THE ROLE OF ADENYLYL CYCLASE SIGNALING PATHWAYS IN DEVELOPMENTAL DENDRITOGENESIS *IN VIVO*

by

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B.Sc., University of California, Davis, 2005

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)

April 2012

Abstract

The period of early brain development involves an exceptional amount of neuronal morphological growth and refinement to form functional brain circuits. Although it is known that neural activity influences dendrite morphogenesis, the molecular pathways which convert a neural activity input to changes in morphology are not well understood. Here I show that activation of the adenylyl cyclase pathway promotes growth of developing brain neurons *in vivo*, in a neuron maturation-dependent manner. Rapid time-lapse two-photon imaging of single neuron growth within the developing vertebrate brain and pharmacological manipulations reveal a synergistic role for PKA and Epac in growth downstream of β-adrenergic receptors and adenylyl cyclase. Inhibition of the protease calpain increases axonal and dendritic filopodial density, but only in axons is this effect downstream of PKA. Furthermore, experiments indicate that PKA localization by AKAPs may be important in its regulation of dendritogenesis. Together, the results presented here outline multiple steps of a signaling pathway important in dynamic dendritogenesis and axogenesis *in vivo*.

Preface

All experiments in this thesis were performed and analyzed by myself. The analysis software used for dendritic morphometrics was programmed by Dr. Jamie Boyd and Shay Neufeld.

All experimental procedures were conducted according to the guidelines of the Canadian Council on Animal Care, and were approved by the Animal Care Committee of the University of British Columbia, certificate number A11-0332.

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List of Abbreviations

5-HT: Serotonin

8-CPT: 8-CPT-2'-O-Me; 8-(4-chlorophenylthio)-2'O-methyl-cyclic monophosphate

AC: Adenylyl cyclase

A-CREB: Dominant negative CREB

ADF: Actin depolymerizing factor

AKAP: A-kinase anchoring protein

ALLM: N-Acetyl-L-leucyl-L-leucyl-L-methioninol

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ARF: ADP-ribosylation factor

Arp2/3: Actin-related protein 2/3

β-AR: β adrenergic receptor

BDNF: Brain-derived neurotrophic factor

BFA: Brefeldin A

BMP-7: Bone morphogenic protein 7

BTN: Branch tip number

CaMKII: Calcium/calmodulin-dependent protein kinase II

CaMKIV: Calcium/calmodulin-dependent protein kinase IV

cAMP: Cyclic adenosine monophosphate

cAPK: cAMP-dependent protein kinase

cGMP: Cyclic guanosine monophosphate

CICR: Calcium-induced calcium release

CNG: Cyclic nucleotide gated ion channel

CPN: Calpain

CPN2: Calpain 2 overexpression construct

CREB: Cyclic-AMP-responsive-element-binding protein

CREB-DIEDML: Constitutively active CREB

CREST: Calcium-responsive transactivator

D1/D5: Dopamine receptor isoform 1 / isoform 5

da: Dendritic arborization

DCC: Deleted in colorectal cancer

ddFsk: Dideoxyforskolin

Dia2: Diaphanous-related formin 2

DIV: Days *in vitro*

DRG: Dorsal root ganglion

dTC: d-Tubocurare

Ena: Enabled

Epac: Exchange protein activated by cAMP

Epac2ΔCBR: Constitutively active Epac

EpacG114E/G422D: Dominant negative Epac

EPSP: Excitatory post-synaptic potential

ERK: Extracellular-signal regulated kinase

ES: External sensory neuron

fEGFP: Farnesylated enhanced green fluorescent protein

Fsk: Forskolin

FRET: Fluorescence resonance energy transfer

GABA: Gamma-aminobutyric acid

GAP: GTPase-activating protein

GAP43: Growth associated protein 43

GCF: Growth cone filopodia

GEF: Guanine exchange factor

GPCR: G-protein coupled receptor

GRIP1: Glutamate receptor-interacting protein 1

HCN: Hyperpolarization-activated cyclic nucleotide gated ion channel

HDAC5: Histone deacetylase 5

Ht31: AKAP St-Ht31 inhibitor peptide

Ht31P: AKAP St-Ht31P control peptide

IF: Interstitial filopodia

Iso: Isoproterenol

JGI: Joint genome institute

LTD: Long-term depression

LTP: Long-term potentiation

MAG: Myelin-associated glycoprotein

mAKAP: Muscle-specific A-kinase anchoring protein

MAP2: Microtubule-associated protein 2

MAPK: Mitogen-activated protein kinase

MD: Multidendritic neuron

mDia2: Mammalian diaphanous-related formin 2

mGluR: Metabotropic glutamate receptor

Mena: Mammalian homologue of Ena

MS-222: Tricane methansulfonate; 3-aminobenzoic acid ethyl ester

NGF: Nerve growth factor

NLG: Neuroligin

NMDAR: N-methyl-D-aspartate receptor

NMJ: Neuromuscular junction

NR2A: NMDA receptor subunit 2A

NR2B: NMDA receptor subunit 2B

NRX: Neurexin

NT-4: Neurotrophin-4

N-WASP: Neuronal Wiskott-Aldrich syndrome protein

PACAP: Pituitary adenylate cyclase-activating polypeptide

PDE: Phosphodiesterase

PKA: Protein kinase A

PKC: Protein kinase C

PKMζ: Protein kinase Mζ

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2A

PSD-95: Postsynaptic density protein 95

RGC: Retinal ganglion cell

RIF: Rho in filopodia

Rp-cAMPS: Adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer

SAP97: Synapse-associated protein 97

SCE: Single-cell electroporation

Sema3A: Semaphorin 3A

SGC: Soluble guanylate cyclase

shot: Short stop/kakapo

SQ22536: 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine

Sp-cAMPS: Adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer

TDBL: Total dendritic branch length

VASP: Vasodilator-stimulated phosphoprotein

VGCC: Voltage-gated calcium channel

VIP: Vasoactive intestinal peptide

WAVE: WASP family verprolin homology protein

Acknowledgements

First and most importantly I would like to thank my supervisor, Dr. Kurt Haas, for all the years of support as I worked on this project. I could never have done this without you. Thanks also to my supervisory committee members, Dr. Shernaz Bamji, Dr. John Church, and Dr. Jane Roskams, for their support, time, advice, and reagents. Thank you to Dr. Tim O'Connor and Dr. Wun Chey Sin for advice and reagents.

Thanks to my lab mates both past and present – Sharmin Hossain, Simon Chen, Kasper Podgorski, Xuefeng Liu, Derek Dunfield, and Sesath Hewapatharine – your help was invaluable; to Jamie Boyd and Shay Neufeld for programming the analysis software; to my lunch buddies from the BRC, especially Ted Dobie and Kevin She, who kept me laughing even when my experiments were failing; to Kelley Bromley-Brits, who is an inspiration (I loved our therapeutic coffee dates and book swaps!); to the undergrads who came through the lab, both for all the help and because I enjoyed teaching you; and, of course, my friends, the Gong Show, who kept me sane and happy, which wasn't always easy to do.

Special thanks to my parents, who supported me both emotionally and financially, and my sister Nicola, mostly because I love you but also because you cried when I got permission to write up (which even I didn't do). That meant a lot. I've never been good at the sentimental bits so I'll just stop there, but thank you guys, and I love you.

For my parents…*obviously*

Chapter 1: General introduction

1.1 Dendritogenesis – an overview

The period of early brain development involves an exceptional amount of neuronal morphological growth and plasticity. To create functional neural circuits, newly formed neurons must extend long and often complex axonal and dendritic arbors to contact distant neurons and to increase contact area for synapse formation. Neuronal signals in the form of action potentials propagate from the cell body, or soma, through the axon to presynaptic terminals. At the synapse the signal is transmitted to the dendrite of the downstream neuron, integrated in the dendritic arbor, and transmitted to the soma where it may contribute to the firing of a new action potential. Neural function is intimately connected with neuronal morphology, and errors in axogenesis or dendritogenesis during development can have devastating long term consequences to the functioning of neuronal circuits.

Not only do axons and dendrites subserve unique functions within the cell, they also exist as unique cellular compartments with defined internal structures and distinct patterns of protein expression (Craig and Banker, 1994). For example, both axons and dendrites have an inner skeleton composed of microtubules, but contain different microtubule binding proteins; tau in axons, and microtubule-associated proteins 2 (MAP2) and CHO1/MKLP1 in dendrites (Kosik and Finch, 1987; Sharp et al., 1997). Similarly, Growth Associated Protein 43 (GAP43) is present in axons and axonal growth cones but absent from dendrites and their growth cones (Goslin et al., 1988), while dendrites contain outposts for Golgi trafficking which are absent from axons (Horton et al., 2005).

Dendritic morphology itself varies among cell types (Wong and Ghosh, 2002). The Purkinje cells of the cerebellum have a highly branched and extremely complex dendritic arbor. Pyramidal neurons, in contrast, have two distict components of their dendritic arbor, termed the apical and basal dendrites, which originate on opposite sides of the soma and receive connections from different sets of inputs. Neurons of the bird or reptile nucleus laminaris have two highly branched, short dendritic arbors specialized for sound localization. Each dendrite receives input from either the contralateral or ipsilateral ear, but not both, and the length of the dendrite is dependent on the frequency of sound the cell receives (Jan and Jan, 2003; Kubke and Carr, 2000). While these cell-type specific morphologies are under the control of intrinsic factors, extrinsic factors such as neural activity also play an important role in the ultimate shape of a dendritic arbor. For example, in the absence of their presynaptic partners, Purkinje neurons show characteristic initial branching patterns and spine formation, but have greatly reduced higher order branching and disrupted orientation. When cultured with granule cells, their presynaptic partners, Purkinje neurons develop more normal morphologies. These experiments indicate that intrinsic factors can guide basic aspects of dendritic development, but that extrinsic factors such as synaptic input are required to achieve normal complexity (Baptista et al., 1994; Scott and Luo, 2001).

Dendritic morphology determines a number of functional properties of the neuron. The size and shape of the dendritic arbor determines the number and type of presynaptic inputs a cell can receive. Dendritic morphology also influences how electrical signals propagate from a synapse to the axon hillock at the soma for action potential induction (Magee, 2000). Typically, single excitatory post-synaptic potentials (EPSPs) are not sufficient for axon potential generation. Rather, large numbers of synaptic signals occurring

all across the dendritic arbor must be integrated, first through specific electrical properties within dendritic branches, before summation within the soma. The size and diameter of any given dendritic branch, the expression of ion channels, and the dendritic branching pattern all play a role in determining the contribution of any given EPSP to action potential generation (Magee, 2000).

Studies imaging dendritic arbor growth have provided valuable information on how dendrites grow and integrate into functional neural circuits. Neurons can be grown in low-density culture allowing for imaging using light microscopy. A cultured neuron initially extends a number of "neurites", a term which refers to processes which have not yet adopted an axonal or dendritic fate. After one neurite differentiates into an axon, the remaining neurites differentiate into dendrites and begin to elaborate into a dendritic arbor (Craig and Banker, 1994). Alternatively, non-neuronal cells, often tumor derived, such as PC12 or PC6 cells, extend neurites when exposed to growth factors, allowing study of extrinsic factors regulating neurite growth. The major caveats of these studies, however, are that cultured neurons do not exist within their natural environment, and that cultured non-neuronal cells such as PC12 cells do not become polarized or form synapses and may not respond to the same signals as true neurons. Development of *in vivo* labeling and imaging techniques has allowed imaging of dynamic dendritic arbor growth of developing neurons within their natural environment. Together these studies, both *in vitro* and *in vivo*, have allowed scientists to begin to piece together the processes underlying dendritic growth.

Brain neural circuit formation occurs through several stages (McAllister, 2000; Wong and Ghosh, 2002). First, neurons are born and migrate to their appropriate terminal target sites. Next, axons sprout from the cell body, search for targets, and elaborate their terminal

branches. Concurrent with or following axonal development, dendrites sprout either from a separate site on the soma, or from the base of the axon, depending on the neuron type. The overall pattern of axonal and dendritic arborization is often determined by intrinsic, cell-type specific patterns. In sensory systems, it has been demonstrated that the period of maximum dendritic arbor growth and remodeling occurs concurrently with periods of afferent innervation and synapse formation, suggesting that neural transmission and synapse formation may contribute to regulation of dendritic growth.

In vivo imaging of *Xenopus laevis* tectal neurons demonstrates that dendritic arbor development in these neurons progresses through three distinct phases. During the first phase, while axonal growth and arbor elaboration is taking place, the dendritic arbor is composed of short filopodial processes that exhibit high turnover without formation of longer branches. In the second phase, filopodia transition into longer branches and the dendritic arbor expands rapidly. This growth is accompanied by high levels of refinement, with rapid extension and retraction of branches. In the third phase, the rate of growth slows down and the dendritic arbor becomes relatively stable (Cline, 2001; Wong et al., 2000; Wu et al., 1999). During these phases dendritic growth graded synaptic maturation confers graded structural stabilization, and nascent processes which fail to form synaptic contacts are removed (Chen and Haas, 2011; Liu et al., 2009; Niell et al., 2004).

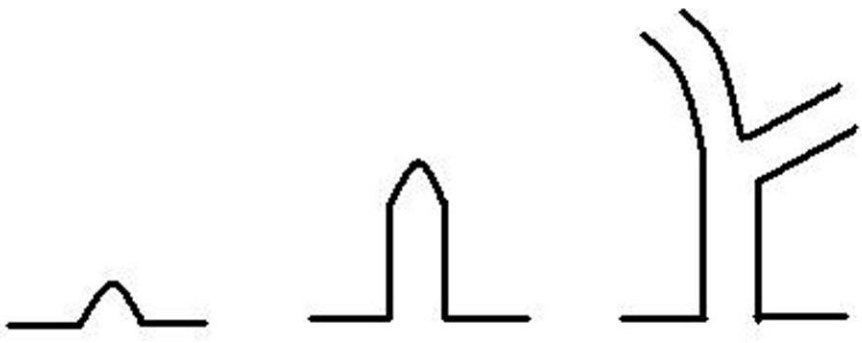
Early in development dendrites are studded with small, highly motile, actin-rich protrusions called filopodia. Filopodia differ from dendritic branches in that they are smaller, averaging 100-300 nm in diameter and are typically less than 10 μm in length (Faix et al., 2009; Mattila and Lappalainen, 2008) and have a different cytoskeletal structure. Filopodia are actin rich, while the branches are composed primarily of microtubules (Mattila and

Lappalainen, 2008; Scott and Luo, 2001). Filopodia are also more highly motile than branches. Rapid time-lapse imaging studies suggest that filopodia are added and retracted on the order of minutes (Cline and Haas, 2008; Hossain et al., 2011). They can exist either along the shaft of a dendritic branch (interstitial filopodia), or at the end of a branch as part of a dendritic growth cone (growth cone filopodia). Evidence from dye-filled growing pyramidal neurons in acute hippocampal slices suggests that interstitial filopodia respond to activity-dependent cues while growth cone filopodia respond to activity-independent cues (Portera-Cailliau et al., 2003).

One role of filopodia is to search the area surrounding the neuron for potential synaptic contacts (Wong et al., 2000). In some types of more mature neurons, filopodia are precursors of dendritic spines, which can serves as the sites of excitatory postsynaptic inputs (Hotulainen and Hoogenraad, 2010). In developing neurons during dendritogenesis, synapses can form on filopodia (Niell et al., 2004). Filopodia also serve as the precursors of branches which form the overall shape of the dendritic arbor (Niell et al., 2004). However, despite many years of study, neither the precise function of dendritic filopodia nor mechanism by which they form are well understood.

Improper dendritogenesis during development can have lasting detrimental effects on neural function. Several animal models of developmental brain disease have uncovered dendritic abnormalities that may contribute to behavioral symptoms. For example, induced seizure activity in developing *X. laevis* tadpoles leads to long-term changes in dendritic structure (D. Sesath Hewapathirane and K. Haas – unpublished results). Disrupting the cell adhesion molecules neurexin and neuroligin, which have been linked to Autism Spectrum Disorders (ASDs) (Feng et al., 2006; Jamain et al., 2003), destabilizes dendritic filopodia and

reduces synapse density (Chen et al., 2010b). Dendritic abnormalities are strongly associated with mental retardation, including the disorders Down syndrome, Rett syndrome, and Fragile X (Kaufmann and Moser, 2000). Postmortem studies of the brains of schizophrenic humans show dendritic and synaptic abnormalities as well as abnormalities in neuron number, density, and morphology, indicating that early neural development may be disrupted (Arnold, 1999; Bunney et al., 1995). An overview of the current knowledge of dendritic morphogenesis is presented in the sections below (Fig. 1).



	Initiation/ branching	Extension	Stabilization/ maturation
Extrinsic	BDNF *CPG15* Slit/Robo	BDNF *CPG15* Calcium	BDNF *CPG15* Calcium
Synapse-associated	NMDA receptor	NMDA receptor D1 receptor	*AMPA receptor* *Homer 1b* *NRX-NLG*
Signaling	CaMKII PKA	PKA	*CaMKII* *PKMζ* PKA
Gene expresion	MAPK CREB	CREB	CREB
Cytoskeletal reorganization	CHO/MKLP1 MAP2 *Rac* *Cdc42*	Myosin II MAP2 *Rho*	

Figure 1: Factors implicated in filopodial growth and dendritic development
Shown are several factors potentially involved in dendrite growth, grouped by functional class and stage of filopodial development. Factors in red are known to be part of the adenylyl cyclase signaling pathway. In the case of proteins which are known to be upstream of multiple signaling cascades, for instance BDNF, a signaling link through adenylyl cyclase for regulation of dendritogenesis has always been definitively proven. Factors in italics have been verified in *X. laevis* tadpoles, an *in vivo* model of dendritic development. While a number of factors have been implicated in filopodial initiation and extension, few have been verified *in vivo*.

1.1.1 Intrinsic factors governing dendritogenesis

A number of genetic factors have been implicated in cell-type specific dendritogenesis. This is particularly well-studied in the fruit fly *Drosophila melanogaster*, where the relative ease of creating genetic mutants, and ability to identify specific neurons between individuals has revealed genes critical to proper dendrite growth. For example, the dendritic arborization (da) neurons fall into four distinct morphological classes: da class 1 neurons which have small, simple arbors; da class II and III neurons which have intermediate arbors; and da class IV neurons which have a complex dendritic morphology and tile the larval body wall (Grueber et al., 2002). Whether a da neuron grows as a class I, II, III, or IV neuron depends on the level of expression of three transcription factor genes: *abrupt*, *cut*, and *spineless* (Grueber et al., 2003; Kim et al., 2006a; Li et al., 2004; Sugimura et al., 2004). Only da class I neurons express *abrupt* (Li et al., 2004; Sugimura et al., 2004). Dendritic arborization class II, III, and IV neurons all express *cut*, but at low, high, and intermediate levels, respectively (Grueber et al., 2003). Loss of Cut reduces dendritic growth and terminal branching and converts class III and IV neurons to neurons resembling class I and II (Grueber et al., 2003). Ectopic expression of Abrupt has a similar effect, reducing branch number in class IV neurons (Li et al., 2004; Sugimura et al., 2004). Hamlet, another transcription factor, is capable of converting a highly complex multidendritic neuron (MD) to an external sensory (ES) neuron, which has only a single simple dendrite. When expressed in a committed MD neuron, *hamlet* reduces dendritic growth (Moore et al., 2002). Other genes which affect dendritogenesis when mutated include those encoding guidance molecules, receptors, cytoskeletal proteins or proteins which regulate the behavior of the cytoskeleton,

cell adhesion molecules, and protein kinases (Furrer et al., 2003; Gao and Bogert, 2003; Gao et al., 1999; Liu et al., 2000; Ou et al., 2008; Reuter et al., 2003).

1.1.2 Extrinsic factors governing dendritogenesis

1.1.2.1 Guidance cues

Guidance cues are molecules that direct the growth and target tissue localization of axons. At the terminal of a developing axon is an organelle known as a growth cone covered with receptors which bind to soluble or membrane-bound guidance cue ligands in the extracellular space or bound to other cells or the extracellular matrix. Guidance cue receptor-ligand binding induces attractive or repulsive growth responses in the growth cone to guide axonal growth through complex tissue to appropriate target cells. Whether growing dendrites also respond to guidance cues, and even whether dendrites express growth cones similar to axonal growth cones, are poorly addressed questions.

One of the few studies to investigate dendritic responses to known guidance cues examined midline crossing in *D. melanogaster*. Netrin and its receptor Deleted in Colorectal Cancer (DCC; known as Frazzled in *D. melanogaster*) have been identified as attractive cues for axon growth, allowing axonal crossing at the midline (Furrer et al., 2003; Hiramoto et al., 2000; Kolodziej et al., 1996). The dendrites of *Drosophila* aCC motoneurons also exhibit midline-crossing. Interestingly, these dendrites elaborate ipsilaterally when Frazzled or netrin are mutated (Furrer et al., 2003). The guidance cue Slit has been shown to be a chemorepellant for axons (Furrer et al., 2007; Whitford et al., 2002). Knockdown of the Slit receptor Robo in single aCC neurons leads to reduced dendritic growth, while genetic

deletion of Slit results in a complete loss of aCC dendrites (Furrer et al., 2007). Slit and Robo positively regulate dendritic growth and branching in mammalian cortical neurons (Whitford et al., 2002). They also mediate an attractive growth response in axons of *X. laevis* retinal ganglion cells (RGCs) which synapse onto the dendrites of neurons within the optic tectum (Hocking et al., 2010). However, Slit does not appear to regulate RGC dendritic guidance, but does increase dendritic branching, thus in the same cell Slit functions in axon guidance and in dendritic growth, but not dendritic guidance (Hocking et al., 2010).

Semaphorin 3A (Sema3A) has also been identified as a guidance cue with distinct effects in axons and dendrites. Sema3A was originally identified for its repulsive effects on axon growth (Chedotal et al., 1998; Polleux et al., 1998). It has also been found to be both necessary and sufficient to orient the apical dendrites of cortical neurons towards the pial surface as a chemoattractant, and disrupting Sema3A signaling by blocking its receptor Neuropilin1 results in disoriented apical dendrites (Polleux et al., 2000). This opposition of effects in axons and dendrites of the same cortical pyramidal neurons has been attributed to the asymmetric distribution of soluble guanylate cyclase (SGC). High levels of SGC in the dendrite confer a chemoattractive response to Sema3A through the SGC-dependent elevation of cyclic guanosine monophosphate (cGMP) levels and subsequent activation of the protein kinase G signaling pathway (Polleux et al., 2000). Together, these studies show that dendrites can respond to guidance cues, and that this response can be different from that observed in axons.

1.1.2.2 Neurotrophic factors

Neurotrophic factors have also been found to regulate dendritogenesis, although the effects are dependent on the system, neuron, and neurotrophin being studied. Work from the Katz lab has shown that the response of cortical neurons to neurotrophins is dependent on both cortical cell layer and domain of the dendritic arbor (McAllister et al., 1995). In the postnatal ferret visual cortex, apical dendrites respond to a wide range of neurotrophins, with the degree of response depending on cell layer. Basal dendrites, in contrast, respond most strongly to a single neurotrophin, with the particular neurotrophin depending on cell layer. Pyramidal neurons in layer 4 respond to brain derived neurotrophic factor (BDNF), while layer 5 and 6 neurons respond to neurotrophin-4 (NT-4) (McAllister et al., 1995).

BDNF is typically reported to increase dendritic growth. For example, BDNF induces expression of neuritin, also known as CPG15 (Naeve et al., 1997). When expressed in *X. laevis* tectal neurons, neuritin/CPG15 increases dendritic size, growth rate, and complexity (Nedivi et al., 1998). However the growth-promoting effects of BDNF are not universal. Work from the Cohen-Cory lab has shown that microinjection of BDNF into the *X. laevis* optic tectum increases the number of postsynaptic specializations but does not influence dendritic arbor morphology (Sanchez et al., 2006). Dendritic morphologies of RGCs change in response to BDNF, but that response is dependent on the source of the BDNF (Lom et al., 2002; Lom and Cohen-Cory, 1999). When BDNF was inserted into the retina using neurotrophin-coated microspheres, RGC dendritic arborization decreased. The opposite was observed when BDNF was inserted into the optic tectum, indicating that not only can BDNF retrogradely enhance dendritic arborization, but that dendritic response to a cue can be

dependent on whether the cue is local or target-derived (Lom et al., 2002; Lom and Cohen-Cory, 1999).

1.1.3 Neuronal activity regulates dendritogenesis

1.1.3.1 Glutamatergic synaptic transmission

Glutamatergic synaptic transmission through the N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors influences dendrite growth. In the retinotectal system of *Xenopus laevis* tadpoles, proper dendritogenesis is dependent on the activity of NMDA receptors during early stages of development and both NMDA and AMPA receptors later in development (Rajan and Cline, 1998). Blocking NMDA receptors in immature neurons results in decreased branch additions and retractions, resulting in a smaller arbor overall (Rajan et al., 1999). Growth of immature optic tectal neurons is increased by visual stimulation and this growth is blocked by NMDA or AMPA receptor antagonists (Sin et al., 2002). Furthermore, reduction of AMPA receptor expression at synapses in individual growing *X. laevis* tectal neurons results in reduced dendritic filopodial stabilization (Haas et al., 2006). Neurons with reduced glutamatergic transmission, growing into a normal brain circuit, retracted dendrites in response to visual stimulation that induced growth in cells with normal transmission (Haas et al., 2006), suggesting a complex role of intra-neuronal competition based on strength of glutamatergic synaptic transmission.

Glutamate has been implicated in dendritogenesis in other animal models. Blockade of glutamatergic transmission in the chick retina reduces dendritic growth motility as well as

the size and complexity of the dendritic arbor (Wong et al., 2000). BDNF-induced elaboration of layer 4 pyramidal neurons in the ferret visual cortex is dependent on both NMDA and AMPA receptors (McAllister et al., 1996). In rat organotypic hippocampal slices, NMDA receptor activity is required for activity-induced growth of new dendritic filopodia (Maletic-Savatic et al., 1999). A role for glutamate in dendritic filopodia dynamics has also been shown in acute slices of developing mouse neocortex, where blocking AMPA and NMDA receptors decreases filopodia turnover and density, while focal glutamate application increases filopodia length (Portera-Cailliau et al., 2003).

1.1.3.2 Calcium transients

Increased intracellular calcium levels brought about by synaptic activity may be an important signaling mechanism regulating dendritic development (Lohmann and Wong, 2005). Upon synaptic stimulation, calcium influx into the cell can occur via NMDA receptors or voltage-gated calcium channels (VGCCs). In addition, synaptically-induced increases in calcium levels can initiate further intracellular calcium increases by stimulating the release of calcium from intracellular stores, a process known as calcium-induced calcium release (CICR). Calcium increases can occur on both the global and local level within a cell. Local calcium transients, often associated with putative synaptic sites, are first observed when a filopodium emerges from a dendrite, increase in frequency as filopodial growth accelerates, and at their peak levels are associated with filopodial stabilization (Lohmann et al., 2005). Imaging growth of chick retinal ganglion cells in culture has shown that neurotransmitter-evoked local calcium transients and CICR stabilize dendritic filopodia, while blockade of calcium transients leads to filopodial destabilization and retraction (Lohmann et al., 2002).

These observations that calcium levels regulate filopodial motility suggest that calcium may act, in part, through regulation of the cytoskeleton. One possible mechanism involves the calcium/calmodulin-dependent protein kinase II (CaMKII), which has recently been shown to bind to actin, both sequestering monomeric actin to reduce polymerization, and bundling actin filaments, increasing their rigidity (Sanabria et al., 2009).

Alternately or concurrently, calcium may affect dendritic growth by influencing gene transcription. Calcium influx through VGCCs acts through the calcium/calmodulin-dependent protein kinase IV (CaMKIV) and the transcription factor cyclic-AMP-responsive-element-binding protein (CREB) to increase dendritic growth (Redmond et al., 2002). Calcium can also act through CaMKII and mitogen-activated protein kinase (MAPK), which is also known to activate CREB, to induce dendrite formation (Redmond et al., 2002; Vaillant et al., 2002). Mice knockouts for the calcium-responsive transactivator (CREST), a protein which interacts with CREB, show reduced brain neuron dendritic length, branching, and complexity (Aizawa et al., 2004). Calcium can also indirectly activate CREB via calcium-activated isoforms of adenylyl cyclase (AC) (Sadana and Dessauer, 2009), the potential morphological consequences of which will be discussed in more detail later in this chapter.

1.1.3.3 Sensory stimulation

Early studies into the role of sensory information on neural development revealed a critical role for visual and aural information in neural growth within the cortical regions receiving input from these sensory organs. Unilateral ear plugging results in shortened dendritic arbors in high-frequency regions of the nucleus laminaris and lengthened dendritic

arbors in low-frequency regions (Smith et al., 1983). Visual deprivation, either through dark rearing or monocular deprivation, decreases dendrite growth in the visual processing regions of the brain (Coleman and Riesen, 1968; Wiesel and Hubel, 1963). Monocular deprivation can also lead to dendritic remodeling. In layer 4 spiny stellate cells, monocular deprivation causes dendrites to reorient to ocular dominance columns representing the open eye (Kossel et al., 1995). More recently, the Cline lab has shown that visual stimulation increases growth in *X. laevis* tectal neurons dependent on NMDA and AMPA receptor activity (Haas et al., 2006; Sin et al., 2002). Furthermore, housing experimental animals in an enriched environment also increases higher-order branching of pyramidal neuron dendrites compared to littermates reared individually in an unenriched environment (Volkmar and Greenough, 1972).

1.1.4 Mechanisms of dendritogenesis downstream of neural activity

1.1.4.1 Synaptotropic model of dendritogenesis

The 'synaptotropic model' of dendritogenesis proposed by Vaughn (Vaughn, 1989) posits that stabilization of dendritic filopodia and branches is conferred by synapse formation. Through an iterative process of activity-dependent synaptogenic filopodial stabilization, and subsequent extension to form persistent branches that support further filopodia addition, dendrites arborize into regions of neuropil with appropriate innervation. Inadequate afferent innervation leads to synapse weakening, morphological destabilization, and branch retraction from regions of neuropil with inappropriate input. This model is supported by *in vivo* imaging of single neurons expressing a fluorescently-tagged marker for

synapses (postsynaptic density protein 95, PSD-95) within brains of developing zebrafish (Niell et al., 2004). Imaging of neuronal morphological growth along with PSD-95 puncta formation demonstrated that synaptogenesis occurs concurrently with dendritogenesis and that puncta formation is directly correlated with filopodial stabilization.

One weakness of the synaptotropic model in this form was that it provided no explanation for how growth is eventually halted when neurons reach maturation. Taken to its logical conclusion, the hypothesis implies that neurons will continue to grow ad infinitum, as neural activity does not stop in mature cells. This contradicts experimental observations that arbor growth rate slows with neuronal maturation (Wu et al., 1999). In fact, there are a number of studies suggesting that synaptic activity can, in certain situations, decrease dendritic growth, particularly as cells mature (Tripodi et al., 2008; Wu and Cline, 1998), and expression of the scaffolding protein Homer 1b in tectal neurons decreases arbor development while also driving synaptic maturation (Van Keuren-Jensen and Cline, 2008). A recent study by Liu and Haas helped address this issue by proposing a modified version of the synaptotropic model (Liu et al., 2009). This study examined the role of protein kinase Mζ (PKMζ), a constitutively active isoform of protein kinase C (PKC), in dendrite growth in individual neurons within the intact *X. laevis* optic tectum. PKMζ promotes glutamatergic synapse maturation. Overexpressing PKMζ stabilized dendritic filopodia and reduced arbor extension, while inhibiting PKMζ destabilized filopodia and promoted arbor extension while at the same time destabilizing synapses (Liu et al., 2009). Based on these data, the authors proposed an expanded synaptotropic model where graded levels of synapse maturation confer similarly graded levels of morphological stabilization. In this way, nascent, immature synapses stabilize labile processes to prevent retraction while allowing them to extend into

longer branches. Strong mature synapses, however, produce strong stabilization that restricts all morphological growth. This mechanism explains the slowing of arbor growth that accompanies neuronal maturation. Further support for this expanded synaptotropic hypotheses came from evidence that increasing interactions of the cell adhesion molecules neuroligin (NLG) and neurexin (NRX) stabilizes dendritic filopodia and increases synaptic specializations (Chen et al., 2010b). Expression of a NLG mutant which is able to bind NRX but not recruit PSD-95 only transiently stabilized filopodia and decreased postsynaptic specializations.

1.1.4.2 Cytoskeletal remodeling underlying developmental dendritogenesis

The processes of branching, extension, and retraction which underlie filopodial behaviour and dendritic growth are intimately linked with the behaviour of the actin cytoskeleton. Each of the three major morphological structures of dendrites, branches, filopodia, and growth cones, have a unique cytoskeletal identity.

The dendritic shaft is composed of filamentous actin distributed along the cortex region directly inside the plasma membrane, and an interior core comprised of microtubules (Scott and Luo, 2001). In contrast to axons, which have unidirectional plus-end-distal microtubule arrangements, dendrites have both plus-end distal and minus-end distal arrangements (Baas et al., 1988; Burton, 1988), and distinct microtubule-binding proteins are expressed in axons, such as tau, and dendrites, such as CHO1/MKLP1 and MAP2 (Kosik and Finch, 1987; Sharp et al., 1997).

At the terminal end of dendritic branches are the growth cones, which exist either in a collapsed state or as a thin (100-200 nm), spreading sheet of membrane known as a

lamellipodium which is composed of a network of branching actin filaments and microtubules (Mattila and Lappalainen, 2008). Lamellipodia are also more commonly observed as part of axonal growth cones, or in non-neuronal cells where they play a role in cell migration. Dendritic, axonal, and non-neuronal lamellipodia all contain highly motile filopodia which aid in sampling the surrounding environment for growth cues and in cell migration.

Filopodia can also be found distributed along the dendritic shaft. Filopodia are highly motile actin-rich structures averaging 100-300 nm in diameter and typically less than 10 μm in length (Faix et al., 2009; Mattila and Lappalainen, 2008). Evidence indicates that shaft, or interstitial, filopodia are functionally different from dendritic growth cone filopodia. Growth cone filopodia exhibit more dynamic growth behaviours (Hossain et al., 2011) and respond to activity-independent cues, while interstitial filopodia respond to activity-dependent cues (Portera-Cailliau et al., 2003).

Until recently, the structural composition of dendritic filopodia was unclear. Studies performed in non-neuronal, migrating cells provided the bulk of information on cytoskeletal behaviour and filopodia dynamics. Two recent studies, utilizing electron microscopy (Korobova and Svitkina, 2010) and fluorescence microscopy of cells in culture (Hotulainen et al., 2009), have shown that dendritic filopodia in hippocampal neurons have key structural differences from conventional, or non-neuronal filopodia (Hotulainen and Hoogenraad, 2010). Neuronal filopodia consist of both branched and straight actin filaments, while conventional filopodia consist of stiff bundles of straight actin filaments. Non-neuronal stiff bundles are held together and strengthened by the protein fascin (Faix et al., 2009; Mattila and Lappalainen, 2008), which is absent from dendritic filopodia (Korobova and Svitkina,

2010). In non-neuronal filopodia, protrusion and growth is driven by actin polymerization at the filopodial tip (Mallavarapu and Mitchison, 1999), while active filament disassembly by cofilin occurs at the base of the filopodium (Faix et al., 2009). Filaments in dendritic filopodia, in contrast, are polymerized from both the tip and the base (Hotulainen et al., 2009). These filaments adopt a unipolar array in non-neuronal filopodia but a bipolar array in dendritic filopodia (Hotulainen et al., 2009; Korobova and Svitkina, 2010). Finally, myosin II, an actin-dependent motor which contracts actin filaments, is present in dendritic but not non-neuronal filopodia (Korobova and Svitkina, 2010).

A host of cytoskeletal regulatory proteins translate external inputs into dynamic changes in the growth behaviours of the distinct compartments of the dendritic arbor, including interstitial filopodia, growth cones, and the dendritic shaft. Mutation studies in *D. melanogaster* have revealed a number of cytoskeletal proteins which are necessary for regulating dendritogenesis. *Dhc64* and *lis1*, homologues of mammalian dynein heavy chain, are both required for dendrite growth and branching (Liu et al., 2000), while mutants of the dynein light chain homologue *roadblock* have reduced dendritic size and complexity (Reuter et al., 2003). Mutants of *enabled*, a protein which binds profilin in order to regulate actin filament dynamics, have altered dendritic growth and guidance (Gao et al., 1999). Mutants of the actin filament-stabilizing protein tropomyosin II show increased dendrite length, number, and field sizes (Li and Gao, 2003). The result of a mutation can be specific to the cell type studied, as is observed in mutants of *short stop/kakapo (shot)*, a protein which crosslinks cytoskeletal elements such as actin and microtubules. *Shot* mutants show projection of abnormal dendritic branches in mushroom body neurons in the *Drosophila* olfactory system (Reuter et al., 2003). However, these same mutants show reduced dendrite growth and

branching in the peripheral multiple dendrite neurons, which are thought to be touch receptors or proprioceptors (Gao et al., 1999), and in RP3 motoneurons (Prokop et al., 1998).

Studies in vertebrate neurons have also identified a number of cytoskeletal factors as potential regulators of dendritic growth. One such factor is a family of three closely related proteins, Enabled (Ena; homologue of the *Drosophila* gene *enabled*), vasodilator-stimulated phosphoprotein (VASP), and Mena (the mammalian homologue of Ena), known collectively as the Ena/VASP proteins. Ena/VASP proteins promote filopodial growth in a number of non-neuronal cell types including *Dictyostelium discoideum* (Han et al., 2002) and mouse melanoma cell culture (Mejillano et al., 2004). Elimination of all three proteins results in reduced filopodia and neurite formation in neurons (Dent et al., 2007; Kwiatkowski et al., 2007). Ena/VASP proteins increase formation and elongation of filopodia in neurite shafts and growth cones of primary hippocampal neurons downstream of netrin-1 and protein kinase A (PKA) (Lebrand et al., 2004). Whether Ena/VASP proteins also function in filopodia growth in dendrites is unknown. Actin binding proteins appear to be involved not just in increasing growth but in regulating retraction rates. For example, knocking out gelsolin, a protein which severs and caps actin filaments, reduces filopodia retraction in neurite shafts and growth cones of primary hippocampal neurons (Lu et al., 1997). Microtubule binding proteins also appear to be important, which is unsurprising given that microtubules fill the center of, and provide structural integrity for dendrites. Neurons with reduced levels of the microtubule-associated proteins CHO1/MKLP1 or MAP2 have reduced length of dendrites or neurites or fail to form processes (Caceres et al., 1992; Harada et al., 2002; Sharp et al., 1997), or fail to correctly differentiate dendrites and axons (Yu et al., 2000). Inhibition of the contractile protein myosin IIB in cultured hippocampal neurons

results in increased generation of filopodia-like protrusions and loss of mushroom-body spines (Ryu et al., 2006)

1.1.4.2.1 The RhoGTPases

One group of molecules which regulates the actin cytoskeleton demonstrated to be intimately involved in dynamic dendritogenesis is the Rho family of GTPases. This family consists of three members: Rho, Rac, and Cdc42. RhoGTPases cycle between active (GTP bound) and inactive (GDP bound) states. They are activated by guanine exchange factors (GEFs), which catalyze the exchange of GDP for GTP, and inactivated by GTPase-activating proteins (GAPs). A number of studies have examined the role of the RhoGTPases in filopodial formation, although most of these studies have been in non-neuronal cells such as epithelial or dendritic cells (Hall, 2005; Jaffe and Hall, 2005). The general consensus appears to be that each of the three well-described RhoGTPases controls a particular cellular function in migrating, non-neuronal cells: Rho regulates contractile actin-myosin filaments, and stress fibre and focal adhesion formation; Rac controls lamellipodia formation by regulating actin polymerization at the cell periphery; and Cdc42, also acting at the cell periphery, promotes actin filament assembly and filopodia formation and controls cell polarity (Hall, 2005). While studies in non-neuronal cells or in cell types that are capable of extending neurites under certain stimuli are valuable in teasing out the roles of different signaling pathways in neural growth, they are not necessarily indicative of what occurs physiologically in the dendrites of the living animal, particularly considering the observed differences between non-neuronal and neuronal filopodia (Hotulainen et al., 2009; Korobova and Svitkina, 2010). Studies directly examining dendrites, either in cultured neurons or within the intact animal,

are therefore key to understanding the role signaling pathways and regulation of the cytoskeleton play in dendritogenesis.

In *D. melanogaster*, both *dcdc42* and *drac1* (homologues of mammalian Cdc42 and Rac1, respectively) overexpression mutants have stunted primary branches and fewer secondary branches compared to wild type controls (Gao et al., 1999), while loss of Cdc42 leads to increased total dendrite length, misguided dendrites, and defects in dendrite calibre (Scott et al., 2003). In cultured cortical neurons, dominant negative versions of Rac and Cdc42 reduce dendrite number while constitutively active versions have the opposite effect (Threadgill et al., 1997). Blocking Rac activity reduces dendritic number and, correspondingly, total arbor length in mammalian hippocampal neurons (Rosso et al., 2005), and in neuroblastoma cells, expression of constitutively active Rac increases filopodia initiation rate (Korobova and Svitkina, 2008). These data support a role for Rac and Cdc42 in dendrite initiation, with secondary effects on total dendritic arbor length which may be the result of changes in total dendrite or filopodia number.

Rho, on the other hand, appears to be involved in regulating the extension and motility of already initiated filopodia, rather than in the formation of filopodia and lamellipodia themselves. Activation of RhoA or its effector p160ROCK leads to neurite retraction in NG108 cells (Sakisaka et al., 2004), *Aplysia* bag cell neuron growth cones (Zhang et al., 2003), and NIE-115 neuroblastoma cells (Hirose et al., 1998). Reductions in expression of the actin-related protein 2/3 (Arp2/3) complex results in RhoA activation in primary hippocampal neurons and differentiated neuroblastoma cells, and induces impaired lamellipodia and filopodia formation, and decreased filopodial extension and motility (Korobova and Svitkina, 2008). *Drosophila* neurons lacking functional RhoA results in

overextension of dendrites, while expressing active RhoA reduces dendritic complexity (Lee et al., 2000). A similar effect is observed in mammalian hippocampal neurons, where expression of constitutively active RhoA produces less complex dendritic arbors (Nakayama et al., 2000; Pilpel and Segal, 2004).

In the dendrites of chick retinal ganglion cells, RhoA and Rac1 have reciprocal effects on dendritic growth, with Rac increasing filopodial motility and turnover and Rho decreasing motility while increasing the fraction of persistent processes. In stage 46 *X. laevis* optic tectal neurons imaged *in vivo*, Rac expression decreases filopodial stability, causing an increase in both addition and retraction rate, but does not alter filopodial length (Li et al., 2000). RhoA in contrast, decreases branch extension when activated and increases branch extension when inhibited, but does not affect additions or retractions (Li et al., 2000). In these same neurons, visual stimulation increases both dendritic growth rate and branch number in a manner dependent on active Rac and Cdc42, and inhibited RhoA (Sin et al., 2002). In these neurons, endogenous RhoA activity is high, and activity-induced inhibition of Rho allows for increased branch elongation. However, in RGCs of stage 18-20 tadpoles, constitutively active, dominant negative, and wild-type overexpression manipulations of all three RhoGTPases decrease both length of longest dendrite and number of dendrite tips, indicating that cell type or animal age may be important to the morphological result of altered GTPase activity (Ruchhoeft et al., 1999).

There are distinct classes of interstitial filopodia based on neuronal type and maturational stage. In immature neurons during dynamic dendritogenesis, filopodia are precursors of longer dendrites (Hossain et al., 2011), while in more mature mammalian pyramidal neurons, filopodia are precursors of dendritic spines. RhoGTPases have also been

implicated in regulating spine formation and morphology. Neurons expressing constitutively-active Rac1 show decreased spine length but increased spine number (Luo et al., 1996; Tashiro et al., 2000), while those expressing dominant-negative Rac1 show decreased spine density (Nakayama et al., 2000). Active RhoA has the opposite effect, reducing both spine length and formation (Tashiro et al., 2000).

Based on these studies, it appears that Rac and Cdc42 have a variety of effects on dendritic elaboration, but are primarily involved in branching and stability, and Rac has the additional role of inducing lamellipodia formation (Pilpel and Segal, 2004). Rho, however, appears to be involved primarily in limiting the extension of dendrites, although it should be noted that there is some overlap with these effects, for instance constitutively active Rho increases primary dendrite number in cortical neural culture (Threadgill et al., 1997).

The morphological effects of altered RhoGTPase activity are likely due to changes in cytoskeletal organization. For example, Cdc42 and Rac both indirectly activate the Arp2/3 complex, which promotes actin polymerization and filopodia formation (Eden et al., 2002; Machesky and Insall, 1998; Millard et al., 2004; Takenawa and Miki, 2001). The importance of the Arp2/3 complex in process initiation is supported by data showing that loss of Arp2/3 in growth cones of both primary hippocampal neurons and differentiated neuroblastoma cells impairs the formation of both lamellipodia and filopodia (Korobova and Svitkina, 2008). In these neuroblastoma cells, expression of constitutively active Rac increases filopodia initiation rate – an effect that is blocked with co-transfection of an siRNA against Arp2/3 (Korobova and Svitkina, 2008).

It has been suggested that RhoA might act by directing, through its effectors, the phosphorylation of myosin light chains and, subsequently, actomyosin contractility (Hirose et

al., 1998; Kimura et al., 1996; Zhang et al., 2003). Inhibition of the Rho effector p160ROCK in NIE-115 neuroblastoma cells induces neurite extension via the assembly of microtubules and intermediate filaments (Hirose et al., 1998). Conversely, activated p160ROCK induces neurite retraction through the phosphorylation of myosin light chain (MLC) to increase actomyosin contractility (Hirose et al., 1998).

Although RhoA, Rac, and Cdc42 are the most commonly studied GTPases in terms of filopodia formation, a number of other GTPases have been linked to dendritic or neuritic growth. Overexpression of Rho in filopodia (RIF) acts through diaphanous-related formin 2 (Dia2) to induce the formation of elongated filopodia in non-neuronal cells (Ellis and Mellor, 2000; Pellegrin and Mellor, 2005), and in neuronal cells depletion of mammalian Dia2 (mDia2) reduces filopodia number (Hotulainen et al., 2009). Overexpression of another Rho-related protein, RhoG, increases neurite addition in PC12 cells upstream of Rac and Cdc42 (Katoh et al., 2000). Cortical pyramidal neurons of mice lacking GTPase Dbl have significantly shortened dendrites (Hirsch et al., 2002). Activation of R-Ras promotes lamellipodia formation and inhibits filopodia formation in non-neuronal cell culture; inhibition of R-Ras has the opposite effect (Ada-Nguema et al., 2006). Rap1 can inhibit RhoA (Krugmann et al., 2004), and may therefore be involved in filopodial or lamellipodial dynamics.

1.1.4.3 Intracellular signaling pathways

While it is clear that both neural activity and cytoskeletal reorganization are involved in dendrite morphogenesis, our understanding of the molecular pathways underlying developmental synaptic and morphological plasticity are rudimentary. However, recent

studies have indentified signaling pathways which may be involved in transmitting a synaptic signal to a morphological phenotype.

In vivo studies of neuronal growth in *X. laevis* tadpoles have implicated CaMKII in developmental dendritogenesis (Wu and Cline, 1998). CaMKII is a calcium- and activity-regulated kinase implicated in synaptic long-term potentiation (LTP) and learning and memory in the mature brain (Lisman et al., 2002). In immature *X. laevis* tectal neurons, overexpression of CaMKII both promotes synapse maturation and increases dendritic filopodial stability, while blocking CaMKII activity decreases filopodial stability (Wu and Cline, 1998). A role for CaMKII in dendritic growth has been confirmed in both sympathetic and hippocampal neurons, which have identified the Ras-MEK-ERK pathway as the transducer of the CaMKII signal (Vaillant et al., 2002; Wu et al., 2001). Interestingly, inhibition of either CaMKII or ERK inhibited dendrite formation, but inhibition of both was required for dendrite retraction (Vaillant et al., 2002). It's possible that there may be another activity-dependent pathway upstream of ERK, for instance cAMP and its effectors PKA and Epac, which acts in concert with CaMKII to prevent dendritic retraction. Evidence supports CaMKII regulation of the cytoskeleton, since it directly interacts with both actin (Sanabria et al., 2009) and microtubules (Quinlan and Halpain, 1996a, b; Sanchez et al., 2000; Vaillant et al., 2002), although these interactions can be age-dependent (Quinlan and Halpain, 1996a).

Another kinase implicated in dendrite growth is protein kinase C (PKC). PKC is involved in learning and memory in *Drosophila* and rodents, and in maintenance of hippocampal LTP (Drier et al., 2002; Ling et al., 2002; Pastalkova et al., 2006). Studies in Purkinje cells show that activating PKC inhibits, while inhibiting PKC promotes, dendritic growth (Metzger and Kapfhammer, 2000). A constitutively-active isoform of PKC, PKMζ,

when overexpressed in developing *X. laevis* neurons, stabilizes dendritic filopodia and restricts arbor expansion (Liu et al., 2009). These results imply that PKC is a negative regulator of dendrite growth, most likely by directing synapse and arbor maturation.

How these signaling molecules induce a morphological phenotype is not clear. Presumably they act, through downstream effectors, to either regulate the cytoskeleton or to increase synaptic strength or stability, although these possibilities are not mutually exclusive. Regulation of synaptic strength could then lead to increased growth, as described by the synaptotropic hypothesis, or to reduced growth and morphological stabilization as the cell matures. The adenylyl cyclase signaling pathway has been linked to both cytoskeletal effectors and to synaptic plasticity, however studies of a direct role in dendritogenesis are rudimentary. This pathway is therefore an attractive target when searching for potential regulators of developmental dendritogenesis *in vivo*.

1.2 Adenylyl cyclase – a signaling pathway potentially involved in dendritogenesis

One pathway which could potentially regulate dendritogenesis *in vivo* is the adenylyl cyclase (AC) pathway. There are ten isoforms of AC – nine membrane-bound and one soluble (sAC) – which are activated or inhibited in response to specific types of activity. AC regulators include calcium, PKA, PKC, and stimulatory or inhibitory G proteins (Cooper and Crossthwaite, 2006; Sadana and Dessauer, 2009; Willoughby and Cooper, 2007). When activated, AC converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), setting off a signaling cascade which has numerous effects within the cell.

While there has been little direct demonstration of a role for AC in dendrite growth *in vivo*, AC has been shown to play a role in LTP in the mature brain (Chetkovich et al., 1991;

Storm et al., 1998; Villacres et al., 1998; Wang et al., 2004; Wang et al., 2003; Weisskopf et al., 1994; Wong et al., 1999; Wu et al., 1995) and in learning and memory. Studies in *Drosophila melanogaster* have shown that mutation of the gene *rutabaga,* which encodes an AC isoform, causes learning and memory deficits (Dudai, 1985; Dudai and Zvi, 1985; Levin et al., 1992; Livingstone et al., 1984). Pharmacological activation of AC induces LTP in the hippocampus (Villacres et al., 1998), increases synaptic potentiation at the *Drosophila* NMJ (Cheung et al., 2006), and increases NMDA receptor-mediated EPSPs in the amygdala (Huang et al., 1993) and hippocampus (Raman et al., 1996). Mutations of *Adcy1,* or AC1, are responsible for the mouse *barrelless* mutation (Abdel-Majid et al., 1998). Layer 4 of the mouse somatosensory cortex contains anatomically distinguishable areas called "barrels" which form a topographic map responding to information from the vibrissae, or whiskers (Woolsey and Van der Loos, 1970). This system has long been used as a model for experience-dependent neural plasticity. Mice carrying the *barrelless* mutation lack this barrel structure. *Barrelless* synapses remain in an immature state, with impaired AMPA receptor trafficking to synapses and poor LTP/LTD induction as a result of reduced cAMP levels (Lu et al., 2003).

Activation of AC induces neurite growth in PC12 (Ravni et al., 2008; Richter-Landsberg and Jastorff, 1986), AS583.8 cells (Kwon et al., 1996) and cultured striatal neurons (Schmidt et al., 1998), and induces both neurite growth and synaptogenesis in cultured hippocampal neurons (Tominaga-Yoshino et al., 2002) and spinogenesis in cultured cortical pyramidal neurons (Kwon and Sabatini, 2011). ACs have also been implicated in axonal growth and growth cone dynamics. In cultured rat DRG neurons, application of netrin-1 activates sAC, causing an increase in growth cone filopodia and axon length (Wu et

al., 2006). The evidence for an AC role in synaptogenesis, synaptic plasticity, and neurite outgrowth *in vitro* imply that AC may be involved in dendritogenesis *in vivo*. Pathways upstream and downstream of AC which are implicated in synaptic plasticity, cytoskeletal remodeling, or neurite outgrowth are summarized in Fig. 2.

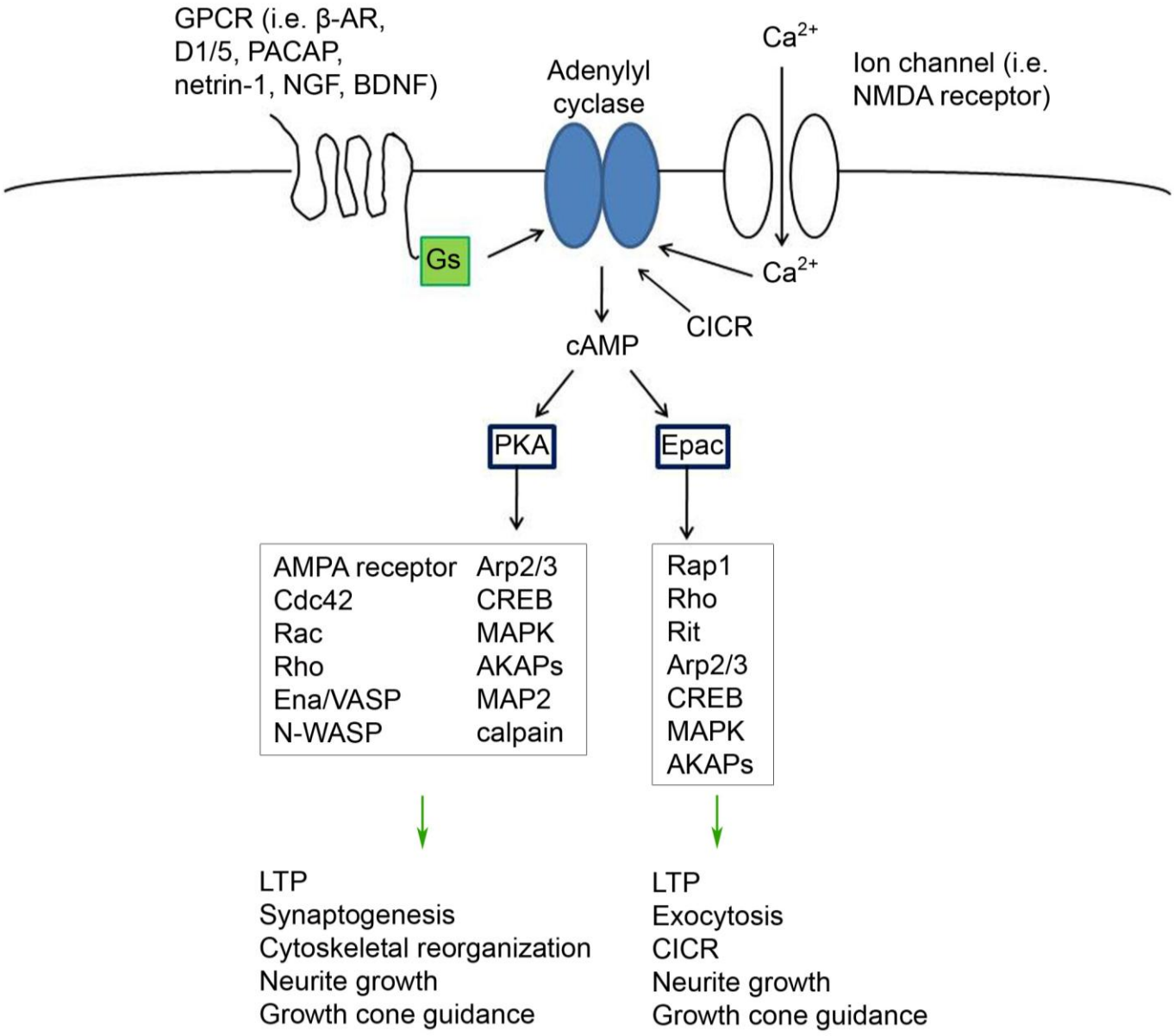


Figure 2: Pathways upstream and downstream of AC implicated in neurite outgrowth.
A schematic showing the activators of AC known to be upstream of PKA or Epac, and to be involved in neurite growth, synaptic plasticity, or cytoskeletal remodeling. PKA and Epac both activate a number of downstream effectors, with results that imply a potential role in dendritogenesis. The link between AC and developmental

dendritogenesis *in vivo*, however, remains unproven. Further discussion of each of these pathways is provided in the text of the introduction.

1.2.1 Upstream activators and inhibitors of adenylyl cyclase

1.2.1.1 Calcium

As discussed above, calcium transients are an important factor regulating developmental dendritogenesis (see section 1.1.3.2). This effect of calcium could be mediated in part by the AC signaling pathway. AC1 and AC8 are activated by calcium in a calmodulin-dependent manner (Cooper and Crossthwaite, 2006; Willoughby and Cooper, 2007). In hippocampal neurons, these AC isoforms can be activated by Ca^{2+} influx associated with NMDA receptor activity (Chetkovich et al., 1991; Chetkovich and Sweatt, 1993). Submicromolar concentrations of free calcium can also inhibit ACs, as in the case of AC5 and AC6, which is thought to be involved in generating oscillating cAMP signals (Sadana and Dessauer, 2009; Willoughby and Cooper, 2007).

1.2.1.2 G-protein coupled receptors

AC activity can be regulated by G-protein coupled receptors (GPCRs). GPCRs positively coupled to AC via stimulatory G proteins include all β adrenergic receptor (β-AR) isoforms (β1, β2, and β3) (Bylund et al., 1994), dopamine receptor isoforms D1 and D5 (Yao et al., 2008) and serotonin receptor isoforms 5-HT4 and 5-HT7 (Araki et al., 2005; Bard et al., 1993; Dumuis et al., 1988; Lovenberg et al., 1993; Ruat et al., 1993). For these receptors, much more is known about their role in synaptic plasticity than in neurite or dendrite growth. For example, β-AR activation induces a PKA-dependent enhancement of NMDA receptor responses in the amygdala (Huang et al., 1993) and hippocampus (Raman et al., 1996;

Thomas et al., 1996). Stimuli that are normally insufficient to induce LTP can induce both early, non-protein synthesis-dependent (Thomas et al., 1996; Winder et al., 1999) and late, protein-synthesis-dependent (Gelinas and Nguyen, 2005; Huang and Kandel, 2007) forms of LTP following β-AR activation. Activation of both the D1 and D5 receptors induces protein-synthesis dependent late-phase LTP in the hippocampus (Huang and Kandel, 1995) and amygdala (Huang and Kandel, 2007). D1/D5 activation also induces an AC-dependent increase in early LTP (Otmakhova and Lisman, 1996), an AC- and PKA-dependent reduction of synaptic depotentiation (Otmakhova and Lisman, 1998), and PKA-dependent enhancement of NMDA- and AMPA-receptor mediated currents (Gonzalez-Islas and Hablitz, 2003; Seamans et al., 2001; Yang, 2000). Serotonin application causes cAMP-dependent increases in synaptic potential in the crayfish lateral giant interneuron (Araki et al., 2005) and in mouse spinal dorsal horn neurons (Wang and Zhuo, 2002). Hippocampal LTP is significantly reduced in 5-HT7 knockout mice (Roberts et al., 2004), and 5-HT4 receptors are involved in PKA- and protein synthesis-dependent LTP in the amygdala (Huang and Kandel, 2007).

Although the GPCR literature primarily focuses on their role in synaptic plasticity, there is evidence that β2-ARs and D1/D5 receptors also play a role in neural morphogenesis *in vitro*. β2-AR activation induces rapid, protein synthesis-independent process outgrowth in cultured AS583.8 cells (Kwon et al., 1996; Kwon et al., 1998). This phenotype is associated with increased microtubule dynamics and actin redistribution, suggesting that adrenergic signals may act, in part, through cytoskeletal reorganization. In cultured striatal neurons, dopamine stimulation through D1/D5 receptors causes a protein synthesis-, cAMP- and PKA-dependent increase in neurite length and complexity, and in growth cone number

(Schmidt et al., 1996; Schmidt et al., 1998). In contrast, overexpression or pharmacological activation of the D1A subunit in mouse cortical neurons reduces dendritic extension and increases MAP2 phosphorylation, also dependent on PKA (Reinoso et al., 1996; Song et al., 2002). Although there is no demonstrated role for 5-HT4 and 5-HT7 receptors in altering dendritogenesis, increasing serotonin levels in the hippocampus by blocking serotonin reuptake increases neurogenesis (Malberg et al., 2000) and spine density (Hajszan et al., 2005). These effects have not been attributed to any specific serotonin receptor isoform. This role in neurite growth *in vitro* implies that GPCRs positively coupled to AC may play a role in dendritogenesis *in vivo*.

Not all GPCRs are positively coupled to AC. The Group II and Group III metabotropic glutamate receptors (mGluRs) are linked to inhibitory G-proteins, and thus reduce AC activity and cAMP levels (Knopfel and Grandes, 2002). Activity of Group II mGluRs has been linked to hippocampal mossy fiber development (Koyama et al., 2002), but it is unknown whether these receptors are also involved in dendrite growth. Inhibition of AC can lead to neurite extension, as has been shown with dopamine receptor isoform 2 (D2) in cortical neural culture (Reinoso et al., 1996).

1.2.2 Downstream effectors of adenylyl cyclase

1.2.2.1 cAMP

Cyclic AMP (cAMP) is a molecule produced by active AC. Cyclic AMP in turn activates three known downstream targets: PKA, Epac, and the cyclic nucleotide-regulated cation channels, which consist of the cyclic nucleotide-gated channels (CNGs) and the

hyperpolarization-activated cyclic nucleotide gated channels (HCNs) . In rat DRG neurons, cAMP levels are high during development and decline during maturation. In these neurons, cAMP has been implicated in axonal growth and reduced expression with maturation, which has been hypothesized to underlie a concomittant loss in ability of axons to regenerate (Cai et al., 2001). Cyclic AMP levels are regulated within the cell by phosphodiesterases (PDEs), most notably phosphodiesterase 4 (PDE4), which breaks down cAMP to limit the time course of the cAMP signal. Tight regulation of the magnitude and time course of cAMP signaling is critical for proper neural functioning, as evidenced by *D. melanogaster* mutants of the cAMP PDE *dunce*, which have increased cAMP levels and deficits in learning and memory (Davis, 1996).

The effects of artificially increasing activity in cAMP-dependent pathways by application of non-specific cAMP analogues, such as 8-Br-cAMP, largely mimic the effects of AC activation. For example, 8-Br-cAMP-dependent dephosphorylation of the actin-binding proteins actin depolymerizing factor (ADF) and cofilin is accompanied by neurite extension in PC12 cells (Meberg et al., 1998), similar to the extension effects observed when AC is activated either through its upstream effectors or through direct pharmacological means (see sections1.2 and 1.2.1.2). Potential effects of cAMP on dendritic outgrowth will be discussed in more detail in the following sections, separated based on downstream effectors.

1.2.2.2 PKA

The cAMP-dependent protein kinase, or protein kinase A (cAPK or, more commonly PKA), is a protein kinase which, following activation by cAMP, phosphorylates and thereby alters the activity of target proteins (Nguyen and Woo, 2003). PKA is expressed as a

tetrameric holoenzyme containing two regulatory subunits (isoforms RIα, RIβ, RIIα, and RIIβ) and two catalytic subunits (isoforms Cα, Cβ, and Cγ). Under basal conditions, the PKA regulatory subunits block the active site on the catalytic subunit. Upon binding of two cAMP molecules, one to each regulatory subunit, these subunits detach to expose the catalytic active sites and allow PKA-dependent phosphorylation of substrate proteins.

PKA is involved in both the induction (Abel and Nguyen, 2008; Calixto et al., 2003; Nguyen and Woo, 2003) and maintenance of LTP (Abel and Nguyen, 2008; Calixto et al., 2003; Esteban et al., 2003; Nguyen and Woo, 2003) and plays an important role in learning and memory in a number of model systems, including *Aplysia* (Kandel, 2001), *Drosophila* (Davis, 1996; McGuire et al., 2005), and rodents (Abel and Nguyen, 2008). Activation of PKA increases neurite length in cultured *X. laevis* spinal neurons and NG108-15 cells (Kao et al., 2002), and increases neurite and varicosity number in NG108-15 cells (Tojima et al., 2003a). Forskolin activation of AC induces neurite outgrowth in cultured rat motoneurons in a PKA-dependent manner (Aglah et al., 2008). In primary hippocampal cultures, PKA activity increases both process length and number of branch points (Fujioka et al., 2004). Mouse knock-outs of MAP2, a PKA anchoring protein, have reduced PKA expression and reduced dendrite length in hippocampal and cortical neurons (Harada et al., 2002). The hormones corticotropin-releasing factor and urocortin cause a PKA-dependent increase in neurite number in rat cerebellar Purkinje cells (Swinny et al., 2004).

While most studies on PKA have revealed a positive effect on growth, a few studies have focused on the ability of PKA to restrict growth. In *Drosophila* aCC motor neurons, expression of a dominant-negative form of PKA results in dendritic overgrowth. Overexpression of PKA in these same neurons was able to rescue overgrowth in mutants

with reduced synaptic transmission (Tripodi et al., 2008). In another study, activation of PKA caused repulsive growth cone turning in dorsal root ganglion (DRG) axons (Murray et al., 2009a). These studies highlight an important consideration to keep in mind when studying the action of intracellular signaling pathways on developmental dendritogenesis: that often the morphological result of pathway activation is dependent on the type or maturational state of the cells being examined.

There are several pathways through which PKA can hypothetically affect dendritogenesis. For example, PKA activates Rac (O'Connor and Mercurio, 2001) and Cdc42 (Feoktistov et al., 2000) and inhibits Rho (Dong et al., 1998; Ellerbroek et al., 2003; Lang et al., 1996), providing a key linkage point to cytoskeletal regulation. PKA can also act on proteins which directly affect the cytoskeleton, for instance PKA phosphorylates VASP (Howe, 2004), thereby increasing formation and elongation of growth cone filopodia (Lebrand et al., 2004).

PKA can be activated under a wide range of conditions and acts on a large number to downstream effectors, each of which have distinct and sometimes contradictory effects on cell function. Thus, cellular mechnisms are required to maintain specificity to particular PKA signals. One such mechanism is the use of A-kinase anchoring proteins (AKAPs), which anchor PKA to specific activators and effectors in order to both temporally and spatially restrict signaling by minimizing diffusion and to ensure that activated PKA activates only certain of its downstream effectors in order to realize a specific effect. AKAPs also anchor PKA and AC in close proximity to proteins such as PDE4 or protein phosphatases, which limit the time course of the signal by either breaking down cAMP or dephosphorylating PKA targets, respectively. A large number of proteins have been discovered to be AKAPs,

including MAP2 and WAVE1 (Wong and Scott, 2004). Disrupting PKA binding to AKAPs has effects on synaptic activity (Davare et al., 2001; Westphal et al., 1999; Zhong et al., 2009), growth cone turning (Han et al., 2007), and neurite formation (Caceres et al., 1992; Dinsmore and Solomon, 1991). MAP2 knock-out mice show reduced PKA expression and reduced CREB phosphorylation in response to forskolin, and reduced length of hippocampal dendrites (Harada et al., 2002), indicating a role for proper PKA localization in the regulation of dendrite growth.

1.2.2.3 Epac

For many years, PKA was thought to be the sole effector of cAMP. This changed with the 1998 discovery of the Exchange Protein Activated by cAMP, or Epac (de Rooij et al., 1998; Kawasaki et al., 1998). It has since become clear that several of the pharmacological cAMP analogues thought to activate PKA also activate Epac, forcing re-evaluation of the pathways downstream of cAMP.

Epac is a guanine-nucleotide exchange factor (GEF) which has been identified as a cAMP-dependent, PKA-independent activator of the small GTPases Rap1, Rap 2, and R-Ras (Bos, 2006; Holz et al., 2006; Kawasaki et al., 1998; Kopperud et al., 2003; Roscioni et al., 2008). Unlike PKA, Epac exists as a single subunit, which in its inactive state is folded so that the active site is inaccessible. Upon binding of cAMP, Epac changes its confirmation, revealing the active site (Rehmann et al., 2006). As an exchange protein, Epac acts by exchanging GDP for GTP on GTPases, thus converting GTPases to an active form and enabling them to activate downstream effectors. There are two isoforms of Epac, Epac1 and Epac2. The most notable differences between the two isoforms are that Epac2 has a second,

weak binding site for cAMP, but the functional significance is unknown (Murray and Shewan, 2008), and that Epac1 has a more ubiquitous expression profile than Epac 2, which is limited to the brain and endocrine tissues (Kawasaki et al., 1998). Both isoforms are expressed in the brain and are developmentally regulated (Kawasaki et al., 1998; Murray and Shewan, 2008).

Epac activation enhances protein-synthesis dependent LTP in hippocampal slices (Gelinas et al., 2008a) and increases synaptic potentiation at the Calyx of Held (Kaneko and Takahashi, 2004) and the crayfish (Zhong and Zucker, 2005) and *Drosophila* neuromuscular junctions (NMJs) (Cheung et al., 2006). Epac induces neural differentiation of PC12 (Christensen et al., 2003; Kiermayer et al., 2005) and PC6 cells (Shi et al., 2006) and neurite outgrowth in PC12 cells (Christensen et al., 2003). Epac activation increases neurite length in organotypic slices of the mouse pre-Bötzinger complex (Mironov et al., 2011). Rap1, a downstream target of Epac (de Rooij et al., 1998), regulates neurite length and number in PC12 cells in an AC-dependent, PKA-independent manner (Ravni et al., 2008). Epac activation significantly increases neurite outgrowth in cultured rat dorsal root ganglion (DRG) neurons (Murray and Shewan, 2008). In this study, PKA activators were unable to enhance neurite outgrowth when Epac was knocked down.

Epac causes release of calcium from intracellular stores and mediates calcium-induced calcium release (CICR) (Kang et al., 2003). A direct link between β-adrenergic receptors, Epac, and CICR has been demonstrated in cardiac myocytes (Oestreich et al., 2007). Regulation of intracellular calcium levels may be one mechanism by which Epac affects dendritogenesis. In chick retinal ganglion cells for example, CICR prevents dendritic retraction (Lohmann et al., 2002).

As with PKA, the effects of Epac activation on dendritic growth are not always straightforward. One example of this is the case of the pituitary adenylate cyclase-activating polypeptide (PACAP), which activates AC through the G-protein coupled vasoactive intestinal peptide (VIP) receptor. PACAP inhibits bone morphogenic protein 7 (BMP-7)-induced dendritic growth in cultured sympathetic neurons, most likely through increased cAMP activity, although the specific cAMP effector was not identified (Drahushuk et al., 2002). In contrast, PACAP has been positively linked to neurite growth in PC12 (Ravni et al., 2008) and PC6 cells (Shi et al., 2006), through an Epac-dependent, PKA-independent pathway. As with PKA, the cell type in question may be a primary factor in determining response to Epac.

As a GEF for small GTPases, Epac is potentially linked to key regulators of the cytoskeleton (see section 1.1.4.2.1 – the RhoGTPases). Epac acts directly on Rap1 and 2 as well as R-Ras, Rims, and possibly Rit, and through Rap Epac indirectly activates Rac and inhibits Rho (Bos, 2006; Holz et al., 2006; Kawasaki et al., 1998; Kopperud et al., 2003; Maillet et al., 2003; Roscioni et al., 2008; Zieba et al., 2011). Epac has also been shown to interact with microtubules and affect microtubule polymerization (Gupta and Yarwood, 2005; Mei and Cheng, 2005) Activation of Epac leads to microtubule polymerization which is not increased further by the addition of exogenous cAMP (Mei and Cheng, 2005; Sehrawat et al., 2008).

As with PKA, Epac signaling is regulated by anchoring proteins (Gloerich and Bos, 2010; McConnachie et al., 2006). The muscle-specific A-kinase anchoring protein (mAKAP) clusters Epac1 with PKA, phosphodiesterase 4D3, and ERK5 to regulate cAMP signaling in

the heart (Dodge-Kafka et al., 2005). Whether Epac targeting to AKAPs is involved in neural function is unknown.

1.2.2.4 PKA versus Epac: independence, synergy, and opposition

How a cell responds to a cAMP signal can depend on the relative activation levels of PKA and Epac. The degree to which a cAMP signal activates PKA versus Epac is regulated through both differential anchoring with AKAPs and through the magnitude of the cAMP signal. It has previously been reported that cAMP has a higher affinity for PKA than Epac (Dao et al., 2006; Ponsioen et al., 2004; Poppe et al., 2008). PKA- and Epac-based FRET probes show different responses to cAMP signals, with PKA activating at lower cAMP concentrations but with slower activation kinetics than Epac (Nikolaev et al., 2004; Ponsioen et al., 2004). These studies have been questioned by data showing that PKA and Epac have similar affinities for cAMP (Dao et al., 2006). The authors of this study suggest that dissociated PKA regulatory subunits do have a higher affinity for cAMP then Epac, but when bound to a catalytic subunit PKA/cAMP affinity drops and becomes more comparable with that of Epac. The PKA-based cAMP sensors, however, have all domains intact (Ponsioen et al., 2004), so the question of the relative affinities remains unanswered.

Both Epac and PKA play a role in neurite outgrowth, and there are several different lines of evidence regarding the relative roles played by these two factors. In some cases, only one of these two proteins is implicated in a growth phenotype. For example, cAMP increases neurite length in the pre-Bötzinger complex of the MeCP2-/y mouse, which is a model for Rett syndrome. This effect is mimicked by Epac activation but independent of PKA activity

(Mironov et al., 2011). Similarly, the effects of PACAP on neuritogenesis are reported to be Epac-dependent and PKA-independent (Ravni et al., 2008; Shi et al., 2006).

In most cases however, hypothesizing a mechanism whereby only PKA or Epac is responsible for a given phenotype is overly simplistic. Often the two proteins have opposing or even synergistic effects. For example, one study of PC12 cells reported that Epac increased neural differentiation while PKA increased cell proliferation (Kiermayer et al., 2005). Another study using these same cells reported that Epac and PKA act synergistically to promote neurite outgrowth (Christensen et al., 2003). Using these and other data, Gerdin and Eiden (Gerdin and Eiden, 2007) proposed a model whereby AC-induced cAMP increase activates two pathways: an Epac-dependent pathway and a PKA-dependent pathway. The Epac-dependent pathway acts through Rap1 or Rit to induce neuritogenesis and the PKA-dependent pathway acts through ERK/MAPK to control other aspects of differentiation, such as ion channel expression. However, it should be noted that this model is based on neurite growth in PC12 cells which are derived from rat adrenal medulla (Greene and Tischler, 1976), and thus may not reflect what happens in developing dendrites *in vivo*, and that the pathways activated downstream of Epac and PKA varied with the mode of AC activation. The two proteins can also act in opposition, in a maturational state-dependent manner. A study from the Shewan lab showed that Epac regulates netrin-1 and cAMP-dependent attractive axonal growth cone turning in DRG neurons from embryonic rats. In neurons from adult rats, however, netrin-1 induced repulsive growth cone turning was dependent on PKA (Murray et al., 2009b), suggesting that differential activation of PKA versus Epac could underlie the maturational switch in growth cone responses to cAMP. More research is needed in order to fully tease apart the relative roles of PKA and Epac in the regulation of neurite

growth. Whether they act individually or in concert may depend on, among other things, the system used, the experimental set-up – such as whether neurons were cultured on cells expressing myelin associated glycoprotein (MAG), which promotes or inhibits cAMP-dependent axonal regeneration dependent on cell maturational state (Domeniconi and Filbin, 2005), the mode of AC activation, or the spatiotemporal control of AC signaling within the cell.

1.2.2.5 Cyclic nucleotide-regulated cation channels

Cyclic AMP can also activate two subtypes of ion channels, allowing for cation influx and membrane depolarization: the cyclic nucleotide gated (CNG) channels and the hyperpolarization-activated cyclic nucleotide gated (HCN) channels.

1.2.2.5.1 Cyclic nucleotide gated channels

Cyclic nucleotide gated ion channels (CNGs) require either cAMP or cGMP for their opening, but have a higher affinity for cGMP than for cAMP. They are permeable to Na^+, K^+, and Ca^{2+} (Biel, 2009; Biel and Michalakis, 2009). CNGs are primarily involved in sensory transduction in the visual and olfactory systems. Cyclic GMP-sensitive CNGs are expressed in photoreceptors, where they remain open during the dark and hyperpolarize in response to light-induced hydrolysis of cGMP (Biel, 2009). In the olfactory system, binding of odorants to their receptors causes the synthesis of cAMP or cGMP and subsequent opening of CNGs (Biel, 2009; Biel and Michalakis, 2009). CNGs are expressed in the brain, although the functional significance is poorly understood (Biel and Michalakis, 2009; Kaupp and Seifert, 2002). One olfactory subtype, CNG2A, has been localized in murine hippocampus.

Hippocampal LTP induced by theta burst stimulation is reduced in CNG2A knockout mice, although neither basal synaptic activity nor LTP induced by other stimulation protocols differ significantly from wild type (Parent et al., 1998). There has been no detection of memory impairment in these mice (Biel and Michalakis, 2009). CNG3A knockout mice show normal basal synaptic transmission but enhanced hippocampal LTP in response to theta stimulation. Despite the enhanced LTP, these mice show no changes in hippocampus-dependent learning (Michalakis et al., 2011). As of this writing, there is no demonstrated role for the CNGs in dendritic morphogenesis.

1.2.2.5.2 Hyperpolarization-activated cyclic nucleotide gated channels

Hyperpolarization-activated cyclic nucleotide gated (HCN) channels are a family of cAMP-sensitive ion channels expressed in the heart and brain and known for their ability to generate rhythmic activity. They are primarily permeable to Na^+ and K^+ but can also weakly conduct Ca^{2+} (Biel, 2009). HCNs are encoded by four genes, HCN1-4, with HCN2 and 4 being the most sensitive to cAMP and HCN 1 and 3 being only weakly responsive to cAMP (Wahl-Schott and Biel, 2009). Although membrane hyperpolarization is a requirement for HCN opening, binding of cAMP to HCNs speeds channel opening and shifts voltage-dependence of activation to more positive voltages (DiFrancesco and Tortora, 1991).

All four HCN isoforms are expressed in the brain, where they mediate pacemaking rhythmic or oscillatory activity. HCNs are involved in dendritic integration of synaptic signals in the hippocampus by normalizing the time course of EPSPs generated at distal and proximal inputs (Robinson and Siegelbaum, 2003). There is also evidence for a role of HCNs in the regulation of synaptic activity. Most effects of HCNs on synaptic activity seem to be

inhibitory in nature. Mice lacking the HCN1 gene show increases hippocampal learning and memory and LTP, but impaired motor learning (Nolan et al., 2004; Nolan et al., 2003). HCN1 channels can control calcium spiking by setting the resting membrane potential, and it has been suggested that this is the mechanism by which HCNs limit LTP (Tsay et al., 2007; Wahl-Schott and Biel, 2009). It should be noted that cAMP facilitates, but is not absolutely required for the opening of HCN1 channels. Further, HCN1 is only weakly responsive to cAMP, so this phenomenon may not be dependent on cAMP activity. At this time, there is no evidence of a role for HCNs in dendritic morphogenesis. Given the inhibitory role of HCN in LTP and calcium spiking, and the evidence of roles for PKA or Epac in synaptic activity and neurite growth, HCNs are not as likely a candidate for mediating a potential cAMP-dependent effect on dendritogenesis as PKA or Epac.

1.3 The albino *Xenopus laevis* tadpole retinotectal system as a model for developmental dendritogenesis.

A number of methods have been developed to study neuronal morphological development. Neural culture allows imaging of axons and dendrites using light microscopy. Non-neuronal cells capable of adopting a neuronal fate allow for easy manipulation of intracellular signaling pathways which potentially regulate neuron growth. However, these techniques are limited in that they are not a true reconstruction of what occurs in an intact, developing brain.

The retinotectal system of the *Xenopus laevis* tadpole is a powerful system for studying dynamic dendritic arbor growth of developing vertebrate brain neurons *in vivo* (Cline, 2001). Using the technique of single-cell electroporation (Haas et al., 2001), it is

possible to introduce a wide range of molecules into individual neurons within an otherwise normally functioning brain circuit. These include plasmid DNA for the expression of fluorescent proteins or constitutively-active or dominant-negative mutated versions of proteins of interest, small peptide inhibitors, and fluorescent dyes and indicators. The permeability of the tadpole allows for non-invasive bath application of drugs. Further, it is possible to inject compounds directly into the brain with minimal damage to the animal. Tadpoles are albino, and transparent at early stages of development, allowing for non-invasive subsequent *in vivo* imaging of morphological growth plasticity of neurons within their natural environment. Direct time-lapse two-photon imaging of single neurons can be performed over time periods ranging from minutes to days.

Experiments are performed in the optic tectum, which corresponds to the superior colliculus in mammals. Optic tectal neurons are born in a proliferative zone in the medial section of the tectum, then elaborate arbors laterally toward the neuropil, where they recieve direct innervation from the retinal ganglion cells of the contralateral eye. Optic tectal axons terminate locally, or extend into the contralateral tegmentum or down the spinal cord. Dendritic arbor development in these neurons progresses through three distinct phases. During the first phase, while axonal growth and arbor elaboration is taking place, the dendritic arbor is composed of short filopodial processes that exhibit high turnover without formation of longer branches. In the second phase, filopodia transition into longer branches and the dendritic arbor expands rapidly. This growth is accompanied by high levels of refinement, with rapid extension and retraction of branches. In the third phase, the rate of growth slows down and the dendritic arbor becomes relatively stable (Cline, 2001; Wong et

al., 2000; Wu et al., 1999). These three distinct, recognizable phases allow for examination of age-dependent growth mechanisms.

The natural features of the *X. laevis* tadpole allow for dynamic imaging of dendritic growth behavior in an intact, developing animal. This organism is a powerful vertebrate *in vivo* model system for investigating the molecular mechanisms underlying neural development and circuit plasticity.

1.4 Hypothesis and aims

Although much work has been done on the signals and associated receptor systems, both activity-dependent and activity-independent, which regulate dendritic growth, little is known about the intracellular mechanisms which translate a pertinent signal into a morphological phenotype. It is known that the processes of filopodial initiation and extension require cytoskeletal remodeling and that new filopodia can initiate in response to cues like growth factors. Later in development, synaptogenesis guides dendritic growth and graded synaptic maturation provides graded structural stabilization. However, the intracellular signaling pathways which link extracellular signals to cytoskeletal remodeling and altered morphology are poorly understood.

The AC signaling pathway is activated by activity dependent signals such as calcium and the GPCRs, and, through its effectors, can alter the activity of actin-binding proteins leading to cytoskeletal remodeling, however a direct link between AC and developmental dendritogenesis remains unproven. ***I hypothesized that, given the links between AC and processes related to dendrite growth, that AC and its associated signaling pathways may be key regulators of dendritogenesis in vivo.*** In my Ph.D. thesis research, I proposed to test

whether AC-dependent signaling pathways are involved in dendritic growth in the optic tectum of the albino *Xenopus laevis* tadpole. First, I tested whether manipulating AC or cAMP activity alters dendritic growth. Using drugs designed to increase or decrease cAMP production, I characterized the role of cAMP in developmental dendritogenesis.

PKA and Epac are two downstream effectors of AC. Both PKA and Epac have been implicated in synaptic plasticity, cytoskeletal remodeling, neurite growth in cultured neurons or non-neuronal cells capable of adopting a neuronal fate. ***I hypothesized that either PKA or Epac are responsible for transducing a cAMP signal to a morphological phenotype in developing neurons in vivo.*** I used pharmacological manipulations to activate or inhibit PKA or Epac in the whole brain, or single-cell electroporation of DNA constructs to increase or decrease the activity of target proteins in individual neurons. With time-lapse two-photon imaging of labeled neurons I was able to characterize the role of PKA and Epac in the developing brain, over time courses ranging from minutes to days.

Several GPCRs linked to AC have been implicated in synaptic plasticity and neurite growth, but of these, the evidence suggesting a role for β-AR activity in dendritogenesis is most convincing. ***I hypothesized that β-adrenergic receptor activity influences dendritic growth through the AC/cAMP and either PKA or Epac.*** Using time-lapse two-photon imaging of labeled neurons I outlined a pathway from β-ARs through PKA and Epac for the regulation of dendritic growth dynamics.

PKA has a wide rance of effects within the cell, which are not limited to its roles in synaptic or morphological plasticity (Shabb, 2001). AKAPs anchor PKA close to its activators and effectors, applying temporal, spatial, and mechanistic specificity to particular PKA signals. AKAP-dependent anchoring of PKA close to its activators and effectors is

critical to PKA's role in synaptic activity. ***I hypothesized that this PKA-AKAP anchoring is also critical to PKA-dependent dendritic growth.*** I use direct tectal infusion of an inhibitory peptide designed to abolish PKA localization to test this hypothesis.

While much *in vivo* work has been done on the stages of dendritic growth which require synaptic maturation and structural stabilization (Chen et al., 2010b; Liu et al., 2009; Wu and Cline, 1998), little is known about the earlier stages of growth, during which nascent filopodia are first initiated. It seems clear, however, that cytoskeletal remodeling resulting from actin branching and polymerization is a key step in filopodial initiation. Previous studies have shown that PKA-dependent inhibition of the protease calpain regulates axonal filopodial initiation *in vitro* upstream of the actin-binding protein cortactin (Mingorance-Le Meur and O'Connor, 2009). ***I hypothesized that PKA-dependent inhibition of calpain regulates filopodial initiation in both axons and dendrites of developing neurons in vivo.*** Using both pharmacological and genetic manipulations, I examined the role of calpain in both axonal and dendritic growth *in vivo*.

Chapter 2: Adenylyl cyclase activation increases dendritogenesis in optic tectal neurons of *Xenopus laevis* tadpoles

2.1 Introduction

The period of early brain development involves an exceptional amount of neuronal morphological growth and refinement to form functional brain circuits. Growing neurons must extend long and often complex axonal and dendritic arbors to contact distant neurons and to increase contact area for synapse formation. The process of dendritogenesis is highly dynamic, involving the rapid extension and retraction of small filopodial processes that are precursors of longer and persistent branches (Hossain et al., 2011). This process requires both cytoskeletal reorganization and synaptogenesis (for reviews see: (Cline and Haas, 2008; Cline, 2001; Craig and Banker, 1994; Faix et al., 2009; Jan and Jan, 2003; Lohmann and Wong, 2005; McAllister, 2000; Scott and Luo, 2001; Wong and Ghosh, 2002). Although it is known that neural activity is involved in dendrite morphogenesis, the downstream molecular pathways which convert neural transmission into changes in growth are not well understood.

The cyclic AMP (cAMP) pathway has been implicated in synaptogenesis (Kwon and Sabatini, 2011; Tominaga-Yoshino et al., 2002) and synaptic plasticity in the mature brain (Chetkovich et al., 1991; Chetkovich and Sweatt, 1993; Storm et al., 1998; Villacres et al., 1998; Wang et al., 2004; Wang et al., 2003; Weisskopf et al., 1994; Wong et al., 1999; Wu et al., 1995). Cyclic AMP activity, acting either directly or through downstream effectors, has also been demonstrated to regulate actin binding proteins, the actin cytoskeleton (Harada et al., 2002; Howe, 2004; Lebrand et al., 2004; Meberg et al., 1998; Mingorance-Le Meur and O'Connor, 2009), microtubules (Dave et al., 2009; Gupta and Yarwood, 2005; Leterrier et al.,

2009; Mei and Cheng, 2005; Sehrawat et al., 2008), and small GTPases known to influence cytoskeletal dynamics (Bos, 2006; Dong et al., 1998; Ellerbroek et al., 2003; Feoktistov et al., 2000; Holz et al., 2006; Kawasaki et al., 1998; Kopperud et al., 2003; Lang et al., 1996; Maillet et al., 2003; Mei and Cheng, 2005; O'Connor and Mercurio, 2001; Roscioni et al., 2008; Shi et al., 2006). Although both synaptic activity and cytoskeletal dynamics are known to play a role in developmental dendritogenesis, the role of cAMP in these processes is poorly understood. Such a developmental role is implicated by *in vitro* studies showing AC/cAMP influences on neurite growth in PC12 cells (Ravni et al., 2008; Richter-Landsberg and Jastorff, 1986), NG108-15 cells (Tojima et al., 2003b), murine embryonic stem cells (Cazillis et al., 2004), and cultured striatal neurons (Schmidt et al., 1998). Activation of cAMP also increases dendrite length and number in immature neurons in the adult hippocampus *in vivo* (Fujioka et al., 2004).

Despite this evidence, a role for cAMP in developmental dendritogenesis *in vivo* has yet to be demonstrated. In order to determine whether cAMP is involved in developmental dendritogenesis *in vivo*, I used rapid time-lapse two-photon imaging of the albino *Xenopus laevis* tadpole to examine the growth of neurons within the intact and unanesthetized developing brain while altering AC activity levels. I find that activation of AC increases dendritic filopodial length and motility dependent on cell age. To my knowledge, this is the first demonstration of a role for AC/cAMP in developmental dendritogenesis *in vivo*.

2.1.1 AC isoforms are developmentally expressed in *X. laevis* and *X. tropicalis*

Prior to performing experiments, the expression patterns of AC isoforms were determined for both *X. laevis* and *X. tropicalis* with searches of published literature and

genomic databases. Unlike *X. laevis*, the *X. tropicalis* genome has been fully sequenced (Hellsten et al., 2010), and can provide insights into *X. laevis* genetics until such time that the *X. laevis* genome becomes available. A recent study of expressed RNAs has shown a high level of conservation between the two species (Yanai et al., 2011). There are 9 membrane-bound isoforms of AC and one soluble isoform which are differentially regulated by distinct effectors, including calcium, PKA, PKC, and stimulatory or inhibitory G proteins (Cooper and Crossthwaite, 2006; Sadana and Dessauer, 2009; Willoughby and Cooper, 2007). All membrane-bound isoforms except AC2 have been cloned from *X. tropicalis*, including the brain-expressed transcripts AC1, AC3, AC4, AC8, and AC9 (Bowes et al., 2008). All nine membrane-bound ACs are developmentally expressed in murine brains, although AC4 is only expressed in the blood vessels (Visel et al., 2006; Willoughby and Cooper, 2007). Transcripts which are predicted to match AC6, AC7, AC8, and AC9 have been cloned from *X. laevis* (Bowes et al., 2008), and AC3, AC4, AC7, and AC9 have been identified in microarray analysis of expressed *X. laevis* mRNAs (Yanai et al., 2011). Developmental expression has been confirmed for all membrane-bound isoforms except AC2 and AC5 (Bowes et al., 2008; Yanai et al., 2011). *X. tropicalis* transcripts show homologies to human transcripts ranging from 22-94%, depending on the isoform (Bowes et al., 2008; Flicek et al., 2011). These findings are summarized in Table 1.

Table 1: Expression patterns of AC isoforms in *X. laevis* and *X. tropicalis*.
JGI: Information obtained in part from the Joint Genome Institute at the United States Department of Energy.

Isoform	**Regulation**	***Xenopus* Expression**	***X. laevis***	***X. tropicalis***	**Stage**	**Homology**	**Mammalian Function**	**References**
1	activated by Gsα, Ca^{2+}/ calmodulin, PKCα; inhibited by G(i,z,o)α, Gβγ, CaMKIV	brain, mesonephric kidney, whole organism		gene sequenced, mRNA expressed	35/36 to adult	chicken: 91%; human: 77%; mouse: 76%; zebrafish: 71%	learning and memory, synaptic plasticity and LTP, opiate withdrawal, barrel patterning, pain response, excitotoxicity, protection from activity-blockade induced neurodegeneration	(Abdel-Majid et al., 1998; Bowes et al., 2008; Cooper and Crossthwaite, 2006; Flicek et al., 2011; Sadana and Dessauer, 2009)
2	activated by Gsα, Gβγ, PKCα							(Sadana and Dessauer, 2009)
3	activated by Gsα, Golf, Ca^{2+}, PKCα; inhibited by Gβγ, CaMKII	brain, heart, head, eye, testes, whole organism	mRNA expressed	gene sequenced, mRNA expressed	28 to adult	chicken: 76%; human: 72%; mouse: 72%; zebrafish: 55%	olfaction, sperm function	(Bowes et al., 2008; Cooper and Crossthwaite, 2006; Flicek et al., 2011; Sadana and Dessauer, 2009; Yanai et al., 2011)

Isoform	Regulation	*Xenopus* Expression	*X. laevis*	*X. tropicalis*	Stage	Homology	Mammalian Function	References
4	activated by Gsα, Gβγ; inhibited by PKCα	brain, central nervous system, fat body, heart, intestine, liver, lung, mesonephric kidney, oviduct, skin, spleen, stomach, tail, testis, whole organism	mRNA expressed	gene sequenced, mRNA expressed	10.5 to adult	chicken: 56%; human: 58%; mouse: 58%; zebrafish: 40%		(Bowes et al., 2008; Flicek et al., 2011; Sadana and Dessauer, 2009; Yanai et al., 2011)
5	activated by Gsα, Gβγ, PKCα, PKCζ; inhibited by free Ca^{2+}, G(i,z)α, PKA			gene sequenced		chicken: 96%; human: 94%; rat: 94%; zebrafish: 90% (JGI)	cardiac contraction, motor coordination, opiate dependency, pain response	(Bowes et al., 2008; Cooper and Crossthwaite, 2006; Sadana and Dessauer, 2009)
6	activated by Gsα, Gβγ; inhibited by free Ca^{2+}, G(i,z)α PKA, PKCδ, PKCε	testis, whole organism	gene predicted	gene sequenced, mRNA expressed	25 to adult	human: 23%; mouse: 22%; zebrafish: 24%	cardiac contraction, calcium sensitivity, opiate dependency	(Bowes et al., 2008; Cooper and Crossthwaite, 2006; Flicek et al., 2011; Gao et al., 2007; Sadana and Dessauer, 2009)

Isoform	**Regulation**	***Xenopus* Expression**	***X. laevis***	***X. tropicalis***	**Stage**	**Homology**	**Mammalian Function**	**References**
7	activated by Gsα, Gβγ, PKCα	egg, lung, testis, thymus, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 32%; human: 29%; mouse: 27%; zebrafish: 30%	ethanol dependency	(Bowes et al., 2008; Flicek et al., 2011; Sadana and Dessauer, 2009; Yanai et al., 2011)
8	activated by Gsα, Ca^{2+}/ calmodulin; inhibited by Gβγ	brain, eye, whole organism	gene predicted	gene sequenced, mRNA expressed	10 to adult	chicken: 79%; human: 78%; mouse: 78%; zebrafish: 53-76% (predicted sequences) (JGI)	learning and memory, synaptic plasticity and LTP, opiate withdrawal, protection from activity-blockade induced neurodegeneration	(Bowes et al., 2008; Cooper and Crossthwaite, 2006; Sadana and Dessauer, 2009)
9	activated by Gsα; inhibited by Ca^{2+}/ calcineurin, PKC; insensitive to Fsk	brain, eye, intestine, spleen, testis, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	10 to adult	chicken: 72%; human: 70%; mouse: 70%; zebrafish: 57%		(Bowes et al., 2008; Flicek et al., 2011; Hacker et al., 1998; Sadana and Dessauer, 2009; Yanai et al., 2011)
soluble AC	activated by Ca^{2+}, bicarbonate						sperm function, fertilization	(Sadana and Dessauer, 2009)

2.2 Methods

2.2.1 Animals

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°C on a 12 h light-dark cycle. Experiments were conducted at room temperature (22°C). All experimental procedures were conducted according to the guidelines of the Canadian Council on Animal Care, and were approved by the Animal Care Committee of the University of British Columbia.

2.2.2 Fluorescent labelling of neurons within the intact tadpole brain

Individual immature neurons within the tadpole optic tectum were fluorescently labeled using *in vivo* single-cell electroporation (SCE) (Haas et al., 2001) of plasmid DNA encoding farnesylated enhanced green fluorescent protein (fEGFP, 2 μg/μl endotoxin-free plasmid DNA in dH_2O) (Fig. 3A,B). For electroporation, tadpoles were briefly anesthetized by immersion in 0.02% tricane methanesulfonate (also known as 3-aminobenzoic acid ethyl ester; MS-222, Sigma-Aldrich, St. Louis, MO). Under visual guidance using an upright stereomicroscope, a sharp glass pipette (tip diameter ~0.6 μm) filled with plasmid DNA solution was inserted into the proliferative zone of the optic tectum. An Axoporator 800A (Axon Instruments, Union City, CA) was used to deliver a brief train of short-duration square wave pulses between the silver wire within the pipette and an external bath electrode. Stimulus parameters were: pulse intensity = 1.5 μA; pulse duration = 1 msec; pulse frequency = 300Hz; train duration = 360 msec.

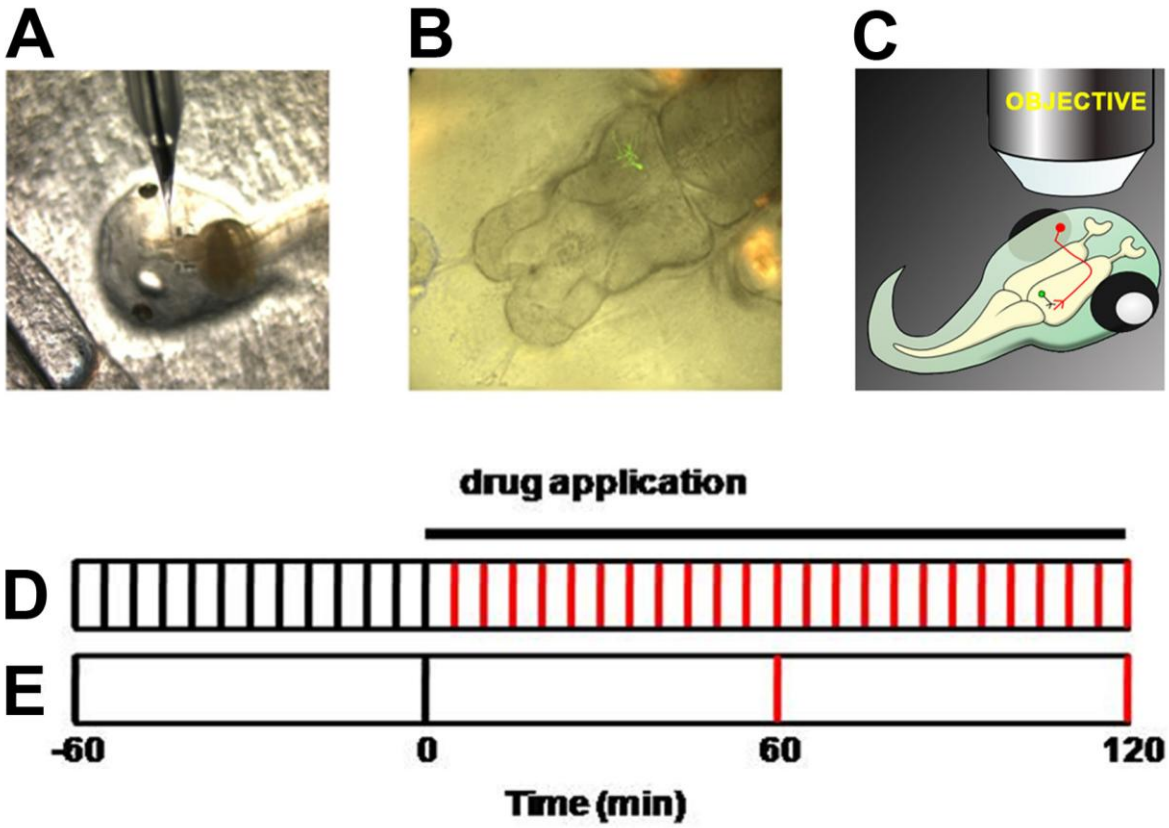


Figure 3: Summary of experimental protocols.
(A) Single-cell electroporation of plasmid DNA into the *X. laevis* tadpole optic tectum. (B) A single, electroporated, fEGFP-expressing neuron within the optic tectum. (C) Tadpoles whose brains contain single labeled neurons are imaged using two-photon microscopy. (D,E) Neurons were imaged for 1 hr of baseline (black) and 1-2 hr of drug application (red). (D) Rapid time-lapse – 5 min interval. (E) 1 hr interval.

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

For rapid time-lapse imaging, tadpoles were paralyzed by bath application of the reversible paralytic agent d-tubocurare (dTC; 2-5mM in 10% Steinberg's rearing solution, Sigma-Aldrich, St. Louis, MO) and then embedded under a thin layer of agarose (1%, prepared in 10% Steinberg's rearing solution) in an imaging chamber continuously perfused with oxygenated rearing solution. Tubocurare was chosen from a range of several available curare-based reversible paralytics. Alternate paralytics, for instance pancuronium dibromide, can potentiate norepinephrine signaling in the cardiovascular system (Kobayashi et al., 1987;

Pinaud and Souron, 1984; Sai et al., 1998; Sato et al., 1999; Vercruysse et al., 1979), and adrenergic signaling can affect neurite growth and synaptic plasticity (see section 1.2.1.2). The imaging chamber was mounted on the stage of a custom-built two-photon laser-scanning microscope, constructed from a BX51 microscope (Olympus, Center Valley, PA) equipped with an Olympus FV300 confocal system and 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) captured the entire dendritic arbors of labeled tectal neurons using an Olympus LUMFI 60X, 1.1 NA, water immersion objective, and Fluoview software (Olympus, Center Valley, PA). In order to capture changes to the dendritic arbor morphology over time, due to intrinsic growth and effects of drug exposure, repeated full-arbor image stacks were acquired at 5 min intervals. Following 1 hr baseline imaging at 5 min intervals, drugs were either added to the rearing solution or directly injected into the optic tectum, after which tadpoles were imaged for a further 1-2 hr at 5 min intervals. Anesthetics were not used during continuous rapid time-lapse imaging experiments to circumvent confounding effects of prolonged anesthetics on normal patterns of neural circuit activity. In experiments examining dendritic arbor growth over 1 hr intervals, tadpoles were briefly anesthetized with 0.02% MS-222, rapidly imaged and returned to their chambers where they recovered from anesthesia within 1-3 min. These protocols are summarized in Fig. 3C, D, and E.

2.2.4 Drug solutions and application

Drugs used for this experiment were as follows: forskolin (Fsk, 50 μM, Sigma-Aldrich, St. Louis, MO); dideoxyforskolin (ddFsk, 50 μM, Enzo Life Sciences, Farmingdale, NY); 9-(Tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ22536, 100 μM, Tocris Bioscience,

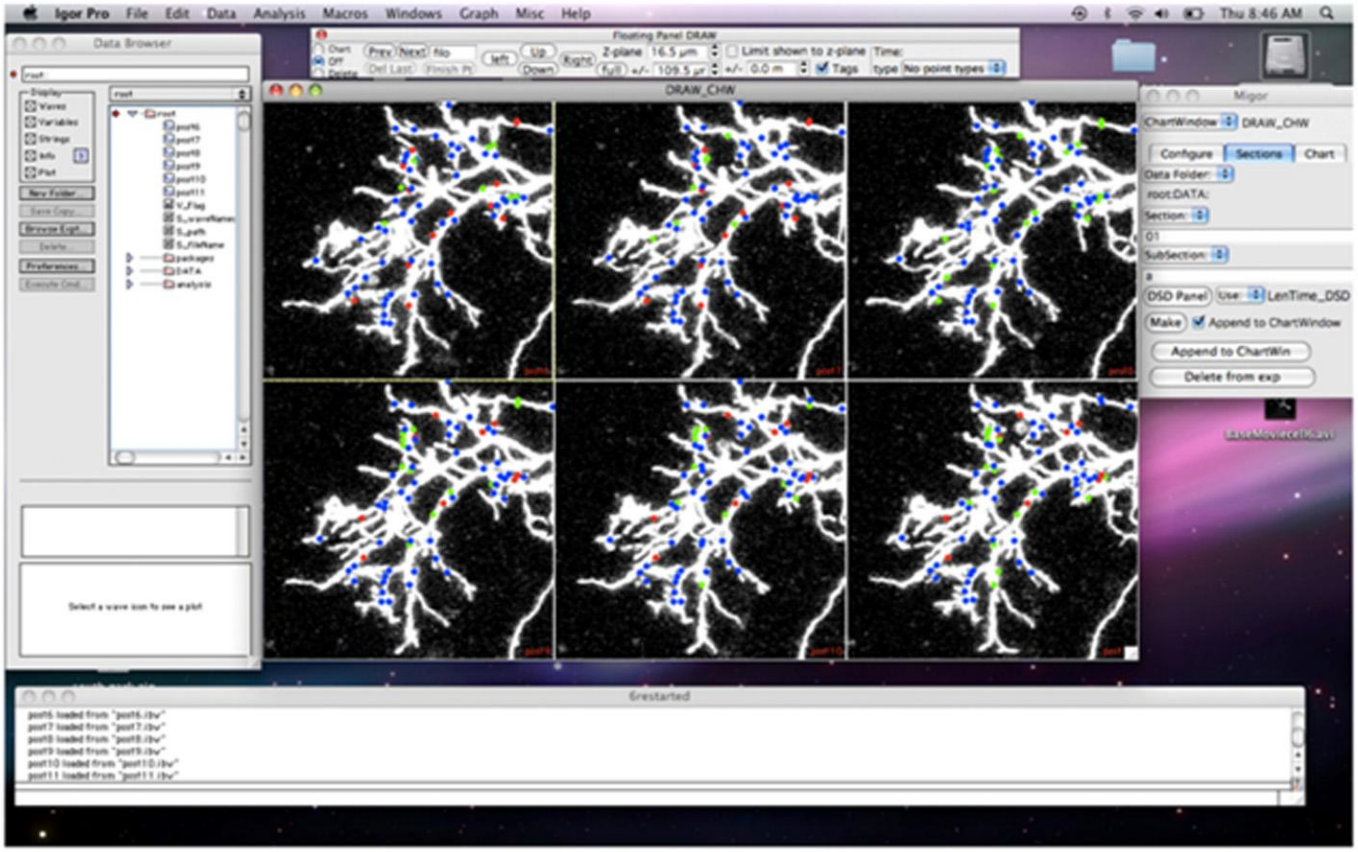
Ellsville, MO); and 4-(3-(Cyclopentyloxy)-4-methoxyphenyl)pyrrolidin-2-one (rolipram, 10 μM, Tocris Bioscience, Ellsville, MO and Enzo Life Sciences, Farmingdale, NY). Forskolin, dideoxyforskolin, and rolipram were dissolved in DMSO (1:1000 concentration in dH_2O), all other compounds were dissolved in dH_2O. Forskolin, dideoxyforskolin, SQ22536, and rolipram were diluted to working solution in normal rearing medium and applied to the tadpole bath. For rapid time-lapse imaging, bath-applied drugs were continuously perfused through the imaging chamber following baseline imaging. For direct injection of drugs into the optic tectum, rolipram was dissolved to working concentrations in Amphibian Ringer's solution (116 mM NaCl, 1.2 mM KCl, 1.0 mM $CaCl_2$, 2.7 mM $NaHCO_3$). Under visual guidance using an upright stereomicroscope, a small incision was made in the skin lateral to the tegmentum of tadpoles anesthetized in 0.02% MS-222. A sharp glass pipette filled with drug solution was inserted through this small cut and into the tegmentum. A Picospritzer II (General Valve Co., Fairfield, NJ) was used to deliver a low pressure, continuous air pulse into the pipette allowing injection of the drug solution into the tegmentum and tectum at a slow rate to prevent damage. No statistically significant differences between bath application and tectal injection of drugs were found, so the results from both experiments were pooled. Anesthesia was maintained until after injection was complete.

2.2.5 Dendrite morphometric analysis

Dynamic dendrite growth dynamics were analyzed using custom-designed procedures (modified from software created by Dr. Jamie Boyd, University of British Columbia, Vancouver, BC) running on the software platform IGOR Pro 6 (WaveMetrics, Portland, OR) (Fig. 4). This software allows identification and measurement of all filopodia and branches

within the entire neuronal dendritic arbor in three dimensions, as well as tracking each filopodium over time. Individual filopodia are manually identified and traced, allowing for detailed calculation of filopodial length, motility, and addition and retraction rates. Only structures with lengths less than 10 μm at the start of each imaging period were considered to be filopodia and thus included in analysis (Mattila and Lappalainen, 2008). Any structure that grew longer than 10 μm was considered to transition from filopodia to branch. These structures continued to be analyzed for the remainder of the imaging period. However, if the transition occurred during the baseline period, the structure was discarded from analysis at the start of the subsequent experimental period. Structures below 1 μm at all observed time points could not be reliably distinguished from imaging artifacts and so were discarded from analysis. Any structure within 5 μm of a branch ending was considered to be a growth cone filopodia (GCF); all others were considered interstitial filopodia (IF). Due to evidence that these two filopodial subtypes respond differently to growth cues and show distinct patterns of behavior (Hossain et al., 2011; Portera-Cailliau et al., 2003), GCF and IF were analyzed separately. Lifetime calculations included all newly added filopodia which were first detected in the second through tenth time points of each hour of five minute interval imaging, and which retracted within that hour.

A



B

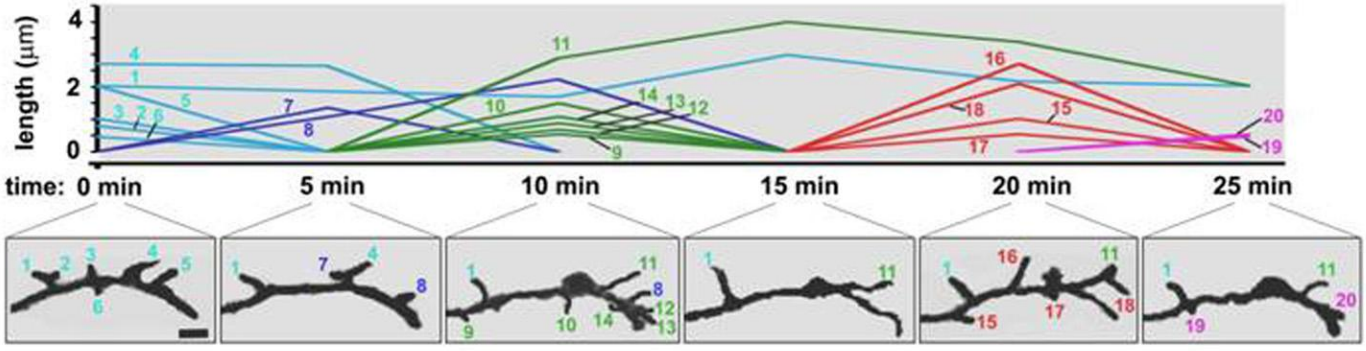


Figure 4: Dynamic morphometric comprehensive quantification of rapid growth behavior.
(A) Custom-written software on the Igor platform allows tracking of every single filopodium on the entire dendritic arbor at every time point. (B) Measures including filopodial length, motility, and addition and retraction rates can be calculated, allowing for highly detailed reconstruction of dendritic growth dynamics.

2.2.6 Statistical analysis

For data analysis, treatment hours were compared both to control cells and to internal baselines. For rapid-time lapse five-minute and one-hour imaging, two-way ANOVAs with Bonferroni post-tests were performed for comparison of growth under pharmacological agents to growth in control neurons. Student's T-tests were performed for comparison of treatment hours to internal baseline within each group of cells. T-tests were paired for stable filopodia, otherwise they were unpaired. In figures, significant differences from control neurons are indicated with an asterisk (*) and significant differences from internal baseline are indicated with plus-signs (+). */+: $p<0.05$; **/++: $p<0.01$. Numbers of replicates are listed in the legend for each figure.

2.3 Results

2.3.1 Manipulating cAMP levels alters dendritic length, motility, and addition rate

In order to determine the effects of AC on dendritic development, single neurons within the *X. laevis* tadpole optic tectum were electroporated (Haas et al., 2001) for delivery of plasmid encoding farnesylated enhanced green fluorescent protein (fEGFP). Tadpoles expressing fEGFP in single neurons were paralyzed with d-tubocurare (dTC, 5mM) and placed in an imaging chamber with continuous perfusion of Steinburg's solution. Baseline growth was measured by imaging the labeled neuron for 1 hr at 5 min intervals using time-lapse two-photon microscopy. Immediately following the baseline period the AC activator forskolin (Fsk, 50μM), which targets every membrane-bound AC isoform except AC9 (Hacker et al., 1998; Sutkowski et al., 1994), was applied to the perfusion solution and the

cell was imaged for an additional 1-2 hr at 5 min intervals. Control neurons were imaged continuously without the addition of any additional compound. Custom-written analysis software was used to measure the growth dynamics of all dendritic filopodia in the cell over the entire imaging period. Immature neurons were selected for initial experiments in order to examine effects on actively growing dendritic arbors. Immature neurons were defined as those with initial total dendritic branch length (TDBL) below 400 μm (Hossain et al., 2011; Wu et al., 1999). Filopodia, which were defined as processes less than 10 μm long (Hossain et al., 2011; Mattila and Lappalainen, 2008), were not included in the TDBL calculation. For measures of dynamic filopodial growth, only filopodia more than 5 μm from a branch tip (defined as interstitial filopodia) were analyzed, due to evidence that interstitial filopodia behave differently from and respond to different cues than filopodia within 5 μm of a branch tip (defined as growth cone filopodia) (Hossain et al., 2011; Portera-Cailliau et al., 2003).

Over 1 hr of continuous imaging, control neurons displayed dynamic addition and retraction of filopodia but no detectable change in number or length (Fig. 5A). Fsk application induced a significant increase in average filopodial length (Fig. 5B,C) and increased the average motility of both extending (Fig. 5F) and retracting (Fig. 5G) filopodia. These results indicate a role for AC in regulating the length and motility but not overall number of dendritic filopodia.

In order to verify that these effects were under the control of AC, a second, 1hr interval imaging protocol was used. Neurons were imaged once per hour for 2 hrs. The first hour interval served as the baseline, and immediately following the second image drugs were applied and neurons were imaged again 1 hr later. No significant differences in growth were detected for control or Fsk neurons between the two imaging protocols (imaging every 5 min

versus every hr, *data not shown*). In order to determine whether the effects of AC activation on filopodial length is specific to nascent, or long-lasting filopodia which are likely to posses synapses (Chen et al., 2010b; Niell et al., 2004), filopodia existing at the end of each hour were split into two groups: "stable" (those present at both the beginning and end of each hour), and "newly added" (those not present at the beginning of that hour). Fsk application increased length of both stable (Fig. 5D) and newly added filopodia (Fig. 5E), indicating that the presence of a synapse on a given filopodium may not be necessary for AC activity to alter the length of that filopodium. As forskolin has been reported to reduce potassium currents (Baxter and Byrne, 1990), dideoxyforskolin (ddFsk, 50μM), which also reduces potassium currents but has no effect on AC activity, was used as a control. Dideoxyforskolin had no effect on filopodial length (Fig. 5D,E), ruling out the possibility that reduced potassium currents contribute to the observed effects. The effect of Fsk was blocked by the non-isoform specific AC-blocker SQ22536 (100 μM), confirming that Fsk is acting on AC to increase filopodial length and motility. SQ22536 alone had no effect, indicating no role for basal levels of AC on filopodial length in these cells. Rolipram (10 μM), a PDE4 inhibitor, only increased length of newly added filopodia. Unlike Fsk, rolipram does not increase the formation of new cAMP molecules, but rather prevents degradation of cAMP, thus prolonging existing cAMP signals. It is possible that stronger, externally induced cAMP signals, such as those induced by Fsk, may be necessary to increase the growth of stable filopodia above basal levels.

Fsk had no effect on filopodia density, turnover, or lifetime (Table 2, Fig. 6). However, SQ22536 decreased the number of filopodia additions, indicating that basal AC activity is necessary to maintain basal addition rates (Table 2).

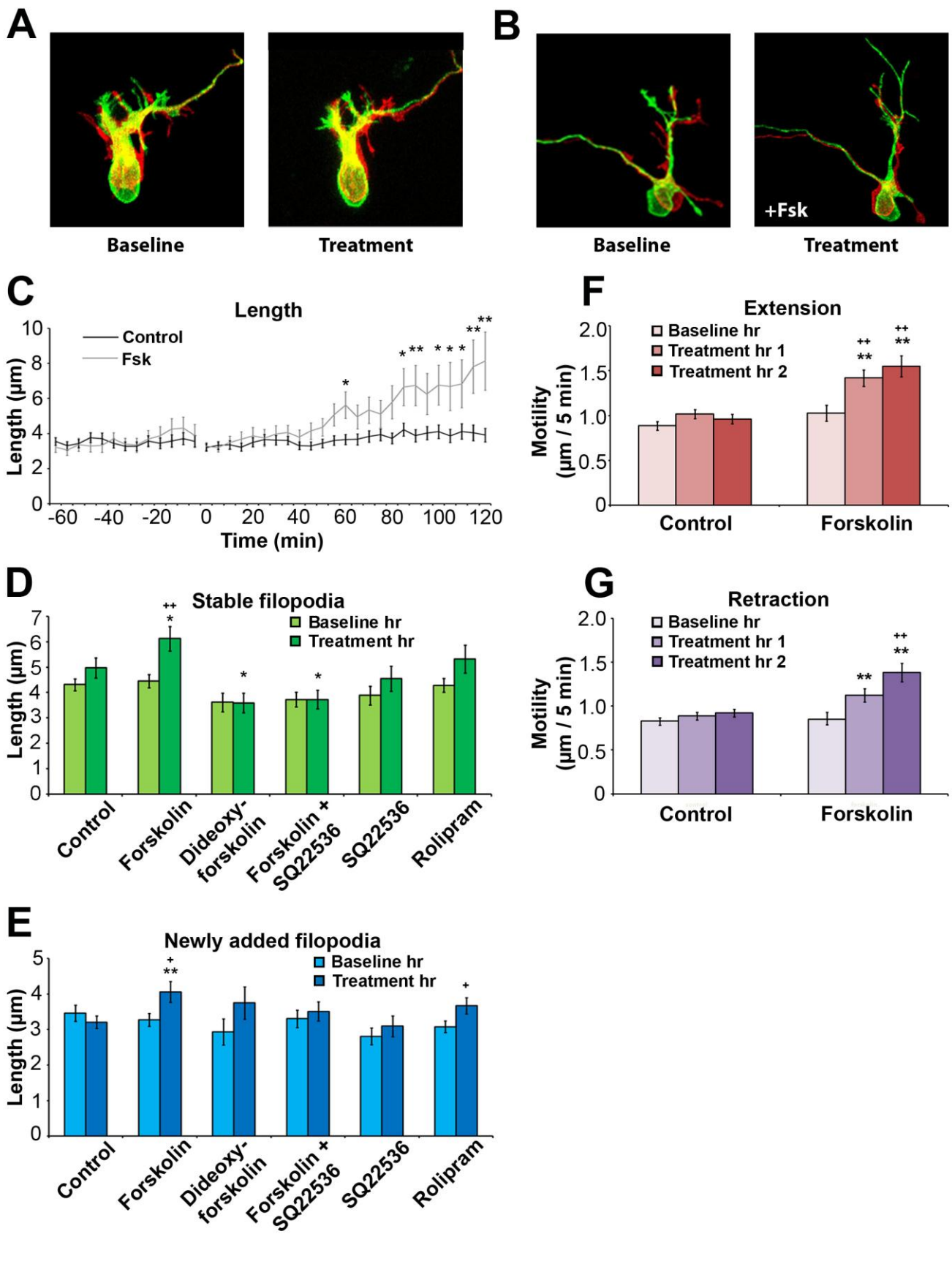

A
Baseline
Treatment
B
Baseline
+Fsk
Treatment
C
Length
Control
Fsk
Length (µm)
Time (min)
F
Extension
Baseline hr
Treatment hr 1
Treatment hr 2
Motility (µm / 5 min)
Control
Forskolin
D
Stable filopodia
Baseline hr
Treatment hr
Length (µm)
Control
Forskolin
Dideoxy-forskolin
Forskolin + SQ22536
SQ22536
Rolipram
G
Retraction
Baseline hr
Treatment hr 1
Treatment hr 2
Motility (µm / 5 min)
Control
Forskolin
E
Newly added filopodia
Baseline hr
Treatment hr
Length (µm)
Control
Forskolin
Dideoxy-forskolin
Forskolin + SQ22536
SQ22536
Rolipram

Figure 5: Manipulating cAMP levels increases dendritic filopodial length and motility.
(A) Representative images of a control neuron imaged every 5 min for 2 hr. Image on the left corresponds to the baseline hr, image on the right corresponds to the treatment hr. Shown are overlays of the first (red) and last (green) images of each hour. (B) Representative images of an experimental neuron imaged every 5 min for 2 hr. Image on the left corresponds to the baseline hr, image on the right corresponds to 1 hr treatment with Fsk. Shown are overlays of the first (red) and last (green) images of each hour. (C) Fsk increases average filopodial length. (D) Fsk increases length of stable filopodia. This effect is blocked by SQ22536 and not mimicked by ddFsk. (E) Fsk and rolipram both increase length of stable filopodia. This effect is blocked by SQ22536 and not mimicked by ddFsk. (F) Fsk increases filopodial extension. (G) Fsk increases filopodial retraction. Replicate numbers: Rapid time lapse imaging: Control: N=5; n= 287 (baseline hr); 350 (treatment hr 1); 373 (treatment hr 2); Fsk: N=5 (2 hr total imaging), 3 (3 hr total imaging); n= 163 (baseline hr); 201 (treatment hr 1); 81 (treatment hr 2); 1 hr interval imaging: Control: N=17; n (stable) = 100; n (newly added) = 220 (baseline hr); 219 (treatment hr); Fsk: N=11; n (stable) = 79; n (newly added) = 155 (baseline hr); 145 (treatment hr); ddFsk: N=3; n (stable) = 47; n (newly added) = 43 (baseline hr); 31 (treatment hr); Fsk+SQ22536: N=5; n (stable) = 56; n (newly added) = 103 (baseline hr); 96 (treatment hr); SQ22536: N=4; n (stable) = 39; n (newly added) = 83 (baseline hr); 49 (treatment hr); Rolipram: N=8; n (stable) = 66; n (newly added) = 113 (baseline hr); 122 (treatment hr); N = number of individual cells; n = number of individual filopodia.

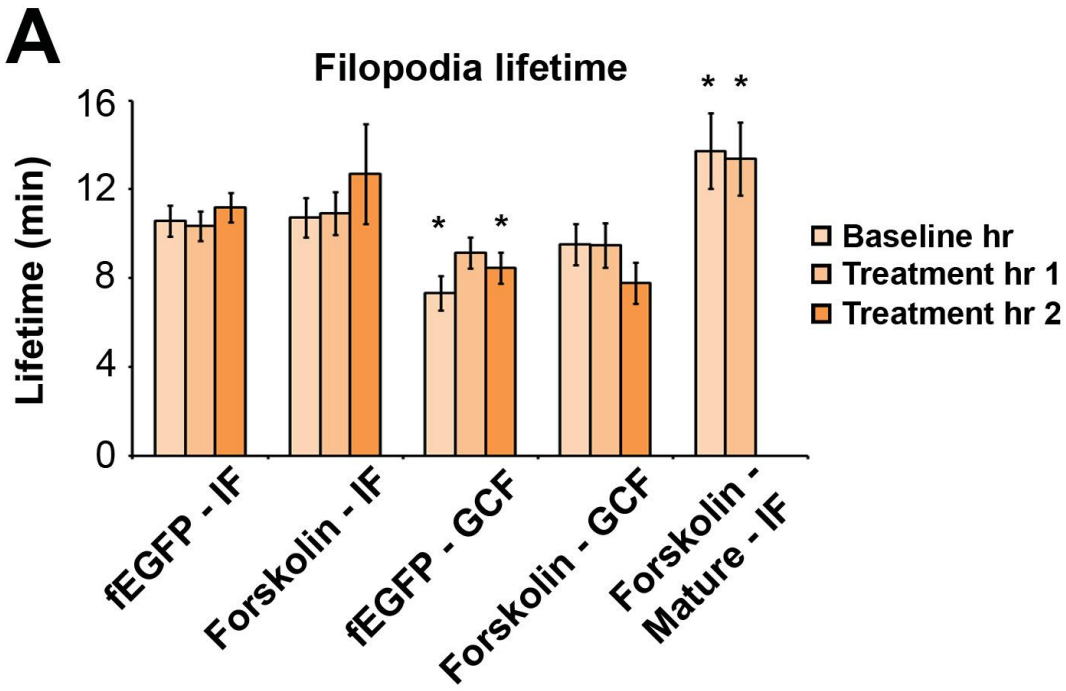


Figure 6: Effect of cell age, filopodia type, and adenylyl cyclase activation on filopodia lifetime.
(A) Lifetime of transient filopodia. Unless otherwise stated, filopodia are interstitial and cells are immature (TDBL < 400μm). Growth cone filopodia have shorter lifetimes than interstitial filopodia, and filopodia lifetime increases with cell age. Replicate numbers: fEGFP – IF: N=5; n= 122 (baseline hr); 164 (treatment hr 1); 149 (treatment hr 2); Fsk – IF: N=5 (2 hr total imaging), 3 (3 hr total imaging); n= 68 (baseline hr); 88 (treatment hr 1); 28 (treatment hr 2); fEGFP – GCF: N=5; n= 58 (baseline hr); 82 (treatment hr 1); 68 (treatment hr 2); Fsk – GCF: N=5 (2 hr total imaging), 3 (3 hr total imaging); n= 53 (baseline hr); 30 (treatment hr 1); 18 (treatment hr 2); Fsk – mature – IF: N=3; n= 43 (baseline hr); 49 (treatment hr 1); N = number of individual cells; n = number of individual filopodia.

Table 2: Effects of cAMP manipulation on filopodia density, additions, and retractions

Neurons were imaged for 2 hr at 1 hr intervals. Displayed are filopodia density per 100 μm of TDBL or number of additions or retractions per 100 μm of TDBL. Unless otherwise stated, values refer to interstitial filopodia on immature cells. For ANOVAs, IF comparisons are vs. fEGFP – IF, and GCF comparisons are vs. fEGFP – GCF. Replicate numbers: fEGFP control (IF and GCF): N=17; Fsk: N=11; Fsk (mature cells): N=3; ddFsk: N=3; Fsk+SQ22536: N=5; SQ22536: N=4; Rolipram: N=8.

	Filopodia / 100 μm		Additions / 100 μm		Retractions / 100 μm	
	Baseline hr	Treatment hr	Baseline hr	Treatment hr	Baseline hr	Treatment hr
fEGFP control (IF)	7.49 ± 0.75	7.65 ± 0.75	4.94 ± 0.62	5.32 ± 0.66	5.12 ± 0.63	4.75 ± 0.58
fEGFP control (GCF)	1.87 ± 0.32	2.09 ± 0.44	1.50 ± 0.24	1.74 ± 0.36	1.86 ± 0.34	1.50 ± 0.21
Forskolin (IF)	8.03 ± 1.27	6.86 ± 0.69	5.31 ± 0.94	4.68 ± 0.58	6.44 ± 1.28	4.98 ± 0.70
Forskolin (GCF)	1.64 ± 0.37	1.46 ± 0.25	1.50 ± 0.33	1.15 ± 0.24	1.54 ± 0.31	1.30 ± 0.27
Forskolin (mature cells)	4.33 ± 1.70	3.19 ± 0.97	2.74 ± 1.16	0.99 ± 0.24	1.75 ± 0.72*	2.61 ± 1.13
Dideoxyforskolin (IF)	15.63 ± 6.40	13.32 ± 5.73	9.94 ± 4.42*	9.94 ± 6.77	8.04 ± 2.95	10.90 ± 5.87
Dideoxyforskolin (GCF)	1.94 ± 0.88	1.75 ± 0.34	1.79 ± 0.84	1.78 ± 0.54	2.62 ± 1.43	1.82 ± 0.86
Forskolin + SQ22536 (IF)	9.69 ± 2.29	7.05 ± 1.93	6.27 ± 1.35	5.81 ± 1.15	4.56 ± 1.70	6.65 ± 2.13
Forskolin + SQ22536 (GCF)	2.02 ± 1.33	1.44 ± 0.87	2.05 ± 1.47	1.26 ± 0.75	0.54 ± 0.26	1.85 ± 1.23
SQ22536 (IF)	8.37 ± 1.80	8.50 ± 1.83	6.51 ± 0.43	$3.59 \pm 0.55^{++}$	5.02 ± 1.01	5.57 ± 1.15
SQ22536 (GCF)	1.06 ± 0.36	1.34 ± 0.52	1.03 ± 0.30	1.18 ± 0.40	0.92 ± 0.34	0.93 ± 0.30
Rolipram (IF)	9.35 ± 1.32	8.35 ± 1.21	5.22 ± 1.00	6.06 ± 1.22	4.75 ± 0.97	6.68 ± 1.25
Rolipram (GCF)	1.24 ± 0.39	1.28 ± 0.35	1.03 ± 0.41	1.34 ± 0.32	1.29 ± 0.28	1.18 ± 0.41

2.3.2 Response to cAMP is dependent on cell age

In order to determine whether cellular response to AC activation is dependent on cell maturational state, the responses of mature cells (TDBL above 400 μm) to Fsk were compared to the responses of immature cells (TDBL below 400 μm). As with immature cells, there was no change in filopodia density, additions, or retractions when Fsk was applied to mature cells (Table 2). A significant maturational effect on basal filopodial growth dynamics was observed. Filopodia on mature cells exhibited shorter filopodia, increased lifetimes, and decreased retractions compared to immature cells when baseline hours were compared (Table 2, Fig. 6,7). In contrast to immature cells, both length (Fig. 7A) and motility (Fig. 7B,C) of mature cell filopodia decreased in response to AC activation. These results indicate that AC activation results in unique and opposite cellular responses based on cell maturational state – increasing growth and motility in immature neurons, while decreasing growth and motility in mature neurons. In order to focus experiments on early stages of dendritogenesis, and to avoid maturational changes in cellular responses, only immature neurons were used in the remainder of the experiments.

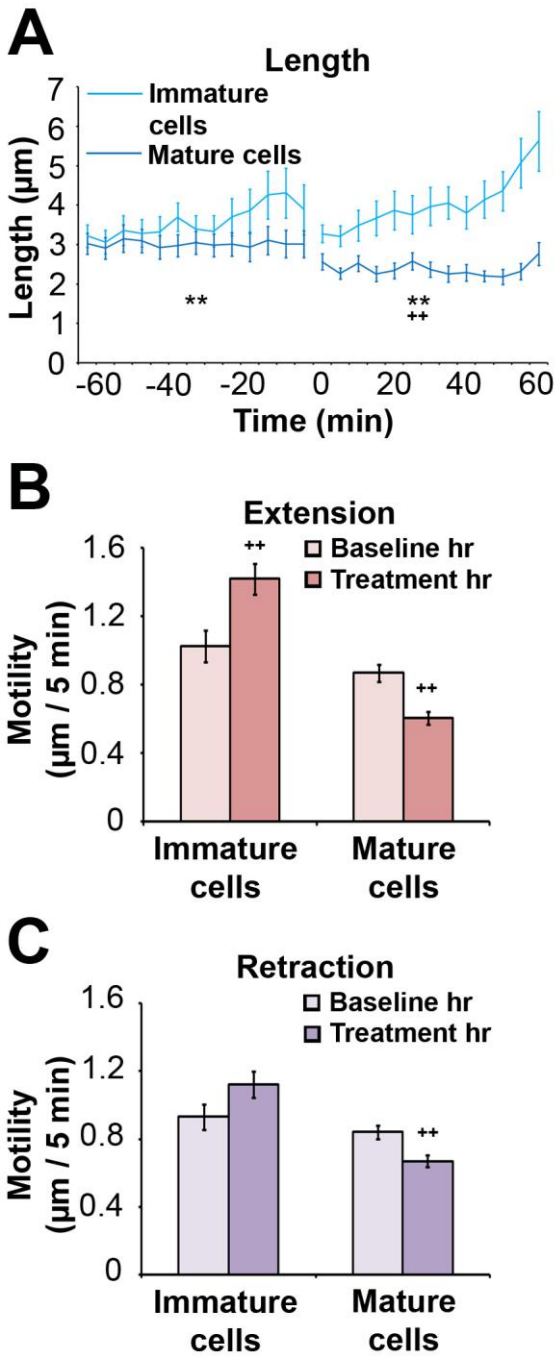


Figure 7: Adenylyl cyclase activation decreases filopodial length and motility in mature cells.
(A) Fsk application increases filopodial length in immature neurons but decreases filopodial length in mature neurons. Mature neurons, measured during the baseline hour, have shorter filopodia than immature neurons. (B) Fsk decreases filopodial extension in mature cells. (C) Fsk decreases filopodial retraction in mature cells. Replicate numbers: Immature cells: N=5; n= 163 (baseline hr); 201 (treatment hr); Mature cells: N=3; n= 145 (baseline hr); 124 (treatment hr); N = number of individual cells; n = number of individual filopodia.

2.3.3 Interstitial and growth cone filopodia show distinct responses to cAMP manipulation

Past research has suggested that filopodia associated with growth cones (within 5 μm of a branch tip, or growth cone filopodia, GCF), respond differently to extracellular signals than filopodia located along the branch shaft, termed interstitial filopodia (IF) (Portera-Cailliau et al., 2003). We tested whether GCF respond differently to AC activation than IF. The same group of immature cells analyzed in Fig. 1 was used for GCF analysis.

Over 2 hr of dynamic, 5 min interval imaging during exposure to Fsk there was a small but significant increase in average GCF length (Fig. 8A), but the effects were more variable and less significant than IF (Fig. 5C). As with IF, there was a significant increase in GCF extension motility (Fig. 8D), but there was no change in GCF retraction motility (Fig. 8E). As with IF, AC activation did not change the lifetime of transient GCF filopodia (Fig. 6). However, GCF did show decreased lifetimes compared to IF when measured during the baseline hour (Fig. 6), providing evidence to support the hypothesis that IF and GCF are behaviourally and functionally distinct.

Following experiments in which neurons were imaged over 1 hr intervals while cAMP-manipulating drug combinations were applied, GCF were separated into two groups: stable and newly added. Unlike IF, no effect of cAMP manipulation on GCF was observed for either the stable or newly added filopodia groups (Fig. 8B,C). There was also no effect of cAMP manipulation on filopodia density, or rates of addition or retraction (Table 2). It should be noted, however, that GCF are highly dynamic structures and therefore the number of stable GCF over the course of 1 hr is very small, leading to low statistical power when analyzing stable filopodia.

GCF show a wider range in length than IF – from short filopodia at branch tips not associated with lamellipodia, to elongated filopodia typically associated with large lamellipodia. In order to test whether these two populations responded differently to AC manipulation, filopodia were separated into subgroups based on their initial lengths (greater than, or smaller than 3 μm, chosen based on the average GCF length at the start of imaging). Splitting filopodia in this manner revealed that Fsk application induced an increase in average filopodia length in initially short but not long filopodia, implying that short GCF, which are less likely to be associated with lamellipodia, are more responsive to AC activation than long GCF, or that there is a maximal length of GCF (Fig. 9A,B). Although this response had a higher magnitude than that observed with IF, it was also far more variable. No significant changes were found in length of stable or newly added filopodia following AC manipulation in either subgroup of GCF (Fig. 9C,D,E,F). Because GCF responded differently to AC activation than IF, and because the IF response was more robust and less variable than the GCF response, only IF were analyzed in subsequent chapters.

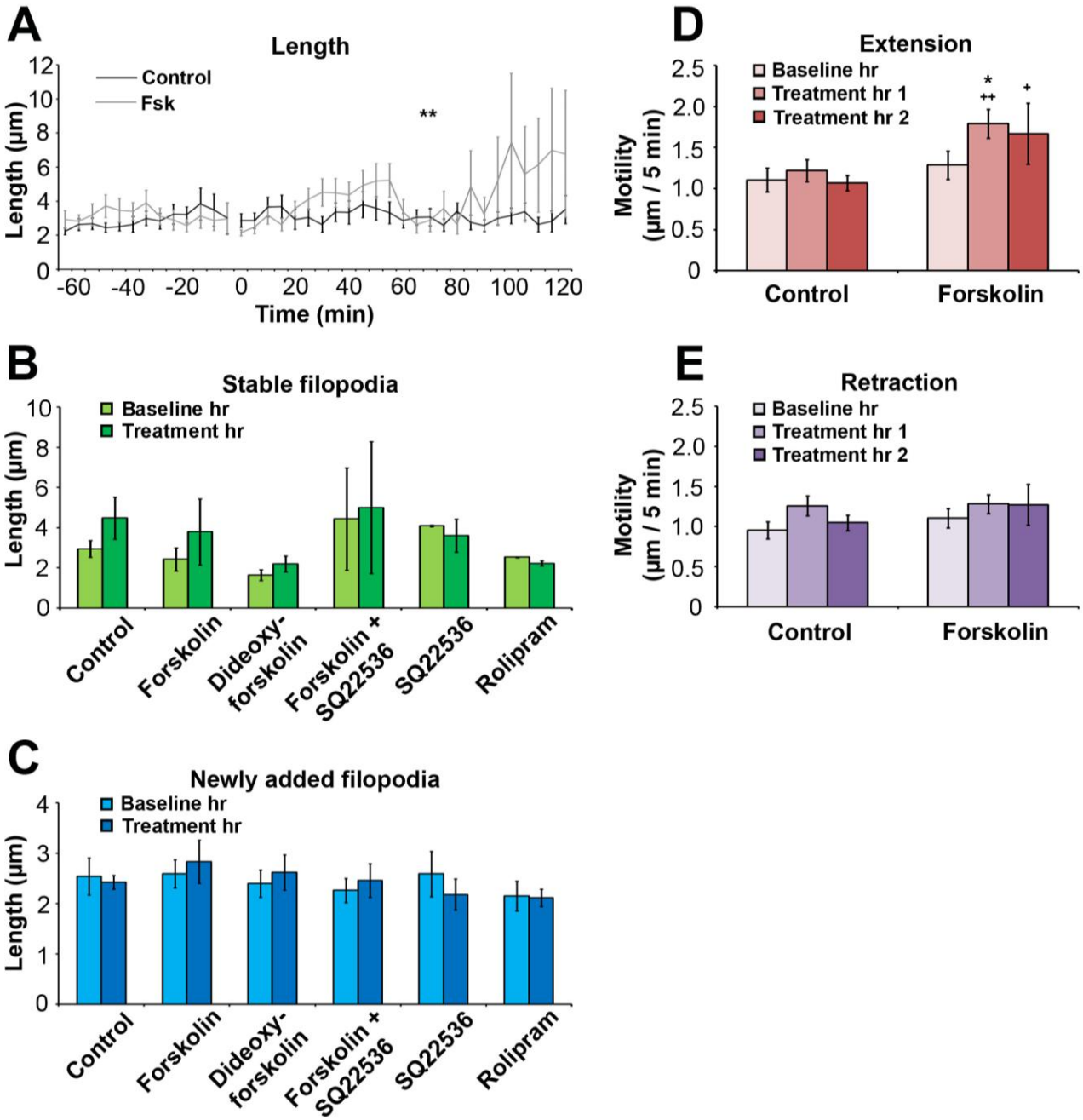


Figure 8: Growth cone filopodia respond more weakly to AC activation than interstitial filopodia. (A) Fsk slightly increases average filopodial length. (B) Stable growth cone filopodia do not respond to manipulations of cAMP levels. (C) Newly added growth cone filopodia do not respond to manipulations of cAMP levels. (D) Fsk increases filopodial extension. (E) Fsk has no effect on filopodial retraction. Replicate numbers: Rapid time lapse imaging: Control: N=5; n= 111 (baseline hr); 143 (treatment hr 1); 126 (treatment hr 2); Fsk: N=5 (2 hr total imaging), 3 (3 hr total imaging); n= 87 (baseline hr); 84 (treatment hr 1); 38 (treatment hr 2); 1 hr interval imaging: Control: N=17; n (stable) = 7; n (newly added) = 58 (baseline hr); 75 (treatment hr); Fsk: N=11; n (stable) = 4; n (newly added) = 42 (baseline hr); 38 (treatment hr); ddFsk: N=3; n (stable) = 3; n (newly added) = 21 (baseline hr); 16 (treatment hr); Fsk+SQ22536: N=5; n (stable) = 2; n (newly added) = 20 (baseline hr); 15 (treatment hr); SQ22536: N=4; n (stable) = 2; n (newly added) = 13 (baseline hr); 17 (treatment hr); Rolipram: N=8; n (stable) = 2; n (newly added) = 21 (baseline hr); 29 (treatment hr); N = number of individual cells; n = number of individual filopodia.

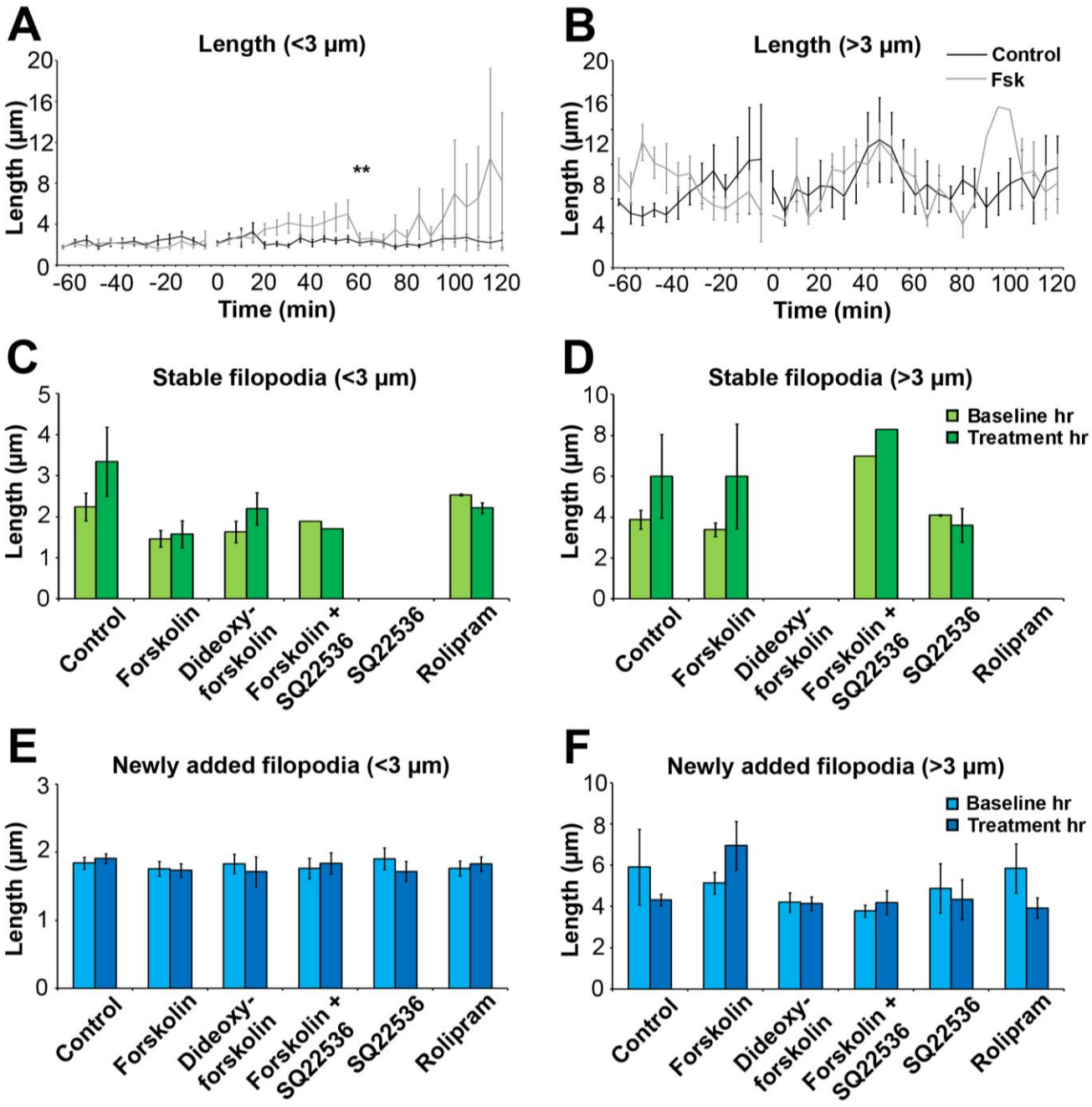


Figure 9: Effects of AC manipulation on growth cone filopodia based on initial filopodia length
(A,B) Effect of Fsk on average filopodial length of short (A) and long (B) growth cone filopodia. (C,D) Effect of AC manipulation on length of initially short (C) and long (D) stable growth cone filopodia. (E,F) Effect of AC manipulation on length of short (E) and long (F) newly added growth cone filopodia. Replicate numbers: Filopodia < 3µm: Rapid time lapse imaging: Control: N=5; n= 88 (baseline hr); 117 (treatment hr 1); 101 (treatment hr 2); Fsk: N=5 (2 hr total imaging), 3 (3 hr total imaging); n= 68 (baseline hr); 69 (treatment hr 1); 29 (treatment hr 2); 1 hr interval imaging: Control: N=17; n (stable) = 4; n (newly added) = 48 (baseline hr); 59 (treatment hr); Fsk: N=11; n (stable) = 2; n (newly added) = 32 (baseline hr); 30 (treatment hr); ddFsk: N=3; n (stable) = 3; n (newly added) = 16 (baseline hr); 10 (treatment hr); Fsk+SQ22536: N=5; n (stable) = 1; n (newly added) = 15 (baseline hr); 11 (treatment hr); SQ22536: N=4; n (stable) = 0; n (newly added) = 10 (baseline hr); 14 (treatment hr); Rolipram: N=8; n (stable) = 2; n (newly added) = 19 (baseline hr); 25 (treatment hr);

Filopodia > 3μm: Rapid time lapse imaging: Control: N=5; n= 23 (baseline hr); 26 (treatment hr 1); 25 (treatment hr 2); Fsk: N=5 (2 hr total imaging), 3 (3 hr total imaging); n= 19 (baseline hr); 15 (treatment hr 1); 9 (treatment hr 2); 1 hr interval imaging: Control: N=17; n (stable) = 3; n (newly added) = 10 (baseline hr); 16 (treatment hr); Fsk: N=11; n (stable) = 2; n (newly added) = 11 (baseline hr); 8 (treatment hr); ddFsk: N=3; n (stable) = 0; n (newly added) = 5 (baseline hr); 6 (treatment hr); Fsk+SQ22536: N=5; n (stable) = 1; n (newly added) = 5 (baseline hr); 4 (treatment hr); SQ22536: N=4; n (stable) = 2; n (newly added) = 3 (baseline hr); 3 (treatment hr); Rolipram: N=8; n (stable) = 0; n (newly added) = 2 (baseline hr); 4 (treatment hr); N = number of individual cells; n = number of individual filopodia.

2.4 Discussion

2.4.1 AC activation increases filopodial length and motility without altering filopodial stability

In this study we report that AC activation causes an increase in dendritic filopodial length and motility in developing neurons *in vivo*, which is blocked by AC inhibition and partially mimicked by PDE4 inhibition. These results agree with previously published data showing a role for AC and its effectors in neurite extension in cell culture (Aglah et al., 2008; Christensen et al., 2003; Fujioka et al., 2004; Kao et al., 2002; Kiermayer et al., 2005; Kwon et al., 1996; Murray and Shewan, 2008; Ravni et al., 2008; Richter-Landsberg and Jastorff, 1986; Schmidt et al., 1998; Shi et al., 2006; Tojima et al., 2003b; Vogt Weisenhorn et al., 2001), and in dendrite length in newborn neurons in an *in vivo* adult system (Fujioka et al., 2004). These effects are specific to young neurons and constitute the first *in vivo* evidence of a role for AC in developmental dendritogenesis.

We report no increase in filopodial density or addition as a result of Fsk application. This result agrees with *in vitro* studies which reported an effect of cAMP on neurite elongation but not on neurite number or stability (Murray and Shewan, 2008). However, a number of studies have shown a role for AC activation in both neurite length and in the addition of new neurites (Aglah et al., 2008; Kwon et al., 1996; Ravni et al., 2008; Richter-

Landsberg and Jastorff, 1986; Shi et al., 2006; Tojima et al., 2003b). It should be noted that these studies were looking at neurite initiation at the point when cells were first adopting a neuronal fate, while our study examines dendritic filopodia on differentiated neurons which already have distinct axonal and dendritic processes. Difference in protocols of drug application may also be a factor in results between studies. For example, in a study by Aglah et al. cultured rat motor neurons treated with 3 μM Fsk for 12 hr showed increased neurite length, but 24 hrs of treatment was required in order to increase the number of motor neurons which developed neurites (Aglah et al., 2008). It is possible that, in our experimental system, longer continuous treatment with Fsk, or observation of neurons several hours or days after acute Fsk administration, would reveal a previously undetected increase in filopodia addition.

A recent study by Kwon and Sabatini reported that Fsk was unable to induce spine formation in mature hippocampal neurons. When Fsk was co-applied with glutamate there was a large increase in spine number greater than that induced by glutamate alone (Kwon and Sabatini, 2011). This effect was dependent on NMDA receptors, PKA, and MAPK activity, and there was no concomitant increase in spine length. Spines are functionally distinct from filopodia in a number of ways, such as the presence versus absence of synapses, the degree of motility, and the cell age at which they primarily appear. In fact, local glutamate application to developing hippocampal neurons increases filopodia length (Portera-Cailliau et al., 2003), an effect which was not observed in the Kwon and Sabatini study on spines, and glutamate receptor activity is upstream of several AC isoforms (Cooper and Crossthwaite, 2006; Sadana and Dessauer, 2009; Willoughby and Cooper, 2007). It is possible that Fsk has a growth promoting, length-extending effect in the filopodia in immature neurons, while in

older neurons it induces synaptogenesis or spinogenesis without simultaneously increasing process length.

We detected a decrease in filopodial additions when AC activity is reduced below basal levels, suggesting that although increased AC activity does not cause increased filopodial additions above baseline, basal AC activity levels are necessary to maintain normal addition rates during development. This may be the result of reduced activity in the NMDAR/Ca^{2+}-stimulated AC1 or AC8, which would agree with previous data showing that the NMDAR antagonist APV reduces branch additions in *X. laevis* tectal neurons (Rajan and Cline, 1998; Rajan et al., 1999; Sin et al., 2002).

The effect of AC activation is distinct from effects reported when other signaling pathways are activated. CaMKII, PKC, and NRX-NLG interactions, which have all been implicated in synaptic plasticity and growth-promotion in other experimental systems, have been studied in this same experimental system with results supporting a role in developmental dendritogenesis (Chen et al., 2010b; Liu et al., 2009; Wu and Cline, 1998). Increasing activity in these pathways in *X. laevis* tectal neurons *in vivo* is associated with increased filopodial stability, indicated by reduced rates of addition and retraction, motility, and growth, and the precocious adoption of a mature morphological phenotype characterized by high complexity with little structural change over time. In two of these studies (Chen et al., 2010b; Liu et al., 2009), dendritic effects are associated with enhanced synaptogenesis. These studies and others (Niell et al., 2004; Vaughn, 1989) have led to the development and refinement of the 'synaptotropic hypothesis of dendritogenesis', which posits that the formation of synapses guides dendritic growth. Following pharmacological activation of AC, another pathway known to be involved with synaptic plasticity and growth-promotion, here I

observe increased filopodial length and motility and no effects on rates of addition and retraction. This represents a distinct phenotype from that observed by activating CaMKII or PKC or increasing NRX-NLG interactions. Based on the differences between my results and other results reported in this system, I conclude that the data presented herein do not support the hypothesis that AC activation increases filopodial growth through a synaptotropic mechanism. I do not suggest that my data is evidence against the well-supported synaptotropic hypothesis, but rather evidence of an alternate, concurrent mechanism guiding dendritic growth.

If AC-induced growth is not being guided synaptotropically, what then is the underlying physiological mechanism? I propose that AC may be acting through regulation of the cytoskeleton in order to induce its effects, possibly through altered GTPase activity levels. There have been several reported cases of cytoskeletal or GTPase manipulations which result in increased filopodial length without concurrent changes in rates of filopodial addition or retraction (Hirsch et al., 2002; Lee et al., 2000; Li et al., 2000). Genetic screens in *Drosophila* support the hypothesis that dendrite growth and dendrite branching can be regulated separately. For example, a study by Ou et al. identified a number of mutants with defects in dendrite growth but not branching (Ou et al., 2008). *X. laevis* provides a system for testing this hypothesis through pharmacological manipulation or the expression of dominant-negative or constitutively-active genetic constructs within an intact *in vivo* setting.

2.4.2 Response to AC activation is dependent on neuronal maturational state

This study found that the response of developing neurons to AC activity is dependent on neuronal maturational state. AC activation increases filopodial length and motility in

immature cells (TDBL < 400 μm) but has the opposite effects in mature cells (TDBL > 400 μm). Even in the absence of pharmacological AC activation, filopodia on mature cells behave differently from those on immature cells, with shorter lengths, increased lifetimes and decreased retractions.

Age-dependent changes in the behaviour of *X. laevis* optic tectal dendrites have previously been demonstrated (Hossain et al., 2011; Wu et al., 1999). Tectal neurons progress through three distinct phases of growth. During phase 1, newly differentiated neurons extend an axon to the lateral tegmentum. The dendritic arbor consists of highly dynamic filopodia which fail to stabilize and transition to longer branches, so little net change in dendritic arbor growth occurs. Phase 2 is a period of rapid dendritic arbor growth due to the stabilization of filopodia and their transition to branches, and extensive branch elongation. During phase 3, the dendritic arbor achieves a mature and stable state, in which there is no net change in total branch number or length.

Past studies have also demonstrated that dendritic morphogenesis in response to activity is dependent on neuronal maturation. Developing neurons shift their relative proportions of glutamatergic receptor types as they age. The relative levels of NMDA and AMPA receptor expression are well studied, with young neurons showing high levels of NMDA receptor and low levels of AMPA receptor which reverses to high AMPA receptor expression through mechanisms requiring synaptic activity as the neuron matures (Isaac et al., 1997; Liao et al., 1995; Wu et al., 1996). Additionally, NMDA receptor subunit expression depends on cell age, with younger neurons expressing high levels of the NR2B subunit and more mature neurons expressing high levels of the NR2A subunit (Kew et al., 1998; Monyer et al., 1994; Roberts and Ramoa, 1999; Shi et al., 1997). Both of these

switches have been confirmed in *X. laevis*. Phase 1 neurons show no evoked synaptic responses, phase 2 neurons have low ratios of evoked synaptic AMPA/NMDA receptor-mediated currents, and phase 3 neurons have high AMPA/NMDA ratios (Rajan and Cline, 1998; Wu et al., 1996; Wu et al., 1999). Tectal neurons expressing exogenous NR2B, and thus electrophysiologically immature, are more motile than exogenous NR2A-expressing, electrophysiologically older cells (Ewald et al., 2008). The present study confirms the finding that mature cells are less motile than immature cells by finding increased lifetimes and decreased retractions in cells with TDBL above 400 μm.

In this study we find that that activation of the AC pathway promotes growth in young cells but stabilization in more mature cells. Cells with TDBL below 400 μm, or phase 1 and 2 neurons, show increases in filopodial length in response to AC activation. Mature neurons behaved opposite to immature neurons when AC was activated, showing decreased length and motility. It is not clear why this age-dependent switch occurs in response to AC activation. It may be due to a decrease in NMDA receptor current during maturation (Carmignoto and Vicini, 1992; Hestrin, 1992), changes in the composition of GPCR subtypes (Reis et al., 2005), or to changes in the expression or response patterns of downstream effectors of AC (Bowes et al., 2008; Murray et al., 2009b).

2.4.3 Interstitial and growth cone filopodia show distinct responses to AC activation

In this study we demonstrate that GCF respond to AC activation in a manner distinct from IF. This is consistent with previously published findings that dendritic GCF and IF have distinct growth behaviours and respond differently to extracellular signals (Hossain et al., 2011; Portera-Cailliau et al., 2003). A study by Portera-Cailliau, et al., showed that IF behave

differently from GCF in pyramidal neurons in acute slices from the developing mouse neocortex. Specifically, IF are sensitive to manipulations affecting synaptic transmission, calcium levels, and glutamate receptor transmission, while GCF responded to none of these manipulations (Portera-Cailliau et al., 2003). We find that, while in our system GCF do behave differently than IF, they also show a weak response to AC activation. If GCF are not responsive to activity-dependent signals (Portera-Cailliau et al., 2003), why do we observe effects from activation of AC, a protein known to be at the head of activity-dependent signaling pathways? The answer may lie in specific isoforms of AC which respond not to calcium or to neurotransmitters like norepinephrine and dopamine, but rather to hormones and growth factors such as PACAP, NGF, or BDNF (Cazillis et al., 2004; Chen et al., 2010a; Drahushuk et al., 2002; Herbst et al., 2011; Ravni et al., 2008; Shi et al., 2006; Ster et al., 2009; Wang and Zheng, 1998). Axonal growth cones are capable of responding to cAMP, as evidence has shown that cAMP, PKA, and Epac can all regulate growth cone turning and collapse (Han et al., 2007; Kim and Wu, 1996; Lankford et al., 1988; Lebrand et al., 2004; Lohof et al., 1992; Murray et al., 2009a; Murray and Shewan, 2008; Song et al., 1997; Wang and Zheng, 1998). The result reported herein, implies that dendritic growth cones *in vivo* also respond to cAMP.

Chapter 3: PKA and Epac act synergistically to increase dendritogenesis in optic tectal neurons of *Xenopus laevis* tadpoles

3.1 Introduction

The process of dendritic arbor growth during development is a highly complicated one, requiring both synaptogenesis and cytoskeletal remodeling, and occurring in response to both activity-dependent and activity-independent signals. Much work has gone into teasing out the specific mechanisms involved in dendritogenesis, but the role of intracellular signaling pathways remains poorly understood. In the previous chapter I indentified adenylyl cyclase (AC) and its product cyclic adenosine monophosphate (cyclic AMP; cAMP) as a potential regulator of dendritic growth. Pharmacological activation of AC in developing *X. laevis* optic tectal neurons *in vivo* caused an increase in dendritic filopodial length and motility without changing filopodial stability. This effect was specific to young neurons and more robust in IF than in GCF. However, the downstream pathways which transmit a cAMP signal into a morphological phenotype in these neurons remain undetermined.

Two distinct signaling pathways downstream of cAMP have been implicated in neurite growth, synaptogenesis, and cytoskeletal reorganization, and may therefore play a role in developmental dendritogenesis *in vivo*. One well studied effector of cAMP is protein kinase A (PKA) (Nguyen and Woo, 2003; Shabb, 2001), while a second effector recently identified is the exchange protein activated by cAMP (Epac) (de Rooij et al., 1998; Kawasaki et al., 1998). PKA and Epac both have established roles in synaptic plasticity (Abel and Nguyen, 2008; Calixto et al., 2003; Cheung et al., 2006; Esteban et al., 2003; Gelinas et al., 2008a; Kaneko and Takahashi, 2004; Nguyen and Woo, 2003; Sakaba and Neher, 2003;

Tojima et al., 2003a; Zhong and Zucker, 2005), but little is known about their role in dendritogenesis. It is known that activation of PKA induces neurite outgrowth in NG108-15 (Tojima et al., 2003a) and PC12 cells (Christensen et al., 2003)**,** and in cultured rat motoneurons (Aglah et al., 2008). PKA activation also increases neurite length of cultured *X. laevis* spinal neurons (Kao et al., 2002) and neurite length and branching of newborn neurons in adult hippocampus (Fujioka et al., 2004). Epac induces neural differentiation of PC12 (Christensen et al., 2003; Kiermayer et al., 2005) and PC6 (Shi et al., 2006) cells, and promotes neurite outgrowth of PC12 cells (Christensen et al., 2003) and cultured rat dorsal root ganglion (DRG) neurons (Murray and Shewan, 2008).

How PKA and Epac transmit neural activity signals into a morphological phenotype is just beginning to be understood. A number of studies have linked PKA and Epac to filopodial dynamics *in vitro* through the action of proteins associated with the cytoskeleton, including cortactin (Mingorance-Le Meur and O'Connor, 2009), Ena/VASP (Lebrand et al., 2004; Lin et al., 2007), syndecan-2 (Lin et al., 2007), and the small GTPases Rac, Rho, Cdc42, and Rap (Bos, 2006; Dong et al., 1998; Ellerbroek et al., 2003; Feoktistov et al., 2000; Holz et al., 2006; Kawasaki et al., 1998; Kopperud et al., 2003; Lang et al., 1996; Maillet et al., 2003; O'Connor and Mercurio, 2001; Roscioni et al., 2008). The interplay between PKA and Epac in these pathways is complicated, and evidence indicates that both the pathways recruited and morphological outcome of activating PKA versus Epac is dependent on cell type, age, and the method used to activate AC. PKA and Epac have been shown to act synergistically (Christensen et al., 2003; Hewer et al., 2011; Hochbaum et al., 2008; Kiermayer et al., 2005; Kwan et al., 2007; Lee et al., 2011; Li et al., 2007; Petersen et

al., 2008), in opposition (Kiermayer et al., 2005; Mei et al., 2002; Murray et al., 2009b), and in an age-dependent manner (Murray et al., 2009b).

The involvement of PKA and Epac in synaptic plasticity and neurite growth *in vitro* suggests they may regulate dendritogenesis *in vivo* during early brain development. Here, we employ *in vivo* rapid time-lapse two-photon imaging of neurons within the developing brain to demonstrate a dual role for PKA and Epac in neuron development. We find that PKA and Epac act synergistically to increase dendritogenesis in the developing brain.

3.1.1 PKA and Epac are developmentally expressed in *X. laevis* and *X. tropicalis*

Prior to performing experiments, the expression patterns of PKA and Epac isoforms were determined for both *X. laevis* and *X. tropicalis* with searches of published literature and genomic databases. Epac1 has been cloned from both *X. laevis* and *X. tropicalis*, while Epac2 has only been cloned from *X. tropicalis* (Bowes et al., 2008; Lee and Han, 2005). Epac1 is expressed in developing *X. laevis* embryos (Lee and Han, 2005), which agrees with data showing developmental Epac1 expression in the rat (Murray and Shewan, 2008). In fact, both Epac isoforms are known to be expressed in the brain and developmentally regulated (Kawasaki et al., 1998; Murray and Shewan, 2008). The RIα, Cβ and Cγ subunits of PKA have been cloned from both *X. laevis* and *X. tropicalis*, while the RIIα and RIIβ subunits have only been cloned from *X. tropicalis* (Bowes et al., 2008). The PKA RIα, RIIα, RIIβ, Cα and Cβ subunits and Epac1 have been identified in microarray analysis of expressed *X. laevis* mRNAs (Yanai et al., 2011). *X. tropicalis* transcript homologies with human transcripts for PKA subunits range from 60-80%, while Epac1 and Epac2 have homologies of 58% and

83%, respectively (Bowes et al., 2008; Flicek et al., 2011). Several studies have examined the role of PKA in aspects of *X. laevis* development (Kao et al., 2002; Song et al., 1997).

As multi-day imaging experiments involved manipulating the levels of the transcription factor CREB, searches were also done to determine the patterns of CREB expression in *X. laevis* and *X. tropicalis*. *Xenopus* species express 3 isoforms of CREB, CREB1, 3, and 5, but only CREB1 and 3 are expressed in the brain and during development (Bowes et al., 2008; Yanai et al., 2011). CREB1 corresponds to the version of CREB known to be involved in learning and memory (Bowes et al., 2008; McKusick, 2007). *X. tropicalis* sequences have 93% and 29% sequence homology with human CREB1 and CREB3, respectively (Bowes et al., 2008; Flicek et al., 2011). Findings for PKA, Epac, and CREB are summarized in Table 3.

Table 3: Expression patterns of PKA, Epac, and CREB subunits in *X. laevis* and *X. tropicalis*.
JGI: Information obtained in part from the Joint Genome Institute at the United States Department of Energy.

Protein	Expression	*X. laevis*	*X. tropicalis*	Stage	Homology	References
PKA RIα	brain, eye, head, heart, limb, lung, mesonephric kidney, oocyte, ovary, spleen, testis, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult, brain from stage 45 to adult.	chicken: 61%; human: 60%; mouse: 60%; zebrafish: 61%	(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)
PKA RIβ	brain, central nervous system, egg, eye, mesonephric kidney, oocyte, spleen, testis, thymus, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	human: 64%; mouse: 62% (JGI)	(Bowes et al., 2008)
PKA RIIα	Keller explant, brain, ectoderm, egg, endomesoderm, eye, fat body, head, heart, intestine, lung, mesonephric, kidney, oocyte, ovary, skin, spleen, tail, testis, thymus, whole organism	mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 82%; human: 72%; mouse: 72%; zebrafish: 56%	(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)
PKA RIIβ	animal cap, brain, ectoderm, egg, eye, spleen, testis, upper blastopore lip, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 84%; human: 73%; mouse: 73%; zebrafish: 69%	(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)

Protein	Expression	*X. laevis*	*X. tropicalis*	Stage	Homology	References
PKA Cα	brain, central nervous system, egg, endomesoderm, eye, fat body, heart, intestine, liver, lung, oocyte, oviduct, skeletal muscle, spleen, testis, thymus, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	human: 71%; mouse: 83%; zebrafish: 83%	(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)
PKA Cβ	brain, central nervous system, ectoderm, egg, oocyte, ovary, oviduct, skeletal muscle, spleen, tail, testis, thymus, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 82%; human: 80%; mouse: 79%; zebrafish: 90%	(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)
PKA Cγ	not identified in *Xenopus*					(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)
Epac1	hatching gland, upper blastopore lip, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	10 to 66	human: 58%; mouse: 56%; zebrafish: 57%	(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)

Protein	Expression	*X. laevis*	*X. tropicalis*	Stage	Homology	References
Epac2	brain, mesonephric kidney, testis, whole organism		gene sequenced, mRNA expressed	25 to adult	chicken: 81%; human: 83%; mouse: 82%; zebrafish: 74%	(Bowes et al., 2008; Flicek et al., 2011)
CREB1	Keller explant, brain, cardiac mesoderm, dorsal marginal zone, ectoderm, egg, endoderm, endomesoderm, eye, fat body, fused heart primordium, head, heart, intestine, lung, mesoderm, neuroectoderm, ovary, oviduct, skin, spleen, testis, thymus, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 96%; human: 93%; mouse: 93%; zebrafish: 90%	(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)
CREB3	brain, ectoderm, endomesoderm, eye, fat body, head, heart, intestine, lung, ovary, oviduct, stomach, testis, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	10 to adult	chicken: 46%; human: 29%; mouse: 28%; zebrafish: 29%	(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)

Protein	**Expression**	***X. laevis***	***X. tropicalis***	**Stage**	**Homology**	**References**
CREB5	testis	mRNA expressed	gene sequenced, mRNA expressed	adult	chicken: 91%; human: 89%; mouse: 89%; zebrafish: 77%, 43% (predicted sequences)	(Bowes et al., 2008; Flicek et al., 2011; Yanai et al., 2011)

3.2 Methods

Animal care, fluorescent labeling of neurons within the intact tadpole brain, 1 hr and 5 min interval *in vivo* two-photon fluorescence imaging of dendritic growth, and statistical analysis were performed as described in Chapter 2.

3.2.1 Drug solutions and application

Drugs used for this experiment were as follows: forskolin (Fsk, 50 μM, Sigma-Aldrich, St. Louis, MO); Adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-cAMPS, 50 μM, BIOLOG Life Science Institute, Bremen, Germany); Adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS, 100 μM, BIOLOG Life Science Institute, Bremen, Germany and Biomol International Inc., Farmingdale, NY); 8-(4-Chlorophenylthio)-2'O-methyl-cyclic monophosphate (8-CPT, 100 μM, BIOLOG Life Science Institute, Bremen, Germany and Tocris Bioscience, Ellsville, MO); and brefeldin A (BFA, 100 μM, Tocris Bioscience, Ellsville, MO). Forskolin and brefeldin A were dissolved in DMSO (1:1000 concentration in dH_2O), all other compounds were dissolved in dH_2O. Forskolin, Rp-cAMPS, Sp-cAMPS, 8CPT, and brefeldin A were diluted to working solution in normal rearing medium and bath applied to the tadpole. For rapid time-lapse imaging, bath-applied drugs were continuously perfused through the imaging chamber following baseline imaging. For direct injection of drugs into the optic tectum, Sp-cAMPS, 8-CPT, brefeldin A, and Rp-cAMPS were dissolved to working concentrations in Amphibian Ringer's solution (116 mM NaCl, 1.2 mM KCl, 1.0 mM $CaCl_2$, 2.7 mM $NaHCO_3$). Under visual guidance using an upright stereomicroscope, a small incision was made lateral to the tegmentum of tadpoles anesthetized in 0.02% MS-222. A sharp glass pipette filled with drug

solution was inserted through this small cut and into the tegmentum. A Picospritzer II (General Valve Co., Fairfield, NJ) was used to deliver a low pressure, continuous air pulse into the pipette allowing injection of the drug solution into the tegmentum and tectum at a slow rate to prevent damage. No statistically significant differences between bath application and tectal injection were found for Rp-cAMPS, 8-CPT, Sp-cAMPS or brefeldin A, so the results from both experiments were pooled.

3.2.2 DNA constructs

Constructs used for multi-day imaging were as follows: Dominant-negative PKA (PKA RIα in the MT-REV vector, a gift from Dr. G. Stanley McKnight, University of Washington, Seattle, WA) (Rogers et al., 1990); constitutively-active PKA (PKA Cα, a gift from Dr. G. Stanley McKnight, University of Washington, Seattle, WA) (Uhler et al., 1986); dominant-negative Epac2 (EpacG114E/G422D in the pFLAG-CMV2 vector, a gift from Dr. Susumu Seino, Kobe University, Kobe, Japan) (Ozaki et al., 2000); constitutively-active Epac (Epac2ΔCBR in the Clontech pEGFP-C1 vector, a gift from Dr. Lawrence A. Quilliam, Indiana University, Indianapolis, IN) (Li et al., 2006); dominant-negative CREB (A-CREB in the CMV500 vector, a gift from Dr. Charles Vinson, National Institutes of Health, Bethesda, MD) (Ahn et al., 1998); and constitutively-active CREB (CREB-DIEDML in the Invitrogen pRc/RSV vector, a gift from Dr. Richard H. Goodman, Oregon Health Sciences University, Portland, OR) (Cardinaux et al., 2000). Constructs were co-electroporated along with fEGFP (1 μg/μl each to a final concentration of 2 μg/μl) into single neurons within the *X. laevis* tadpole optic tectum. For simultaneous expression of two dominant-negative or two constitutively-active constructs, plasmids encoding each at 1 μg/μl were combined with a

plasmid encoding fEGFP at 0.5 μg/μl for a final plasmid concentration of 2.5 μg/μl. All construct groups were electroporated into tadpoles from the same clutches of eggs.

3.2.3 Multi-day imaging

One day following electroporation with genetic constructs manipulating PKA, Epac, or CREB activity, tadpoles were briefly anesthetized with 0.02% MS-222, rapidly imaged, and returned to their chambers where they recovered from anesthesia within 1-3 min. This protocol was repeated every 24 hr for 5 days.

3.2.4 Dendrite morphometric analysis

Image stacks collected at 5 min or 1 hr intervals were analyzed as described in Chapter 2 (see section 2.2.5). As IF showed a stronger response to AC activation than GCF (see section 2.3.4), only IF were included in the current study.

Multi-day images were analyzed using the same software as for dynamic growth dynamics. The entire dendritic arbor of the cell, minus any structures less than 10 μm long (filopodia) was drawn and the total dendritic branch length (TDBL) and total number of dendritic branches (branch tip number – BTN) was determined. All drawings were done blind, and only cells with a TDBL below 400 μm on the first day of imaging were included in multi-day analysis.

3.3 Results

3.3.1 Neither PKA nor Epac alone are responsible for mediating the AC effect on filopodial growth

Cyclic AMP primarily mediates its effects through either PKA or Epac, so we tested whether increasing or decreasing PKA or Epac levels mimics or blocks the effects of Fsk, respectively. The PKA activator Sp-cAMPS (50 μM) did not increase filopodial length (Fig. 7A) but did increase motility of both extending (Fig. 10B) and retracting filopodia (Fig. 10C), indicating that PKA activation partially mimics the effects of AC activation on dendrite growth. When imaged at 1 hr intervals, Sp-cAMPS did not increase length of either stable (Fig. 10D) or newly added filopodia (Fig. 10E). While the PKA inhibitor Rp-cAMPS (50 μM) did not block Fsk-induced increases in stable filopodia length, it did block increases in newly added filopodia length, indicating that PKA activity is necessary but not sufficient to influence growth of newly added filopodia. Unexpectedly, application of Rp-cAMPS alone increased stable filopodia length.

The Epac activator 8-CPT-2'-O-Me-cAMPS (8-CPT, 100 μM) increased newly added filopodia length (Fig. 10E) but had no effect on stable filopodia length (Fig. 10D). The Epac inhibitor brefeldin A (BFA, 100 μM) blocked the Fsk-induced increase in stable filopodia length, indicating that Epac activity is necessary but not sufficient for this phenotype. BFA did not block the Fsk-induced increase in newly added filopodia length. BFA alone had no effect on filopodial growth, indicating no role for basal Epac activity levels on filopodial length in these cells. The distinct responses of stable and newly added filopodia to manipulations of PKA and Epac suggests different mechanisms regulating the growth of

these two groups of filopodia. Similar to manipulations of cAMP, altering PKA or Epac function had no effect on filopodial density, turnover, or lifetime (*data not shown*). Together, these results indicate that while both PKA and Epac play a role in the regulation of filopodial growth and motility, the activity of each alone cannot fully account for the effects of AC activation.

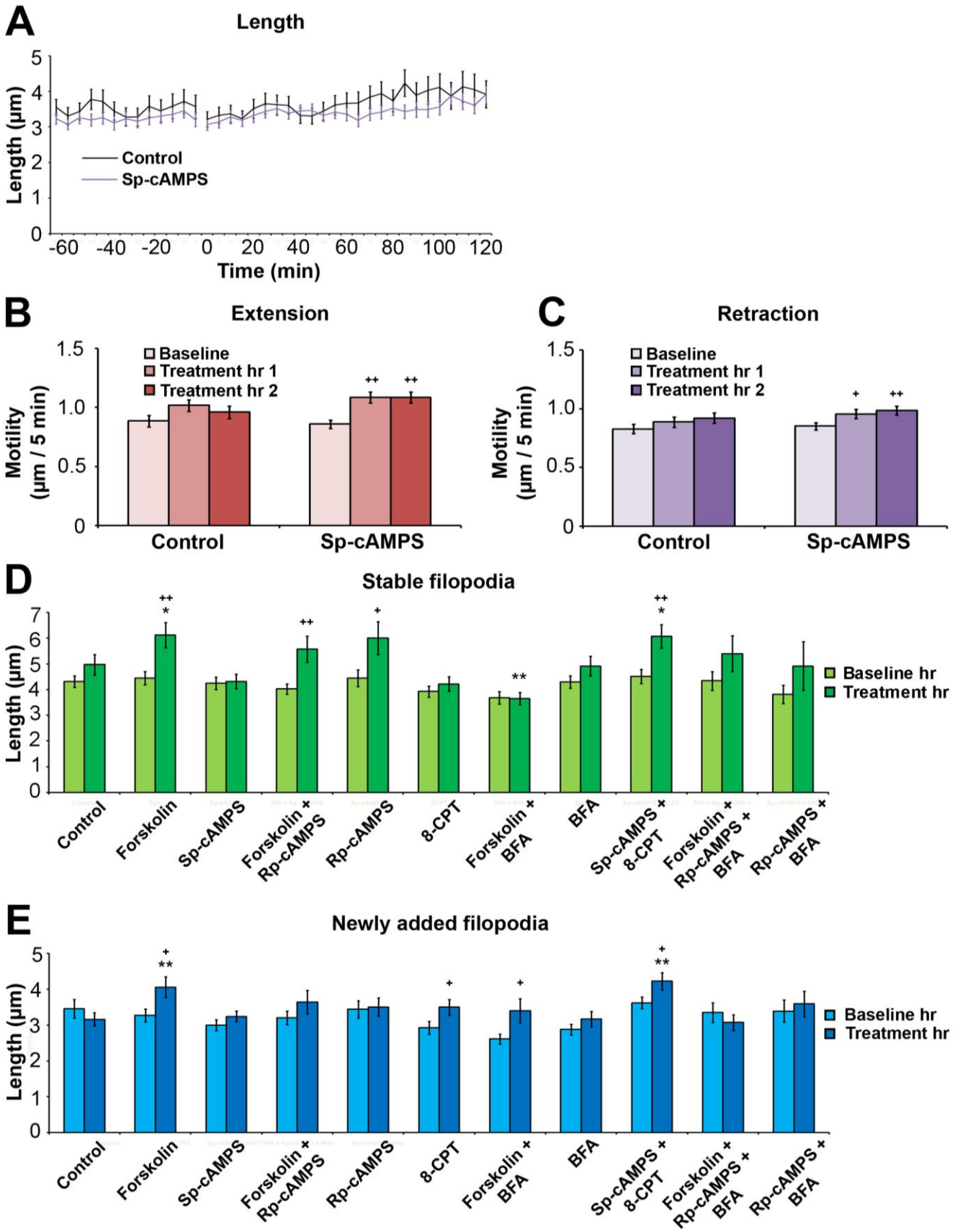

A
Length
Length (µm)
Time (min)
Control
Sp-cAMPS
B
Extension
Motility (µm / 5 min)
Baseline
Treatment hr 1
Treatment hr 2
Control
Sp-cAMPS
C
Retraction
Motility (µm / 5 min)
Baseline
Treatment hr 1
Treatment hr 2
Control
Sp-cAMPS
D
Stable filopodia
Length (µm)
Baseline hr
Treatment hr
Control
Forskolin
Sp-cAMPS
Forskolin + Rp-cAMPS
Rp-cAMPS
8-CPT
Forskolin + BFA
BFA
Sp-cAMPS + 8-CPT
Forskolin + Rp-cAMPS + BFA
Rp-cAMPS + BFA
E
Newly added filopodia
Length (µm)
Baseline hr
Treatment hr
Control
Forskolin
Sp-cAMPS
Forskolin + Rp-cAMPS
Rp-cAMPS
8-CPT
Forskolin + BFA
BFA
Sp-cAMPS + 8-CPT
Forskolin + Rp-cAMPS + BFA
Rp-cAMPS + BFA

Figure 10: PKA and Epac act synergistically to increase filopodial growth.
(A) Sp-cAMPS has no effect on average filopodial length. (B) Sp-cAMPS increases filopodial extension. (C) Sp-cAMPS increases filopodial retraction. (D) The effect of manipulating PKA and/or Epac activity levels on length of stable filopodia. (E) The effect of manipulating PKA and/or Epac activity levels on length of newly added filopodia. Replicate numbers: Rapid time lapse imaging: Control: N=5; n= 287 (baseline hr); 350 (treatment hr 1); 373 (treatment hr 2); Sp-cAMPS: N=6; n= 571 (baseline hr); 541 (treatment hr 1); 614 (treatment hr 2); 1 hr interval imaging: Control: N=17; n (stable) = 100; n (newly added) = 220 (baseline hr); 219 (treatment hr); Fsk: N=11; n (stable) = 79; n (newly added) = 155 (baseline hr); 145 (treatment hr); Sp-cAMPS: N=13; n (stable) = 102; n (newly added) = 162 (baseline hr); 192 (treatment hr); Fsk+Rp-cAMPS: N=11; n (stable) = 113; n (newly added) = 140 (baseline hr); 99 (treatment hr); Rp-cAMPS: N=8; n (stable) = 41; n (newly added) = 114 (baseline hr); 139 (treatment hr); 8-CPT: N=9; n (stable) = 113; n (newly added) = 145 (baseline hr); 142 (treatment hr); Fsk+BFA: N=9; n (stable) = 114; n (newly added) = 129 (baseline hr); 78 (treatment hr); BFA: N=12; n (stable) = 97; n (newly added) = 164 (baseline hr); 158 (treatment hr); Sp-cAMPS+8-CPT: N=15; n (stable) = 124; n (newly added) = 269 (baseline hr); 231 (treatment hr); Fsk+Rp-cAMPS+BFA: N=7; n (stable) = 40; n (newly added) = 70 (baseline hr); 62 (treatment hr); Rp-cAMPS+BFA: N=5; n (stable) = 34; n (newly added) = 89 (baseline hr); 84 (treatment hr); N = number of individual cells; n = number of individual filopodia.

Pharmacological manipulations are a powerful way to test the behaviour of proteins within a cell, but there are several caveats to their use. Drugs can have off target effects, for instance BFA also inhibits GEFs which activate ADP-ribosylation factor (ARF), thus interfering with vesicular transport from the Golgi apparatus (Chardin and McCormick, 1999; Zhong and Zucker, 2005). Also, bath-application of the drugs to the tadpole can have potentially unpredictable systemic effects which could influence neuronal function, such as the sympathomimetic effects of pancuronium dibromide (Kobayashi et al., 1987; Pinaud and Souron, 1984; Sai et al., 1998; Sato et al., 1999; Vercruysse et al., 1979). Direct tectal injection of drugs partially overcomes this issue, but still involves swamping an entire neural structure with a pharmacological agent. Another important consideration is the effective concentration of drug within brain cells. Although the applied drug concentration may be accurately measured, the concentration which reaches intracellular compartments of neurons in the intact brain is affected by its pharmacokinetics, including metabolism, tissue distribution, and ability to cross the blood-brain barrier and cell membranes.

In order to address these questions, we designed complementary experiments to genetically regulate PKA and Epac function using constitutively-active and dominant-

negative constructs. These constructs circumvent the issue of systemic effects by only influencing target activity within individual transfected neurons, allowing examination of cell-autonomous effects within an otherwise unaltered brain. Single-cell electroporation mediated transfection results in persistent exogenous expression, allowing measures over multiple days as neurons develop. Because these constructs inhibit or enhance target protein function via mechanisms distinct from pharmacological manipulations, these experiments control for nonspecific drug effects. One limitation of this approach, however, is the difficulty in regulating levels of exogenous constructs, which can only roughly be modulated by use of weak or strong promoters.

In order to test whether genetic inhibition of PKA or Epac blocks the effects of Fsk, individual neurons were electroporated with a combination of plasmids encoding fEGFP and a construct encoding a dominant-negative version of either PKA (PKA RIα) or Epac (EpacG114E/G442D). PKA RIα encodes a version of the regulatory subunit with mutated cAMP binding sites, preventing activation of the kinase (Rogers et al., 1990). EpacG114E/G442D encodes a version of Epac2 with mutated cAMP binding sites (Ozaki et al., 2000). Alternatively, tectal neurons were electroporated with plasmids encoding constitutively-active versions of PKA and Epac, termed PKA Cα (Uhler et al., 1986) and Epac2ΔCBR (Li et al., 2006), respectively. Both constructs encode the catalytic subunit with no inhibitory regulatory subunit, and are designed to swamp the cell with active PKA or Epac.

Twenty-four hours following electroporation, neurons were imaged at 1 hr intervals using *in vivo* two-photon microscopy. For dominant-negative constructs, the first hour of imaging served as a measure of baseline growth, after which Fsk was applied and neurons

were imaged again 1 hr later. Constitutively-active constructs were only imaged for 1 hr and not exposed to Fsk at any time. Fsk did not increase length of either stable (Fig. 11A) or newly added filopodia (Fig. 11B) when applied to neurons expressing either PKA RIα or EpacG114E/G442D. This confirms the effect of BFA on stable filopodia and Rp-cAMPS on newly added filopodia (Fig. 10D,E). There is discrepancy between the results from pharmacological and construct experiments however, as Fsk increased length of newly added filopodia in the presence of BFA but not EpacG114E/G442D, and of stable filopodia in the presence of Rp-cAMPS but not PKA RIα. It could be that in these cases the concentration of pharmacological inhibitor within the cell is insufficient to block the effects of AC activation. Alternatively, the effect of prolonged inhibition caused by the genetic constructs could result in a more powerful phenotype than the 1 hr pharmacological inhibitions. Constitutively-active constructs did not alter filopodial length compared to fEGFP controls (*data not shown*). PKA RIα, PKA Cα, and EpacG114E/G442D did not change filopodial density, addition, or retraction (Table 4), confirming effects observed with pharmacological manipulations (Table 2). Epac2ΔCBR, however, did increase filopodia density compared to fEGFP-expressing controls (Table 4).

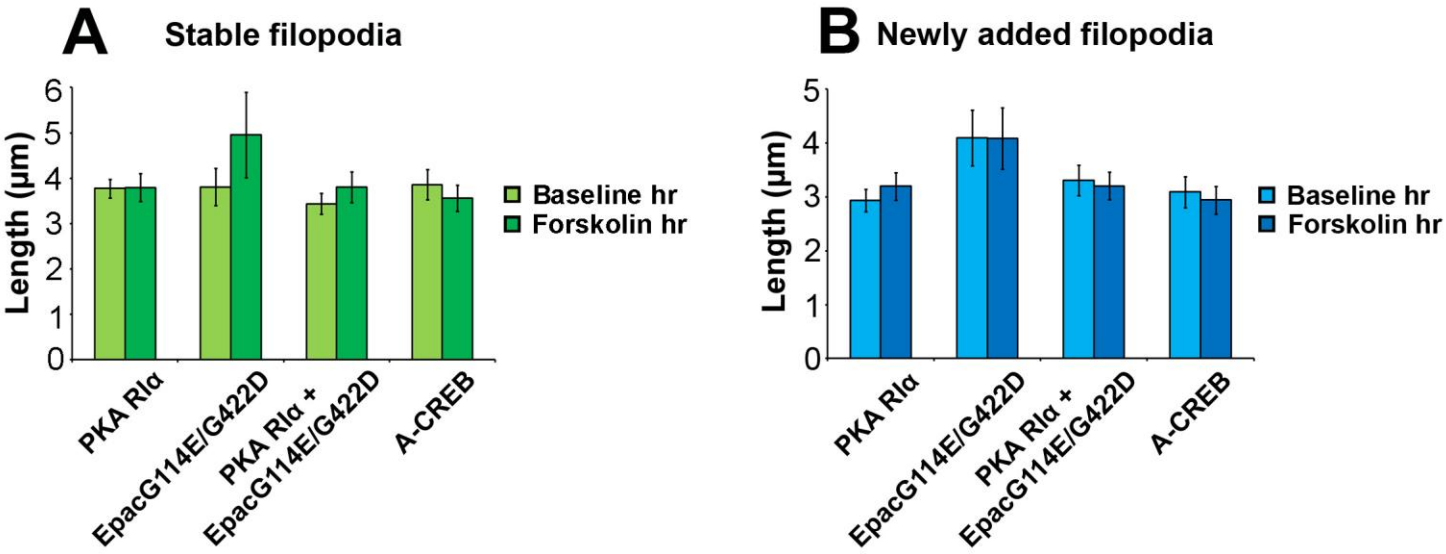


Figure 11: Dominant negative versions of PKA, Epac, or CREB prevent Fsk-induced dendritic growth. (A) Fsk does not increase stable filopodia length when constructs inhibiting PKA, Epac, or CREB are expressed. (B) Fsk does not increase newly added filopodia length when constructs inhibiting PKA, Epac, or CREB are expressed. Replicate numbers: 1 hr interval imaging: PKA RIα: N=7; n (stable) = 95; n (newly added) = 142 (baseline hr); 117 (treatment hr); EpacG114E/G422D: N=4; n (stable) = 22; n (newly added) = 60 (baseline hr); 56 (treatment hr); PKA RIα + EpacG114E/G422D: N=5; n (stable) = 74; n (newly added) = 66 (baseline hr); 65 (treatment hr); A-CREB: N=4; n (stable) = 50; n (newly added) = 84 (baseline hr); 72 (treatment hr); N = number of individual cells; n = number of individual filopodia.

Table 4: Effects of long-term PKA, Epac, or CREB manipulation on filopodia density, additions, and retractions
Neurons were either imaged for 1 hr or for 2 hr at 1 hr intervals. In the second hr of imaging, neurons expressing dominant-negative constructs were treated with Fsk. Displayed are filopodia density per 100 μm of TDBL or number of additions or retractions per 100 μm of TDBL N/A: Not applicable. There was no treatment hr for these neurons. Replicate numbers: fEGFP: N=17; PKA RIα: N=7; PKA Cα: N=8; EpacG114E/G422D: N=4; Epac2ΔCBR: N=3; PKA RIα + EpacG114E/G422D: N=5; PKA Cα + Epac2ΔCBR: N=4; A-CREB: N=4; CREB-DIEDML: N=9.

	Filopodia / 100 μm		Additions / 100 μm		Retractions / 100 μm	
	Baseline hr	Treatment hr	Baseline hr	Treatment hr	Baseline hr	Treatment hr
fEGFP	7.49 ± 0.75	7.65 ± 0.75	4.94 ± 0.62	5.32 ± 0.66	5.12 ± 0.63	4.75 ± 0.58
PKA RIα	10.19 ± 1.38	8.89 ± 1.35	5.88 ± 1.02	4.99 ± 0.93	6.38 ± 1.06	6.29 ± 0.99
PKA Cα	8.43 ± 1.48	N/A	6.19 ± 0.89	N/A	4.69 ± 0.81	N/A
EpacG114E/ G442D	7.34 ± 1.34	7.03 ± 0.76	4.93 ± 1.85	4.98 ± 0.84	5.36 ± 0.83	5.29 ± 1.42
Epac2ΔCBR	13.44 ± 4.32*	N/A	8.76 ± 4.50	N/A	6.44 ± 2.89	N/A
PKA RIα + EpacG114E/G442D	11.18 ± 2.21	10.92 ± 2.06	6.39 ± 1.92	5.63 ± 1.42	5.38 ± 1.53	5.89 ± 1.28
PKA Cα + Epac2ΔCBR	9.15 ± 1.42	N/A	5.43 ± 1.53	N/A	5.59 ± 1.52	N/A
A-CREB	8.76 ± 1.88	8.77 ± 2.29	4.63 ± 0.67	6.44 ± 3.47	4.80 ± 1.37	5.63 ± 1.31
CREB-DIEDML	9.10 ± 1.08	N/A	5.50 ± 0.96	N/A	4.45 ± 0.87	N/A

3.3.2 PKA and Epac act synergistically to increase filopodial growth

As neither PKA nor Epac alone account for the effects of adenylyl cyclase, I examined the possibility that simultaneous activity of both pathways is required for AC-mediated filopodial growth. When Sp-cAMPS and 8-CPT were co-applied, length of both stable (Fig. 10D) and newly added filopodia increased (Fig. 10E). The effects of Fsk were blocked by both co-application of Rp-cAMPS and BFA (Fig. 10) and by simultaneous expression of PKA RIα and EpacG114E/G442D (Fig. 11), indicating that PKA and Epac act synergistically to increase filopodial growth. Co-application of Rp-cAMPS and BFA in the absence of Fsk had no effect on filopodial length, confirming no role for basal activity levels in the AC pathway in filopodial length. Neither simultaneous construct expression (Table 4) nor pharmacological manipulation (*data not shown*) of PKA and Epac altered filopodial density, turnover, or lifetime.

Both PKA and Epac are known to be upstream of the cyclic-AMP-response-element-binding protein (CREB), a transcription factor with established roles in learning, memory, and neural development (Chen et al., 2010a; Davis, 1996; Fujioka et al., 2004; Harada et al., 2002; Lee et al., 2011; Nguyen and Woo, 2003; Redmond et al., 2002; Sands and Palmer, 2008; Schmidt et al., 1998; Shabb, 2001; Shi et al., 2006; Tojima et al., 2003a; Vo et al., 2005; Wayman et al., 2008). CREB is developmentally expressed in both *X. laevis* and *X. tropicalis*. (Bowes et al., 2008; Yanai et al., 2011). In order to test whether the synergistic effect of PKA and Epac is mediated by CREB, tectal neurons were electroporated with either a dominant-negative or constitutively-active version of CREB, termed A-CREB and CREB-DIEDML, respectively. A-CREB has disrupted DNA binding (Ahn et al., 1998), while

CREB-DIEDML has a modified domain mimicking the ser133 phosphorylated state (Cardinaux et al., 2000). CREB-DIEDML did not change filopodial length compared to fEGFP-expressing controls (*data not shown*). Similar to PKA RIα and EpacG114E/G422D, Fsk increased neither stable nor newly added filopodia length when A-CREB was expressed (Fig. 11), indicating that the increase in length seen with AC activation may be due to CREB-dependent gene expression downstream of PKA or Epac activation. Phenotypes dependent on new gene expression and protein synthesis generally take more time to express than phenotypes which don't require these extra steps. A dependence on gene expression could explain why it takes almost 50 mins for an AC-induced change in length to first be detected (Fig. 5C). CREB constructs had no effect on filopodial density, additions, or retractions (Table 4).

3.3.3 Simultaneous genetic manipulation of PKA and Epac alters long-term dendritic growth

In order to examine the long-term effects on manipulating PKA and Epac, PKA RIα, PKA Cα, EpacG114E/G442D, or Epac2ΔCBR were expressed in individual neurons using single-cell electroporation along with plasmid encoding fEGFP. Starting 24 hr following electroporation, neurons were imaged every 24 hr for 5 days. Long-term changes in dendritic branch number and length were analyzed using custom-written analysis software.

Similar to short-term pharmacological manipulations, long-term manipulation of either PKA or Epac activity with genetic constructs had no effect on either the total dendritic branch length (TDBL) (Fig. 12A) or branch tip number (BTN) (Fig. 12B). Because simultaneous pharmacological activation of PKA and Epac was required to elicit a change in

filopodial length, a similar experiment was conducted using genetic constructs. Individual neurons in the optic tectum were co-electroporated for expression of fEGFP and either both constitutively-active constructs or both dominant-negative constructs. Simultaneous activation or inhibition of PKA and Epac both resulted in increased TDBL and BTN 24 hr following electroporation (Fig. 12C,D). Despite causing an initial increase in BTN, the cells expressing both PKA RIα and EpacG114E/G442D showed no further branch addition, resulting in a statistically significant reduction in BTN by the fourth and fifth days (Fig. 12D). These results indicate that activity of either PKA or Epac is necessary for proper dendritogenesis over the long term, and that loss of function of both proteins inhibits long-term dendritogenesis.

We next asked whether this long-term effect on dendritic growth is dependent on CREB. Single neurons were electroporated with constructs encoding fEGFP and either A-CREB or CREB-DIEDML. We found that, as with simultaneous manipulation of PKA and Epac, manipulation of CREB activity increased both TDBL and BTN in the first 24 hr following electroporation (Fig. 12E,F), indicating that the effect seen with PKA and Epac is partially under the control of CREB.

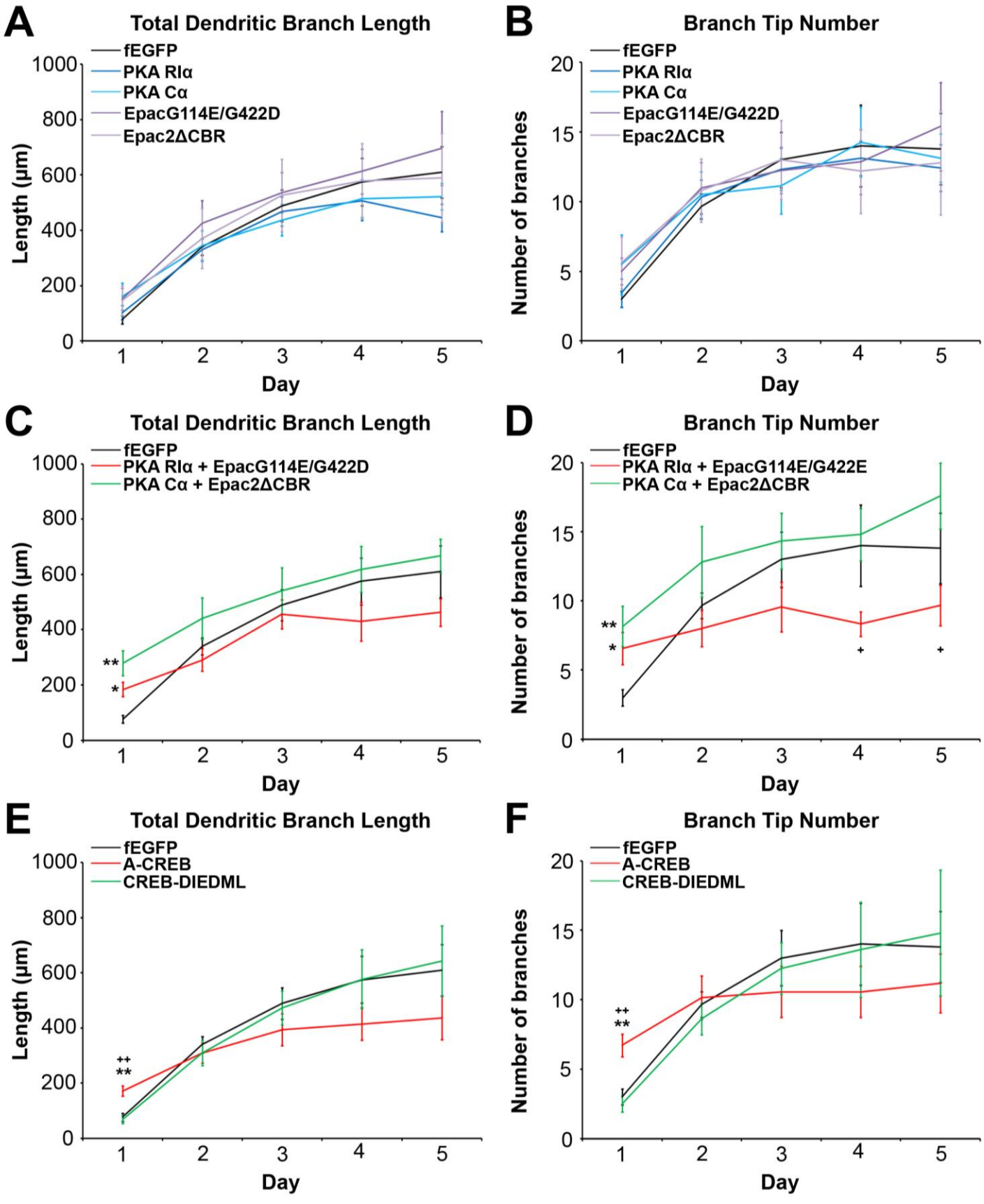

A
Total Dendritic Branch Length
fEGFP
PKA RIα
PKA Cα
EpacG114E/G422D
Epac2ΔCBR
Length (μm)
Day
B
Branch Tip Number
Number of branches
C
Total Dendritic Branch Length
PKA RIα + EpacG114E/G422D
PKA Cα + Epac2ΔCBR
D
Branch Tip Number
PKA RIα + EpacG114E/G422E
E
Total Dendritic Branch Length
A-CREB
CREB-DIEDML
F
Branch Tip Number

Figure 12: Simultaneous manipulation of PKA and Epac activity alters long-term dendritic growth. (A) Dominant-negative or constitutively active versions of PKA and Epac have no effect on TDBL over several days. (B) Dominant-negative or constitutively active versions of PKA and Epac have no effect on BTN over several days. (C) Simultaneous transfection of either both constitutively active PKA and Epac constructs or both dominant negative PKA and Epac constructs causes an initial increase in TDBL that is not maintained. (D) Simultaneous transfection of either both constitutively active PKA and Epac constructs or both dominant negative PKA and Epac constructs causes both short- and long-term changes in BTN. (E) A-CREB causes an initial increase in TDBL that is not maintained. (F) A-CREB activity causes an initial increase in BTN that is not maintained. *: $p<0.05$, **: $p<0.01$ vs. fEGFP; +: $p<0.05$, ++: p,0.01 dominant negative vs. constitutively active. Replicate numbers: fEGFP: N=6; PKA RIα: N=9; PKA Cα: N=8; EpacG114E/G422D: N=8; Epac2ΔCBR: N=5; PKA RIα + EpacG114E/G422D: N=7; PKA Cα + Epac2ΔCBR: N=6; A-CREB: N=7; CREB-DIEDML: N=8.

3.4 Discussion

3.4.1 PKA and Epac act synergistically to increase dendritic morphogenesis

In this study we find that synergistic activation of PKA and Epac is required to increase dendritic growth in developing neurons imaged *in vivo*. Although a synergistic role for PKA and Epac has been reported *in vitro* in non-neuronal cells (Hewer et al., 2011; Hochbaum et al., 2008; Kwan et al., 2007; Lee et al., 2011; Li et al., 2007; Petersen et al., 2008) or in neuritogenesis in cultured cells such as PC12 cells (Christensen et al., 2003; Gerdin and Eiden, 2007; Kiermayer et al., 2005), this is the first demonstration of this effect in neurons *in vivo*.

3.4.2 PKA and Epac have distinct effects on different subtypes of filopodia

One interesting finding of this study was that stable and newly added filopodia responded differently to manipulations of PKA and Epac. Persistent stable filopodia are predicted to contain stabilizing synapses (Niell et al., 2004; Vaughn, 1989). This would suggest that PKA and Epac may have distinct effects on filopodia based on whether they bear synapses. Inhibition of Epac but not PKA blocked the Fsk-induced increase in stable

filopodia length, while the opposite was observed in newly added filopodia. Furthermore, activation of Epac mimicked the Fsk-induced increase in newly added but not stable filopodia length. It is possible that Epac enhances synaptogenesis, creating a stabilizing point on a newly added filopodium from which new length can be added. This is supported by our data showing that long-term activation of Epac increases filopodia density, as it had been reported that synaptogenesis supports the addition of new branch points (Niell et al., 2004). Alternatively, inhibition of Epac may supress synaptogenesis, resulting in filopodial destabilization and failure to increase in length. However, no changes in filopodial density, additions, or retractions were observed, with the exception of the effect on density by Epac2ΔCBR. Lack of stabilization implies that the observed phenotype is not mediated by synaptogenesis, as has been discussed in Chapter 2. Additional experiments directly measuring synapse formation are required in order to conclude what mechanisms underlie the distinct effects of PKA and Epac on stable versus newly added filopodia.

In this study we report no role for Epac in the regulation of dendritic arbor growth under basal conditions over short time periods, demonstrated by the failure of BFA to induce a dendritic phenotype when applied alone. We do, however, observe an increase in length of stable filopodia observed when Rp-cAMPS was applied alone, indicating a role for basal levels of PKA in limiting arbor growth. This result is strange for a number of reasons. First, based on the AC data reported in Chapter 2 and previously published results, for example a study from the Ghosh lab on rat cortical cultures which found no role for PKA in basal growth (Redmond et al., 2002), we would expect that Rp-cAMPS would either reduce stable filopodia length or have no measurable effect. Second, activation of both PKA and Epac induces an increase in length of stable filopodia similar to that seen with inhibition of PKA.

This suggests that an optimal level of PKA activity is necessary to maintain stable filopodia length. It is possible that reduction of PKA activity below basal levels unmasks an alternate pathway, such as relieving PKA-mediated inhibition on a non-AC related pathway regulating neural growth. Alternatively, reduction in PKA activity could signal a lack of incoming inputs, causing neurons to enter a search mode, where they extend processes farther in order to search for potential synaptic contacts. Similar dichotomous effects of altered PKA activity have been reported for other cell functions. For example, both activation and inhibition of PKA have been reported to decrease cell migration (Edin et al., 2001; Howe, 2004; Ydrenius et al., 1997), and activation of PKA has also been reported to increase cell migration (O'Connor and Mercurio, 2001). Such results suggest a complex interaction with multiple signaling cascades.

3.4.3 Potential off-target effects of pharmacological manipulations

The use of pharmacological methods to distinguish between signaling pathways has a number of caveats. It is possible that some of the effects observed are due to non-specific interactions with other cellular proteins or imperfect specificity for PKA versus Epac. Sp-cAMPS was chosen over alternate means of activating PKA due to its relative lack of off-target effects. Other commonly used PKA activators, such as 8-Br-cAMP, 8-pCPT-cAMP (lacking the 2'-O-methyl group), and DB-cAMP can also activate Epac or have other significant off-target effects (Christensen et al., 2003; Poppe et al., 2008; Yusta et al., 1988). Although Sp-cAMPS has been regarded as a selective PKA agonist, recent studies have suggested that Sp-cAMPS can activate Epac (Christensen et al., 2003; Murray et al., 2009b; Rehmann et al., 2003). 8-CPT has been reported to be completely specific for Epac as

opposed to PKA (Christensen et al., 2003; Enserink et al., 2002; Gloerich and Bos, 2010; Holz et al., 2008; Rehmann et al., 2003). However, 8-CPT can bind to and inhibit PDE1, -2, and -6, thus indirectly increasing cAMP, which provides a mechanism by which 8-CPT could indirectly increase PKA activity (Poppe et al., 2008). When considering the potential ability of Sp-cAMPS or 8-CPT to activate the alternate target, it should be noted that separate application of Sp-cAMPS and 8-CPT herein results in distinct effects. This suggests that Sp-cAMPS and 8-CPT have different activation profiles. This could be further verified with the application of other PKA activators such as 6-Bnz-cAMP which have also been reported to have a minimum of off-target effects (Poppe et al., 2008).

As with Sp-cAMPS, Rp-cAMPS was chosen over alternate PKA inhibitors due to its relative lack of off-target effects. Other commonly used inhibitors, H89 and KT5720, also alter the activity of other proteins including PKB, PKC, β-ARs and other GPCRs, Rho kinase, MAPK, calcium activity, and alter cell excitability by affecting potassium and sodium channel conductance (Davies et al., 2000; Murray, 2008; Murray et al., 2009b; Penn et al., 1999). While numerous studies have shown Rp-cAMPS to be specific for PKA as opposed to Epac (Christensen et al., 2003; Poppe et al., 2008), a recent study has suggested that Rp-cAMPS is non-specific and may inhibit Epac as well as PKA (Rehmann et al., 2003). However, this study was done using cell-free systems. In cell models Rp-cAMPS has been unable to block the effects of Epac activation with 8-CPT (Holz et al., 2008; Roscioni et al., 2008). It's possible that an intracellular environment affects response to pharmacological activators and inhibitors.

Brefeldin A is the only commercially available inhibitor of Epac. However, BFA has an off-target effect which poses a significant concern. It also inhibits GEFs which activate

ADP-ribosylation factor (ARF), thus interfering with vesicular transport from the Golgi apparatus (Chardin and McCormick, 1999; Zhong and Zucker, 2005). One such GEF, ARF6, enhances dendritic branching when inhibited with a dominant-negative construct. This effect is mediated by Rac1 (Hernandez-Deviez et al., 2002). Horton et al. applied BFA to hippocampal neural cultures with the intent of inhibiting secretory trafficking and disassembling the Golgi apparatus (Horton et al., 2005). When BFA was applied to young neurons (2-3 DIV) dendrite length decreased, but recovered within 48 hr of drug washout. Application of BFA to mature neurons (22 DIV) also decreased dendrite length, indicating that BFA negatively affects both arbor growth and arbor maintenance (Horton et al., 2005).

3.4.4 Multi-day manipulation of PKA and Epac

In this study, we find that simultaneous manipulation of PKA and Epac with genetic constructs alters dendrite growth over multiple days. One day after electroporation, simultaneous activation of PKA and Epac, or simultaneous inhibition of PKA and Epac both cause an increase in TBDL without a corresponding increase in filopodial length. These findings suggest that long-term manipulations of PKA and Epac may be involved in increasing branch growth rather than filopodial growth. This is likely due to extension of existing branches. PKA and Epac constructs may also cause extension of filopodia to lengths longer than 10 μm, as was observed when cAMP levels or PKA and Epac activity were pharmacologically increased. However, this was only observed during the initial periods of PKA and Epac activation. It is possible that, after several hours of continuous PKA and Epac activity, compensatory mechanisms are activated to prevent filopodial overgrowth. This may be why increases in filopodial length were observed with acute pharmacological

manipulations but not long-term genetic manipulations. This hypothesis assumes that the mechanisms underlying branch extension and filopodia extension are different, which is possible given the unique cytoskeletal identity of the two structures. Alternatively, it is possible that the single neuron manipulations of genetic constructs cause different filopodial effects than bath application of a drug which affects the entire neural tissue. Electroporated neurons, growing in a normal brain circuit, may have a different morphological response to altered PKA and Epac activity then neurons growing in a drug-exposed circuit.

We also observed an increase in branch tip number in individual neurons with dually activated or inhibited PKA and Epac, which was not observed with pharmacological manipulations. This suggests that BTN changes may only occur as a result of prolonged alterations in PKA and Epac activity. The finding that simultaneous activation or inhibition of PKA and Epac both promote overgrowth after one day was surprising, however it does agree with data showing an effect for Rp-cAMPS in increasing length of stable filopodia. No significant differences from controls were observed at longer time periods following the first day after electroporation. Although we saw no change compared to controls, we do see a significant difference between the BTNs of neurons expressing dominant-negative constructs versus those expressing constitutively-active constructs.

3.4.5 MAPK and CREB as a potential mechanism for AC-, PKA-, and Epac-dependent control of dendritic growth

PKA and Epac both activate CREB. PKA-dependent activation occurs either directly through phosphorylation and or indirectly through the intermediate MAPK. Epac also activates MAPK through its downstream effectors. Thus PKA and Epac can converge at the

level of MAPK in order to regulate gene expression or to act on MAPK targets that aren't transcription factors. Like PKA and Epac, MAPK and CREB have both been linked to increases in neurite and dendrite length and number (Aizawa et al., 2004; Chen et al., 2010a; Fujioka et al., 2004; Gerdin and Eiden, 2007; Kiermayer et al., 2005; Ravni et al., 2008; Redmond et al., 2002; Schmidt et al., 1998; Shi et al., 2006; Tojima et al., 2003a; Vaillant et al., 2002; Vo et al., 2005; Wu et al., 2001).

Here we report that electroporation of individual tectal neurons *in vivo* for expression of a dominant-negative form of CREB (A-CREB) increases TDBL and BTN one day following transfection. This phenotype is similar to that observed with co-expression of dominant-negative forms of PKA and Epac. By the second day after transfection growth increases slow such that both BTN and TDBL are similar to control levels. Rat cortical cultures, transfected with a different dominant-negative version of CREB (KCREB) at 2 DIV, showed no change in total dendritic length 2 days following transfection (Redmond et al., 2002), which agrees with results reported here. As with the PKA and Epac double dominant-negative, growth appears to slow after the first day of expression. These data imply that the initial increase in growth caused by synergistic knockdown of PKA and Epac is mediated by reduced CREB activity. Electroporation of a constitutively-active form of CREB does not mimic the effects observed when cells are simultaneously electroporated with constitutively-active forms of PKA and Epac. In this case, PKA and Epac may be acting through other pathways, such as cytoskeletal proteins and the RhoGTPases, synaptic proteins, or other signaling cascades. These results imply the recruitment of multiple signaling pathways based on the activity levels of PKA and Epac in the control of dendritic development. Based on the combined pharmacological and genetic PKA, Epac, and CREB

data, I propose that basal PKA and Epac activity levels maintain CREB activity levels in order to prevent abnormal overgrowth. When activated for prolonged periods of time, PKA and Epac initially promote growth until compensatory mechanisms normalize growth back to control levels.

Chapter 4: β-Adrenergic receptors act through both PKA and Epac to increase dendritogenesis

4.1 Introduction

In Chapter 3, I demonstrated that adenylyl cyclase increases dendritic filopodial length and motility through the synergistic activation of both PKA and Epac. However, Fsk activates all AC isoforms except AC9 (Hacker et al., 1998; Sutkowski et al., 1994), while under physiological conditions distinct AC isoforms are activated or inhibited based on specific neural signals (Table 1). ACs 1 and 8 are activated by calcium influx through channels such as the NMDA receptor. Other AC isoforms are activated by neurotransmitters such as epinephrine, dopamine, or serotonin, or hormones such as PACAP. Intracellular signaling proteins such as PKC, PKA, CaMKII, and CaMKIV can regulate AC activity level (Cooper and Crossthwaite, 2006; Sadana and Dessauer, 2009; Willoughby and Cooper, 2007). Although my experiments show a role for AC activity in the regulation of developmental dendritogenesis, they do not provide information as to which upstream activators of AC regulate dendritogenesis *in vivo*. I therefore designed experiments to determine whether targeted activation of specific ACs would recapitulate the results observed with Fsk administration.

Of the various physiological activators of AC, the β-adrenergic receptor (β-AR) system is a promising candidate as a regulator of dendritic development. β2-AR activity has been implicated in neurite growth, inducing a rapid, protein synthesis-independent process outgrowth in cultured AS583.8 cells, an embryonic rat basal forebrain cell line. This growth is associated with increased microtubule dynamics and the redistribution of filamentous actin

from the cell body into the tips of growing processes (Kwon et al., 1996; Kwon et al., 1998). β-ARs regulate synaptic plasticity by lowering the threshold for LTP induction (Gelinas and Nguyen, 2005; Huang and Kandel, 2007; Thomas et al., 1996; Winder et al., 1999). β-AR activation induces a PKA-dependent enhancement of NMDA receptor responses in the amygdala (Huang et al., 1993) and hippocampus (Raman et al., 1996; Thomas et al., 1996). β-ARs are known to signal through both PKA and Epac (DiPilato et al., 2004; Gelinas et al., 2008b; Gloerich and Bos, 2010; Huang and Hsu, 2006; Huang and Kandel, 2007; Kassel et al., 2008; Oestreich et al., 2007; Raman et al., 1996; Schmidt et al., 2001), which are themselves linked to neurite growth (Aglah et al., 2008; Christensen et al., 2003; Fujioka et al., 2004; Kao et al., 2002; Kiermayer et al., 2005; Lebrand et al., 2004; Mingorance-Le Meur and O'Connor, 2009; Murray and Shewan, 2008; Shi et al., 2006; Tojima et al., 2003a), and which, in Chapter 3 of this thesis, were shown to be involved in developmental dendritogenesis *in vivo*.

The evidence linking β-AR activity to neurite growth, cytoskeletal regulation, synaptic plasticity, and signaling pathways known to be involved in dendritic growth imply a role for β-ARs in dendritogenesis *in vivo*. In this chapter, I test whether agonists or antagonists of β-ARs alter dendritic growth in the intact, developing brain. I find that β-AR activation recapitulates some, but not all of the effects of direct AC activation with Fsk, and that this recapitulation is dependent on both PKA and Epac.

4.1.1 Beta-adrenergic receptors are developmentally expressed in *X. laevis* and *X. tropicalis*

Prior to performing experiments, the expression patterns of β-AR isoforms were determined for both *X. laevis* and *X. tropicalis* with searches of published literature and genomic databases. β1-AR and β2-AR have both been cloned in *X. tropicalis* and *laevis* (Bowes et al., 2008) and β2-AR has been identified in microarray analysis of expressed *X. laevis* mRNAs (Yanai et al., 2011). β-ARs have also been identified in the brains of another frog species, the American bullfrog (*Rana catesbeiana*) (Bachman et al., 1998) and in mouse and rat (Harmar et al., 2009). *X. laevis* myocardial cells express β2-adrenergic receptors which are both coupled to AC and pharmacologically similar to mammalian β2-adrenergic receptors (Port et al., 1992). *X. tropicalis* transcripts have sequence identities with *H. sapiens* transcripts of 58% for β1-AR and 62% for β2-AR (Bowes et al., 2008; Flicek et al., 2011). These findings are summarized in Table 5.

Table 5: Expression patterns of beta-adrenergic receptor isoforms in *X. laevis* and *X. tropicalis*.
JGI: Information obtained in part from the Joint Genome Institute at the United States Department of Energy.

Isoform	Expression	*X. laevis*	*X. tropicalis*	Stage	Homology	Mammalian function	References
β1-AR	fat body, head, heart, lung, skin, whole organism	gene predicted	gene sequenced, mRNA expressed	15 to adult	human: 58%; mouse: 60%; zebrafish: 58% (JGI)	increase cardiac rate and contraction force, relaxation of coronary arteries and gastrointestinal smooth muscle, enhancement of LTP	(Bowes et al., 2008; Bylund et al., 1994; O'Dell et al., 2010)
β2-AR	egg, endomesoderm, head, heart, intestine, lung, ovary, skeletal muscle, skin, spleen, tail, whole organism	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 68%; human: 62%; mouse: 64%; zebrafish: 50%	smooth muscle relaxation, modulation of norepinephrine release from sympathetic nerve terminals, enhancement of LTP	(Bowes et al., 2008; Bylund et al., 1994; Flicek et al., 2011; O'Dell et al., 2010; Yanai et al., 2011)
β3-AR						lipolysis in white adipose tissue, thermogenesis in brown adipose tissue, insulin secretion	(Bylund et al., 1994)

4.2 Methods

Animal care, fluorescent labeling of neurons within the intact tadpole brain, *in vivo* two-photon fluorescence imaging of dendritic growth, dynamic morphometric analysis, and statistical analysis were performed as described in Chapter 2.

4.2.1 Drug solutions and application

Drugs used for this experiment were as follows: isoproterenol (Iso, 15 μM, Sigma-Aldrich, St. Louis, MO, a gift from Dr. John Church, University of British Columbia, Vancouver, BC); 9-(Tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ22536, 100 μM, Tocris Bioscience, Ellsville, MO); Adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS, 100 μM, BIOLOG Life Science Institute, Bremen, Germany and Biomol International Inc., Farmingdale, NY); and brefeldin A (BFA, 100 μM, Tocris Bioscience, Ellsville, MO). All compounds were dissolved in dH_2O, diluted to working solution in normal rearing medium, bath applied to the tadpole, and continuously perfused through the imaging chamber following baseline imaging.

4.3 Results

4.3.1 β-adrenergic receptor activation influences growth upstream of both Epac and PKA

Next we examined the role of a potential upstream physiological activator of AC, the β-adrenergic receptor (β-AR). The β-AR agonist isoproterenol (also known as isoprenaline) (Iso, 15 μM), which targets all three β-AR isoforms (Harmar et al., 2009), was applied to

tadpoles under both the 5 min and 1 hr interval time-lapse imaging protocols. β-AR activation had no effect on filopodial growth motility (Fig. 13A) but did increase filopodial retraction motility (Fig. 13B), similar to Sp-cAMPS and Fsk. Iso did not increase the length of stable filopodia (Fig. 13C) but did increase length of newly added filopodia, an effect which was blocked by inhibitors of PKA, Epac, and AC (Fig. 13D), indicating that the effect is dependent on activity of both PKA and Epac. Iso also increased filopodial density (Fig. 14). An increase in filopodial density was previously observed following chronic activation of Epac (Table 4). Although the effects of Iso are dependent on both PKA and Epac, they do not fully mimic the phenotype observed when PKA and Epac are simultaneously activated.

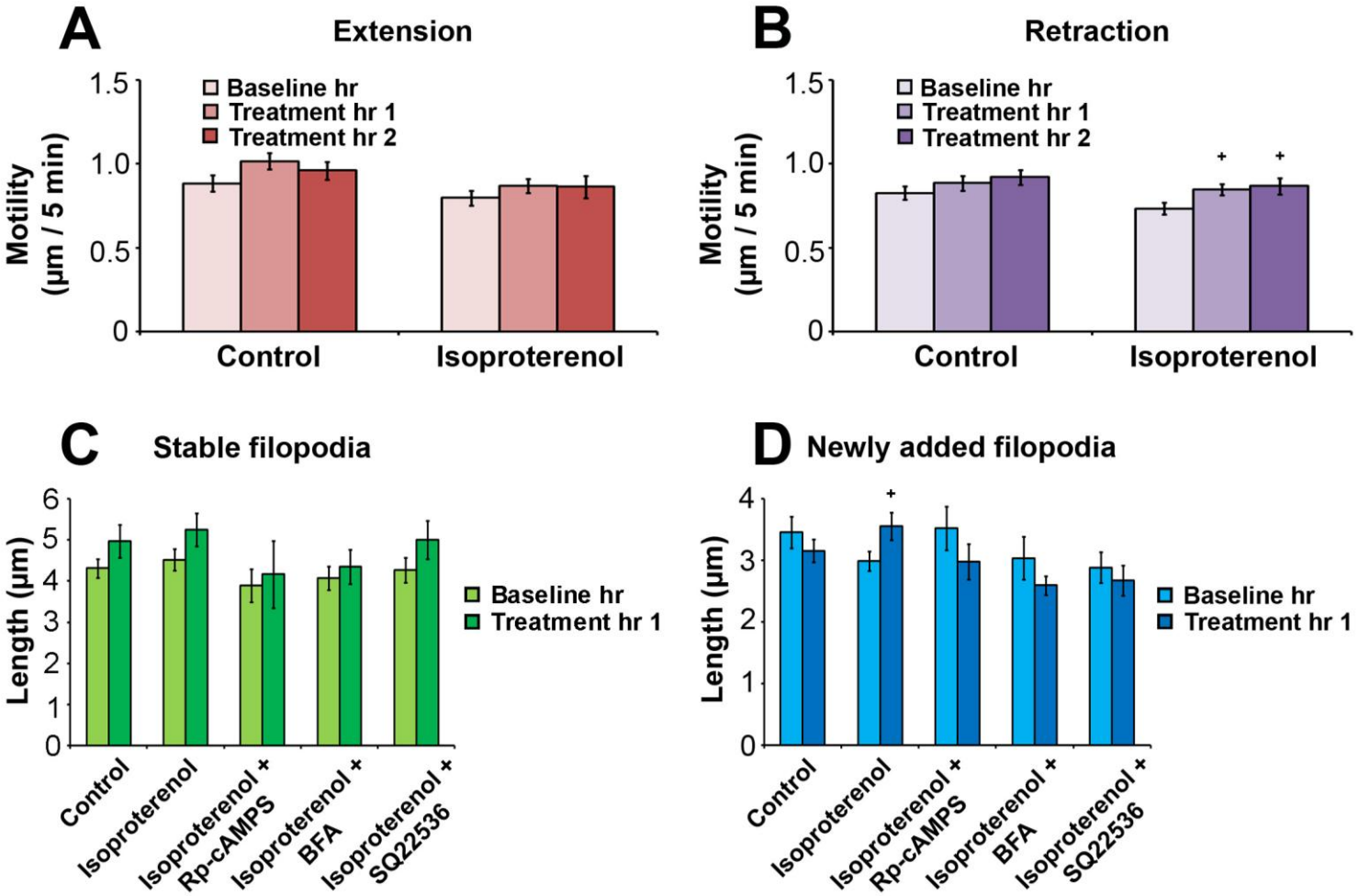


Figure 13: β-Adrenergic receptor activation only partially mimics the effects of adenylyl cyclase activation, but these effects are under the control of both PKA and Epac.
(A) Iso does not increase filopodial extension motility. (B) Iso increases filopodial retraction motility. (C) β-AR activity has no effect on stable filopodia length. (D) Iso increases newly added filopodial length. This effect is dependent on PKA, Epac, and AC activity. Replicate numbers: Rapid time lapse imaging: Control: N=5; n= 287 (baseline hr); 350 (treatment hr 1); 373 (treatment hr 2); Iso: N=3 (2 hr total imaging), 1 (3 hr total imaging); n= 315 (baseline hr); 370 (treatment hr 1); 125 (treatment hr 2); 1 hr interval imaging: Control: N=17; n (stable) = 100; n (newly added) = 220 (baseline hr); 219 (treatment hr); Iso: N=9; n (stable) = 93; n (newly added) = 137 (baseline hr); 135 (treatment hr); Iso+Rp-cAMPS: N=4; n (stable) = 31; n (newly added) = 53 (baseline hr); 45 (treatment hr); Iso+BFA: N=5; n (stable) = 56; n (newly added) = 55 (baseline hr); 62 (treatment hr); Iso+SQ22536: N=6; n (stable) = 58; n (newly added) = 59 (baseline hr); 55 (treatment hr); N = number of individual cells; n = number of individual filopodia.

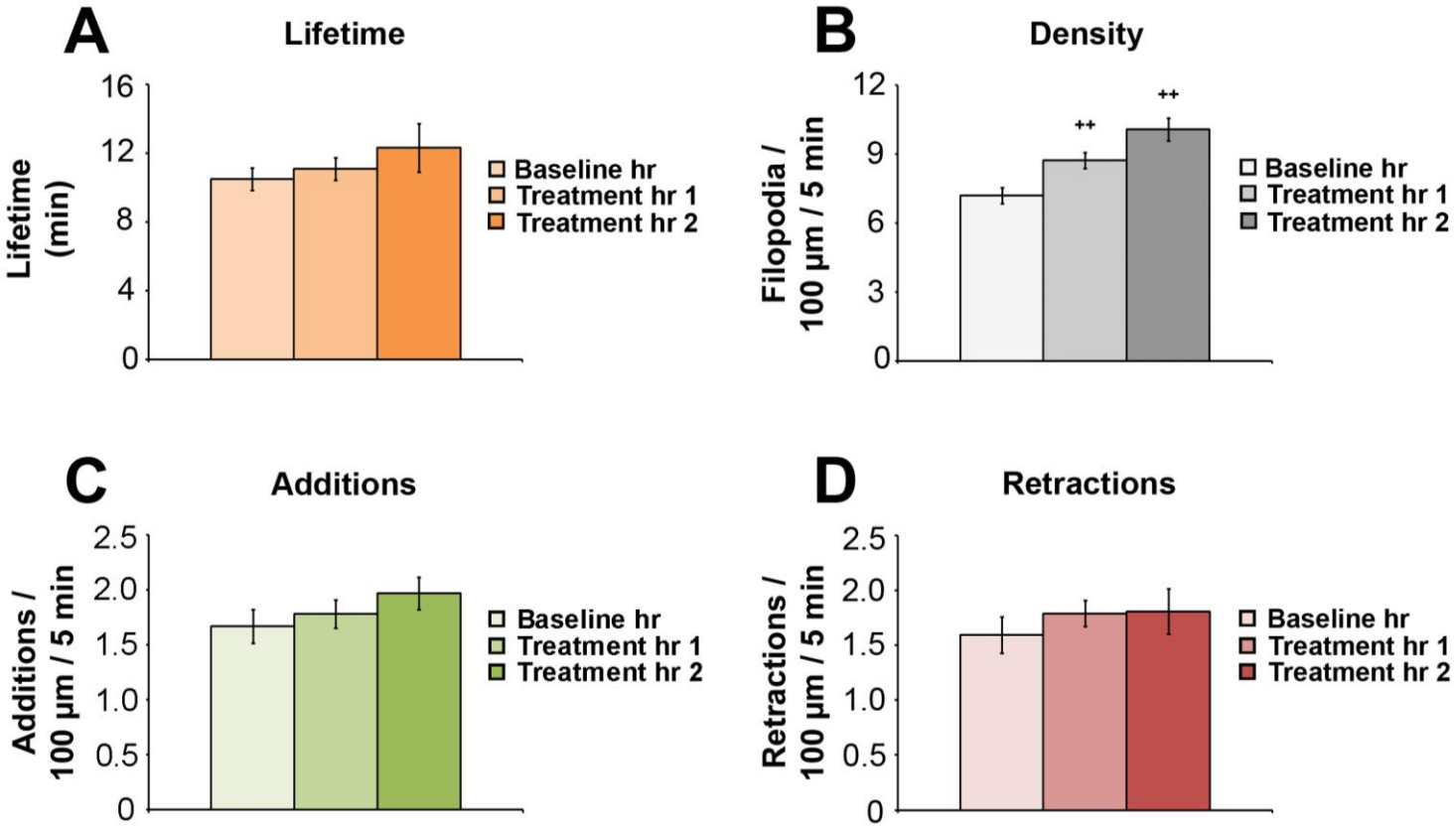


Figure 14: Effect of isoproterenol addition on filopodia stability.
(A) Lifetime of transient filopodia. (B) Iso increases filopodia density. (C) Filopodia additions. (D) Filopodia retractions. Replicate numbers: Rapid time lapse imaging: N=3 (2 hr total imaging), 1 (3 hr total imaging); n= 142 (baseline hr); 166 (treatment hr 1); 54 (treatment hr 2); N = number of individual cells; n = number of individual transient filopodia for lifetime calculation.

4.4 Discussion

4.4.1 Activation of β-adrenergic receptors only partially mimics the effects of AC activation on dendritic growth.

We find that β-AR activation only partially mimics the effects of Fsk on developmental dendritogenesis, but that these effects are dependent on both PKA and Epac. These results are the first observation of β-AR-dependent regulation of dendritic growth in developing neurons *in vivo*. With these data I outline a pathway from β-ARs through AC, PKA, and Epac for the control of dendritogenesis within the intact, developing brain.

Although the effects of Iso are similar to activation of AC, PKA, and Epac, and dependent on active AC, PKA, and Epac, they do not fully mimic the phenotype observed when PKA and Epac are simultaneously activated. There are several possible explanations for this. One is that only a subset of effects are physiologically under the control of β-ARs, the rest being under the control of calcium-activated ACs or other GPCRs such as dopaminergic or serotonergic receptors. Another explanation is that PKA and Epac do not reach the same levels of activation under β-AR activation as they do when directly activated pharmacologically. 8-CPT has a higher affinity for its target than does cAMP (Poppe et al., 2008; Rehmann et al., 2003) but Sp-cAMPS has a lower affinity (Bertinetti et al., 2009; Dostmann et al., 1990; Rehmann et al., 2003). Another important consideration is the effective concentration of drug within brain cells, which depends on factors such as metabolism, tissue distribution, and ability to cross the blood-brain barrier and cell membranes. These factors may affect Iso, Sp-cAMPS, and 8-CPT differently, and thus influence the relative activation level of their targets. If, in this experimental system, Sp-

cAMPS and 8-CPT result in a higher level of target activation than Iso, then the effects under β-AR activation would likely be less robust. Finally, although both Sp-cAMPS and 8-CPT have been reported to be highly specific, and there have been no reported off-target effects of Iso aside from a very low affinity for α-adrenergic receptors (Harmar et al., 2009; Overgaard and Dzavik, 2008), this does not rule out the possibility of other, as yet unidentified off-target effects.

4.4.2 Activation of β-adrenergic receptors increases dendritic filopodial density – an effect not observed when AC is activated.

In this study we report that β-AR activation increases filopodial density, which was not observed when AC, PKA, or Epac were activated pharmacologically. With only one manipulation have we observed this effect – when neurons were electroporated with a constitutively-active Epac construct and imaged the next day. Long-term manipulations of either CREB or PKA and Epac simultaneously increased BTN, but this is a measure of branches, not filopodia. It is possible that selective activation of β-ARs increases filopodial density through activation of Epac, but further experiments are necessary in order to confirm this result.

4.4.3 Physiological significance of a role for β-ARs in dendritic development

As discussed above, β-ARs have an established role in the enhancement of LTP (Gelinas and Nguyen, 2005; Huang et al., 1993; Huang and Kandel, 2007; Raman et al., 1996; Thomas et al., 1996; Winder et al., 1999). They are also known to be involved in fear conditioning (Bush et al., 2010; Rodrigues et al., 2009), anxiety disorders (Davidson, 2006;

Schelling, 2008), and emotional memory (Cahill et al., 1994). The finding that β-ARs regulate dendritogenesis implies a role for emotionally salient stimuli in the control of neural development. Through the β-adrenergic pathway, brain development can be shaped to experience to produce lasting changes in brain function.

Chapter 5: Blocking interactions between PKA and A-kinase anchoring proteins alters filopodial stability and has effects which are distinct from PKA activation and inhibition

5.1 Introduction

PKA has an extremely wide range of effects within cells, raising the question of how a neuron can apply any specificity to a PKA signal. A-kinase anchoring proteins (AKAPs) anchor PKA in the vicinity of both its activators and specific effectors, allowing the cell to control which pathways are activated downstream of a given PKA signal. AKAPs also anchor protein phosphatases, which can "turn off" the PKA signal by dephosphorylating PKA target proteins. In this way AKAPs provide both spatial and temporal specificity to a PKA signal.

AKAPs provide a link between PKA and the cytoskeleton. The dendritic microtubule associated protein MAP2 was originally described as a protein which, in cell free systems, binds to and promotes the polymerization and elongation of microtubules (Herzog and Weber, 1978; Kim et al., 1979; Sloboda et al., 1976) and bundling of actin filaments (Sattilaro, 1986; Selden and Pollard, 1983) dependent on its phosphorylation state. It was later discovered to be an AKAP for PKA (Davare et al., 2001; Harada et al., 2002; Lohmann et al., 1984; Wong and Scott, 2004). MAP2 has four isoforms: MAP2A, B, C, and D. MAP2B, C, and D are developmentally expressed in the nervous system while MAP2A is primarily expressed in the adult (Sanchez et al., 2000). MAP2 is localized to cell bodies, dendrite shafts, and dendritic spines (Caceres et al., 1983; Morales and Fifkova, 1989;

Sanchez et al., 2000). MAP2 provides a direct mechanism of linking dendritic PKA to the cytoskeleton.

Experiments from the Hell lab have described one such MAP2/PKA signaling complex which exists in neurons (Davare et al., 2001). The study's authors showed that MAP2B clusters PKA with the voltage-gated calcium channel $Ca_V1.2$, protein phosphatase 2A (PP2A), the β2-adrenergic receptor, an unidentified AC isoform, and a heterotrimeric G protein. Patch-clamp analysis showed that the diffusion of the β2-AR-stimulated cAMP signal was able to increase calcium channel activity only if it occurred in the vicinity of the channel. In this way, the AKAP can regulate local calcium influx through spatial regulation of the cAMP signal. Another study (Zhong et al., 2009) found that MAP2 binds to PKA and sequesters it in dendritic shafts. Cyclic AMP elevation with forskolin and the PDE inhibitor IBMX or norepinephrine caused the translocation of the catalytic subunit of PKA into spines. The ability of norepinephrine to modulate LTP induction was impaired in mice with disrupted PKA-MAP2 binding (Zhong et al., 2009).

In addition to anchoring PKA, MAP2 is also phosphorylated by PKA (Harada et al., 2002; Sloboda et al., 1975; Vallee, 1980; Walaas and Nairn, 1989). The phosphorylation state of MAP2 is developmentally regulated and increases with dendritic arborisation (Diez-Guerra and Avila, 1993, 1995). Antisense oligonucleotides against MAP2 suppress neurite formation in embryonic carcinoma cells (Dinsmore and Solomon, 1991) and cerebellar macroneurons (Caceres et al., 1992). MAP2 knock-out mice show reduced length of hippocampal dendrites, reduced PKA expression, and reduced CREB phosphorylation in response to forskolin (Harada et al., 2002).

Several other AKAPs are associated with neural proteins. Yotiao, also known as AKAP9 and a splice variant of AKAP350, clusters PKA and protein phosphatase 1 (PP1) to the NMDA receptor (Westphal et al., 1999). Yotiao facilitates cAMP-dependent enhancement of NMDA receptor currents dependent on both active PKA and the ability of PKA to bind Yotaio. Yotiao also negatively regulates cAMP signaling through inhibition of ACs 2 and 3 (Piggott et al., 2008). AKAP79/150, also known as AKAP5, is enriched in postsynaptic densities and dendritic spines (Carr et al., 1992). AKAP79/150 is capable of assembling multiple, distinct clusters within the postsynaptic density, which include proteins such as PKA, PP2B, PKC, the GluR1 subunit of the AMPA receptor, the NR2B subunit of the NMDA receptor, PSD-95, and SAP97 (Colledge et al., 2000; Klauck et al., 1996; Wong and Scott, 2004). The AKAP gravin is enriched in the filopodia of human erythroleukemia cells, binds PKA and PKC, and targets PKA to the neuromuscular junction (Nauert et al., 1997; Wong and Scott, 2004). WAVE1, a protein known to interact with the Arp2/3 complex, has been identified as an AKAP which regulates lamellipodia formation in response to Rac activation (Machesky and Insall, 1998; Miki et al., 1998; Westphal et al., 2000). WAVE1 is located at the leading edge of lamellipodia but is absent from filopodia (Nozumi et al., 2003). With the exception of WAVE1, none of these AKAPs has a demonstrated role in dendritic morphology. Loss of WAVE1 in striatal neurons, either through expression of a siRNA or through gene knockout, causes a decrease in density of mature spines and an increase in filopodia. In these same neurons, forskolin increases the number of both mature spines and filopodia, but only the increase in mature spines is dependent on WAVE1 (Kim et al., 2006b). A study by Han et al. showed that PKA is anchored in growth cone filopodia by an unidentified AKAP, and that blocking PKA

localization prevents cAMP-induced attractive growth cone turning (Han et al., 2007). Currently, however, little is known about the role of AKAPs and PKA localization in dendritic morphogenesis.

We conducted pilot experiments to determine whether PKA localization to AKAPs is important to dendritic development *in vivo*. Abolishing PKA-AKAP binding increased filopodial lifetime and decreased retractions without effects on filopodial length or motility. These preliminary experiments demonstrate a potential role for PKA localization in dendrite growth.

5.1.1 AKAPs are developmentally expressed in *X. laevis* and *X. tropicalis*

Next we examined the role PKA localization in the regulation of dendritic growth. A number of AKAPs have been isolated in the nervous system in other animals, where they play a role in ion channel regulation, synaptic plasticity, and cytoskeletal dynamics. Prior to performing experiments, the expression patterns of these AKAPs were determined for both *X. laevis* and *X. tropicalis* with searches of published literature and genomic databases. *X. tropicalis* AKAP transcripts show a wide range of homologies with human transcripts, ranging from 5% with Yotiao to 83% with WAVE1 (Flicek et al., 2011). MAP2 is developmentally expressed in the X. tropicalis brain and present in the optic tectum of adult *X. laevis* (Bowes et al., 2008; Guo et al., 2001). These findings are summarized in Table 6.

Table 6: Expression patterns of AKAPs in *X. laevis* and *X. tropicalis*.

AKAP	Expression	Known binding partners	*X. laevis*	*X. tropicalis*	Stage	Homology	References
AKAP7 (AKAP15, AKAP18)	brain, egg, head, lung, oocyte, spleen, whole organism	PKA, voltage-gated Ca^{2+} channel, voltage-gated Na^{+} channel	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 39% human: 19% mouse: 36% zebrafish: 26%	(Bowes et al., 2008; Flicek et al., 2011; Wong and Scott, 2004; Yanai et al., 2011)
AKAP10	Keller explant, brain, ectoderm, egg, head, oocyte, spleen, testis, whole organism	PKA	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 73% human: 70% mouse: 69% zebrafish: 64%	(Bowes et al., 2008; Flicek et al., 2011; Wong and Scott, 2004; Yanai et al., 2011)
AKAP11	brain, central nervous system, egg, eye, head, lung, mesonephric kidney, ovary, skin, spleen, tail, testis, whole organism	PKA, PP1, GSK3β	mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 42% human: 45% mouse: 42% zebrafish: 38%	(Bowes et al., 2008; Flicek et al., 2011; Wong and Scott, 2004; Yanai et al., 2011)

AKAP	Expression	Known binding partners	*X. laevis*	*X. tropicalis*	Stage	Homology	References
AKAP79/ 150 (AKAP5)		PKA, PKC, PP2B, NMDAR, AMPAR, PSD-95, KCNQ2 channel, L-type Ca^{2+} channel, aquaporin channel					(Wong and Scott, 2004)
Gravin (AKAP12, AKAP250)	Keller explant, anterior neural tube, blastopore, brain, central nervous system, chordoneural hinge, dorsal, dorsal marginal zone, ectoderm, endomesoderm, eye, fat body, fused heart primordium, head, intestine, limb, lung, mandibular crest, neuroectoderm, notochord, oviduct, roof plate, spinal cord, spleen, tail, testis, ventral, whole organism	PKA, PKC, β2-AR	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	gastrula to adult		(Bowes et al., 2008; Wong and Scott, 2004; Yanai et al., 2011)

AKAP	Expression	Known binding partners	*X. laevis*	*X. tropicalis*	Stage	Homology	References
mAKAP (AKAP6)	central nervous system, spleen, testis	PKA, PDE43D, PP2A, Erk5, Epac1		gene sequenced, mRNA expressed	58 to adult	chicken: 35% human: 36% mouse: 36% zebrafish: 32%	(Bowes et al., 2008; Flicek et al., 2011; McConnachie et al., 2006; Wong and Scott, 2004)
MAP2	brain, central nervous system, eye, head, skin, whole organism	PKA, tubulin, $Ca_V1.2$, PP2A, β2-AR, AC	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	28 to adult	chicken: 18% human: 58% mouse: 18% zebrafish: 13%	(Bowes et al., 2008; Davare et al., 2001; Flicek et al., 2011; Wong and Scott, 2004; Yanai et al., 2011)
WAVE1	bone tissue, brain, central nervous system, egg, endomesoderm, eye, head, lung, mesonephric kidney, oocyte, ovary, stomach, testis, whole organism	PKA, Abl, Rac, Arp2/3	gene predicted, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 83% human: 83% mouse: 83% zebrafish: 52%	(Bowes et al., 2008; Flicek et al., 2011; Wong and Scott, 2004; Yanai et al., 2011)

AKAP	Expression	Known binding partners	*X. laevis*	*X. tropicalis*	Stage	Homology	References
Yotiao (AKAP9)	egg, intestine, mesonephric kidney, skin, spleen, stomach, testis, whole organism	PKA, AC1, AC2, AC3, AC9, PP1, NMDAR, KCNQ1 channel, inositol-1,4,5,-triphosphate receptor	mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 5% human: 5% mouse: 4% zebrafish: 1%	(Bowes et al., 2008; Flicek et al., 2011; Piggott et al., 2008; Wong and Scott, 2004; Yanai et al., 2011)

5.2 Methods

Animal care, fluorescent labeling of neurons within the intact tadpole brain, *in vivo* two-photon fluorescence imaging of dendritic growth, dynamic morphometric analysis, and statistical analysis were performed as described in Chapter 2.

5.2.1 Ht31 peptide application

Pressure injection of peptide solution into the tegmentum and tectum was performed as described in Chapter 2. The peptide injected was either the inCELLect AKAP St-Ht31 Inhibitor Peptide or the inCELLect AKAP St-Ht31P Control Peptide (Ht31 or Ht31P, respectively, 20 μM, Promega, Madison, WI).

5.3 Results

5.3.1 Blocking PKA-AKAP interactions has no effect on filopodial length or stability, increases filopodial lifetime, and temporarily reduces rates of filopodial retraction

In order to determine whether PKA anchoring is involved in developmental dendritogenesis, single neurons within the intact *X. laevis* optic tectum were electroporated with a construct encoding fEGFP and the rapid time-lapse imaging protocol was used. Following 1 hr of baseline imaging at 5 min intervals, peptides designed to interfere with PKA anchoring were infused directly into the tectum and tegmentum, after which tadpoles were returned to the imaging chamber and imaged for a further 2 hr at 5 min intervals. Peptides used were Ht31 (20 μM), which binds to the anchoring domain of the regulatory subunit of PKA, preventing PKA from binding to AKAPs, and Ht31P (20 μM), which

contains a proline substitution in the binding motif and is therefore unable to bind PKA. Ht31 has previously been shown to be effective in disrupting PKA-AKAP binding in *X. laevis* (Corstens et al., 2006; Han et al., 2007; Isoldi et al., 2010). As with the PKA activator Sp-cAMPS, Ht31 had no effect on filopodial length (Fig. 10A,15A). Ht31 also had no effect on filopodial motility (Fig. 15B,C). These results are distinct from those seen with PKA activation, which caused increases in both extension and retraction motility (Fig. 15B,C). This effect is also distinct from that observed with PKA inhibition, which over 1 hr increased length of stable filopodia (Fig. 10D) but had no other detectable effects.

Ht31 increased filopodial stability. Although it did not alter density or addition rate (Fig. 16B,C), it caused an increase in filopodial lifetime that became significant in the second hour of treatment (Fig. 16A). Ht31 application initially caused a decrease in filopodial retraction which was significant for the first hour of treatment, after which retraction rates returned to baseline levels. As with length and motility, these effects are different from those observed under either acute PKA activation or inhibition.

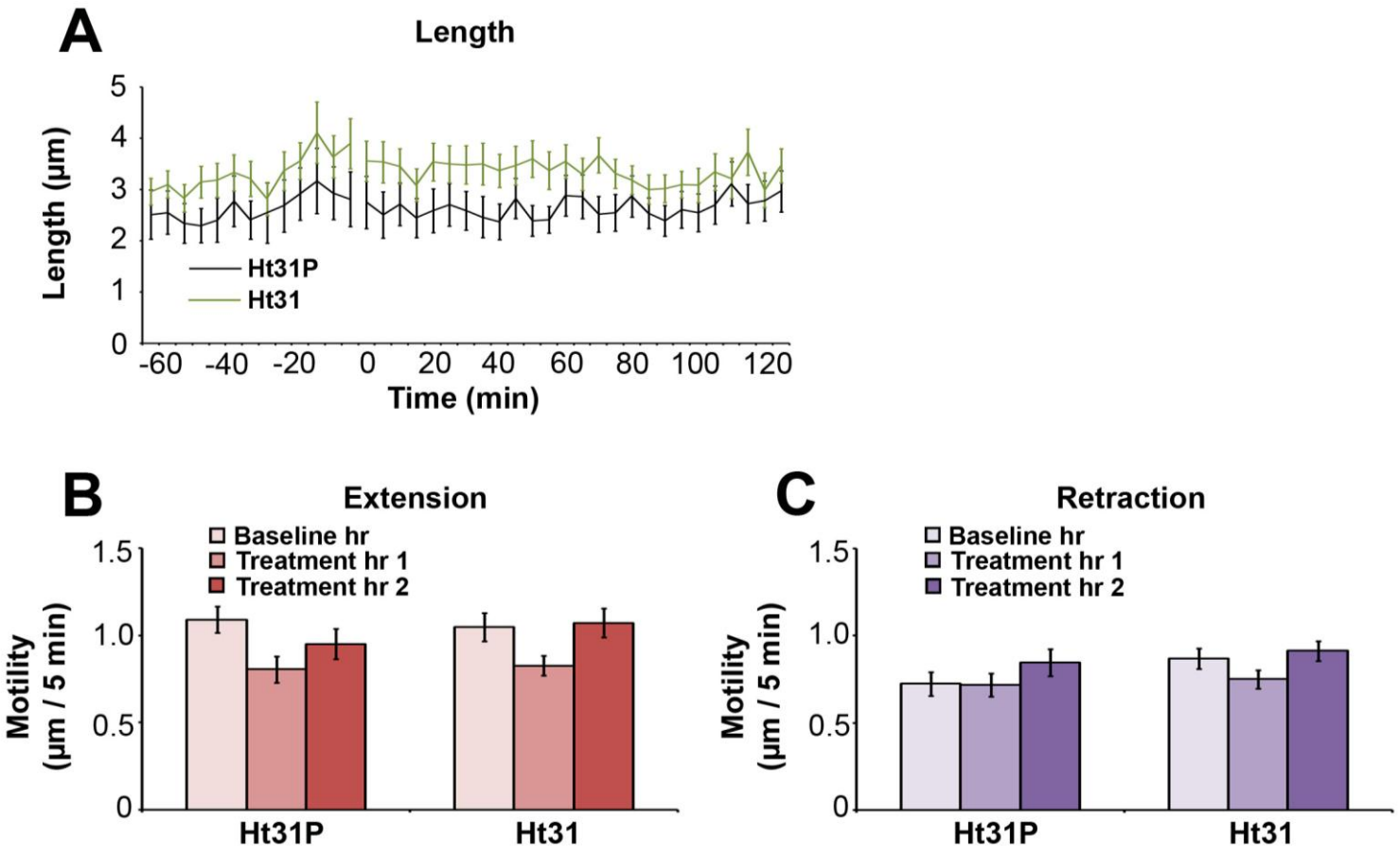


Figure 15: Blocking PKA-AKAP interactions has no effect on filopodia length or motility
(A) Ht31 does not alter average filopodia length. (B) Ht31 does not alter filopodia extension. (C) Ht31 does not alter filopodia retraction. Replicate numbers: Ht31P: N=2; n= 113 (baseline hr); 88 (treatment hr 1); 91 (treatment hr 2); Ht31: N=3; n= 163 (baseline hr); 113 (treatment hr 1); 151 (treatment hr 2); N = number of individual cells; n = number of individual filopodia.

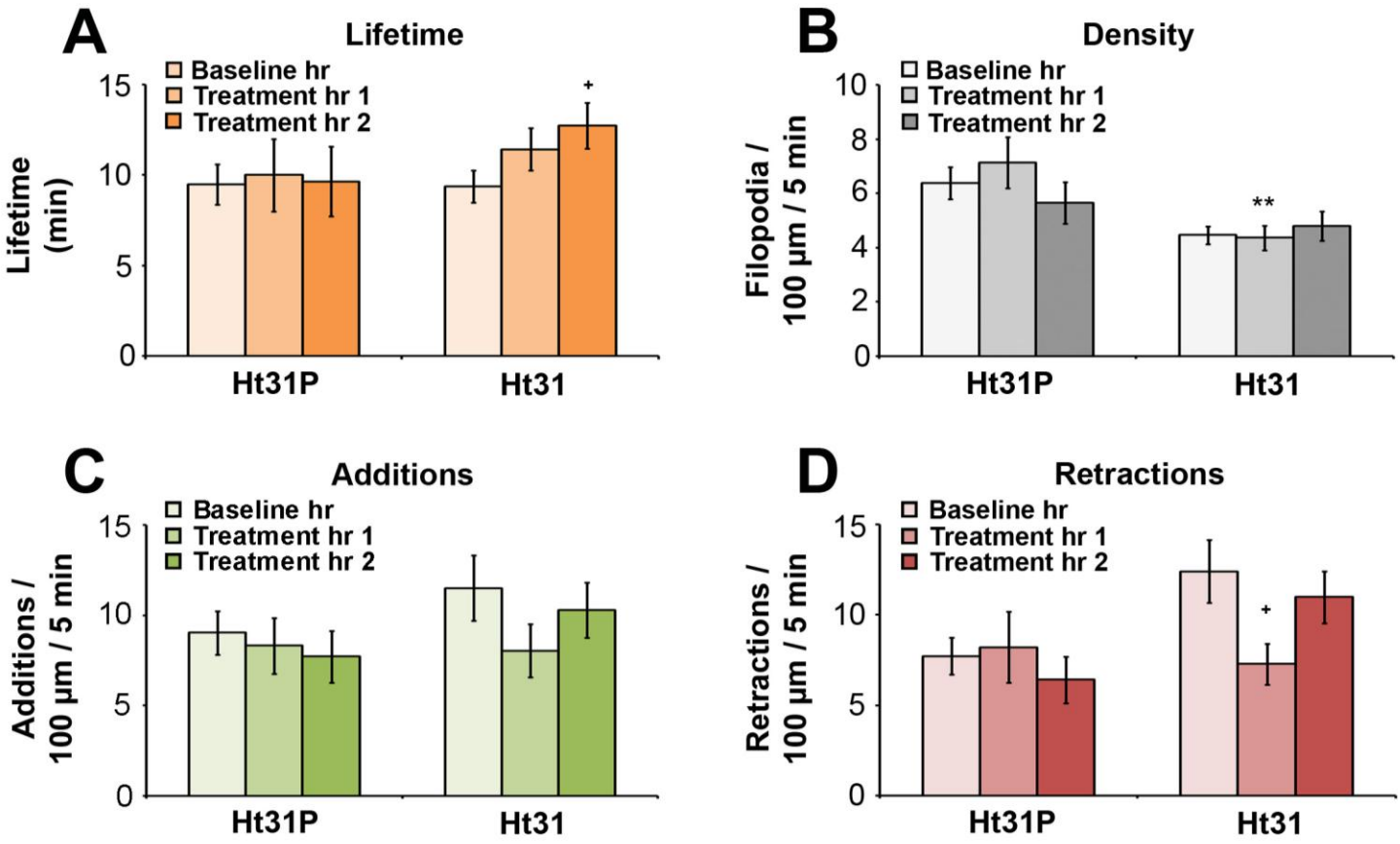


Figure 16: Blocking PKA-AKAP interactions increases filopodia lifetime and temporarily decreases filopodia retractions.
(A) Ht31 increases filopodia lifetime. (B) Ht31 does not alter filopodia density. (C) Ht31 does not alter filopodia additions. (D) Ht31 temporarily decreases filopodia retractions. Replicate numbers: Rapid time lapse imaging: Ht31P: N=2; n= 39 (baseline hr); 20 (treatment hr 1); 29 (treatment hr 2); Ht31P: N=3; n= 79 (baseline hr); 42 (treatment hr 1); 75 (treatment hr 2); N = number of individual cells; n = number of individual transient filopodia for lifetime calculation.

5.4 Discussion

5.4.1 Blocking PKA interactions with AKAPs has effects which are unique from pharmacological PKA activation or inhibition

In this pilot study I report that blockade of PKA-AKAP interactions increases filopodial lifetime while temporarily decreasing retractions, but has no effect on filopodial length or motility. These effects are unique from those observed under either PKA activation

or PKA inhibition. This the first evidence of a role for PKA-AKAP binding in dendritic development in *X. laevis* brain neurons imaged *in vivo*.

I hypothesized that abolishing PKA localization would have a negative effect on growth. Blocking PKA-AKAP interactions negatively affects growth cone turning (Han et al., 2007), and synaptic activity (Davare et al., 2001; Westphal et al., 1999; Zhong et al., 2009), and knocking MAP2 down or out suppresses neurite and dendrite formation (Caceres et al., 1992; Dinsmore and Solomon, 1991; Harada et al., 2002). Here, however, we report an increase in filopodial lifetime and a decrease in retraction, indicating a potential stabilizing effect, but no change in length. This is different from the phenotype observed when basal levels of PKA were inhibited over 1 hr with Rp-cAMPS. The mechanisms of the two techniques are different – Rp-cAMPS is a competitor for the cAMP binding site on PKA, and Ht31 prevents PKA localization while not affecting the ability of cAMP to activate PKA – and therefore they may not lead to the same morphological result.

One possible experiment would be to apply Ht31 to cells stimulated with forskolin or isoproterenol. Blocking basal PKA levels had a minimal effect on dendrite growth (see Chapter 3). It is possible that the effects observed here were minimal because basal PKA levels are not heavily involved in regulating filopodial and dendritic morphogenesis in these cells at this developmental stage. Blocking PKA was able to block some of the effects of forskolin and isoproterenol application, indicating a role for PKA under increased neural activity. It is possible that, in order to observe an effect with Ht31, cAMP or PKA levels must first be increased, either through activation of AC or a GPCR such as the β-ARs, or through direct activation of PKA with Sp-cAMPS.

5.4.2 Non-specificity of Ht31

One potential confounding factor in these experiments is the non-specificity of Ht31, which binds to the AKAP-targeting domain on PKA, thus preventing PKA from binding to any AKAP. If different AKAP complexes serve different roles in the cell, then interfering with all AKAPs may create compounding or possibly opposing effects, which would make it difficult to tease out any clear, interpretable phenotype. One way to control for this would be to electroporate cells with mutated versions of the AKAPs which have all domains intact except for the PKA binding domain, and thus would presumably fill the same intracellular niche as the endogenous protein without participating in PKA anchoring and signaling. This would allow us to determine the role of individual AKAPs in developmental dendritogenesis.

Chapter 6: Calpain has distinct effects on axonal and dendritic development downstream of β-adrenergic receptors and PKA

6.1 Introduction

Data presented in this thesis outlines a pathway from β-ARs through AC, PKA, and Epac for the regulation of dendritogenesis within developing brain neurons *in vivo*. These data also support a synergistic role for PKA and Epac in dendritic growth. However, the question remains as to what the mechanism is downstream of PKA and Epac. How do PKA and Epac induce a morphological change? One candidate protein which interacts with both the synapse and the cytoskeleton and which links PKA signaling to filopodial dynamics in cultured neurons is the calcium-activated protease calpain.

The effects of calpain at the synapse are not straightforward, at times enhancing and at other time reducing synaptic response. Calpain cleaves both NMDA and AMPA receptors. While the effects of NMDA receptor cleavage are unclear (Wu and Lynch, 2006), cleavage of the AMPA receptor leads to removal of the receptor from postsynaptic densities (Bi et al., 1997; Lu et al., 2000; Wu and Lynch, 2006). Reduction of AMPA receptor stability is augmented by calpain-dependent cleavage of the postsynaptic density proteins synapse-associated protein 97 (SAP97), and glutamate receptor-interacting protein 1 (GRIP1) (Jourdi et al., 2005). Furthermore, calpain inhibition blocks the formation of long-term depression (LTD) (Hrabetova and Sacktor, 1996). In other cases, calpain seems to enhance synaptic response. NMDA receptor activation increases calpain-dependent cleavage of L-type calcium channels, resulting in increased calcium influx in response to synaptic activity (Hell et al., 1996). Calpain cleavage of NMDA and AMPA receptors has been hypothesized to lead to

long-term potentiation (Lynch, 1998), although not all the available data supports this hypothesis (Goll et al., 2003; Wu and Lynch, 2006).

Calpain also acts on cytoskeletal components including those involved in linking the cytoskeleton to the cell membrane and intermediate proteins important for actin cross-linking (Goll et al., 2003). This implies a potential role for calpain in the formation of filopodia and lamellipodia (Hotulainen and Hoogenraad, 2010; Hotulainen et al., 2009; Korobova and Svitkina, 2010; Mattila and Lappalainen, 2008). Calpain cleaves RhoA into a dominant-negative form (Kulkarni et al., 2002), and RhoA inhibition is associated with dendritic overextension, while RhoA activation leads to dendritic retraction, simplification, and loss of motility (Hirose et al., 1998; Korobova and Svitkina, 2008; Lee et al., 2000; Li et al., 2000; Nakayama et al., 2000; Pilpel and Segal, 2004; Sakisaka et al., 2004; Sin et al., 2002; Wong et al., 2000; Zhang et al., 2003). Calpain can also cleave MAP2. Phosphorylation of MAP2 by PKA and PKA binding to MAP2 provide mutual protection from calpain proteolysis (Alexa et al., 1996; Johnson and Foley, 1993). Through its interactions with the cytoskeleton, calpain is able to alter filopodial and lamellipodial motility of non-neuronal, migrating cells (Dourdin et al., 2001; Franco et al., 2004; Kulkarni et al., 2002; Potter et al., 1998), but studies in neurons are limited.

Whether calpain is involved in dendritic development *in vivo* is unknown, although the available evidence suggests a potential role. Removal of the autoinhibitory domains of CaMKII and PKC by calpain produces catalytically active fragments (Al and Cohen, 1993; Hajimohammadreza et al., 1997; Kishimoto et al., 1983; Kishimoto et al., 1989; Wu and Lynch, 2006), and both CaMKII and PKMζ, a catalytically active fragment of PKC, have been implicated in dendritic growth in *X. laevis* (Liu et al., 2009; Wu and Cline, 1998).

Calpain may also play a role in recovery and regrowth following injury. Calpain activity is necessary for growth cone formation in *Aplysia* neurons following axotomy through a process requiring cytoskeletal rearrangement (Gitler and Spira, 1998, 2002; Spira et al., 2003), and for recovery of cortical neuron dendrites following excitotoxic injury (Faddis et al., 1997). Whether calpain plays any role in dendritic growth during development, however, is unknown.

There are multiple potential cross-talk points between calpain and the AC pathways. Calpain is activated by calcium, which is also a known activator of ACs 1, 3 and 8 and inhibitor of ACs 5, 6, and 9 (Table 1), suggesting that calpain activation can occur concurrently with both increases and decreases in cAMP pathway signaling, depending on the subset of ACs expressed. PKA phosphorylates and inactivates calpain 2 (Shiraha et al., 2002; Smith et al., 2003). Furthermore, PKA-dependent phosphorylation of the calpain-specific inhibitor calpastatin alters its interaction with calpain, increasing its inhibition of calpain 2 but decreasing its inhibition of calpain 1, while dephosphorylation of calpastatin has the opposite effect (Salamino et al., 1994). β-adrenergic receptor agonists increase calpain 2 and calpastatin and decrease calpain 1 activity in mammalian skeletal muscle (Bardsley et al., 1992; Kretchmar et al., 1989). In the case of calpain 2 and calpastatin, this appears to be due to an increase in mRNA levels (Parr et al., 1992), and the promoter for the calpastatin gene is known to contain a cAMP-responsive element (Cong et al., 1998), implying a role for CREB in the regulation of calpain activity.

It has recently been shown that inhibition of calpain by PKA causes increased axonal filopodial density in cultured neurons (Mingorance-Le Meur and O'Connor, 2009). This study showed that under basal conditions calpain is active along the axonal shaft, and inhibits

the actin binding protein cortactin, thus preventing the extension of new filopodia. Upon inhibition of calpain by active PKA, inhibition on cortactin is relieved, allowing filopodial addition. This effect was specific to the axonal shaft, as calpain was not basally active in the growth cone. We tested whether calpain plays a similar role in dendrites *in vivo*, and whether calpain inhibition can account for the effects we observed with PKA activation. We find that calpain inhibition recapitulates the motility-promoting effect of PKA activation in dendritic filopodia. Furthermore, calpain inhibition increases filopodial density in both axons and dendrites, but only in axons is this effect downstream of PKA activation. These results are the first demonstrated effects of calpain on neural morphology *in vivo*, and the first demonstration of distinct signaling pathways regulating calpain in axons versus dendrites.

6.1.1 Calpain isoforms are developmentally expressed in *X. laevis* and *X. tropicalis*

Prior to performing experiments, the expression patterns of calpain isoforms were determined for both *X. laevis* and *X. tropicalis* with searches of published literature and genomic databases. The calpains form a family of calcium-activated cysteine proteases, the best studied of which are the ubiquitously expressed calpains 1 and 2, also the only isoforms known to be expressed in neural tissue. Both isoforms consist of a large catalytic subunit and a smaller regulatory subunit (Wu and Lynch, 2006). Both calpain 1 and 2 have been cloned and sequenced from both *X. tropicalis* (Bowes et al., 2008) and *X. laevis*, although the *X. laevis* sequences are limited to the large subunit genes (Cao et al., 2001; Klein et al., 2002). Both calpains have also been identified in microarray analysis of expressed *X. tropicalis* and *X. laevis* mRNAs (Yanai et al., 2011). *X. laevis* calpain 2 has between 40-60% amino acid identity with calpains from other species, including chicken, rat, and human (Cao et al.,

2001), while sequence identity between *X. tropicalis* and *H. sapiens* is 77% for calpain 1 and 63% for calpain 2 (Bowes et al., 2008; Flicek et al., 2011). Both calpains 1 and 2 are widely expressed in the *X. laevis* embryo at stage 48 (Moudilou et al., 2010), and spinal neurons from stage 22 neural tubes express calpain 1 (Robles et al., 2003). Evidence for a potential role for calpains in *X. laevis* development comes from their high expression level during early developmental stages and organogenesis which decreases during morphogenesis. Adult *X. laevis* show relatively low calpain expression in all tissues except the intestinal brush border (Moudilou et al., 2010). Overexpression of *X. laevis* calpain 2 into the dorsal blastomeres of the developing embryo results in severe neural defects, including microcephaly, indistinguishable prosencephalon and mesencephalon, reduced ventricle size, and smaller or completely absent eyes and otic vesicles (Cao et al., 2001). Evidence for a developmental role for calpains in a non-*Xenopus* system comes from studies showing that calpain 1 and 2 are expressed in the neurite shafts of young cultured hippocampal neurons (Mingorance-Le Meur and O'Connor, 2009) and that mice lacking both calpain 1 and calpain 2 activity die at embryonic day 11.5 (Arthur et al., 2000; Zimmerman et al., 2000). These findings are summarized in Table 7.

Table 7: Expression patterns of calpain isoforms in *X. laevis* and *X. tropicalis*.

Isoform	Expression	*X. laevis*	*X. tropicalis*	Stage	*X. tropicalis* Homology	References
CPN1	bone tissue, brain, extoderm, egg, endomesoderm, eye, fatbody, head, heart, intestine, limb, liver, lung, oocyte, oviduct, skeletal muscle, skin, spleen, tail, testis, thymus, whole organism	large subunit sequenced, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 67%; human: 77%; mouse: 74%; zebrafish: 69%	(Bowes et al., 2008; Flicek et al., 2011; Klein et al., 2002; Yanai et al., 2011)
CPN2	Keller explants, brain, egg, endomesoderm, head, liver, lung, mesonephric kidney, oviduct, skin, spleen, stomach, testis, thymus, whole organism	large subunit sequenced, mRNA expressed	gene sequenced, mRNA expressed	unfertilized egg to adult	chicken: 65%; human: 63%; mouse: 63%; zebrafish: 57% (subunit a), 54% (subunit b)	(Bowes et al., 2008; Cao et al., 2001; Klein et al., 2002; Yanai et al., 2011)

6.2 Methods

Animal care, fluorescent labeling of neurons within the intact tadpole brain, *in vivo* two-photon fluorescence imaging of dendritic growth, and statistical analysis were performed as described in Chapter 2.

6.2.1 DNA constructs

For experiments examining the effects of overexpression of calpain, a construct (CPN2; a gift from Dr. Tim O'Connor, University of British Columbia, Vancouver, BC) encoding the full length version of mouse calpain 2, subcloned into the pEGFP-N1 vector (Clontech, Mountain View, CA) (Mingorance-Le Meur and O'Connor, 2009), was co-electroporated along with fEGFP (1 μg/μl each to a final concentration of 2 μg/μl) into single neurons within the *X. laevis* tadpole optic tectum. Transfected neurons were imaged 24 hr later.

6.2.2 Drug solutions and application

Drugs used for this experiment were as follows: N-Acetyl-L-leucyl-L-leucyl-L-methioninol (ALLM, 25 μM, a gift from Dr. Tim O'Connor, University of British Columbia, Vancouver, BC); isoproterenol (Iso, 15 μM, Sigma-Aldrich, St. Louis, MO, a gift from Dr. John Church, University of British Columbia, Vancouver, BC); adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-cAMPS, 50 μM, BIOLOG Life Science Institute, Bremen, Germany). All compounds were dissolved in dH_2O, diluted to working solution in normal rearing medium, bath applied to the tadpole, and continuously perfused through the imaging chamber following baseline imaging.

6.2.3 Morphometric analysis

Dynamic dendrite growth dynamics were analyzed as described in Chapter 2. As IF showed a stronger response to AC activation than GCF (see section 2.3.3), only IF were included in the analysis.

Axonal morphometrics were analyzed using the same software as for dendrites. Due to imaging limitations, it was not possible to image the axon to its fullest extent. Therefore, as much of the axon as possible was collected while imaging the dendrite, and the length of this axonal segment was measured. As this varied between cells, all axon filopodial measurements for a given cell were normalized to the collected length of that cell's axon.

6.3 Results

6.3.1 Calpain inhibition mimics the effects of PKA but not adenylyl cyclase activation

Next we examined the role of a calpain, a protein which could potentially link cAMP/PKA signaling to the cytoskeleton for regulation of dendritogenesis. Similar to PKA and AC activation, inhibition of calpain with ALLM (25 μm) increased the motility of both growing (Fig. 17A) and retracting filopodia (Fig. 17B). Electroporation of a calpain overexpression construct (CPN2) (Mingorance-Le Meur and O'Connor, 2009) did not change filopodial motility compared with fEGFP controls (Fig. 17A,B). Sp-cAMPS was still able to increase filopodial motility even with calpain overexpressed. It is possible that Sp-cAMPS activates PKA to levels high enough that the excess calpain is still inhibited to levels low enough for a measurable motility change. Alternately, motility may be under the control of

two distinct pathways – one dependent on inhibition of calpain but independent of PKA, and one dependent on PKA but not calpain inhibition. ALLM, CPN2, and Sp-cAMPS on a CPN2 background were all unable to increase length of both stable (Fig. 17C) and newly added filopodia (Fig. 17D). No change in lifetime of transient filopodia was observed with any manipulation (Table 8).

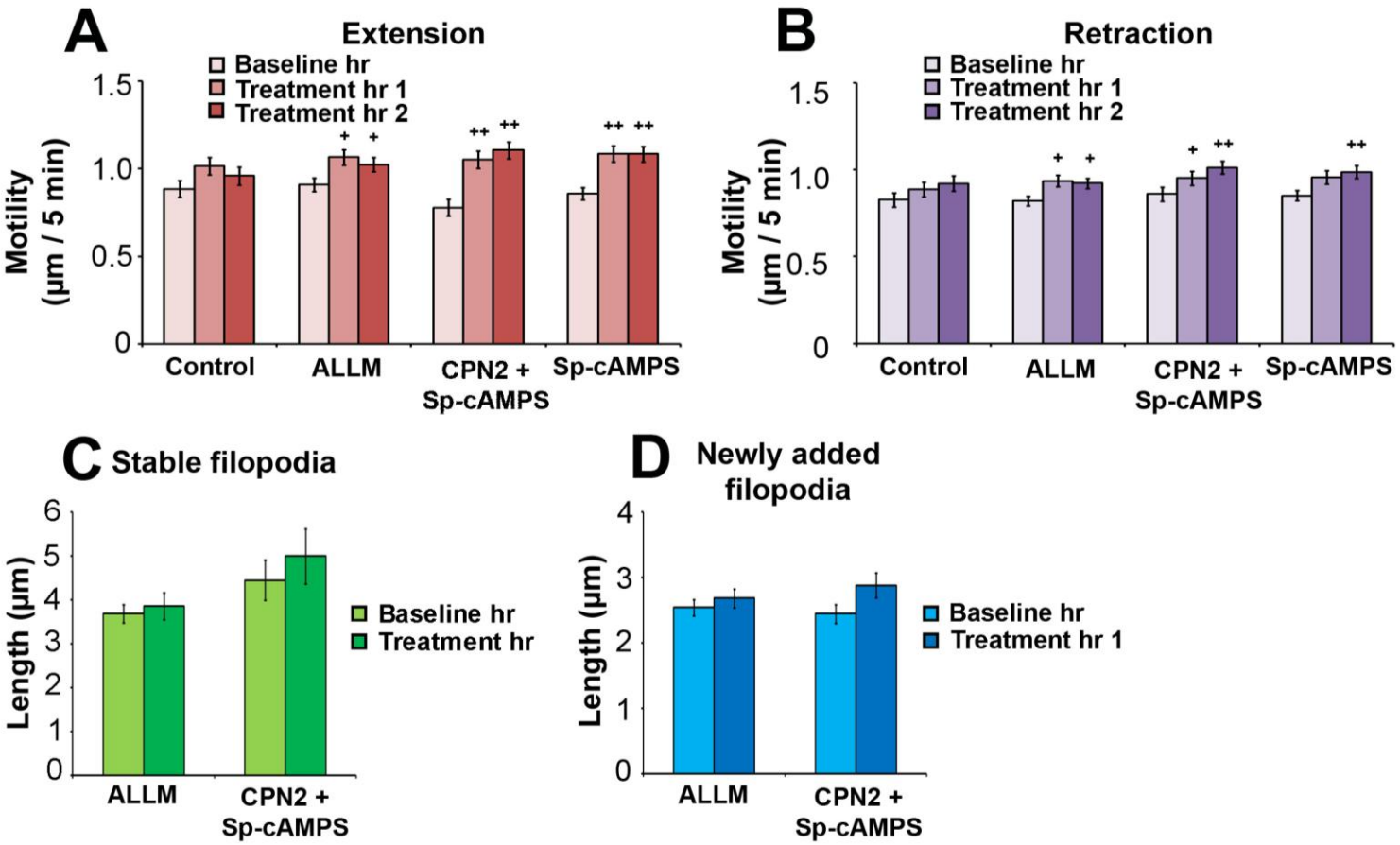


Figure 17: Calpain inhibition mimics the effects of adenylyl cyclase activation on filopodial motility but not length, and mimics the effects of PKA activation on both motility and length.
(A) ALLM increases filopodial extension. Sp-cAMPS increases filopodial extension on both an fEGFP and calpain-overexpression background. (B) ALLM increases filopodial retraction. Sp-cAMPS increases filopodial retraction on both an fEGFP and calpain-overexpression background. (C) Neither inhibition or overexpression of calpain alters length of stable filopodia. Sp-cAMPS does not increase stable filopodia length when calpain is overexpressed. (D) Neither inhibition or overexpression of calpain alters length of newly added filopodia. Sp-cAMPS does not increase newly filopodia length when calpain is overexpressed. Replicate numbers: Rapid time lapse imaging: Control: N=5; n= 287 (baseline hr); 350 (treatment hr 1); 373 (treatment hr 2); ALLM: N=5; n= 551 (baseline hr); 707 (treatment hr 1); 773 (treatment hr 2); CPN2+Sp-cAMPS: N=5; n= 393 (baseline hr); 462 (treatment hr 1); 518 (treatment hr 2); Sp-cAMPS: N=6; n= 571 (baseline hr); 541 (treatment hr 1); 614

(treatment hr 2); 1 hr interval imaging: ALLM: N=9; n (stable) = 92; n (newly added) = 169 (baseline hr); 198 (treatment hr); CPN2+Sp-cAMPS: N=5; n (stable) =30; n (newly added) = 83 (baseline hr); 89 (treatment hr); N = number of individual cells; n = number of individual filopodia.

Table 8: Manipulation of either calpain or PKA has no effect on lifetime of transient filopodia.
Replicate numbers: Control: N=5; n= 122 (baseline hr); 164 (treatment hr 1); 149 (treatment hr 2); ALLM: N=5; n= 231 (baseline hr); 350 (treatment hr 1); 347 (treatment hr 2); CPN2+Sp-cAMPS: N=5; n= 167 (baseline hr); 236 (treatment hr 1); 260 (treatment hr 2); Sp-cAMPS: N=6; n= 248 (baseline hr); 233 (treatment hr 1); 266 (treatment hr 2); N = number of individual cells; n = number of individual filopodia.

	Baseline hr	**Treatment hr 1**	**Treatment hr 2**
Control	10.57 ± 0.69	10.34 ± 0.66	11.17 ± 0.67
ALLM	10.39 ± 0.59	11.06 ± 0.49	11.18 ± 0.50
CPN2+Sp-cAMPS	10.09 ± 0.61	9.98 ± 0.50	10.69 ± 0.50
Sp-cAMPS	11.01 ± 0.60	9.85 ± 0.51	11.52 ± 0.53

6.3.2 Calpain influences dendritic and axonal morphology under two distinct pathways

Inhibition of calpain with ALLM increased density of both dendritic and axonal filopodia *in vivo* (Fig. 18A,B,C, 19A,B). In axons (Fig. 18A,B, 19B), this effect was mimicked by application of Sp-cAMPS, and Sp-cAMPS was unable to induce increased density on a CPN2 background, confirming *in vitro* results reported by Mingorance-Le Meur and O'Connor (2009). Activation of β-ARs with Iso also increased axonal filopodial density, implying a path from β-ARs through PKA-dependent inhibition of calpain to increase density. In dendrites (Fig. 18A,C, 19A), ALLM increased and CPN2 decreased filopodial density. The effect of ALLM was mimicked by Iso but not Sp-cAMPS, implying two separate pathways, one PKA-dependent and one PKA-independent, underlying calpain-inhibition-dependent control of filopodial density in axons versus dendrites respectively.

ALLM application resulted in less stable axons and dendrites, as measured by both increased additions (Fig. 18B,C, 19C,D) and retractions (Fig. 18B,C, 19E,F). Despite

increased retractions, the magnitude of additions was high enough to result in increased density. This effect was mimicked by Sp-cAMPS in axons but not dendrites, confirming distinct pathways in the two compartments.

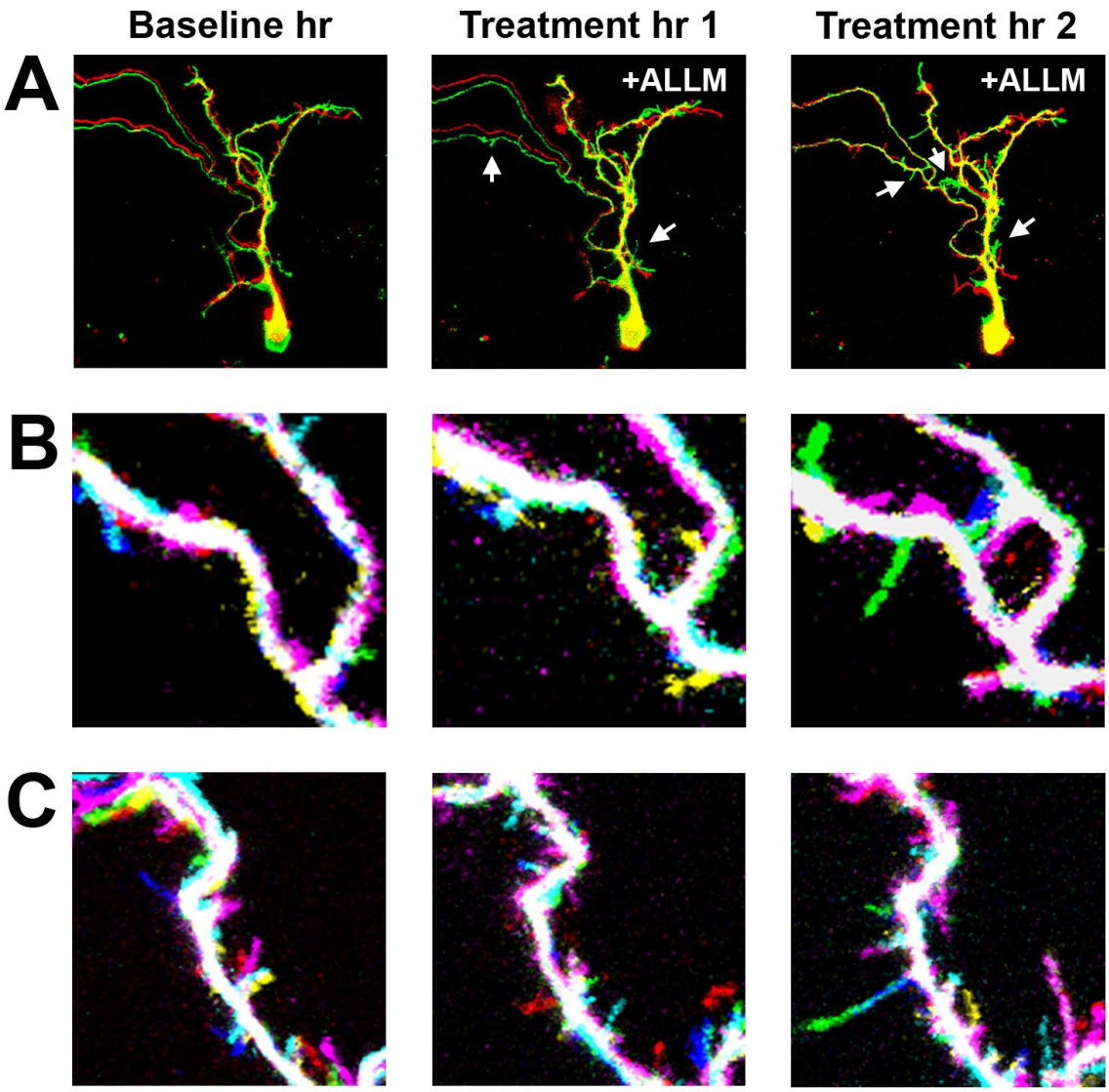


Figure 18: Calpain inhibition increases filopodial density and turnover in both axons and dendrites. Shown is a representative neuron imaged every 5 min over 3 hr. The first hr served as baseline, after which ALLM was applied for 2 consecutive hrs. (A) ALLM increases filopodial density. Images are overlays of the first (red) and last (green) image of each hr. Arrows indicate regions of increased filopodial density in both axons and dendrites. (B,C) ALLM increases filopodial addition and turnover. Shown are zoomed-in sections of the axon (A) and dendrite (B). Colors correspond to 10-min interval frames taken within the hour of imaging. White indicates regions of morphological stabilization.

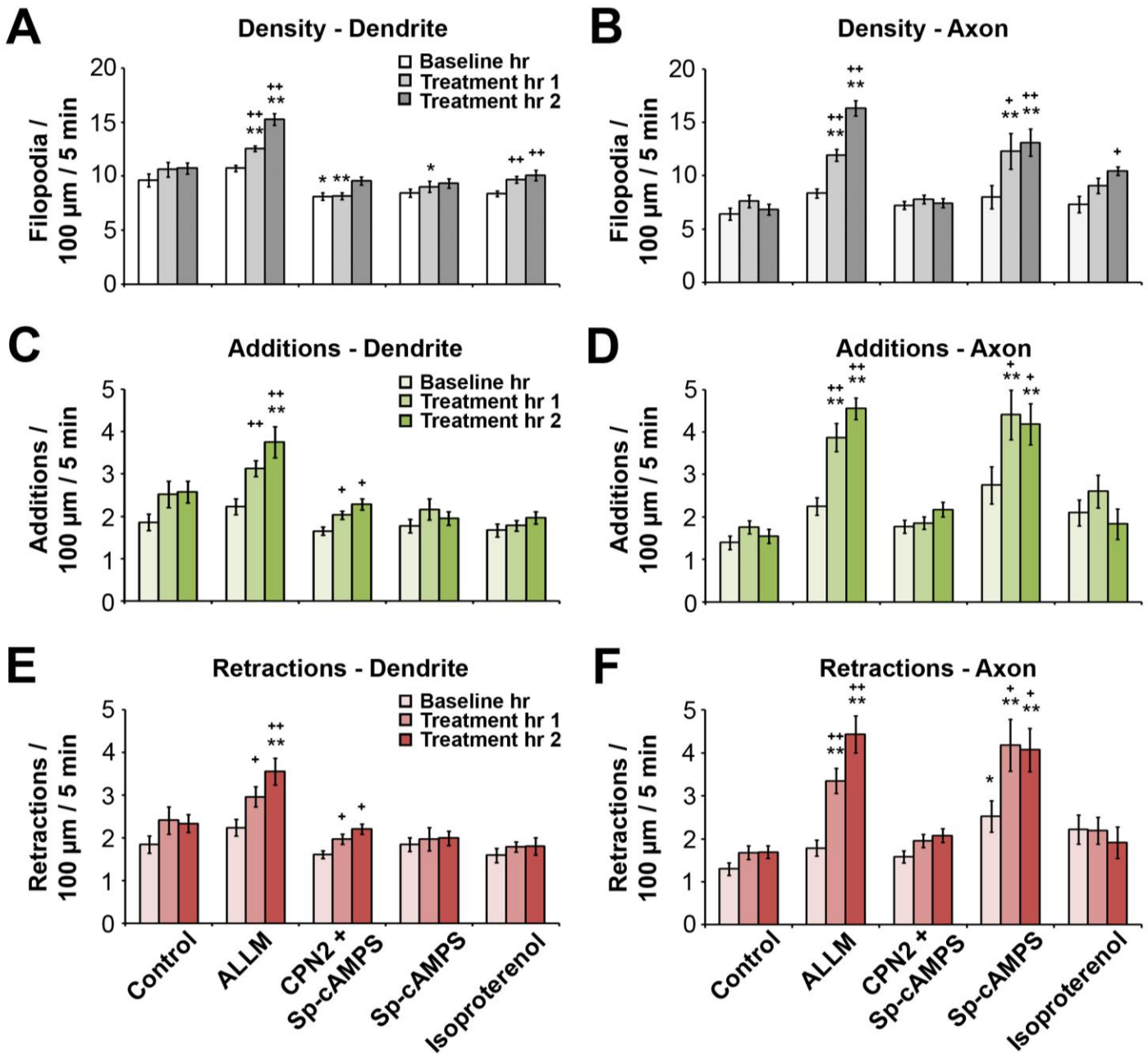


Figure 19: Calpain inhibition increases filopodial density in both axons and dendrites, but downstream of unique pathways.
(A) ALLM increases and CPN2 decreases dendritic filopodial density. Iso also increases filopodial density, but Sp-cAMPS has no effect. (B) Iso, Sp-cAMPS, and ALLM increase axonal filopodial density. Sp-cAMPS is unable to increase density when calpain is overexpressed. (C) ALLM increases dendritic filopodial additions. Sp-cAMPS is only able to increase additions when calpain is overexpressed. (D) Sp-cAMPS and ALLM both increase axonal filopodial additions. Sp-cAMPS is unable to increase additions when calpain is overexpressed. (E) ALLM increases dendritic filopodial retractions. Sp-cAMPS is only able to increase retractions when calpain is overexpressed. (F) Sp-cAMPS and ALLM both increase axonal filopodial retractions. Sp-cAMPS is unable to increase retractions when calpain is overexpressed. Replicate numbers: DENDRITES: Control: N=5; n= 287 (baseline hr); 350 (treatment hr 1); 373 (treatment hr 2); ALLM: N=5; n= 551 (baseline hr); 707 (treatment hr 1); 773 (treatment hr 2); CPN2+Sp-cAMPS: N=5; n= 393 (baseline hr); 462 (treatment hr 1); 518 (treatment hr 2);

Sp-cAMPS: N=6; n= 571 (baseline hr); 541 (treatment hr 1); 614 (treatment hr 2); Iso: N=3 (2 hr total imaging), 1 (3 hr total imaging); n= 315 (baseline hr); 370 (treatment hr 1); 125 (treatment hr 2); AXONS: Control: N=5; n= 253 (baseline hr); 294 (treatment hr 1); 279 (treatment hr 2); ALLM: N=5; n= 219 (baseline hr); 360 (treatment hr 1); 440 (treatment hr 2); CPN2+Sp-cAMPS: N=5; n= 193 (baseline hr); 226 (treatment hr 1); 269 (treatment hr 2); Sp-cAMPS: N=6; n= 168 (baseline hr); 248 (treatment hr 1); 250 (treatment hr 2); Iso: N=3 (2 hr total imaging), 1 (3 hr total imaging); n= 145 (baseline hr); 165 (treatment hr 1); 33 (treatment hr 2); N = number of individual cells; n = number of individual filopodia.

6.4 Discussion

6.4.1 The effects of calpain inhibition are distinct from the effects of PKA activation

In this study we demonstrate a role for calpain in regulating the density and turnover of dendritic filopodia in developing brain neurons imaged *in vivo*. To our knowledge, this is the first demonstration of calpain involvement in the control of dendritic morphology. The effects of calpain inhibition showed only partial similarity with the effects of PKA activation. This is expected, as not all PKA-dependent effects are mediated by calpain. Neither calpain inhibition nor PKA activation altered the length of stable or newly added filopodia. Calpain inhibition and PKA activation both increased filopodial motility, but the effect of PKA on motility was not proven to be dependent on its ability to inhibit calpain. Calpain inhibition increased filopodia density, additions, and retractions, an effect not observed when PKA was activated. Furthermore, PKA activation was able to increase additions and retractions, but only when active calpain was highly overexpressed. These results imply multiple signaling pathways regulating dendritic growth, which are outlined in Figure 20.

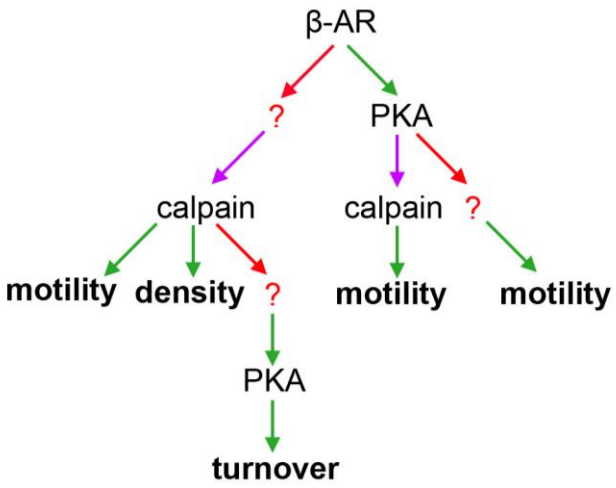


Figure 20: A preliminary pathway linking β-ARs, calpain, and PKA for the regulation of dendritic filopodia dynamics
Green: activation. Purple: inhibition. Red: unknown.

This pathway is far from complete, but there are several possible experiments which can help clear up some of the question marks. The first unknown is whether PKA is increasing motility through inhibition of calpain, or whether there are two separate pathways regulating motility. Although an overexpression calpain construct was used, we are unable to rule out the possibility that Sp-cAMPS activated PKA to high enough levels to inhibit all present calpain. In order to rule out this possibility, a non-phosphorylatable calpain mutant can be used (Shiraha et al., 2002). Second, there is an unknown intermediate between β-ARs and calpain for the regulation of motility. Epac is a clear candidate, although a link between Epac and calpain has not been established. It should be noted that while our data suggests a role for β-ARs in this pathway, it does not definitively prove that role.

PKA was able to increase filopodia turnover, but only when calpain was overexpressed. It is possible that calpain cleavage leads to increased PKA expression through direct or indirect downstream interactions with factors that regulate transcription or translation. Calpain cleaves β-catenin, causing its nuclear translocation and increased

expression of target genes (Abe and Takeichi, 2007). In another study, calpain-dependent cleavage of PKCα caused the nuclear export of histone deacetylase 5 (HDAC5) and increased transcription of genes related to apoptosis (Zhang et al., 2011). Given that calpain can regulate gene expression, it remains an unproven possibility that the PKA gene is one of its targets. Overexpression of calpain could lead to very high levels of PKA activity, unmasking an effect of PKA which would otherwise be undetectable. Alternatively, the construct used was an overexpression of the wild-type form of calpain, not a constitutively-active. It is possible that the expressed calpain was basally inactive in the absence of an exogenous, Ca^{2+}-increasing signal, and that the observed PKA effect was therefore completely unrelated to calpain activity levels. This is unlikely though, as the ability of ALLM to induce a strong phenotype and the fact that CPN2 overexpression decreases density, as well as the findings of the O'Connor lab (Mingorance-Le Meur and O'Connor, 2009), imply that calpain is basally active.

6.4.2 Manipulating calpain has distinct effects on axons versus dendrites

In this study we show that inhibiton of calpain increases filopodial density in both axons and dendrites but through PKA-dependent and PKA-independent pathways, respectively. This is the first demonstration of calpain having the same role in the two compartments, but downstream of different effectors.

The functional differences between axons and dendrites are accompanied by differences in protein expression and response to signaling factors. Cytoskeletal proteins are differentially expressed in these two compartments. For example, the microtubule binding protein tau is highly expressed in axons while MAP2 is expressed in dendrites (Kosik and

Finch, 1987). Axons and dendrites also respond differently to the guidance cue Sema3A (Chedotal et al., 1998; Polleux et al., 1998; Polleux et al., 2000). There are also differences in how signaling pathways regulate morphology. For example, RhoA is required for dendritic, but not axonal morphogenesis (Lee et al., 2000).

How does calpain induce the same effect, but downstream of different pathways, in these two compartments? One possibility is that calpain is anchored to different upstream effectors through proteins such as AKAPs. So far there is no reported case of AKAP-dependent calpain clustering, but calpain is known to interact with the AKAP MAP2. PKA phosphorylation protects MAP2 from calpain-dependent proteolysis (Alexa et al., 1996; Johnson and Foley, 1993). It has been suggested that calpain may transduce its effects on neuroplasticity and neuropathology in part through regulation of MAP2 (Sanchez et al., 2000). However, it is not known whether MAP2 functions as an AKAP for calpain. Differential calpain binding to AKAPs in axons versus dendrites may explain the differences we observed.

6.4.3 Signaling from β-adrenergic receptors to calpain for the regulation of dendritic and axonal growth

Here we show that both activation of β-ARs and inhibition of calpain increases filopodia density in both axons and dendrites. This implies a signaling link between β-ARs and calpain, but that link has not yet been proven. The consequences of β-AR signaling to calpain are poorly understood. Global ischemia promotes cell death in the hippocampus which is accompanied by increased calpain activity, proteolysis of calpain targets, and reduced cAMP levels. Activation of the β2-AR prior to ischemia rescues cAMP levels and

reduces cell death and calpain activity (Rami et al., 2003), implying role for β2-AR activity in protection from proteolysis associated with cell death. That increased cAMP levels are associated with reduced calpain activity indicates that this may be mediated by PKA-dependent phosphorylation and inhibition of calpain. β-AR agonists increase calpain 2 and calpastatin and decrease calpain 1 activity in mammalian skeletal muscle (Bardsley et al., 1992; Kretchmar et al., 1989). The decrease in calpain 1 activity is suggested to underlie β-AR-dependent skeletal muscle hypertrophy (Kim and Sainz, 1992). In general, however, studies into this signaling link are lacking, although the established ability of β-ARs to activate PKA, and for PKA to inhibit calpain, imply that this link is present and likely has more functional consequences than we currently understand. Further experiments that would definitively prove the potential observed link between β-ARs and calpain would provide some of the first evidence of this signaling pathway being active in neurons, and the first evidence of a role in development and morphology.

Chapter 7: General discussion

7.1 Summary of findings

The molecular mechanisms underlying the morphological development of neurons remains poorly understood. Unanswered questions include how new filopodia initiate on immature dendrites, and the processes that guide transition of an immature dendrite to a mature one. Mounting evidence supports the hypothesis that molecules involved in synaptic plasticity or cytoskeletal rearrangement may also be involved in dendritic morphogenesis in early developmental stages. Adenylyl cyclase (AC), an enzyme at the head of neural activity-dependent pathways linked to synapse strengthening and the cytoskeleton, is one such molecule. For my Ph.D. thesis research, I studied the role of AC in dendrite growth using the albino *Xenopus laevis* tadpole as an *in vivo* vertebrate model of early neural development. In this process, I employed rapid time-lapse two photon imaging of dynamic dendritogenesis in single neurons within the intact, unanesthetized brain. Tracking of all filopodia within a neuron's entire dendritic arbor across brief, 5 min time points over multiple hours of imaging allowed for detailed reconstruction of neural growth dynamics. I applied pharmacological agents designed to increase or decrease AC activity or levels of its product, cAMP, in order to examine the effects of AC on dendritic growth. I find that AC activation increases filopodial length and motility in immature neurons, and decreases filopodial length and motility in mature neurons. This effect was specific to filopodia located along the dendritic shaft as opposed to filopodia located in dendritic growth cones. I find that AC activation has little effect on filopodial stability. These results indicate that AC has a distinct role in development compared to other manipulations associated with increased neural activity, such

as increasing levels of PKMζ or CaMKII or increasing neurexin-neuroligin interactions (Chen et al., 2010b; Liu et al., 2009; Wu and Cline, 1998), which cause increased filopodial stability associated with synaptogenesis and morphological maturation. This is one of the first reported *in vivo* examples of an intracellular signaling pathway resulting in different morphological effects dependent on cell age (Fig. 21).

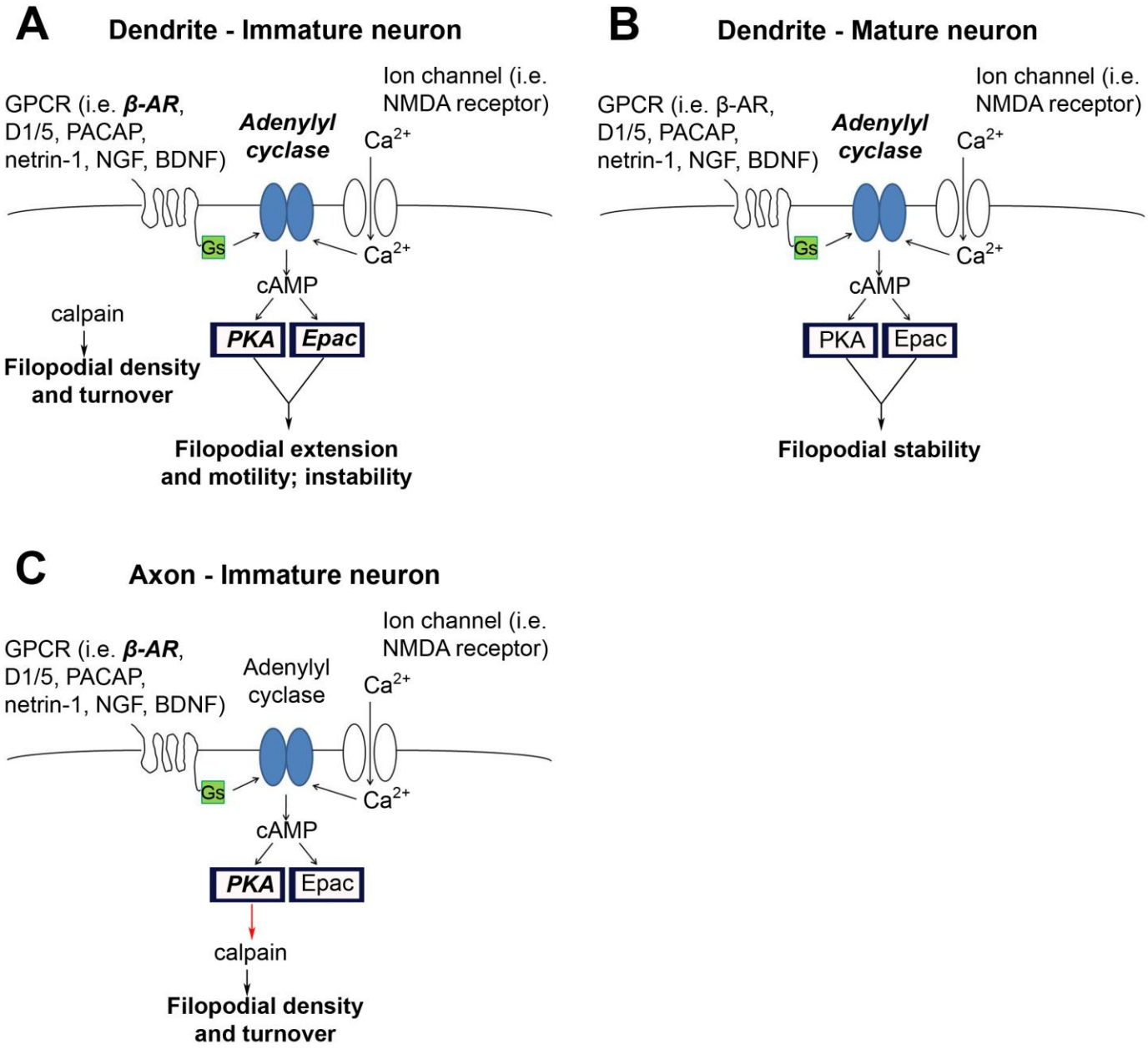


Figure 21: Summary of the morphological results of adenylyl cyclase signaling pathway activation

(A) Dendritic filopodia in immature neurons. (B) Dendritic filopodia in mature neurons. (C) Dendritic filopodia in immature neurons. Bold-italics indicates molecules and pathways elucidated in this study.

Cyclic AMP transduces its effects through the kinase PKA or the GEF Epac. I performed experiments designed to determine whether PKA or Epac were responsible for mediating the effects of cAMP on dendritic growth. Pharmacological activators and inhibitors altered PKA or Epac activity within the entire brain. Constitutively-active and dominant-negative constructs allowed examination of the role of PKA or Epac within single neurons existing within an otherwise unaltered brain circuit. I find that PKA and Epac act synergistically to regulate dynamic dendritic growth, and that simultaneous inhibition of PKA and Epac has long-term effects on morphology. I also electroporated single neurons with constructs encoding constitutively-active or dominant-negative versions of the transcription factor CREB and found the effects of long-term simultaneous PKA and Epac inhibition are possibly mediated by reduced activation of CREB and, therefore, altered patterns of gene expression. These results are the first evidence of a synergistic role for PKA and Epac in dendritic growth *in vivo*. Given the low cAMP-binding affinity for Epac compared to PKA, this may indicate that high levels of neural activity are necessary for PKA and Epac to have a morphological effect. Manipulations which reduced activity in these pathways caused an increase in length of stabilized filopodia and branches that was only observable in time periods of one day or less, indicating that basal activity may actually be involved in restricting growth early in development, and that the mechanisms underlying growth with PKA and Epac activation versus inhibition may be distinct from each other.

Physiologically, AC is activated either by GPCRs or by calcium influx through channels such as the NMDA receptor. I chose the β-adrenergic GPCR, which is linked to neurite growth, synaptic plasticity, and the cytoskeleton, and tested whether activation of this

receptor recapitulates effects observed when AC was pharmacologically activated. I used pharmacological compounds to activate or inhibit β-ARs while imaging growth of single neurons within the intact brain. I found that basal β-AR activity is not involved in dendritic growth *in vivo*, but that activation of β-ARs partially recapitulates the effects of AC activation, and that this effect is dependent on both PKA and Epac. These results support a synergistic role for PKA and Epac in dendrite growth. They also indicate that β-ARs are not solely responsible for all effects that can be attributed to AC, implying a role for other GPCRs or calcium-activated ACs.

PKA has a wide range of effects within the cell, and is activated by a wide range of inputs. Spatial and temporal specificity of PKA signaling is imparted by AKAPs, which anchor PKA in immediate proximity with its upstream activators and downstream effectors. Previous studies have implied a role for AKAPs in synaptic plasticity (Davare et al., 2001; Westphal et al., 1999; Zhong et al., 2009). I performed a pilot experiment to determine whether proper PKA localization by AKAPs is important to dendritic morphogenesis and filopodia dynamics. A peptide designed to block PKA-AKAP binding was infused into the optic tectum in *X. laevis* tadpoles, and single neurons expressing fEGFP were imaged using rapid time-lapse two photon microscopy. Preliminary results revealed minor effects of PKA-AKAP binding on filopodia stability, but future experiments are necessary in order to confirm and expand on results.

How a growth signal is transmitted into a morphological phenotype downstream of PKA or Epac is poorly understood. In my final set of experiments I conducted experiments to determine whether calpain, a protease inhibited by PKA and shown to regulate the protrusion of axon shaft filopodia (Mingorance-Le Meur and O'Connor, 2009), is involved in dynamic

dendritic and axonal growth *in vivo*. Using a pharmacological agent to inhibit calpain and a plasmid encoding the wild type version of calpain for protein overexpression, I found that calpain increases filopodial density in both axons and dendrites *in vivo*. Through activation of PKA and β-ARs I found that calpain acts downstream of PKA in axons, confirming previously published results (Mingorance-Le Meur and O'Connor, 2009), but not in dendrites. I outline a pathway from β-ARs through PKA to calpain for the regulation of filopodia protrusion in axons. Neural activity through β-ARs activates PKA, which inhibits calpain, relieving calpain-dependent proteolysis of proteins such as cortactin, which alter cytoskeletal branching and elongation, allowing the protrusion of new filopodia. With these experiments I provide the first *in vivo* evidence that calpain regulates development of both axons and dendrites, and outline a pathway from a neurotransmitter receptor down to a cytoskeletal regulator for the regulation of neural morphogenesis (Fig. 21). Proteins and molecular pathways studied in this thesis are summarized in Table 9.

Table 9: Summary of morphological effects of proteins studied.
Unless otherwise stated, effects refer to dendritic filopodia on immature neurons

Protein	Morphological effects
AC	• Increase filopodial extension and motility in immature neurons. • Increase filopodial stabilization in mature neurons.
PKA	• Increase filopodial motility • Necessary and but not sufficient for growth of newly added filopodia. • Increase filopodial extension when co-activated with Epac • Along with Epac, regulates long-term dendritic branch growth. • Increase axonal filopodia density and turnover.
Epac	• Necessary and but not sufficient for growth of stable filopodia. • Increase extension of newly added filopodia. • Increase filopodial extension when co-activated with PKA. • Increase filopodial density when activated over extended periods of time. • Along with PKA, regulates long-term dendritic branch growth.

Protein	Morphological effects
CREB	• Necessary for AC-induced filopodial extension • Regulates long-term dendritic branch growth similar to PKA and Epac.
β-AR	• Increase length of newly added filopodia upstream of AC, PKA, and Epac. • Increase density of both axonal and dendritic filopodia.
PKA-AKAP binding	• When disrupted, results in increased filopodial stability.
calpain	• In axons, increase filopodial density and turnover downstream of inhibition by PKA. • In dendrites, increase filopodial density and turnover independently of PKA.

7.2 Overall significances

7.2.1 Albino *Xenopus laevis* tadpoles as a model for vertebrate development

The *Xenopus laevis* tadpole is a powerful system for studying vertebrate development *in vivo* (Cline, 2001). Using the technique of single-cell electroporation (Haas et al., 2001), it is possible to introduce a wide range of molecules into individual neurons within an otherwise normally functioning brain circuit. These include plasmid DNA for the expression of fluorescent proteins or constitutively-active or dominant-negative mutated versions of proteins of interest, small peptide inhibitors, and fluorescent dyes and indicators. The permeability of the tadpole allows for non-invasive bath application of drugs. Further, it is possible to inject compounds directly into the brain with minimal damage to the animal. Neurons within the optic tectum are innervated by retinal ganglion cells (RGCs) from the contralateral eye. Visual stimulation is an easy way to induce a physiological, activity-induced growth response without surgery or pharmacological methods.

Tadpoles are albino, and transparent at early stages of development, allowing for non-invasive imaging of neurons within their natural environment. Using reversible paralytic

agents it is possible to image the growth of single neurons over prolonged time periods while avoiding the dampening effects of anaesthetics on neural activity. The technique of two-photon imaging lets us capture three-dimensional images of each neuron with high temporal and spatial resolution. We have recently developed custom-written computer software (Dr. Jamie Boyd, Kasper Podgorski, and Shay Neufeld, University of British Columbia), with which we can follow the behaviour of each individual filopodium over the entire imaging period, allowing for reconstruction of filopodial dynamics in unprecedented detail.

The natural features of the *X. laevis* tadpole, together with the technologies developed by our lab and others, have turned this organism into a powerful vertebrate *in vivo* model system for investigating the molecular mechanisms underlying neural development and circuit plasticity.

7.2.2 Highlighting the functional differences between mature and immature neurons

In this study I report that the morphological effects of AC activation are dependent on neuronal maturational state. Mature and immature neurons are functionally different from one another, for example, mature neurons have higher AMPA/NMDA ratios and higher expression levels of CaMKII, and show different behavioral patterns (Hossain et al., 2011; Rajan and Cline, 1998; Wu et al., 1996; Wu et al., 1999). In previous studies, increasing or decreasing activity in certain pathways guides maturation (Chen et al., 2010b; Liu et al., 2009; Wu and Cline, 1998); for example, increasing CaMKII activity in young neurons causes precocious dendritic stabilization, while decreasing CaMKII activity in mature neurons leads to dendritic instability and a return to more immature patterns of filopodial behavior (Wu and Cline, 1998). In this study however, I report an entirely different effect.

Activation of AC does not drive dendritic maturation, or lead to stabilization regardless of cell age. Nor does inhibition of AC shift neuronal behaviors to a more immature fate. Instead, AC activation has distinct morphological effects in neurons depending on their maturational state (Fig. 22). It promotes extension and motility in immature neurons, and promotes stability in mature neurons. In other words, increasing AC activity promotes and magnifies the natural growth tendencies of a neuron at a given age.

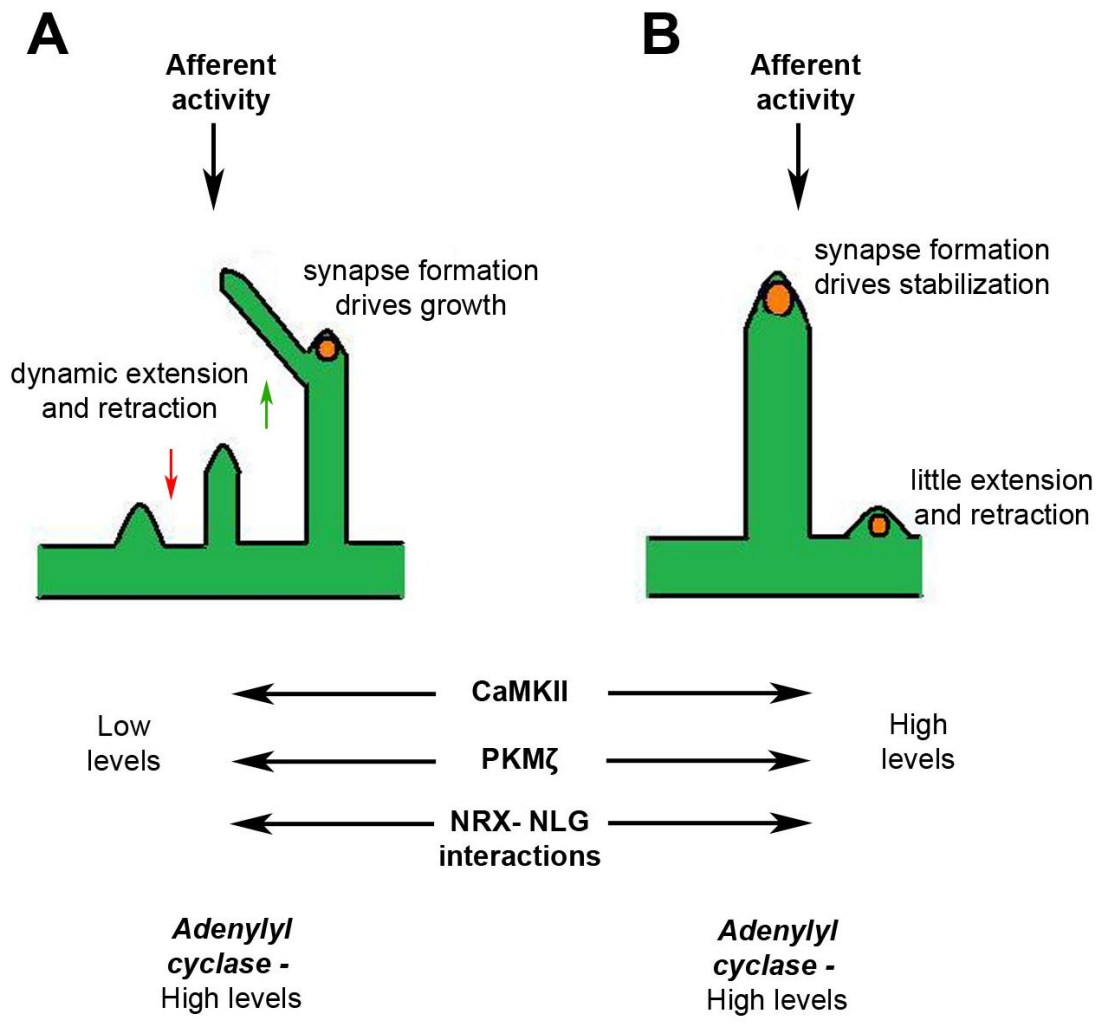


Figure 22: Schematic of the differences between immature and mature neurons.
(A) Immature neuron, characterized by dynamic dendritic growth. (B) Mature neuron, characterized by structural stabilization and a high level of synapses. Increasing activity in the CaMKII or PKMζ pathways, or increasing NRX-NLG interactions, drives neurons towards a more stable state characteristic of maturity. Decreasing activity in these pathways leads to structural destabilization characteristic of immaturity. AC, in contrast, promotes extension and motility in immature neurons and promotes stability in mature neurons.

This effect could be due to differences in the activity levels of downstream effectors or the expression patterns of GPCR subtypes. Axons of immature but not mature rat spinal neurons are capable of regenerating, which is believed to be due to a developmental decrease in cAMP levels (Cai et al., 2001). In these same neurons, axonal growth cones switch their response from to cAMP gradients from attractive in juveniles to repulsive in adults. This is reported to be the result of a developmental switch in signaling downstream of AC: from Epac-mediated in juveniles, to PKA-mediated in adults (Murray et al., 2009b). The present study extends these findings to an intact, *in vivo* system, providing support for the hypothesis that the morphological results of AC activation are developmentally regulated. These results have implication for understanding the processes of process regeneration after injury and the developmental loss in neural plasticity. It is conceivable that by altering the expression levels of proteins downstream of AC, it would be possible to force mature neurons to behave like immature neurons which are more capable of developmental or injury-induced plasticity.

7.2.3 Identification of a role for AC in early development

Previous studies have identified a role for AC in growth cone guidance, neurite development in cultured cells, and synaptic plasticity, but evidence of a role in dendritic development *in vivo* was lacking. In this study I identify AC as a regulator of dendritogenesis upstream of both PKA and Epac.

The evidence implying a potential role for PKA in development is much greater than that for Epac. PKA is more convincingly linked to synaptic plasticity and LTP and cytoskeletal dynamics. Less is known about the function of Epac, which was discovered

more recently. That most cAMP analogues used to activate PKA also activate Epac confounds interpretation of results, especially in studies which pre-date the identification of Epac. It is only in the last few years that Epac has been identified as an important regulator of cellular function in both neuronal and non-neuronal cells. For example, Epac is involved in secretion, which in neurons translates to pre-synaptic vesicle priming and release. Even less is known about the role of Epac in development. My data expands on a growing body of evidence that Epac is far more involved in brain neuron development and function than previously thought.

One interesting aspect of this study is that the results differed from other studies in *X. laevis* where activity was increased in a pathway associated with neural plasticity. Increasing levels of PKMζ or CaMKII or increasing neurexin-neuroligin interactions are hypothesized to influence growth and maturation through a synaptotropic mechanism (Chen et al., 2010b; Liu et al., 2009; Wu and Cline, 1998). Instead of stabilizing filopodia, slowing growth, and driving maturation, as would be predicted if AC were also acting through a synaptotropic mechanism, I observe increased filopodia length and motility.

What is the functional significance of increased dendritic filopodia or branch length? Current evidence from mammalian pyramidal cells demonstrates that dendritic structure affects functional integration (Katz et al., 2009; Kleindienst et al., 2011; Makino and Malinow, 2011). By altering dendritic shape during development, AC activity in the *X. laevis* optic tectal system can affect synaptic integration and action potential firing. Other examples of dendritic shape affecting function come from both the well-studied visual and auditory systems. Starburst amacrine cells have dendrites which function in direction selectivity of visual stimuli (Euler et al., 2002). It has been suggested that, in these cells, longer dendrites

aid direction selectivity by increasing the chance of a moving image overlapping with, and thus exciting, an individual dendrite (Jan and Jan, 2003). In the nucleus laminaris of the auditory system of reptiles and birds, dendritic tree length, and thus presumably branch length, is related to the sound frequency to which the neuron responds (Jan and Jan, 2003; Kubke and Carr, 2000). Projection neurons in the *X. laevis* optic tectum receive direct afferent input from RGCs in the eye. Increased dendritic length may increase the number of RGCs which a tectal neuron contacts. This would allow neurons to respond to a wider range in incoming stimuli. Increased retinal field size, however, can have the drawback of reduced resolution. Other activity dependent pathways, such as the PKC pathway, may be responsible for refining contact area with maturation. The cell may need a balance of activity in both extension-promoting and stability-promoting pathways in order to contact a physiologically appropriate number of RCGs, and activation of the AC pathway shifts this balance towards increased extension. Neural maturation shifted the response to AC activation from extension to stability, possibly as a result of altered expression patterns of proteins downstream of PKA and Epac during different stages of development.

7.2.4 Emotionally salient stimuli as a potential regulator of dendritic development

My data support a role for β-AR activity in both dendritic and axonal development. β-ARs are known to be involved in fear conditioning (Bush et al., 2010; Rodrigues et al., 2009), anxiety disorders (Davidson, 2006; Schelling, 2008), and emotional memory (Cahill et al., 1994), and have an established role in lowering the threshold for LTP (Gelinas and Nguyen, 2005; Huang et al., 1993; Huang and Kandel, 2007; Raman et al., 1996; Thomas et al., 1996; Winder et al., 1999). By lowering the threshold for LTP, fearful or otherwise

highly emotional stimuli are more likely to be remembered, which is beneficial for animal survival. The finding that β-ARs regulate dendritogenesis implies a role for emotionally salient stimuli in the control of neural development. Fearful stimuli could lead to long term changes in neural structure and function which last into adulthood. This has implications for children exposed to a traumatic event. Blockade of β-AR activity has been tested as a treatment for post-traumatic stress disorder in adults with variable results (Ravindran and Stein, 2010; Schelling, 2008; Strawn et al., 2010). There is the possibility that β-ARs are a potential therapeutic target to minimize long-term behavioral and emotional consequences in children who experience a traumatic event. There is some evidence that the β-AR antagonist propranolol may be effective in treating post-traumatic stress disorder in children and adolescents, but the studies are currently in their infancy (Stamatakos and Campo, 2010; Strawn et al., 2010). The young brain is far more plastic than the adult brain however, and giving β-blockers to children may have unforeseen long-term morphological and synaptic consequences.

7.2.5 Insights into neurological disorders

Improper dendritogenesis during development can have lasting detrimental effects on neural function. Abnormal neural morphologies are associated with epilepsy (Caplan, 1995), schizophrenia (Arnold, 1999; Bunney et al., 1995), and mental retardation, including the disorders Down syndrome, Rett syndrome, and Fragile X (Kaufmann and Moser, 2000). This includes dendritic and synaptic abnormalities as well as abnormalities in neuron number, density, and morphology, indicating that early neural development may be disrupted.

Calpain has been associated with both neural dysfunction and injury recovery. Calpain levels increase following injury, and calpain activity is necessary for recovery following axotomy or excitotoxicity (Faddis et al., 1997; Gitler and Spira, 1998, 2002; Spira et al., 2003), but calpain-dependent proteolysis has also been linked to cell death following global ischemia (Rami et al., 2003). AKAPs may also be involved in recovery following injury, as MAP2 expression increases during the recovery phase following status epilepticus in juvenile and adult rats (Jalava et al., 2007; Pereno and Beltramino, 2010). In this study I identified the AC pathway as an important regulator of both dendritic and axonal morphology in early developmental stages. Proper signaling through this pathway may be necessary to prevent dendritic abnormalities associated with neurological disorders.

7.3 Future directions

7.3.1 The RhoGTPases as a potential downstream signaling mechanism for AC-, PKA-, and Epac-dependent control of dendritic growth

In this study I report that AC, PKA, and Epac, activated over a period of 1-2 hr, increase dendritic filopodia length without concomitant changes in filopodial number or turnover. The downstream mechanism which regulates this is unclear, but cytoskeletal reorganization by the RhoGTPases, particularly Rho, seems a likely candidate.

Rho inhibition increases neurite extension while Rho activation causes neurite retraction (Hirose et al., 1998; Korobova and Svitkina, 2008; Lee et al., 2000; Nakayama et al., 2000; Pilpel and Segal, 2004; Sakisaka et al., 2004; Zhang et al., 2003). Manipulations of Cdc42 and Rac, in contrast, alter branching and process addition (Gao et al., 1999; Korobova

and Svitkina, 2008; Rosso et al., 2005; Scott et al., 2003; Threadgill et al., 1997). In *X. laevis* optic tectal neurons imaged *in vivo*, inhibition of RhoA increased branch extension while activation of RhoA had the opposite effect. Neither manipulation had any effect on rates of addition or retraction. Furthermore, inhibition of RhoA was able to rescue the decrease in arbor growth caused by NMDA receptor inhibition (Li et al., 2000). As NMDA receptors can activate AC, and PKA and Epac both inhibit RhoA (Bos, 2006; Dong et al., 1998; Ellerbroek et al., 2003; Lang et al., 1996; Roscioni et al., 2008; Zieba et al., 2011), it is conceivable that the extension phenotype of Rho inhibition is regulated by AC-dependent mechanisms. Future experiments will test whether AC-induced increases in filopodia length are mediated by Rho inhibition. Electroporation-mediated expression of dominant-negative or constitutively-active Rho mutants, or application of drugs such as lysophosphatidic acid could be used to alter Rho activity levels in optic tectal neurons *in vivo*, followed by rapid time-lapse two photon imaging and dynamic morphometric analysis.

7.3.2 The effects of AC on synaptogenesis

The data I present here support a role for ACs in dendritic filopodial growth dynamics, but do not support any conclusions regarding synaptogenesis or synapse maturation. Filopodia are thought to be the precursors of synapses (Hotulainen and Hoogenraad, 2010; Niell et al., 2004), so it seems logical that a manipulation which alters filopodial dynamics may also alter synapse formation. AC activation is associated with synaptogenesis in other systems (Kwon and Sabatini, 2011; Tominaga-Yoshino et al., 2002), but the results presented herein do not support a synaptotropic mechanism. In order to test whether AC is involved in synaptogenesis in developing optic tectal neurons *in vivo*, tectal

neurons could be transfected with a construct encoding a fluorescently-tagged version of PSD-95 and two-photon imaging and pharmacological manipulations could be used to test whether altering AC activity changes the number and distribution of PSD-95 puncta. Putative synaptic sites can be confirmed with immunohistochemical staining for the post-synaptic marker PSD-95 and the pre-synaptic marker SNAP-25, and with electrophysiological recording of tectal synaptic responses.

7.3.3 Alternate physiological methods of AC activation

β-AR activation was not able to fully account for all observed effects of AC activation. It is likely that other methods of activating AC are employed physiologically, such as other GCPRs or calcium. I propose testing the role of other GPCRs, specifically the D1/D5, 5HT-4, and 5HT-7 receptors, in filopodial dynamics. Each of these receptors is positively coupled to AC, modulates synaptic strength, and is the subject of preliminary data indicating a potential role in neurite growth (see Section 1.2.1.2 – G-protein coupled receptors). One can use pharmacological methods to test whether these receptor systems are involved in dynamic dendritogenesis *in vivo*.

Visual stimulation, which has previously been shown to affect *X. laevis* neuron growth and circuit plasticity (Dunfield and Haas, 2009; Sin et al., 2002), may increase AC activity in optic tectal neurons. One can expose tadpoles to a patterned visual stimulus, reconstructing a more natural experience than pharmacological methods will allow. Two-photon imaging and dynamic morphometric analysis can determine whether visual stimulation acts through cAMP in order to increase growth. Finally, it will be interesting to determine whether AC activity affects function of entire tectal circuits as well as the

morphology of single neurons. Bulk loading of a calcium indicator dye followed by visual stimulation allows quantification of visually-induced calcium spikes in the entire optic tectum. Performing calcium imaging in tandem with pharmacological manipulation of AC activity will allow one to test whether AC alters circuit plasticity as well as individual neuron morphology.

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