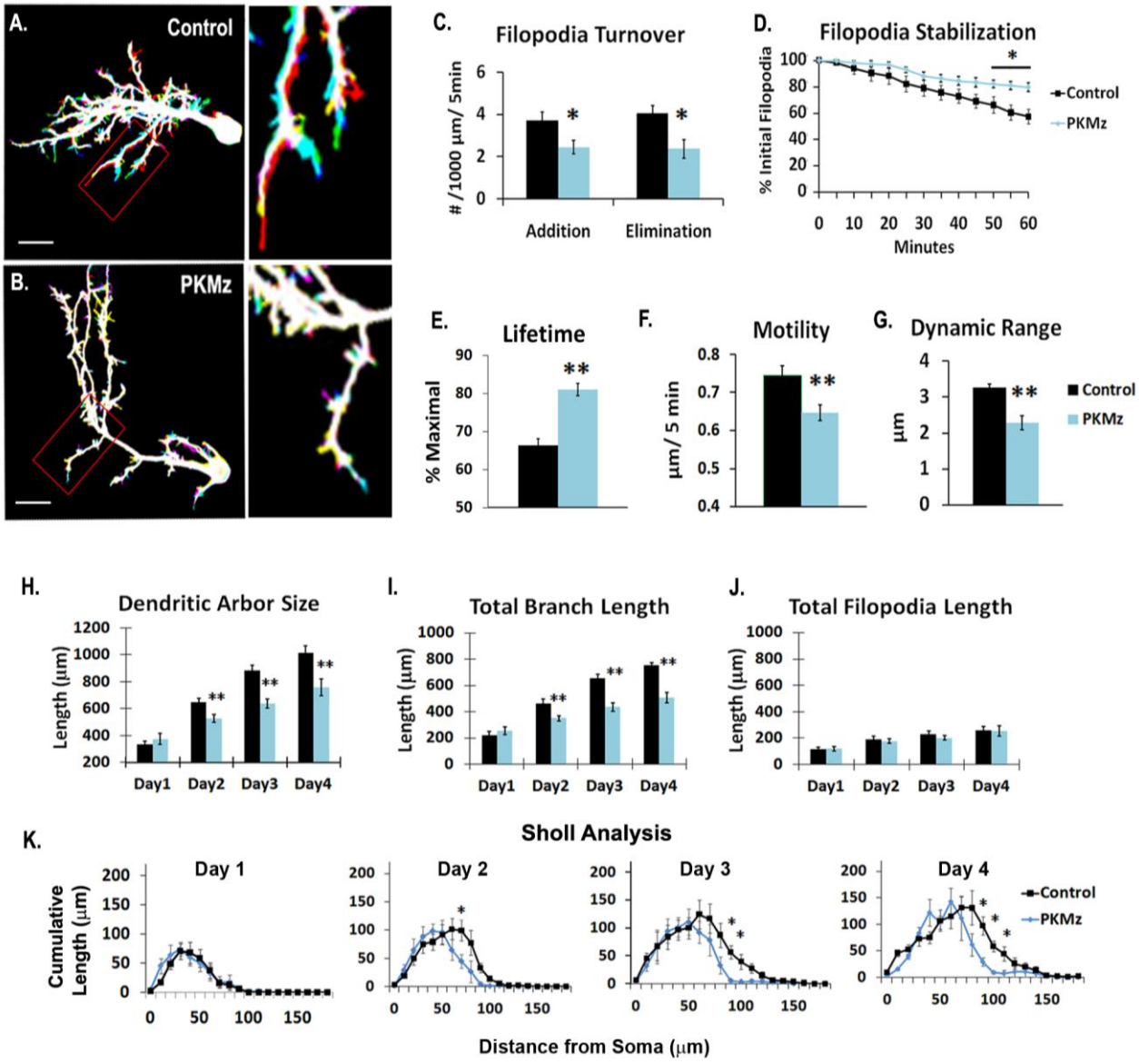

Figure 2.2 | PKMz overexpression stabilizes dendritic filopodia and restricts arbor extension. Superimposed images of six successive time-points (each a different color, with overlap = white) at 10 min imaging intervals of a neuron expressing exogenous PKMz (**A**), and a control neuron (**B**). Area within the boxed region is enlarged on the right. Bars represent 20 µm. (**C-G**) Morphometric analysis of dendritic filopodial dynamics of PKMz neurons (N=6 cells, n=269 filopodia) and Control neurons (N=7 cells, n=255 filopodia). PKMz over-expression significantly decreases the rates of filopodial addition and elimination (**C**); increases the fraction of filopodia that persist for 1h (**D**) and mean filopodial lifetime (**E**); decreases filopodial motility (**F**) and dynamic range (**G**). (**H-K**) 3D morphometric analysis of tectal neurons expressing GFP alone (control, n =12), and GFP with PKMz (n=10) 1–4 days following electroporation. PKMz over-expression restricts daily addition of dendritic arbor size (**H**), due to decreased extension of branches (**L**) but not filopodia (**J**). (**K**) 3D Sholl analysis of dendritic arbor complexity 1–4 days

after transfection. * p<0.05, ** p<0.01.

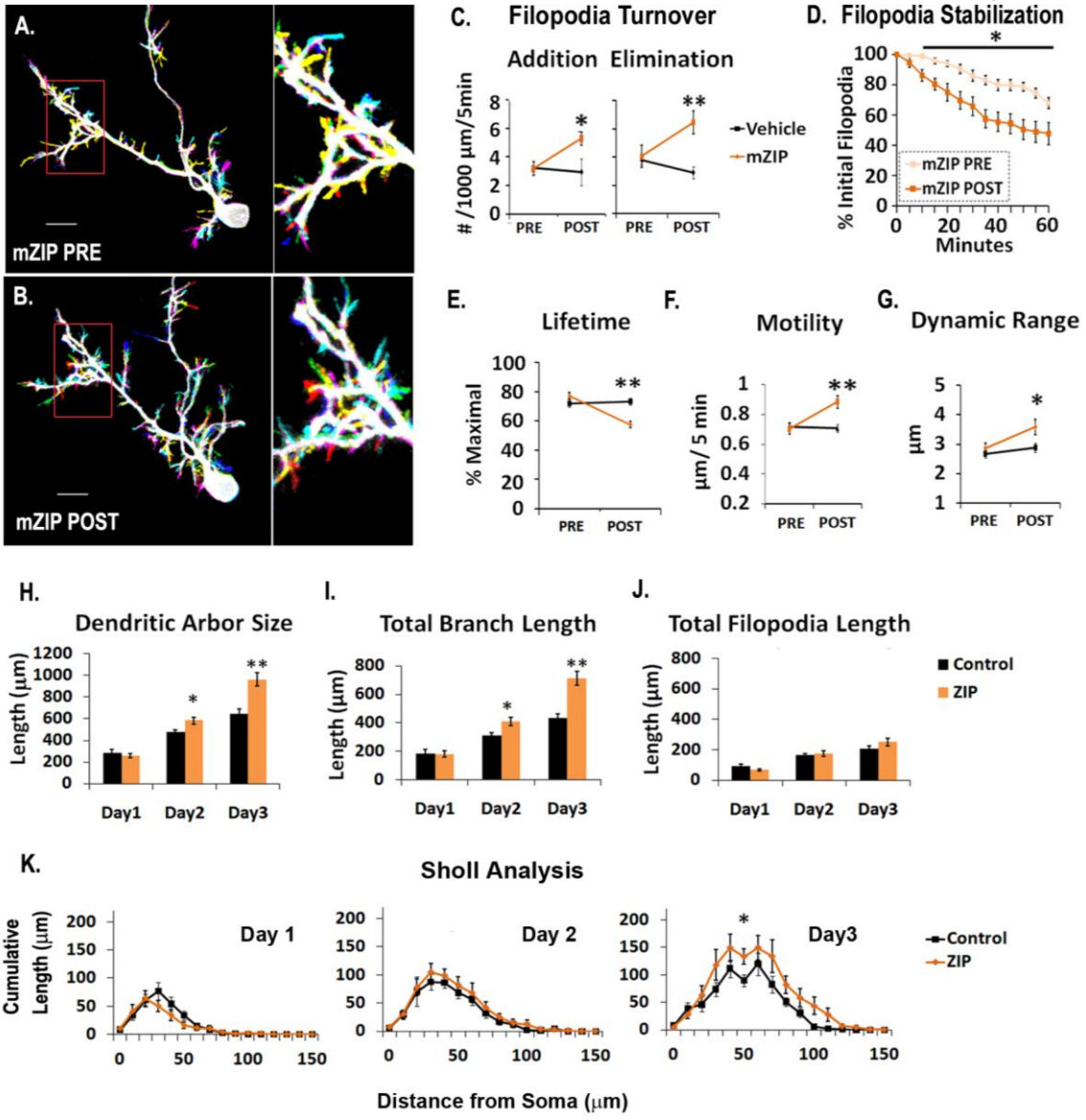

Figure 2.3 | ZIP destabilizes dendritic filopodia and promotes arbor extension.
Overlay of six successive images at 10 minutes of a GFP neuron before (**A**) and 4h after mZIP treatment (**B**). White portions of the arbor are stable, and colored portions are dynamic over 1 h imaging period. The area within the boxed region is enlarged on the right. Bar represents 20 µm. (**C-G**) Morphometric analysis of dendritic filopodial dynamics of neurons prior to and after mZIP delivery to tectum (N=5 cells, n=213 filopodia for PRE and 215 filopodia for POST) and vehicle (N=5 cells, n=221 filopodia for PRE and 210 filopodia for POST). mZIP treatment significantly increases the rates of filopodial addition and elimination (**C**), decreases the proportion of stabilized pre-existing filopodia (**D**) and mean filopodial lifetime (**E**); increases the filopodial motility (**F**) and dynamic range (**G**). (**H-K**) 3D morphometric analysis of tectal neurons labeled with Alexa Fluor 488 dye alone (control, n=12), and with ZIP (n=13) 1–3 days after

electroporation. ZIP increases total dendritic arbor size (**H**) and branch length (**I**), but not total filopodial length (**J**). (**K**) 3D Sholl analysis of dendritic arbor complexity 1–3 days after electroporation. * p<0.05, ** p<0.01.

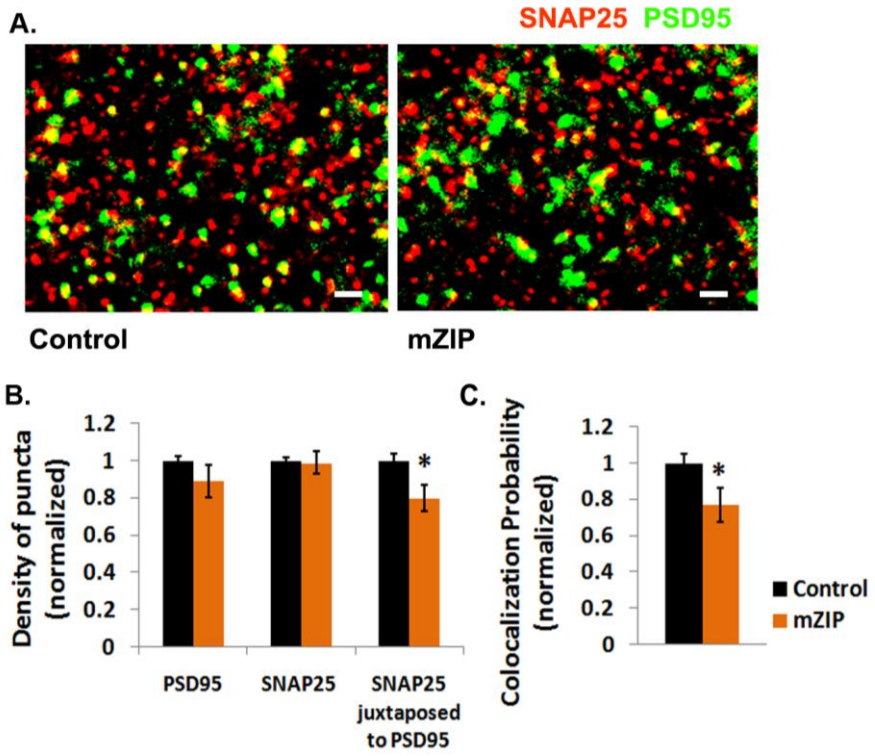

Figure 2.4 | mZIP impairs maintenance of retinotectal synapses *in vivo.* (**A**) Representative immunostaining of the tectal neuropil from a horizontal brain section with antibodies against SNAP-25 (red) and PSD-95 (green) 4h following tectal infusion of vehicle (control) and mZIP. Yellow puncta represent the overlap of SNAP-25 and PSD-95 puncta. Scale bars represent 1 µm. (**B,C**) Quantitative analysis of the density of SNAP-25 puncta, PSD-95 puncta and juxtaposed puncta **(B)**, as well as colocalization probability **(C)**, normalized by the average value of the corresponding controls (n = 5 tadpoles for each condition, * p<0.05).

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3 PKMZ REGULATES MATURATION OF GLUTAMATERGIC SYNAPSES OF OPTIC TECTAL NEURONS [2]

3.1 Introduction

Synaptogenesis is a multistage process initiated by formation of axon-dendritic contacts, or "pseudo synapses", then following synaptic integration of transmitter release mechanisms and receptors results in functional and mature synapses (Garner et al., 2002). Specifically, maturation of glutamatergic synapses is evident from gradual synaptic integration of AMPA receptors, rendering the synapse with higher fidelity of glutamate transmission (Wu et al., 1996). Mounting evidence supports the conservation of molecular mechanisms underlying this developmental plasticity with well-studied pathways mediating synaptic plasticity in mature brain (Constantine-Paton and Cline, 1998; Zhao et al., 2006). One example is CaMKII, an activity-dependent kinase associated with induction of long-term potentiation (LTP) of glutamatergic synapses(Malinow et al., 1989; Hayashi et al., 2000). During early brain development, CaMKII activity was found to promote synaptogenesis and synapse maturation (Wu et al., 1996).

[2]A version of this chapter will be submitted for publication: Liu XF, Haas K (2010) PKMz regulates maturation of glutamatergic synapses of optic tectal neurons.

Recently, another plasticity-associated kinase shown to underlie plasticity in mature systems has been connected to developmental synaptotropic dendritogenesis. A recently identified isoform of PKCz, PKMz has been intensively studied for its roles in adult synaptic and neural plasticity. PKMz has been found to be sufficient and necessary for maintenance of long-term synaptic potentiation (Ling et al., 2002). PKMz activity underlies the late phase of LTP through trafficking of AMPA receptors at synapses (Ling et al., 2006). In rodents and *Drosophila* PKMz is necessary for long-term memory, but not learning or short-term memory (Drier et al., 2002; Pastalkova et al., 2006; Shema et al., 2007).

Furthermore, during critical periods of early brain development, neurons face the challenging task of establishing appropriate elaborations of dendritic and axonal structures, and formation of correct synaptic connections in order to achieve mature brain circuit function. It is becoming clear that these processes of developmental neuronal morphogenesis and synaptogenesis are interrelated (Niell et al., 2004). The association between synaptogenesis and morphological growth has been postulated as the synaptotropic dendritogenesis which suggests that synapses confer morphological stabilization to growing processes (Vaughn, 1989).

In our previous studies, we showed that endogenous expression of PKMz in *Xenopus* tadpole brain follows developmental stages of synaptogenesis and neuronal morphological growth. We found that over-expression or inhibitory peptide-mediated reduction in PKMz kinase activity interferes with normal dendritic arbor growth patterns (Liu et al., 2009). However, additional evidence is necessary to determine whether PKMz mediates a synaptotropic growth

mechanism by regulating synapse maturation. Here, we employ synthetic morpholino oligonucleotides to selectively knock-down PKMz and patch clamp electrophysiology in order to test effects on developmental maturation of glutamatergic synapses, as well as concurrent effects on dendritic arborization.

3.2 Methods

3.2.1 Animals

Freely swimming Stage 47 albino *Xenopus laevis* tadpoles (Nieuwkoop and Faber, 1994) were reared in 10% Steinberg's solution (1X Steinberg's: 10 mM HEPES, 60 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO3)2, 0.83 mM MgSO4, pH 7.4), and housed at room temperature, 22 ^{o}C, on a 12hour light/dark cycle. Experiments were conducted at room temperature. All experimental procedures were conducted according to the guidelines of the Canadian Council on Animal Care, and approved by the Animal Care Committee of the University of British Columbia's Faculty of Medicine.

3.2.2 Reduced endogenous PKMz expression with morpholinos

To achieve specific down-regulation of endogenous PKMz, synthetic morpholino oligonucleotides (moPKMz; 5'-ATATGCTTTTTACAGGTCAGCGGGA-3') were designed against a sequence including the last 23 nucleotides from the 5' UTR region and the first 2 nucleotides from the coding region of Xenopus PKMz mRNA (Liu et al., 2009). A control morpholino (moCTRL; CCTCTTACCTCAGTTACAATTTATA) was constructed based on a random sequence

that does not correspond to any known Xenopus mRNA. Both morpholinos were tagged with lissamine red fluorescent dye (Gene Tools, Philomath, OR).

3.2.3 *In vivo* delivery of exogenous compounds to tectal neurons

For electrophysiological studies, bulk electroporation was used to deliver morpholinos to multiple neurons (Haas et al., 2001). Tadpoles were briefly anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222), and moPKMz or moCTRL was infused under low pressure into the tectum using a picospritzer. Following infusion, brief trains of electric pulses (200 ms trains of 1 ms square pulses, 200 Hz, 10-15 V) were delivered through platinum plate bipolar electrodes placed externally, yet touching the skin flanking the tectal region. Approximately 10-20% of tectal neurons receive delivery compounds by this method.

For studies of neuronal morphological development, single-cell electroporation (SCE) was used to deliver compounds to individual neurons within intact brain (Haas et al., 2001). SCE of a combination of Alexa Fluor 488 dextran (3 mM) and morpholinos (1 mM) was used to fluorescently label treated neurons. An Axoporator 800A (Molecular Devices, Sunnyvale, CA) was used to deliver electric pulses using the stimulus parameters: pulse intensity = 1.5 μA; pulse duration = 1 msec; pulse frequency = 300Hz; train duration 30 msec.

3.2.4 *In vivo* imaging and morphometric analysis of dendritic growth

In vivo 3D images of neurons were captured using a custom-built two-photon microscope consisting of a modified Olympus Fluoview 300V confocal scan box mounted on an Olympus

BX50WI microscope coupled to a Coherent Chameleon Ti:Sapphire laser. An Olympus LUMPlanFl_IR 60X, water-immersion objective (1.1 NA) was used, with a z-axis step size of 1.5 µm. Dendrite growth was imaged at 24h intervals over 4 days. Stacks of images through the entire dendritic arbor were captured by briefly anaesthetizing tadpoles with 0.02% MS222. Following imaging, tadpoles were returned to rearing solution where they rapidly recovered from anesthesia. Three-dimensional skeletonized reconstructions of entire dendritic arbors were constructed using Neurolucida software (MicroBrightfield, Williston, VT) and used to analyze dendritic branch length, branch number, and 3D Sholl analysis. All morphometric data analysis was tested for significance with unpaired Student's t-tests.

3.2.5 Electrophysiology

For *in vivo* whole-cell patch clamp recording of synaptic responses, tadpoles were paralyzed in 3mM panacurarium dibromide (Tocris Biosciences, MO), embed under a thin layer of 0.8% agarose in a recording chamber continuously perfused with HEPES-buffered extracellular solution (115mM NaCl,4mM KCl, 3mM $CaCl_2$, 3mM $MgCl_2$, 5mM HEPES, 10mM glucose,10mM glycine, pH7.2 with NaOH, osmolality 255 mOsm). The skin above tadpole brain was retracted to expose tectal cells for whole-cell patch clamp electrophysiology using an internal solution of: 80mM cesium methane sulfonate,5mM $MgCl_2$, 20mM tetraethylammo- nium chloride, 10mM EGTA, 20mM HEPES, 2mM ATP, 0.3mM GTP, pH7.2 with CsOH, osmolality 255mOsm. Recordings of spontaneous AMPA mEPSC were made at a membrane holding potential (Vh) of -70mV, with 1 µM tetrodotoxin (AlomoneLabs, Jerusalem, Israel) and 100 µM

picotoxin (Tocris Biosciences, MO) added to the extracellular solution. mEPSC signals were analyzed using miniAnalysis software (Synaptosft, GA) and tested for significance using Student's t-test. In order to record evoked synaptic currents, tadpole brains were sectioned along the dorsal midline and a bipolar stimulating electrode (MicroProbes for Life Science, MD) was placed at the optic chiasm to stimulate the optic nerve using stimulus parameters as described previously (Wu et al., 1996). 100 μM picotoxin (Tocris Biosciences, MO) were added into the extracellular solution to block GABA receptor mediated responses. Evoked AMPAR synaptic currents were recorded at Vh = -70 mV and amplitudes measured as the peak current during a 2-to-5ms window and subtracting the amplitude of the baseline current immediately before stimulation. NMDAR-mediated events were captured using Vh = +55 mV, and amplitude measured as the mean response amplitude during a 10ms window 20 ms after response onset, and subtracting baseline value obtained immediately before stimulation. At 20 ms after response onset, the AMPAR component has decreased to less than 5% of the total current. For measuring the paired-pulse ratio, pairs of stimuli (25Hz, 25–50trials) were delivered, and the peak AMPA amplitude ratio (EPSC2/EPSC1) was calculated. Amplitude analysis was performed using Clampfit software and tested for significance using Student's t-test. Neurons had input resistances in a range of 1–4GΩ and series resistances less than 100MΩ. Series resistance was measured throughout experiments and only cells were analyzed that remained within these bounds. All recordings were made with an Axopatch200B amplifier and digitized using a Digidata1322 analog-to-digital board. Stimulation and data acquisition were performed with pClamp9.2 software and digitized at 10 kHz. All equipment and software are from Axon

Instruments/Molecular Devices.

3.3 Results

3.3.1 PKMz down-regulation reduces miniature EPSCs amplitude of developing glutamatergic synapses

In order to employ morpholino knock-down of endogenous PKMz in Xenopus tadpole brain, we first tested the effects of moPKMz and moCTRL on HEK293 cells transfected with a Xenopus PKMz plasmid, which was constructed by inserting a sequence correspondent to the last 83 nucleotides from the 5' UTR regions as well as the entire coding region from Xenopus PKMz mRNA into a pCDNA expression vector. moPKMz, but not moCTRL, dramatically decreased the expression of PKMz in HEK cell (Fig. 3.1A).To investigate the roles of endogenous PKMz in the development of glutamatergic synapses, we examined AMAPR-mediated mEPSCs from moPKMz- and moCTRL-treated neurons using *in vivo* whole-cell patch clamp recordings (Fig. 3.1B). mEPSC amplitude recorded from moPKMz neurons was significantly reduced (moPKMz: n=18 neurons) compared to controls (moCTRL: n=16 neurons) (Fig. 3.1C-D). However, we found no significant difference in mEPSC frequency or kinetics, including rise and decay time constants between these two groups (data not shown). To test if the effect was contributed by presynaptic mechanisms, we delivered stimulation through the tadpole optic chiasm, and measured paired-pulse ratio on tectal neurons (Fig. 3.1E). Results show that though amplitudes of both evoked responses from moCTRL neurons are larger than moPKMz group, no significant difference is found in the paired-pulse ratio between

two groups (moPKMz: n=16 neurons; moCTRL: n=16 neurons) (Fig. 3.1F), suggesting PKMz acts at postsynaptic sites.

3.3.2 PKMz is critical for maturation of glutamatergic synapses.

To test whether PKMz is critical for synapse maturation, we conducted *in vivo* whole-cell patch clamp recording of tectal neurons while stimulating the optic chiasm to evoke retinotectal glutamatergic synaptic transmission. Tectal cell membrane potential (Vh) was held at –70mV to record evoked AMPAR-mediated synaptic currents and at +55mV to record combined AMPAR- and NMDAR-mediated responses (moPKMz: n=14 neurons; moCTRL: n=18 neurons) (Fig. 3.2A). Only monosynaptic responses with onset within 5 ms after stimulation were kept for analysis. Since the transition from 'silent' NMDAR-only synapses to 'functional' synapses containing both NMDARs and AMPARs is a hallmark of glutamatergic synapse maturation, the ratio of the amplitudes of AMPAR- and NMDAR-mediated responses has been widely accepted as a measure of synapse maturity. We find that down regulation of endogenous PKMz by moPKMz significantly reduces AMPA/NMDA ratios compared to moCTRL neurons (Fig. 3.2B). Further analysis shows that this decrease is due to reduction in amplitude of AMPAR-mediated responses, while NMDAR-mediated responses are unaltered (Fig. 3.2C).

3.3.3 PKMz down regulation leads to excessive dendritic expansion

Glutamatergic synaptic transmission has been found to regulate dendritic arborization of growing tectal neurons (Rajan and Cline, 1998; Sin et al., 2002; Haas et al., 2006).

Furthermore, neuronal morphological maturation, evidence from gradual stabilization of dendritic morphology, is concurrent with synaptic maturation (Wu et al., 1996). Therefore, we tested whether morpholino-mediated down-regulation of endogenous PKMz, associated with decreased glutamatergic synapse maturation interferes with dendritic arbor development. Immature tetcal neurons treated with either moPKMz or moCTRL were imaged daily over three days, spanning the dynamic phase of dendrite growth. We find that moPKMz promotes dynamic dendritic outgrowth, leading to larger dendritic arbors with more dendritic branches compared to controls (moPKMz: n=11 neurons, moCTRL: n= 10 neurons) (Fig. 3.3A-C). Further morphometrical analysis demonstrates increased growth of both filopodia (processes less than 10 μm) and branches (Fig. 3.3D-G), and increased complexity from Sholl analysis (Fig. 3.3H).

3.4 Discussion

Using a combination of *in vivo* electrophysiological recording and *in vivo* time-lapse single-neuron imaging in the intact developing brain, we have investigated the roles of endogenous PKMz in regulating synaptic and structural development. While a role has been described for PKMz in synapse plasticity and learning and memory in the mature brain, it remains unknown whether PKMz plays a similar role during early brain development. During early brain development, newly differentiated neurons undergo a rapid period of morphological growth in which complex axonal and dendritic arbors are established (Wu et al., 1999). Once neurons mature, their dendritic arbor morphologies become stabilized. This period of structural stabilization is concomitant with periods of glutamatergic synapse maturation (Wu et al., 1996).

Here, our results demonstrate that specific down-regulation of PKMz in developing tectal

neurons impairs maturation of glutamatergic retinotectal synapses, and produces unrestricted dendrite growth and dendritic arbor expansion. We find that PKMz knock-down by moPKMz reduces the amplitude of AMPAR-mediated mEPSCs, suggesting that endogenous PKMz plays a critical role in trafficking and insertion of AMPARs into developing synapses. However, it may not be involved in the initial conversion of silent to functional synapses, as we didn't find an effect of moPKMz on AMPAR mEPSC frequency. Our results are consistent with previous findings obtained from hippocampal neurons, where PKMz is implicated in mediating late-phase long-term potentiation (Ling et al., 2006; Yao et al., 2008). At retinotectal synapses, PKMz may play a similar role, and therefore not influence the number of nascent synapses, but the strength of each synapse.

The AMPA/NMDA ratio is commonly used as a measure of glutamatergic synapse maturation (Wu et al., 1996; Chiu et al., 2008). Our findings of a dramatic reduction in the AMPA/NMDA ratio at retinotectal synapses by moPKMz treatment provides further evidence that endogenous PKMz is critical for postsynaptic maturation. Moreover, PKMz down-regulation specifically reduces AMPAR-mediated transmission without altering the NMDAR component. Although some studies have reported a role for kinases in the PKC family in NMDAR trafficking (Lan et al., 2001; Haas et al., 2006), our results suggest that PKMz is specific to AMPARs. Altogether, our results on PKMz's role in synapse development provide additional evidence supporting shared molecular mechanisms underlying synaptic plasticity in developmental and mature brain tissues.

When investigating synapse maturation during early brain development, it is important to understand the dynamic interconnection between synaptogenesis and structural growth. Since synapse formation confers stabilization to new and dynamic dendritic processes, structural growth is in part directed by synapse formation. Evidence for a strong role of synaptic transmission in dendritic arbor growth further supports a role for activity-dependent synapse maturation in this process. Here, by interfering with endogenous PKMz expression, we examine the effects of reduced synapse maturation on structural growth. Immature tectal neurons treated with moPKMz were able to form glutamatergic synapses, yet synapse maturation was deterred. Interestingly, this results in enhanced dendrite outgrowth and a general promotion in arbor expansion. Combined with previous results, we propose that formation of immature synapses is sufficient to provide structural stabilization required to prevent new dendritic process retraction and support further dendritic outgrowth. Further synapse maturation may confer further stabilization that restricts growth plasticity, and may underlie the maturation shift from dynamic to stable dendritic arbors. PKMz appears to be central to this synapse maturation in the developing brain. When PKMz activity is blocked by moPKMz, synapses remain immature and abnormal growth continues unabated.

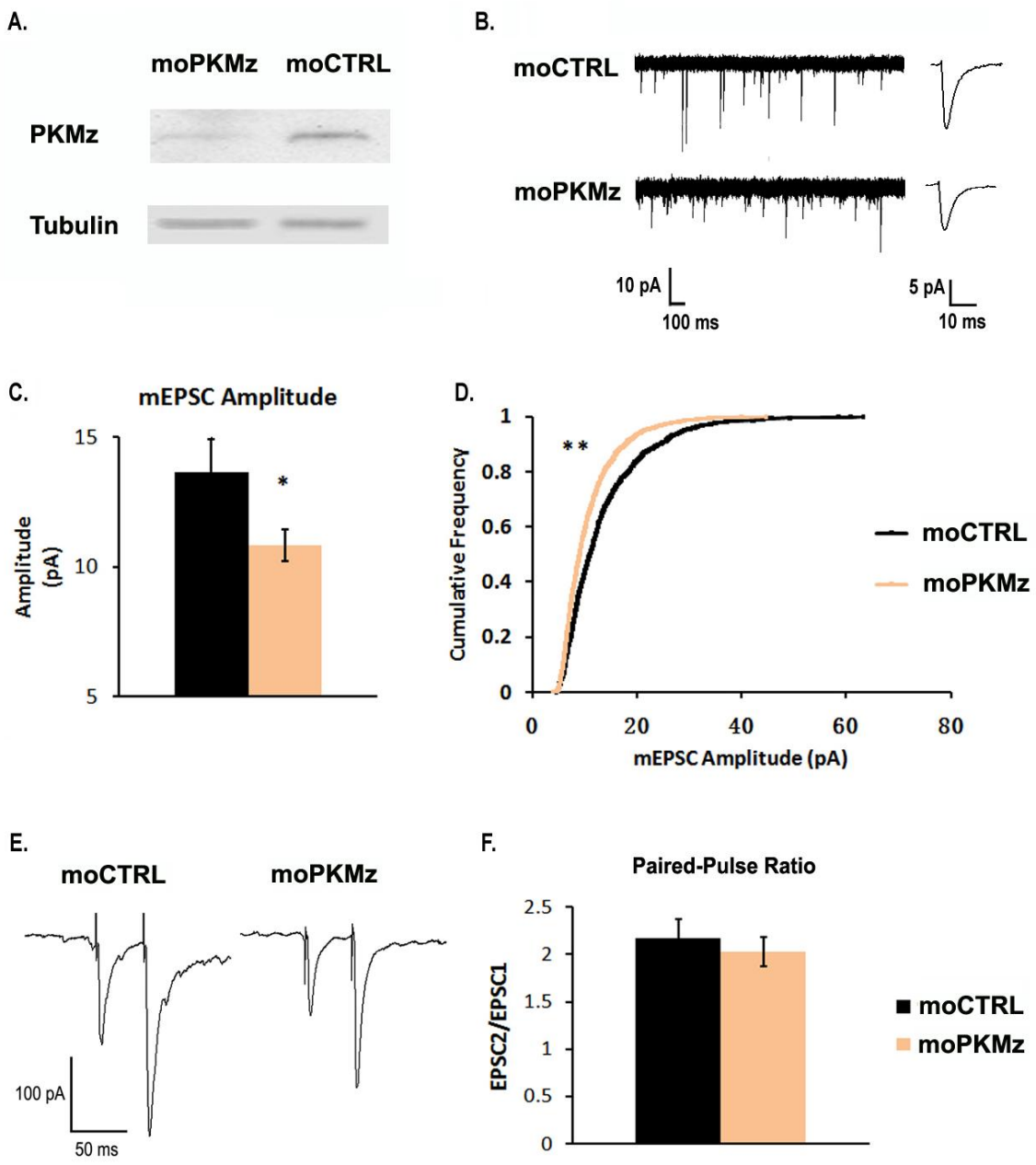

Figure 3.1| PKMz down-regulation reduces miniature EPSCs amplitude of developing glutamatergic synapses (A) Verification of specificity and potency of moPKMz. (B) Representative traces of miniature EPSCs recorded from tectal neurons transfected with moPKMz and moCTRL, respectively. Averaged single events are shown at right. (C.D) moPKMz transfection significantly reduced the mEPSC amplitude. (E) Representative traces of EPSCs evoked by paired stimulation. Though moPKMz transfection reduced amplitudes of the two responses, there is no difference on the ratio between two groups. Data are represented as Mean± SEM. * $p<0.05$, ** $p<0.01$.

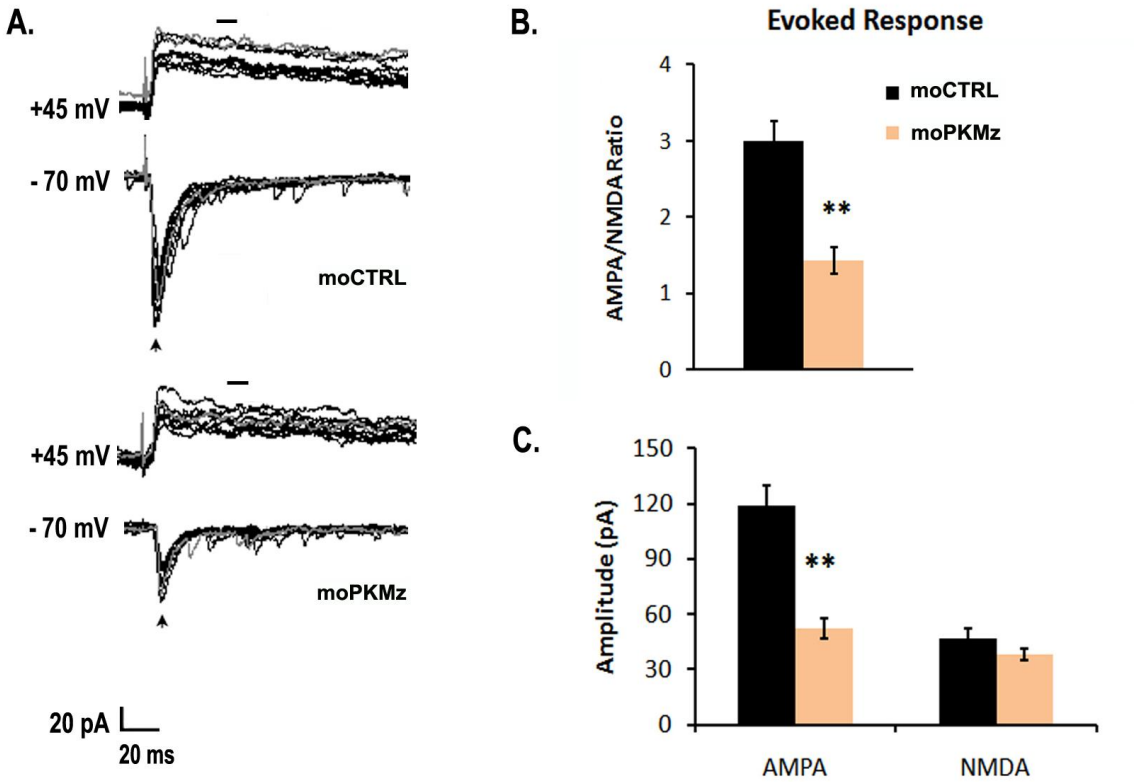

Figure 3.2 | **PKMz is critical for maturation of glutamatergic synapses** (A) Overlays of 10 evoked EPSCs by retinal axon stimulation, recorded at -60 and +45. Arrows and bars showed when AMPA and NMDA responses were measured, respectively. (B) Amplitude ratio of AMPA/NMDA responses is significantly lower in moPKMz transfected tectal neurons. (C) Analysis of separate components finds that the reduction is mainly attributed to smaller AMPA EPSC amplitude of moPKMz transfected neurons. No significant difference is found on NMDA EPSC amplitude between two groups. Data are represented as Mean± SEM. * $p<0.05$, ** $p<0.01$.

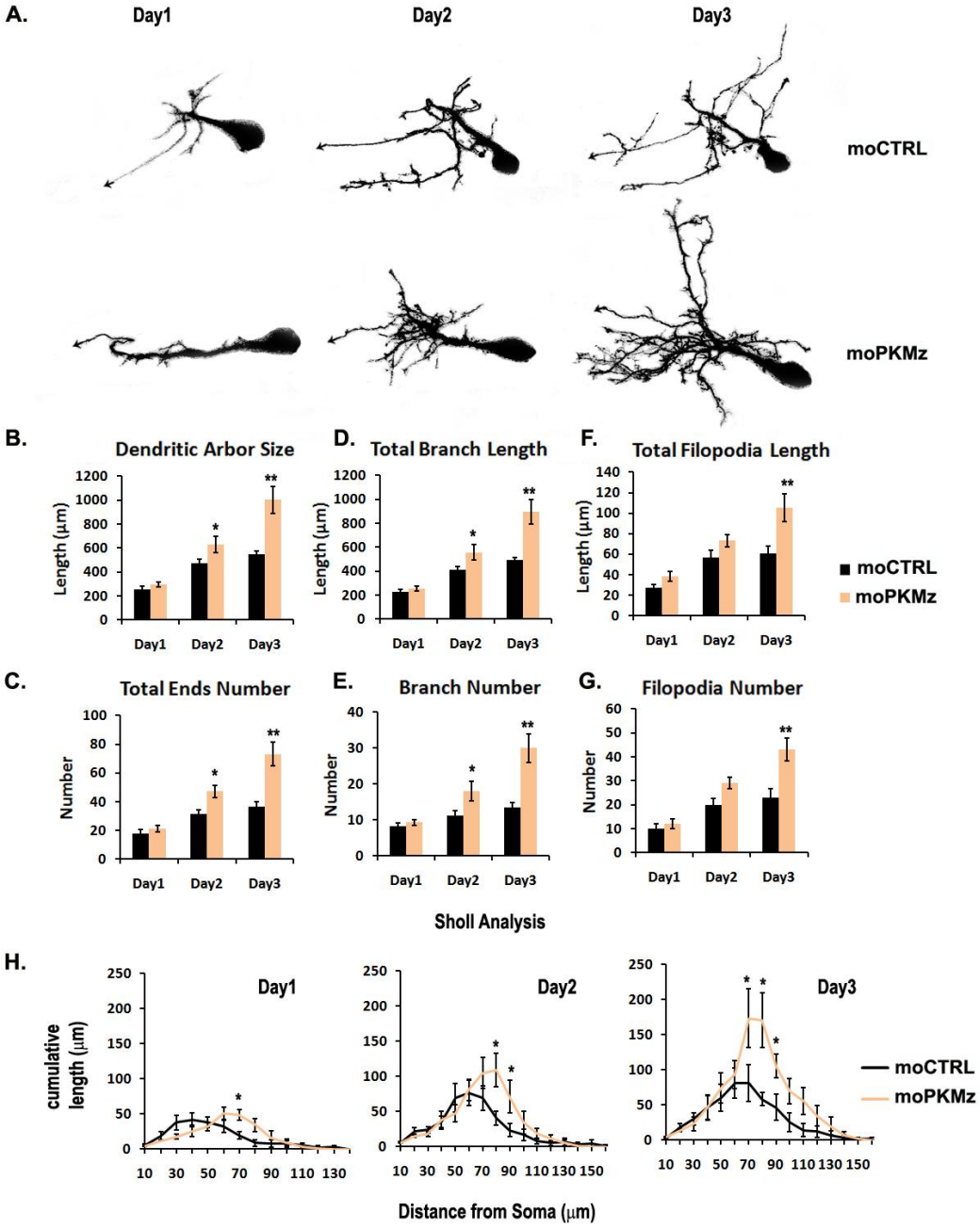

Figure 3.3 | PKMz down regulation leads to excessive dendritic expansion (A) Images of representative tectal neurons labeled with Alexa Fluor 488 dye with moCTRL, and with moPKMz 1–3 days after electroporation. (B-G) 3D morphometrical analysis of dendritic growth over three days. MoPKMz transfection increases both size and number of total dendritic arbors (B,C), major branches (D,E), total filopodia (F,G). (H) 3D Sholl analysis of dendritic arbor complexity 1–3 days after electroporation. * $p<0.05$, ** $p<0.01$.

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4. PKMZ MEDIATES SEIZURE AFFECTED DENDRITOGENESIS[3]

4.1 Introduction

The immature brain's inherent enhanced susceptibility for seizure activity (Haas et al., 1990; Sperber et al., 1991; Haas et al., 1992), together with epileptogenic factors common in early life (Jensen and Baram, 2000) results in an exceptionally high prevalence of seizures during infancy and early childhood (Moshe, 1993; Ben-Ari and Holmes, 2006; Thibeault-Eybalin et al., 2009). This period of high seizure occurrence is concomitant with critical periods of heightened brain plasticity when patterned neuronal activity directs both the structural and functional maturation of nascent neural circuits (Katz and Shatz, 1996). Whether early-life seizures disrupt ongoing programs of activity-dependent circuit formation is of significant clinical relevance since the establishment of abnormal neural circuits may produce persistent deficits in cognitive function and potentially predispose individuals to neurological disorders later in life (Stafstrom et al., 2000; Swann, 2004; Holmes, 2005). Indeed, clinical studies have established correlations between early-life seizures and persistent deficits in brain function (Burnham, 2002; Austin and Caplan, 2007)**,** as well as an increased susceptibility to subsequent seizures (Lee, 1989; Vestergaard et al., 2007) and other neurological disorders (Gaitatzis et al., 2004; Vestergaard et

[3] A version of this chapter will be submitted for publication: Hewapathirane DS*, Liu XF *, Chen S, Yen W, Karimi Tari P, Neufeld S, Haas K (2010) Developmental seizures inhibit dynamic dendritogenesis *in vivo* by excessive AMPA receptor-mediated transmission. * co-first author

al., 2005; Ekinci et al., 2009). Controlled, experimentally induced seizures in animal models have similarly found lasting deficits in learning, memory and cognitive tasks, as well as heightened susceptibility to further seizures, suggesting a direct causal effect of seizures rather than separate neuropathological origins (Holmes et al., 1988; Huang et al., 1999; Jensen and Baram, 2000; Lynch et al., 2000; Rutten et al., 2002). The developing brain exhibits remarkable resistance to seizure-induced neuronal damage and death seen in adults, however, it remains poorly understood how early-life seizures alter developing brain circuits to produce lasting functional deficits.

The structural development of neuronal dendritic arbors is an important component of neural network formation and refinement since dendritic arbor size and shape directly influences circuit connectivity, complexity, and function (Häusser et al., 2000; Wong and Ghosh, 2002; Cline and Haas, 2008). Direct *in vivo* time-lapse imaging of growing brain neurons has found that dendritogenesis is a highly dynamic process involving continuous extension and retraction of both branches and shorter filopodia, which are precursors to branches at this stage of maturation. The high motility and turnover of dendritic structures is proposed to enable growing dendrites to explore the surrounding extracellular space for appropriate presynaptic partners, a theory supported by findings that glutamatergic transmission (Haas et al., 2006) and synapse formation (Niell et al., 2004) promote stabilization of dendritic filopodia. Neuronal activity-dependent, synaptotropically-driven dendritogenesis therefore may direct arbor growth into regions of appropriate afferent innervation, thereby optimizing circuit morphology and connectivity to process specific afferent activity (Cline and Haas, 2008). Correlation between

aberrent seizures activity during development and abnormal dendritic morphology has been found in *in vitro* and post-mortem histology studies (Jiang et al., 1998; Swann et al., 2000; Nishimura et al., 2008), yet it remains unclear whether and how early-life seizures directly interfere with ongoing dynamic patterns of dendrite growth.

Here, we utilize a novel model of early-life seizures based on the transparent vertebrate albino *Xenopus laevis* tadpole (Hewapathirane et al., 2008; Hewapathirane and Haas, 2009) and *in vivo* two-photon time-lapse imaging in combination with comprehensive 3D analysis of dynamic and protracted dendrite growth patterns to examine whether seizures alter dendritogenesis during critical periods of early brain development. Using rapid imaging with 5 min intervals over 5 hours, encompassing periods before and during seizures, and tracking and analyzing all dendritic branches and filopodia over time, we precisely characterize the subtle effects of seizures on dendritogenesis within the intact and awake developing brain. We identify two distinct yet opposing effects of seizures: the rapid destabilization of dendritic processes existing prior to seizures and hyper-stabilization of those emerging during seizures. Moreover, such effects can be blocked by reducing AMPA receptor-mediated transmission or by knocking-down the plasticity-associated protein kinase, PKMz that has been shown to be involved in synapse maturation. Seizures also significantly reduce synapse number and function, further establishing that early-life seizures lead to a lasting reduction in neural circuit complexity. Longer-interval imaging over hours to days allows identification of persistent effects on neural circuit structures, and we find that seizures cause lasting stunting of dendritic arbors over many days. Together, these results support a model in which seizures influence growth through

activating endogenous mechanisms of activity-dependent growth and identify morphological substrates potentially underlying persistent neural circuit dysfunction associated with early-life seizures.

4.2 Methods

4.2.1 Animals and seizure induction

Stage 47 albino *Xenopus laevis* tadpoles(Nieuwkoop and Faber, 1994) were maintained in 10% Steinberg's solution (1X Steinberg's: 10 mM HEPES, 60 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, pH 7.4), and housed at room temperature (22^{o}C) on a 12 h light/dark cycle. Seizures were induced by bath application of either pentylenetetrazol (15 mM, PTZ, Sigma-Aldrich), a non-competitive GABA receptor antagonist; pilocarpine (75 mM, Tocris, Ellisville, MO), a non-selective muscarinic receptor agonist; or 4-aminopyridine (1 mM, Tocris), a potassium channel blocker. At these doses, each convulsant reliably induces behavioral and electrographic seizures in freely swimming, and in agarose-immobilized tadpoles (Hewapathirane et al., 2008). Drugs were dissolved in normal rearing medium adjusted to pH 7.4. All experimental procedures were approved by the Animal Care Committee of the University of British Columbia

4.2.2 *In vivo* single-cell electroporation for fluorescent labeling and targeted transfection of developing neurons

Individual immature neurons within the tadpole optic tectum were fluorescently labeled

using *in vivo* single-cell electroporation (Haas et al., 2001a; Hewapathirane and Haas, 2008) of the fluorescent dye Alexa Fluor 488, 3000MW (1mM in dH_2O, Molecular Probes, Eugene, OR), or a plasmid vector encoding membrane-targeted, farnesylated enhanced green fluorescent protein (fEGFP, 2μg/μl endotoxin-free plasmid DNA in dH_2O). For electroporation, tadpoles were briefly anesthetized by immersion in 0.02% 3-aminobenzoic acid ethyl ester (MS-222, Sigma-Aldrich, St. Louis, MO). A pulled glass micropipette (tip diameter 0.6μm) filled with dye or plasmid DNA solution was inserted into the cell-body layer of the optic tectum, and an Axoporator 800A (Axon Instruments, Union City, CA) was used to deliver brief trains of square wave pulses between a silver wire within the pipette and an external bath electrode. For plasmid DNA, pulse stimulus parameters were: intensity = 1.5 μA, duration = 200 μsec, frequency = 300Hz, train duration = 300 msec. For Alexa Fluor 488 dye, stimulus parameters were: pulse intensity = 2 μA; pulse duration = 1 msec; pulse frequency = 300Hz; train duration = 20 msec. In experiments testing the role of AMPA receptor-mediated neurotransmission in seizure-induced effects, a plasmid vector encoding the carboxy-terminal domain of the *Xenopus laevis* AMPA receptor subunit GluR1 (residues 809–889; GluR1Ct) fused to the carboxy-terminal of EGFP was delivered by single-cell electroporation, and cells were imaged 48h post-transfection (Haas et al., 2006).

4.2.3 *In vivo* two-photon fluorescence imaging of dendritic growth

For rapid time-lapse imaging of dynamic dendritic growth behavior, tadpoles were initially paralyzed by bath application of the reversible paralytic pancuronium dibromide (2mM in normal

rearing medium, Tocris, Ellisville, MO), embedded under a thin layer of agarose (1%, prepared in normal rearing medium), and immersed in an imaging chamber continuously perfused with oxygenated rearing medium (Hewapathirane et al., 2008). The imaging chamber was mounted on the stage of a custom-built two-photon laser-scanning microscope, constructed from an Olympus FV300 confocal system (Olympus, Center Valley, PA) with a Chameleon XR laser light source (Coherent, Santa Clara, CA). Using an excitation wavelength of 910 nm, stacks of images (*z*-axis step size = 1.5 μm) of labeled neurons were captured using an LUMFI 60X water immersion objective (Olympus, NA = 1.1) and images were recorded using Fluoview software (Olympus). In experiments employing imaging intervals of 4 or 24 hours, tadpoles were briefly anesthetized with 0.02% MS-222, placed in a Sylgard (Dow Corning, Midland, MI) imaging chamber, coverslipped, and imaged. After imaging, tadpoles were immediately returned to their chambers where they fully recovered from anesthesia within 5 min.

4.2.4 Dendrite morphometric analysis

Custom-designed software (based on a program created by Dr. Jamie Boyd, University of British Columbia), running on the IGOR Pro 6 platform (WaveMetrics, Portland, OR) was developed to comprehensively analyze both rapid and protracted dendritic growth events. Every dendritic filopodium and branch within a 3D stack encompassing the full dendritic arbor were identified, measured, and tracked through successive 5 min time points over the entire 5 h imaging period. In experiments with long-interval (4 and 24 h) time-lapse imaging paradigms, dendritic arbors were manually drawn using computer-assisted 3D rendering software

(Neurolucida, MicroBrightField, Williston, VT).

4.2.5 Immunohistochemistry of synaptic markers

Fluorescence immunohistochemistry was performed on horizontal 20 µm cryostat sections of whole tadpoles fixed with 4% paraformaldehyde (Sigma-Aldrich, diluted in 0.1M phosphate buffer pH 7.4 (PB)). Experimental and control group tissue sections were yoked, with identical numbers of tissue sections in experimental and control groups mounted onto each microscope slide. Sections were pre-incubated in blocking solution consisting of 5% (v/v) normal goat serum (NGS) in 0.1M PB containing 1% Triton X-100 (Acros Organics, Geel, Belgium), for 1 h at room temperature (RT). Sections were then rinsed and incubated for 24 h at 4^{o}C with primary antibodies to both rabbit α-SNAP-25 (Stressgen, Ann Arbor, MI), and mouse α-PSD-95 (Millipore, Billerica, MA) diluted 1:400 and 1:200, respectively, in blocking solution. Sections were subsequently rinsed and incubated in the dark for 1 h at RT with secondary antibodies goat α-rabbit Alexa-568 and goat α-mouse Alexa-633 (Molecular Probes, each diluted 1:200 in blocking solution). Immunofluorescence within a randomly selected region of the tadpole optic tectal neuropil region was visualized and imaged with an Olympus FV1000 confocal system, using a PlanApo N 60X oil immersion objective (Olympus, 1.42 NA). Dual-channel images were obtained with sequential fluorophore excitation using the 543 nm and 633 nm laser lines, and emitted signal was filtered using a 560–600 nm bandpass filter and a 650 nm long-pass filter. Following acquisition, blinded images were subjected to background subtraction and immunostained puncta with sizes in the range of 0.2-3.0 µm^2 (Ruthazer et al., 2006; Lim et al., 2008) were quantified and subjected to colocalization analysis. In order to assess whether colocalization of fluorescence signals was due to actual juxtaposition of pre- and postsynaptic elements as opposed to random colocalization of fluorescent puncta, analyses were repeated where one image was rotated by 90 degrees. In all experiments, rotated images showed a significant decrease in colocalization densities to ~10% those of original values. Image analysis

was performed using NIH ImageJ software.

4.2.6 Statistical analyses

Statistical tests were conducted using PASW Statistics 17 software (SPSS Inc., Chicago, IL). Multiple-way repeated measures ANOVA comparisons followed by Tukey's post-hoc analysis were used to examine both within- and between-group differences, unless otherwise noted. Within-group differences compared baseline values to subsequent measurements in individual subjects of a given experimental group, while between-group differences compared time-matched measurements between two or more experimental groups. Kolmogorov-Smirnov comparisons were used to compare differences between cumulative proportion datasets.

4.3 Results

4.3.1 Seizure-induced inhibition of dendritic growth is due to excessive AMPA receptor activation

Single-cell electroporation was used to introduce the fluorescent dye Alexa Fluor 488 into individual immature neurons within the tadpole optic tectum, a midbrain structure corresponding to the mammalian superior colliculus. Dendritic growth of labeled neurons was imaged at 4 h intervals over 8 h, with the first 4 h used as an internal control of baseline growth for a subsequent 4 h period in which seizures were induced. All neurons were re-imaged 1 day following experiments to confirm cell viability following seizures. Three chemoconvulsants with distinct mechanisms of action were utilized in order to distinguish seizure-induced effects from potential unrelated drug-specific effects. Following imaging of baseline growth, freely-swimming tadpoles were exposed to Pentylenetetrazol (PTZ) (15mM), pilocarpine (75 mM), or

4-Aminopyridine (4-AP) (1mM) for 1 h (Hewapathirane et al., 2008). In this and all subsequent experiments, control tadpoles were exposed to vehicle solution composed of normal rearing medium. Dendritic arbor growth rates were similar during the first 4 h baseline period across groups, with neurons in all groups demonstrating ~20% increase in total arbor length, and ~13% increase in the combined number of branches and filopodia, relative to each individual neuron's initial values (Fig. 4.1A and 4.1B). However, neurons in tadpoles experiencing seizure activity lasting 1 h showed no increase in dendritic arbor size during the second 4 h period (4-AP: –1.41 ± 5.45%, n=8 neurons; pilocarpine: 0.00 ± 5.61%, n=8 neurons; PTZ: –1.27 ± 8.55%, n=9 neurons; control: 17 ± 6.34% n=10 neurons), and an overall loss of branch and filopodial tips (4-AP: –8.79 ± 9.91%; pilocarpine: –7.00 ± 5.87%; PTZ: –15.08 ± 12.32%; controls: 12.42 ± 7.99%). A separate group of tadpoles was exposed to PTZ (15mM) for 4 h to examine the effects of extended seizure activity on dendritic growth. Seizure activity lasting 4 h significantly reduced dendritic length, and numbers of dendritic processes (–21.64 ± 5.52% and –19.58 ± 6.95%, respectively; n=8 neurons), decreasing to values below each individual neuron's initial values. Although 1 or 4 h seizures interfered with normal patterns of growth, dendritic blebbing was not observed, and all labeled neurons were viable 24 h following seizures. These data demonstrate a duration-dependent effect of seizures on dendritogenesis *in vivo*, with 1 h of seizure activity retarding growth and 4 h of seizure activity inducing dendritic arbor atrophy. The similar effects of seizures induced by chemoconvulsants with different mechanisms of action indicate that the observed effects are directly caused by seizures, as opposed to drug-specific effects not related to their seizure-inducing activity. For this reason, PTZ was used for seizure induction in subsequent experiments.

Previous studies have implicated excessive excitatory glutamatergic neurotransmission as a potential mediator of seizure-induced dendritic spine loss and arbor atrophy (Swann et al., 2000; Nishimura et al., 2008). To investigate the role of AMPARs in seizure affects on dendritogenesis without altering seizure activity, we employed single-cell electroporation to

selectively reduce synaptic AMPAR-mediated transmission only in the individual developing tectal neurons being imaged (Haas et al., 2006). This cell-autonomous genetic manipulation, within the otherwise unaltered brain, circumvents systemic and circuit-level effects of pharmacological approaches. Dendrite growth of individual transfected neurons was imaged at 4 h intervals over 8 h as above, with tadpoles in the seizure group being exposed to PTZ (15mM) for 1 h in the second 4 h period. No significant difference in arbor growth rates was observed between groups during the baseline 4 h period (~11% increase in arbor length and ~13% increase in dendrite tip number; n = 12 neurons/group; Fig. 4.1C and 4.1D). Strikingly, individual neurons with reduced AMPAR transmission in tadpoles that experienced seizures also continued to grow at rates similar to controls (arbor growth: 10.05 ± 1.61%; Fig. 4.1C). Moreover, seizures do not promote filopodial elimination as seen in control EGFP neurons treated with PTZ for 1 h (dendritic tip number increase: 13.45 ± 1.68%; Fig. 4.1D), supporting our model that reduced synaptic AMPAR-mediated transmission prohibits filopodia from rapid elimination and hyper-stabilization. These results strongly implicate AMPAR-mediated excitatory input as a critical cell-autonomous mediator of seizure-induced effects on dendritic growth, rather than systemic factors.

4.3.2 Seizures increase rates of filopodial and branch elimination

The effects of ongoing seizures on dynamic dendritic growth were examined using rapid time-lapse imaging within intact and awake tadpoles. Anesthetics were not used during continuous rapid time-lapse imaging experiments to avoid interference with seizure progression and endogenous activity-dependent processes. Images of single EGFP labeled neurons were taken at 5 min intervals over 5 h, comprising of an initial 1 h baseline period followed by either 4 h of PTZ-induced seizures or 4 h exposure to control solution (n=5 neurons/group; Fig. 4.2A).

Control neurons demonstrated linear and significant increases in total branch length, increasing after 5 h to 132.87 ± 15.85% relative to initial values (Fig. 4.2B). In contrast, while neurons in tadpoles experiencing seizures exhibited a similar rate of branch growth during the baseline period, the mean total branch length following seizure induction significantly decreased over the subsequent 4 h, with final lengths of 78.21± 8.25% relative to initial values (Fig. 4. 2B). A significant reduction in total branch number was also observed during seizures, whereas control neurons continued to add new branches over the entire course of imaging (Fig. 4.2C). Seizure-related reduction in branch number was due to a significant increase in branch eliminations compared to control neurons (Fig. 4.2D). However, neurons continued to add new branches during seizures demonstrating continued capacity for growth (Fig. 4.2E).

Total filopodial numbers on each arbor were analyzed at 5 min intervals over the entire 5 h time-course of imaging as a measure of dynamic dendritic growth (a total of 3275 filopodia were analyzed in all neurons; n=5 neurons/group). All dendritic filopodia on a developing dendritic arbor were identified, measured and tracked across each 5 min time point over 5 h using custom-designed software. Seizures induced a decrease in total filopodia number, reaching levels significantly less than control values 85 min following seizure induction, after which values continued to decline (Fig. 4.2F). Notably, filopodial density did not significantly differ from controls, due to the concomitant decrease in total branch length (Fig. 4.2G). Filopodial addition and elimination rates were measured to determine which aspect of filopodial turnover contributed to the overall loss of filopodia during seizures. Rates of filopodial addition significantly decreased during the first hour of seizure activity, and continued to decrease over

time to a final value of 47.32 ± 12.70%, relative to the baseline rate (Fig. 4.2H). Seizures did not affect the rate of filopodial elimination over the first hour post-induction (114.24 ± 11.32 of baseline values), however, eliminations significantly declined over the following 3 h, reaching a final value of 60.98 ± 11.02 % relative to the baseline rate (Fig. 4.2I). Although both the rates of additions and eliminations are significantly reduced during seizures when compared to control group, the rate of addition is reduced to a larger degree, resulting in an overall net loss of filopodia over time (Fig. 4.2J). Collectively, these data indicate that seizure-induced inhibition of growth and the atrophy of developing dendritic arbors over long periods are due to an inhibition of branch elongation, an increase in the rate of branch elimination relative to addition, and an overall loss of dendritic filopodia. While dendrites continue to exhibit growth behavior during seizures, including addition of new branches and filopodia, larger increases in the rates of retraction result in an overall retardation of growth.

4.3.3 Seizures induce rapid elimination and subsequent stabilization of specific filopodial sub-populations

Next, we examined whether seizure activity interferes with normal patterns of filopodial turnover and stabilization. Analysis of the maintenance of filopodia present prior to seizure induction, referred to here as ‘pre-seizure’ filopodia, reveals a rapid loss of filopodia over the first 15 min following PTZ exposure (Fig. 4.3A). Using extracellular electrophysiological recordings, we previously demonstrated that PTZ-induced seizures (15mM) typically initiate within 10-15 min following drug application (Hewapathirane et al., 2008). The observed rapid destabilization

of pre-existing filopodia therefore reflects events occurring within minutes of seizure onset. Over the subsequent 20-170 min, the proportion of pre-seizure filopodia remaining was similar across groups, with values steadily declining over time. Notably, however, over the final 65 min period (175-240 min post-seizure induction), seizure neurons retained significantly more pre-seizure filopodia than control neurons, indicating hyper-stabilization of a subpopulation of pre-seizure filopodia during seizures (Fig. 4.3A, see inset). Conversely, filopodia generated *during* seizures were found to be hyper-stabilized when compared to control filopodia generated over the same period (Fig. 4.3B). Correspondingly, filopodia from seizure neurons showed significant lower motility (Fig. 4.3C), and the mean lifetime of filopodia generated during seizures was significantly greater than control values (Fig. 4.3D). Together, these results demonstrate that seizures induce two distinct, yet opposing, effects on filopodia: (i) destabilization occurring rapidly at seizure onset, and (ii) hyper-stabilization of a subset of pre-seizure filopodia and those that are added during the seizure event.

4.3.4 PKMz mediates hyper-stabilization of dendritic filopodia during seizures

PKMz is a kinase that has been found to be upregulated by tetanus stimulation, and critical in maintaining late-phase long-term potentiation and memory (Drier et al., 2002; Ling et al., 2002; Pastalkova et al., 2006; Shema et al., 2007). Recently, PKMz has been found to direct dendritic filopodial stabilization and structural plasticity (Liu et al., 2009). Over-expressing PKMz in growing neurons hyper-stabilizes dendritic filopodia and restricts arbor expansion, while blocking endogenous PKMz destabilizes dendritic filopodia and promotes excessive dendrite growth. We thus propose a second component of our model that filopodial hyper-stabilization

generated during seizures results from activation of plasticity pathways downstream of strong AMPAR transmission involving PKMz. To investigate this hypothesis, western blotting was performed on brain lysates from tadpoles that were exposed to PTZ for 30 min, 1 h, 2 h, or 4h to examine effects on expression of PKMz, as well as CaMKIIα as a control protein associated with synapses (Fig. 4.4A, 4.4B). Western blot analysis finds that PKM levels significantly decreased in the first 30 min, and then gradually increased to the baseline level in 2 to 4 h. CaMKIIα level maintained at reduced level after the first 30 min, which is consistent with reductions in dendritic arbor size and filopodial number due to seizure activity (Fig. 4.4 C). Increase of the relative amount of PKMz per dendrite filopodia is therefore implied. To test a role of PKMz activation in seizure-induced morphological changes, we selectively knocked-down endogenous *Xenopus* PKMz using synthetic morpholino oligonucleotides (MO-PKMz, sequence: ATATGCTTTTTACAGGTCAGCGGGA; Gene Tools, Philomath, OR). Single-cell electroporation was used to introduce lissamine-tagged MO into individual neurons along with the fluorescent dye Alexa Fluor 488 dextran, and labeled neurons were imaged at 1 h intervals over 5 h, with tadpoles in the seizure group exposed to PTZ (15mM) starting in the second hour. All dendritic filopodia were identified, measured, and tracked across time points to characterize the filopodial maintenance rate. PTZ treated EGFP neurons demonstrate hyper-stabilization of filopodia generated during seizure, evident from increased filopodial maintanence ratio during every hour period (Fig. 4.4D, 4.4E). Strikingly, MO-PKMz neurons were immune from the hyper-stabilization effect by seizure, and showed same morphological changes as control group (Fig. 4.4F, 4.4G). These results support a role for PKMz in enhancing synapse strengthen leading to the hyper-stabilization of new filopodia generated during seizure

4.3.5 Seizure-induced inhibition of dendritic growth is persistent

To determine whether the acute and short-term seizure effects on dendritic growth have persistent consequences to neuronal morphology, we imaged newly differentiated Alexa Fluor

488-labeled tectal neurons daily for 4 days, a period spanning tectal dendritic arbor maturation (Wu et al., 1999; Haas et al., 2006). Tadpoles were subjected to a single 1 h PTZ-induced seizure after acquisition of the day 2 image, an early time-point during dendritic maturation (n=14 neurons/group; Fig. 4.5A). Over the first 2 days, total dendritic arbor length and dendritic tip number increased at similar rates across groups (controls: 342.20 ± 49.42µm arbor growth and 30.96 ± 5.35 additional tips; seizure group: 387.25 ± 49.33 µm arbor growth and 27.08 ± 4.47 additional tips, Fig. 4.5B and 4.5C). However, while control neuron arbors continued to grow over the following 2 days, exhibiting arbors 320.62 ± 64.01 µm larger with 19.23 ± 3.53 additional dendritic tips, arbors of neurons experiencing seizures did not continue to grow over days 2 to 4, demonstrating significantly reduced dendritic arbor length and dendritic tip number on days 3 and 4 compared to age-matched control cells. Similarly, while control neurons significantly increased dendritic arbor field volume after day 2, field volumes of seizure neurons failed to significantly increase after day 2, with day 3 and 4 volumes remaining significantly lower than age-matched controls (Fig. 4.5D). Dendritic complexity, as assessed using 3D Sholl analysis, showed similar increases across groups in the dendritic length added over the first 2 days of imaging, within the 3D space corresponding to a spherical shell 40-100µm from the cell body (Fig. 4.5E). Seizure neurons showed no significant increase in complexity over the days subsequent to the seizure episode, contrasting with control neurons which significantly increased dendritic complexity within the 3D space 60-80µm radial to the cell body over the equivalent time period. These finding indicate that seizure-induced inhibition of dendrite growth persists well beyond the duration of the seizure event, resulting in persistently stunted arbors.

4.3.6 Seizures persistently decrease excitatory synapse densities and mEPSC frequency

We examined whether seizures alter the density of excitatory synapses within the neuropil region of the optic tectum using immunohistochemical colocalization analysis of the pre- and

postsynaptic markers SNAP-25 and PSD-95, respectively (Lim et al., 2008) (Fig. 4.6A and 4.6C). To examine short-term effects, tadpoles were either exposed to PTZ for 1 h and sacrificed 3 h later, or exposed to PTZ for 4 h and sacrificed immediately. Persistent changes were examined in tadpoles exposed to PTZ for 1 h and sacrificed after either 24 or 48 h (Fig. 4.6D). While the density of SNAP-25 puncta remained unchanged across all groups, a significant reduction in the density of both colocalized puncta and total PSD-95 puncta were observed after seizures, compared to controls (Fig. 4.6E). Although densities of PSD-95 puncta colocalized with SNAP-25 puncta, and total PSD-95 puncta densities significantly increase after intervals of 24 and 48 h post-seizure, suggesting partial recovery from the acute effects of seizures, values remained significantly lower than controls. Changes in puncta colocalization were solely due to changes in PSD-95 puncta numbers, since the relative proportion of PSD-95 puncta that were colocalized did not differ across groups (Fig. 4.6F). Whole-cell patch clamp electrophysiology of tectal neurons *in vivo,* employed to test the physiological effects of reduced PSD-95 puncta levels finds a significant decrease in AMPAR-mediated mEPSC frequency following 4 h of PTZ seizure, with no change in amplitude (Fig. 4.6G-I) . Collectively, those findings indicate seizure activity reduces excitatory synapses in tadpole tectum.

4.4 Discussion

Previous attempts to identify structural correlates of developmental seizure-induced neural circuit dysfunction have been limited, in part, by the inability to detect subtle alterations in neuronal morphogenesis due to the use of single time-point or long-interval time-lapse examinations (Jiang et al., 1998; Swann et al., 2000; Nishimura et al., 2008). By employing a developmental model system that allows direct *in vivo* short and long intervals imaging of neuronal growth before, during and after seizures, we have comprehensively characterized the acute and persistent effects of seizures on the structural maturation of developing neurons. Furthermore, the examination of seizure-related effects on neuronal morphogenesis within the

intact and anaesthetized brain maintains physiologically relevant micro- and macro-cellular environments during seizures, while avoiding the confounding effects of prolonged anesthetic exposure on neural growth, circuit function, and seizure expression. We find that early-life seizures induce a complex effect on dynamic growth behavior which culminates in persistent stunting of dendritic arbor development.

Extended *in vivo* rapid time-lapse imaging at 5 min intervals over 5 h, followed by tracking and measurement of all dendritic filopodia and branches in 3D reveals bi-directional effects of seizures on dendritic growth dynamics. Seizures rapidly induce a significant retraction of pre-existing dendritic filopodia within minutes of seizure onset. In contrast, filopodia that arise during seizures exhibit hyper-stabilization, demonstrated by decreased motility and prolonged lifetimes. As seizures progressed, branch elongation decreases and rates of branch elimination increased. Together, these effects produce significant seizure-induced retardation of dendritic arbor growth, resulting in an overall reduction in arbor size. Critically, long-interval imaging over days reveals that the effects of individual seizures are persistent, resulting in stunting of subsequent arbor growth. Reduced arbor size and complexity likely has enduring functional consequences, given that dendritic size and shape influences dendritic integration of synaptic inputs, intrinsic excitability based on input resistance, and the number and types of pre-synaptic sites that may be contacted (Häusser et al., 2000; Wong and Ghosh, 2002; Cline and Haas, 2008). In support for such decreased circuit complexity, we find that seizures persistently decrease the density of excitatory synaptic numbers, shown by reduced synaptic markers and AMPAR-mediated mEPSC frequency. In light of substantial evidence demonstrating that altered neural activity during critical periods of development modifies neural circuit formation leading to lasting effects on function and plasticity (Crowley and Katz, 2002; Hensch and Fagiolini, 2005; Taha and Stryker, 2005), our findings reveal a mechanism through which early-life seizures may induce lasting brain dysfunction.

In order to identify mechanisms mediating seizure-induced alterations in dendritogenesis,

we tested the effects of various chemoconvulsants working through distinct molecular targets. Since seizures induced by either enhancing glutamatergic or cholinergic transmission, inhibiting potassium channels, or inhibiting GABA receptors each produced similar effects on dendritogenesis, we conclude that altered growth results from seizure activity rather than drug-specific effects unrelated to seizure induction.

We have applied targeted single-neuron transfection to test potential mechanisms underlying seizure-induced morphological changes. We test the role of direct excessive glutamatergic neurotransmission in seizure-induced growth inhibition by selectively reducing AMPAR-mediated synaptic transmission in individual neurons within otherwise unaltered brains through single-cell expression of a peptide construct that interferes with AMPAR trafficking to synapses (Shi et al., 2001; Haas et al., 2006). We find that reduced AMPAR-mediated transmission within individual neurons protects those neurons from seizure-induced growth inhibition. These results support a direct role for excessive glutamatergic receptor activity on altered dendritic growth rather than secondary systemic factors, such as metabolic distress or abnormal extracellular pH and ion concentrations. Excessive AMPAR-mediated activity during seizures has been linked to memory deficits in immature rats subjected to prolonged seizures (Mikati et al., 1999), establishing a potential link between seizure-induced higher-order functional deficits and the dendritic structural abnormalities reported here.

It remains unclear how excessive glutamatergic transmission alters dendrite growth, yet two distinct processes may be involved: sub-lethal cellular injury induced by excitotoxicity, and/or the pathological activation of endogenous activity-dependent, competition-based synaptotropic dendrite growth mechanisms. Excitotoxicity-induced neuronal death is a well-documented hallmark of prolonged seizures (Meldrum, 1991). While the immature brain demonstrates remarkable resistance to seizure-induced neuronal loss (Haas et al., 2001b; Thibeault-Eybalin et al., 2009), it is possible that seizures activate sub-lethal excitotoxic processes that interfere with normal cellular growth patterns. Sustained membrane depolarization during seizures

facilitates activation of calcium permeable NMDA receptors and voltage-gated calcium channels which, in turn, lead to calcium-dependent release of intracellular calcium stores (Olney, 1990). Cumulatively, these events may induce focal or cell-wide increases in intracellular calcium concentrations sufficient to activate excitotoxicity-related pathways (Swann et al., 2000). However, such injury-related responses might not be expected to result in filopodial hyper-stabilization, but rather in an overall decrease in filopodial lifetimes. Further, seizures did not eliminate ongoing growth processes, since new branches and filopodia continue to be added during seizures, even after extended periods of seizure activity.

Alternatively, excessive synchronous excitatory neurotransmission during seizures may activate endogenous, competition-based programs of synaptic plasticity which underlie synaptotropic stabilization and elimination of nascent dendritic processes. Mounting evidence supports the synaptotropic model of dendritogenesis in which synapse formation and maturation, driven by activity-dependent competition between afferent inputs, confers morphologic stabilization to newly extended labile dendritic filopodia, while synapse loss is associated with process retraction (Vaughn et al., 1988; Niell et al., 2004; Cline and Haas, 2008). Indeed, experimental manipulations in individual *Xenopus* tadpole optic tectal neurons which promote maturation of synapses induce dendritic morphological stabilization (Wu and Cline, 1998), while those that reduce synapse maturation result in filopodial destabilization (Haas et al., 2006). Here, we identified a plasticity-related protein kinase, PKMz that has been shown to regulate synapse maturation and filopodial stabilization in *Xenopus* optic tectal neurons, to play a role in regulating the filopodial hyper-stabilization during seizures. We find that knocking-down PKMz expression in individual neurons prohibits filopodia from hyper-stabilization during seizures; supporting our hypothesis that excessive synchronous excitatory neurotransmission during seizures promotes synapse maturation, which hyper-stabilizes dendritic filopodia. Together, our results suggest a model in which strong seizure-associated afferent input out-competes endogenous circuit activity, leading to synapse weakening and the morphological destabilization and elimination of

dendritic process which were integrated into endogenous circuits. Existing or new synapses involved in circuits transmitting seizure activity, however, would be expected to strengthen, resulting in stabilization of the dendritic process within which they reside. This model is supported by evidence linking increases in seizure susceptibility following neonatal seizures to AMPA-receptor-mediated synaptic potentiation (Rakhade et al., 2008), further implicating our observations of altered dendritic structure with negative consequence of early-life seizures.

Conducting comprehensive morphometrical analysis of dynamic growth behavior is necessary to detect and decipher the subtle, yet functionally significant changes to neural circuit structural formation caused by developmental seizures. Our results demonstrate two distinct consequences of early-life seizures on circuit function. First, the rapid destabilization of existing labile dendritic structures observed within minutes of seizure onset indicates a loss of morphologic processes formed and stabilized by pre-seizure endogenous activity. Seizure-induced loss of dendritic processes and associated synapses involved in normal circuit function may result in deficits in processing of endogenous activity. Secondly, seizures promote the hyper-stabilization of dendritic processes that arise during seizures, presumably by the stabilization and strengthening of synapses contained within these structures. The persistence of such structures and their constituent synapses suggests that seizures may hardwire seizure-associated circuits. These abnormal neural circuit structures may be more prone to support future seizure activity and/or underlie abnormal network activity. Thus, we find evidence for morphological substrates potentially underlying deficits in normal circuit function, and the possible origin of subsequent neuropathology later in life. While dendritic abnormalities have long been observed in patients with epilepsy (Wong, 2008), it has remained unclear whether these changes are directly induced by seizures. Our results demonstrate that early-life seizures directly induce dramatic and persistent morphological abnormalities in developing neurons, thereby interfering with normal brain circuit development.

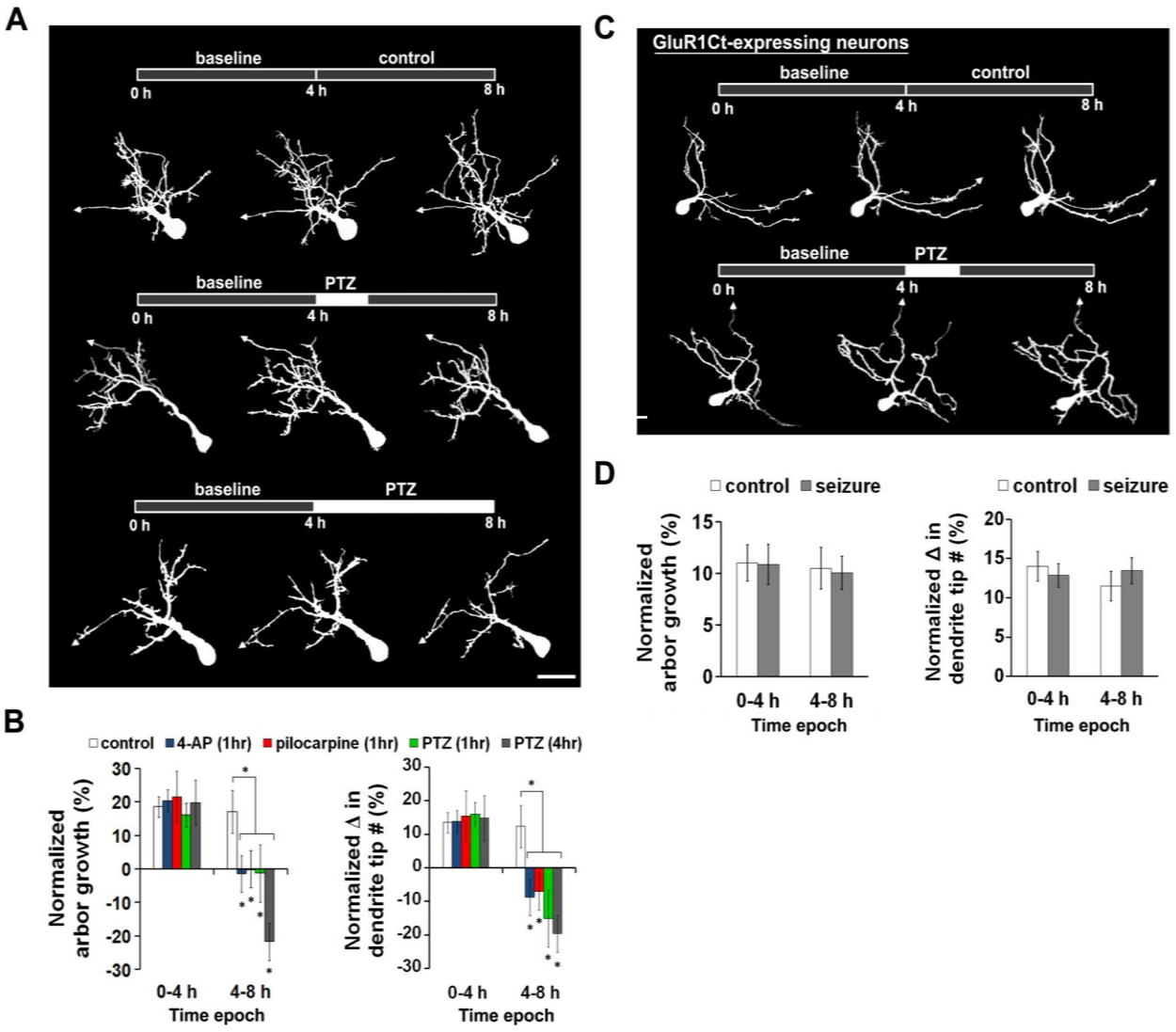

Figure 4.1 | Developmental seizures inhibit dendrite growth through excessive AMPA receptor activation. Two-photon imaging of single growing neurons within the intact brain was used to examine dendritic arbor growth of individual fluorescently labeled neurons. **(A)** Arbor growth over the initial 4 h baseline period was compared to growth during the subsequent 4 h period in which seizures were induced. Pentylenetetrazol (PTZ)-induced seizures lasting 1 h inhibited further arbor growth, while prolonged seizures lasting 4 h induced marked branch retraction leading to arbor atrophy. Arrowheads denote axons. Scale bar = 20 µm. **(B)** Quantification of arbor growth (*left,* change in total dendritic length) and changes in dendrite tip number (*right*, branch and filopodial tips) reveals that 1 h of seizure activity induced by either 4-aminopyridine (4-AP, 1mM), pilocarpine (75mM), or PTZ (15mM) inhibits further arbor growth and leads to an overall loss of dendritic tips. 4 h of PTZ-induced seizures induced overall arbor atrophy. n≥8 neurons/group. **(C)** Single neurons with reduced AMPA receptor-mediated

transmission (GluR1Ct-expressing neurons) within the otherwise unaltered brain continue to elaborate their dendritic arbors even after being subjected to 1 h of PTZ-induced seizure activity. Scale bar = 20 μm (A); arrowheads denote axons. **(D)** No significant differences in arbor growth (*left*) or tip number (*right*) was found, indicating that reduced AMPA receptor-mediated transmission protects developing neurons from seizure-induced inhibition of dendritic growth (n=12 neurons/group). *p<0.05, multiple-way repeated measures ANOVA. Data presented as means ± SEM.

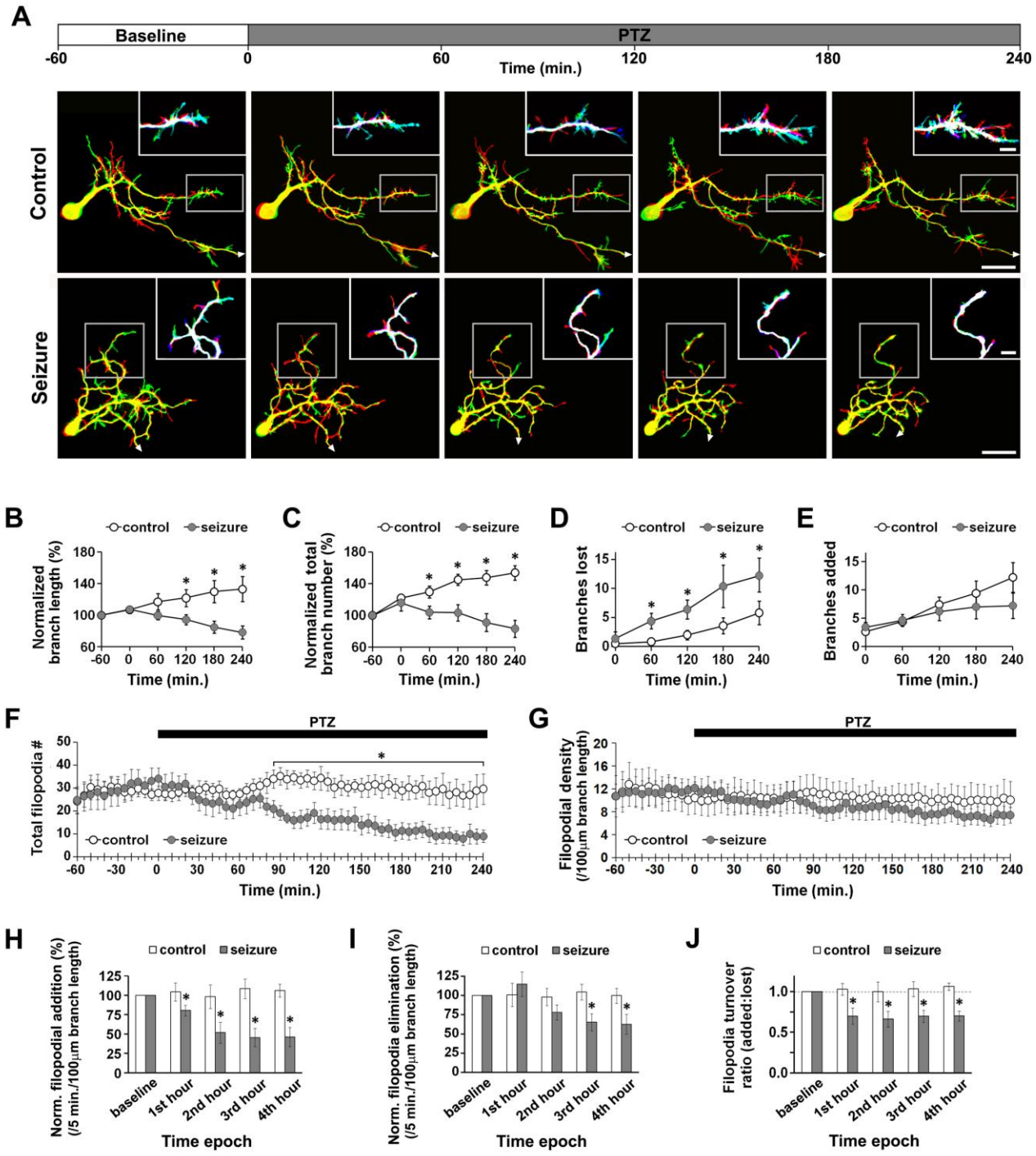

Figure 4.2 | Rapid time-lapse imaging of dendrite growth during seizures in the intact and unanesthetized brain. (A) 3D images of neurons were taken over 5 min intervals for 5 h, comprising of an initial 1 h baseline period followed by 4 h of PTZ-induced seizures. Representative images are color coded to highlight dendritic segments that are added (green; t = 1 h) and retracted (red; t = 0 h) during each hourly period (arrowheads denote axons; scale bar = 20 μm). Boxed regions are shown at higher magnification as insets, displaying overlays of

4 color-coded images spaced by 15 min intervals (red, blue, green and cyan; overlap = white; scale bar for insets = 5 µm). **(B-E)** Each dendritic branch was identified, measured and tracked individually over the entire period of imaging (n=5 neurons/group). Branch length (excluding constituent filopodia) significantly decreased during seizures, while control neuron branches exhibited elongation. Seizures also induced a significant reduction in branch number (C), due to a significant increase in branch elimination (D, cumulative branches lost/hour epoch) compared to controls. Seizures did not significantly alter branch addition rates (E, cumulative branches added/hour epoch). **(F-J)** Seizures induced a significant decrease in total filopodia number, with values steadily decreasing over time (F). Filopodial density was not significantly decreased (G), presumably due to concomitant branch retraction. Seizures also significantly reduced the rate of filopodial addition, which continued to decline over time (H). Seizures did not alter the rate of filopodial retraction over the first hour post-induction, however, rates of elimination gradually declined over the subsequent 3 h to values significantly less than controls (I). The net loss of filopodia during seizures was found to be due to a relative increase in filopodial retraction rates compared to addition rates within individual neurons, demonstrated by significant decrease in filopodial addition:elimination ratio during seizures (J). *$p<0.05$, multiple-way repeated measures ANOVA. Data presented as means ± SEM.

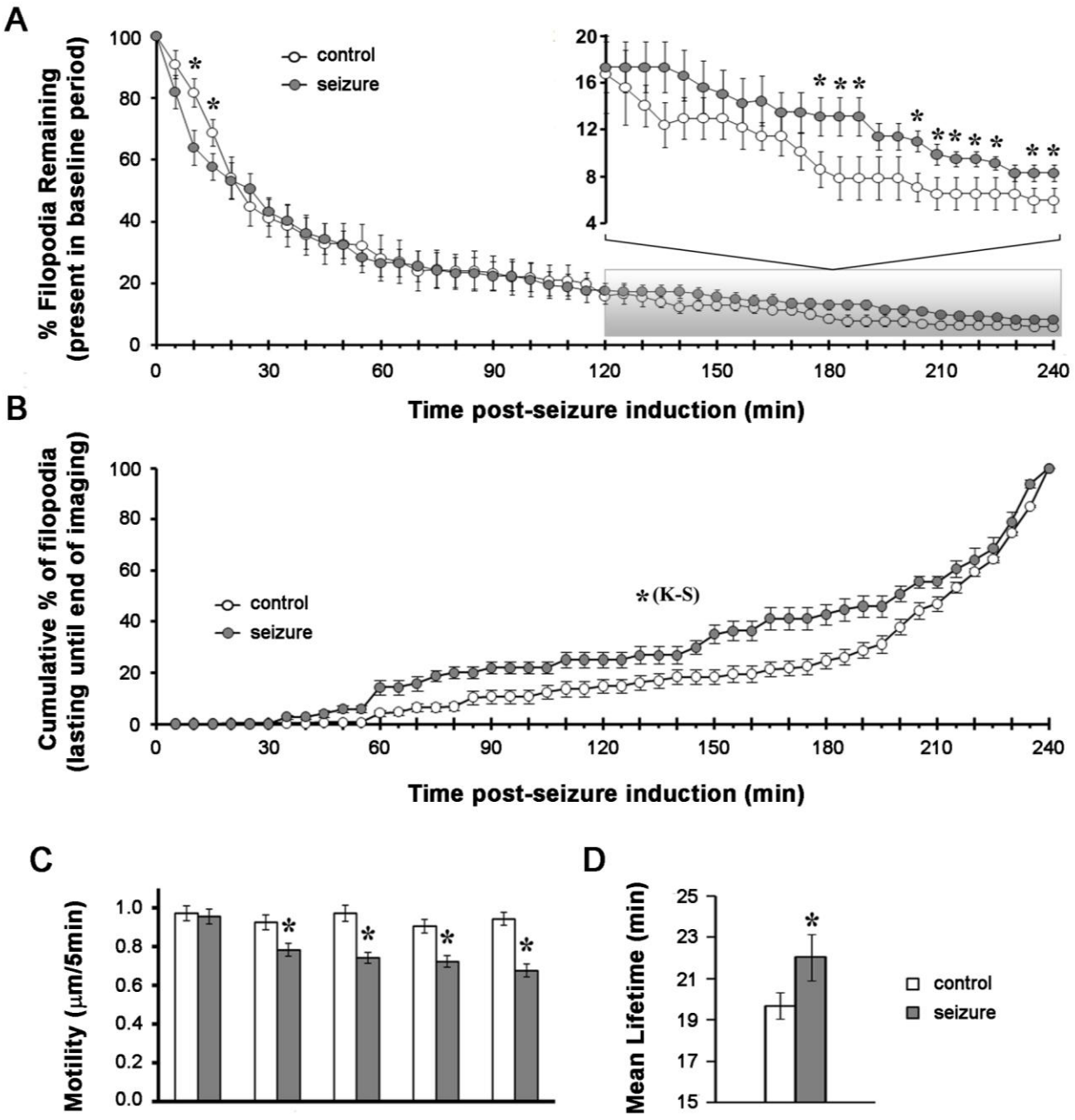

Figure 4.3| Seizures rapidly eliminate existing and hyper-stabilize new filopodia during seizures. Images of individual neurons were taken every 5 min for 5 h *in vivo*, within intact and unanesthetized tadpoles. Each dendritic filopodium was identified, measured and tracked individually over time, spanning 1 h baseline followed by 4 h of pentylenetetrazol (PTZ)-induced seizures (n=3275 filopodia from 5 neurons/group). **(A)** Seizures rapidly induced filopodial destabilization, seen as an increase in the rate of elimination of filopodia present prior to seizure induction (pre-seizure filopodia), after which values matched those of controls until ~120 min post-seizure induction. Over the subsequent period (highlighted as inset with expanded y-axis), the proportion of remaining pre-seizure filopodia was significantly elevated in seizure neurons,

indicative of a hyper-stabilization effect. *p<0.05, unpaired Student's t-test. **(B)** Cumulative plot of the proportion of filopodia generated after the baseline period which remained until the end of imaging. Filopodia generated during seizures were significantly hyper-stabilized, with a greater proportion of those lasting until the end of imaging being added at earlier time-points compared to controls (*p<0.001, two sample Kolmogorov-Smirnov (KS) comparison, *D* =0.1590). **(C)** Seizures induced a significant decrease in the absolute motility of filopodia (mean |Δ length|) compared to controls (* p<0.05, multiple-way repeated measures ANOVA). **(D)** Filopodia generated during seizures exhibited a significant increase in average lifetime, compared to control neuron filopodia generated over the same time period, (* p<0.05, unpaired Student's t-test). Data presented as means ± SEM.

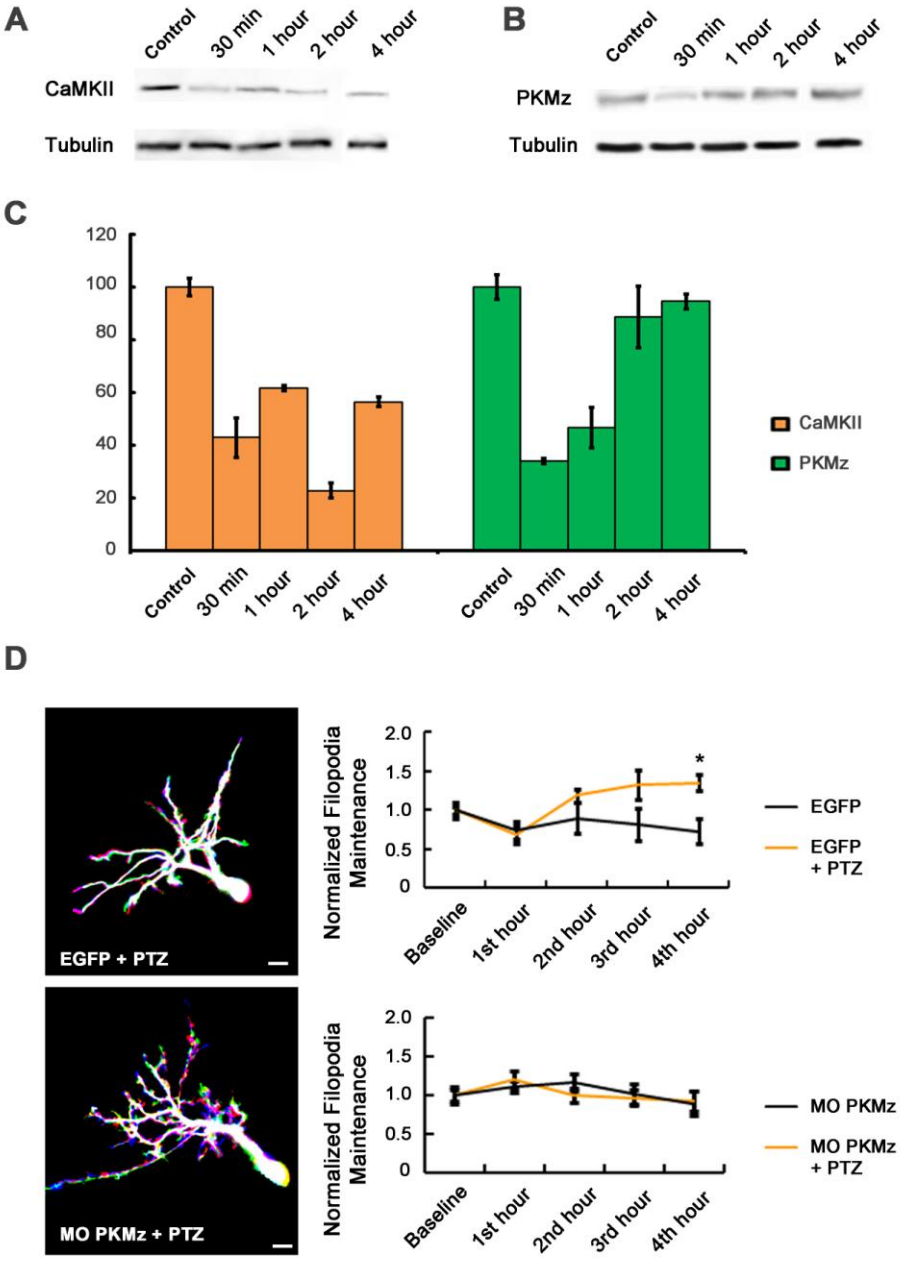

Figure 4.4 | PKMz mediates seizure induced hyper-stabilization of dendritic filopodia. Immunoblots of CaMKIIα (A) and PKMz (B) from brain lysates of tadpoles that were exposed to PTZ for various durations. (C) Quantitative analysis of immunoblotting results. The intensity value of each CaMKIIα (left) or PKMz (right) blot was normalized to tubulin, and then normalized to the averaged values of control group (n=3 for each time point). **(D,F)** Superimposed images from the last three successive time points of a 5 hour imaging session at one hour intervals (each of a different color, white=overlap) of either an EGFP neuron (D) or MO-PKMz neuron (F)

treated with PTZ. Bar = 20 μm. PTZ induced seizure leads to hyper-stabilization of dendritic filopodia in an EGFP neuron, represented by higher ratio of filopodia maintenance per hour, normalized to baseline level (Control, n=10; PTZ, n=5) **(E)**, while the hyper-stabilization effect was absent in MO-PKMz treated neurons (Control, n=5; PTZ, n=5) **(G)**. * p<0.05, unpaired Student's t-test. Data presented as means ± SEM.

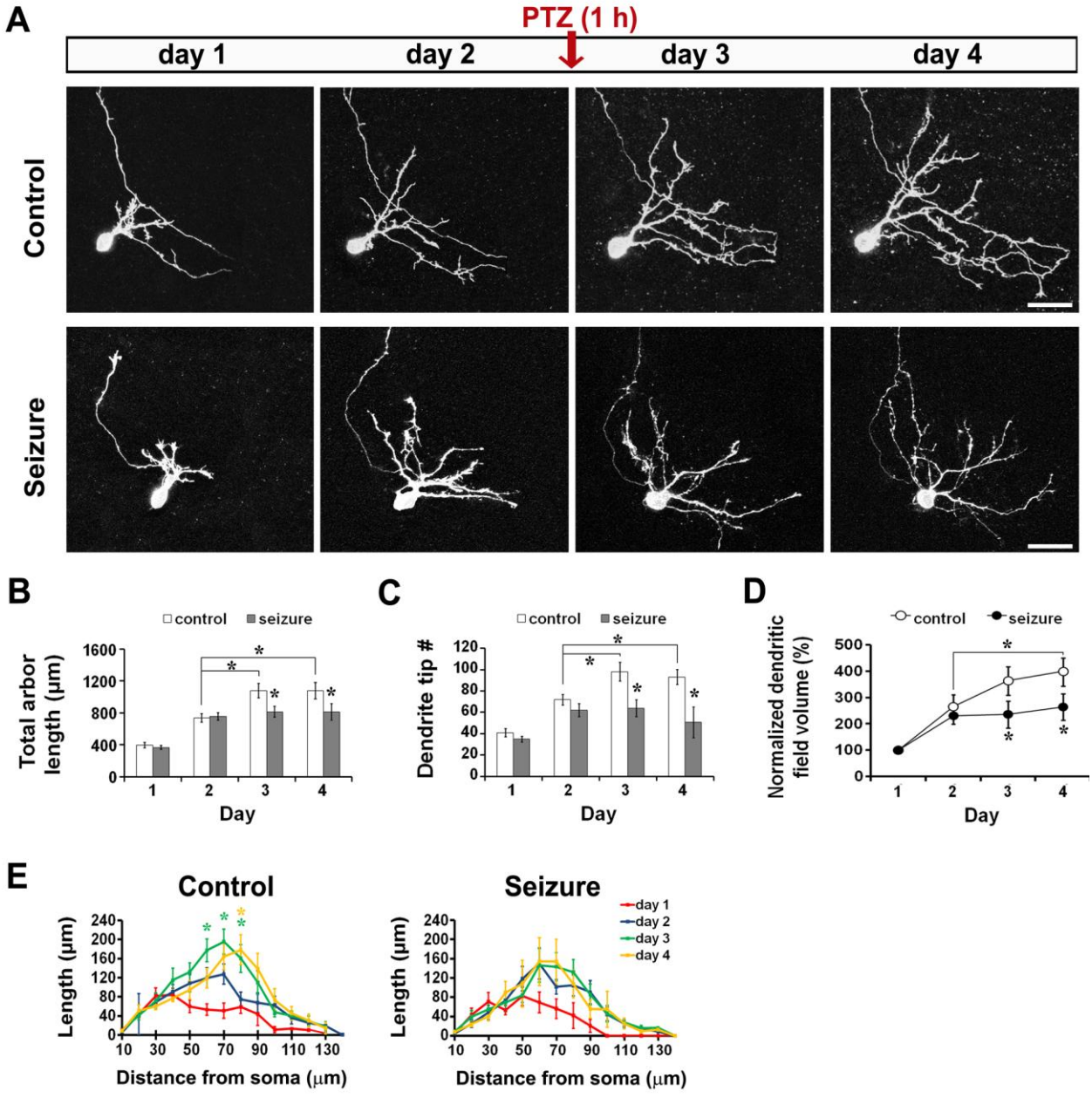

Figure 4.5 | Seizures persistently stunt further arbor growth and elaboration *in vivo.* Individually labeled neurons were imaged daily within the intact brain over 4 days, a period spanning tectal neuron dendritic maturation. Tadpoles were subjected to a single 1 h

PTZ-induced seizure after acquisition of the day 2 image to examine long-term effects on dendritogenesis. **(A)** Neurons experiencing a single seizure showed no significant increases in arbor size or complexity over the following 2 days. Scale bar = 20 µm; arrowheads denote axons. **(B-E)** A single seizure restricted subsequent increases in arbor size (B), dendritic tips (C; branches and filopodia), dendritic field volume (D), and branch complexity as assessed by 3D Sholl analysis (E), over the subsequent 2 days post-seizure. n=14 neurons/group; *p<0.05, multiple-way repeated measures ANOVA. In D, markers indicate significant differences from day 2 values. Data presented as means ± SEM.

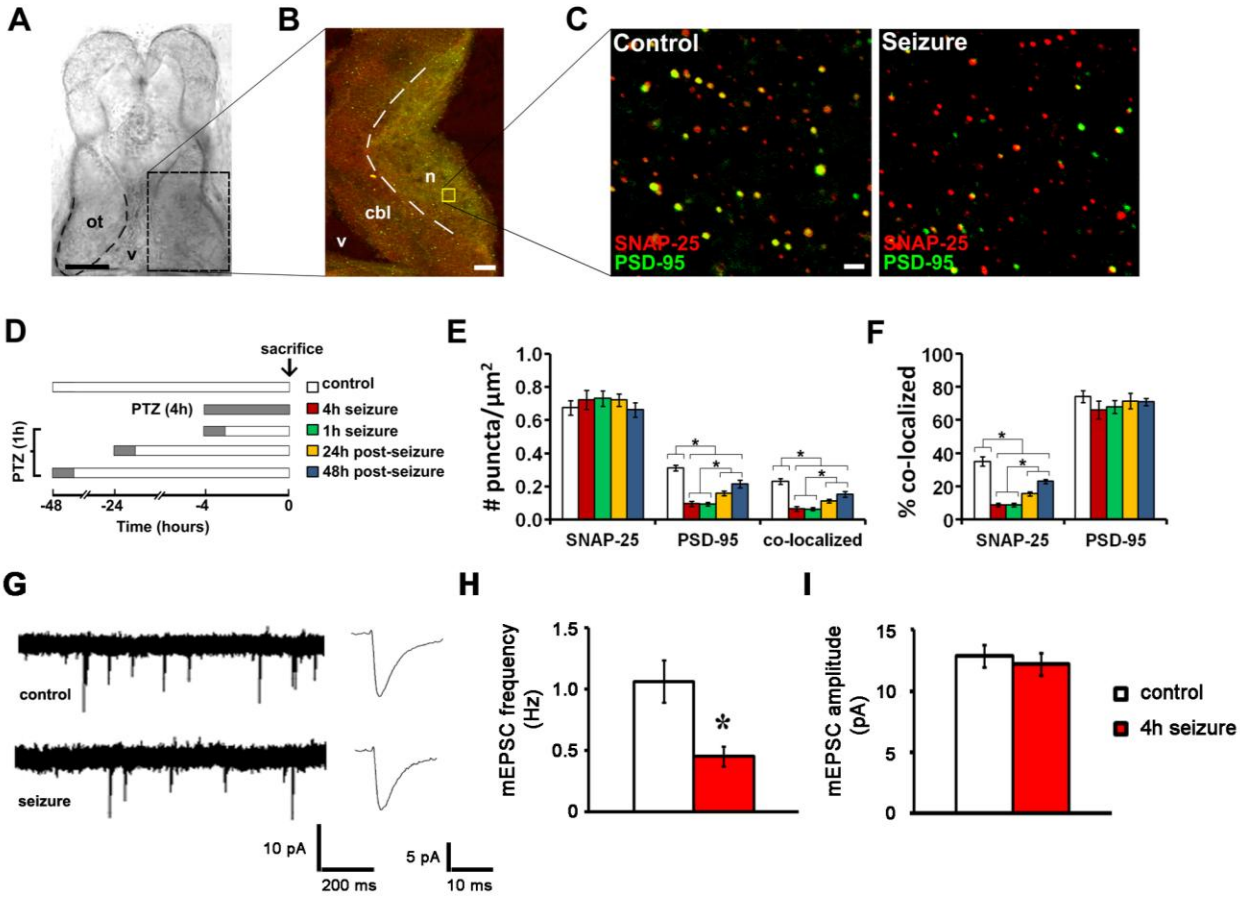

Figure 4.6 | Seizures persistently reduce synapse density. (A) Dorsal view of the albino *Xenopus laevis* tadpole brain (stage 47, rostral end towards top of image). *ot* – optic tectum; *v* – ventricle. Scale bar = 150µm. **(B)** Horizontal cryostat sections from fixed tadpoles were immunostained with antibodies against pre- and post-synaptic elements SNAP-25 (red) and PSD-95 (green), respectively. The dotted line demarcates border between tectal cell body layer (*cbl*) and neuropil (*n*), relative to the ventricle (*v*). For each section (n= 4 sections/tadpole; 3-4 tadpoles per group), a random region of neuropil was analyzed (boxed). Scale bar = 30µm. **(C)** Higher magnification images of immunolabeled tectal neuropil demonstrate that 4 h of PTZ

seizures decreases the density of colocalized SNAP-25 and PSD-95 puncta (yellow). Scale bar = 2μm. **(D)** Experimental paradigm used to test both the acute and persistent effects of seizures on synapse numbers. **(E)** Both total PSD-95 and colocalized punctum densities were significantly reduced after seizures. Densities slightly recovered with longer intervals post-seizure, however, values remained significantly lower than controls. SNAP-25 punctum densities remained unchanged across groups, indicating that changes in the numbers of colocalized puncta reflect changes in PSD-95 punctum numbers. **(F)** The percentage of PSD-95 puncta that are colocalized remains invariant despite changes in total PSD-95 punctum densities across groups. Colour key for histograms as in D. *$p<0.05$, one-way ANOVA. Data presented as means ± SEM. **(G)** Representative traces of AMPA mEPSC. **(H)** 4 hour seizures significantly reduced the frequency of mEPSC recorded on tectal neurons *in vivo*. **(I)** mEPSC amplitude was not affected. * $p<0.05$, unpaired Student's t-test. Data presented as means ± SEM.

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5. A SYNAPTOTROPIC MODEL OF DENDRITIC GROWTH INCORPORATING MATURATION-INDUCED STABILIZATION[4]

5.1 Introduction

Correct morphological development of brain neurons, including elaboration of complex dendritic arbors is a critical component of early brain circuit development. *In vivo* time lapse imaging of brain neuron growth has found that dendritic arborization is a highly dynamic process, involving rapid and continuous addition and elimination of dendritic filopodia, branches and synapses (Wu et al., 1999; Niell et al., 2004). This high turnover suggests searching the local environment for appropriate presynaptic partners and a "trial-and-error" process with a high threshold for testing and selecting contacts to maintain. The "synaptotropic model of dendritogenesis" first proposed by Vaugh (1989) based on observations of fixed tissue of rat spinal cord, suggests that dendrite arbor growth is directed by synapse formation(Vaughn, 1989). In this model, dynamically searching dendrites either form appropriate synapses and become stabilized, or fail to establish partners and retract and are eliminated. Furthermore, this model suggests that synaptically-stabilized dendritic processes retain morphologic plasticity and can support further growth from synaptic sites. In this manner, dendrites can grow iteratively from

[4]A version of this chapter will be submitted for publication: Liu XF, Haas K (2010) A synaptotropic model of dendritic growth incorporating maturation-induced stabilization

established synapses and arbor morphology is directed towards regions of appropriate innervation. *In vivo* time-lapse imaging of dendrite growth and synapse formation in zebrafish tectal neurons provided direct evidence supporting this model (Niell et al., 2004). By expressing a fluorescently tagged postsynaptic marker protein, PSD-95, it was found that the presence of postsynaptic specializations on filopodia confers stabilization to these highly motile and labile structures, prevented retraction beyond the synaptic site.

Following this seminal study by Neil and Smith confirming synaptotropic dendritogenesis, a number of *in vivo* imaging studies involving altered glutamatergic synaptic transmission and maturation have yielded results that veer significantly from a simple interpretation of the synaptotropic model. Manipulations that reduce AMPA receptor trafficking to synapses in growing neurons were found to destabilize dendritic filopodia, as predicted, yet produced an unexpected excessive elongation of remaining dendritic branches (Haas et al., 2006). Similar effects were found when two kinases critically involved in glutamatergic synaptic maturation, CaM Kinase II (CaMKII) and Protein Kinase M zeta (PKMz), were inhibited. Conversely, over-expressing CaMKII or PKMz resulted in a dramatic reduction in filopodial dynamics, and restricted arbor elaboration over long periods (Wu and Cline, 1998; Liu et al., 2009). A direct interpretation of the synaptotropic model would suggest decreased dendrite growth with manipulations that reduced synaptogenesis and increased growth with those that increase synaptogenesis.

Given the wealth of high resolution *in vivo* imaging data of dynamic and long-term dendritic arbor growth, mathematical modeling and computer simulation can be applied as valuable tools

to identify underlying principles of morphological development. To date, various biologically plausible models have been proposed to examine processes ranging from neurite initiation to refinement of mature and stable dendritic structures, including models attempting to mechanistically explain and predict dendritic arborization via subcellular processes, such as cytoskeletal dynamics (van Veen and van Pelt, 1994) and intracellular signaling (Graham and van Ooyen, 2004), as well as models simulating dendritic branching and dynamics as a probabilistic or stochastic process guided by explicit rules such as synaptotropic mechanism (Kiddie et al., 2005; Niell, 2006).

Although previous models of synaptotropic mechanism could simulate dendritic arborization resulting from iterative synapse formation and filopodia maintenance, they consider synapses as static all-or-none physical structures, and therefore, these models are not able to incorporate roles of synapse plasticity or maturation on filopodia stabilization and arbor growth. To address this shortcoming, here we propose a new expanded model, and show how an elaborated synaptotropic mechanism can better direct dendritic arborization in regions of synaptic connections and drive further refinement and consolidation of optimal dendritic structures.

5.2 Methods

We have implemented a Matlab (Mathworks)-based simulation of the dynamic growth of dendritic arbors using a discrete stochastic model. Our analysis was restricted to two-dimensions for simplicity, but the principles and results could be applied to three-dimensional situations to represent neurons within real brain environments. The simulated arbor consisted of three types of objects: stable branches, dynamic branches or filopodia, and

synapses. A strict synaptotropic rule was applied to direct an iterative process of synapses formation and filopodial stabilization and branching. A synapse could form on a branch, either stable or dynamic with a probability of pSyn per length of the branch, with an initial strength of 1. A synapse could be potentiated with the probability of Ppot or depressed with the probability of Pdep, with a change of 1 unit in strength value per time. A synapse is eliminated when its strength is 0, and it could not be potentiated above Smax = 5. New filopodia were formed from synaptic sites with a probability of pFilo per branch length per time. The motility of a filopodial tip was negatively correlated with the strength or maturity of the neighboring synapse (s), and was determined as [3/(1+exp(-1/s^2))-1.5] μm/min , based on a putative mechanism that calcium transients from local synaptic transmission regulate filopodial motility (Lohmann et al., 2002; Lohmann et al., 2005). However, the fundamental conclusions of the simulation will not be affected by other forms of equations based on other putative mechanism such as synaptic cytoskeletal complex stabilizing filopodia, as long as the inverse correlation between synaptic maturation and filopodia dynamics is represented. For simplicity, only bifurcation of dendritic branches was permitted. The transition between filopodial extension and retraction occurred with a probability of pTr per time. During filopodia retraction, if a filopodium retracted to the point of a synapse, the filopodium was converted to a stable branch, which could not further change in length. Conversely, if the tip synapse of a stable branch was eliminated, the branch was converted into a dynamic branch again. If a filopodium retracted to length of 0, it was eliminated. The angle at which new filopodia extended from a branch was determined randomly with a value between 0 and 90 degrees. The specific range was set to comply with the natural appearance of

dendritic arbor structure, and had no effect on the quantification. An initial condition for simulation was one stable branch with 5 synapses. The initial values of parameters are: pFilo = 0.03 μm-1 min-1. pTr = 0.1 min-1, which were determined based on previous observations (Portera-Cailliau et al., 2003; Niell et al., 2004; Liu et al., 2009).

5.3 Results

5.3.1 Discreet simulation of synaptotropic dendritic arborization

We tested the general performance of this simulation using a field of homogenous probability of synapse formation at 0.02 μm-1 min-1, with equal Ppot and Pdep. As demonstrated in the simulation (Fig. 5.1A), new branches were created, elongated, retracted and eliminated randomly, and synapses were formed and eliminated randomly. Dendritic arbors elaborated relatively slowly during initial stages, with rates increasing over time to rapidly increased arbor size and complexity (Fig. 5.1B). Changes in synapse number showed a similar pattern (Fig. 5.1C). Average branch length per synapse has been used as a measure of arborization efficiency (Niell, 2006). We found that as arbors elaborate, the average branch length per synapse gradually decreased. This growth pattern is consistent with *in vivo* observations of dendritogenesis in tadpole tectal neurons, and also assimilates the previous simulation developed by Neill. We also tested the 'synaptotropic' property of this simulation, which is the ability of dendritic arbor growth towards regions with a higher probability of synapse formation. The simulated dendritic arbor was placed in a field with a probability of synapse formation following normal distribution (Fig. 5.1E). Initial dendritic arborization was highly

dynamic and random, with dendritic process protruding in all directions. Over time, more dendritic branches that were closer to the 'hot region' were maintained, and those further away were lost, leading to gradual arborization towards the preferred region. As the synaptotropic principle directs growth through short range guidance, we quantified the efficiency of directed growth by measuring time or branch length required for forming 100 synapses, with varying distances between the arborization origin and the location of peak synapse formation probability. Branch length and time increased with larger distance (Fig. 5.1F, G).

5.3.2 Synaptic maturation regulates arborization by affecting local filopodial motility

We next examined how arborization is affected by different probabilities of synaptic plasticity. Simulations were performed in fields of uniform probability of synapse formation, but varying probability of synapse plasticity. ΔThreshold (dT) was introduced to indicate the shift of probability from a baseline situation when each synapse formed were subject to equal Ppot and Pdep (dT = 0. Fig. 5.2B). Positive value of dT therefore means shift toward higher Ppot (Fig. 5.2A), and negative dT means higher probability of synapse depression accordingly (Fig. 5.2C). As demonstrated by the representative simulations, higher dT (dT=0.2) led to reduced dynamic dendritic arborization, prohibiting arbor expansion. This pattern mimics the observed dendritic growth pattern of neurons with perturbations that promote synapse potentiation or maturation, such as exogenous overexpression of PKMz or CaMKII. Conversely, decreasing Ppot by decreasing dT (dT=-0.2) led to increased dendritic arbor expansion, which is consistent

with observed dendritic growth of neurons with PKMz or CaMKII activity inhibition (Fig. 5.2C). Branch length required for forming 100 synapses decreased with larger dT (Fig. 5.2D). However, time required for forming 100 synapses showed single-bottom curve over dT, reaching a minimal level at dT around zero, but increased as dT further increased (Fig. 5.2E). A possible reason for this difference is that although potentiation makes a synapse less likely to be eliminated, it also decreases filopodia motility and leads to stunted filopodia extension. Since synapse formation is dependent on filopodia length, promoted synapse potentiation may actually prohibit formation of new synapse. In other words, synapse maturation may promote spatially efficiency of arborization in the expense of temporal efficiency.

5.3.3 Dendritic arborization in fields of heterogeneous probability of synapse maturation

To illustrate how our elaborated synaptotropic model directs dendritic arborization towards formation of most appropriate synaptic connections, we simulated dendritic arborization in a field with spatially heterogeneous probability of synaptic plasticity represented by red (dT=0.2) and blue (dT=-0.2) regions, resembling simplified distribution of correlated and non-correlated synaptic partners, respectively. In this simulation, the arbor initially expanded in every direction, with rapid increase in both arbor size and synapse number. Filopodia appeared more stabilized in the left red region, where synapses were more likely to be strengthened, leading to arbor stabilization. In contrast, filopodia in the right blue region appeared more dynamic as synapses were generally weak in this region. The high motility allowed the arbor to extend further into the

red region, where the arbor elaborated and became stabilized (Fig. 5.3A). Interestingly, although arbor grew dramatically larger in a field of homogenously lower synapse maturation probability, demonstrated in Fig. 5.2, in a heterogeneous field the major proportion of the arbor was present in regions of higher synapse maturation probability, indicating effects from competition between synaptic inputs. Further competition was enforced by applying a maximal value of total synaptic strength the arbor sustains(Turrigiano and Nelson, 2004; 250 for the simulation showed in Fig. 5.3). When this limit was reached, a synapse could not be formed or potentiated before another synapse is depressed or eliminated. When total synaptic strength reached the limit, growth rate of arbor size gradually decreased. Enforced competition drove further elimination of synapses in blue region, leading the arbor complexity to shift into red regions and stabilize with minimal net changes in arbor size and synapse number (Fig. 5.3 B, C).

5.4 Discussion

The synaptotropic model of dendritogenesis describes the inter-relationship between synaptogenesis and dendritic filopodia maintenance, which leads to an iterative process guiding directional dendritic arbor elaboration into regions of appropriate presynaptic partners (Vaughn, 1989). Given the ever-increasing volume and quality of data obtained from imaging real neuronal dendritic arbor growth within intact systems, we have the opportunity to continuously advance our models of these processes and compare them to real neurons. Optimal models do not simply mimic known experimental findings, but can be utilized to conduct virtual experiments. Utilizing a discreet stochastic simulation, here we demonstrate how random growth behaviors of dendritic branches and synapses can be orchestrated by a simple and local rule to produce

complex dendritic arbors. Dendrites function to receive and integrate synaptic inputs. Appropriate dendrite growth is defined as the formation of structures allowing creation of correct synapses to mediate each neuron's computational function. Here, we mimic these synaptic partners as a localized 'hot' region of synapse formation. We demonstrate that the efficiency of the synaptotropic effect is limited by the distance between the hot region and the arbor origin, suggesting that this mechanism acts locally to guide dendritic arborization.

The conventional synaptotropic model represented by previous simulations considers synapses as uniform and bi-modal, present/not present component (Niell, 2006). However, in reality, synapses exist in varying strengths both in their ionic signal transduction and their complement of cytoskeletal scaffolding proteins (Sheng and Hoogenraad, 2007; Holtmaat and Svoboda, 2009). Synaptic strength is likely to be constantly modified by potentiation or depression (Montgomery and Madison, 2004). Also, dynamic dendritic growth is not uniform, and direct observations of dendritic growth find that dendritic filopodial motility is highly variable(Portera-Cailliau et al., 2003; Liu et al., 2009). *In vivo* imaging studies suggest that initial nascent synapse formation is sufficient and critical for filopodial maintenance and allows further extension(Niell et al., 2004). However, synapse maturation is associated with restricted growth, probably by locally regulating growth dynamics of the filopodia on which these synapses reside (Wu and Cline, 1998; Liu et al., 2009). By incorporating graded levels of synapse maturity and strength conferring relative levels of morphological stabilization into a new synaptotropic model, we have been able to represent more realistic patterns of growth. In this new model, formation of nascent synapses confers maintenance to local filopodia, and subsequent increases in synaptic

maturity or strength decrease filopodial motility. Simulations based on this elaborated model show that changing the probability of synapse potentiation dramatically affects arborization. This test simulates real experiments of *Xenopus* tectal neuron growth in which molecular cascades promoting synapse potentiation have been enhanced. *In vivo* imaging and our simulations demonstrate that synapse maturation acts to restrict growth and mediates cessation of dendritogenesis as neurons mature. Mature synapses are associated with decreased filopodial motility and retraction, so further expansion is restricted. Conversely, weak synapses are more prone to filopodial retraction leading to elimination. However, increased filopodial motility allows more probing of the local environment in order to find optimal presynaptic partners to create 'appropriate' circuits.

This notion was further illustrated by simulating dendritic arborization in fields of heterogeneous probability of synapse maturation. Dendritic processes growing into appropriate or 'potentiating' region are stabilized, and further structural rearrangement is restricted. On the other hand, weak synapses present in less appropriate regions allow nascent dendritic processes to probe the environment more actively to reach appropriate targets. The inverse correlation between local structural plasticity and synapse maturity allows only appropriate, or mature synaptic connections to achieve persistent stabilization. This dynamic process suggests competition between uncorrelated and correlated synaptic connections, and results in dendritic arbors shifting towards potentiated region, which was observed in the simulation, especially after the maximal synaptic strength was reached. The resulting patterned dendritic field in our simulation resembles what has been observed in developing neural circuits, including

sensorimotor, auditory, olfactory and visual pathways, and establishing patterned dendritic fields in those regions were found to require activity-dependent competition between synaptic inputs (Wong and Ghosh, 2002; Parrish et al., 2007), suggesting our elaborated synaptotropic mechanism may be a candidate mechanism.

By incorporating synapse maturation, our model is able to simulate the relationship between synaptogenesis and dendrite growth, yet further elaborations are required to create a more realistic model. Dendrite growth is a complicated process involving numerous factors. For example, intrinsic genetic programs and external guidance cues play fundamental roles in directing dendritic orientation and basic dendritic arbor patterning, which may be independent of synaptogenesis (Jan and Jan, 2003). Furthermore, effects triggered through synapses may not be restricted to local actions as implied by the synaptotropic mechanism, but instead may act globally to regulate general dendritic arborization, such as synaptic activity-induced gene transcription(Konur and Ghosh, 2005). In addition, synapse maturation in real neurons *in vivo* is dependent on dynamically changing molecular cascades mediating plasticity, including a developmental increase in expression of CamKII and PKMz (Wu et al., 1996; Liu et al., 2009). As models incorporating more complex biologically-based factors emerge, we will achieve more realistic simulations.

Acknowledgement: I would like to thank Dr. Christopher Niell for help on Matlab programming.

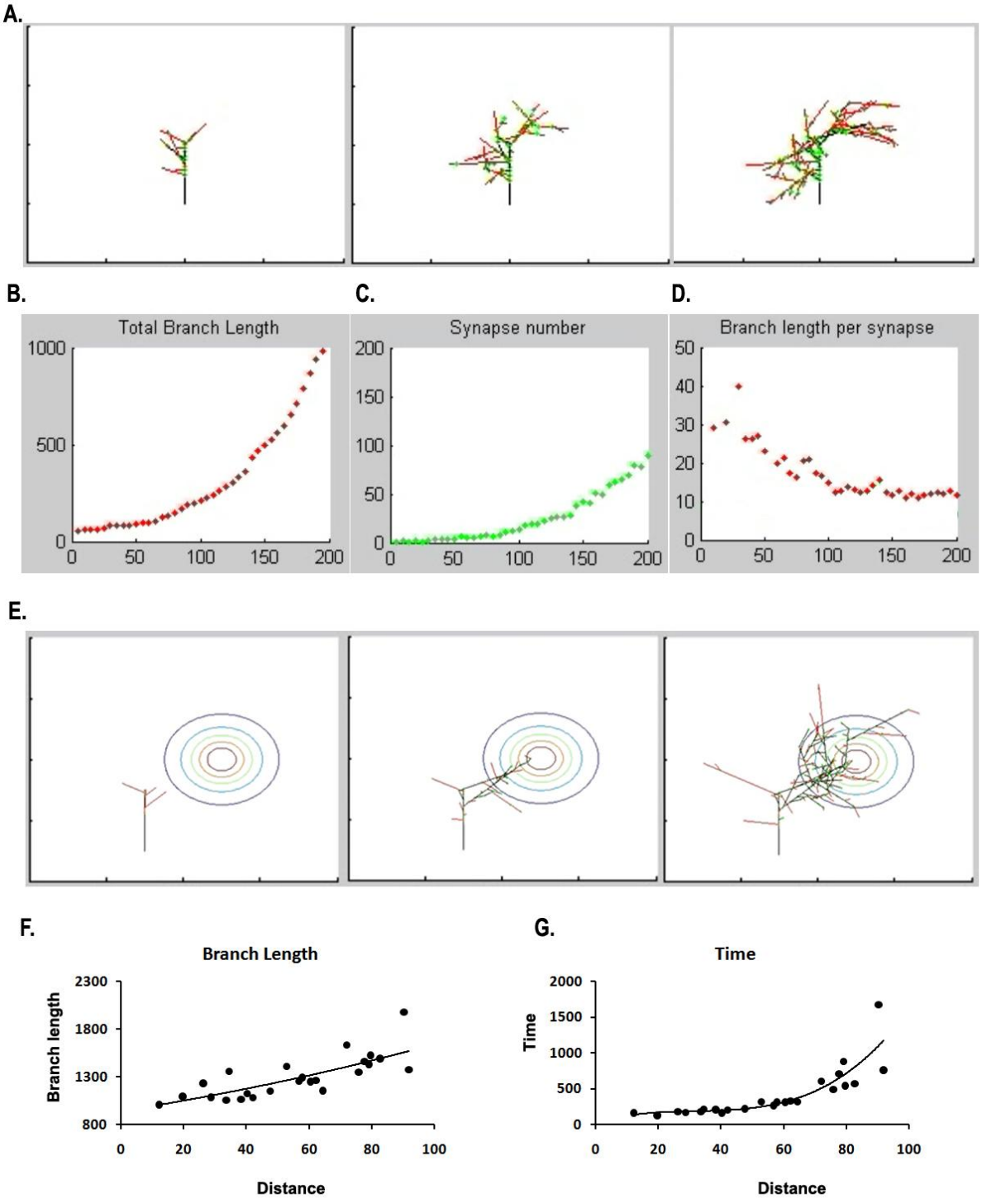

Figure 5.1 | Discreet simulation of synaptotropic dendritic arborization (A) Snapshots from stochastic simulation of synaptotropic dendritic arborization in field of homogenous synapse formation probability. Three images (from left to right) represent early, middle, and final stages of

dendritic arbor. Plots of dendritic arbor length vs. time (B) and total synapse number vs. time (C) indicates increasing rate of dendritic arbor growth. (D) Average branch length per synapse decreases rapidly at early phase and then stabilizes. (E) Snapshots from stochastic simulation of synaptotropic dendritic arborization towards increasing probability of synapse formation. Green dots represent synapses. Red lines represent motile branches, and black lines represent stable branches. The contour map represents normal distribution of synapse formation probability in the field. (F)Branch length or time (G) required for forming 100 synapses with different distances between the arbor origin and the location of maximal synapse formation probability. Each data point represents average value from 3 replications.

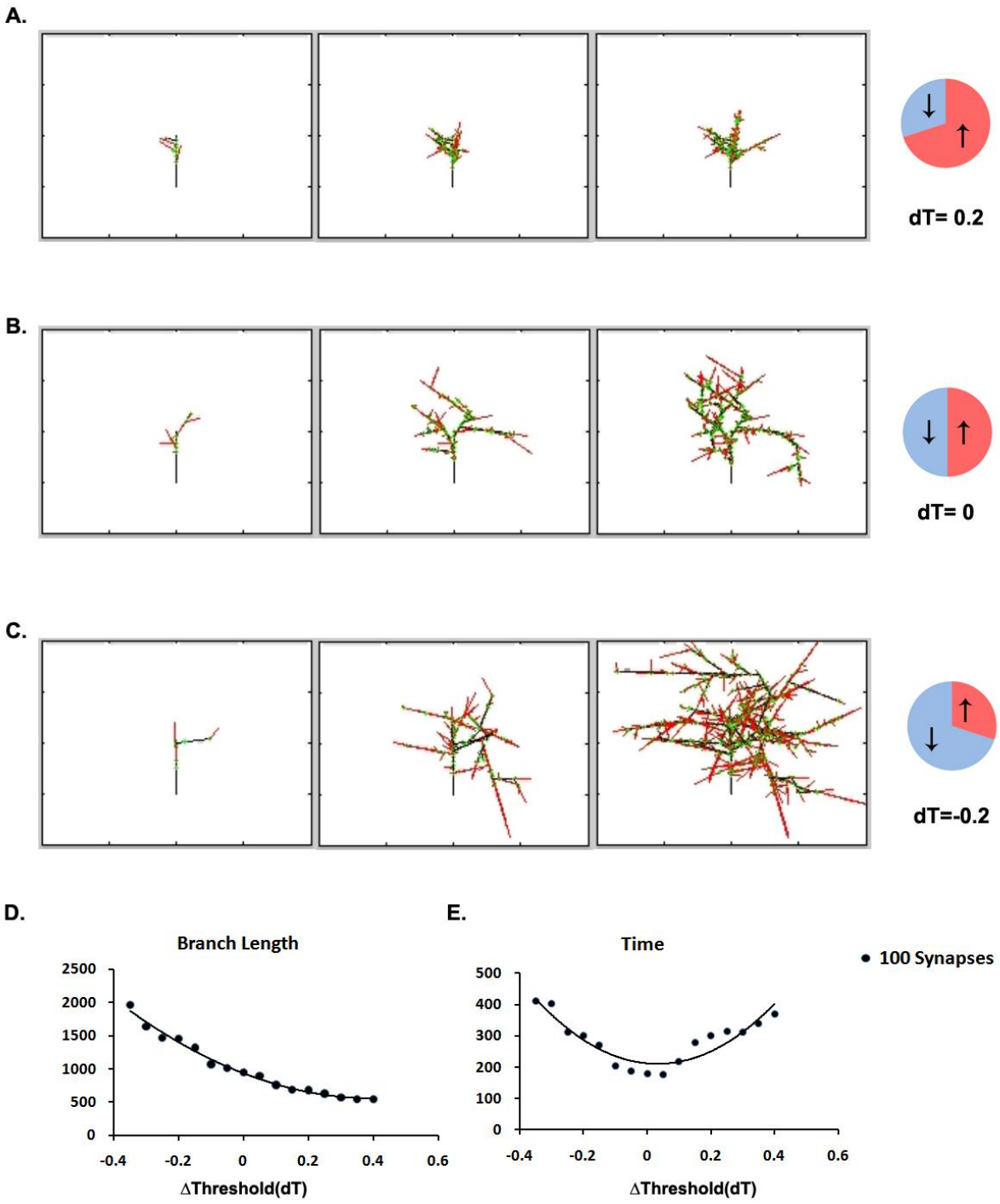

Figure 5.2 | Synaptic maturation regulates arborization by affecting local filopodial motility (A-C) Snapshots from simulation of dendritic arborization with different but spatially homegenous probability of synapse potentiation and depression, which is indicated by the pie charts at the right of each row. Probability of synapse potentiation is represented in red pie, and depression in blue pie. dT (ΔThreshold) indicates probability value shifted from equal probability of synapses potentiation and depression (dT= 0). (D) Time and (E) branch length required for

forming 100 synapses.

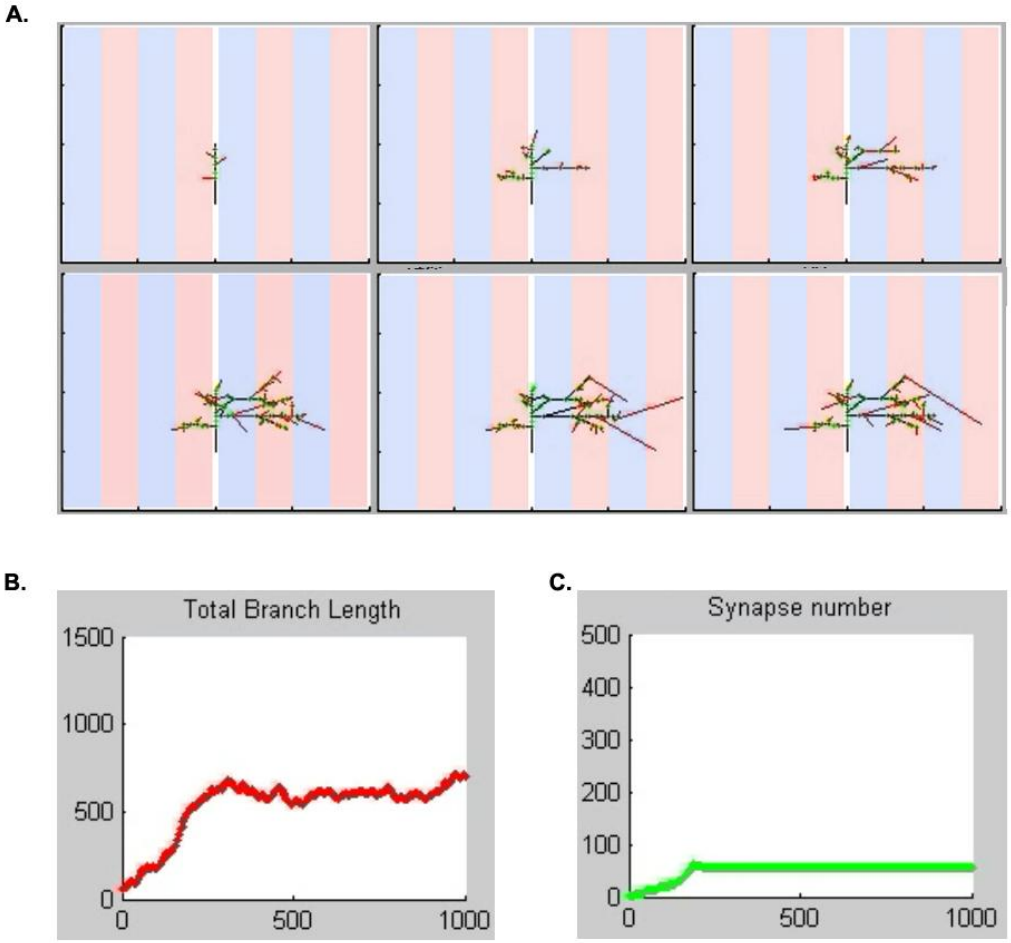

Figure 5.3 | Dendritic arborization in fields of heterogeneous probability of synapse maturation (A) Snapshots from simulation of dendritic arborization with spatially heterogeneous probability of synapse plasticity. dT=0.2 in red regions, and -0.2 in blue regions. (B) Plots of total branch length over time. (C) Plots of total synapse number over time. Maximal total synapse strength was 250 units.

5.5 References

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6. GENERAL DISCUSSION

6.1 Summary of findings

The molecular mechanisms underlying activity-dependent neural circuit growth and plasticity during early brain development remain poorly understood. Mounting evidence supports roles for many molecular components and pathways that have been identified as being involved in adult synaptic transmission and plasticity to also affect various aspects on neuronal development (Rajan and Cline, 1998; Wu and Cline, 1998; Sin et al., 2002; Haas et al., 2006). PKMz, a recently identified endogenous constitutively-active kinase associated with late-phase long-term synaptic potentiation and memory in the mature brain, may be one such molecule (Madronal et al., ; Drier et al., 2002; Ling et al., 2002; Pastalkova et al., 2006; Shema et al., 2007). For my Ph.D. thesis research, I initiated studies to determine the roles of PKMz in neuronal development by using the albino *Xenopus laevis* tadpole as a vertebrate model of early central neural system development. In this process, I cloned *Xenopus* PKMz from tadpole brain lysate and found its putative amino acid sequence is highly homologous to PKMz from other species, ranging from mouse to human. I also discovered that the expression of PKMz is developmentally regulated in the embryonic *Xenopus* retinotectal system with progressively increasing levels from Stages 35 to 50 corresponding to peak periods of dendrite growth and synaptogenesis. I employed *in vivo* rapid and long-interval two-photon time-lapse imaging of actively growing *Xenopus* tectal neurons and post-imaging comprehensive three-dimensional tracking of dynamic and persistent dendritic growth behavior. From these studies I found that

altered PKMz activity affects morphologic growth by regulating dendritic filopodia stabilization. Exogenous over-expression of PKMz within single neurons stabilizes dendritic filopodia evident by increased dendritic filopodial lifetimes and decreased filopodial additions, eliminations, and motility. By using long-term *in vivo* imaging I found that these alterations in dynamic growth behavior culminate to restrict dendritic arbor expansion of the mature neuron. In contrast, I found that blocking endogenous PKMz activity in individual growing tectal neurons with an inhibitory peptide, zeta-inhibitory peptide (ZIP) acutely destabilizes dendritic filopodia and over long periods promotes excessive arbor expansion.

Previous work on adult rat brain has found that PKMz activity potentiates glutamatergic synapses by promoting synaptic integration of AMPARs (Ling et al., 2006; Yao et al., 2008). Therefore, I conducted studies to determine whether and how endogenous PKMz is involved in the development of retino-tectal synapses in tadpole brain. I found that inhibiting endogenous PKMz by tectum infusion of ZIP decreases colocalization of the immunostained presynaptic and postsynaptic markers, SNAP-25 and PSD-95, respectively, suggesting impaired synapse maintenance. Moreover, using *in vivo* patch clamp electrophysiological recording methods, I directly examined how endogenous PKMz activity affects functional development of retino-tectal synapses in tadpole brain. I found that down-regulation of endogenous PKMz in optic tectal neurons, by introducing PKMz specific morpholino synthetic oligonucleotides into the tectum, impairs AMPAR-mediated transmission. I found that blocking PKMz results in a significant decrease in AMPAR mEPSCs amplitude, indicating a decrease in synapse maturation. From conducting evoked paired-pulse stimulation of the optic nerve, I found no change in the ratio of

paired-pulse responses, indicating no presynaptic change in probability of release. Moreover, I examined evoked synaptic AMPAR and NMDAR responses and I found that the ratio of the amplitudes of evoked AMPAR and NMDAR responses (AMPA/NMDA) is significantly reduced following PKMz knock-down by Morpholino treatment, indicating synapse maturation is deterred by PKMz knock-down. Further analysis shows that this ratio change is due to a selective reduction in the AMPAR response, with no change in NMDAR responses. My long-term time-lapse imaging finds that morphologically, PKMz knock-down by morpholino treatment leads to increased or unrestricted, dendrite growth in tectal neurons. Together, these results demonstrate critical roles of PKMz in promoting synapse maturation, which may contribute to synaptotropic stabilization of growing dendritic processes underlying maturation-associated halting of dendritic arbor expansion.

I further explored the above model by examining how PKMz is involved in dendritogenesis of tectal neurons in tadpoles exposed to PTZ-induced seizure activity. Rapid imaging reveals that within minutes of seizure onset, existing labile dendritic processes are destabilized and eliminated, while new processes emerging during seizures become hyper-stabilized. Notably, individual neurons within the seizing brain can be protected from seizure-induced hyper-stabilization by reducing synaptic AMPAR expression or by reduction of PKMz. PKMz expression was initially reduced during seizure onset, but recovered to baseline levels within 2 hours. Since seizures significantly reduce brain neuron arbor size and synapse number during this same period, we believe that this results in an increase in PKMz concentration relative to the number of synapses or dendrite area remaining. Together, these results indicate that excessive

excitatory synaptic transmission during seizure may promote up-regulation of PKMz at dendrites, which may account for hyper-stabilization of dendritic filopodia during seizures.

The synaptotropic model of dendritogenesis provides a potential mechanism for highly complex neural networks to form by self-organization driven by patterned neuronal activity through these developing circuits. In this model dendritic arbor growth proceeds in an iterative fashion through local refinement by synaptogenesis in a trial-and-error manner (Vaughn, 1989). Previous *in vivo* time-lapse Imaging of dendrite growth, as well as our work on PKMz has provided evidence for such a model (Niell et al., 2004; Cline and Haas, 2008). Here, I carried out a computational analysis to further investigate and advance this model. Using discreet stochastic simulation, I show that as synapse formation guides growth of dendritic branches towards appropriate targets by promoting local filopodia maintenance, synapse maturation regulates arbor expansion rates by affecting local filopodia motility. Furthermore, by applying the simulation in an enforced competitive environment, I show this elaborated synaptotropic mechanism could direct efficient growth and consolidation of dendritic arborization towards preferred targets.

6.2 Overall significances

6.2.1 Identification of PKMz's function during brain development

Previous studies have identified important roles of PKMz in neural plasticity and function in mature neural circuits. My thesis work expands our understanding of PKMz's function during development. It has been hypothesized that signaling mechanisms underlying maturation of

developing synapses are shared with well-studied pathways mediating synaptic plasticity in mature neural systems (Constantine-Paton and Cline, 1998). Accordingly, molecular components essential for induction of synaptic LTP, such as CaMKIIα, have been found critical for developmental glutamatergic synapse maturation (Wu et al., 1996). My research on PKMz therefore provides additional evidence for this hypothesis by showing conservation of molecular mechanisms mediating late-phase LTP. Furthermore, this work is the first time that the morphological effects from PKMz activity have been studied in neurons. By utilizing short- and long-interval imaging and comprehensive post-imaging quantification of dendrite growth, I show that PKMz activation affects growth through stabilization of dendritic filopodia, and restricts dendritic arborization over longer periods. My findings are surprising as they counter the original model that suggests that enhanced synaptic maturation would promote enhanced dendritogenesis. This discrepancy has led me to modify the synaptotropic model incorporating a graded effect of synapses on morphological stabilization, in which immature synapses prevent filopodial retraction, but mature synapses act as a “stop” signal to prohibit filopodia dynamics, stabilizing the appropriate synaptic connections. Gradual strengthening of synapses on a neuron may confer the stabilization of entire arbor as the neuron matures.

It is possible that my findings on the morphological roles of PKMz may also shed light on the structural mechanism underlying its critical roles in adult brain function, such as memory maintenance. Previous studies suggest that persistent storage of information may rely on structural stabilization (Lamprecht and LeDoux, 2004).

6.2.2 Insights on the origins of neurological disorders

There is mounting evidence that many common neurological and psychiatric disorders emerging later in life, including schizophrenia and epilepsy have origins during early brain development (Caplan, 1995; Opler and Susser, 2005). It is possible that some of these disorders arise from abnormal activity-dependent growth and synaptogenesis, which produce aberrant neural networks susceptible to emergence of dysfunction. My work on PKMz demonstrates one example of how perturbed synaptic activity affects long-term circuit formation. Furthermore, using a seizure model based on *Xenopus* tadpoles, we directly investigated how excessive neural activity during seizures affects endogenous PKMz expression, and how PKMz activity is involved in the aberrant dendritogenesis induced by seizure activity. We find that seizures may hyper-stabilize dendritic filopodia through activation of PKMz. In this way, seizure activity may strengthen synapses and dendritic structures that are activated by the abnormal seizure activity. Our findings that these abnormal growth patterns are persistent suggest a mechanism by which an early-life seizure has lasting effects on circuit dysfunction that may underlie development of future brain disorders including epilepsy.

6.2.3 Elaboration of a theoretical model of dendritogenesis

The synaptotropic model of dendritogenesis has been successful in describing mechanisms mediating formation of optimized dendritic arbor growth based on external environmental input. The conventional mechanism underlying synaptotropic-directed dendritic growth focuses on the interrelationship between filopodia maintenance and synapse turnover (Niell, 2006), and does

not explain how dendritic arbors become stabilized with neuronal maturation. By directly examining concurrent effects of PKMz activity on synapse maturation and dendritic arborization, my research provides additional evidence supporting a modification of this model. In this elaborated model, while nascent synapses may confer filopodial maintenance, synapse maturation by PKMz directs filopodial stabilization. Gradual maturation of appropriate synapses leads to consolidation of optimal dendritic structure as a neuron matures. In my work, I have used a computational model to illustrate this elaboration of the synaptotropic model. The simulation is more biologically realistic than the original model and can reproduce more natural patterns of dendritic arborization.

6.3 Future directions

6.3.1 Downstream signaling mechanism linking synapse maturation with cytoskeleton dynamics

My thesis work provides evidence that PKMz activity underlies developmental dendritic arbor morphological plasticity by promoting maturation of retinotectal synapses. However, the downstream molecular mechanisms linking PKMz-induced synapse maturation to enhanced cytoskeleton stability remain poorly understood. One candidate mechanism, as proposed from studies on rat hippocampal slice cultures, is that synapse strengthening may leads to enhanced local calcium transients, which in turn activate local Rho GTPases to stabilize the filopodia cytoskeleton (Lohmann et al., 2002; Lohmann et al., 2005). In future studies, this possibility could be examined by monitoring dendritic localized calcium transients using *in vivo* calcium

imaging in neurons with experimentally-altered levels of PKMz activity, or examining if inhibitors of internal calcium release block the stabilization effect induced by PKMz over-expression.

6.3.2 PKMz's effect on functional properties of tectal neurons

My research has focused on how PKMz affects synapse development and dendritic morphology of individual growing tectal neurons. One future direction of this work would be to examine the functional significance of the effects I have characterized, either on individual optic tectal neurons or the larger visual circuit. For example, one could measure visual stimulation induced composite currents from tectal neuron with perturbed PKMz activity, and examine how PKMz affects recruitment of neurons into the visual circuit (Chiu et al., 2008). Furthermore, receptive field properties of tectal neurons were found to undergo activity-dependent developmental refinement, characterized by a gradual reduction in size and increase in response amplitude. Also, there is evidence that receptive field refinement of tadpole tectal neurons shares mechanism with activity-dependent synapse maturation (Lim et al., 2010; Dong et al., 2009). Therefore, it will be interesting to examine whether PKMz activity is involved in tectal neuron receptive field refinement. One potential experiment would be to map changes of receptive field refinement of neurons with altered levels of PKMz activity, or examine how tectal infusion of the PKMz inhibitory peptide ZIP affects performance of behaviors associated with visual circuit development (Dong et al., 2009).

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APPENDIX A: A NON-RADIOACTIVE ASSAY FOR PROTEIN KINASE/PHOSPHATASE ACTIVITY [5]

A.1 Introduction

Protein kinases are enzymes that catalyze the transfer a phosphate from a donor molecule, such as ATP, to a target protein, in a process called 'phosphorylation'. Protein kinases recognize unique amino acid sequences and structural motifs on their target proteins, termed the kinase substrate. Kinases are one of the largest groups of enzymes, and approximately three to four percent of the human genome encodes genes for protein kinases, with more than 500 kinases already identified (G. Manning et al., 2002). Protein phosphatases appose the action of kinases by removing a phosphate from a phosphorylated protein, and represent another large group of enzymes. It has been estimated that humans have approximately 1,000 protein phosphatase genes (T. Hunter, 1995). The recent genome sequencing projects and developments in molecular biology techniques have led to the continuous discovery of new kinases with uncharacterized biochemical properties and substrate specificity.

Due to their physiological relevance, variety and ubiquitous expression, protein kinases and phosphatases have become two of most important and widely studied families of enzymes in biochemical and medical research. Studies have shown that protein kinase/phosphatase

[5] A version of this chapter will be submitted for publication: Liu XF, Haas K (2010) A non- radioactive assay for protein kinase/phosphatase activity

regulation of proteins are key to many cell functions, including signal transduction, transcriptional regulation, cell motility, and cell division(E. H. Fischer, 1997, B. M. Sefton, 2001, S. Shenolikar, 2007). Several oncogenes have also been shown to encode protein kinases/ phosphatases, suggesting implication of protein phosphorylation with cancer (B. Drobic et al., 2004, J. E. Hutti et al., 2009, H. H. Yeh et al., 2009) .

A sensitive assay measuring the activity and substrate motif profile of existing and newly identified kinases/phosphatases therefore, would be of great value. Traditional protein kinase assay methods can be divided to two major types: radioactive and non-radioactive. Radioactive methods involve the use of radiolabeled ATP as a phosphate donor, and a synthetic peptide as substrate containing the respective kinase recognition motif (B. Deus and H. E. Blum, 1974, D. Tritsch et al., 2004). Following the kinase reaction the substrate peptide is immobilized on a phosphocellulose paper via ion exchange, followed by wash to remove free radioactive ATP. Radioactivity incorporated into the substrate peptide is detected by scintillation counting. Radioactivity-based assays are relatively simple, and reasonably sensitive. However, radiometric assays have several drawbacks, including the generation of radioactive waste and the short half-life of radioactive ATP. To avoid problems in dealing with radioactivity, some non-radioactive kinase assay methods have been developed, which rely on detection of phosphorylation by specific phosphopeptide antibodies (H. Togame et al., 2005, H. H. Versteeg et al., 2000). However, sensitivity, specificity and target kinase range of use are usually limited by qualities of antibodies and kinetics of antibody binding.

In the method we described here, we use a ferric iron (Fe3+) conjugated molecule as a

universal probe for phosphorylation. Ferric iron is well known to interact with phosphoproteins, phosphopeptides, organophosphates and other phosphorylated (R-PO3) molecules. The affinity of ferric ion to phospho-groups is extremely high, with a Ka reported to be greater than 10^{13} /M. Ferric iron bound to solid phase affinity chromatography resins has formed the basis for phosphocompound purification since the mid-1980's(L. Andersson and J. Porath, 1986, M. Belew and J. Porath, 1990). Ferric iron (Fe 3+) is usually introduced in such reactions in a chelated form either by nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA). When labelled with HRP, the conjugate can act as a probe for phosphorylated molecules. High binding affinity, smaller size, and simply detection mechanism make it an ideal probe for kinase/phosphatase assays. Beside phosphate group, carboxyl group could also bind the iron derivatives (M. Zachariou and M. T. Hearn, 1995). Therefore, a blocking process that converts free carboxyl group into terminal amino groups using ethylenediamine dihydrochloride is necessary when significant amount of free carboxyl groups are present in the reaction system, such as the substrate peptide.

It is worth noting that assay methods for kinases and phosphatases are usually identical only except that kinase assay measures phosphate transfer onto an initially non-phosphoylated substrate, while phosphatase assay measures phosphate removal from an initially phosphorylated substrate.

A.2 General protocol

<*Note*> ***This protocol is for kinase assay. Adjust for phosphatase assay as follows:***

1. Change the immobilized substrate peptide from kinase substrate to phosphatase substrate, which is a phosphorylated peptide.

2. Buffer recipe and condition for phosphatase reaction should be changed accordingly. No nucleoside triphosphate is needed.

3. The results should be interpreted in opposite to what is acquired from kinase assay. The negative control always has the highest reading. The lower the signal, the higher the activity of phosphatases is.

A.2.1 Material

Equipment:

- Streptavidin or avidin coated microplate (e.g. SigmaScreen™ Streptavidin coated plates);
- Microplate reader for absorbance measurement (e.g. BioTek absorbance microplate reader);
- Multichannel pipettes (e.g. Eppendorf Research® Multichannel Pipettes);
- Microplate shaker (e.g. Cole-Parmer Compact Economy Orbital Microplate Shaker);
- Incubator (e.g. Thermo Precision Economy Incubator);

Reagent:

- Biotinylated synthetic peptide substrate;
- TBS buffer: 50 mM Tris Base, 0.9% NaCl, pH 7.6;
- TBS/T buffer: TBS with 0.05% Tween-20;

- Reaction buffer: use according recipe based on specific properties of the kinase being assayed ;

  <Note> e.g. PKC reaction buffer: PKC reaction buffer: 20mM HEPES (pH 7.4), 1.5mM CaCl2, 1mM DTT, 10mM MgCl2

- Nucleoside triphosphate (ATP or GTP);
- MES buffer: 0.1 M MES (2-[N-morpholino]ethanesulfonic acid), 0.9% NaCl; pH 7.4;
- Blocking Buffer: MES Buffer plus 0.5 M Ethylenediamine Dihydrochloride (EDA) and 25 mM 1-ethyl-3'- [3dimethylaminopropyl]-carbodiimide hydrochloride (EDC) ;
- Horseradish peroxidase conjugated phosphorylation probe: (Fe3+) (IDA) –HRP (INDIA^{TM}Phosphoprobe-HRP, Pierce, IL)
- Quench Buffer: MES Buffer plus 50 mM EDA ;
- Acetate Wash Buffer: 0.1 M sodium acetate, 1.0 M NaCl; pH 5.0 ;
- Detection Reagent: 3,3', 5,5"-tetramethylbenzidine(TMB) solution ;
- 1M H_2SO_4;

A.2.2 General procedure

1. Coating

   Make solution of biotinylated peptide substrate in TBS buffer at concentration of 2.5 µM. For maximal capacity, 200 µL of solution will be added to each well of a 96 well streptavidin coated plated. Coating can be done at 4℃ overnight or at room temperature for 1 hour on a shaker.

<Note> *High Capacity microplate is desired for larger range of measurement*

2. Aspirate and wash 3 times with TBS/T buffer to remove unbound peptide
3. Reaction

   Add solutions containing kinase, reaction buffer and nucleoside triphosphate in coated wells and enable reaction in desired conditions.

   <Note> *Moderate shaking is desired to facilitate reaction.*
4. Aspirate and wash 3 times with MES buffer

   <Note> *A thorough wash is critical for reducing background.*
5. Blocking

   Add 200 µL freshly made Blocking Buffer in each well and incubate at room temperature for 30-60 min.

   <Note> *Optional if purified kinase and carboxyl free substrate are used*

   <Note> *Optimal blocking time should be determined for each reaction.*
6. Aspirate and wash 3 times with Quench Buffer, rinse once with Acetate Wash Buffer.
7. Probe binding

   Solve the phosphorylation probe in Acetate Wash Buffer at concentration of 10 µg/mL supplemented with 0.05% Tween-20. Aspirate the wash buffer from the plates and add 200 µL of the solution in each well and incubate at room temperature for 45 min.
8. Aspirate and wash with Acetate Wash Buffer for more than 4 times

   <Note> *A thorough wash is critical for reducing background.*

9. Aspirate and add 200 µL Detection Reagent, incubate on a shaker with plate protected from light for 15 min. Add 1M H_2SO_4 to stop development, and read the absorbance at A_{450} nm using a microplate reader. Also measure background value at $A_{550.}$ (Fig.A.1)

   <Note> *Simultaneous addition of Detection Reagent is desired. Use a multichannel pipette if available.*

   <Troubleshooting>

10. Relative kinase activity is represented by A_{450}-A_{550}.For actual kinase activity. Fit the value in a stand curve acquired in parallel experiments using pure kinase. (Fig. A.2)

A.2.3 Trouble shooting

Problems (Step 9):

1. Low signal

Solution: Increase concentration of iron probe; Change reaction buffer; Use fresh reagent, e.g. ATP; Decrease blocking time;

2. High background

Solution: Decrease concentration of iron probe; Wash thoroughly for extra rounds; Increase blocking time;

A.3 Other implementations

The above general protocol could be modified for other specific purposes, and standardized procedure could be developed accordingly:

A.3.1 For screening kinases affected by a treatment

Biological activities involve action of multiple kinases. Therefore, it is of interest to dissect kinases affected by a certain treatment or physiological process. For this purpose, individual biotinylated substrate peptides for kinases to be profiled are immobilized onto each well of a microplate. After desired physiological or pharmacological treatments, the biological sample is homogenized. Tissue extracts or cell lysates from both control and treatment groups, either crude or partially purified, are added to neighbor wells to enable kinase reaction. The plate is then processed as described in the general procedure. Kinases that are affected could be assessed by compare the colormetric response between two neighbor wells coated with same correspondent substrate.

<Note> Use universal kinase reaction buffer if possible.

A.3.2 For profiling substrate motif of a known or novel kinase

For this application, a number of peptides are synthesized based on putative substrate motif sequences, biotinylated and immobilized on each well of the microplate. Standard procedure is then performed with the purified kinase and proper reaction conditions. Phosphorylation efficiency of each motif can be assessed based on respective colormetric readings.

<Note> *Length of the peptide affects reaction efficiency. Include 10 spacing amino acids between putative phosphorylation site and biotin terminal if possible.*

A.3.3 For screening candidate modulators for a kinase

One of the most important applications for a high throughput kinase assay platform is to screen modulators (activators or inhibitors) of a target kinase. For this purpose, a specific substrate peptide is biotinylated and immobilized on a microplate. Following addition of modulator candidates into individual wells, kinase reaction mix containing purified kinase, nucleoside triphosphate and proper reaction buffer is added and incubated with the immobilized substrate. Following procedures as described in the general protocol, efficiency of each modulator can be assessed based on its colormetric readings.

<Note> *Concentration of ATP or other ingredients in buffer may affect efficiency of some modulators. For an example, see Fig. A.3.*

A.4 Discussion

Here we described a novel non-radioactive assay method for kinases and phosphatases. This method has several advantages compared to other currently available methods. Compared to conventional radioactive assay methods, this method has a higher safety margin and is more environmentally friendly. Also, since this assay can be performed in microplates, it is easy to be automated, and therefore has a high throughput screening (HTS) capability. Compared to non-radioactive assay methods that utilize specially designed and treated substrate peptides to satisfy the special condition required for detection, this method uses common, well-established peptide substrates, so specificity and efficiency of kinase reaction is increased. Also, biotinylated forms of those peptides are readily available, making this method more accessible.

Compared to other non-radioactive assay methods that utilize specially designed antibodies to detect phosphorylated residues, the iron derivative probe used in our method has several benefits: First, the iron probe has much higher affinity to phosphorylated target than antibodies, and is therefore more sensitive. Second, the smaller size of the probe leads to less steric hindrance which can prohibit reaction between a kinase molecule and its immobilized substrate. Third, compared to the high cost of an antibody, the iron probe is more cost effective. Moreover, the same probe can be used for all kinds of phosphorylated targets, making this method a universal solution.

However, the efficiency of this novel assay is affected by various factors, which requires careful consideration and further optimization. The blocking process is important for reducing false positive signals caused by free carboxyl units on substrate peptides. However, excessive blocking leads to decreased signal and assay range. In addition to pre-experiments to determine proper blocking conditions, it is also helpful to optimize peptide design to reduce sources of noise. Furthermore, we found blocking is not necessary if purified kinase/phosphatase is used in the reaction. To facilitate reaction between enzyme and its immobilized substrate, it is useful to include a spacing region in the peptide to reduce steric hindrance.

A limitation for this method is that it currently can only conveniently be employed for endpoint measurements. For measurements of enzyme reaction kinetics, multiple parallel reactions can be terminated at different time points. Then kinetic curves can be reconstituted by assaying phosphorylation of those reactions.

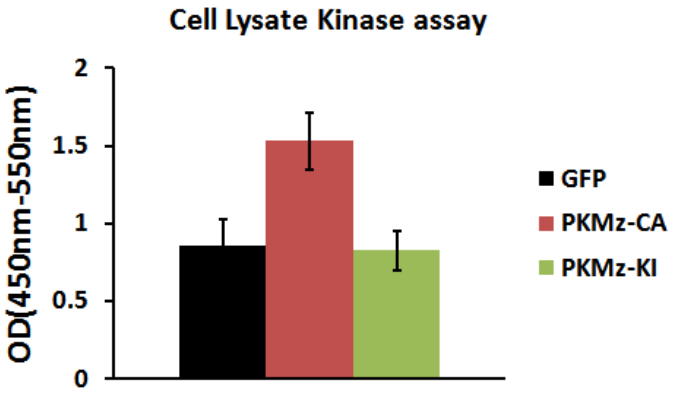

Figure A.1 | Assay of Protein Kinase M zeta (PKMz) activity from HEK293 cell lysates. A specific PKMz substrate peptide was used. PKMz activity of lysates from HEK293 cells expressing green fluorescent protein (GFP), PKMz (PKMz-CA), and inactive PKMz (PKMz-KI). The graph showed dramatic increase of PKMz activity in PKMz-CA group, compared to GFP and PKMz-KI groups.

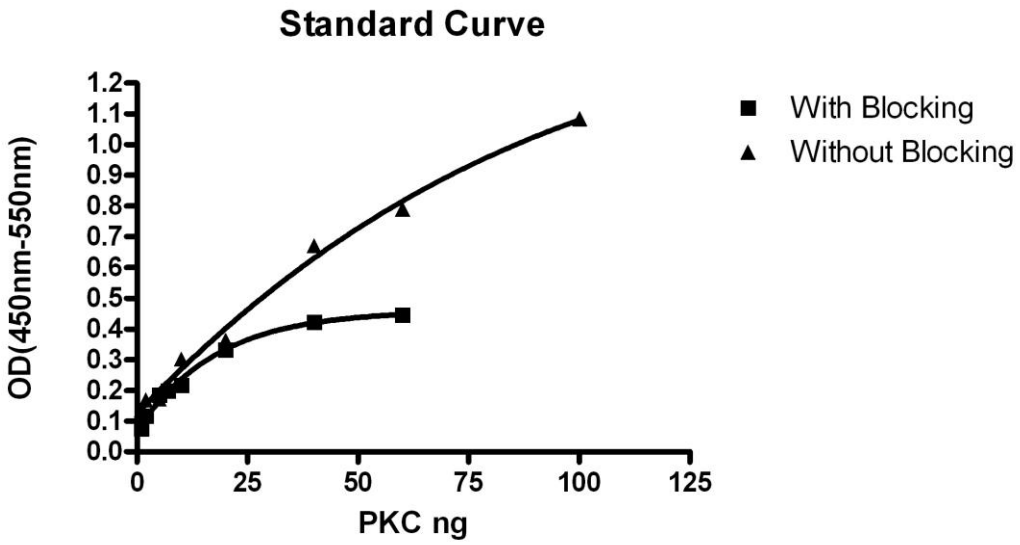

Figure A.2 | Standard curve of PKC (Promega, WI) activity, with or without blocking process. (Biotin)- RFARKGSLRQKNV (Anaspec, CA) was used as substrate. A higher linear range was achieved without blocking process.

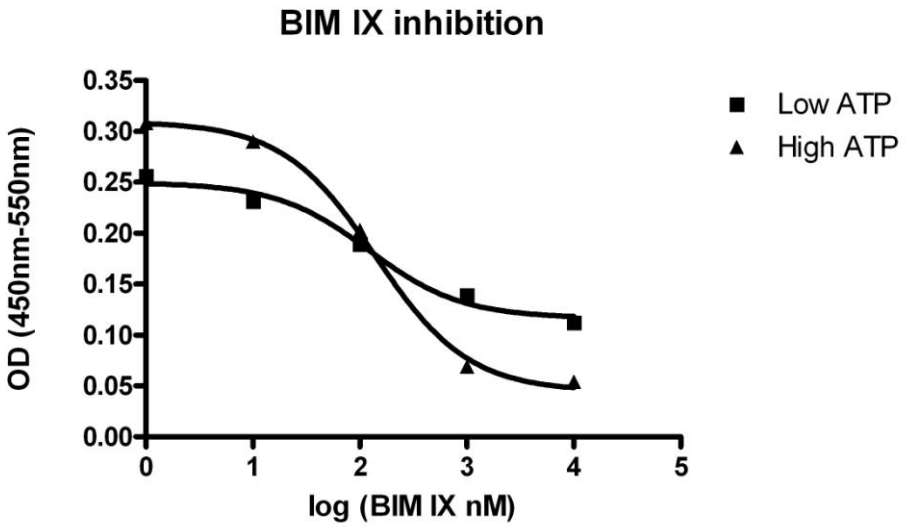

Figure A.3 | Inhibition curve of bisindolylmaleimide IX (BIM IX, Lclabs, MA) at low (1mM) or high (10 mM) ATP concentration. 25 ng PKC was used in each reaction. (Biotin)-RFARKGSLRQKNV was used as substrate. BIM IX inhibits PKC by competitively binding to its ATP binding site. Therefore its potency is affected by ATP concentration.

A.5 References

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APPENDIX B. ETHICS BOARD CERTIFICATES

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A09-0021

Investigator or Course Director: Kurt Haas

Department: Cellular & Physiological Sc.

Animals:

Frogs Xenopus Laevis 60

Approval Date: February 12, 2009

Funding Sources:

Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	In vivo imaging stimulation-evoked dendritic arbor growth and synaptogenesis in the CNS: role of synaptic transmission and downstream signalling in brain circuit development
Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	Role of NMDA receptor-mediated transmission on dendritic growth in the intact brain - implications for the developmental origin of schizophrenia and epilepsy
Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	An in vivo model of abnormal neuronal circuit information: the role of glutametergic synaptic transmission in dendritic arbor growth and synaptogenesis
Funding	Michael Smith Foundation for Health Research

Agency:	Michael Smith Foundation for Health Research
Funding Title:	In vivo imaging of activity-dependent synaptogenic events between dynamic axonal and dendritic filopodia within the developing brain - connections to the development of schizophrenia and highly common neonatal siezures
Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	An in vivo model of abnormal neuronal circuit formation: The role of glutamatergic synaptic transmission in dendritic arbor growth and synaptogenesis
Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	An in vivo model of abnormal neuronal circuit formation: The role of glutamatergic synaptic transmission in dendritic arbor growth and synaptogenesis
Funding Agency:	EJLB Foundation
Funding Title:	In vivo imaging of the formation of dysfunctional brain circuits: critical role of glutameatergic transmission in dedritic growth and synaptogenesis
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above breeding program.

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0042

Investigator or Course Director: Kurt Haas

Department: Cellular & Physiological Sc.

Animals:

Frogs Xenopus laevis tadpoles 20000

Start Date: **February 17, 2007** **Approval Date:** **June 22, 2009**

Funding Sources:

Funding Agency:	Epilepsy Canada
Funding Title:	Summer Studentship/Simon Chen/Effects of excessive activity on dendritic arbor growth
Funding Agency:	UBC Human Early Learning Partnership (HELP)
Funding Title:	Real-time Imaging of Brain Circuit Formation within the Developing Brain
Funding Agency:	British Columbia Ministry of Children and Family Development
Funding Title:	HELP: Direct measurements of how environmental stimuli sculpt developing brain structure and function
Funding Agency:	EJLB Foundation
Funding Title:	In vivo imaging of NMDA receptor-mediated control of dedritic growth and synaptogen
Funding	

Funding Agency:	National Alliance for Research (US)
Funding Title:	In vivo imaging of dendritic structural development A test of the glutamate hypofunction model of schizophrenia
Funding Agency:	UBC Faculty of Medicine
Funding Title:	Start up grant
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Facility for imaging and recording of neuronal development and synaptogenesis
Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	An in vivo model of abnormal neuronal circuit formation: The role of glutamatergic synaptic transmission in dendritic arbor growth and synaptogenesis
Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	An in vivo model of abnormal neuronal circuit formation: The role of glutamatergic synaptic transmission in dendritic arbor growth and synaptogenesis
Unfunded title:	n/a

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

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