MECHANISMS OF DEPOLARIZATION INDUCED DENDRITIC GROWTH OF *DROSOPHILA* MOTOR NEURONS

by

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ABSTRACT

The study of the cellular mechanisms underlying dendritic growth contributes to our understanding of nervous system development, function and disease. Electrical activity is a fundamental property of neurons, and this property is utilized to influence the mechanisms involved in dendrite formation and maturation. Here we employ the *Drosophila* transgenic system to quantify dendritic growth of identified motor neurons using both *in vitro* and *in vivo* techniques. Two novel techniques are introduced: one a system to visualize and measure dendritic outgrowth in cultured neurons using reporter proteins, and the other using 3D reconstruction to measure the arborization of identified motor neurons *in vivo*. Both transgenic manipulation of K^+ channel function and depolarizing concentrations of K^+ in the culture medium result in an acceleration of dendritic outgrowth. Depolarization induced outgrowth is dependent on *Plectreurys* Toxin (PLTX)-sensitive voltage-gated calcium current and protein synthesis in cultured motor neurons. Depolarization leads to direct induction of fos, a protein that heterodimerizes with jun to make the functional transcription factor, AP-1. Fos, but not jun, is necessary for basal levels of dendritic growth, while both are necessary for depolarization induced outgrowth. Over-expression of AP-1 in control cells is sufficient to cause dendritic outgrowth. The transcription factor Adf-1 is also necessary for basal and depolarization induced growth, but unlike AP-1 is not sufficient to cause outgrowth when over-expressed. Another transcription factor CREB, on the other hand, is not necessary for basal levels of dendritic growth, but is necessary for depolarization induced dendritic growth. Over-expression of CREB, like Adf-1, is not sufficient to cause

dendritic outgrowth. These findings present exciting new techniques for the study of the field of dendritic regulation and contribute to our understanding of the cellular mechanisms underlying dendritic growth.

CHAPTER 1 DENDRITIC PLASTICITY

This dissertation concerns factors influencing the growth and plasticity of dendrites of identified motor neurons in *Drosophila*. The first three chapters are a broad overview of the literature in this field spanning many different systems, developmental time points, and techniques involved in the study of dendritic plasticity. This chapter begins with a discussion on structural and functional diversity of dendrites, embryonic and post-embryonic dendritic plasticity, and age and disease related dendritic disorders. Some topics only briefly mentioned here will be covered in more depth in the subsequent chapters. Important mechanisms that carry out plasticity in dendrites and factors involved in the establishment of dendritic identity and polarity of a neuron are outlined in Chapter 2. The questions that this dissertation will address and the reasons *Drosophila* is a good model for the study of dendritic growth are covered briefly in Chapter 3. Chapters 4, 5, and 6 are manuscripts to be submitted for publication. They investigate mechanisms of activity - or more specifically, depolarization - induced motor neuron dendritic growth. Chapter 7 includes applications and concluding remarks.

Dendrites are essential components of neuronal networks. Once thought of as static integrators of synaptic inputs only passively transmitting signals as they receive them, dendrites have been shown to amplify these signals through generating action potentials, back-propagate signals to communicate via dendrodendritic synapses, and undergo dynamic structural change that can be independent of afferent activity (Paulsen and Sejnowski, 2000). The dynamic nature of dendrites is essential to proper nervous system development and function. Both intrinsic genetic factors and extracellular cues

govern dendritic morphology during the development of the nervous system as well as in the mature nervous system.

During embryogenesis, the dendrites of neurons become distinct from axons, then grow and acquire identifying unique characteristics. The development of neuronal polarity is covered in Chapter 2. After the formation of a dendrite, it must undergo maturation, which includes growth, branching and pruning. Dendrites are also able to undergo plastic change after embryonic development and maturation, however, and this has major ramifications for the function of the mature nervous system. This enables an organism to adapt to influences on the mature nervous system, such as stress, learning and memory formation, motor function adaptation, and behavior (Lewis, 2004).

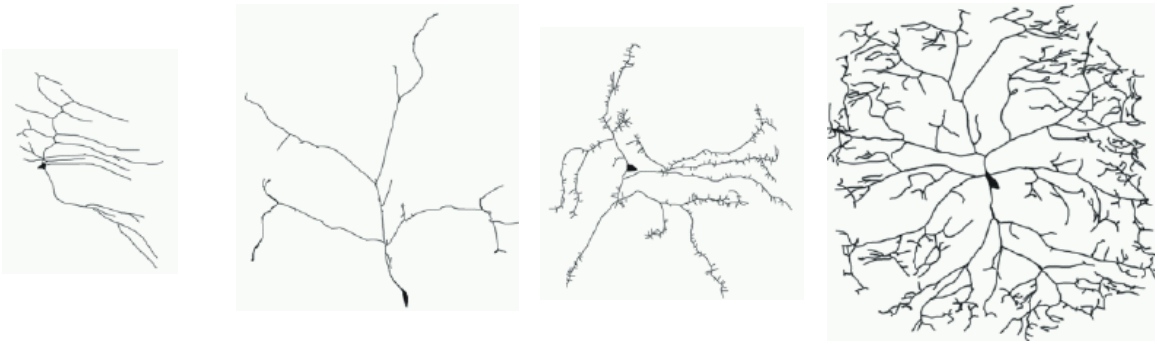
It is likely that there are similar molecular mechanisms governing dendritic formation during embryogenesis and plasticity of the mature nervous system. Thus, the genetic programs that have been implicated during original dendrite formation, may be involved in post-embryonic plasticity. Chapter 2 will discuss the factors involved in dendritic plasticity and formation, and point out some parallels between post-embryonic and embryonic mechanisms.

New emerging technologies have enabled rigorous analysis of the factors influencing dendrite plasticity during the normal development of the nervous system and post-embryonic modifications. Dendritic alterations also occur during the normal aging process of the brain; dendrite abnormalities have been implicated in many neurological disorders, such as certain forms of mental retardation and degenerative disease.

Therefore, insights derived from studies of dendritic development will enhance our understanding of these disorders.

1.1 Structural and Functional Dendritic Diversity

Each neuron type has a stereotypical dendritic morphology, but neurons within one class can have diverse variations of the theme. *Drosophila* tactile sensory neurons perfectly illustrate this point and will be referred to in subsequent sections of this dissertation. These neurons are split into 4 classes; class I and II have simple dendrites with a small field and no contact with each other, while class III has many short dendritic spikes and IV has complex dendrites with large fields. These sensory neurons share a common precursor cell, known as the external sensory single ectodermal precursor (Brewster and Bodmer, 1995). These neurons also have similar functions, innervation of the epidermis where they serve as touch receptors (Brewster *et al*., 2001), but their dendritic morphologies vary widely (see Fig 1 on next page). Both class III and IV neurons display distinct patterns of dendritic tiling, in which dendrites of like neurons cover the entirety of the body wall but do not overlap, much like the tiles of a kitchen floor. Many things, including type specific patterns of gene expression and cell-cell interaction, have been found to influence the consistent patterns of dendritic morphology and tiling of these sensory neurons (Jan and Jan, 2003).



Class I Class II Class III Class IV

Figure 1 Different dendritic morphologies of *Drosophila* sensory neuron classes. Individual GFP positive neurons were identified through the development of MARCM clones. 2D confocal images were reconstructed through tracing using Adobe Photoshop 7.0 software to produce the images shown here (Grueber et al., 2003).

Some neurons have different dendritic regions that comprise distinct compartments. The synaptic inputs of pyramidal neurons of the rat hippocampus and cerebral cortex are partitioned to have an apical (see arrowheads on Fig 2) and a basolateral (emerging from the base of the soma) set of dendrites that display dendritic spines (Dailey and Smith, 1996). Dendrite plasticity of rat hippocampal neurons has been extensively studied using organotypic slice preparations and cell culture techniques (Fukunaga *et al*., 1996; Banker and Cowan, 1979). Many factors influence the length, branch number or spine density of one specific dendritic compartment without affecting the other compartment within the same neuron. For example, brain derived neurotrophic factor (BDNF) treatment of CA1 hippocampal slice preparations increases the dendritic length of apical, but not basolateral dendrites of pyramidal neurons through activation of a MAPK cascade (Alonso *et al*., 2004). Other external influences on dendritic properties will be discussed in the next chapter.

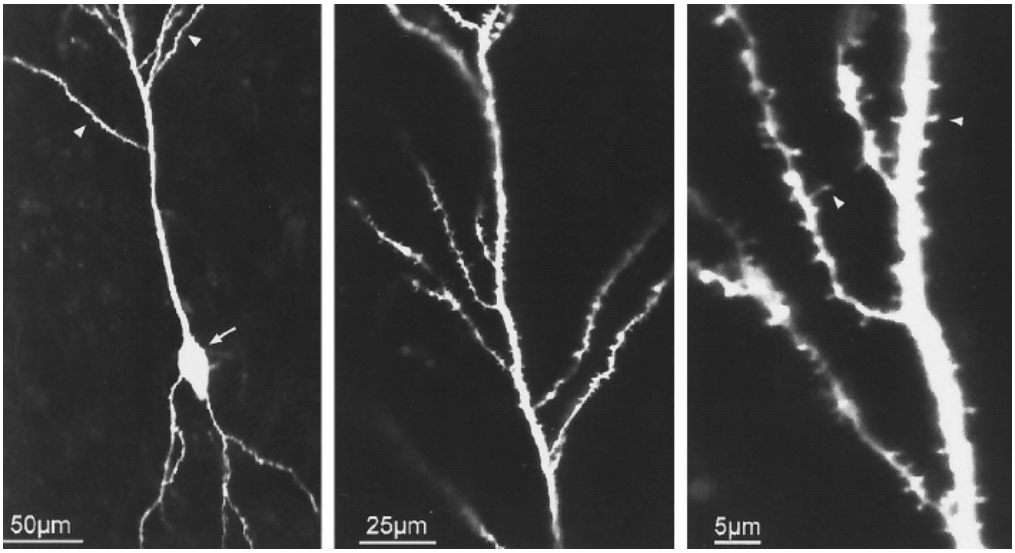


Figure 2 Dendrites of CA1 hippocampal pyramidal neurons in organotypic slice prepartations visualized through post-fixation labeling with DiI. Low magnification confocal image reveals apical (arrowhead) and basolateral dendrites. Higher magnification shows dendritic spines on the apical dendrites (Dailey and Smith, 1996)

The morphology of dendrites complements their function within a circuit. The dendritic shape of neurons involved in rabbit olfaction illustrates this point. The axons of many olfactory receptor neurons for a single odor converge onto a glomerulus composed of the apical dendrites of specific mitral cells. Mitral cells, in turn, excite an inhibitory granule neuron that does not have an axon. This granule neuron inhibits the mitral cell as well as close mitral cell neighbors through dendrodendritic synapses (see Fig 3). This results in lateral inhibition and, moreover, dendritic fields of neurons with closely related odor receptors often neighbor each other and are confined within a glomerulus to create an odor-topic map (Yokoi *et al*., 1995). This lateral inhibition from the dendrite rich granule cells enables heightened sensitivity during olfaction as individual mitral cells will exhibit excitatory responses to a strictly defined range of odor molecules and inhibitory responses to molecules exciting their close neighbors.

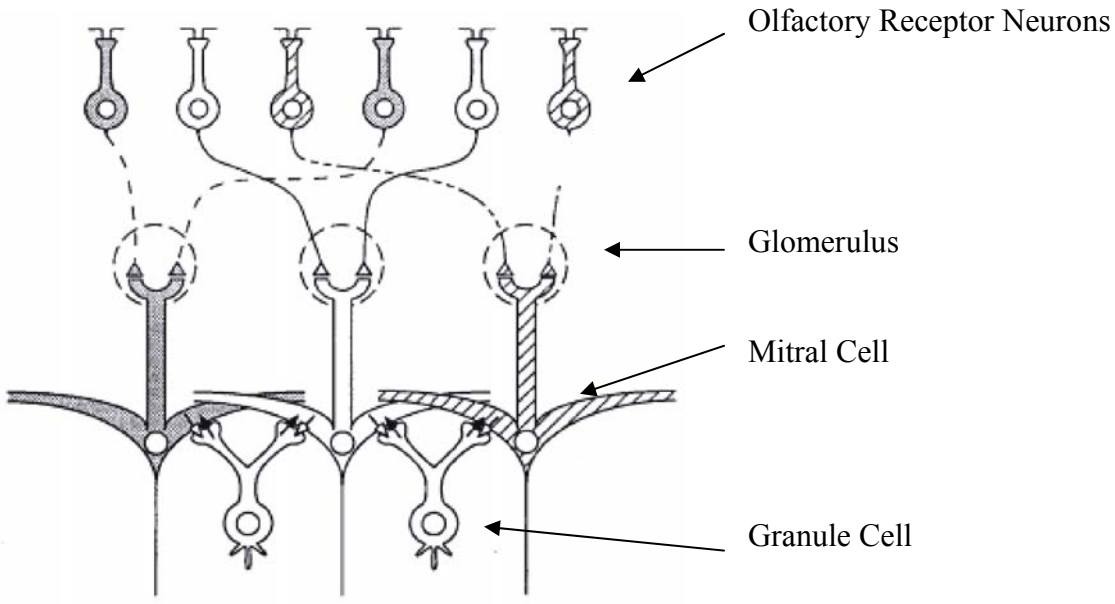


Figure 3 Proposed model of excitatory and inhibitory contacts of mitral cells within the olfactory bulb circuit. Olfactory receptor neurons that respond to related odors converge excitatory synapses onto glomeruli of neighboring mitral cells. Lateral inhibition between mitral cells is provided by inhibitory inputs of granule cells (Yokoi *et al*., 1995).

How the structure of a neuron dictates its function is also illustrated by the striking morphology of the Purkinje cells of the cerebellar cortex. Purkinje cell dendrites form complex arborizations that receive excitatory inputs via parallel fibers of granule cells within the cortex. These dendrites have a flat, two dimensional fan shape (Fig 4) and are stacked like dominos within the cerebellar cortex. This enables the parallel fibers, which run perpendicularly across the dendritic fields of many Purkinje neurons, to make synaptic connections with multiple Purkinje cell dendrites along the way. The Purkinje cell soma receives inputs from climbing fibers from the medulla. Purkinje neurons send inhibitory output to the deep cerebellar nuclei to dictate motor coordination. Purkinje cell dysfunction can lead to loss of coordination and motor impairment as is the

case with Spinocerebellar ataxia type 1, hallmarked by degeneration of cerebellar Purkinje cells (Cummings *et al*., 1999)

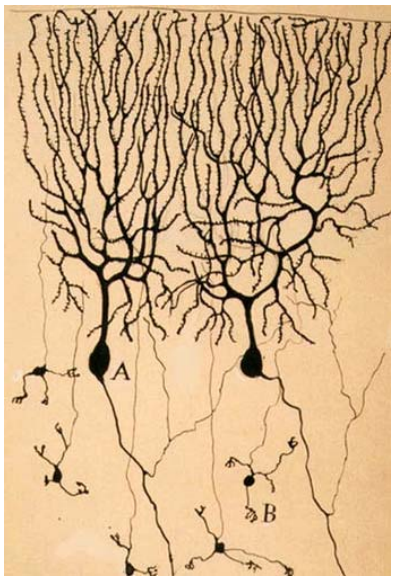


Figure 4 Drawing of golgi stained pigeon cerebellar Purkinje (A) and granule (B) cells by Ramon y Cajal, 1899 (Instituto Sanitago Ramon y Cajal, Madrid, Spain)

Dendritic function also depends on the distribution of molecules and organelles that contribute to the electrical properties of the neuron. The localization of synapse and calcium sequestering proteins along the Purkinje cell dendrites, for example, determine the strength with which they respond to different excitatory inputs. A single Purkinje cell forms thousands of synapses with parallel fibers while only forming a single synapse with a climbing fiber. Outside of the intrinsic pacemaker activity of Purkinje neurons, parallel fibers make weak synapses to spines in the Purkinje cell dendrites that result in action potentials (simple spikes), whereas climbing fibers provide strong excitatory input to the cell soma that result in plateau potentials (complex spikes) (Miyakawa *et al*., 1992). Climbing fiber and parallel fiber synaptic potentials both evoke a voltage gated calcium current. However, there is a high density of calcium sequestering proteins in the dendrites of Purkinje cells, including a plasma membrane calcium pump. The rapid reduction in intracellular calcium concentrations following Purkinje cell activity due to this pump along with other calcium binding proteins in the

Purkinje cell dendrites accounts for the simple spikes evoked by the parallel fibers (Talamoni *et al*., 1993).

Johnston and colleagues (Johnston et al., 1996) have characterized the ion channel distribution of post-embryonic CA1 hippocampal pyramidal neuron apical dendrites using cell attached patch and single channel recordings in organotypic slice preparations. These dendrites contain 4 types of voltage gated K^+ channels, including uniformly distributed delayed rectifier K^+ channels and A-type channels (Chen and Johnston, 2004), ubiquitous TTX sensitive voltage gated Na^+ channels, as well as 3 types of voltage gated Ca^{+2} channels (Magee and Johnston, 1995). These include low voltage activated rapidly inactivating Ca^{+2} channels, as well as high voltage activated moderately inactivating and high voltage activated non-inactivating channels with large conductance. The types of Ca^{+2} current resulting from these different channel types and functional differences imposed by this current is important in dendritic growth and will be discussed in section 2.3. This illustrates how the channel distribution of dendrites is an important factor for determining their excitable properties, and I will discuss why their excitability is important for neuronal function and development.

1.2 Post-Embryonic Plasticity

Although mature dendrites display specialized and elaborate structures and functions, there can be extensive post-embryonic modifications. This phenomenon has been termed “plasticity”. For example, dendritic growth and pruning takes place more than once in certain circumstances. Established dendrites of some invertebrate motor

neurons of *Manduca* and *Drosophila* undergo 3 phases of developmental plasticity: first the larval dendritic field expands in concert with growth of the larval nervous system, second the larval dendrites regress during early metamorphosis as the larval target structures degrade, and third adult dendrites regrow as the motor neuron innervates newly formed adult structures (Truman and Reiss, 1988). The dendritic arbor is often larger and more complex in the adult to accompany growth of the nervous system and changes in synaptic interactions (Levine and Truman, 1985).

Post-embryonic dendritic modifications can be cell type specific. Retinal ganglion cells are extensively remodeled during early postnatal development of rabbits and cats. Some ganglion cells undergo dendritic growth, thickening, and increased branching, as is the case with small field type I ganglion cells. Other ganglion cells, like large field type 1 cells, undergo dendritic growth during this time, but reduce the number of branches dramatically (Wong, 1990). The small field cells maintain their dendritic spines from birth to adulthood, whereas large field cells lose 100% of their spines by adulthood.

Post-embryonic modifications can take place to accompany learning. Alterations in spine density of mature hippocampal slice preparations have been observed in response to associative memory formation. Trace conditioned rats (paired stimuli of white noise and periorbital shock) display an increased spine density in the basal dendrites of pyramidal cells in the CA1 region of the hippocampus compared to rats presented with

unpaired stimuli (Leuner *et al*., 2003). More on post-embryonic dendritic plasticity will be covered in the next chapter.

1.3 Dendritic Abnormalities Related to Aging, Addiction, and Disease

Changes to dendritic morphology related to normal aging and age-associated disease can have great implications for the function of the nervous system. Superficial pyramidal neurons of the rat medial frontal cortex undergo regression after 18 months of age (Grill and Riddle, 2002). The basilar dendrites of pyramidal neurons in the human prefrontal cortex also show an increased dendritic regression with age (de Brabander *et al*., 1998). Age has been shown to influence dendritic spines as well. Pyramidal apical and basal dendrites lose spine density in aged macaque monkeys (Duan *et al*., 2003).

Neuron loss and changes in dendritic architecture are often associated with age related pathology, as is the case with the symptoms associated with Alzheimer's disease. Not only does excess neuronal loss take place among dentate gyrus granule cells as well as CA1 pyramidal neurons, but both suffer substantial dendritic degeneration in Alzheimer's patients compared to aged-matched controls (Anderton *et al*., 1998). These regional alterations in dendritic structure and neuron loss affect not only the cells involved, but the circuits in which they participate. The dendritic degeneration of hippocampal neurons has been correlated with the level of dementia experienced by Alzheimer's patients in many studies (Laakso *et al*., 1998).

Other states besides aging can influence the structure of dendrites, as is the case with addiction. Amphetamine, cocaine, nicotine and morphine all have been shown to

induce structural plasticity in brain areas associated with incentive motivation, judgment, and inhibitory control of behavior (Robinson and Kolb, 2004). Nicotine, a common (and legal!) drug of abuse increases spine density and dendritic branching in pyramidal neurons of the medial frontal cortex (Brown and Kolb, 2001), and these morphological changes persist up to 21 days after exposure to the drug. It can take up to 3.5 months for complete neuronal repair to occur. These structural changes are implicated in the cognitive enhancement observed with nicotine use and occur in brain areas associated with the reinforcing and craving effects of drugs of abuse. These long lasting, but reversible plastic changes to dendritic architecture give evidence that recovery from addiction can take substantial amounts of time.

These examples highlight just a few systems and organisms in which dendritic plasticity has been observed. Overall, the dynamic nature of dendrites has been studied in depth, because of its importance in the normal function of the nervous system. Investigators are now left with the task of examining the complex mechanisms involved in neural plasticity. These mechanisms can be divided into several major categories: gene expression, signal cascades, activity, growth factors, and hormones. There is evidence of crosstalk between the mechanisms that lead to plasticity in the living organism.

There is a diversity of known mechanisms underlying plasticity that are neuron-type specific, but often involve common themes amoung neurons. In many cases, one factor can have opposite effects on different neuron types. For example, gonadal steroid

hormones increase the dendritic tree the motor neurons of the spinal nucleus of the bulbocavernosus (Goldstein and Sengelaub, 1994), while preventing spine formation in arcuate neuroendocrine neurons (Danzer *et al*., 1998). The next chapter considers the many aspects found to influence dendritic plasticity, bearing in mind cell type specificity.

CHAPTER 2 MECHANISMS OF DENDRITIC PLASTICITY

The process of dendritic development and plasticity are a result of both intrinsic neuronal programs and external factors. Initial dendritic development occurs during embryogenesis while plasticity is considered to be a modification of the dendritic architecture of post-embryonic neurons. Dendritic sprouting and identity is determined during embryogenesis and will be covered at length at the end of this chapter. Many of the factors involved in the initial development of polarity can come into play later during post-embryonic plasticity, as well. Thus later stages of dendritic development are characterized by the expression of proteins that are involved in dendritic elongation, spine formation, and maturation. The regulators of dendritic growth, branching, and plasticity of post-embryonic neurons will be covered in this chapter.

The mechanisms governing dendritic plasticity can be divided into two categories: intrinsic and extrinsic. These can then be divided into subcategories, with intrinsic mechanisms including lineage dependent genetic programs, activity, and signal cascades. Extrinsic mechanisms include growth factors, neurotransmitters, hormones, and cell-cell contact. All of these mechanisms ultimately involve the expression of genes that regulate the growth and differentiation of dendrites. Crosstalk occurs between many of these pathways during growth and plasticity (see Figure 1). Each mechanism will be considered, in turn, and examples of crosstalk between pathways will be highlighted in each section.

Intrinsic Regulation

2.1 Lineage Dependent Genetic Program Regulation

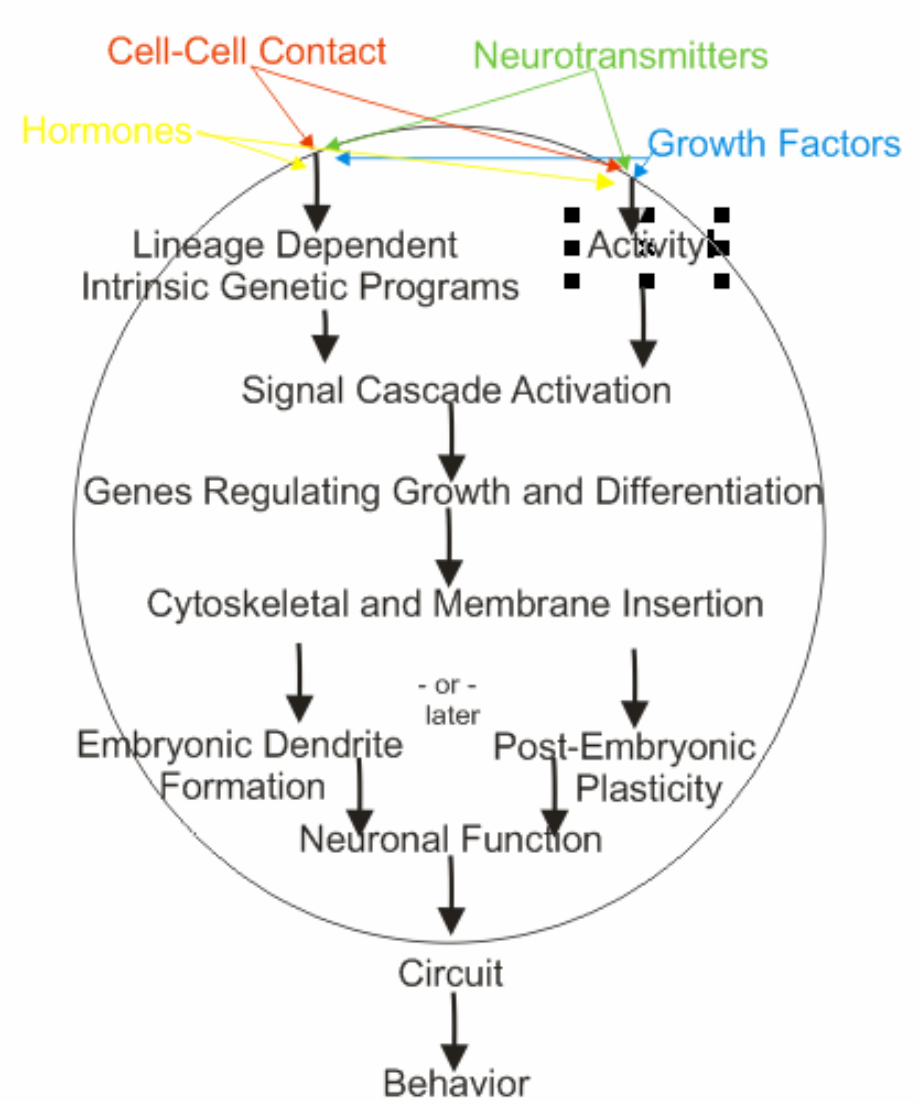


Figure 1 Mechanisms of Dendritic Growth and Plasticity. Many external and internal influences of neuron dendritic growth and plasticity have been discovered. There is much crosstalk occurring between intracellular pathways. Activity refers to changes in membrane potential that can lead to depolarization, changes in spiking frequency or spiking patterns.

Lineage dependent intrinsic genetic programs are a large part of determining dendritic morphology and function. Many neurons, like embryonic *Drosophila* motor neurons that innervate body wall muscles, develop a stereotypical dendritic field. In this case, the dendrites create a myotopic map within the ganglion (see Figure 2). The patterning of these dendritic domains is controlled by an intrinsic mechanism independent of muscle innervation or glial cell input. When the embryos are made to transgenically express proteins that interrupt the development of either target muscles or surrounding glia, the motor neurons still develop the characteristic dendritic shape. It is also apparent that cell-cell competition is not involved for the observed dendritic tiling (in which dendritic fields of neighboring neurons do not overlap), as transgenic ablation of neighboring motor neurons does not result in an overgrowth of dendrites from remaining neurons (Landgraf *et al*., 2003).

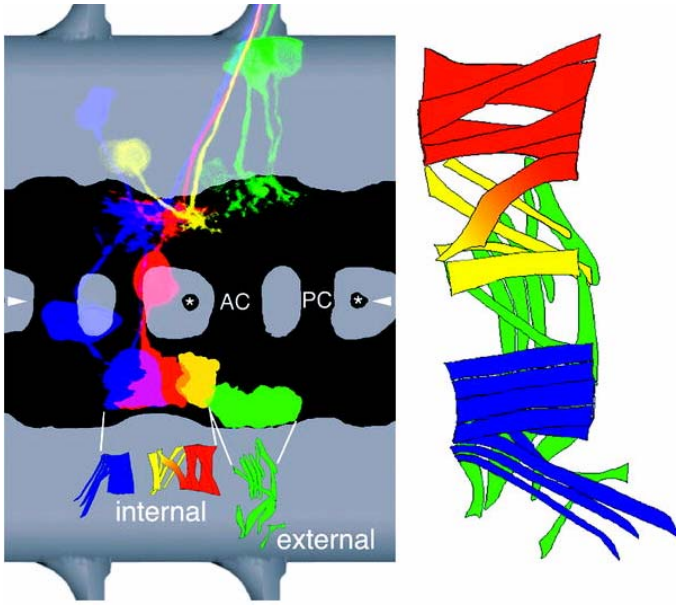


Figure 2 Digital reconstruction of identified late 3rd instar *Drosophila* motor neurons and their corresponding dendritic fields. Each colored dendritic field corresponds with matching colored muscles of the larval body wall, creating a "myotopic map" within the ganglion (Landgraf *et al*., 2003).

The ability of neurons to form stereotypical dendritic morphologies arises from the temporal expression of lineage dependent transcription

factors. Several genes have been shown to be present in *Drosophila* neuronal precursor cells, also known as neuroblasts. Some genes are expressed before cell division, while others activated upon cytokinesis or cell cycle progression. The intrinsic gene hierarchy is what regulates the cell type specific characteristics of a neuron within the same class (Cui and Doe, 1995). *Even skipped*, one of the genes activated during cell cycle progression, is only activated in a single neuroblast (Cui and Doe, 1995). This results in expression of the *Even skipped* transcription factor in a select group of RP2 and aCC motor neurons in the ganglion (McDonald *et al*., 2003; Garces and Thor, 2006). In Chapter 6, the promoter region of this transcription factor is utilized with the *Drosophila* transgenic system to gain spatial control of GAL4 expression in a small population of motor neurons.

Intrinsic genetic programs also allow for neurons to take on a stereotypical dendritic shape in culture. Embryonic rat hippocampal pyramidal neurons develop a morphology that is strikingly similar to their counterparts *in vivo*. The cell soma becomes triangular and they develop an apical and basal set of dendrites after one week in culture (Banker and Cowan, 1979). Intrinsic programs also persist *in vitro* to direct certain proteins to compartments consistent with their location *in situ*. Microtubule associated protein 2 (MAP2) is located in dendrites of neurons in culture and *in vivo* (Caceres *et al*., 1986).

Intrinsic genetic programs also allow post-embryonic expression of transcription factors that regulate the protein synthesis needed to carry out neuron-specific dendritic

growth. Class I, II, IV, and III sensory neurons mentioned earlier have non-detectable, low, medium, and high levels of *Cut* (a homeodomain containing transcription factor) expression, respectively, throughout embryonic and larval development in *Drosophila*. A loss-of-function *Cut* mutation causes stunted dendritic growth in class III neurons, while ectopic expression of *Cut* in class I and II neurons causes the dendrites to take on the appearance of class III arbors, including dendritic spikes (Grueber *et al*., 2003). *Cut* controls the distinct, class-specific patterns of dendritic morphology. The authors suggest that *Cut* expression is upstream of key regulators of dendrite branching.

Other transcription factors that are not as specific as *Cut*, but are expressed in many neuron types, have been found to be important for the development of dendritic arborization as well. *Sequoia*, a zinc finger protein transcription factor, is necessary for normal dendritic growth of *Drosophila* neurons. Unlike most transcription factors studied, *Sequoia* is not neuron type specific. It has pan-neural expression in the nervous system, suggesting that it controls the expression of a universal dendritic developmental pathway (Brenman *et al*., 2001).

Translational control has also been found to be important for dendritic growth, and has shown to take place independent of the soma. Electron microscopy studies have revealed the presence of ribosomes in dendrites (Stewart and Fass, 1983) as well as several mRNAs (Glanzer and Eberwine, 2003). This suggests that protein synthesis takes place in dendrites. Crino and Eberwine (1996) discovered that mRNAs targeted to dendritic space are actively translated. In Drosophila, two interacting RNA binding

proteins, *Nanos* and *Pumilo*, are suggested to form a translational complex that is targeted to sensory neuron dendrites and is necessary for normal dendrogenesis (Ye *et al*., 2004). However, it still remains unknown if active translation occurs in insect dendrites.

Depolarization and certain growth factors, both of which will be discussed in more detail shortly, have been shown to increase dendrite specific translation in vertebrate neurons. Mouse cerebellar purkinje cell cultures chronically exposed to elevated levels of K^+ demonstrate an increase of mRNA levels in their dendrites as well as and increase in protein expression for a particular mRNA that encodes a 99 amino acid peptide called L7/pcp-2. A similar effect was observed when the neurons were exposed to the γ-aminobutyric acid $(GABA)_A$ antagonist picrotoxin (Wanner *et al*., 2000). Cultured hippocampal neurons with a GFP reporter protein fused to the dendritically localizing CAMKII mRNA display an increase in GFP signal when exposed to brain derived neurotrophic factor. The increased signal occurs at the site of translation, the dendrites (Aakalu *et al*., 2001).

2.1a Genetic Dendrite Defects in Humans

Developmental dendritic abnormalities are often associated with inherited disorders that lead to cognitive deficits. Mental retardation is the most common manifestation of genetically caused dendrite abnormalities. Down syndrome individuals have the chromosomal abnormality of trisomy 21 which results in abnormal facial features, lower than average cognitive function, and deficits with spoken language and fine motor skills. In Down syndrome children and adults, dendritic spines are reduced in

the visual and motor cortex, but Down syndrome fetuses and neonates often have normal or increased dendritic branching and spine numbers in these regions (Takashima *et al*., 1981; Prinz *et al*., 1997). Rett syndrome is characterized by a halt in neural development at one year of age, followed by progressive degeneration of motor and language skills accompanied by stereotypical abnormal behaviors and microcephaly. A hallmark of Rett syndrome is a low level of dendritic spines throughout development in the frontal, temporal, and motor cortices (Armstrong *et al*., 1995). Individuals with Fragile X syndrome have impaired social development and autism. Long dendritic spines with prominent heads are present on the neurons of posterior cingulate and anterior temporal regions and there are an excess number of dendritic spines in many areas of the brain, including the visual cortex of individuals with Fragile X syndrome (Hinton *et al*., 1991; Beckel-Mitchener and Greenough, 2004). Although still largely unknown, it is hypothesized that these specific dendritic abnormalities and the brain regions they occur in are linked to the particular cognitive deficits present in each of these syndromes.

Rett syndrome and Fragile-X syndrome both have a single gene defect, while Down syndrome involves several genes that are over-expressed. Rett syndrome involves a mutation in the X-linked gene encoding methyl-CpG binding protein 2, so it only affects females as it is lethal in hemizygous males. This protein is involved in transcriptional repression (Amir *et al*., 1999). A mutation in the fragile X mental retardation gene that makes the translational repressor RNA binding fragile X mental retardation protein entails Fragile X syndrome (Kaufmann and Reiss, 1999). The loss of this protein leads to an increase of protein synthesis of glutamate receptors (Vanderklish

and Edelman, 2005). Down syndrome involves the dysregulation of several genes contained on the long arm of chromosome 21. Four proteins upregulated in Down syndrome brains and mouse models are NRIP1, GABPA, DYRK1A, and SUMO3 (Gardiner, 2006). NRIP1 is a steroid hormone co-repressor that inhibits many receptors (Castet *et al*., 2004). DYRK1A is a serine-threonine protein kinase and can activate CREB (Gardiner, 2006). SUMO3 makes post-translational modifications to inhibit the protein function of many proteins including the transcription factor Elk-1 (Eaton and Sealy, 2003). GABPA is a transcriptional co-activator and is phosphorylated by MAPK (Fromm and Burden, 2001).

Drosophila homologues exist for some of the proteins that cause dendritic defects in mental retardation. The *dFMR-1* gene is the homologue for the human fragile X mental retardation protein. Mutant flies display deficits in courtship conditioning learning assays and mushroom body malformation. Both can be rescued by treating the animals with mGluR receptor antagonists (McBride *et al*., 2005). This study is consistent with findings in mammalian models of Fragile X syndrome and could lead to beneficial therapies for Fragile X individuals. The established learning assays and transgenic techniques available in flies make it an advantageous model for the study of mechanisms involved in the dysregulation of gene expression in mental retardation.

2.2 Activity Dependent Dendritic Plasticity

The effect of activity dependent effects on dendrite plasticity is the central focus of this dissertation. Electrical signaling is a hallmark of neuronal function, and neurons

harness this specialized capability to regulate their development. Voltage gated calcium influx plays a major role in this regulation as it initiates gene expression through activation of signal cascades.

The literature includes many demonstrations of activity dependent dendrite plasticity. Activity can be considered an extrinsic mechanism, initiated by extracellular factors like neurotransmitters, but it can also be an intrinsic mechanism, as is the case with spontaneous activity. The next section deals with intracellular cascades downstream of activity, so therefore activity will be discussed here. However, the exact definitions of activity, excitability, and depolarization have become muddled as these terms are often used synonymously. This confuses the field of dendritic plasticity as these three terms mean different things. For example, depolarization of the cell membrane in the absence of action potentials can induce dendritic plasticity in the form of growth and branching (Wu *et al*., 2001). However, plasticity can be influenced by the rate and pattern of action potential activity (Mu and Poo, 2006). This thesis will use these terms in the following contexts: activity is the spontaneous or synaptically-driven rate of action potentials firing of a neuron as well as the pattern of spiking; excitability is the propensity of a neuron to fire based on the intrinsic resting membrane properties of the cell; depolarization is a increase in the resting membrane potential of a neuron that doesn't necessarily lead to an action potential. Another widely used term is "experience". This refers to activity in sensory or motor systems that is associated with use. Experience-induced neural plasticity can involve activity, excitability, depolarization, or a combination of these factors (Ren and Dubner, 1999). This section will first address "synaptically driven"

depolarization and activity induced dendritic changes mimicked *in vitro* and point out some inconsistencies in the literature. Then, spontaneous changes in depolarization and activity that lead to dendritic growth will be briefly mentioned followed by examples of experienced based dendritic plasticity.

Synaptic transmission *in vivo* in response to synaptic input or spontaneous activity can be mimicked *in vitro* through alterations in ion concentrations in the culture media, direct stimulation of a cell with an electrode, or exposure of cells to neurotransmitters. For example, Vaillant and colleagues (2002) demonstrated that dendritic elaboration of rat sympathetic cultured neurons could be induced through depolarization with 50mM increase of K^+ in the culture medium, a constant 60mA current delivered at 5 Hz to the cells, or the addition of the cholinergic agonist, carbachol. Similarly, dendritic growth of granule neurons can be induced by increasing K^+ concentrations by 30mM in the cell culture medium (Gaudilliere *et al*., 2004). Addition of 90mM K^+ to cultured hippocampal neurons induces protrusion of new dendritic filopodia within minutes to hours of exposure (Wu *et al*., 2001), while chronic exposure to a 50mM increase in K^+ results in an increase in total dendrite length and branch number by 4-6 days *in vitro* (Szebenyi *et al*., 2005). All of these methods of depolarization-induced growth required chronic depolarization, as growth was reversible after 2 days of no stimulation. Unfortunately, as no recordings were done, the only study in which it is certain that depolarization, as opposed to spike activity, is what induced dendritic plasticity was from Wu *et al*., 2001, who administered TTX to the culture medium to inhibit Na^+ based action potentials. This has important implications on the interpretation of the findings of these

studies as some of the induced growth involves depolarization while other may involve a change in activity as well.

Gaudilliere *et al*., 2004 made claims that their induced dendritic growth was dependent on activity even though they did not use a pharmacological means to block action potentials and did not measure the activity of the neurons. Both Szebenyi *et al*., 2005 and Vaillant *et al*., 2002 depolarized the cells with 50mM KCl, and stated that they were observing "activity dependent plasticity" without the use of action potential recordings or the use of TTX in a depolarized background. It is important not to use the terms activity and depolarization synonymously for several reasons. The experiments were performed in different culture media which will result in different excitability properties, depending on the ion and substrate content. Furthermore, the studies were performed with different neuronal types that may display unique intrinsic excitabilities. A 50mM increase in KCl could result in depolarization in one neuron type and increased spike activity in another, depending on the unique excitable properties of the neuron. This is an important distinction, since low-voltage threshold calcium currents (see below) may be activated in the absence of Na^+-dependent action potentials. It cannot be stated definitively that elevated K^+ induces activity, unless recordings of membrane properties are performed.

Signal cascades that are activated by "depolarization" induce the protein synthesis that is needed for dendritic growth to occur. These will be discussed in great detail in the subsequent section. It is worth mentioning, however, that the level of activity can specify

which cascades are activated within a neuron. For example, in hippocampal neurons, the duration of activity and the firing frequency can induce different signal cascades. A short 0-10 min stimulation can induce the CAMK/CREB cascade while a 60 min duration stimulation is needed to induce the MAPK cascade (Wu *et al*., 2001). Similarly, short action potential frequency (<3 min) activates the MAPK/ERK cascade while longer action potential frequency (>10 min) activates CREB (Fields *et al*., 1997). In this dissertation I show that depolarization can induce the growth of *Drosophila* motor neuron dendrites. Careful interpretation of the existing literature suggests the most likely cascades involved in this growth. This will be discussed in section 2.3.

Some neurons generate spontaneous changes in membrane potential or activity that influence dendritic growth. Neonatal ganglion and amacrine neurons of the retina generate spontaneous Ca^{+2} transients *in vitro* that are not dependent on glutamate release and are hypothesized to play a role in developing precise sets of connections and cell differentiation (Wong *et al*., 1995). Embryonic amphibian spinal neurons generate spontaneous Ca^{+2} transients as well. The spontaneous activity is necessary for neuron differentiation and maturation (Spitzer and Ribera, 1998), as discussed in more depth in the following section. Spontaneous spiking not only leads to dendrite growth. Existing connections can become unstable and degenerate as well. Increased spontaneous spiking of olfactory bulb neurons (upon treatment with bicuculline, a GABA antagonist) in mice induces both the formation and loss of apical dendrites (Mizrahi and Katz, 2003).

Competitive experience-dependent dendrite development often involves synapse elimination. Activity-dependent competitive dendritic plasticity has been well characterized in the peripheral nervous system, including synapse elimination at the vertebrate neuromuscular junction. At birth, individual muscle fibers are polyinnervated by multiple motor axons. The competitive process of synapse elimination occurs until the axon with the strongest synaptic input is the one that remains (Sanes and Lichtman, 1999). This same kind of competitive elimination takes place with immature dendrites as well. Immature mitral cells have dendritic connections to multiple glomeruli of the rat olfactory bulb where they receive sensory synaptic input. With maturation, most dendrites retract while the remaining dominant primary branch simultaneously begins to form higher order branches (Malun and Brunjes, 1996).

Post-embryonic experience based dendritic modifications occur as well, enabling the nervous system to adapt to environmental cues and permitting learning and memory formation. Environmental challenges, such as stress, lead to changes in the morphology of dendrites. Restrained rats demonstrate dendritic retraction and spine loss in the hippocampus and prefrontal cortex and an increase in spine density in the amygdala (Watanabe *et al*., 1992; Radley *et al*., 2005; Vyas *et al*., 2002). Trace conditioning (white noise is paired with a periorbital shock) in rats leads to an increase in dendritic spines in the hippocampus. This associative learning based hippocampal dendritic plasticity does not occur in the presence of an NMDA receptor antagonist (Leuner *et al*., 2003), suggesting that glutamate binding to the neurons of the hippocampus results in depolarization, Ca^{+2} influx, and/or increased activity that catalyzes cascades involved in

the increase in dendritic spines. The molecular mechanisms that are involved in dendritic growth and plasticity will be considered in the next section.

2.2a Influence of Genetic Defects that Alter Activity

Many genetic defects involved in human disease result in altered neuronal activity. Similar to dendritic abnormalities found in Fragile-X mammalian dendrites, mutation in the *Drosophila* homologue of the Fragile-X mental retardation gene *dFMR-1*, results in an increased number of terminal dendritic branches in Type IV dendritic arborization sensory neurons (Lee *et al*., 2003). This mutation also alters larval crawling patterns. The mutant animals have shorter linear paths and more turns than wild type larva. The dFMR protein interacts with *Pickpocket* RNA, the gene for a sensory neuron specific epithelial sodium channel (ENaC) and inhibits its expression. Mutant larvae, with no dFMR, express 76% more *Pickpocket* mRNA (Xu *et al*., 2004). The increased expression of sodium channels in the membrane may lead to an increased activity of the neurons (although direct electrophysiological recording have not been taken) and the authors hypothesize that this activity leads to the erratic crawling behaviors in the mutants. If this gene directly controls the electrical properties of neurons in mammals as well, it could be related to the excess and malformed dendritic spines that are present in Fragile X individuals.

Epilepsy, the dysregulation of activity in the central nervous system, can be the result of a heritable genetic mutation. It is characterized by episodic impairment of nervous system function with or without seizures. Many activity-related mutations have

been found to be responsible for epilepsy in humans, including the acetylcholine receptor (Rozycka and Trzeciak, 2003), metabotropic glutamate receptor (Moldrich *et al*., 2003), and various voltage-gated ion channels (Errington *et al*., 2005) and even GABA synthesis (Treiman, 2001). The specific mutation determines the brain region that is affected and, therefore, the specific symptoms that an individual will experience (Chabolla, 2002). These proteins, therefore, are the targets of therapies for epilepsy. This is the case with GABA synthesis, where the reuptake inhibitor tiagabine has anticonvulsive properties (Sendrowski and Sobaniec, 2005).

2.3 Activity Induced Signal Cascades Involved in Dendritic Plasticity

Activity and the induction of signal cascades go hand-in-hand in the study of neuronal plasticity. Many factors downstream of calcium in activity induced dendritic growth have been discovered (see Figure 3). Calcium regulates the actin (Oertner and Matus, 2005) and microtubule cytoskeleton (Mattson, 1999). Moreover, calcium has been found to regulate the three important signaling pathways that are involved in dendritic plasticity, Rho-GTPase, CaMK and MAPK. Rho-GTPases bind and hydrolyze proteins that influence the assembly and stability of the actin cytoskeleton and microtubules. Calcium-calmodulin dependent kinases (CaMKs) and mitogen activated protein kinases (MAPKs) both activate transcription factors that regulate gene expression (Konur and Ghosh, 2005).

Free calcium levels within the cytoplasm can be increased by influx though calcium channels in the cell membrane or by release from intracellular stores. Neurons have both voltage and neurotransmitter-gated calcium channels, including the NMDA receptor, in the cell membrane. Calcium-induced-calcium-release from intracellular stores occurs through calcium binding to the ryanodine and/or IP-3 receptors of the endoplasmic or sarcoplasmic reticulum. Voltage gated calcium current, release from intracellular stores, and neurotransmitter permitted calcium entry in excitable cells, and the influence each has on the function and plasticity of neurons has been studied extensively (Ghosh and Greenberg, 1995; Konur and Ghosh, 2005). Once calcium levels have risen within the cytoplasm, calcium can bind to calmodulin and activate kinases and transcription factors, it can bind to ryanodine receptors and induce further calcium release, or it can interact with actin regulatory proteins and influence filopodial dynamics.

Cultured Purkinje neurons illustrate how different types of calcium currents work in concurrence to influence neurite outgrowth. Exposure of Purkinje cells to high K^+ medium induces neurite outgrowth and branching that is dependent on intracellular calcium concentration increases through two different mechanisms: R-type calcium current and release from intracellular calcium stores. The authors hypothesized that low voltage threshold activation of R-type calcium channels takes place with the administration of a 10 mM increase in K^+ and that this, in turn, activates calcium-induced-calcium-release (Reitstetter and Yool, 1998).

As mentioned previously in the work by Magee and Johnston (1995), there are multiple types of calcium channels located specifically in dendrites. There are low voltage activated T-type channels that have low conductance and fast inactivation. There are also two types of high voltage activated channels. N-type channels have moderate inactivation and L-type channels which open infrequently, have large conductance and no inactivation. These channels allow the activity of the neuron to regulate the calcium concentration within the cell. As will be discussed, the level of concentration change of calcium within the cell determines which downstream cascade is activated.

In addition to the NMDA glutamate receptor, other types of receptors permit calcium influx. Cultured *Drosophila* neurons that are exposed to acetylcholine demonstrate inward calcium current even in the presence of $CdCl_2$ to block voltage-gated calcium entry (Alshuaib *et al*., 2004). 75% of the neurons tested no longer had intracellular increases in calcium concentration in response to acetylcholine when the cholinergic blocker, curare, was present. The difference in calcium response to acetylcholine application between the neuron types was not recorded in this study, and some calcium channels are not Cd-sensitive. However, it can still be concluded that in some *Drosophila* neurons, cholinergic receptors permit calcium entry.

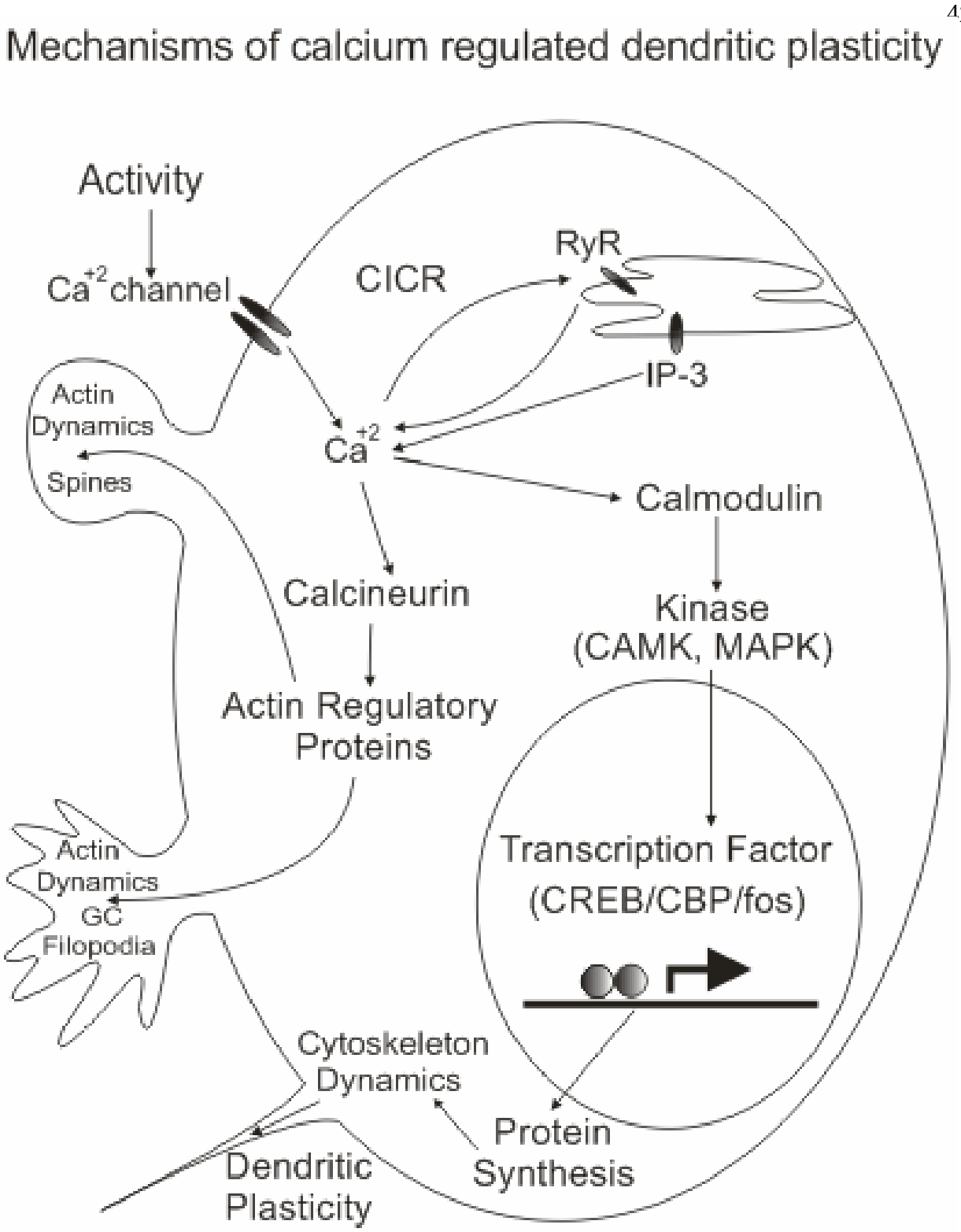


Figure 3 A summary of the findings covered in this section involving Ca^{+2} dependent dendritic plasticity. This covers a range of developmental timepoints and model systems.

Spitzer and colleagues have done a thorough analysis of the presence and types of calcium currents present in embryonic spinal cord neurons, the downstream cascades that are activated, and the morphological and developmental impact of these currents. These neurons have two types of spontaneous elevations in intracellular calcium. Spikes are short in duration with large increases in concentration, whereas and waves are longer in duration with smaller increases in intracellular calcium levels. The waves that are localized to growth cones result in slowed outgrowth through the regulation of calcineurin, a calcium dependent phosphatase. Calcineurin regulates the actin cytoskeleton of growth cones, as activated calcineurin loses its ability to halt neurite outgrowth in the presence of cytochalasin D. By contrast, the spikes regulate the differentiation of GABAergic phenotype through transcriptional activity within the neuron. Calcium spikes, possibly through activation of cAMP, lead to the induction of GAD67 mRNA expression (Spitzer *et al*., 2000; Gorbunova and Spitzer, 2002; Ribera and Spitzer, 1998).

While Spitzer's work focuses on spontaneous depolarization-induced calcium transients in developing neurons, a large body of work involving non-spontaneous depolarization induced calcium current also exists. Three of the high K^+ induced depolarization investigations mentioned in the previous section 2.2 found that calcium current was involved in dendrite growth, as nimodipine (a voltage gated calcium channel blocker) blocked the depolarization induced growth. Calcium leads to the activation of kinases, in the case of the granule cell cultures (Gaudilliere *et al*., 2004), calcium was upstream of CaMKII activation, while in the hippocampal cultures (Wu *et al*., 2001),

calcium was upstream of MAPK (ERK) activation. Is it possible that depolarization induced calcium current leads to the induction of multiple kinases within every neuron to regulate growth? There are several possibilities: specific kinases could be cell type specific, the extent of depolarization could induce one kinase cascade vs. another, some cascades may be initiated earlier than others, etc. It seems in these examples that the kinase activity is cell specific, as the CaMK inhibitor KN-93 did not prevent depolarization induced growth in the hippocampal cells, whereas in the granule cell cultures ERK immunoreactivity, as detected by Western blot, did not increase upon depolarization although CaMKII did.

Although both kinases did not contribute to dendritic plasticity in the previous two studies, in other cases both are involved. CaMKII and MEK-ERK pathways contribute to dendrite stability in the sympathetic neuron study mentioned previously (Vaillant *et al*., 2002). Simultaneous inhibition of both was required for dendrites to retract in a depolarized background. Redmond and colleagues (2002) found that in cultured rat cortical neurons, K^+ induced dendritic growth is dependent on voltage gated calcium entry that activated both MAPK and CaMK. CAMKIV specifically led to dendritic outgrowth through the induction of CREB in this system. Furthermore, a constitutively active form of CAMKIV, but not MEK, was able to induce dendritic outgrowth in the absence of calcium. However, we do not know the extent of depolarization in these latter two studies and how it compares to the previous studies in which only one kinase was activated. It is possible that not only depolarization, but also

spiking occurred in these neurons. Different levels of excitability, therefore, could lead to the activation of different or multiple kinase cascades.

Whether dendritic depolarization related growth regulation takes place through CaMK, MAPK, some other kinase, or a combination of kinases, all of these pathways lead to induction of transcription through activation of transcription factors. This, in turn, initiates the production of the proteins that are necessary to carry out the structural changes underlying dendritic plasticity. There are several examples of calcium mediated kinase activation of transcription factors in neurons. Calcium has not only been linked to calmodulin/CaMK/CREB induced gene expression, (Dolmetsch *et al*., 2001) but ERK/CREB/fos induced gene expression has been described as well (Hardingham *et al*., 2001).

In many studies using *Drosophila*, important aspects of calcium dynamics are reported, but fail to explore the functional role the calcium may have. For example, embryonic *Drosophila* motor neurons RP2 and aCC display voltage sensitive calcium current *in vivo*, although the functional implication of this current is unknown (Baines *et al*., 2001). Pupal *Drosphila* Kenyon cells *in vitro* generate spontaneous increases in intracellular calcium concentration through voltage gated calcium channels, but the developmental implication of these currents has only been speculated (Jiang *et al*., 2005). We have demonstrated that calcium is involved in late 3rd instar *Drosophila* motor neuron growth (as will be presented in Chapter 4). Since these studies detect the presence of calcium currents as well, it would be interesting to investigate the functional roles of the

calcium currents at different developmental time points and in different cell types to compare to our findings.

Calcium activation of kinases will lead to the activation of transcription factors such as AP-1 and CREB. Fos is an activity related protein that heterodimerizes with jun to form the active transcription factor, activity protein 1 (AP-1), which regulates the expression of many genes in a variety of tissue types (Chinenov and Kerppola, 2001). In *Drosophila*, AP-1 is involved in many processes during development, including dorsal closure of the embryo and eye patterning (Kockel *et al*., 2001). The role and activity induction of AP-1 in neural systems has been well characterized in vertebrates. Depolarization of the PC12 neuronal cell line leads to rapid CREB phosphorylation through a cAMP dependent mechanism, to induce transcription of c-fos within 30 min. Furthermore, voltage gated calcium entry initiates the cascade in the PC-12 cells to lead to c-fos induction through cAMP and CREB activation (Ghosh *et al*., 1994). Fos has also been linked to activity induced dendritic changes that are associated with learning. Chicks trained to avoid pecking at bitter-tasting objects display an increase in jun and fos expression within minutes to hours of learning, which is followed by subsequent increases in forebrain spine density, although fos was not linked directly to the dendritic plasticity observed (Rose, 1991). AP-1 influences presynaptic terminal number at the *Drosophila* neuromuscular junction (Sanyal *et al*., 2002). Chapter 6 of this dissertation will present new findings of the influence of AP-1 in depolarization induced dendritic growth.

As mentioned, work done at the *Drosophila* neuromuscular junction has given insight into the transcriptional regulators of neural activity and plasticity because of the simplicity and consistency of measuring the evoked post synaptic response (synaptic strength) and bouton count (synaptic size). Mutations in the *ether-a-go-go* or *shaker* genes (K^+ channels) that render the K^+ channel non-functional, and therefore depolarize the motor neuron or increase spike number, result in increased synaptic strength at the larval neuromuscular junction and increased bouton number (Budnik *et al*., 1990). CREB is necessary for the increased synaptic strength, but not increased synaptic size (Davis *et al*., 1996). *Dunce*, a cyclic AMP phosphodiesterase mutant, disrupts the CREB pathway, suggesting that cAMP modulates synaptic plasticity through the induction of CREB (Zhong *et al*., 1992). Activity induced increases in synaptic size and strength are dependent on AP-1 in a CREB independent fashion (Sanyal *et al*, 2002). Despite the large body of work done at the *Drosophila* neuromuscular junction, and the number of candidate reagents available, there is little information on how the activity dependent pathways that regulate motor neuron axonal plasticity may influence dendrites. The lack of a reliable and reproducible assay for the measurement of individual motor neuron dendritic fields *in vivo*, as well as difficulties in identifying the dendrites *in vitro,* are responsible. These assays, if used in conjunction with the already existing transgenic tools available, would open the possibilities for the study of activity induced motor neuron dendritic plasticity. These goals are fulfilled, in part, by this dissertation as will be discussed in Chapters 4-6.

2.4 Activity Dependent Actin and Tubulin Regulation of Dendritic Plasticity

As briefly mentioned in my discussion of the work by Spitzer, calcium influx stabilizes growth cone filopodia, but the stability of spines and dendrites themselves are also influenced by calcium. In developing retinal ganglion cells, acetylcholine induced calcium influx stabilizes forming synapses and if this calcium current is blocked, rapid retraction of dendrites occurs (Lohmann *et al*., 2002). Cultured hippocampal neuron dendritic spines are dynamic, and in the absence of calcium, their motility increases. The addition of glutamate to the culture halts spine motility in a calcium dependent manner (Oertner and Matus, 2005). Certain proteins, such as gelsolin (a calcium activated protein that caps actin filaments) are necessary for calcium induced spine stabilization (Star *et al*., 2002).

Activity regulated, calcium independent, actin filament and microtubule stabilization occurs as well. The Rho-GTPase family of proteins is important for activity induced dendritic development through control of actin and tubulin dynamics. These proteins bind guanine nucleotides when in their active, GTP bound, state. Three well characterized family members include RhoA (Ras homologue member A), Rac1 (Ras related C3 botullinum toxin substrate 1) and Cdc42 (cell division cycle 42). RhoA is involved in repression of neurite growth, as demonstrated in *Drosophila* mushroom body dendrites by Lee and colleagues (2002) with a constitutively activated form of RhoA that severely reduced dendritic arborization, while loss of RhoA resulted in overextended dendrites. NMDA receptor activation decreases RhoA activity in Xenopus optic tectal neurons and this is required for neurotransmitter induced dendritic growth (Li *et al*.,

2002). Rac1 and Cdc42, however, are important in dendritic branching and growth. Rac1 loss of function mutations result in reduced dendritic branching and length of *Drosophila* mushroom body neurons (Ng *et al*., 2002). Both Rac1 and Cdc42 are needed for activity dependent dendritic growth in *Xenopus* optic tectal neurons as well (Sin *et al*., 2002). However, Rac-1 has also been demonstrated to regulate dendrite growth and branching. Dendritic development of cultured embryonic hippocampal neurons is controlled by the GTP exchange factor ARNO, which localizes to sites of dendrite extension. Loss of ARNO function results in an increased dendritic arborization complexity that can be rescued by over-expression of Rac-1. Therefore, ARNO regulates dendrite growth and branching through Rac-1, and investigators found that ARNO works through ARF-6 to activate Rac-1 (Hernandez-Deviez *et al*., 2002). Rho-GTPases are also involved in neutrophin-dependent dendritic growth, as will be discussed in section 2.5.

2.4a Dendritic Defects Related to the Actin Cytoskeleton

A loss of interaction between Fragile X mental retardation protein and Rac-1 in Fragile X syndrome could explain the phenotype of long dendritic spines with prominent heads in certain areas of the affected brain. It was recently found that the CYFIP protein binds to Rac-1 and FMRP (Schenck *et al*., 2001). It turns out that CYFIP is an effector of Rac-1 that mediates actin nucleation (Eden *et al*., 2002) and CYFIP antagonizes FMRP mediated translational regulation. It is possible that without FMRP, CYFIP activity is tipped toward the effector influence of Rac-1 to over-polymerize actin filaments that leads to the abnormal spines found in Fragile X individuals.

Extrinsic Regulation

2.5 Growth Factors Involved in Dendrite Development

Growth factors are a family of proteins that influence the growth of cells once bound to their receptor on the plasma membrane. Of the many types of growth factors, several are important for dendritic growth and differentiation. These include neurotrophins, bone morphogenic protein (BMP) and transforming growth factor beta (TGF-β). Three neurotrophins that influence dendrites are brain derived neurotrophic factor (BDNF) which binds to TrkB, nerve growth factor (NGF) which binds to TrkA, neurotrophin 3 (NT-3) which binds TrkC and neurotrophin 4/5 (NT-4/5) which also bind TrkB. These neurotrophins are made in neurons, and their release is often dependent on activity.

The tyrosine kinase receptor TrkB is necessary for complex dendritic patterns of layer II/III cortical cells in the mouse visual cortex. Knock-out mice for TrkB display reduced dendritic diameters and higher order branching of pyramidal cell apical dendrites (Xu *et al*., 2000). While this study made contributions to the characterization of the TrkB receptor's role in dendritic growth, it did not determine if the two ligands for TrkB, BDNF or NT-4/5, are both, or individually, responsible for the dendritic phenotype. Specific types of cortical neurons have been found to respond differently to the same neurotrophin. Exogenous BDNF application results in dendritic growth of layer 4, but not layer 6, pyramidal cells in organotypic slices of the ferret visual cortex, whereas NT-4/5 has the opposite effect (McAllister *et al*., 1997).

How is it possible that the same receptor can have such diverse effects on different cell types? Perhaps it isn't the same receptor. The Trk receptor has different isoforms: full length and truncated. Transfection of each receptor type into ferret cortical neuron cultures resulted in isoform specific modes of dendritic growth. Full length Trk regulates dendritic branching while truncated Trk regulates dendritic outgrowth from existing branches. Both of the receptors responded to the two known ligands for Trk, NT-4/5 and BDNF (Yacoubian and Lo, 2000). Another possibility is that the ligands may have specificity in activating downstream signal cascades. Trk has shown to activate two mechanisms, Ras/PI3K/Akt and MEK/MAPK to regulate cell survival and dendritic plasticity. There is evidence that BDNF activates the MEK pathway and that NT-4/5 activates the Ras pathway (Bonni *et al*., 1999 and Minichiello *et al*., 1998). We have seen in this overview that these downstream cascades are involved in a plethora of signaling mechanisms.

The Eph receptor and ephrins, another type of tyrosine kinase receptor and its ligand, are vital for neuronal development and post-embryonic modifications in the adult brain. The Eph receptor is divided into two classes, EphA and EphB, and each class has several isoforms in mammalian systems. Cultured hippocampal cells of triple knock-out mice for EphB1, EphB2 and EphB3 are unable to generate dendritic spines and excitatory synapses. These receptors have redundant functions in spine formation, as a single mutation does not result in a phenotype while different combinations of double mutations results in varying amounts of dendritic spine loss (Henkemeyer *et al*., 2003). These triple mutant animals are viable and able to reproduce. They have abnormal headless or small-

headed spines and a significant decrease in spine density in pyramidal neurons of the hippocampus *in vivo*. It would be interesting to investigate the behavioral consequences of these mutations as the hippocampus is involved in memory and spatial navigation.

The inability of mutant hippocampal cells to generate excitatory synapses may stem from the Eph receptor regulation of NMDA receptors. Ligand bound EphB enhances glutamate stimulated calcium influx through NMDA channels of cultured cortical neurons. This calcium then induces the expression of fos and BDNF genes in a CREB dependent manner. (Takasu *et al*., 2002). The EphB receptor may potentiate the effects of NMDA signaling in developing synapses through the initiation of activity dependent signal cascades required for protein synthesis that is involved in strengthening of a synapse.

2.6 Neurotransmitters and Dendrite Morphology

In addition to their well-known electrical effects on the post-synaptic cell, neurotransmitters may evoke metabolic changes. Neurotransmitters may activate signal cascades directly, with the influx of calcium, or through the activation of G-protein coupled signal cascades. The major excitatory neurotransmitters of the central nervous system are acetylcholine and glutamate. The major inhibitory neurotransmitters are GABA and glycine, although during maturation GABA and glycine may act as excitatory neurotransmitters due to the high level of intracellular chloride in developing neurons. Serotonin and dopamine receptors are coupled to G-proteins, with the exception of one 5-

HT receptor isoform which is a cation channel. GABA, glutamate and acetylcholine, work through both ionotropic and metabotropic receptors.

These neurotransmitters have similar functions and homologues in the mammalian and *Drosophila* nervous system, with a few distinguishing features. Glutamate is the major excitatory neurotransmitter of the central nervous system in vertebrates, with acetylcholine as the major excitatory neurotransmitter of the peripheral nervous system, including the neuromuscular junction. In *Drosophila*, however, glutamate is released at the neuromuscular junction instead of acetylcholine. I will discuss the glutamatergic properties of *Drosophila* motor neurons in Chapter 4. Glutamate, acetylcholine, serotonin, dopamine and GABA all function as central neurotransmitters in *Drosophila.*

Cline and colleagues have studied the role of excitatory neurotransmission and activity on the growth and development of dendrites. Using time lapse imaging of live *Xenopus* optic tectal neurons *in vivo*, they demonstrated that NMDA receptor antagonists severely reduce arborization during development, but AMPA receptor block does not. A similar arborization occurs during development in response to visual stimulation in a NMDA receptor dependent manner (Cline, 2001).

Cline went on to show that in these immature tectal neurons, NMDA receptor expression takes place first at the sites of putative synapses. As the dendrites mature, AMPA receptors are incorporated into the synapses to amplify the post synaptic response. Immature synapses that do not express AMPA receptor retract. This

phenomenon is specific to the developmental period, as blocking of NMDA and AMPA receptors in mature tectal neurons does not influence their arborization characteristics, only their branch length (Rajan and Cline, 1998).

NMDA is necessary for plasticity in the mature *Drosophila* brain as well. Xia and others (2005) cloned and mutated an NMDA receptor subunit which is located on interneurons that project to the mushroom body calyx, as well as to other areas of the fly brain. Both chronic and conditional disruptions of NMDAR activity block Pavlovian olfactory learning (described in 7.2). This study links NMDA dependent learning in *Drosophila* to prior studies of the vertebrate hippocampus. In the *Drosophila* system it will be possible to examine the proteins downstream of NMDAR activation that are ultimately necessary for learning to occur, crosstalk between pathways that are involved in plasticity, and architectural changes occurring in neurons as learning takes place.

2.6a Neurotransmitters and Mental Retardation, Addiction or Disease

Neurotransmitters are involved in certain forms of mental retardation. *Post-mortem* analysis done on human brains of Rett syndrome patients reveals, as mentioned earlier, a loss of dendrites and dendritic spines in layer II and III pyramidal neurons of the frontal cortex. Interestingly, cholinergic basal forebrain neurons innervate these cells and others of the cerebral cortex, and there were fewer and smaller cholinergic cells in Rett syndrome patients. Furthermore, acetylcholine vesicular transport uptake activity was reduced in the putamen and thalamus, which receive cholinergic input from the brainstem. This finding is counterintuitive in relation to other syndromes, like

Alzheimer's disease and Down syndrome, in which the loss of cholinergic cells is associated with a compensatory increase in acetylcholine uptake activity (Wenk and Mobley, 1997; Wenk, 1997). The link, if any, this altered neurotransmitter activity has to the gene defect of methyl-CpG binding protein 2 (MeCP-2), the mutation responsible for Rett syndrome, is not known. It is possible that transcriptional regulation is involved because MeCP-2 binds to the promoter region of genes that are subject to transcriptional silencing (Amir *et al*., 1999). Perhaps the loss of MeCP-2 is tied to an increase in gene expression specific to apoptosis of cholinergic neurons, or the increased gene expression changes their identity.

Addiction can imitate the function of neurotransmitters, as synthetic strengthening of circuits occurs with the use of certain drugs of abuse. Cocaine is a dopamine and serotonin reuptake inhibitor that leads to an increase in the spine density and branching of pyramidal neuron apical and basal dendrites of the medial prefrontal cortex and the medium spiny neurons of the nucleus accumbens in rats (Robinson and Kolb, 2004). Other excitatory drugs, like amphetamine, have similar effects on these brain regions. Cocaine induced plasticity is persistent, lasting up to a month after the last dose is administered (Robinson *et al*., 2001). The dendritic alterations take place in brain regions associated with incentive motivation and learning, but the exact mechanisms involved with cocaine addiction and withdrawal are not well understood. There is evidence of dysregulation of the G-protein coupled cascades downstream of dopaminergic stimulation of dendrites that inhibits the firing of glutamatergic neurons in the prefrontal cortex (Kelley and Schiltz, 2004). The authors hypothesize that the reduced firing of

these glutamatergic neurons is involved in the drug seeking behaviors of cocaine exposed rats.

2.7 The Role of Hormones in Dendritic Development

Hormones are chemical messengers produced by many tissues, including the endocrine glands. They fall into 3 classes: steroid, amine, and peptides. Amines and peptides bind to their receptors on the neuron surface to change the membrane properties of a cell or to induce intracellular signal cascades. The most common mechanism of action of lipophilic steroid hormones is to directly regulate transcription, as their receptors are nuclear transcription factors.

The role of the mammalian gonadal steroid hormone family in neural development and plasticity has been studied extensively. Steroids exert their effects on the brain much like they do on other sexually dimorphic tissue in the body. The influence that a steroid hormone can have on neuronal dendrites can be both direct, as in the case with the medial nucleus of the amygdala (Gomez and Newman, 1991), and indirect, as in the case of the CA1 region of the hippocampus (Rudick *et al*., 2003). The medial amygdala houses the posterodorsal subnucleas in which neurons have more extensive dendritic branching and length in males than females. This area is involved in male social and sexual behaviors in rodents, and castrated male hamsters demonstrate a loss in dendritic branching and length to display a female morphology. The medial amygdala is sensitive to circulating levels of androgen, but surprisingly the dendrites are regulated by estrodiol. These cells express estrogen receptor and aromatase enzyme,

directly converting the circulating androgens to estrogens to alter dendritic length in a cell autonomous manner (Cooke *et al*., 2003). The dendritic spines of pyramidal cells of the CA1 region of the hippocampus are also influenced by estrogen levels, however, they themselves do not express estrogen receptor. One theory is that hippocampal GABAergic interneurons that do express estrogen receptor may be involved. Estrogen decreases the evoked inhibitory post synaptic potentials of these GABAergic interneurons (Rudick *et al*., 2003), which may, in turn, decrease their inhibitory inputs onto the dendrites of the pyramidal cells, thereby increasing pyramidal cell activity and causing dendritic elaboration. Furthermore, this theory is supported by the fact that cultured hippocampal neurons have an increased spine density in response to the depletion of GABAergic input (Murphy *et al*., 1988).

The spinal nucleas of the bulbocavernosus (SNB) is a cluster of motor neurons in the spinal chord that innervate the muscles involved in male sexual function (Breedlove and Arnold, 1980). These neurons are larger in males and their dendrites express androgen receptor. Castration of male rats results in a marked degeneration of SNB dendrites that can be rescued with the replacement of androgen (Kurz *et al*., 1986). Another sexually dimorphic region of the brain is the ventromedial hypothalamic nucleus, which is larger in female rats and is responsible for coordinating estrous and lordosis behavior in rodents. The dendrites of these neurons not only vary between males and females, but are different among females depending on the phases of the estrous cycle. The spine density of these dendrites is doubled in proestrous (high estrogen) as compared to diestrous (low estrogen) (Madeira *et al*., 2001).

If hormones can alter the development of dendritic morphology, what is the effect of this altered morphology on the function of the neuron? The differentiating dendrites are ultimately part of a neuronal circuit and a change in one part of the nervous system has functional implications on the rest of the system. This point is illustrated by the regions of the bird brain that are responsible for birdsong. The song nuclei of the thalamus develop in response to androgens in male song birds during development, and thus are sexually dimorphic. The androgens not only result in an increase in neuron size and survival rate, but also increase neural connectivity. A vocal motor pathway from the HVc to the robust nucleus of the arcopallium (RA) develops in males but is lacking in non-androgen exposed females (See Figure 4). Androgen deprived males do not develop this vocal motor pathway and are unable to produce birdsong (Konishi and Akutagawa, 1985).

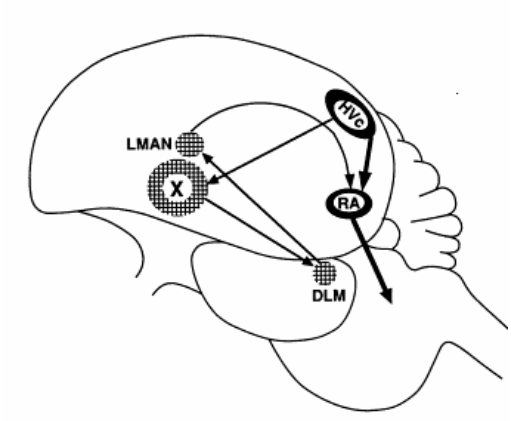


Figure 4 Diagram depicting the vocal motor pathways that develops between the nuclei of the thalamus in male song birds. The neural connectivity does not develop in androgen depleted males (Konishi and Akutagawa, 1985).

Hormone regulated dendritic plasticity has been studied at length in insect metamorphosis. During metamorphosis, larval tissues, including motor neurons, muscles and sensory neurons, either remodel to become functional adult structures (Consoulas *et*

al., 2000) or degenerate because they only serve a function in the larva (Weeks and Truman, 1985). Programmed cell death of tissues fated to die during metamorphosis can be reproduced *in vitro* through administration of ecdysone to cultured cells (Levine and Weeks, 1996) This provides evidence that cell death is controlled by intrinsic genetic programs as isolated cells are able to respond appropriately to hormonal cues. The larval-to-pupal-to-adult transitions involve steroid hormones, including ecdysone and juvenile hormone (Riddiford, 1980). The effects are dependent on developmental time points as the receptors are expressed transiently (Riddiford, 1993). This expression coincides with increases in steroid hormone concentration in the animal (Richards, 1981), but the receptor expression is not directly regulated by its steroid ligand (Zheng *et al*., 2003). Larva deficient in steroid receptors results in lethality.

Class I sensory neurons remodel during metamorphosis to become ddaE sensory neurons in the adult, and juvenile hormone is necessary for this transition (Williams and Truman, 2004). The steroid hormones exert their effects through initiating the expression of several early and late response genes (Hurban and Thummel, 1993). Dendritic remodeling and growth of the larval motor neurons that persist during metamorphosis is dependent on the ecdysone-dependent immediate early gene, *Broad Complex* (Consoulas *et al*., 2005). The *Broad Complex* then initiates the transcription of several late genes, including transcription factors that may eventually be involved in remodeling (Guay and Guild, 1991).

There is evidence of crosstalk between growth factors and steroid hormone receptor expression. As previously mentioned, the Lee group found that mutations of the TGF-β receptor, *Baboon*, result in deficient dendritic remodeling of *Drosophila* mushroom body neurons during metamorphosis. Mutations of the TGF-β downstream effecter, *d-Smad-2*, result in the same phenotype. They further discovered that the expression of ecdysone receptor EcR-B1 at the beginning of metamorphosis requires normal activity of *Baboon* and *d-Smad-2*. Mutational dendritic phenotypes could be rescued through ectopic expression of the EcR-B1 receptor (Zheng *et al*., 2003).

Changes in hormone secretion that regulate dendritic plasticity occur in the mature nervous system as well, and can be a result of external factors. As mentioned previously, restrained rats undergo temporary dendritic spine loss in the hippocampus. This stress also results in a decrease in total length and branch number of apical pyramidal neuron dendrites in the CA3 region of the hippocampus. This effect can be mimicked in a stress free environment with the administration of corticosterone (Watanabe *et al*., 1992).

2.8 Cell-cell Contact

Physical contact between dendrites and the axons that innervate them can lead to the formation of spines and synapses. Dendro-dendritic contacts result in competition that inhibits the overgrowth of dendrites of neighboring cells. The release of signaling molecules may be involved, but it appears that physical interaction is the initial stimulus

for dendritic alterations in some neuron types (Ferreira and Paganoni, 2002; Umeda and Okabe, 2001).

Both *Flamingo*, a G-coupled receptor like protein, and the transcription factor *Hamlet* are responsible for dendritic tiling of type IV tactile sensory neurons in *Drosophila*. Mutant embryos and larvae display an overgrowth of dendrites outside the typical tiling parameters that result in dendritic overlap and a loss of tiling. Ablation of homologous neurons also results in a loss of dendritic tiling, suggesting a homotypic cell contact repellant mechanism. This competitive mechanism keeps the epidermis from being covered by more than one set of sensory neuron dendrites (Gao *et al*., 2000).

Dendritic tiling also takes place in the retina of many organisms. The dendrites of different neuronal subclasses, including several different types of retinal ganglion cells, are found to tile (Logan and Vetter, 2004). Tiling in this system, however, does not necessarily occur from like cell repellant mechanisms, but perhaps from interactions among different neuron types. Using retinal ganglion cell specific mutants, Lin *et al*., (2004) found that ablation of ~80% of retinal ganglion cells did not result in an overgrowth of dendritic fields of the remaining cells compared to controls. They suggest that one possible reason for this is that tiling occurs due to interactions of the retinal neurons with other cell types, including amacrine neurons.

Establishment of Neuron Structure

Thus far, this dissertation has dealt with dendritic growth and plasticity, and the functional and structural differences among dendrites of different neuron types. Now I will discuss the unique properties of dendrites and the processes that are involved in initial dendritic sprouting, growth and maturation. Neurons, like many cell types, are compartmentalized. Just as an absorptive enterocyte has basolateral and apical membranes that each house a unique set of transport proteins vital to its function in the intestine, neuronal axons and dendrites are fundamentally different in structure, function, and protein expression. The establishment of polarity that takes place early during embryogenesis is influenced by both genetic factors and external stimuli.

The polarity of neurons was not immediately apparent with their discovery by Camillo Golgi in 1873, who described the microanatomy of the nervous system as a continuous web, much like that of the circulatory system. Spanish anatomist Santiago Ramon y Cajal, however, drew very different conclusions from the techniques used by Golgi. He proposed the neuron doctrine, which states that the nervous system is composed of individual, polarized cells that communicate with each other through specialized connections (Glickstein, 2006). This set the theoretical foundation for the study of differences in neuronal compartments, establishment of polarity, and the development of the synapse.

2.9 Establishment of Polarity

Just as in the regulation of dendritic plasticity of a mature neuron, intrinsic genetic programs, extrinsic growth factors and cellular interactions have been implicated in the

sprouting and identity of dendrites. The intrinsic properties of the cell that are necessary for polarity to occur include the actin and tubulin networks (the framework of a neuronal process), the placement of certain organelles, the activity of some signal cascades and the presence of cell adhesion molecules on the surface of the developing neurons. Extrinsic polarizing factors include signals of certain growth factors and the extracellular matrix.

A large body of work surrounds the development of rat embryonic hippocampal neuron dendrite development. Pyramidal neurons, when dissected and placed into culture, maintain their polarity, as detected by electron microscopy and compartment specific microtubule-associated proteins (described in section 2.10). Dotti and colleagues (1988) have created a 5 stage assay for the development of their polarity in the hippocampal culture preparations that has become standard in the literature. Stage 1 is characterized by the adherence of the neuron to substrate followed by stage 2 where several short neurites begin to grow. During stage 3, one neurite grows rapidly and begins to take on the appearance of an axon, while the remaining neurites become dendrites. Throughout stage 4 the dendrites as well as the established axon continue to grow and stage 5 is accompanied by the maturity of the neuron as the dendrites develop spines (Craig *et al*., 1993) and synapse formation takes place (Fletcher *et al*., 1991). Using this system, it was found that the cytoskeletal stability determines axon formation, as application of cytochalasin D to the cultures resulted in multiple axon formation and axons forming from established dendrites (Bradke and Dotti, 2000). The investigators found that the localization of microtubule associated proteins changed upon addition of cytochalasin D, ruling out the possibility of non-specific toxic effects. They found that

MAP2 was no longer present in the dendrites that had become axons while Tau was. This suggests that the cells are healthy, under normal regulation and that the dendrites are actually taking on the form of axons. Since the establishment of polarity in these neurons takes place in a cell autonomous fashion, it was assumed that there is an intrinsic genetic program dictating their compartmentalization, although there is evidence of extrinsic control as well.

The centrosomes, golgi apparatus and endosomes are all polarized in these developing neurons and congregate at the site of axon spouting. Cultured hippocampal neurons treated with nocodazole, to prevent microtubule assembly and disrupt trafficking of organelles, or treated with brefeldin A to block protein cycling to the golgi apparatus, do not differentiate (de Anda *et al*, 2005). The investigators then utilized the transgenic system of *Drosophila* to observe the necessity and sufficiency of centrosome placement in polarity development and to investigate the authenticity of the brefeldin A and nocodazole findings. They over-expressed GFP-centrosomin in all neurons *in vitro* and then used chromophore assisted light inactivation to block all centrosome mediated functions through laser illumination of centrosomal-GFP areas in neurons until the GFP signal was reduced to irradiate the centrosomes. They observed lack of axon outgrowth in undifferentiated neurons and lack of axon growth in differentiated cells, suggesting that the centrosome plays an essential role in polarity development.

Just as activity can regulate actin and tubulin dynamics, as discussed in section 2.4, the development of polarity within the central nervous system has been linked to the

regulation of the cytoskeletal and microtubule networks. For example, a mutation in the *Kakapo* gene (AKA the *Short stop*), which is a *Drosophila* actin-microtubule binding cytoskeletal associated protein, is necessary for dendritic sprouting of embryonic *Drosphila* RP3 motor neurons in vivo (Prokop *et al*., 1998). *Roadblock*, a dynein light chain member of the microtubule motor protein family, is necessary for development of normal dendritic density of *Drosophila* mushroom body dendrites (Reuter *et al*., 2003). MAP2 is necessary for normal cytoskeletal dynamics of mouse cerebellar Purkinje cell dendrites *in vivo*. Knock out mice reveal thinner crossbridges and fewer globular structures in the microtubule skeleton compared to controls (Harada *et al*., 2002).

A convoluted signal cascade involved in the regulation of microtubules for axon sprouting and axon identity has been discovered over the course of many different studies. A study from the Jan group describes the polarized distribution of Par3, Par6, and atypical protein kinase C in the axon of the embryonic rat hippocampal neurons and a necessity for their activity in the establishment of polarity. These three proteins form a complex that activates a dynein/kinesin motor complex that is associated with microtubules. They found that the Par3/Par6/aPKC complex is activated by growth factor tyrosine kinase receptor and its downstream activator PI3-kinase, which are both also required for axon sprouting (Shi *et al*., 2003). Yoshimura et al., 2005 then built on this cascade to show, not only that MAPK is upstream of PI3-kinase activation, but that PI-3 kinase also regulates polarity development by activating the glycogen synthase kinase-3 (GSK-3)/collapsing response mediator protein-2 (CRMP-2) cascade, which is necessary for axon sprouting as well. It had been described earlier that CRMP-2 binds to

tubulin dimers to promote their incorporation into the microtubule network and that this is involved in axon identity. Over-expression of CRMP-2 changes a neurite of dendritic morphology to that of an axon (Fukata *et al*., 2002). A possible additional link between these two studies is the finding that CRMP-2 bound to tubulin is transported by kinesin (Kimura *et al*., 2005). Perhaps the Par3/Par6/aPCK complex works with CRMP-2 by activating the kinesin motor protein that will transport it to the axon, where it unloads the tubulin needed for the microtubule structure of the axon to be built.

Extrinsic influences on neuronal compartmentalization involve the extracellular matrix, cell adhesion molecules, and growth factors/guidance molecules. Embryonic hippocampal pyramidal neurons, when grown on certain substrates including neuron-glia cell adhesion molecule (NgCAM) and N-cadherin, are more likely to sprout an axon, and axon growth rate is increased compared to counterparts grown on poly-L-lysine (PLL). This study also found that the largest dendritic arbors and dendritic branch number occurred when neurons were grown on NgCAM, but not N-cadherin or PLL (Esch *et al*., 2000). Axon differentiation is promoted by laminin, which increases the rate of polarization, length of axons and axon branching without an influence on dendrites (Lein *et al*., 1992). Laminin is a part of the extracellular matrix that is recognized by integrins in the neuronal membrane. Dendritic sprouting and growth of rat embryonic sympathetic neurons, in contrast, was enhanced when grown on substrate containing bone morphogenic protein 7 (Lein *et al*., 1992). Other external factors, such as the proteins involved in axon and dendrite guidance, are also important for dendritic sprouting. Normal dendritic sprouting of the RP3 and aCC motor neurons in *Drosophila* require the

Commissureless (membrane receptor involved in attractant/repulsive signaling) and *Frazzled* (a receptor for netrin) proteins, as mutations in these genes resulted in frequent abnormal dendritic sprouting. These proteins work in a cell autonomous manner, as cell specific rescue resulted in normal dendritic sprouting (Furrer *et al*., 2003).

2.10 Dendrite Specific Structural Traits

The structure of dendrites contributes to their functional differences from axons. Dendrites are sites of spines and post-synaptic density that exhibit site specific protein accumulation. Dendritic spines increase the connectivity of neurons, as they are sites of >90% of the excitatory synapses in the mammalian nervous system (Harris, 1999). The extent of dendritic branching can influence the number of sites available for synaptic transmission and enable sophisticated processing by a neuron. The dendritic endoplasmic reticulum provides chemical compartmentalization for Ca^{+2}, and protein trafficking allows the presence of transcriptional machinery and RNA (Pierce *et al*., 2001). Dendrites house the post synaptic density, which is the antithesis to active zones in an axon. While the active zone houses synaptic vesicles filled with neurotransmitters, voltage gated calcium channels, synaptic docking proteins and neurotransmitter reuptake pumps, the dendritic post synaptic density contains neurotransmitter receptors, ion channels, ion pumps, and molecules, such as G-proteins, that are involved in the activation of signal cascades.

How do these specific proteins target to the axon and dendritic compartments? Elegant electron microscopy studies have revealed that the microtubule dynamics of

axons and dendrites vary. Coincidentally, this gives investigators the ability to distinguish neuronal polarity *in vitro* in order to study cell autonomous determinants of polarity. The "hook method" was established in which neurons are exposed to excess tubulin, which binds to existing microtubules in protofilament sheets that look like hooks, off of the microtubules. A clockwise hook indicates that the plus end of the microtubule faces the observer and vice versa (Heidemann and McIntosh, 1980). This method was first shown to determine neuronal polarity by the detection of plus end distal microtubules in axons of cat postganglionic sympathetic fibers *in vivo* (Heidemann *et al*., 1981). Using this method in cultured hippocampal neurons, it was further demonstrated that the most prominent "axon-like" process also has plus-end microtubules directed distally in axons (see Figure 5c). The remaining "dendritic" processes were comprised of a mixture of both plus-and minus-end microtubules directed distally (see Figure 5b). The plus ends of microtubules are dynamic and unstable, while the minus ends are more static (Baas and Black, 1990).

Not surprisingly, microtubule motor proteins segregate between axons and dendrites depending on which direction they are directed along microtubules. Other proteins that are transported by these motor proteins, like MAP2 and Tau, also segregate and this has been used to determine the identity of axon and dendrites *in vitro*. While

electron microscopy studies have not been done with insect neurons to verify

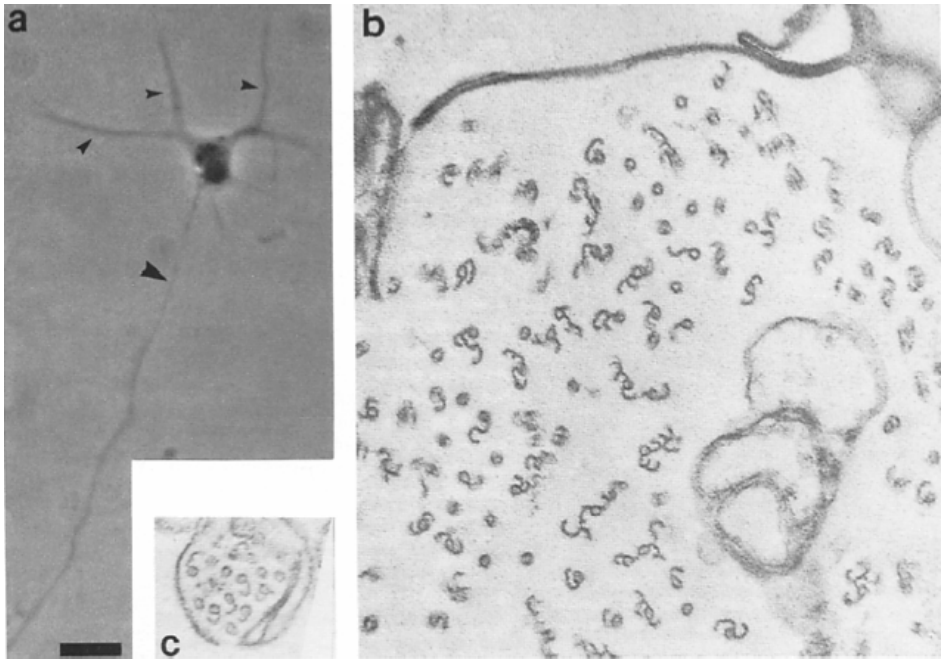


Figure 5 Electron microscopy using the hook method to detect the direction of microtubules in dendrites and axons of cultured rat hippocampal neurons. A) small arrowheads point to dendrites and large arrowhead points to axon. B) a cross section of the axon reveals both clockwise and counter-clockwise hooks representing minus and plus end distal directed microtubules, respectively. C) a cross section of a dendrite displays only clockwise, or minus end distal directed microtubules (Baas et al., 1989).

that the microtubule system is the same, sensory neurons *in vivo* display polarity, with kinesin (plus end directed motor protein) directed to axons (see Figure 6d-f) and nod (minus end directed motor protein) directed to dendrites (see Figure 6a-c). Why kinesin is only transported to the plus end microtubules of axons and not along those of dendrites is not understood. It was recently found that nod, as well as other transport specific molecules, segregate specifically to *Drosophila* MN dendrites *in vivo* as well (Sanchez-Soriano et al., 2005).

While this evidence suggests that the insect microtubule system is similar to that of vertebrate systems, it is not known if insect neurons also maintain polarity in culture. Many of the reagents that are used to verify vertebrate polarity in vitro, such as antibodies for MAP2 (Pennypacker *et al*., 1991) and tau (Mandell and Banker, 1995), are not useful in insects due to limited information about microtubule associated proteins. The

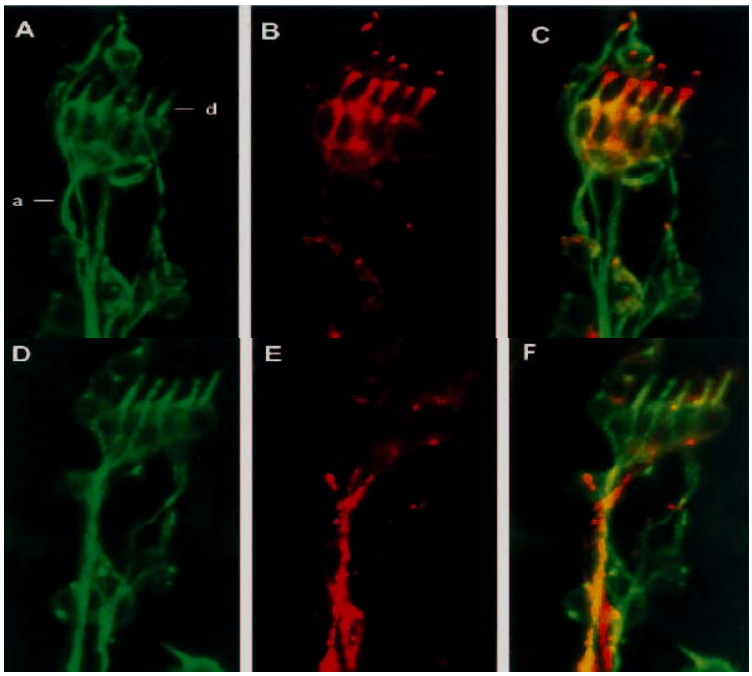


Figure 6 The plus-end directed microtubule motor protein kinesin-LacZ localizes to only the axon of *Drosophila* chordotonal sensory neurons *in vivo* (e) while the minus-end directed microtubule motor protein nod-LacZ localizes to only the dendrites (b) (Clark *et al*., 1997).

detection of insect dendrites in vitro would be a useful and powerful technique for studying the influences on neuronal polarity, dendritic growth and branching when combined with the transgenic system available in *Drosophila*. This issue will be addressed in Chapter 5, where I report the localization of plus and minus-end directed motor proteins in *Drosophila* motor neuron neurites *in vitro*.

CHAPTER 3 RATIONAL AND GOALS OF THIS DISSERTATION

3.1 Important Questions This Dissertation Will Address

We have seen in this overview the importance of dendritic plasticity during embryonic and post-embryonic development and the problems caused when plasticity is not carried out correctly with disease and other disorders. This dissertation will explore the cell autonomous mechanisms of *Drosophila* motor neuron dendritic growth. Given the advantages of the *Drosophila* transgenic system and the extensive work done at the neuromuscular junction, I wanted to characterize motor neuron growth *in vitro* and determine if depolarization through both pharmacological and transgenic means could induce growth in these neurons. This meant first developing a GAL4 line that is specific to motor neurons and establishing a method for quantification in culture. I also wanted to determine if *Drosophila* neurons maintain polarity *in vitro* and to design a methodology for the observation of cell autonomous influences on motor neuron dendritic growth. I next wanted to determine the signaling mechanisms that are involved in basal and depolarization-induced dendritic growth, exploring the candidate proteins that have already been found to be involved in plasticity at the *Drosophila* neuromuscular junction.

Chapter 4 will introduce the transgenic manipulation utilized to gain a motor neuron specific driver. I found that these motor neurons grow in culture, and that depolarization results in neurite outgrowth. I will also present the requirement of calcium current in depolarization induced growth. Chapter 5 characterizes the nature of depolarization induced outgrowth with timeline studies designed to observe chronic vs. acute effects of depolarization. Here I will also present a novel assay for the

differentiation of *Drosophila* axon and dendrites *in vitro* using polarity reporter proteins. Chapter 6 will discuss the effects of depolarization on motor neuron dendrites *in vivo* and explore transcription factor involvement in depolarization induced outgrowth.

3.2 The strengths of *Drosophila* for the study of dendritic plasticity

Like most invertebrate models, the *Drosophila* nervous system provides a simplicity not found in mammals, and most mammalian proteins have homologues in *Drosophila*. *Drosophila* reproduce rapidly and are relatively inexpensive to maintain. *Drosophila,* in particular, provide many advantages not found with other invertebrate models. The entire genome is mapped, there are multiple mutant alleles for many genes and an extensive transgenic system is available. Furthermore, there is a very open and sharing community.

The *Drosophila* GAL4-UAS transgenic system was created by Brand and Perrimon in 1993. The system was designed to ectopically express a protein of interest in a specific tissue type. This system utilizes the transcription factor for the Galactose-4 (GAL-4) protein and its upstream activation sequence (UAS) isolated from yeast. This protein is not endogenous to the fly genome. The GAL4 DNA is placed into the fly genome upstream of a tissue specific promoter in one fly line (AKA "the driver"). The UAS DNA is placed upstream of a gene of interest and placed into a plasmid that is inserted into the genome of another fly line (AKA "the responder"). These lines alone do not result in any ectopic gene expression. The gene downstream of UAS is only expressed when the lines are crossed so that the first generation progeny posses both

Transgene	Purpose	Source
C380-GAL4,UAS-CD8-GFP;;cha-GAL80	C380-GAL4 - Enhancer-trap line used to label motor neurons	Budnik Lab (Sanyal et al., 2003)
	UAS-CD8-GFP - Membrane bound form of GFP	Bloomington Stock Center
	Cha-GAL80 - Block GAL4 activity in cholinergic neurons	Kitomoto, 2002
Ore-R	Provide wild-type copy of genome	Bloomington Stock Center
w;UAS-sh(DN),UAS-eag(DN)	UAS-sh(DN) - dominant negative shaker (truncated form of S1 transmembrane domain)	Mosca et al., 2005
	UAS-eag(DN) - dominant negative ether-a-go-go (truncated hydrophylic N-terminus)	Broughton et al., 2004
UAS-2xEKO	shaker channel with inactivation domain removed	White et al., 2001
w;;UAS-GCaMP	GFP fused to calmodulin for Ca+2 imaging	Reiff et al., 2005
UAS-kin-GFP(III)	axonal reporter protein	Estes, unpublished
yw;;UAS-nod-LacZ	dendritic reporter protein	Clark et al., 1997
w;;UAS-kin-GFP,UAS-nod-LacZ	recombinant line to simultaneously identify axon and dendrites	Estes, unpublished, Clark et al., 1997
GSG-elav-GAL4;;cha-GAL80	GSG-elav-GAL4 - Neuronal GAL4 activated by RU-486	Osterwalder et al., 2001
	Cha-GAL80 - Block GAL4 activity in cholinergic neurons	Kitomoto, 2002
w;UAS-fbz	fos dominant negative (truncated protein including bZIP domain)	Eresh et al., 1997
w;;UAS-jbz	jun dominant negative (truncated protein including bZIP domain)	Eresh et al., 1997
w;UAS-fos,UAS-jun	Overexpression of AP-1	Eresh et al., 1997
w;;UAS-Adf-1	Overexpression of Adf-1	DeZazzo et al., 2000
w;;UAS-Adf-1?1	Adf-1 dominant negative	Tully lab, unpublished
w;UAS-CREB2b	Overexpression of CREB inactivation protein 2b	Eresh et al., 1997
UAS-CREB-2a(III)	Overexpression of CREB activation protein 2a	Boutianella, unpublished
w;RN2-GAL4,UAS-CD8-GFP;act<<spacer<<GAL4,UAS-flp	RN2-GAL4 - eveneskipped driver line	Fujioka et al., 2002
	UAS-CD8-GFP - Membrane bound form of GFP	Bloomington Stock Center
	act<<spacer<<GAL4 - actin GAL4 seperated by flippout sights	Bloomington Stock Center
	UAS-flp - flippase that targets flippout sights	Bloomington Stock Center
w;UAS-sh(DN),UAS-eag(DN);UAS-fbz	UAS-sh(DN) - dominant negative shaker	Mosca et al., 2005
	UAS-eag(DN) - dominant negative ether-a-go-go	Broughton et al., 2004
	UAS-fbz - fos dominant negative	Eresh et al., 1997

Table 1 A list of genetic tools utilized in this dissertation. The list includes the name of the line, its experimental purpose, and its source.

GAL4 and UAS transgenes. The transgenic tools utilized in this dissertation are outlined in Table 1.

Further advances have been made to allow not only spatial tissue specific control of gene expression, but temporal control with the use of conditional GAL4 drivers as well (Fig 1). The Gene Switch system consists of a GAL4 protein fused to the hormone binding portion of the progesterone receptor. This GAL4 only initiates transcription at the UAS site in the presence of RU-486 (Osterwalder *et al*., 2001). The TARGET (temporal and regional gene expression targeting) system utilizes a temperature sensitive GAL80 molecule that binds to GAL4 and prevents its activity at low temperatures (McGuire *et al*., 2004).

The MARCM (mosaic analysis with a repressible cell marker) system was developed to create a small number of homozygous mutant cells in a heterozygous tissue background, through the recombination of single copy mutations in progenitor cells (Lee and Luo, 2001). The mother cell contains one chromosome with a mutation, a GAL4 subunit, a UAS subunit upstream of a reporter protein and another chromosome with a GAL80 protein that prevents GAL4 transcription. The single mutation does not affect this cell because often one wild type allele present in a cell is enough for normal function. During mitotic recombination, the GAL80 region of one set of chromosomes randomly recombines with the GAL4/UAS/mutation region of the other set of chromosomes. This results in a daughter cell homozygous for the mutation with the absence of GAL80 for

the transcription of the reporter protein (see Figure 2). This allows the study of protein function in single cell clones.

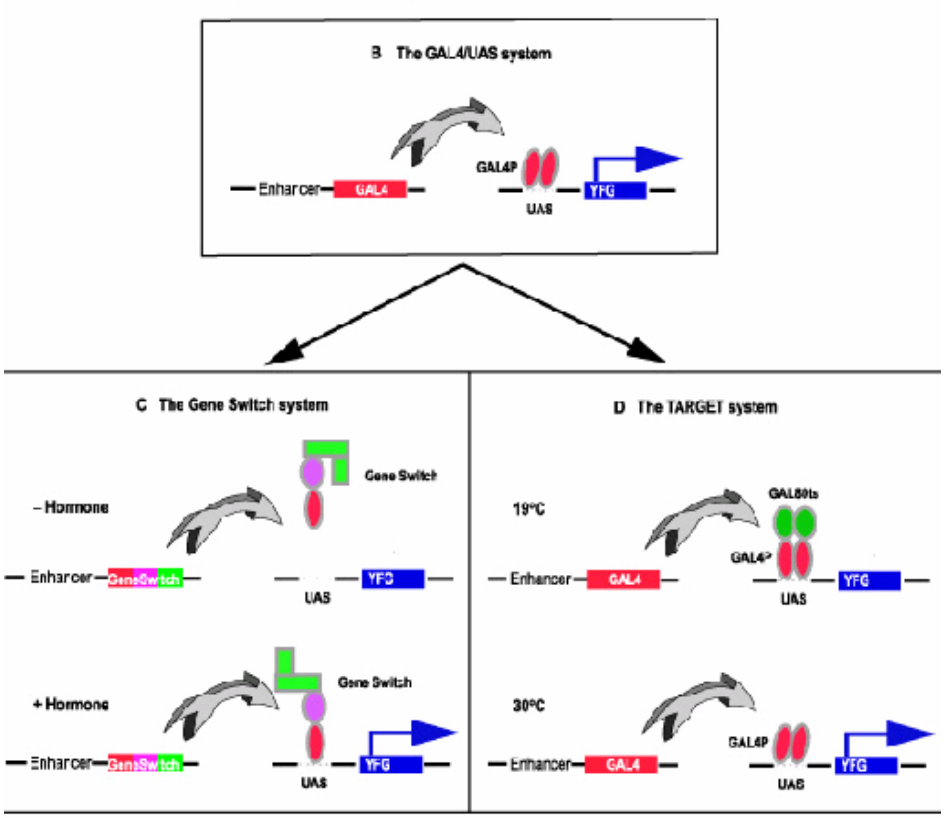


Figure 1 Depiction of the manipulations used to achieve both spatial and temporal gene expression within the *Drosophila* transgenic system. The Gene Switch system utilizes the ligand binding domain of the progesterone receptor that prevents transcription by GAL4 until bound to RU-486, the progesterone antagonist. The TARGET system uses a temperature sensitive GAL80 protein that binds to GAL4 and prevents its activity until heat-shocked (McGuire et al., 2004).

The study of the *Drosophila* model provides many advantages outside of the transgenic system. As touched on in the last three chapters, *Drosophila* motor neurons are well characterized and have extensive and consistent dendritic branching patterns (Landgraf *et al*., 2003; Choi *et al*., 2003). Both intrinsic genetic factors and extracellular signaling molecules are known to control dendrite morphogenesis in *Drosophila* (Gao

and Bogert, 2003), making them ideal to study dendritic plasticity. *Drosophila* neurons *in vitro* maintain their electrical properties and responses to extracellular cues (Jiang *et al*., 2005; Kraft *et al*., 1998; Levine and Weeks, 1996).

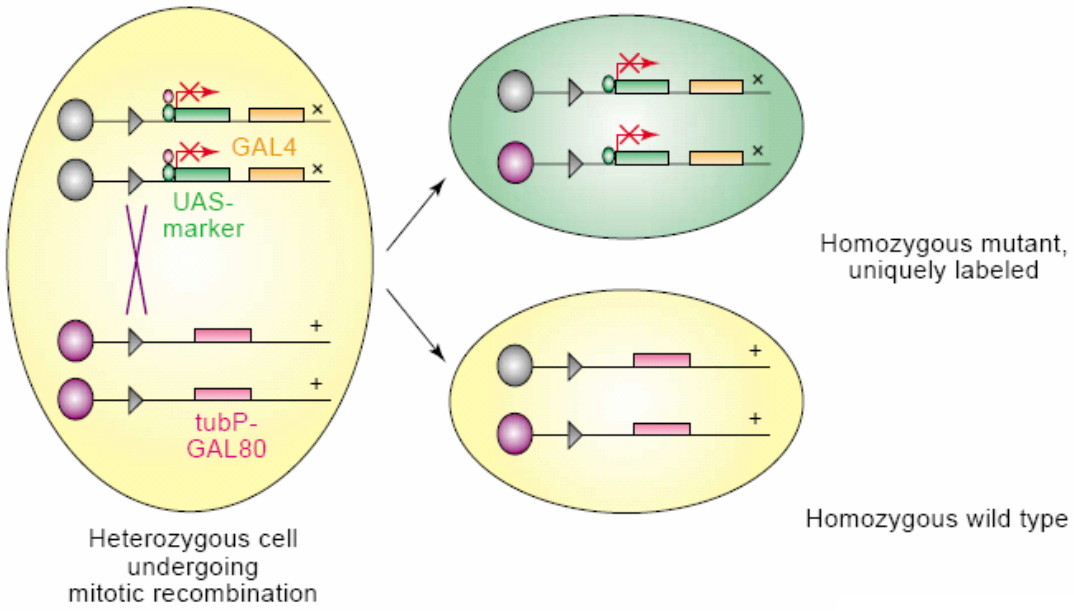


Figure 2 Diagram depicting the MARCM (mosaic analysis with a repressible cell marker) system to generate single cell mutant clones using the *Drosophila* transgenic system. Each cell has a copy of GAL4, UAS ustream of a reporter, a mutation, and GAL80 downstream of a tubulin promoter so that GAL4 activity is blocked in every cell type. This prevents the formation of a homozygous mutant until random recombination lands the GAL4, UAS and mutation all in one cell and excludes the GAL80 (Lee and Luo, 2001).

CHAPTER 4 DEPOLARIZATION INDUCED DENDRITIC GROWTH OF *DROSOPHILA* IDENTIFIED MOTOR NEURONS IS DEPENDENT ON I_{Ca}^{+2}

* *Please note*: All of the electrophysiology work presented here was performed by Richard Levine, Ph.D.

4.1 Abstract

Activity dependent changes in dendrites have been implicated in many essential functions of the nervous system, including learning and memory. Here we demonstrate genetic or pharmacological manipulations that cause depolarization lead to neurite outgrowth of identified *Drosophila* motor neurons *in vitro.* This growth is dependent on I_{Ca}^{+2}. Neurite outgrowth can be induced either by increasing levels of KCl in the culture medium or genetically manipulating the expression of K^+ channels. I_{Ca}^{+2}, not I_{Na}^{+}, is necessary for this depolarization induced growth, as Co^{+2}, not TTX, blocked the observed growth. More specifically, blocking *Plectreurys* toxin sensitive voltage gated I_{Ca}^{+2} leads to significantly reduced dendritic growth. These results suggest that Ca^{+2} entry and the cellular processes it initiates are important for depolarization-dependent growth.

4.2 Introduction

Dendritic plasticity allows precise development of synaptic connectivity and post-embryonic modifications of neuronal function. Intrinsic genetic factors (Jan and Jan, 2003), extracellular factors (Schinder and Poo, 2000), and activity (Chen and Ghosh, 2005) all influence dendritic plasticity. The activity of a neuron can be altered through spontaneous changes in membrane potential, or synaptic input. Ca^{+2} is involved in dendritic plasticity in both scenarios through its activation of diverse signal cascades (Konur and Ghosh, 2005).

There are many examples of dendritic elongation and branching in response to synaptic input (Libersat, 2005). Depolarization in vivo in response to synaptic input can be mimicked in vitro through alterations in the K^+ concentration of the culture medium, direct stimulation of the cell with an electrode, genetic manipulation of ion channel expression, or exposure of the cell to neurotransmitters. For example, Vaillant and colleagues (2002) demonstrated that dendritic elaboration of rat sympathetic neurons could be induced through depolarization with increased K^+ in the culture medium, a constant 60mA current delivered at 5 Hz to the cells, or the addition of the cholinergic agonist, carbachol. This synthetic synaptic input can lead to Ca^{+2} entry through voltage sensitive Ca^{+2} channels or ligand-gated channels. In some cases this initial Ca^{+2} entry leads to calcium-induced-calcium-release from intracellular stores (Lohmann and Wong, 2005). Cytoplasmic Ca^{+2} behaves as an intracellular signaling molecule, and has been shown to activate several kinases and adenylyl cyclases in the nervous system (Ghosh and Greenberg, 1995). This, in turn, can activate the expression of immediate early

genes, such as CREB and fos (Sheng *et al*., 1991) and delayed response genes, such as those encoding certain neurotrophins and growth factors that may be involved in LTP (Ghosh *et al*., 1994; Birren *et al*., 1992).

Depolarization induced dendritic growth has been demonstrated in many different neuron types, including cultured granule neurons (Gaudilliere *et al*., 2004), hippocampal neurons (Wu *et al*., 2001; Szebenyi *et al*., 2005), and sympathetic neurons (Vaillant *et al*., 2002). In each case the depolarization induced growth was reversible after 2 days of no stimulation. Furthermore, I_{Ca}^{+2} was necessary for the depolarization induced dendritic growth, as treatment of the cultures with nimodipine, a voltage gated calcium blocker, prevented growth.

Drosophila is a valuable model for the study of dendritic growth. The extensive GAL4-UAS transgenic system allows the identification of specific neurons *in vitro* and *in vivo* (Brand and Perrimon, 1993). *Drosophila* motor neurons are well characterized and have extensive and consistent dendritic branching patterns (Landgraf *et al*., 2003; Choi *et al*., 2004). Both intrinsic genetic factors and extracellular signaling molecules are known to control dendrite morphogenesis in *Drosophila* (Gao and Bogert, 2003), making them ideal for the study of dendritic plasticity. *Drosophila* neurons *in vitro* are able to maintain their electrical properties and responses to extracellular cues (Jiang *et al*., 2005; Kraft *et al*., 1998; Levine and Weeks, 1996).

Drosophila neurons have I_{Ca}^{+2}. Embryonic *Drosophila* motor neurons display voltage sensitive calcium I_{Ca}^{+2} *in vivo*, although the functional implications of this current

on these specific neurons are unknown (Baines *et al*., 2001) and it is not clear whether the I_{Ca}^{+2} is maintained at later stages. Here we demonstrate that larval motor neurons also have I_{Ca}^{+2} *in vitro* and that these currents are involved in neurite growth. In the discussion we will describe preliminary findings of I_{Ca}^{+2} in larval motor neurons *in vivo* as well. Pupal *Drosophila* Kenyon cells *in vitro* generate spontaneous increases in intracellular calcium levels that are dependent on voltage gated calcium channels rather than release from intracellular stores, however, their functional implications have only been speculated (Jiang *et a*l., 2005).

Calcium has not been directly linked to depolarization dependent growth in *Drosophila* neurons. Here we demonstrate the role of calcium currents in neuronal growth. We describe a system for depolarization induced dendritic outgrowth that is dependent on voltage gated calcium current, independent of sodium based action potentials.

4.3 Methods

Fly Strains

Larvae were selected from the cross of homozygous female C380GAL-4,UAS-CD8-GFP;;chaGAL80 and males of the Ore-R wild-type stock, the *shaker* and *ether-a-go-go* double dominant negative UAS line (UAS-sh-DN/eag-DN) (Mosca et al., 2005; Broughton et al., 2004), or the electrical knock out UAS line (UAS-2xEKO) (White *et al*., 2001). The UAS-shDN/eag-DN line (AKA the EKI line) expresses dominant negative subunits of the *shaker* and *ether-a-go-go* voltage gated K^+ channels, which prevent their normal function and manipulate I_{K^+} of the cell membrane. The electrical knock out (EKO) is a construct of the *shaker* channel that has removed the voltage gated inactivation portion of the protein, resulting in a constitutively open channel that acts as a current shunt. This hyperpolarizes the membrane potential (White *et al*., 2001). We used a line that has two copies of the trangene inserted in chromosome 2. The C380GAL-4,UAS-CD8-GFP;;chaGAL80 driver causes expression of GFP in identified motor neurons of the thoracic abdominal ganglion due to the UAS-CD8-GFP.

Primary Cell Cultures

Culture protocols were as described previously (Kraft *et al*., 1998) with a few modifications. All dissection equipment was soaked in 70% EtOH for 10 min before use. Late 3rd instar larvae were selected and sterilized by placing the animal in 70% EtOH for 1 min. The larvae were rinsed 3 times in sterile water before the brains removed in sterile *Drosophila* Medium (Schneiders medium + 10% fetal bovine serum + 50 μg/ml insulin). The cephalic lobes were discarded and only the thoracic abdominal ganglion was used in

cultures. The ganglia were microdissected to remove ventral lying GFP positive neurons that have not been identified. The ganglia were treated enzymatically in liberase for 1 hour at room temperature (10 µl 2x liberase stock/ 1ml Rinaldinise saline [800 mg of NaCl + 20 mg of KCl + 5 mg of $NaH_2PO_4*H_20$ + 100 mg of $NaHCO_3$ + 100 mg of glucose + 100 mls of H_2O]). The ganglia were rinsed twice in 1 ml *Drosophila* Medium, triturated with a fire polished glass Pasteur pipet, further triturated with an Eppendorf tip and plated on Con-A/Laminin coated dishes in a 100 µl bubble. The cells were plated at a density of 1 microdissected ganglion/dish. The dishes were flooded 24hrs later with 1 ml *Drosophila* Medium and imaged 24hrs after flooding. The cells were maintained in a 25^{o}C, humidified incubator.

The culture dishes were made by drilling an 8 mm hole in 35 mm Petri dishes (Corning 430165). A 12 mm round coverslip (Bellco 1943-10012) was glued under the Petri dish with Sylgard 184 to create a well. The dishes were placed under UV light for 2 hrs to sterilize. The well was coated with 70 µl of ConA (Sigma C-2010) + laminin (Becton-Dickinson 354232) + milliQ H_2O and incubated for 2 hrs at 37^{o}C. Dishes were then rinsed with 6ml sterile H_2O and used within 1 month.

The motor neurons in the high K^+ experiments were fed medium with [K^+] of 32 mM, which is ~10 mM greater than the concentration in the *Drosophil*a culture medium (21 mM). This will theoretically depolarize the neurons by ~10mV according to the Nernst equation (the intracellular [K^+] in insect neurons ranges from 100-400 mM):

Control medium $E_K = 60 \log \frac{21.46}{400} = -76$ mV

High K^+ medium $E_K = 60 \log \frac{32.19}{400} = -67$ mV

change by +10mV

1.6 g/L is the [KCl] in drosophila culture medium

2.4 g/L is the [KCl] in high K^+ experiments

74.55 g/mol is the molecular weight of KCl

Cell Analysis

The cells were imaged live with a Hamamatsu c4742-96 camera using Wasabi software version 1.4 on a Nikon inverted scope at 60x. Both phase/contrast and fluorescent images were obtained for each cell, and the motor neurons had to meet a set of criteria in order to be imaged: alive and healthy with no signs of blebbing or lifting off the dish, bright GFP signal representing a high level of UAS expression, and isolation from neurite interactions with other neurons. 15-20 Tiff image files were then analyzed for each experimental group by tracing the fluorescent image by hand on a Wacom tablet PC. The parameters of total neurite length, total branch number, higher order branching patterns and cell body circumference were recorded using Simple PCI software.

Immunocytochemistry

Immunofluorescent labeling techniques of *Drosophila* tissue have been previously described (Sanyal *et al*., 2002). Tissue was dissected in PBS (milliQ H_2O + 130 mM NaCl + 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4) followed by 3 rinses in PBS. The tissue was fixed in 4% paraformaldehyde in PBS for 1 hr at room temperature. Fixative was

removed with 3x10 min washes of PBS and preparation placed in block (PBS + 2 % bovine serum albumin + .1% Triton X-100 + 5% Goat Serum) for 2 hrs at room temperature. Block was replaced with primary antibody at appropriate dilution in 200 µl block and kept overnight at 18^{o}C. Phalloidin-rhodamine (Mol Probes, R-415) was used to stain peripheral muscle at a concentration of 1:1000, anti-GFP (Mol Probes, A-11120) was used to label the axon of motor neurons with GAL4 expression at 1:50, and anti-VGlut (Daniels *et al*., 2004) was used to label a vescicular glutamate transporter of glutamatergic neurons in culture at 1:5000. The preps were then rinsed 3 quick + 3x10 min washes in PBS before being placed in 500 µl secondary antibody at appropriate dilution (1:1000 used for all secondaries) for 1 hr at room temperature. The secondary was washed with large volumes of TBS (PBS + .1% Triton X-100), 3 quick + 3x10 min washes with the last wash in PBS. The preparation was mounted on Superfrost Plus slides (VWR Scientific, 48311-703) in 80% glycerol +PBS and covered with a glass coverslip. The preparation was stored at -20^{o}C until imaging.

Drug Application

Pharmacological agents were administered to the cultures as previously described (Jiang and O'Dowd, 2005, Baines, 2003): $CoCl_2$ (Co^{+2}, 2 mM, Sigma-Aldrich), *Plectreurys* Toxin (PLTX-II, $5x10^{-2}$ µM, Alomone Labs), Tetrodotoxin (TTX, $1x10^{-1}$ µM, Sigma-Aldrich). Each was added to the *Drosophila* medium and added to neurons after 24 hrs in vitro, cells were imaged 24 hrs later.

Calcium Imaging

C380-GAL4,UAS-CD8-GFP;;chaGAL80 virgins were crossed to males expressing a UAS construct encoding the calcium sensitive fluorescing reporter UAS-GcAMP (Reiff et al., 2005) and imaged with 470 nm excitation at 2 DIV in the presence of regular *Drosophila* medium at 2 second intervals for 3 minutes. The cells were then imaged at 2 second intervals again in the presence of high K^+ medium after being exposed to high K^+ medium for 30 seconds. The images were obtained on an Olympus BX50WI microscope with a Hamamatsu C4742-95 camera and intensity values were measured with SimplePCI software.

Statistics

All data were compared with an ANOVA followed by post-hoc pair wise comparisons. Data were considered to be significant with a p value $\leq .05$. All values are listed as the mean $\pm$ SE.

4.4 Results

Motor neuron specific expression of the C380-GAL4,UAS-CD8-GFP;;chaGAL80 line

Our first goal was to find a driver line that is motor neuron specific. Several *Drosophila* enhancer-trap drivers have been suggested to be motor neuron specific, including the D42-GAL4 and OK6-GAL4 lines (Aberle *et al*., 2002). The C380-GAL4 line also drives expression in a subset of neurons, including several motor neurons (Sanyal *et al*., 2003). Upon careful analysis, however, none of the driver lines published to date were found to be motor neuron specific (data not shown). In order to address this issue the C380-GAL4 transgene was combined with a GAL80 construct downstream of the choline-actetyl transferase (cha) promoter (Kitamoto, 2002), thereby suppressing GAL4 activity in cholinergic neurons. This restricted GAL4 expression to a small population of dorsal motor neurons, with a few unidentified ventral neurons labeled as well (Figure 1a). In order to provide a motor neuron specific population in vitro, these ventral neurons (Figure 1b - see arrows) were microdissected out of the ganglion before plating (Figure 1d - see arrows). This reduced the population of GFP positive neurons even further (Figure 1c). We also recombined a UAS-CD8-GFP transgene onto the first chromosome to drive GFP expression in motor neurons both *in vivo* and *in vitro* (Figure 2). A dorsal confocal projection of the thoracic abdominal ganglion (Fig 3a) depicts the expression pattern of this line. In order to determine which specific motor neurons were labeled with GFP, rhodamine-phalloidin staining of the body wall muscles was used to visualize the peripheral innervation pattern of the fluorescent motor neurons (Figure 3b). Muscles 1-4, 6, 7, 9, 10, 13, 15, 16, 19, and 20 were innervated in each hemisegment

(Figure 4). Based on the central morphology and prior identification of motor neuron muscle innervation patterns (Choi *et al*., 2003), MN1-Ib (aCC) which innervates muscle 1 and has an ipsilateral projecting axon with a contralateral dendritic process (Figure 3c - yellow box, yellow arrow points to process), MNISN-Is (RP2) which innervates muscles 1-4, 9, 10, 19 and 20 and has an ipsilateral projecting axon (Figure 3c – red box), and MNSNb/d-Is (RP5) which innervates muscles 6, 7, and 13 with a contralateral projecting axon (Figure 3c – blue box, blue arrow points to axon) were determined to be the motor neurons labeled by this line.

These neurons can be identified and develop characteristic structural properties *in vitro,* with one dominant process and often a few smaller neurites emerging from the soma (Figure 3d). These cells can be imaged live and the parameters of cell body circumference, total neurite length, total branching number, and higher order branching patterns measured (Fig 3e). Only isolated neurons were imaged and quantified in this study to focus on cell autonomous regulation of motor neuron growth. We wanted to confirm that the cultured motor neurons maintain glutamatergic identity, so we stained cultures with an antibody against a *Drosophila* vesicular glutamate transporter (anti-VGlut, Daniels *et al*., 2004). We found a colocalization of GFP and VGlut (Figure 3f). This identifies our GFP positive population of cells as expressing a protein that is associated with glutamatergic neurons. There was no staining in the no primary control (Figure 3g). There were also GFP negative cells in the culture either stained or not stained with the VGlut antibody (data not shown). This is expected, given that there are many motor neurons in these cultures that do not express the C380 driver.

Depolarization induced motor neuron dendritic growth and morphogenesis

Motor neurons display neurite growth and branch formation in response to depolarization with high K^+ and through expression of transgenes that manipulate K^+ channel function of motor neurons. 2 DIV motor neurons exposed to an increase of 10 mM K^+ in the culture medium for 24 hrs displayed a significant increase in total neurite length, total branch number, and primary branch number when compared to 2 DIV motor neurons maintained in regular medium (Figure 5). Transgenic manipulation of I_K^+ through chronic expression of UAS-shDN/eagDN (EKI), a double dominant negative that interferes with function of the voltage gated *ether-a-go-go* and *shaker* K^+ channels, produced similar results. However, expression of the electrical knock-out (UAS-2xEKO), a *shaker* K^+ channel construct that has been modified to remove the inactivation domain of the channel so that it behaves as a K^+ shunt, did not influence neurite outgrowth significantly compared to controls (Fig 5).

The EKI and EKO transgenes influence the membrane properties of C380 neurons

Since we observed no effect of 2xEKO expression on the neurite outgrowth of C380-GAL4,UAS-CD8-GFP;;chaGAL80 motor neurons, we verified the authenticity of the UAS-2xEKO line through single cell electrophysiological recordings. 2xEKO did have an influence on membrane properties even though it did not influence neurite growth. Expression of the 2xEKO transgene resulted in outward currents that were increased in amplitude compared to control currents (Figure 6a-b). The EKI transgene also influenced motor neuron membrane properties. The motor neurons displayed more

frequent action potentials (Figure 6c – see arrowhead) and a loss of the fast inactivating A-type current that was typically observed in control cells (Fig 6a – see arrow). In addition, current clamp recordings (not shown), revealed an abnormally high frequency of action potentials in response to depolarization in EKI flies. It is interesting to note that current amplitude is similar between EKI and control, and this could represent possible delayed rectifier type compensation.

VGCC I_{Ca}^{+2} is necessary for depolarization induced neurite growth

Depolarization can induce cation influx through both sodium and calcium voltage gated channels. We investigated the role of these currents in activity induced neurite growth by exposing EKI motor neurons *in vitro* to pharmacological blockers. Tetrodotoxin (TTX) has been used to block I_{Na}^{+} current in cultured *Drosophila* neurons (O'Dowd and Aldrich, 1988). TTX also significantly decreases I_{Na}^{+} in embryonic motor neurons *in vivo* (Baines, 2003) and blocks action potentials in larval RP2 *in vivo* (Choi *et al*., 2003). TTX did not influence neurite outgrowth compared to EKI expressing control cells (Figure 7). This suggests that Na^{+} dependent action potentials are not necessary for depolarization induced growth.

I_{Ca}^{+2}, however, is necessary for neurite elongation and branching. Spontaneous Ca^{+2} transients in cultured *Drosophila* Kenyon cells are blocked by both $CoCl_2$ (a non-specific Ca^{+2} channel blocker) and *Plectreurys* toxin (PLTX, a blocker specific to putative T-type voltage sensitive Ca^{+2} channels in insects) (Jiang *et al*., 2005). These agents, when added to EKI expressing motor neurons at concentrations used in the Jiang

et al., study, both resulted in a dramatic decrease in neurite formation and outgrowth (Figure 7). This suggests that voltage gated I_{Ca}^{+2} is necessary for depolarization induced motor neuron neurite outgrowth.

High K^+ medium may induce Ca^{+2} influx in motor neurons

To test whether depolarization caused a chronic increase in intracellular calcium concentration, calcium imaging was performed. The C380-GAL4,UAS-CD8-GFP;;chaGAL80 line was crossed to the UAS-GcAMP reporter line. The GcAMP transgene encodes a calcium-sensitive fluorescent calmodulin protein with GFP fused to its C terminus (Reiff *et al*., 2005). A group of cultured motor neurons were imaged for baseline levels of calcium activity at 2 second intervals for 3 minutes, and the middle 1 minute interval is displayed. The culture was then superfused with high K^+ medium (the same 10mM K^+ increase used for the morphology studies) for 30 seconds and then imaged again while in the depolarizing medium using the same parameters. The baseline fluorescence was slightly increased after the addition of high K^+ medium, but these preliminary results were deemed inconclusive (Figure 8).

4.5 Discussion

We first demonstrated that a motor neuron specific population of cells *in vitro* can be obtained with the combination of fly genetics and microdissection techniques. We combined the putative motor neuron driver C380-GAL4 with a GAL80 construct downstream of a choline-acetyl transferase (ChAT) promoter to block GAL4 activity in cholinergic neurons. However, a few unidentified ventral neurons still remained, which were dissected out before culture. It is possible that these ventral neurons are also motor neurons, although the tissues they innervate could not be deciphered. It is also possible that they are of a different neuron subtype. We confirmed the glutamateric properties of the neurons in culture by labeling GFP positive cells with a vescicular glutamate transporter protein (Daniels *et al*., 2004) that has previously been demonstrated to label motor neurons *in vivo*. This further supports the motor neuron specific nature of our GFP-labeled cultured neurons.

Exposure to a 10mM K^+ increase in the culture medium for 24hrs resulted in a significant increase in total neurite length and branching compared to controls. Likewise, simultaneous expression of dominant negative subunits of the *ether-a-go-go* and *shaker* voltage gated K^+ channels yielded similar results. We expect that the high K^+ is depolarizing the resting membrane potential by ~10 mV, but it may also cause an increase in spontaneous spiking. The EKI transgenes may be influencing the resting membrane potential as well as increasing spike frequencies and action potential duration. This is likely based on work with the *shaker* and *ether-a-go-go* mutants where it was

demonstrated in motor neurons that the *shaker* mutant affects A-type K^+ current while the *ether-a-go-go* reduces delayed rectifier current (Wu *et al.*, 1983).

It is interesting to note that the magnitude of increase in neurite length is much greater in the EKI motor neurons than in high K^+ treatment. This could be due to differences the two methods posed on membrane properties. The high K^+ treatment depolarized the neurons while the EKI animals could have both an increase in spiking frequency and action potential duration. These subtle effects on I_{K}^{+} kinetics could lead to important differences in I_{Ca}^{+2} that could account for the differences in outgrowth. There is an important difference in the duration of depolarization/activity change between the two groups. High K^+ neurons were acutely depolarized for only 24 hrs after placing the larval neurons in culture, while the activity of EKI neurons had been chronically affected throughout embryonic and larval stages by expression of the transgenes. This increased duration could also contribute to the increased length of EKI neurons.

While EKI influenced the magnitude of neurite outgrowth, the branch numbers were comparable to those seen in the high K^+ treatment group. This may represent different mechanisms underlying the formation of branches and the addition of length to a neurite. Neuron sprouting and elongation may depend on the activation of unique signaling pathways that may be influenced differently by external and internal stimuli. There is evidence that axon growth is regulated by proteins like jun, whereas local regulation of branching occurs in the target region (Caroni, 1997). Perhaps the EKI

motor neurons have increased spike frequency that induces cascades involved in growth that aren't activated with high K^+ alone.

We also expect the 2xEKO transgene to hyperpolarize the resting membrane potential as it has been found to silence the electrical activity of neurons (White *et al*., 2001). However, expression of EKO by motor neurons in vitro did not influence their neurite outgrowth or branching. It is possible that a basal level of outgrowth is dictated by intrinsic genetic programs independent of hyperpolarization or a decrease in spiking frequency. However, it is also possible that the 2xEKO hyperpolarization was compensated for by the increase in expression of other channel types, like Na^+ channels. Further electrophysiological experiments are required to distinguish among these possibilities. Although EKO expression did not alter the growth and branching properties of the cell, the EKO expressing neurons did, in general, appear to have neurites smaller in diameter and were not as healthy as control cells. Perhaps our method of quantification did not detect a phenotype imposed by hyperpolarization with EKO.

I_{Ca}^{+2} is necessary for the depolarization induced neurite growth. Blocking all voltage-gated I_{Ca}^{+2} with Co^{+2} or specifically blocking the putative N-type channels with PLTX inhibited depolarization induced motor neuron growth. Na^+ based action potentials may not be involved, as depolarization induced growth took place in the presence of TTX. Preliminary electrophysiological recordings done on these motor neurons both in vivo and in vitro (data not shown) support our findings that these neurons have calcium currents. 2 DIV motor neurons display large outward K^+ currents (see

Figure 6a). At this stage *in vitro*, these neurons do not have Na^{+} based spiking, but do display inward currents that may be Ca^{+2} based. Larval motor neurons *in vivo*, however, do have action potentials that are blocked with TTX (Choi *et al*., 2003). Preliminary voltage clamp experiments reveal Ca^{+2} current as well in larval RP2 (Worrell, personal communication).

What are the possible mechanisms of calcium dependent depolarization induced growth? There is evidence in other systems that depolarization induced calcium influx regulates dendritic plasticity through the activation of kinases. Calcium can be upstream of CaMKII activation (Gaudilliere *et al*., 2004) MAPK (ERK) activation (Wu *et al*., 2001) or both CaMKII and MEK-ERK pathways (Vaillant *et al*., 2002). It is likely, based on evidence from the *Drosophila* neuromuscular junction, that these pathways could be involved in depolarization induced growth. Constitutively activated forms of Ras (a member of the MAPK cascade) and CaMKII induce plasticity at the neuromuscular junction (Koh et al., 2002 and Park *et al*., 2002).

Kinase activation leads to an induction of transcription factors that are involved in the protein synthesis necessary for plasticity. Redmond and colleagues (2002) found that in cultured rat cortical neurons, high K^{+} induced dendritic growth was dependent on calcium entry through voltage gated calcium channels. This calcium induced growth activated both MAPK and CAMK cascades. CAMKIV, specifically, led to dendritic outgrowth through the induction of the transcription factor CREB. Transcription factors, like CREB and AP-1, have also been found to induce plasticity at the *Drosophila*

neuromuscular junction (Davis *et al*., 1996; Sanyal *et al*., 2002). We investigate the roles of CREB and AP-1 in depolarization induced growth in Chapter 6.

What functional implications might activity or depolarization induced motor neuron dendritic plasticity have in the intact animal? There is evidence that crawling behavior is influenced by K^+ channel mutants. This may genetically reproduce foraging during times of starvation, or wandering in search of a pupation site in *Drosophila* larvae. Stride frequency of double mutant *ether-a-go-go*/*shaker* larvae is greater, but average crawling speed is lower than that of controls (Wang *et al*., 2002). Since the Wang *et al.* study was done in mutants, in which all cells were affected, it is difficult to know which neurons were specifically responsible for changes in crawling behavior. The current study demonstrates that activity influences growth in a cell autonomous manner, and it would be interesting to observe any behavioral consequences that this might have. It is demonstrated in Chapter 6 that the genetic manipulations used in this study also affect motor neurons *in vivo*.

The present study demonstrates depolarization induced growth but did not determine whether the duration of depolarization influenced the magnitude of response. Moreover, the study did not determine whether depolarization influenced the rate of growth or the total amount. Another limitation of this study is the inability to differentiate dendritic and axonal compartments *in vitro*. This study only discusses total neurite length and branching, and it would be of interest to distinguish axon and dendrite growth. It would first need to be demonstrated that invertebrate neurons maintain

polarity in culture and that there are reliable methods for detecting this polarity in *Drosophila*. Solutions for these questions will be proposed in Chapter 5.

4.6 Figures

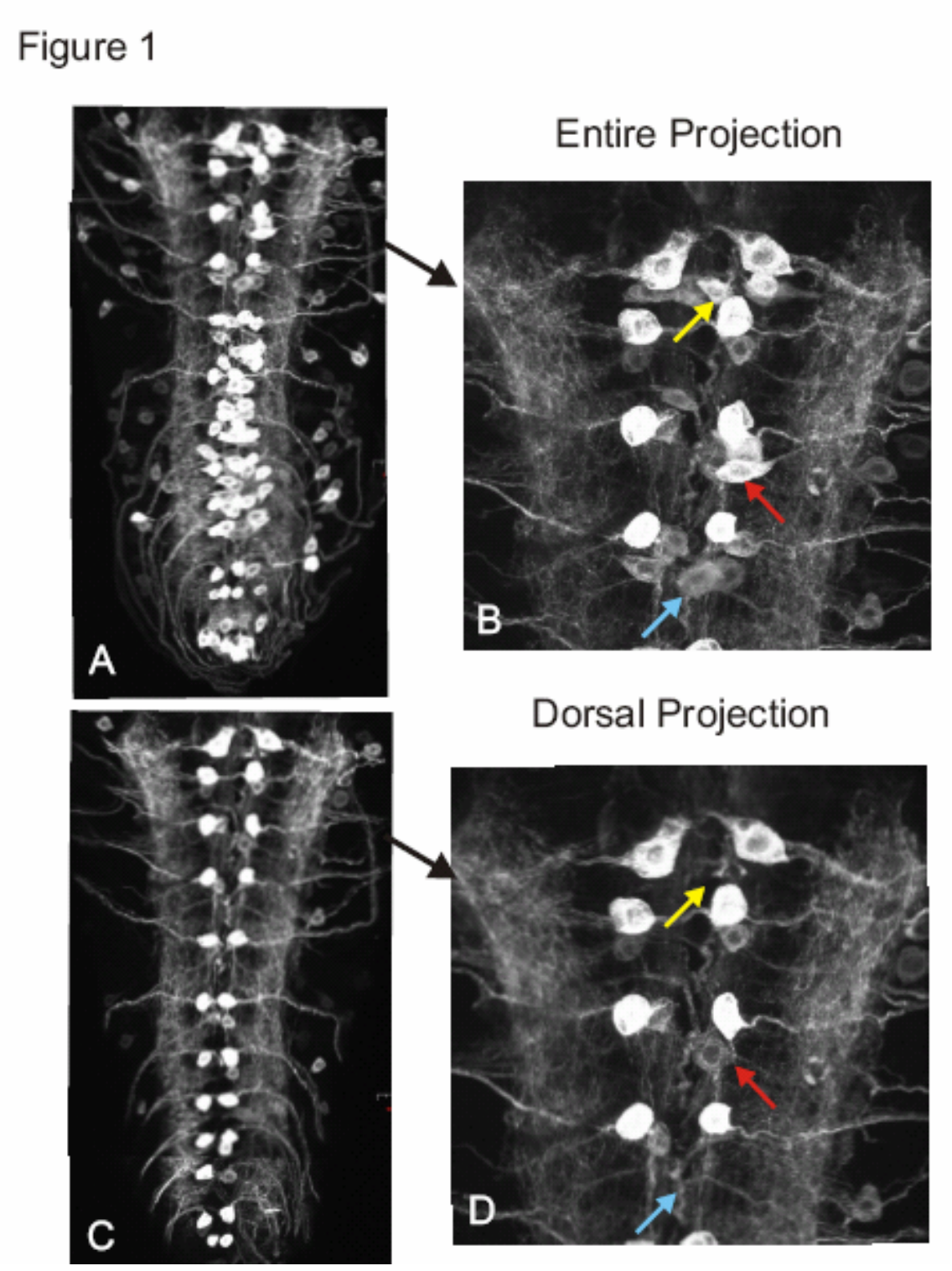
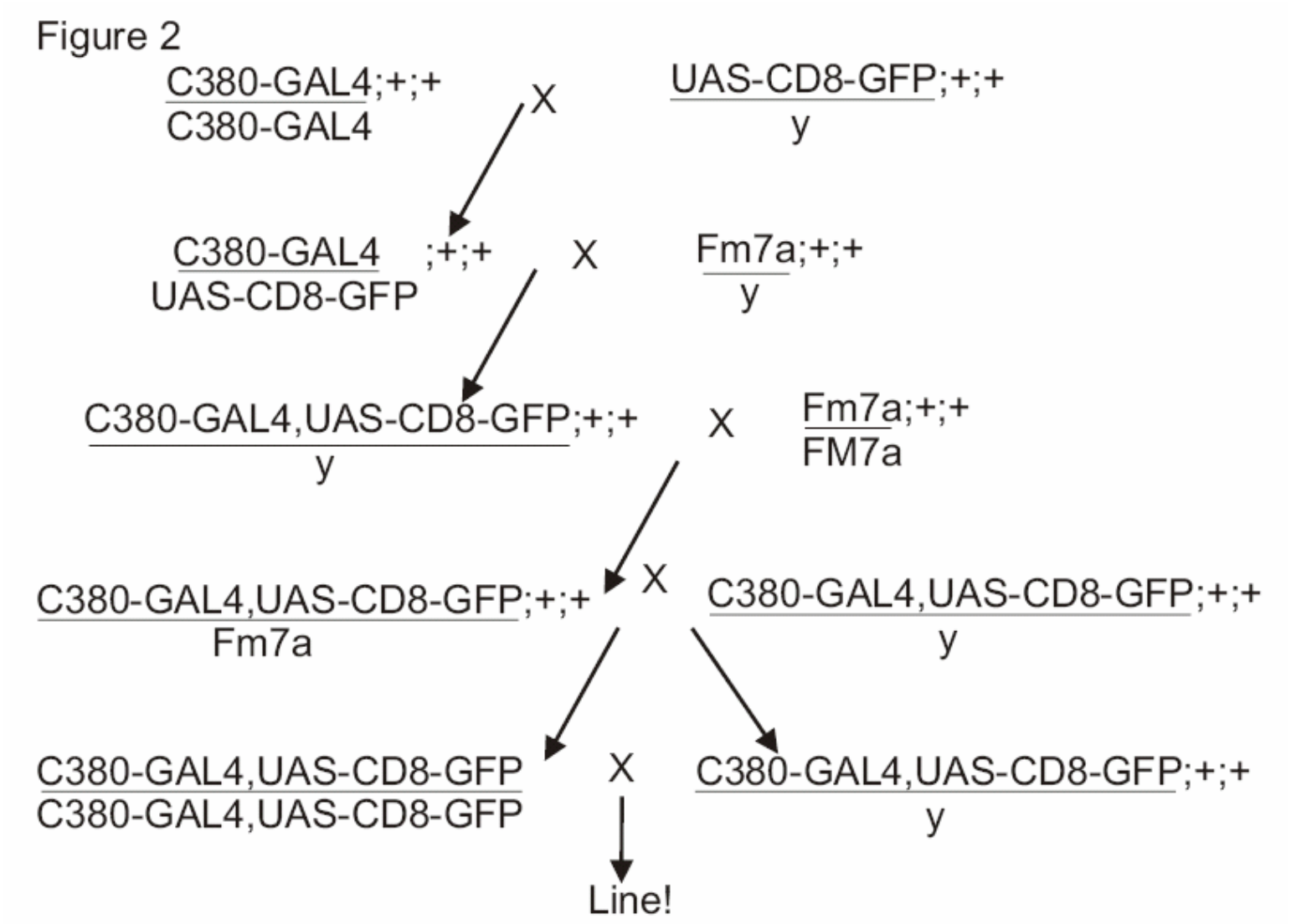


Figure 2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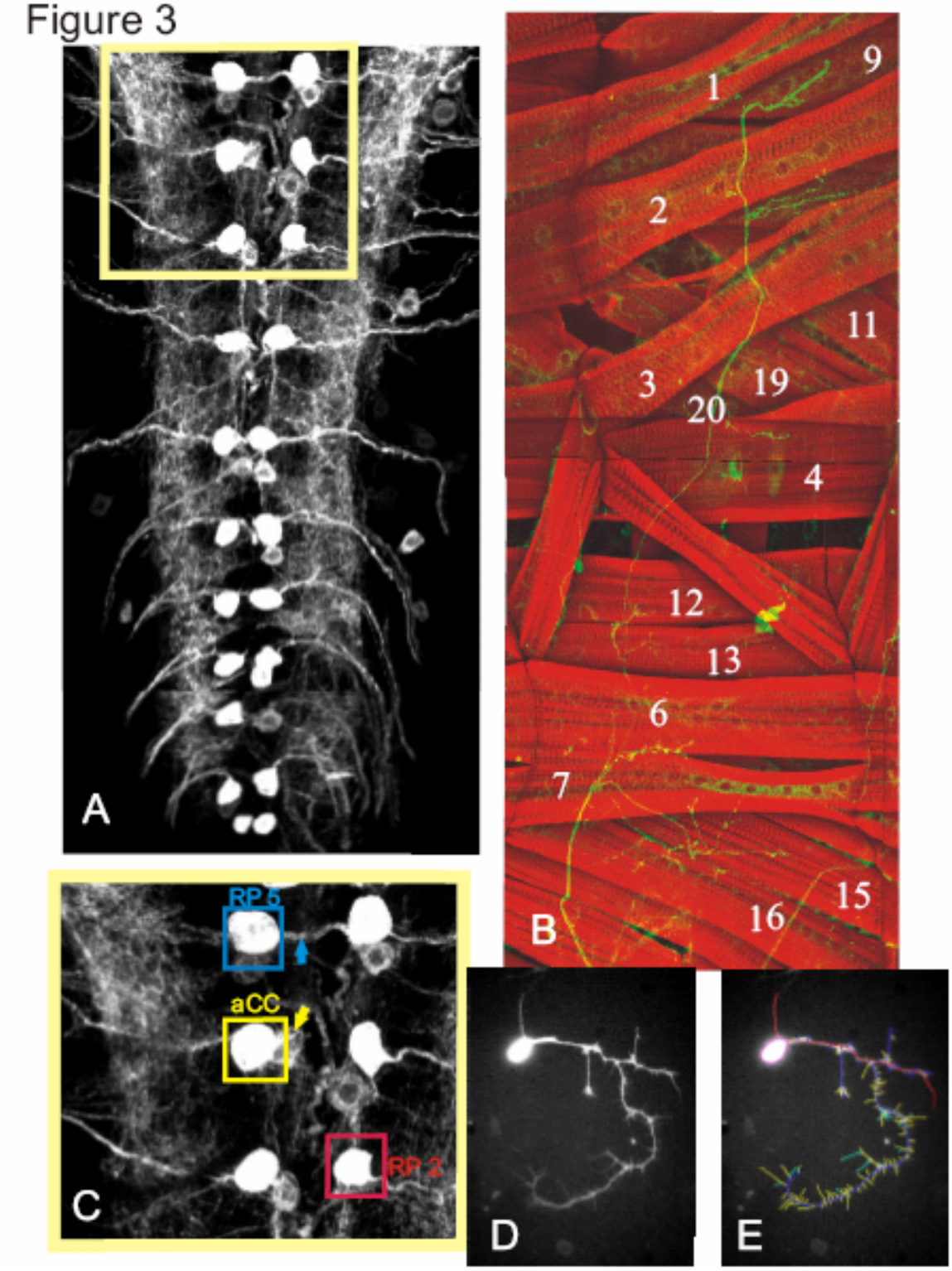
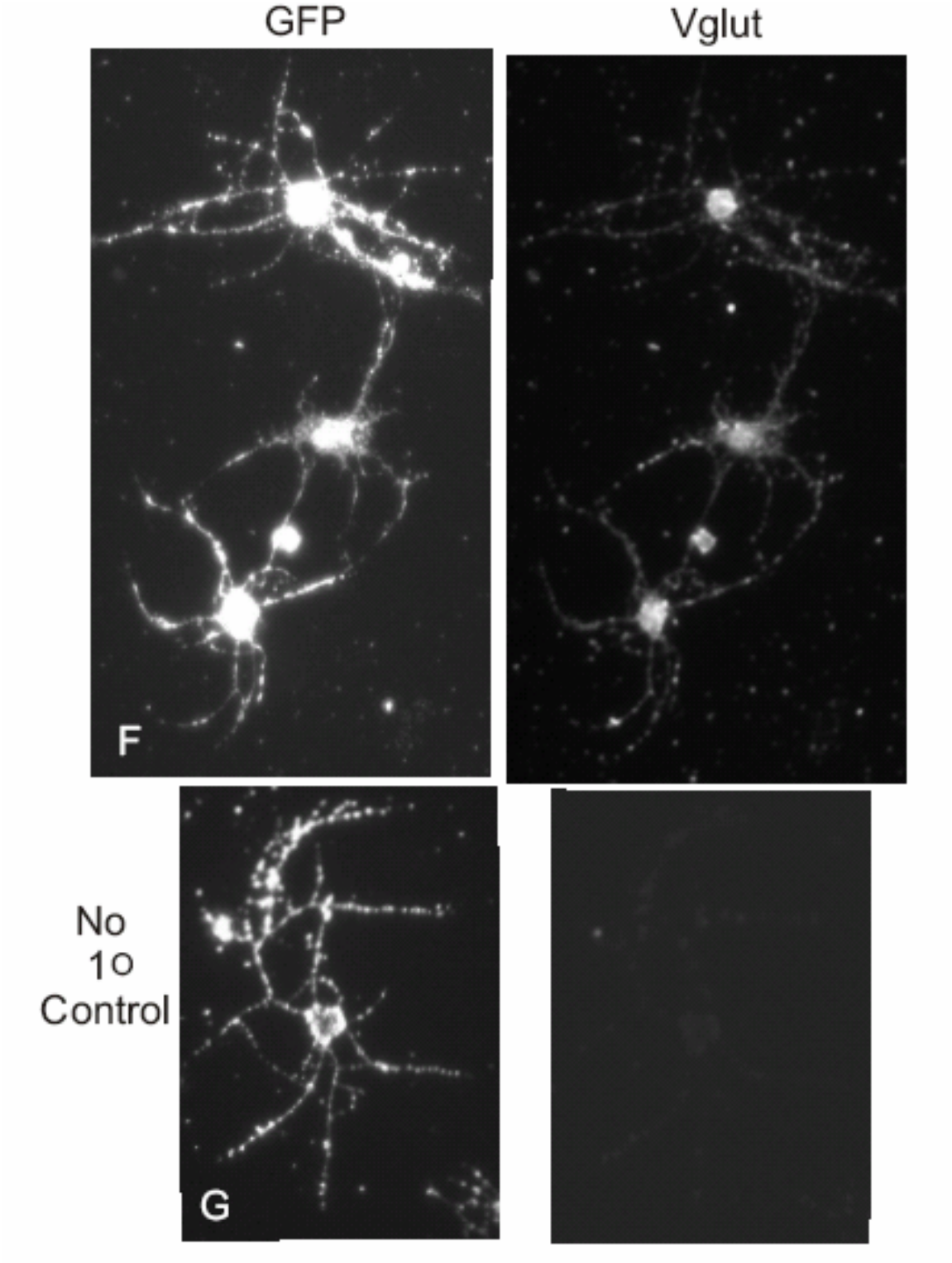
Line!

Figure 3

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Vglut
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No
1o
Control
G

Figure 4

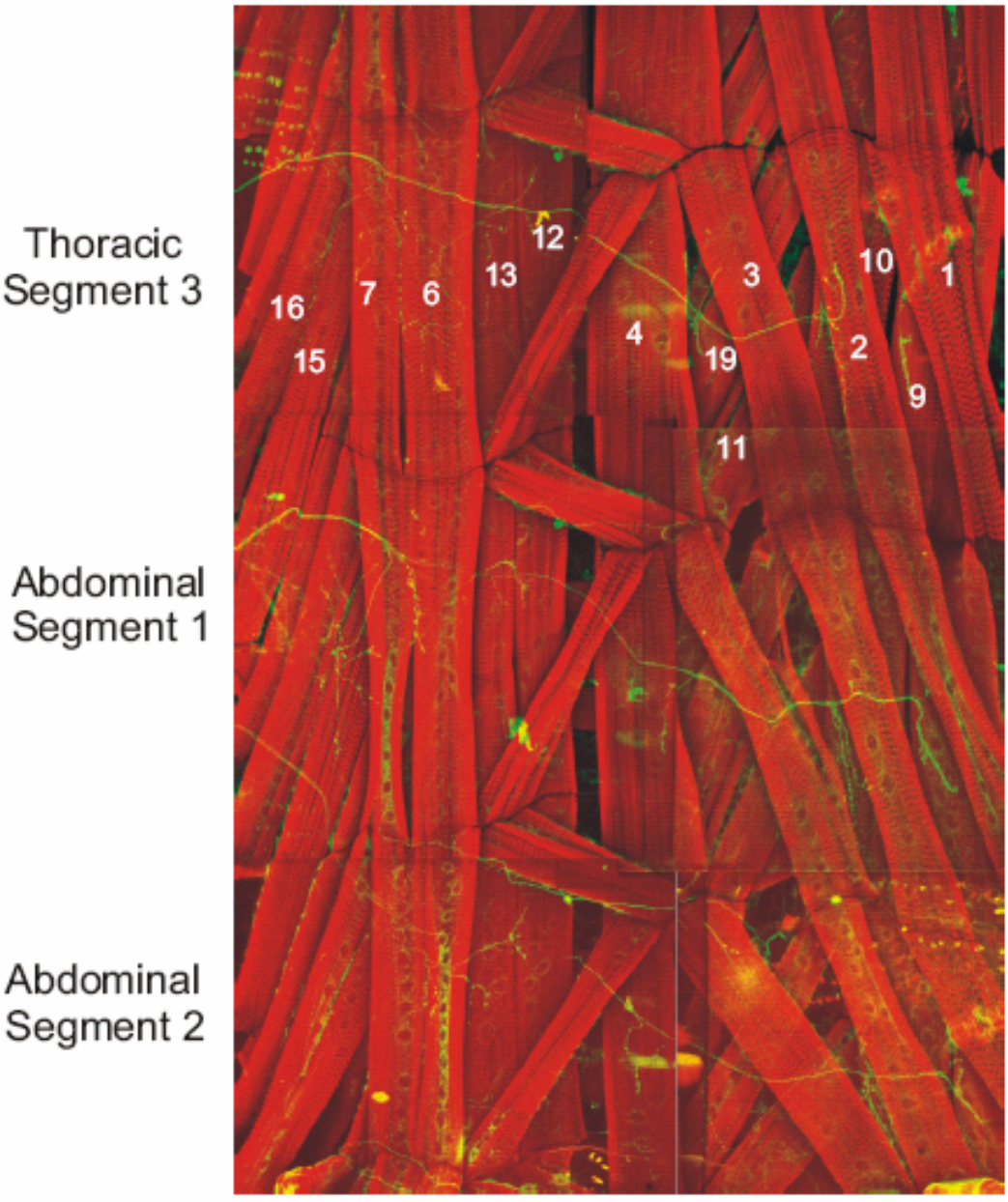
Thoracic Segment 3
Abdominal Segment 1
Abdominal Segment 2
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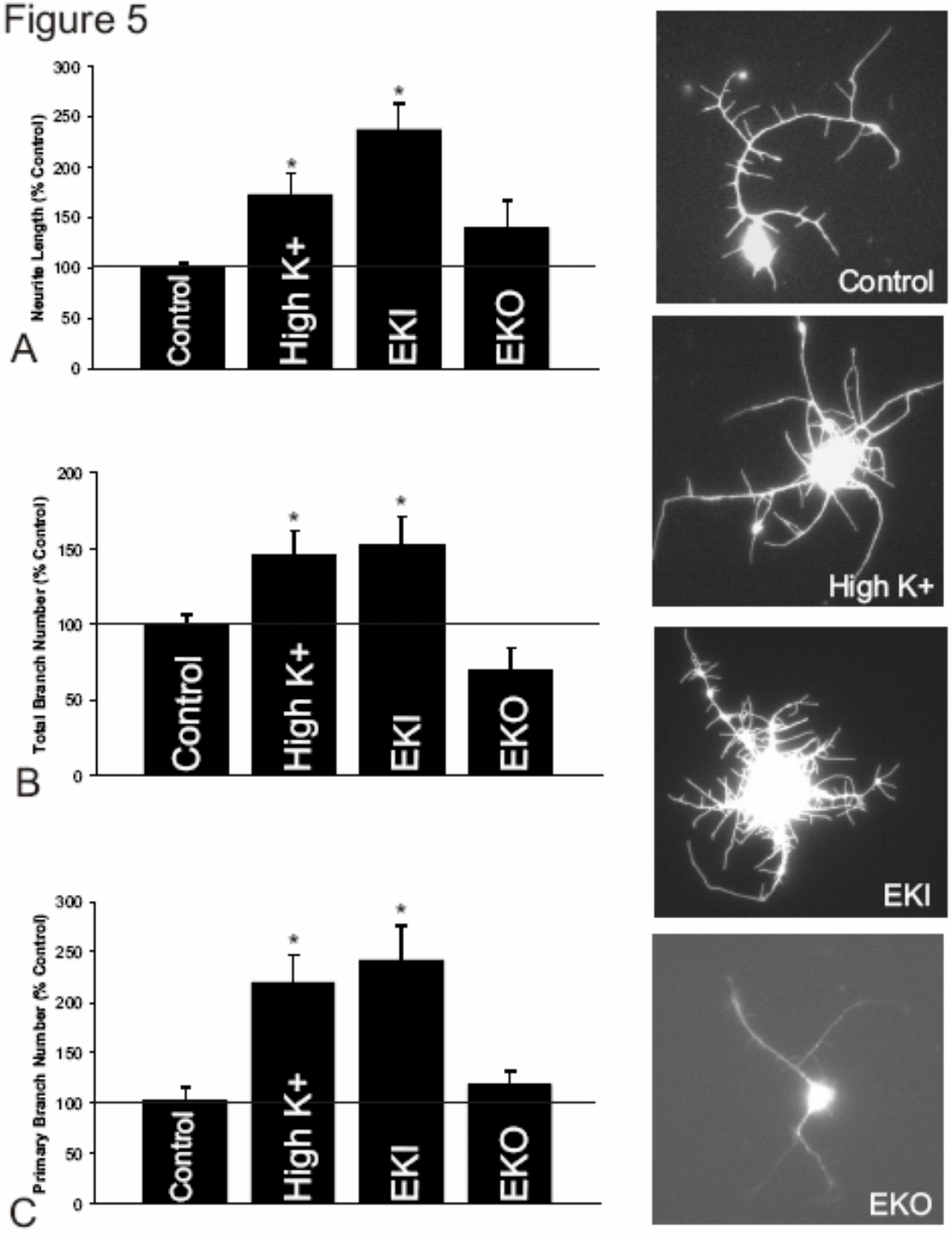
Figure 5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C
Primary Branch Number (% Control)
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High K+
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EKO

Figure 6

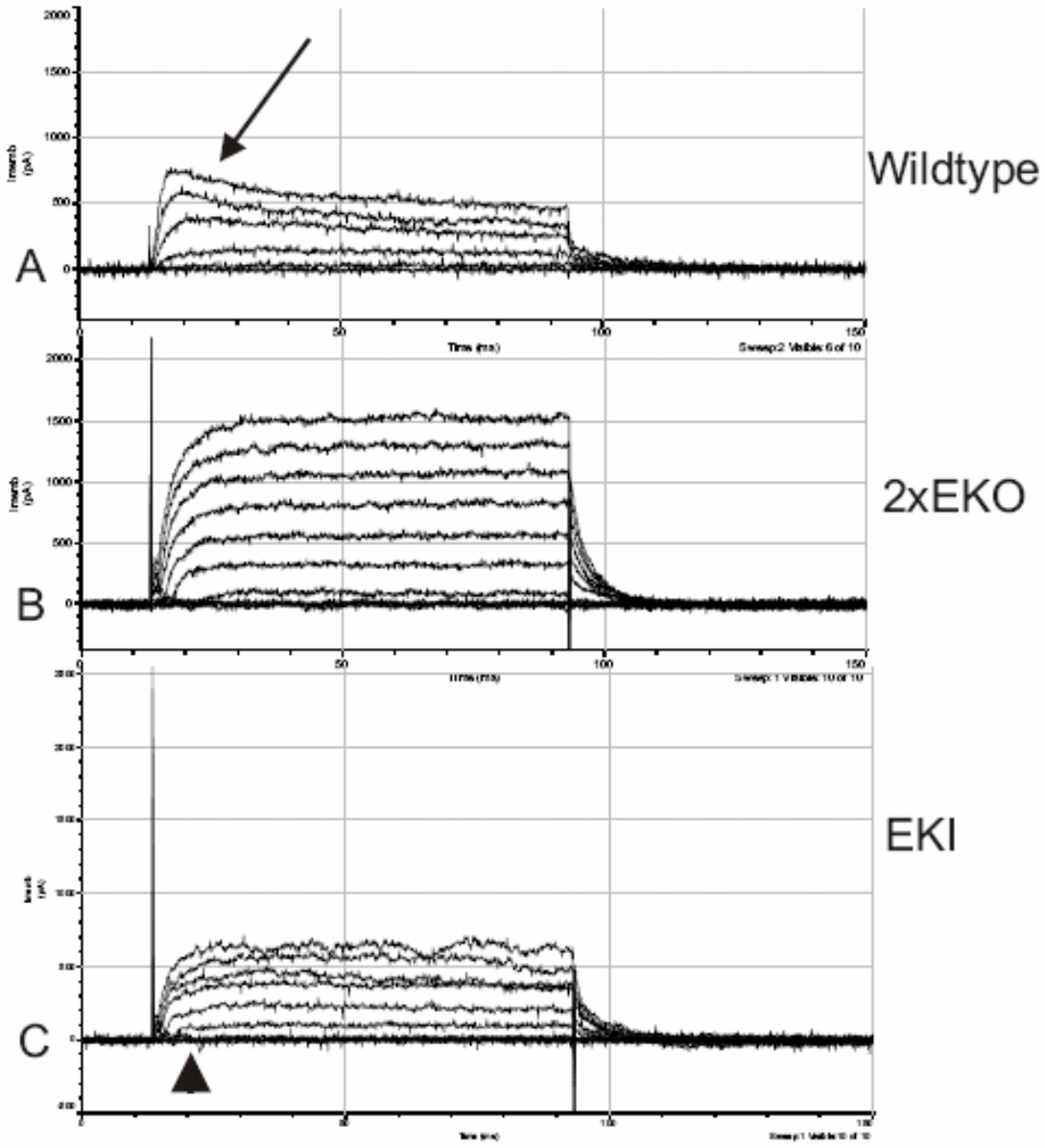
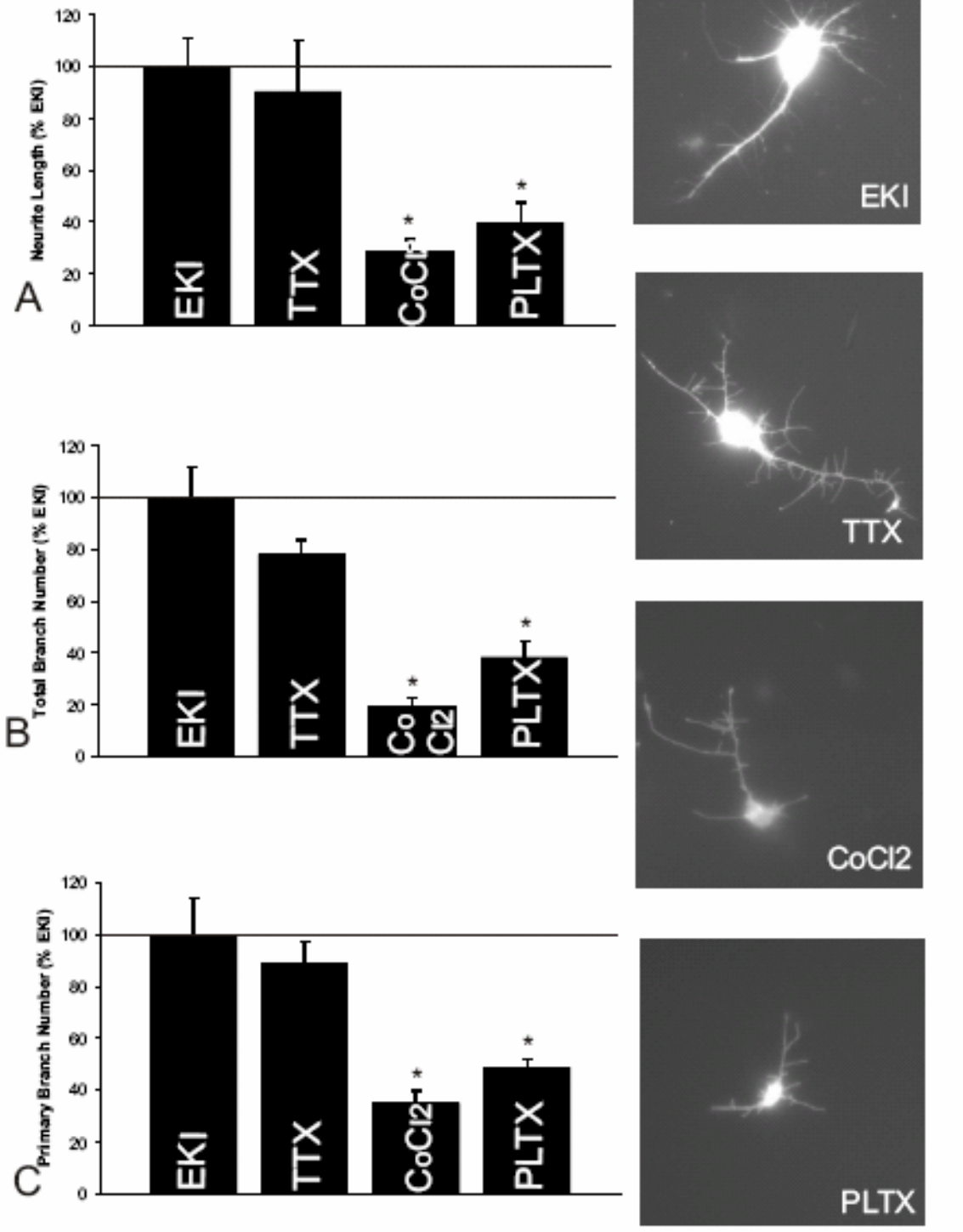


Figure 7


A
Neurite Length (% EKI)
120
100
80
60
40
20
0
EKI
TTX
CoCl-1
PLTX
*
*
B
Total Branch Number (% EKI)
120
100
80
60
40
20
0
EKI
TTX
Co
Cl2
PLTX
*
*
C
Primary Branch Number (% EKI)
120
100
80
60
40
20
0
EKI
TTX
CoCl2
PLTX
*
*
EKI
TTX
CoCl2
PLTX

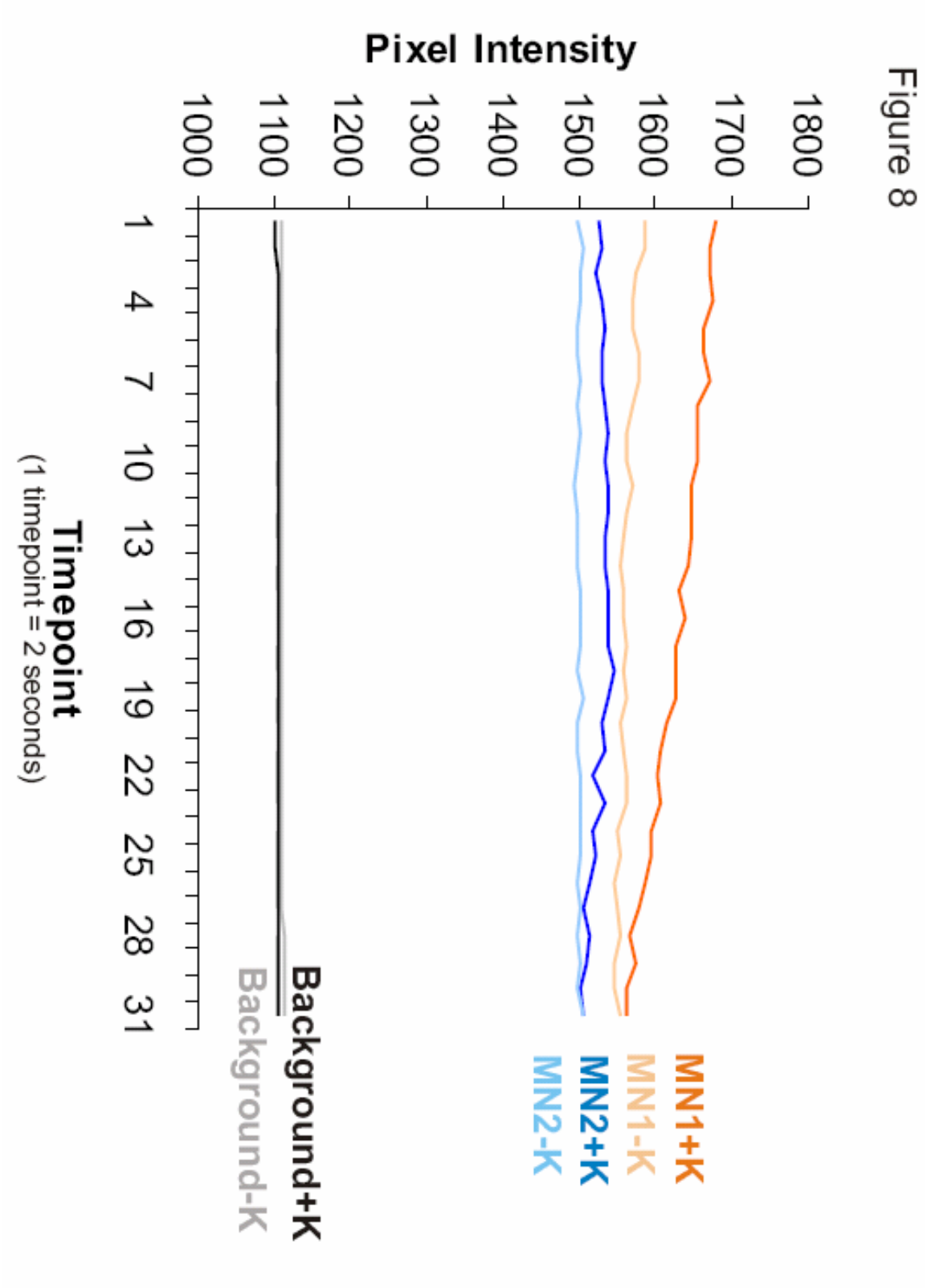


Figure 8
Pixel Intensity
1800
1700
1600
1500
1400
1300
1200
1100
1000
MN1+K
MN1-K
MN2+K
MN2-K
Background+K
Background-K
1 4 7 10 13 16 19 22 25 28 31
Timepoint
(1 timepoint = 2 seconds)

4.7 Figure Legends

Figure 1

C380-GAL4,UAS-CD8-GFP;;chaGAL80 drives expression in dorsal medial identified motor neurons and ventral unidentified neurons. The expression pattern of this driver can be observed in the late 3rd instar thoracic abdominal ganglion using a complete confocal stack of the tissue (A and B) and a dorsal confocal stack (C and D). The unknown ventral neurons are microdissected out of the ganglion before culture preparation for a motor neuron specific GAL4 positive population.

Figure 2

The genetic scheme for creating a C380-GAL4,UAS-CD8-GFP recombinant line. The UAS-CD8-GFP makes a reporter protein that enables the visualization of motor neurons both *in vivo* and *in vitro*.

Figure 3

C380-GAL4,UAS-CD8-GFP;;chaGAL80 drives expression in a subset of identified MNs. A) Confocal microscopy dorsal projection of a late 3rd instar larval thoracic abdominal ganglion. B) Immunocytochemistry of axons (anti-GFP) and body wall muscles (rhodamine-phalloidin) reveals the peripheral innervation pattern of our synthetic motor neuron driver. C) Higher magnification of the thoracic segments indicates MN1-Ib (aCC), MNISN-1s (RP2) and MNSNb/d-1s (RP5) express GFP. D) The motor neurons in culture are visualized live through fluorescent microscopy of the membrane bound form of CD8-GFP. Many motor neurons appear polar in culture with

more secondary branches and fewer primary branches. E) The total process length, branch number, and cell body circumference can be measured by tracing the neurons. Here each group was designated to a different color: teal=circumference, red=1^o branch, blue=2^o branch, yellow=3^o. F) C380-GAL4,UAS-CD8-GFP;;chaGAL80 neurons are glutamatergic, as they stain positive with a V-Glut antibody G) represents the no primary control.

Figure 4

Immunocytochemistry of axons (anti-GFP) and body wall muscles (rhodamine-phalloidin) in three corresponding segments reveals a consistent peripheral innervation pattern of the C380-GAL4,UAS-CD8-GFP;;chaGAL80 motor neuron driver.

Figure 5

A 10 mM increase of KCl in the culture medium increased total neurite length, total branch number, and primary branch number compared to controls. Manipulation of the K^+ channels eag and Sh with double dominant negative transgenes also resulted in neurite growth, phenotypically similar to high K^+ experiments. However, hyperpolarization with the electrical knock-out, 2xEKO, did not influence neurite outgrowth.

Figure 6

Characterization of K^+ channel transgene effects on the membrane properties of motor neurons. Control C380GAL4,UAS-CD8-GFP;;chaGAL80 neurons under voltage clamp at a holding potential of -70 mV and with +20mV step increments display large

outward current with an inactivating component when given depolarizing voltage commands. This current has a fast inactivating current resembling A-type potassium current (see arrow). Expression of 2xEKO results in an increase in outward current compared to controls. Expression of *UAS-ShDN*/*EAGDN* results in a removal of A-type current and action potentials that are difficult to clamp (see arrowhead).

Figure 7

I_{Ca}^{+2}, not I_{Na}^{+}, is necessary for depolarization induced motor neuron neurite outgrowth *in vitro*. The addition of TTX (10^{-8} M) to EKI expressing motor neurons did not significantly change the neurite growth parameters compared to EKI expression alone. Both $CoCl_2$ (2 mM) and PLTX (50 nM) blocked EKI induced motor neuron total neurite outgrowth (A), total branch number (B) and primary branch number (C) compared to EKI controls.

Figure 8

Intracellular calcium levels of 2DIV motor neurons before and after exposure to K^+. Two 2DIV C380GAL4,UAS-CD8-GFP;;chaGAL80 x UAS-GcAMP neurons (designated MN1 and MN2) were imaged at 2 second intervals for 3 minutes, exposed to high K^+ medium for 30 seconds, and then imaged again in the depolarizing medium, with corresponding intensity measurements recorded from areas of the dish not containing cells for background. The high K^+ exposed cells had a slightly higher level of fluorescent intensity.

CHAPTER 5 CHARACTERIZATION OF DEPOLARIZATION INDUCED DENDRITIC GROWTH OF *DROSOPHILA* MOTOR NEURONS *IN VITRO*

5.1 Abstract

The factors influencing dendritic and axonal specification, development, and plasticity of mature structures are diverse and unique to neuron type and time point in the life of the neuron. Here we characterize the growth parameters of depolarization induced dendritic growth and branching of identified *Drosophila* larval motor neurons in culture. We also introduce a technique to distinguish neuron axon and dendrites *in vitro* using established polarity markers (Sanchez-Soriano *et al*., 2005; Clark *et al*., 1997). We are able to detect motor neuron dendrites *in vitro* and measure their growth parameters. Our investigation reveals that depolarization with elevated external K^+ induces an acceleration of dendritic outgrowth compared to controls. Depolarized neurons have more and longer dendritic processes by 2 days *in vitro* (DIV) compared to controls, but this growth reaches a plateau. Control cells "catch up" in growth by 3 DIV. This accelerated growth is dependent on chronic depolarization, as acute exposure to K^+ for 6 hrs of 2 DIV neurons does not result in dendritic outgrowth. 3 DIV neurons exposed to depolarizing medium for 24 hrs and then placed back in control medium for 24 hrs do not display enhanced growth relative to control neurons.

5.2 Introduction

It is well established that many factors, including activity related proteins (Hering and Sheng, 2001) and calcium signaling pathways (Konur and Ghosh, 2005) influence dendritic identity and characteristics during development. Neurons are electrically active structures, and this activity is an essential component to dendrite and axon formation (Cline, 1991) and maintenance (Mizrahi and Katz, 2003). Activity influences the cytoskeleton, regulates signal cascades, and even influence the release of extrinsic factors that are involved in dendritic regulation (Spitzer *et al*., 2000; Zhang and Poo, 2002; Chen and Ghosh, 2004,). Despite the large body of work surrounding this subject, many mechanisms of activity induced dendritic growth remain unexplored, including the crosstalk between activity induced pathways that may be necessary for overall dendritic growth and branching. This question and others could be answered by designing a *Drosophila* culture system to specifically observe dendrites. The influences on dendritic growth could be explored using available *Drosophila* transgenic tools. This research could also contribute to our understanding of human disease with the many *Drosophila* homologues of human disease related proteins.

The ability to identify and characterize dendritic growth *in vitro* allows for the direct application of pharmacological agents and the study of cell autonomous determinants of dendritic growth and sprouting. The rat hippocampal culture system has generated much knowledge about the regulation of dendritic and axonal properties. A five stage assay was created to describe growth in culture (Dotti *et al*., 1988), from stage 1, where only a few neurites sprout from the cell body, to stage 5, where a mature neuron

has defined axon and dendrites and begins to form synapses. The resultant model enabled many observations that were influential to the scientific community, including the microtubule structure of polarized neurons (Baas *et al*., 1989), the stage-dependent localization of certain proteins such as GAP-43 and tau in the axon (Goslin *et al*., 1990; Mandell and Banker, 1995) and MAP2 in dendrites (Caceres *et al*., 1986), and depolarization induced mechanisms involved in dendritic growth (Wu *et al*., 2001).

An equivalent culture system has not been established for *Drosophila*. This would be a powerful tool when combined with genetic approaches. Although an *in vitro* system to study the mechanisms of depolarization induced motor neuron growth was established in the last chapter, it left open many questions about the culture system that this chapter will address. First, what is the time course of depolarization induced growth in this system? Does depolarization increase the total length and branching of motor neuron neurites over time or does it simply accelerate growth to a maximal level eventually reached by all neurons? Is chronic depolarization necessary for significant growth to take place or could brief depolarization lead to the activation of downstream cascades that then carry out neurite growth?

Genetic manipulation of K^+ channels in *Drosophila* motor neurons induces plasticity at the NMJ (Budnik *et al*., 1990; Zhong and Wu, 1991), and as described in the last chapter, depolarization and transgenic manipulation of K^+ channels increase motor neuron neurite growth and branching *in vitro* as well. However, only total neurite growth has been discussed thus far. Do *Drosophila* neurons, like mammalian hippocampal

neurons, maintain polarity *in vitro*? If they do, can we differentiate between axons and dendrites? Classic vertebrate markers for differentiation of neuronal compartments in vitro, like MAP2 (Pennypacker *et al*., 1991) and tau (Mandell and Banker, 1995) are not available for *Drosophila*. The issue will be addressed in this chapter with the use of directional transport proteins.

This study not only confirms the findings of depolarization induced dendritic growth of the last chapter, but characterizes the acute versus chronic depolarization induced growth with a set of timeline studies. We also provide methods for the identification of dendrites and axons using motor proteins that are established polarity markers *in vivo* (Sanchez-Soriano *et al*., 2005; Clark *et al*., 1997). This allowed for the differentiation of depolarization induced effects between axonal and dendritic compartments. The findings enable further study of how the many proteins found to induce changes at the *Drosophila* NMJ (Koh *et al*., 2000; Dubnau and Tully, 1998) may also influence motor neuron dendritic growth.

5.3 Methods

Fly Strains

Late 3rd instar larval stage animals were used for all experiments. Larvae were selected from the cross of homozygous female C380GAL-4,UAS-CD8-GFP;;chaGAL80 and males of the Ore-R line for the initial analysis of depolarization induced neurite outgrowth. The larvae express GFP in identified motor neurons of the thoracic abdominal ganglion (refer to Chapter 4). The C380GAL4;;chaGAL80 line was crossed to either UAS-kin-GFP (III) (Estes, unpublished) or yw::UAS-nod-LacZ (Clark *et al*., 1997) for the polarity studies. The w;;UAS-nod-LacZ, UAS-kin-GFP recombinant line was created to analyze the compartmentalization of these motor proteins when simultaneously over-expressed. The Gene Switch studies were performed with the combined GSG-elav-GAL4;;chaGAL80 line (Osterwalder *et al*., 2001; Kitamoto, 2002) crossed to w;;UAS-nod-LacZ, UAS-kin-GFP. The Gene Switch GAL4 protein is fused to the receptor binding portion of the progesterone receptor and will not initiate transcription at the UAS site until RU-486 is administered to the cultures. This enables conditional expression of GAL4.

Primary Cell Cultures

Culture protocols followed have been described previously with a few modifications (Kraft *et al*., 1998). All dissection equipment was soaked in 70% EtOH for 10 min before dissection. Larvae were selected and sterilized by placing the animal in 70% EtOH for 1 min. The larvae were rinsed 3 times in sterile water before the brains removed in sterile *Drosophila* Medium (Schneiders medium + 10% fetal bovine serum +

50 μg/ml insulin). The cephalic lobes were discarded and only the thoracic abdominal ganglion was used in cultures. The ganglia were microdissected to remove ventral lying GFP positive neurons that have not been identified (see last chapter for more detail). The ganglia were enzymatically treated in liberase for 1 hour at room temperature (10 μl 2x liberase stock/ 1ml Rinaldinise saline [800 mg of NaCl + 20 mg of KCl + 5 mg of $NaH_2PO_4*H_20$ + 100 mg of $NaHCO_3$ + 100 mg of glucose + 100 mls of H_2O]). The ganglia were rinsed twice in 1 ml *Drosophila* Medium, triturated with a fire polished glass Pasteur pipet, further triturated with an Eppendorf tip and plated on Con-A/Laminin coated dishes in a 100 μl bubble. The cells were plated at a density of 1 microdissected ganglion/dish. The dishes were flooded 24hrs later with 1 ml *Drosophila* medium and imaged 24hrs after flooding. The cells were maintained in a 25^{o}C, humidified incubator.

The culture dishes were made by drilling a 8 mm hole in 35 mm Petri dishes (Corning 430165). A 12 mm round coverslip (Bellco 1943-10012) was then glued under the Petri dish with Sylgard 184 to create a well. The dishes were placed under UV light for 2 hrs to sterilize. The well was coated with 70 μl of ConA (Sigma C-2010) + laminin (Becton-Dickinson 354232) + milliQ H_2O and incubated for 2 hrs at 37^{o}C. Dishes were then rinsed with 6ml sterile H_2O and used within 1 month.

The motor neurons in the high K^+ experiments were fed medium with a 10mM increase in [K^+] from 22 mM in normal *Drosophil*a culture medium to 32 mM by the addition of KCl (refer to methods in Chapter 4). Control cells for the high K^+

experiments were fed medium with a similar osmolarity by adding the same concentration of NaCl as KCl to the *Drosophila* medium.

Gene switch cultures were exposed to $9x10^{-10}$ M RU-486 dissolved in EtOH for conditional GAL4 expression at 2DIV. This concentration was used because it is the Kd for progesterone, and a small quantity of progesterone to initiate GAL4 activity to minimize the possible non-specific transcriptional effects of progesterone.

Cell Analysis

The cells were imaged live with a Hamamatsu c4742-96 camera using Wasabi software version 1.4 on a Nikon inverted scope at 60x. Both phase/contrast and fluorescent images were obtained for each cell, and the motor neurons had to meet a set of criteria in order to be imaged: alive and healthy with no signs of lifting off the dish, bright GFP signal representing a high level of UAS expression, and isolation from neurite interactions with other neurons. The Tiff image files were then analyzed by tracing the fluorescent image by hand on a Wacom tablet PC. The parameters of total neurite length, total branch number, higher order branching patterns and cell body circumference were recorded using Simple PCI software.

Immunocytochemistry

Immunofluorescent labeling techniques of *Drosophila* tissue have been previously described (Sanyal *et al*., 2003). Tissue was dissected in PBS (milliQ H_2O + 130 mM NaCl + 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4) followed by 3 rinses in PBS. The tissue was fixed in 4% paraformaldehyde in PBS for 1 hr at room temperature. The fixative was

removed with 3x10 min washes of PBS and place prep in block (PBS + 2 % bovine serum albumin + .1% Triton X-100 + 5% Goat Serum) for 2 hrs at room temperature. The block was replaced with primary antibody at appropriate dilution in 200 µl block and kept overnight at 18^{o}C. Anti-GFP (Mol Probes, A-11120) was used to label the axon of MNs with GAL4 expression at 1:50, β-Gal (Promega, Z3781) used to label nod-LacZ localization in culture at 1:50. The preparations were then rinsed 3 quick + 3x10 min washes in PBS before being placed in 500 µl secondary antibody at appropriate dilution (1:1000 used for all secondaries) for 1 hr at room temperature. The secondary was washed with large volumes of TBS (PBS + .1% Triton X-100), 3 quick + 3x10 min washes with the last wash in PBS. The preparation was mounted on Superfrost Plus slides (VWR Scientific, 48311-703) in 80% glycerol +PBS and covered with a glass coverslip. The preparation was stored at -20^{o}C until imaging.

Statistics

All data were compared with an ANOVA followed by post-hoc pair wise comparisons. Data were considered to be significant with a p value $\leq$ .05. All values are listed as the mean $\pm$ SE.

5.4 Results

Characterization of depolarization induced motor neuron neurite outgrowth

It was described in the last chapter that *Drosophila* motor neurons grow processes *in vitro* that can be quantified and that depolarization induced an increase in total neurite length and branching. Here we verified and built on this finding in a new set of experiments. Depolarization induced dendritic growth has been well characterized for vertebrate culture systems, including extensive work done with rat hippocampal cultures (Wu *et al*., 2001). These studies demonstrate a need for chronic depolarization to induce dendritic outgrowth and branching (Gaudilliere *et al*, 2004; Vaillant *et al*., 2002). A series of depolarization timeline experiments were conducted to address this issue in *Drosophila* (Figure 1a).

Neurons were plated and grown in normal *Drosophila* medium for 24 hrs before being flooded with either high K^+ or control medium (see methods for details). As reported in the last chapter, the 2 DIV group displayed a marked increase in both neurite length and branch number compared to controls after exposure to high K^+ for 24 hrs (Figure 1b and c - Group 3). In order to investigate possible short term influences of depolarization on dendritic growth, 2 DIV motor neurons were exposed to high K^+ for only the final 6hrs in culture. The total length and branching of these neurons was not different from controls, suggesting that brief depolarization is not sufficient for depolarization induced outgrowth (Figure 1b and c - Group 2).

By 3 DIV, control cells were significantly larger than control cells at 2 DIV (Figure 1b – Group 4). However, the 3 DIV cells that had been depolarized for 48 hrs had apparently reached a plateau in growth and were not significantly longer and did not have more branches than the 3 DIV controls (Figure 1b – Group 6). 3 DIV neurons that had been exposed to depolarizing medium for 24 hrs and then were rinsed and placed in control medium for the last 24 hrs were also not significantly different from controls and 48 hr depolarized cells (Figure 1b – Group 5). This suggests that neurons exposed for 24 hrs reached a plateau by 2 DIV, and did not grow significantly during day 3 in culture, similar to Group 6.

A timeline representation of motor neuron neurite length over 3 days illustrates the growth trends of control and depolarized cells (Figure 1d). Although the depolarized neurons appeared to regress slightly from day 2 to day 3, this difference was not significant. The rate of neurite growth is accelerated by depolarization. Control cells, although growing more gradually, reach the same level of growth as depolarized cells by 3 DIV.

The total branch number of depolarized 2 DIV neurons was significantly greater than 2 DIV controls. However, 3 DIV control and depolarized neurons did not have significantly different total branch numbers. These results are very similar to the length findings where depolarization accelerates branch formation by 2 DIV, but then plateaus and control cells reach this plateau one day later.

Drosophila motor neurons maintain polarity *in vitro*

Thus far, only a measurement of total neurite length and branching has been presented. Many of the vertebrate studies mentioned (Wu *et al*., 2001; Gaudilliere *et al*., 2004; Vaillant *et al*., 2002) differentiated between axons and dendrites in vitro with the use of electron microscopy to identify unique microtubule properties or by using antibodies for compartment specific proteins such as MAP2 (Pennypacker *et al*., 1991) and tau (Mandell and Banker, 1995). The structure of microtubules in vertebrate neurons reveals that they maintain polarity in culture, with the plus-end distal microtubule orientation in axons, and dendrites comprised of a mixture of plus and minus-end distal microtubules (Baas *et al*., 1989). The polarity of invertebrate neurons in culture has not been explored to observe if there is homology between their microtubule orientation and that of vertebrates. However, microtubule motor proteins segregate between axons and dendrites in *Drosophila* sensory neurons *in vivo* depending on what direction the motor protein moves along microtubules, with kinesin (plus-end directed motor protein) directed to axons and nod, a member of the kinesin family (minus-end directed motor protein), found specifically in dendrites (Clark *et al*., 1997). It was recently reported that nod, as well as other transport specific molecules, segregates specifically to *Drosophila* motor neuron dendrites *in vivo* (Sanchez-Soriano *et al*., 2005). We have confirmed and extended this finding to identify dendrites *in vitro*. We have found that a nod-LacZ fusion protein localizes in the cell body and all processes except one prominent neurite of cultured motor neurons at 2 DIV (Figure 2a – see blue arrow). A kinesin-GFP fusion protein, on the other hand, was found to localize in only one prominent process of most cultured 2 DIV motor neurons (Figure 2b – see red arrow).

In order to investigate the authenticity of kinesin and nod segregation, a recombinant line was created to express both proteins simultaneously. Chronic expression using the C380-GAL4;;chaGAL80 of both proteins resulted in kinesin still localizing to one major compartment (Figure 3b), but this compartment had a strange morphology I refer to as "blebbing" (Figure 3b - see blue arrow). In addition the nod was no longer excluded from the most prominent neurite (Figure 3c – see blue arrow). Conditional expression of the transgenes with the use of the Gene Switch system (Osterwalder *et al*., 2001) demonstrated that these anomalies were the result of chronic over-expression of the recombinant line. Neurons were administered RU-486 after 2 DIV to provide conditional GAL4 expression of kinesin and nod. 6hrs of exposure to RU-486 led to nod and kinesin synthesis that displayed complete segregation into the presumptive dendritic and axonal compartments (Figure 3d). 12hrs of exposure to RU-486, however, resulted in excess levels of nod spilling into the kinesin domain (Figure 3e – see red arrow). Note that the conditional expression of these proteins prevented the formation of the blebbing phenotype as well. These findings introduce a reliable method for distinguishing *Drosophila* axons and dendrites *in vitro*.

Over-expression of kinesin fusion protein alone, without using the conditional expression system, resulted in more ubiquitous localization with increased time in culture. At 2 DIV most neurons had a single process with kinesin (Figure 4a) whether or not they were depolarized, but a small percentage had multiple processes labeled (Figure 4b). In both control and depolarized 2 DIV cultures, the frequency of multiple kinesin

positive neurites was low, <10-15% of all cells analyzed. By 3 DIV, however, notably more cells had multiple kinesin-containing processes, >30% in both control and depolarized cells, thus making it difficult to decipher which process was the axon (Figure 4c). It is important to note that depolarization did not alter the frequency of kinesin containing processes.

In neurons with only a single kinesin labeled process, the number of neurites sprouting from the soma (i.e. primary processes) at both 2 DIV and 3 DIV was increased with depolarization (Figure 5). This implies that depolarization induces the formation of new, non-kinesin positive primary motor neuron dendrites *in vitro*. However, note in Figure 5 that the number of primary processes among control and depolarized groups did not change between day 2 and day 3. Interestingly, at 2 DIV, the higher order branches per primary neurite (calculated as the total branch number divided by the primary branch number) was higher in depolarized vs. control neurons. Therefore the increase in total branch number caused by depolarization measured on day 2 is due both to an increased in the number of primary and higher order branches per primary branch. However, by 3 DIV the higher order branches per neurite had decreased in depolarized cells, but were increased in controls. Therefore, 3 DIV control neurons catch up in total branch number through higher order branching.

Depolarization induces accelerated growth in motor neuron dendrites

Differentiation of motor neuron axons and dendrites with polarity indicators enabled live cell analysis of depolarization induced growth in separate compartments.

Analysis of the effects of depolarization on neurite length and branching using the kinesin-GFP microtubule motor protein polarity reporter revealed that most of the depolarization induced outgrowth can be attributed to the non-kinesin positive, dendritic compartment. 2 DIV motor neurons with a single prominent process labeled with kinesin were quantified for both control and high K^+. The kinesin positive process, the presumptive axon, was measured to determine length and the number of branches. Similarly, the total length and number of ALL processes were measured for each neuron. Not surprisingly, the total neurite length and branch number were much greater than total axon length and branch number in both control and depolarized neurons (Figure 6a and b). This difference can be attributed to the large dendritic compartment. Axon length and branching were not significantly different in control and depolarized cells (Figure 6a and b). This indicates that depolarization induced growth and branching can be attributed, in large part, to the non-kinesin labeled, dendritic compartment. As reported previously, the total number of branches increased significantly with depolarization. There was not, however, a significant increase in total neurite length with depolarization in this set of experiments (Figure 6a and b).

5.5 Discussion

In this study we verified that depolarization induces an increase in neurite outgrowth of 2 DIV motor neurons as discussed in Chapter 4 and further found that this depolarization specifically accelerates the growth of *Drosophila* motor neuron dendrites. In order to determine this, we first characterized the growth of motor neuron neurites for the first 3 DIV. Depolarization through stimulation by high K^+ accelerates the growth of neurites as measured at 2 DIV. By 3 DIV, however, the control and depolarized cells do not differ significantly in total neurite length and branching. Thus depolarization had accelerated the rate of growth, but not increased the maximum extent of growth by 3 DIV.

2 DIV neurons need greater than 6 hr depolarization in order to observe measurable enhancements in growth. The depolarization took place only 6 hrs before imaging the cells, perhaps not giving the neuron time to initiate the protein synthesis necessary to carry out growth. It would be interesting to briefly depolarize the cells at the beginning of day 2 and then image 18 hrs later. Perhaps the brief pulse of depolarization would initiate signal cascades involved in outgrowth and branching.

Also, 3 DIV neurons depolarized for only 24 hrs, then washed and grown in control medium for the remaining 24 hrs do not vary significantly from 3 DIV control neurons or neurons that were depolarized for 48 hrs. It is likely that these neurons had an acceleration in growth, then reached a plateau by 2 DIV, so that they did not show a net growth during the last 24 hrs in culture. Another possibility is that the removal of depolarizing medium caused a regression in the neurites, but they continued to grow

during day 3 to still reach the same plateau. This can only be deciphered with time-lapse imaging.

I developed an assay for the recognition of axonal and dendritic compartments of cultured motor neurons. The nod and kinesin minus and plus directed microtubule motor proteins, respectively, which have been demonstrated to segregate into dendritic and axonal compartments *in vivo* (Sanchez-Soriano *et al*.; 2005, Clark *et al*., 1997) also segregate *in vitro*. Using kinesin to measure outgrowth of control and depolarized axons *in vitro*, it was determined that axon length and branching did not significantly increase with depolarization. This suggests that the majority of depolarization induced neurite outgrowth observed here and in Chapter 4 is from the dendritic, or non-kinesin positive compartment. In summary, these findings report that a) *Drosophila* neurons maintain polarity in culture, b) depolarization with 10mM K^+ accelerates the growth of neurons such that they reach maximum levels by 2 rather than 3 DIV, and c) the depolarization induced acceleration in neurite growth in 2 DIV motor neurons takes place in the dendritic compartment.

It is interesting to note that when total neurite length was measured in the presence of kinesin-GFP expression, it did not significantly increase under depolarized conditions, which is not consistent with our previous findings. One explanation for this is the anomalies observed with kinesin over-expression. The kinesin-GFP was chronically expressed in this experiment with the C380-GAL4;;chaGAL80 driver for tissue specific expression in motor neurons. Unfortunately, a slight "blebbing" effect was observed in

these cells, although only one dominant process was labeled with kinesin for most cells. Perhaps the congregation of kinesin in this compartment disrupted the microtubule dynamics of the cell, causing more tubulin or other growth substrates to be present here instead of being available for dendritic outgrowth. This also highlights a possible unique mechanism for the development of branches vs. addition of length to a dendrite. The branch number was still significantly increased in this experiment, hinting that perhaps another portion of the cellular matrix, such as the actin cytoskeleton, is involved in branch formation.

The fact that depolarization did not enhance growth beyond a certain plateau level by 3 days, leads us to hypothesize that the depolarization is recruiting cascades that are already involved in basal dendritic growth. These pathways may involve Ca^{+2} dependent cascades, as depolarization induced acceleration of growth is dependent on $I_{Ca}{}^{+2}$ (see previous chapter). Other studies involving Ca^{+2} dependent depolarization induced dendritic growth have shown the activation of CAMK (Gaudilliere *et al*., 2004) and MAPK (Wu *et al*., 2001) or both (Vaillant *et al*., 2002) and activation of the transcription factor CREB (Redmond *et al*., 2002). These pathways promote the protein synthesis necessary for dendritic growth to occur. It would be interesting to investigate the role of kinases and transcription factors that are downstream of Ca^{+2} in this depolarization induced dendritic growth. This will be addressed in Chapter 6.

It is interesting that motor neuron depolarization did not significantly increase axon length and branching. There is ample evidence that manipulation of K^+ channel

function influences motor neuron axon terminal dynamics. Motor neurons *in vivo* in *ether-a-go-go*, *shaker* K^+ channel double mutants have increased synaptic strength and arborization at the neuromuscular junction (Budnik *et al*., 1990; Zhong and Wu, 1991). The difference may reflect that we were observing isolated cells, free from possible synapse formation. It is also possible that in the mutant animals, they observed non-cell autonomous effects, as the mutation affects every cell of the organism. There is evidence that retrograde signals from the muscles innervated by these axons regulate the innervation pattern and transmitter release of the motor neuron (Davis and Goodman, 1998). Perhaps, if the motor neurons were to form synapses *in vitro*, depolarization would influence the complexity of the axonal compartment.

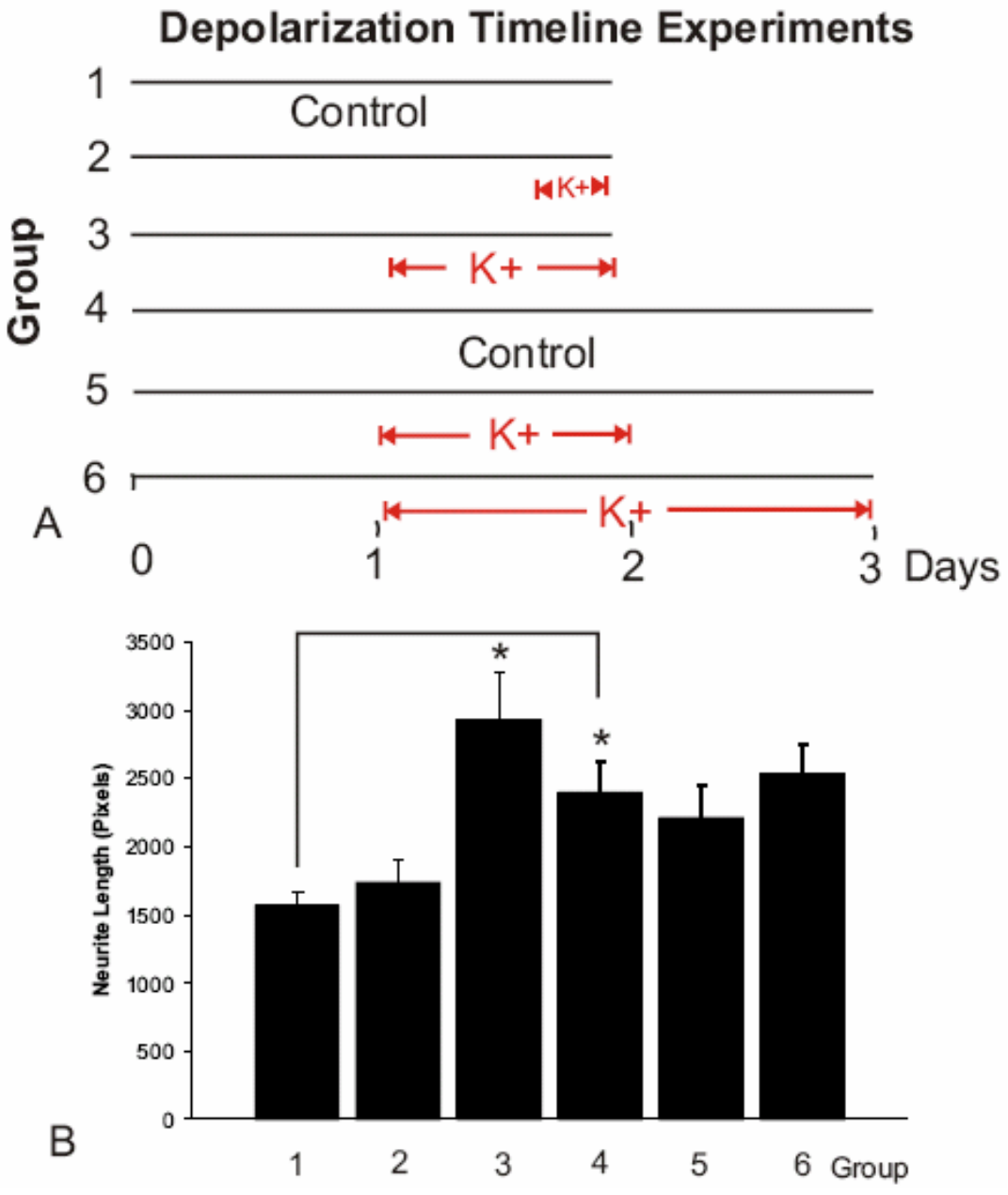
The influences of depolarization on neuron growth and differentiation vary widely between cells, organisms and stage. It would be interesting to see if dendritic growth continues after 3 DIV and if there are other windows of depolarization induced dendritic growth in motor neurons. In addition, this system can be used to investigate the dendritic regulation of many activity related proteins that influence neuromuscular junction plasticity at the same larval stage studied here. This is addressed in the next chapter.

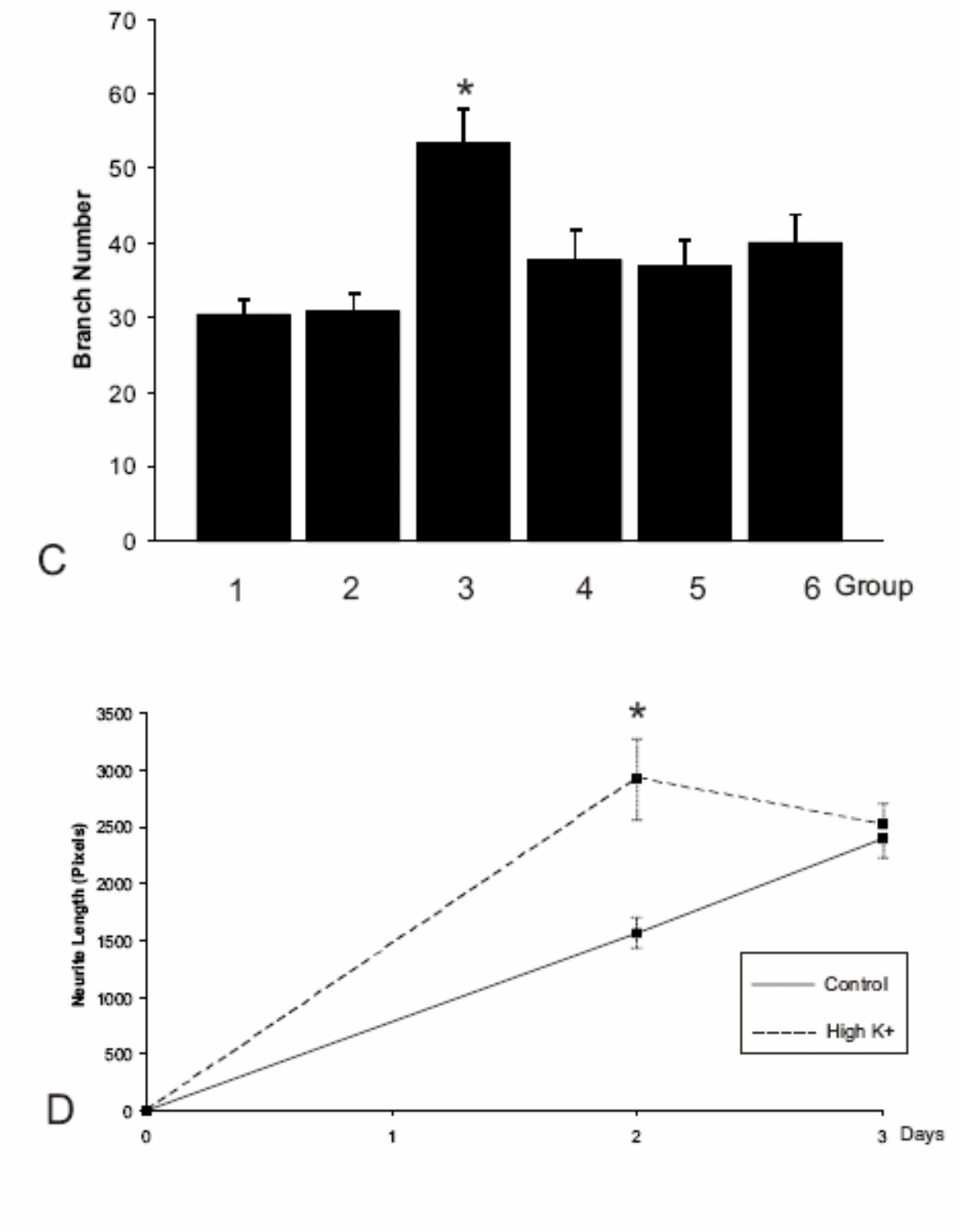
The ability to distinguish the dendritic compartment, when combined with the extensive transgenic system available in *Drosophila,* provides a powerful tool for the study of dendritic growth and branching. However, it is essential to determine if these effects also occur *in vivo*. It is possible that without the inputs that a neuron receives *in vivo*, the typical regulatory cascades are no longer in force. To observe similar effects of

depolarization on dendrites in the intact animal would further justify the use of this system for the study of dendritic growth and branching. The observation of depolarization induced dendritic growth *in vivo* is discussed in Chapter 6, which reveals interesting parallels between the culture and *in vivo* systems.

5.6 Figures

Figure 1

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40
30
20
10
0
Branch Number
*
C
1
2
3
4
5
6
Group
3500
3000
2500
2000
1500
1000
500
0
Neurite Length (Pixels)
*
Control
High K+
D
0
1
2
3
Days

Figure 2

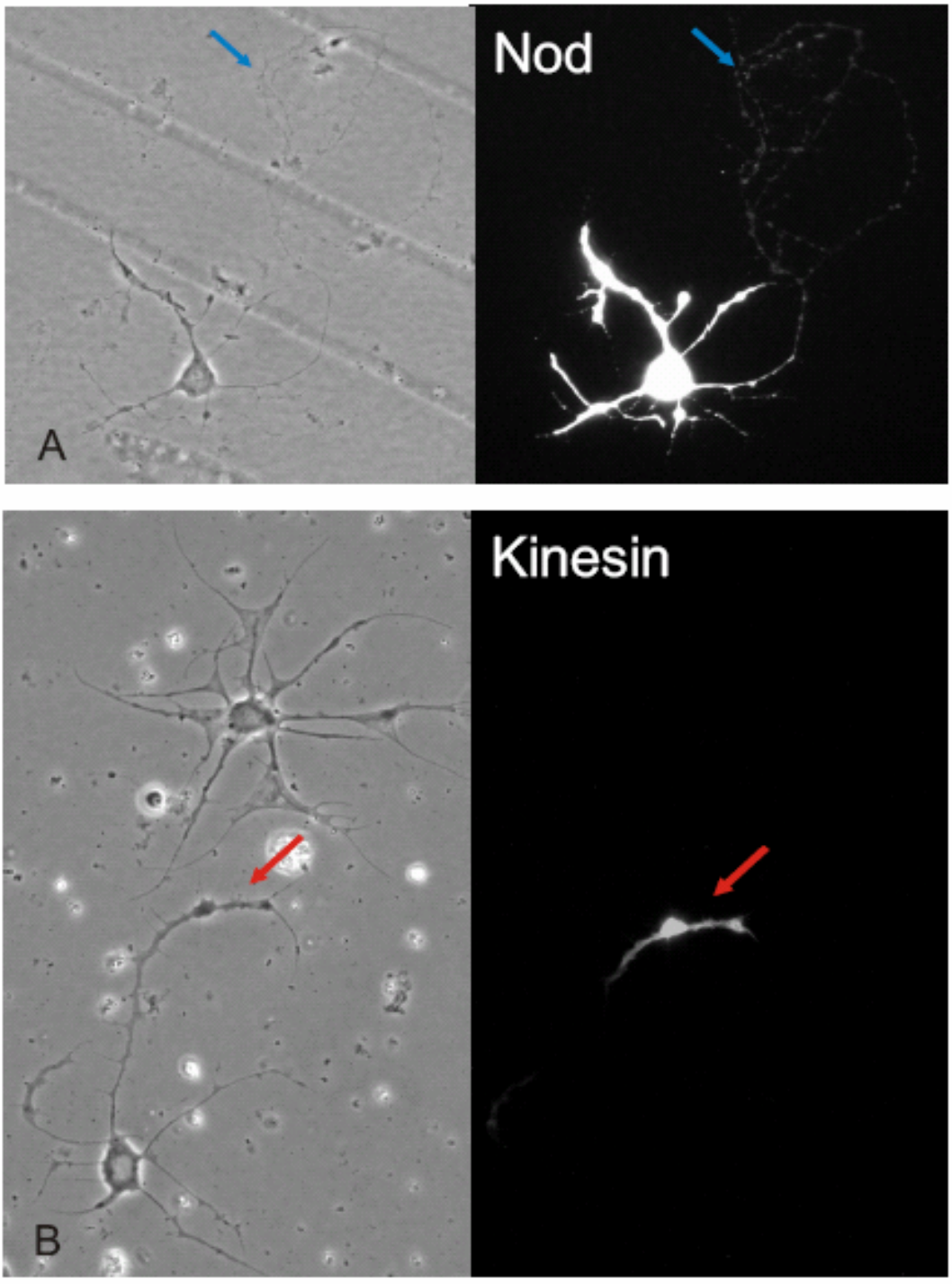


Figure 3

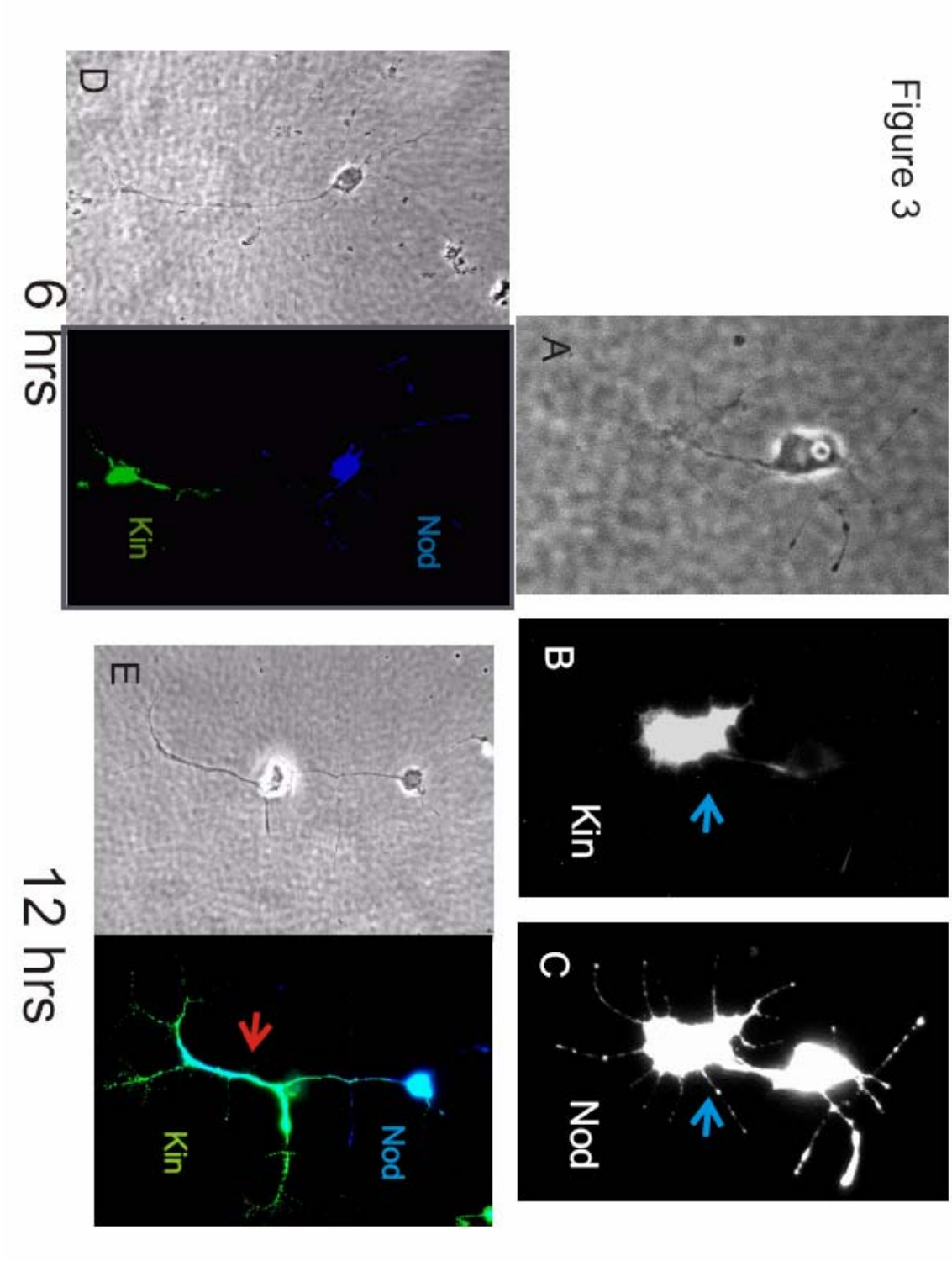


Figure 4

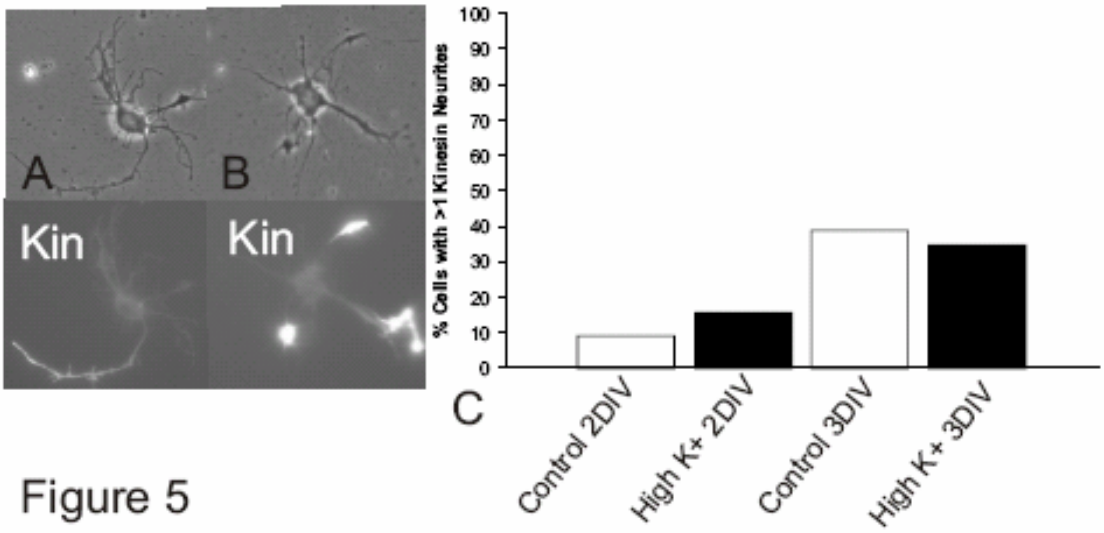


Figure 5

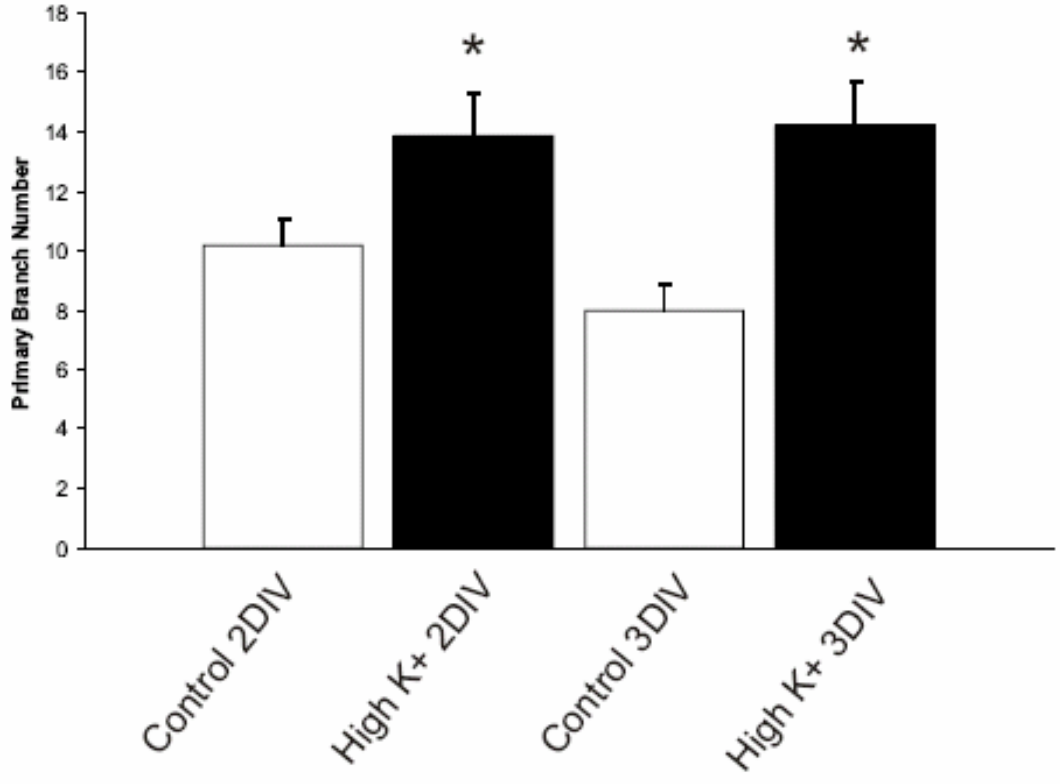
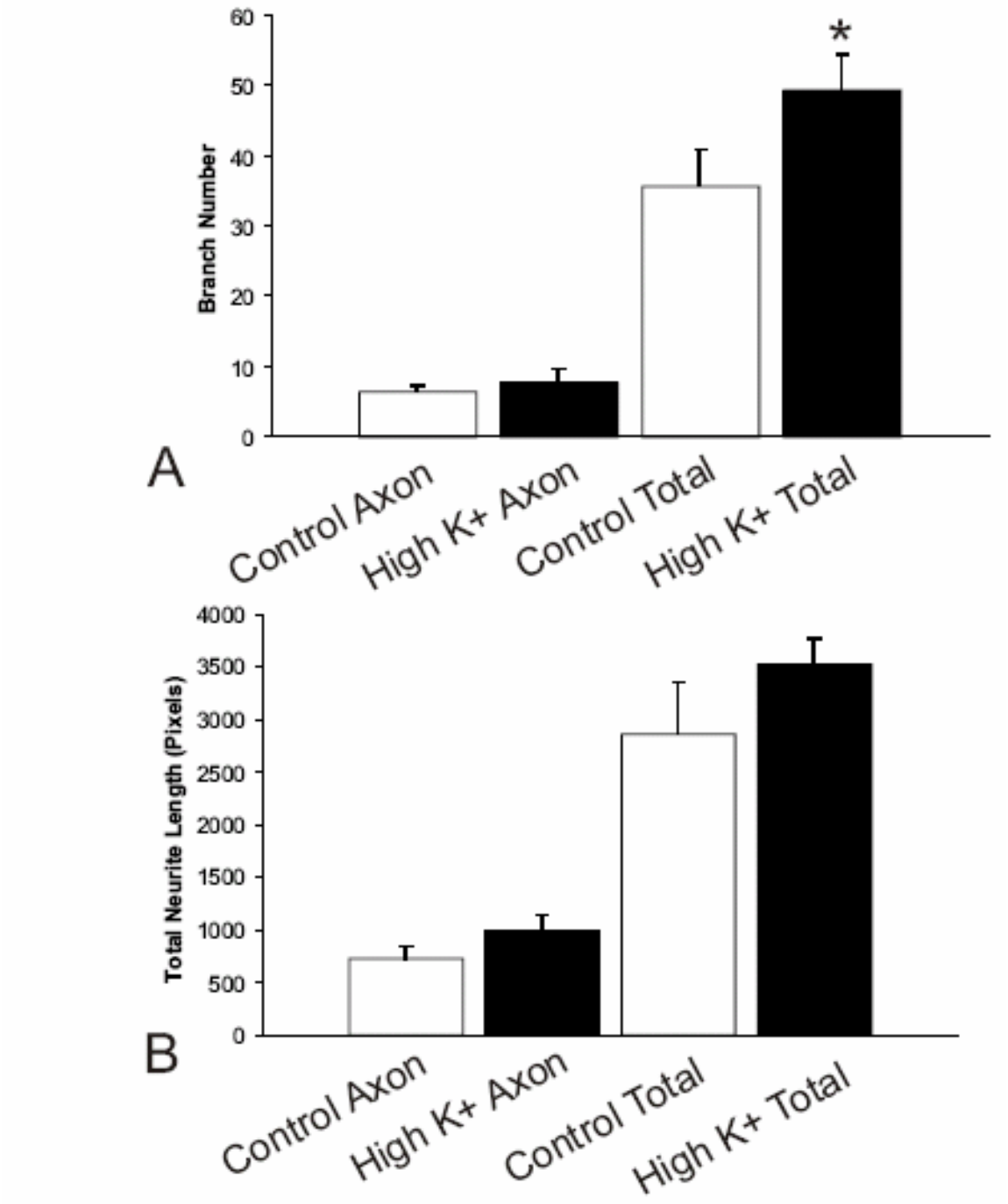


Figure 6


Branch Number
60
50
40
30
20
10
0
*
A
Control Axon
High K+ Axon
Control Total
High K+ Total
Total Neurite Length (Pixels)
4000
3500
3000
2500
2000
1500
1000
500
0
B
Control Axon
High K+ Axon
Control Total
High K+ Total

5.7 Figure Legends

Figure 1

Depolarization accelerates neurite growth. (A) A schematic representation of the motor neuron test groups exposed to depolarization for varying durations. Group 1 represents control neurons grown for 2 DIV. Group 2 represents neurons grown for 42 hrs and then exposed to high K^+ for 6 hrs. Group 3 represents 2 DIV neurons exposed to high K^+ for 24 hrs. Group 3, but not 2, is significantly longer in neurite length (B) and branch number (C) than Group 1. Group 4 represents control cells grown for 3 DIV. These cells have significantly greater length (B), but not branch number (C), than Group 1, indicating that they continue to grow between 2 and 3 DIV. Group 5 represents 3 DIV neurons exposed to 24 hrs of high K^+ followed by 24 hrs of control medium. Group 6 represents 3 DIV neurons exposed to 48 hrs of high K^+. Group 6 length (B) and branch number (C) is not significantly different from Groups 4 and 5. D) The neurite length of Groups 3 and 6 are indicated by the dotted line and of Groups 1 and 4 are indicated by the solid line.

Figure 2

Motor neurons maintain polarity in vitro, as detected with labeled motor proteins. The polarity of neurons in vitro can be detected with the use of the minus end directed microtubule motor protein construct UAS-nod-LacZ (Clark *et al*., 1997) and the plus end directed microtubule motor protein construct UAS-kinesin-GFP (P. Estes, unpublished). Phase contrast imaging is used in conjunction with fluorescent imaging to display localization of each fusion protein within the neurites of motor neurons. A) UAS-nod-

LacZ localizes to all motor neuron neurites except one prominent neurite (see blue arrow) as detected by immunochemistry. B) Live imaging of kinesin-GFP reveals that kinesin is localized to one prominent motor neuron neurite (see red arrow) and not the cell body or other processes.

Figure 3

Recombined nod and kinesin reporter line loses compartmental specificity. C380-GAL4;;chaGAL80 driven expression of the w;;UAS-nod-LacZ,UAS-kin-GFP line (A), kinesin remains in one prominent neurite (B), while nod saturates all compartments (C). Also note the appearance of "blebbing" in the kinesin process (blue arrow). Conditional expression granted with the GSG-elav-GAL4;;chaGAL80 line reveals that both a loss of nod specificity and blebbing are both phenomenon related to transgene over-expression. Cells grown for 2 DIV were exposed to RU486 for varying times. 6 hrs of GAL4 activity allowed the expression of nod and kinesin that were segregated to seperate compartments (D), while 12 hrs of GAL4 expression increased levels of nod that began to spill into the kinesin compartment (E). Also note the typical morphology of the axon is present in the GSG-elav-GAL4;;chaGAL80 neurons (red arrow).

Figure 4

Kinesin compartmentalization dynamics are influenced by extent of expression, but not depolarization. Most motor neurons express kinesin-GFP in one prominent neurite (A), while kinesin-GFP can be detected in more than 1 neurite in a few cells (B). A small percentage of 2 DIV motor neurons express kinesin in >1 neurite in both control

and depolarized cells, but this number notably increases in both control and depolarized groups by 3 DIV (C). The segregation of kinesin to one process of most cells, even in a depolarized background, suggests that any increase in primary neurite number must be the result of formation of new dendrites.

Figure 5

Depolarization induces a significantly increased primary branch number in both 2 DIV and 3 DIV motor neurons that have one kinesin positive process. Thus, the depolarization induced increase in primary neurite number can probably be attributed to dendrite formation.

Figure 6

Depolarization induced dendrite vs. axon growth of 2 DIV motor neurons using the polarity reporter kinesin-GFP to distinguish between neural compartments. Motor neurons with a single kinesin labeled neurite, which was assumed to be the axon, were analyzed in control (A) and high K^+ medium. Axon branch number and total length were not influenced significantly by depolarization. The total number of branches formed by the cell, however, increased significantly with depolarization (C and D). Total neurite length was not increased significantly in this set of experiments.

CHAPTER 6 TRANSCRIPTION FACTOR REGULATION OF DEPOLARIZATION INDUCED DENDRITIC OUTGROWTH OF *DROSOPHILA* MOTOR NEURONS

**Please note:* All of the *in vivo* morphology work presented (figures 1 and 2 and parts of 3 and 4) was performed by Subhabrata Sanyal, Ph.D.

6.1 Abstract

Using new methods to analyze depolarization induced dendritic growth in identified *Drosophila* motor neurons; we investigated the roles of several transcription factors *in vivo* and *in vitro*. Our data indicate that AP-1 and Adf-1 but not CREB are necessary for a basal level of dendritic growth. While all three are necessary for depolarization induced growth, only AP-1 is sufficient to cause dendritic outgrowth when over-expressed. These results suggest that several pathways are involved in dendritic growth, and illustrate the unique yet overlapping roles of activity induced transcription factors.

6.2 Introduction

During learning and memory formation, information flow through networks is modified, at least in part, through structural alterations in neurons. Dendrites, sites of signal integration, are key targets of depolarization dependent modifications. While local mechanisms of dendritic modification are being extensively studied, global mechanisms mediated by nuclear gene expression are relatively poorly understood. This is a particularly important issue, given that long-term neural plasticity in different organisms is critically dependent on transcription factors such as CREB and fos (Lonze and Ginty, 2002; Nestler, 2001). Recent studies, especially of sensory and olfactory pathway neurons, have demonstrated the potential utility of *Drosophila* for understanding how dendritic architecture is genetically specified (Grueber *et al*., 2003; Marin *et al*., 2005). In the current study we describe techniques to induce and measure depolarization-stimulated changes in dendritic fields of identified *Drosophila* motor neurons *in vivo* and in primary culture. We use these methods to determine and demonstrate the role of transcription factors in regulating dendritic growth as well as its necessity during depolarization-dependent dendritic proliferation.

Synthetic regulation, or artificially changing the membrane properties and activity of a neuron *in vitro,* has been used to mimic electrical changes that would be caused by synaptic input *in vivo.* This synthetic regulation can include alterations in ion concentrations in the culture media, direct stimulation of a cell with an electrode, or exposure of cells to neurotransmitters. For example, Vaillant and colleagues (2002) demonstrated that reversible dendritic elaboration of sympathetic neurons of the superior

cervical ganglion could be induced through all three methods. Cerebellar granule neurons (Gaudilliere *et al*., 2004), and CA3/CA1 hippocampal pyramidal neurons (Wu *et al*, 2001), also respond to stimulation with high K^+ concentrations in the culture medium. These studies found that depolarization induced dendritic plasticity is dependent on voltage gated calcium influx, which leads to the activation of MAPK and CAMK cascades. At the *Drosophila* neuromuscular junction genetic manipulation of I_K^+ or depolarization dependent kinases and transcription factors lead to plasticity. The previous two chapters of this dissertation have demonstrated that manipulation of I_K^+ to depolarize motor neurons *in vitro* leads to an acceleration of dendritic growth that is dependent on voltage gated calcium current. However, it is not known if depolarization leads to an induction of dendritic growth *in vivo*. This paper will address the issue by using a new motor neuron specific driver for precise expression of transgenes.

Work done at the *Drosophila* neuromuscular junction gives insights into the molecular players that are involved in depolarization dependent plasticity. An eag-sh double mutant combines mutations in subunits of the *ether-a-go-go* (eag) and *shaker* (sh) voltage gated K^+ channels to interfere with the normal function of the proteins. This results in hyperexcitability and increased synaptic arborization at the neuromuscular junction (Budnik *et al*., 1990). It is suggested that depolarization induced changes at the neuromuscular junction may work through voltage gated calcium influx, as knocking out voltage gated Ca^{+2} channel function with a temperature sensitive α-subunit mutation results in a reduction in synaptic transmission and nerve terminal length (Xing *et al*., 2005). Calcium itself is an established intracellular signaling molecule, initiating many

neuronal cascades through activation of kinases (Ghosh and Greenburg, 1995) and some of these molecular players have also been shown to influence neuromuscular junction size and strength. In *dunce* mutations, a disruption of a phosphodiesterase results in increased cAMP levels that also leads to increases in synaptic strength and number in this system (Zhong *et al*., 1992). The kinases downstream of cAMP, including constitutively activated forms of Ras (part of the MAPK cascade) and CaMKII, increase bouton count alone or both bouton count and synaptic strength, respectively (Koh *et al*., 2002; Park *et al*., 2002). These kinases work through transcription factor activation, including CREB and AP-1. CREB itself only increases synaptic strength, not bouton number (Davis *et al*., 1996). On the other hand, AP-1 increases both synaptic strength and number, independent of CREB (Sanyal *et al*., 2002). Depolarization induced growth in cultured motor neuron dendrites, may be influenced by these pathways as well.

Despite the extensive amount of work done to explore the role of activity and activity induced factors on *Drosophila* motor neuron axon strength and shape, no studies, other than the ones presented in Chapters 4 and 5, have looked at the influence these factors may have on motor neuron dendrites. It is known that *Shortstop*, a cytoskeletal associated protein, and guidance molecules such as *Frazzled*, *Netrin* and *Roundabout*, are necessary for motor neuron dendritic sprouting (Prokop *et al*., 1998; Furrer *et al*., 2003), but these proteins have not been associated with activity. Motivation for the investigation of activity related proteins and dendritic growth stems from the evidence that mechanisms of growth and branching present in a neuron are often involved in influencing both the axon and dendrite (Kim and Chiba, 2004). This report will address

several technical issues that have hindered the study of motor neuron dendrites. GFP labeled motor neuron dendrites of the late 3rd instar larvae are too dense and complex for conventional quantification, like Scholl analysis, that requires the ability to differentiate individual dendritic branches. Motor neuron specific drivers for identifying individual sets of dendrites in vivo have been unavailable until recently. Equivalents of the classic vertebrate markers for dendrites and axons *in vitro*, such as MAP2 (Pennypacker *et al*., 1991) and tau (Mandell and Banker, 1995), are not yet available for *Drosophila*. In the previous chapter, we reported a reliable protocol to detect distinct dendritic and axonal compartments in *Drosophila* motor neurons *in vitro*. These new assays for dendritic growth *in vivo* and *in vitro* will contribute to the discovery of mechanisms underlying dendritic sprouting, guidance, maturation, growth, and plasticity.

6.3 Methods

Fly Strains

Late 3rd instar larval stage animals were used for all experiments. Larvae were selected from the cross of homozygous female C380GAL-4,UAS-CD8-GFP;;chaGAL80 and males of the Ore-R, w;UAS-fbz, w;;UAS-jbz, w;UAS-fos,UAS-jun, w;;UAS-Adf-1, Adf1Δ1(III), w;UAS-CREB2b, and UAS-CREB2a (III) lines for the *in vitro* analysis of depolarization induced neurite outgrowth. The larvae express GFP in identified motor neurons of the thoracic abdominal ganglion (refer to Chapter 4). A more specific motor neuron line, the w;RN2-GAL4,UAS-mCD8-GFP;act<spacer<GAL4,UAS-Flp line (see results for a description of the line) was crossed to Ore-R, EKI (w;UAS-eag(DN),UAS-sh(DN)), w;UAS-fbz, w;UAS-fos,UAS-jun, and EKI-fbz (w;UAS-eag(DN),UAS-sh(DN);UAS-fbz) for the *in vivo* analysis of activity induced dendritic growth.

Primary Cell Cultures

Culture protocols followed have been described previously with a few modifications (Kraft *et al*., 1998). All dissection equipment was soaked in 70% EtOH for 10 min before dissection. Larvae were selected and sterilized by placing the animal in 70% EtOH for 1 min. The larvae were rinsed 3 times in sterile water before the brains removed in sterile *Drosophila* medium (Schneiders medium + 10% fetal bovine serum + 50 µg/ml insulin). The cephalic lobes were discarded and only the thoracic abdominal ganglion was used in cultures. The ganglia were microdissected to remove ventral lying GFP positive neurons that have not been identified. The ganglia were enzymatically treated in liberase for 1 hour at room temperature (10 µl 2x liberase stock/ 1ml

Rinaldinise saline [800 mg of NaCl + 20 mg of KCl + 5 mg of $NaH_2PO_4*H_20$ + 100 mg of $NaHCO_3$ + 100 mg of glucose + 100 mls of H_2O]). The ganglia were rinsed twice in 1 ml *Drosophila* Medium, triturated with a fire polished glass Pasteur pipet, further triturated with an Eppendorf tip and plated on Con-A/Laminin coated dishes in a 100 µl bubble. The cells were plated at a density of 1 microdissected ganglion/dish. The dishes were flooded 24hrs later with 1 ml *Drosophila* Medium and imaged 24hrs after flooding. The cells were maintained in a 25^{o}C, humidified incubator.

The culture dishes were made by drilling a 8 mm hole in 35 mm Petri dishes (Corning 430165). A 12 mm round coverslip (Bellco 1943-10012) was then glued under the Petri dish with Sylgard 184 to create a well. The dishes were placed under UV light for 2 hrs to sterilize. The well was coated with 70 µl of ConA (Sigma C-2010) + laminin (Becton-Dickinson 354232) + milliQ H_2O and incubated for 2 hrs at 37^{o}C. Dishes were then rinsed with 6ml sterile H_2O and used within 1 month.

The motor neurons in the high K^+ experiments were fed medium with a 10mM increase in [K^+] from 21.46 mM in normal *Drosophil*a culture medium to 32.19 mM by the addition of KCl (see Chapter 4 for details). Control cells for the high K^+ experiments were fed medium with a similar osmolarity by adding the same quantity of NaCl as KCl to the *Drosophila* medium.

Drug Application

Cycloheximide was administered to cultures as previously described for the inhibition of protein synthesis (Mizel and Mizel, 1981). Cycloheximide was added at

$1x10^{-2}$ mg/ml to the *Drosophila* medium at flooding and added to 24 hr old neurons, 24 hrs before imaging.

Live Cell Assay

A live cell assay was performed on cycloheximide exposed cells to test their viability using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, L-3224). The culture media was washed with 500 mls of PBS and then placed in 4 M EthD-1 solution in PBS and incubated at room temperature for 20-40 min. The labeled cells were then viewed under a fluorescent microscope and imaged (see cell analysis below).

Cell Analysis

The living cells were imaged with a Hamamatsu c4742-96 camera using Wasabi software version 1.4 on a Nikon inverted scope at 60x. Both phase/contrast and fluorescent images were obtained for each cell, and the motor neurons had to meet a set of criteria in order to be imaged: alive and healthy with no signs of lifting off the dish, bright GFP signal representing a high level of UAS expression, and isolation from neurite interactions with other neurons. The Tiff image files were then analyzed by tracing the fluorescent image by hand on a Wacom tablet PC. The parameters of total neurite length, total branch number, higher order branching patterns and cell body circumference were recorded using Simple PCI software.

Fos Induction Analysis

After staining cell cultures with anti-fos immunocytochemistry, the cell nuclei were imaged using consistent gain (255) and exposure time (120 ms) parameters with a

Hamamatsu c4742-96 camera using Wasabi software version 1.4 on a Nikon inverted scope at 60x. The cells were first identified through GFP expression and then the red nuclear signal was imaged. The images were analyzed for the fos induction experiments by tracing a region of interest around the nucleus of each cell and analyzing the average pixel intensity of signal within the area with Simple PCI software. This blind experiment was repeated twice to confirm the findings.

Dendritic Field 3D Measurements *in vivo*

Isolated RP2 neuron containing segments were selected for imaging. Only segments corresponding to abdominal segments 3-6 were used since the size of the neuropil varies by segment. Images were gathered such that the z steps matched pixel dimensions in the x-y plane to make a cubic voxel for 3D reconstruction. The voltex function in AMIRA was used to construct a 3D image, followed by volume-edit to select only the neuron of interest. Isosurface labeling was used to create the "shell" around the entire dendritic field. The volume contained in this shell was used as a measure of the dendritic field.

Immunocytochemistry

Immunofluorescent labeling techniques of *Drosophila* tissue have been previously described (Sanyal *et al*., 2003). Tissue was dissected in PBS (milliQ H_2O + 130 mM NaCl + 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4) followed by 3 rinses in PBS. The tissue was fixed in 4% paraformaldehyde in PBS for 1 hr at room temperature. The fixative was removed with 3x10 min washes of PBS and place prep in block (PBS + 2 % bovine

serum albumin + .1% Triton X-100 + 5% Goat Serum) for 2 hrs at room temperature. Block was replaced with primary antibody at appropriate dilution in 200 µl block and kept overnight at 18^{o}C. Anti-GFP (Mol Probes, A-11120) was used to label the motor neurons with GAL4 expression at 1:50, topro (Invitrogen, T-3605) used to label neuron nuclei localization in culture at 1:500, anti-HRP (Abcam, 19496) used to label peripheral axons 1:5000. The preps were then rinsed 3 quick + 3x10 min washes in PBS before being placed in 500 µl secondary antibody at appropriate dilution (1:1000 used for all secondaries) for 1 hr at room temperature. The secondary was washed with large volumes of TBS (PBS + .1% Triton X-100), 3 quick + 3x10 min washes with the last wash in PBS. The preparation was mounted on Superfrost Plus slides (VWR Scientific, 48311-703) in 80% glycerol +PBS and covered with a glass coverslip. The preparation was stored at -20^{o}C until imaging.

Generating anti-dFos antibodies

To generate anti-dFos antibodies, we scanned the *Drosophila* fos sequence to select peptides that are unique (no close matches returned with BLAST) and highly antigenic. A 21 amino acid peptide (ERTTKKPAIRKPEDPDPAEED) was synthesized by Fabgennix Inc., Shreveport, LA and after conjugation with KLH, injected into rabbits for antibody production. Following standard immunization and boosting protocols, the antibody was affinity purified against immobilized peptide and used in our assays. Increased fos immunoreactivity was detected in appropriate tissues when fos was expressed transgenically using the GAL4-UAS system.

Statistics

All data were compared with an ANOVA followed by post-hoc pair wise comparisons. Data were considered to be significant with a p value $\leq .05$. All values are listed as the mean $\pm$ SE.

6.4 Results

Several enhancer trap lines (C380, OK6, and D42) have been made that express GAL4 in identified motor neurons of the dorsal medial neuron cluster in the *Drosophila* thoracic abdominal ganglion, although these lines also express GAL4 in unidentified ventral medial and lateral neurons. These lines are sufficient for studies at the neuromuscular junction, as only the axon of these motor neurons is reporter positive in the periphery. However, study of motor neuron dendritic properties requires a line that is much more specific, especially *in vivo* where differentiation of one dendritic compartment from another is necessary for accurate analysis of the influences on dendritic field parameters (Figure 1). We began our pursuit of a motor neuron specific driver line by adding the choline-acetyl transferase (Cha) promoter driven GAL80 to inhibit GAL4 expression in all cholinergic cells. This did dramatically decrease the number of GAL4 positive neurons in the ganglion, but ventral unidentified cells still remain. These lines can be used for culture work, as the ventral cells can be successfully microdissected out of the ganglion to leave a pure GAL4 positive motor neuron population *in vitro* (see Chapter 4 for more details). However, *in vivo* work requires an even more specific line to allow differentiation of individual motor neuron dendritic fields within the ganglion. The w;RN2-GAL4,UAS-mCD8-GFP;act<spacer<GAL4,UAS-Flp line was made to address this issue.

The evenskipped enhancer element (eve^{RRK}) strongly expresses in aCC and RP2 neurons in embryos but is undetectable in later larval stages. We combined this GAL4 line with the reporter UAS-mCD8-GFP, UAS-Flp (a source of flip recombinase), and

act<spacer<GAL4, an actin promoter separated from GAL4 by a spacer DNA fragment flanked by 2 Flp recombinant target (Frt) sites (Figure 2a). The thoracic abdominal ganglion showed a mosaic pattern of GFP expression, owing to random flip-outs of the spacer from act<spacer<GAL4 in aCC and RP2 neurons during early embryonic stages (Figure 2b). We selected segments where RP2 dendrites (Figure 2c), not aCC (Figure 2d), could be imaged cleanly without conflicting labeling from other neurons in adjoining segments. The identification of the motor neurons was confirmed by their innervation pattern where aCC innervates only DA1 (Figure 2e and f) and RP2 innervates DA1-3 among other muscles (Figure 2g and h). We reconstructed the entire dendritic field using AMIRA and measured a surface tightly enclosing the dendritic field (Figure 2i). The internal volume across samples is consistent and sensitive enough to detect changes in dendritic outgrowth.

We used both *in vivo* and *in vitro* systems to test the roles of depolarization and transcription factors known to be involved in activity regulated cascades. We again verified that depolarization of motor neurons *in vitro* with high K^+ (described in more depth in Chapter 5) leads to an increase in dendritic length 2 DIV (Figure 3a). This depolarization induced growth *in vitro* is dependent on protein synthesis as cycloheximide blocks growth (Figure 3c). Note that cycloheximide also inhibits the normal amount of growth in control motor neurons, revealing that protein synthesis is required for basal levels of neurite outgrowth. The GFP labeled motor neurons that were exposed to cycloheximide were not labeled with the red EthD-1 nuclear stain (Figure 3d-

f), indicating that they are viable. Cells that were dying, however, were easily recognizable with this approach (Figure 3g-i).

Figure 5 of Chapter 4 describes how manipulation of K^+ channel function through the expression of the double dominant negative *ether-a-go-go* (eag-DN) and *shaker* (sh-DN) transgenes also results in increased outgrowth *in vitro*. Similarly, when this double dominant negative construct is driven exclusively in RP2 and aCC motor neurons using the w;RN2-GAL4,UAS-mCD8-GFP;act<spacer<GAL4,UAS-Flp line, it leads to an increase in the dendritic field of RP2 *in vivo* (Figure 3b). This verifies that the depolarization induced neurite growth phenotype is not an artifact of cell culture.

The immediate early transcription factor AP-1, a heterodimer composed of fos and jun, has been implicated in activity induced plasticity at the *Drosophila* neuromuscular junction (Sanyal et al., 2002). A dominant negative UAS-construct of fos (fbz) was used to block AP-1 activity in specific motor neurons and, thereby, test its necessity for activity induced dendritic growth. We found that fbz reduced the dendritic field and neurite growth of motor neurons *in vivo* (Figure 4a) and *in vitro* (Figure 4b). In addition, fbz expression blocked the increase in neurite length that is evoked *in vitro* by high K+, as well as the increase in dendrite volume that is evoked *in vivo* by simultaneous expression of dominant negative *ether-a-go-go* (eag-DN) and *shaker* (sh-DN) transgenes. Fbz also reduced the total number of neurite branches in normal and depolarized backgrounds (Figure 4c). A dominant negative UAS-construct of jun (jbz) was also tested in cultured cells (Figure 4d and e). Unlike fbz, jbz did not reduce the

basal levels of neurite outgrowth and branching *in vitro*, however, it did significantly block depolarization induced outgrowth and branching. It is possible that jbz is not completely blocking jun activity and this would explain the difference observed between jbz and fbz. We also tested whether AP-1 was sufficient to cause enhanced outgrowth in the absence of depolarization. Over-expression of AP-1, using a w;UAS-fos;UAS-jun construct, is sufficient to cause dendritic outgrowth similar to depolarized cells both *in vivo* (Figure 4a) and *in vitro* (Figure 4b).

Although Figure 4 demonstrates that fbz blocks depolarization induced outgrowth and that AP-1 over-expression is sufficient to induce outgrowth, we wanted to see if depolarization directly induces AP-1 activity or if AP-1 is involved in a growth cascade not necessarily downstream of depolarization. We investigated this question by observing fos induction through immunolabeling. Motor neurons placed into culture were exposed to high K^+ medium 24 hrs after plating, similar to the previous studies, and then stained with anti-dfos at different time points. High K^+ stimulation rapidly, but transiently, induced fos expression (Figure 5b and c), with highest levels 4 hrs following stimulation, which dropped to baseline by 20 hrs (Figure 5a). This directly links depolarization to AP-1 induction.

CREB was also found to be involved in depolarization induced growth *in vitro*, but had a different mechanism of action than AP-1. Blocking CREB activity with the Drosophila CREB inactivating protein CREB2b had no effect on basal levels of neurite growth (Figure 6a) or branching (Figure 6b) *in vitro*, but did block depolarization induced

growth and branching (note that control and high K^+ data set is the same as in Figure 4) . Over-expression of the CREB activating protein CREB2a was not sufficient to cause motor neuron neurite outgrowth and branching compared to controls.

Alcohol dehydrogenase transcription factor 1 (Adf-1) has recently been described as necessary for proper dendritic elaboration of Drosophila sensory neurons (Parrish *et al*., 2006). Therefore, we explored the influence that Adf-1 might have on depolarization induced motor neuron growth *in vitro*. Adf1Δ1, a dominant negative construct, blocked both basal levels and depolarization induced motor neuron neurite growth (Figure 7a) and branching (Figure 7b) as seen with fbz (note that the control and high K^+ data set it the same as in Figure 4). Unlike AP-1, however, Adf-1 over-expression alone was not sufficient to enhance outgrowth.

6.5 Discussion

Two novel techniques described here, one a system to visualize and measure dendritic arbors of identified motor neurons *in vivo* and the other using highly enriched labeling of motor neurons *in vitro* to directly measure neurite outgrowth, represent widely useful tools. These tools are particularly useful in *Drosophila*, where the methods may be combined, via the GAL4 system, with selective perturbation of UAS-coupled transgenes. Used here to reveal functions of the transcription factor AP-1, the two methods independently lead us to completely consistent conclusions. The function of transcription factors CREB and Adf-1 are also analyzed in culture. It is likely that the depolarization induced changes *in vitro* represent the dendritic compartment. It was demonstrated in Chapter 5, through identification of the axon with a kinesin-GFP construct, that depolarization does not significantly increase the length or branching of cultured motor neuron axons.

It is worth noting that AP-1 is implicated in both the growth that is stimulated by transgenic manipulation, which chronically increased the depolarization throughout life of the motor neuron, and the enhanced outgrowth that occurs in response to more acute depolarization by high potassium addition to cultures. This implies that chronic and acute forms of depolarization function through similar cellular mechanisms to produce dendritic outgrowth. It is likely, however, that high K^+ stimulation and simultaneous expression of dominant negative *ether-a-go-go* (eag-DN) and *shaker* (sh-DN) transgene manipulation of K^+ channel function lead to different levels of depolarization and effects on activity. When both techniques were compared side-by-side in cultured neurons

(Figure 5a, Chapter 4), the magnitude of neurite outgrowth in eag-DN/sh-DN neurons was notably greater than high K^+ stimulation. This may be due to chronic influence on motor neurons through embryonic and larval development. It may also indicate that manipulation of these channels alters activity in a way that differs from mere depolarization. For example, altering the level of function of *shaker* channels may alter spike width and frequency.

AP-1, Adf-1 and CREB are necessary for depolarization-induced dendrite growth. Our data suggest that AP-1 activation alone can largely account for activity-stimulated growth observed in *Drosophila* motor neurons *in vitro* and *in vivo*. Significantly, fos expression is transiently upregulated following depolarization. This suggests a mechanism wherein neural activity initiates transcriptionally driven programs that result in long-term change. Fos expression has been correlated with several models of plasticity in vertebrates (Nestler, 2001; Kaczmarek and Nikolajew, 1990; Pennypacker, 1995), and fos has been shown to be induced by activity (Sheng and Greenburg, 1990).

Many investigations involving the role of AP-1 activity in the neuronal cell line PC-12 have demonstrated the role of fos and jun in neurite outgrowth. Neurite outgrowth of PC-12 neurons is dependent on nerve growth factor activation of AP-1 (Guo *et al*., 2001). Also, if c-jun is over-expressed in these cells, it leads to an increased survival rate and neurite outgrowth (Dragunow *et al*., 2000). A constitutively active form of ERK (MAPK) has been found to upregulate AP-1 expression in these neurons as well (Robinson *et al*., 1998). In addition, and increase in fos induction has been linked to

increased spine density in the forbrain associated with learning (Rose, 1991). The system that we have established allows the study of a direct link between AP-1 and activity induced dendritic growth.

It is interesting to note the differences in the effects of blocking fos and jun activity on basal and depolarization induced motor neuron outgrowth. Jbz did not inhibit the basal levels of outgrowth significantly from controls while fbz did. It is possible that Jbz did not entirely prevent jun function, and this is why it did not inhibit growth in the same manner as Fbz. Jbz was somewhat effective however, as Jbz did significantly mute depolarization induced outgrowth. This suggests that fos plays a different role in neurite outgrowth than jun. The jun promoter regions bind with lower affinity to activators like CREB than fos (Nakabeppu *et al*., 1988). Furthermore, fos can homodimerize to initiate transcription (Porte *et al*., 1997). It is possible that these or other related mechanisms may be involved in fos and jun dynamics in this system making fos more influential than jun during growth.

CREB and AP-1 have been found to influence the *Drosophila* neuromuscular junction in different ways. CREB regulates synaptic strength while AP-1 regulates both synaptic strength and synaptic number (Davis *et al*., 1996; Sanyal *et al*., 2002). This suggests that AP-1 is involved in growth mechanisms that CREB does not participate in. Our data in culture illustrates this point. A basal level of neurite growth requires AP-1 activity, but not CREB (Figure 4b and Figure 5a). However, both are required for

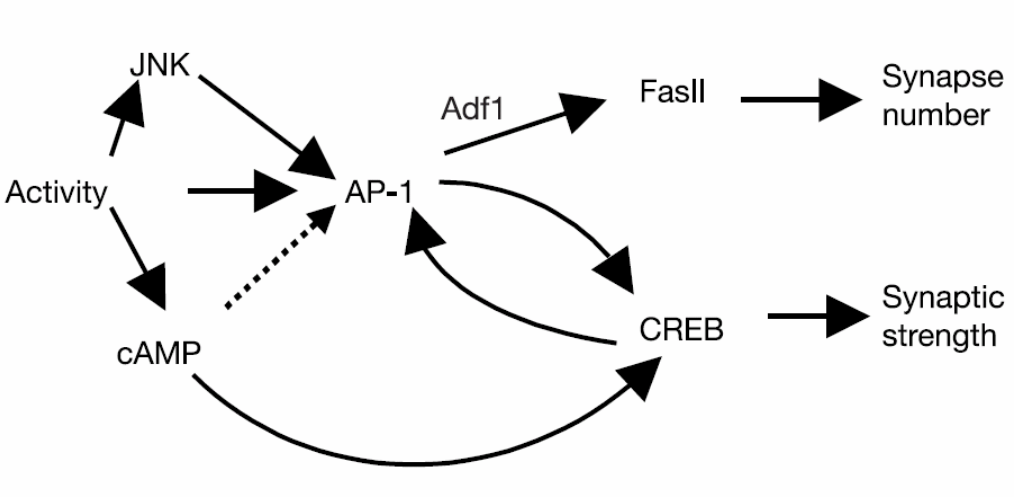
depolarization induced growth. This tells us that AP-1 is involved in both basal and depolarized growth while CREB is only activated during depolarization induced growth.

Adf-1, while it has yet to be studied at the neuromuscular junction, is necessary for growth and branching of Drosophila sensory neurons (Parrish *et al*., 2006). Adf-1, like AP-1, is necessary for both basal and depolarization induced growth (Figure 7), but unlike AP-1, it is not sufficient to induce growth when over-expressed in control medium. This implies that Adf-1 may be downstream of AP-1 and that it and other factors activated by AP-1 are needed to induce depolarization dependent neurite outgrowth.

It is interesting that all of the transcription factors studied *in vitro* had similar influence on neurite growth and branching. For example, fbz affected basal and depolarized levels of both neurite outgrowth and neurite branching. This trend was consistent for jbz, CREB2b, and Adf1Δ1 as well. This suggests that these factors are all involved in a signal cascade that influences both neurite growth and branching.

These findings are surprisingly consistent with a model previously proposed by Sanyal and colleagues (2002) in which they demonstrated the role of AP-1 at the neuromuscular junction. They hypothesized that AP-1 leads to the induction of CREB to influence synaptic strength and that it also induces Adf-1 in a different pathway to increase synapse number (see on next page). Our data fit this hypothesis that CREB and Adf-1 are part of separate pathways. AP-1 and Adf-1 are necessary for basal levels of growth while CREB is not. This is consistent with the NMJ work, as CREB does not

influence synapse arborization. AP-1 over-expression can induce growth on its own because it activates both Adf-1 and CREB, but these two proteins on their own cannot induce a depolarization like outgrowth. In our studies of dendritic growth, we did not investigate a correlate of synaptic strength as was studied at the neuromuscular junction. Perhaps depolarization induced dendritic outgrowth requires both structural changes (Adf-1) and an increase in synaptic architecture (CREB) and AP-1 regulates both mechanisms.



(Sanyal et al., 2002)

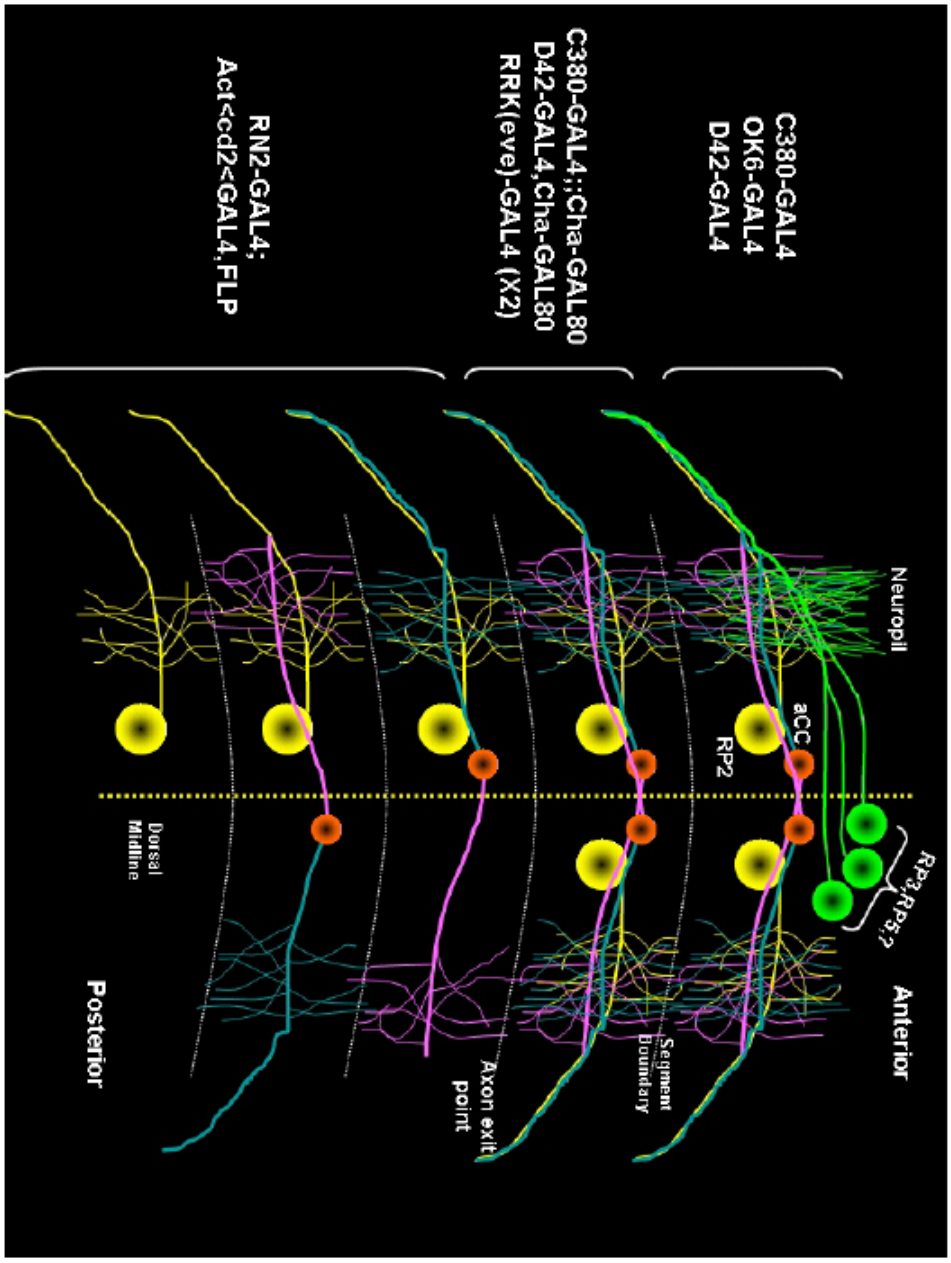
There are some caveats to this conclusion that must be addressed with further experiments. The Adf-1Δ1 and fbz dominant negative contructs interfere with transcription factor activity through competition at the DNA binding domain while CREB2b is an inactivator of CREB activity. It is not clear whether the CREB2b is completely blocking CREB activity. This could be why there is no influence of CREB2b on basal levels of neurite outgrowth. This could be addressed several ways, through either pharmacological blockade of CREB activity in culture, the creation of a CREB

dominant negative, or using a CREB mutant. However, we could also combine CREB2b and AP-1 expression in the same neuron and see if AP-1 is no longer sufficient to induce outgrowth. If this is the case, it would support our model hypothesis and prove that CREB2b is adequately blocking CREB activity. Likewise, we could combine Adf-1Δ1 with AP-1 over-expression and see if outgrowth is blocked or not. If it is, this would also support our hypothesis that AP-1 is upstream of both Adf-1 and CREB and both must be activated to induce depolarization dependent growth.

Ca^{+2} may be upstream of fos activation in this system. It has been demonstrated in vertebrates that voltage gated calcium entry leads to the induction of several early response genes, including c-fos (Ghosh *et al*., 1994). We have also demonstrated in Chapter 4 (Figure 7) that depolarization induced dendritic growth is dependent on voltage gated I_{Ca}^{+2}. As we have further demonstrated here (Figure 4c) that depolarization directly leads to fos induction, it is possible that Ca^{+2} is part of the cascade involved in fos induction. While further experiments will be required to address the interplay between signaling cascades and transcriptional regulation in long-term plasticity, this study indicates an important role for the transcription factors AP-1, Adf-1 and CREB in cell-wide control of activity dependent dendritic plasticity.

6.6 Figures

Figure 1



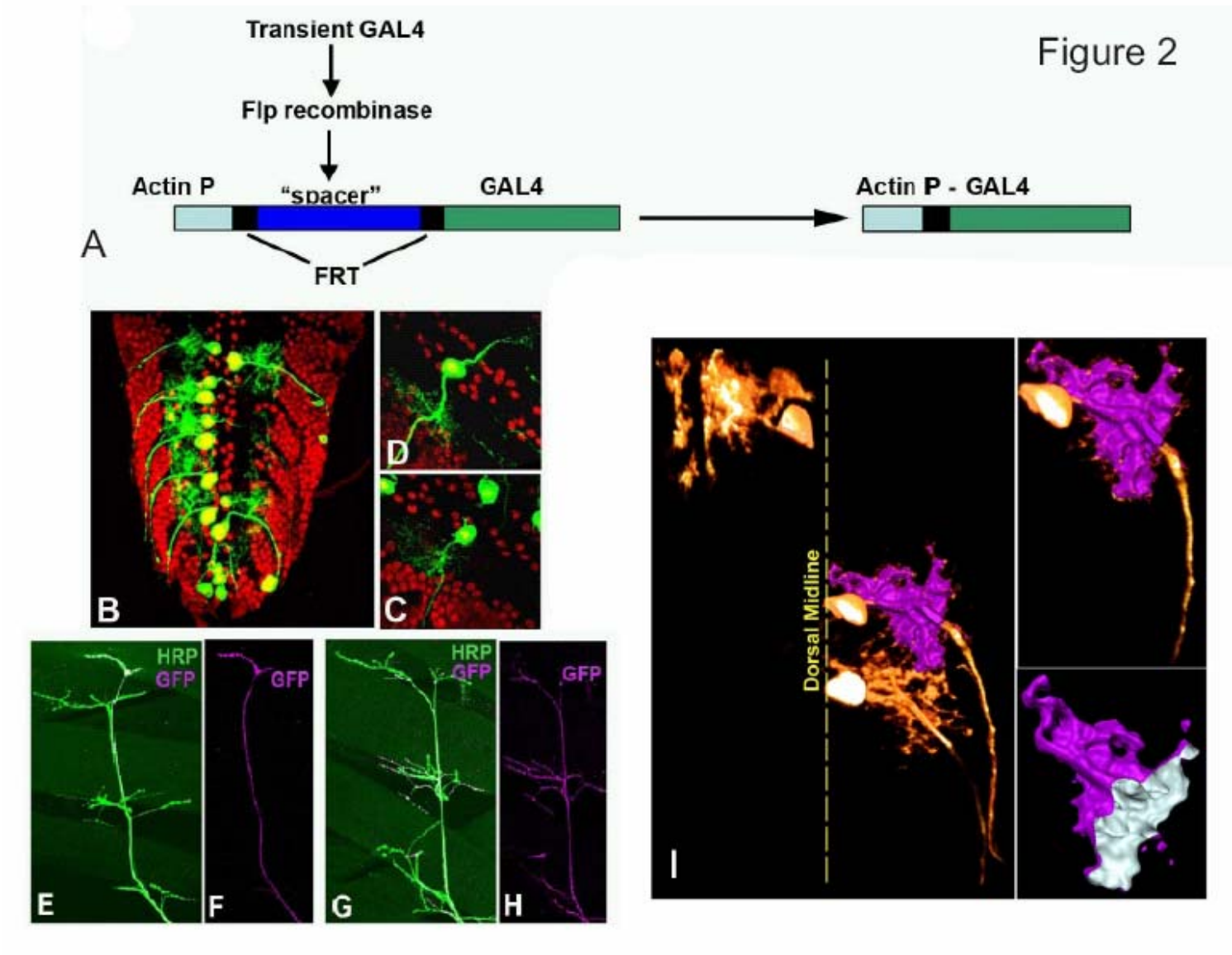
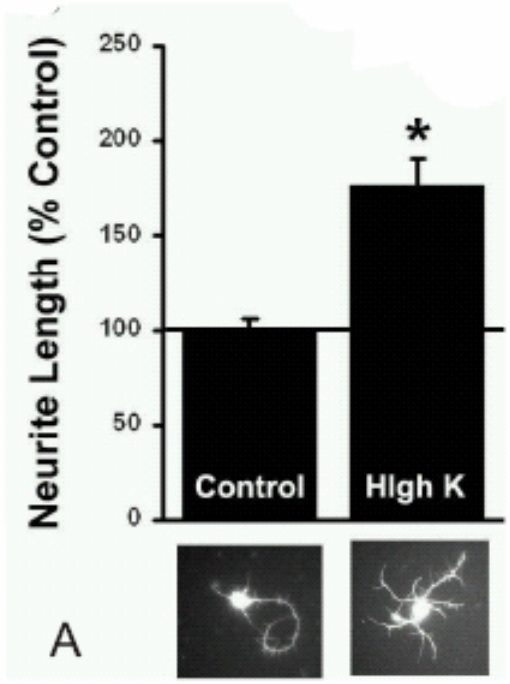
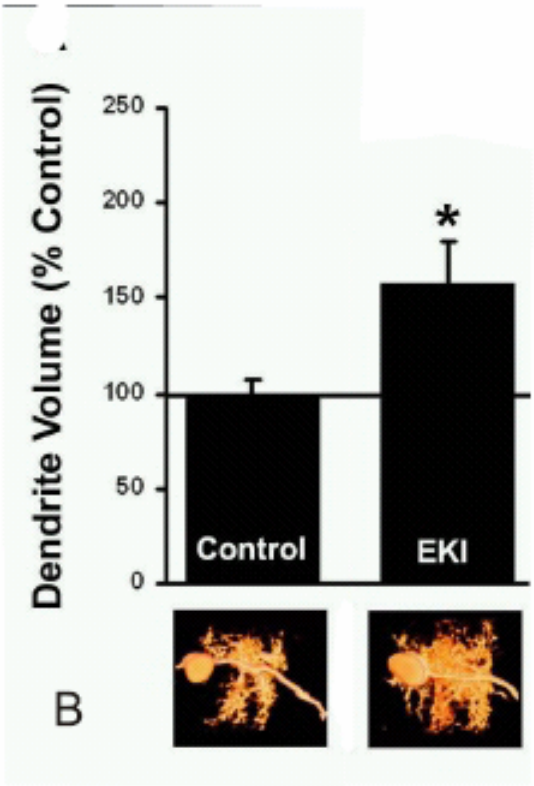
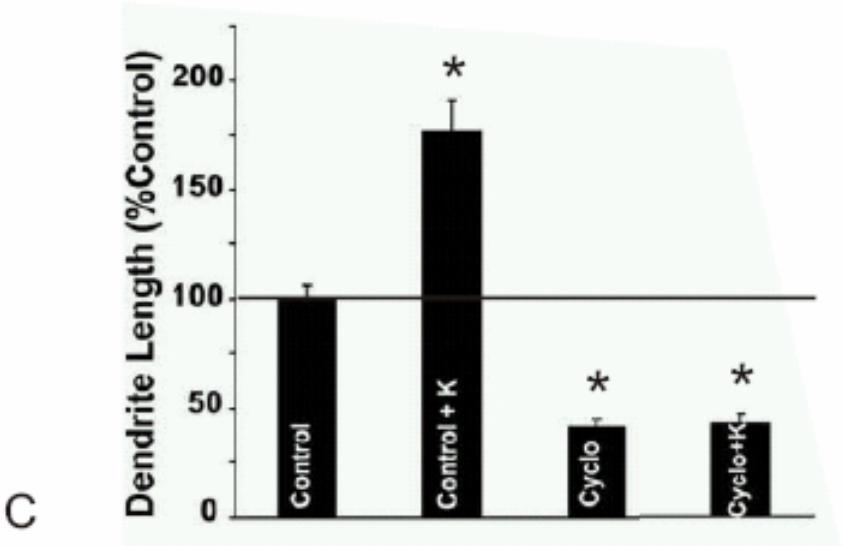


Figure 2
Transient GAL4
Flp recombinase
Actin P
"spacer"
GAL4
Actin P - GAL4
FRT
A
B
C
D
HRP
GFP
GFP
E
F
G
H
Dorsal Midline
I

Figure 3


Neurite Length (% Control)
250
200
150
100
50
0
*
Control
High K
A


Dendrite Volume (% Control)
250
200
150
100
50
0
*
Control
EKI
B


Dendrite Length (%Control)
200
150
100
50
0
*
*
*
Control
Control + K
Cyclo
Cyclo+K
C

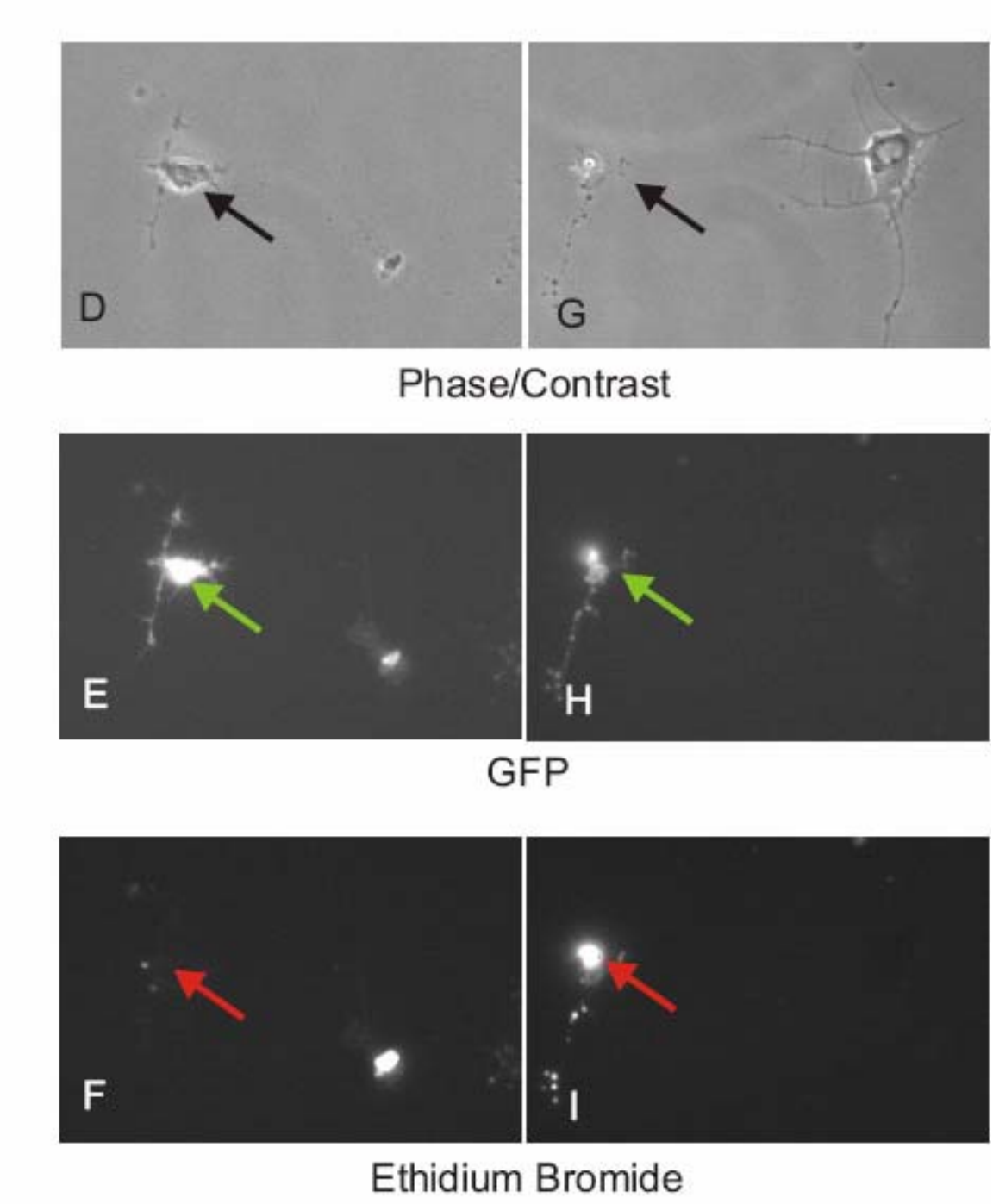
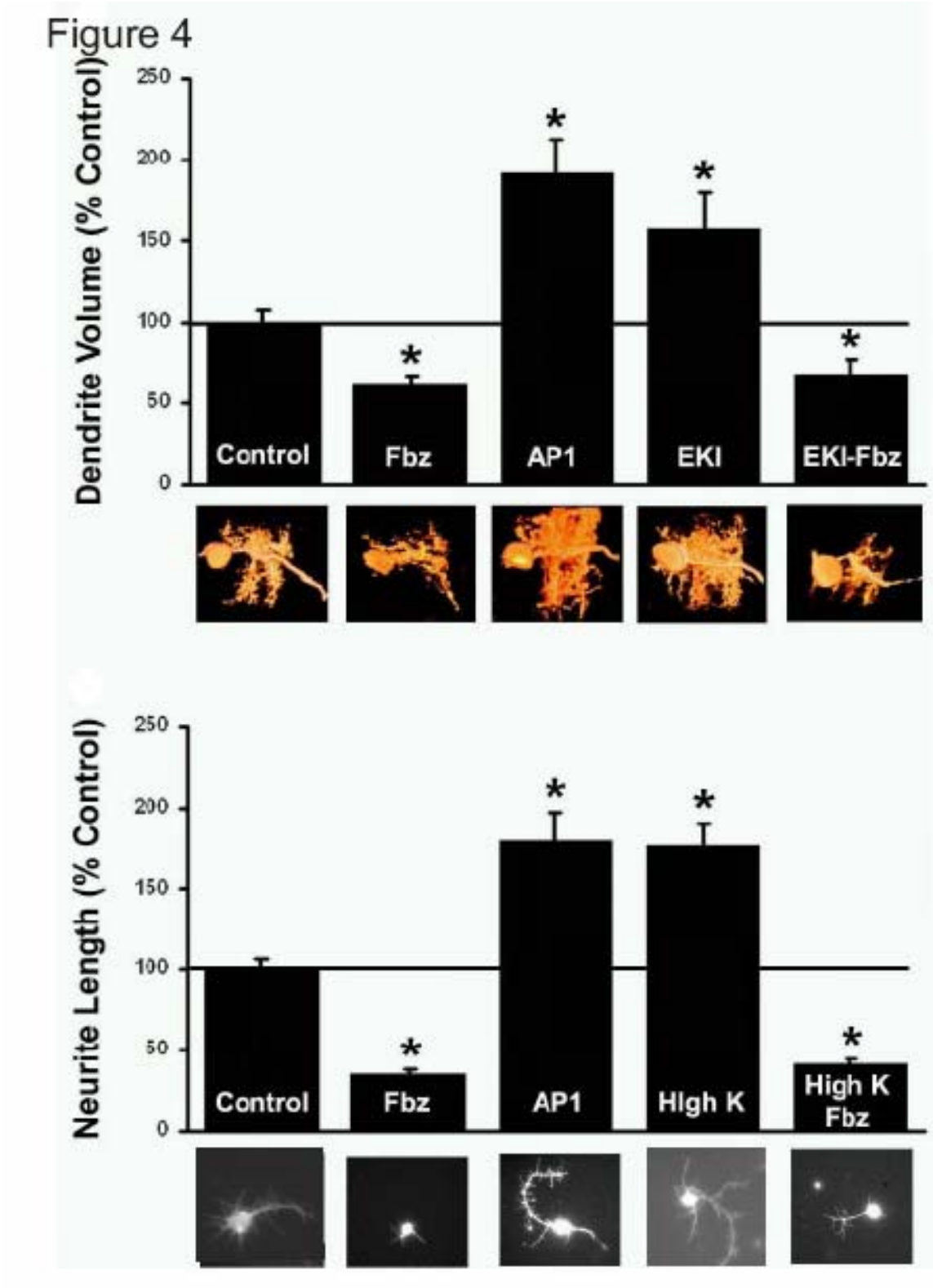

D
G
Phase/Contrast
E
H
GFP
F
I
Ethidium Bromide

Figure 4


Dendrite Volume (% Control)
250
200
150
100
50
0
Control
Fbz
AP1
EKI
EKI-Fbz
Neurite Length (% Control)
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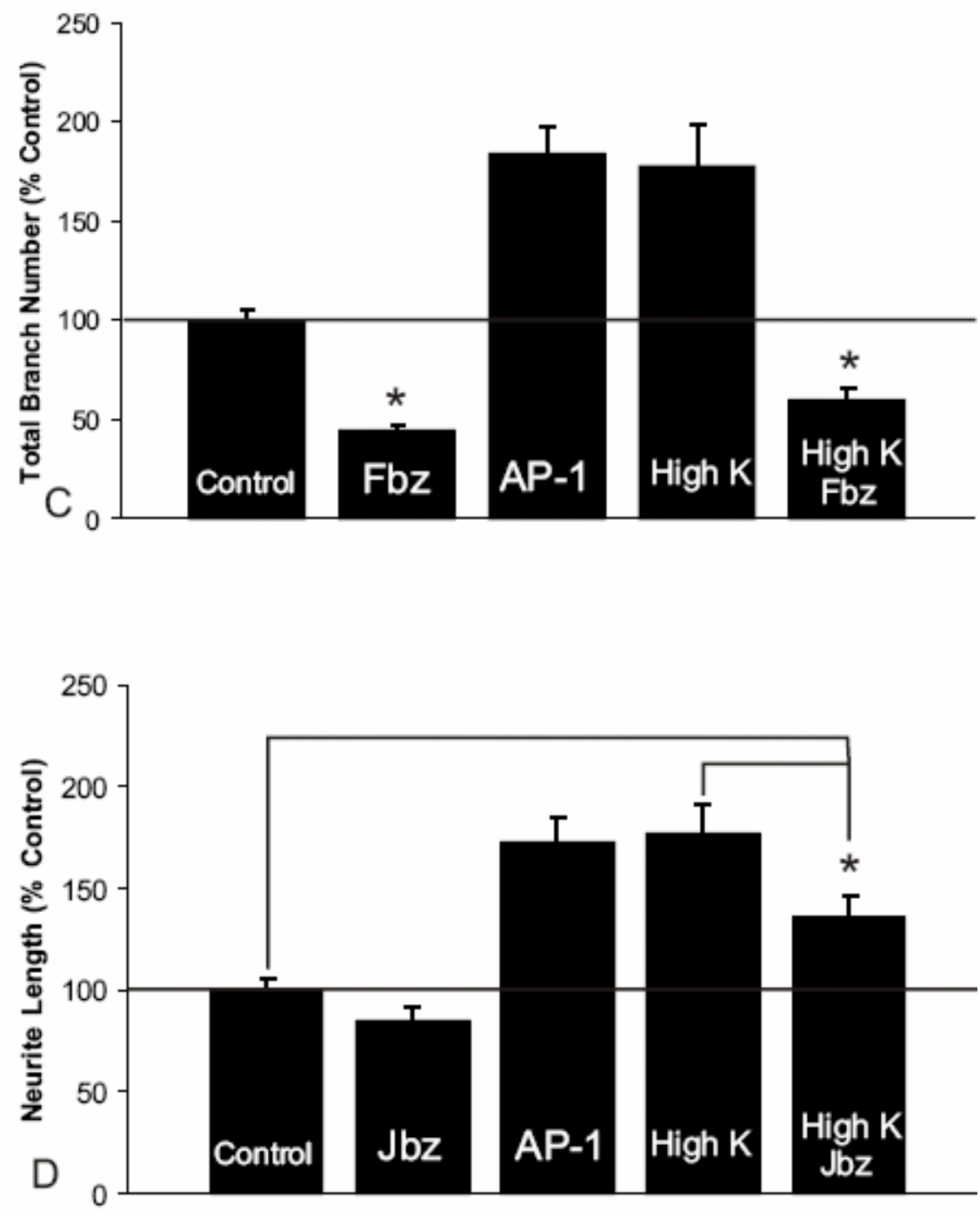
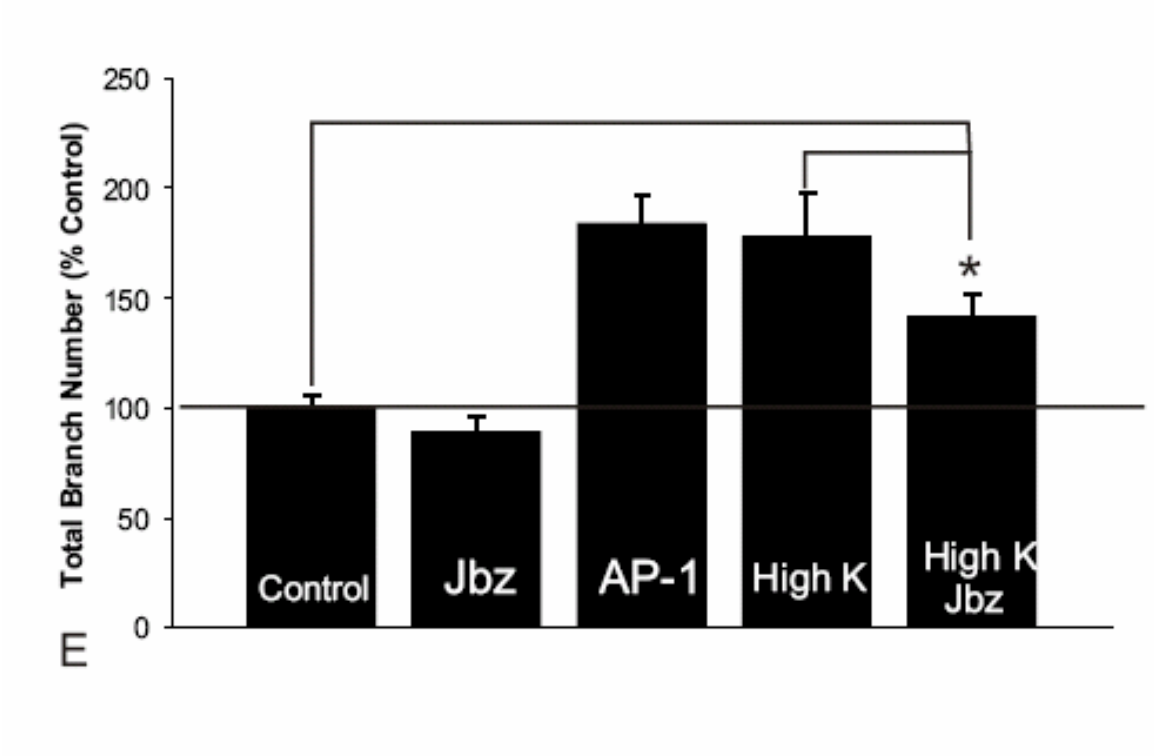
200
150
100
50
0
Control
Fbz
AP1
HIgh K
High K
Fbz


Total Branch Number (% Control)
250
200
150
100
50
0
C
Control
Fbz
AP-1
High K
High K Fbz
*
*
Neurite Length (% Control)
250
200
150
100
50
0
D
Control
Jbz
AP-1
High K
High K Jbz
*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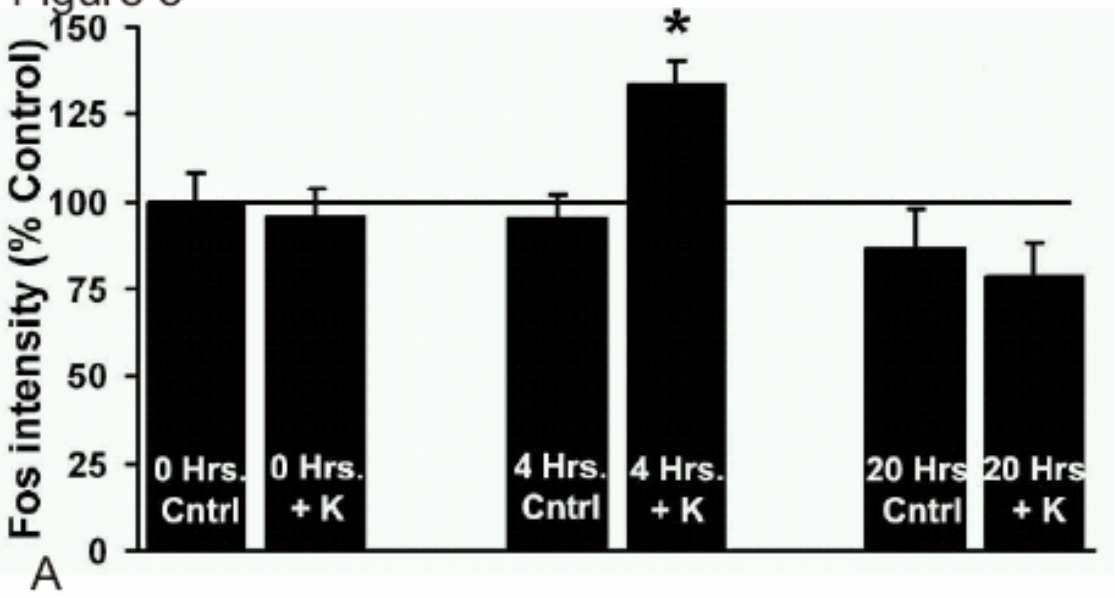
200
150
100
50
0
Control
Jbz
AP-1
High K
High K
Jbz
*

Figure 5


Fos intensity (% Control)
150
125
100
75
50
25
0
*
0 Hrs. Cntrl
0 Hrs. + K
4 Hrs. Cntrl
4 Hrs. + K
20 Hrs Cntrl
20 Hrs + K
A

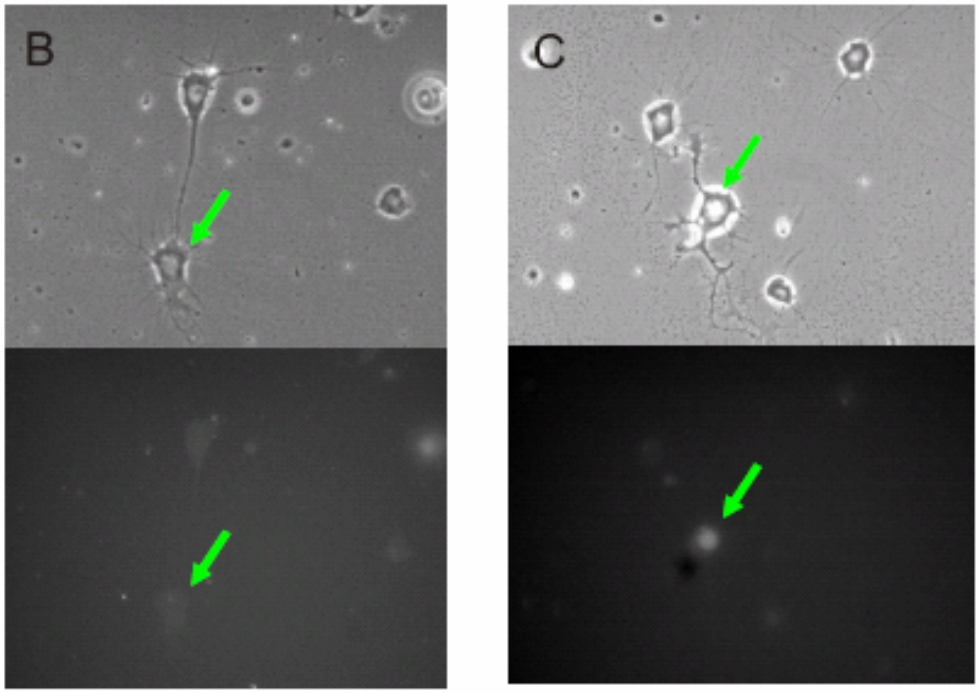

B
C
4 hrs +
4 hrs +K

Figure 6

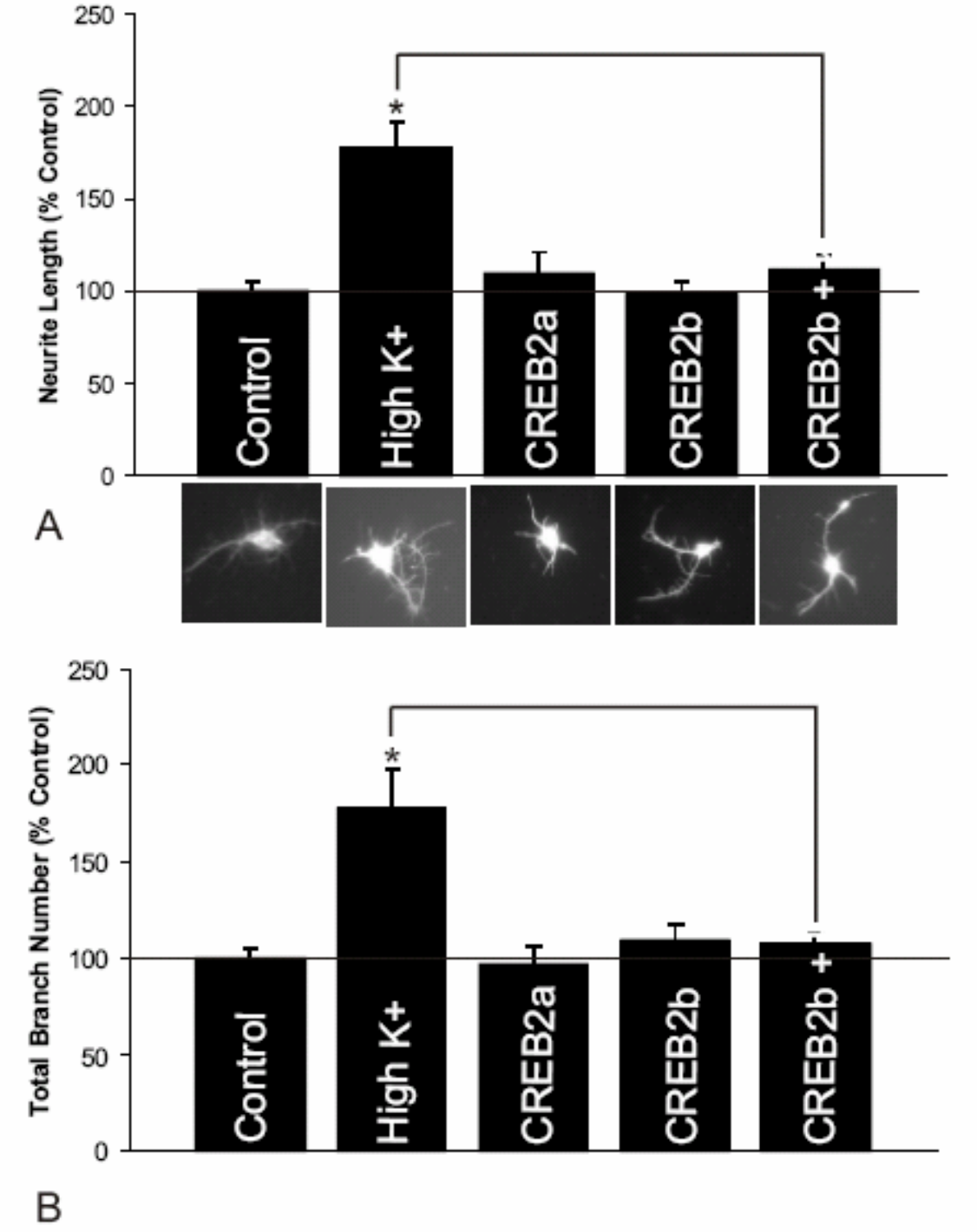
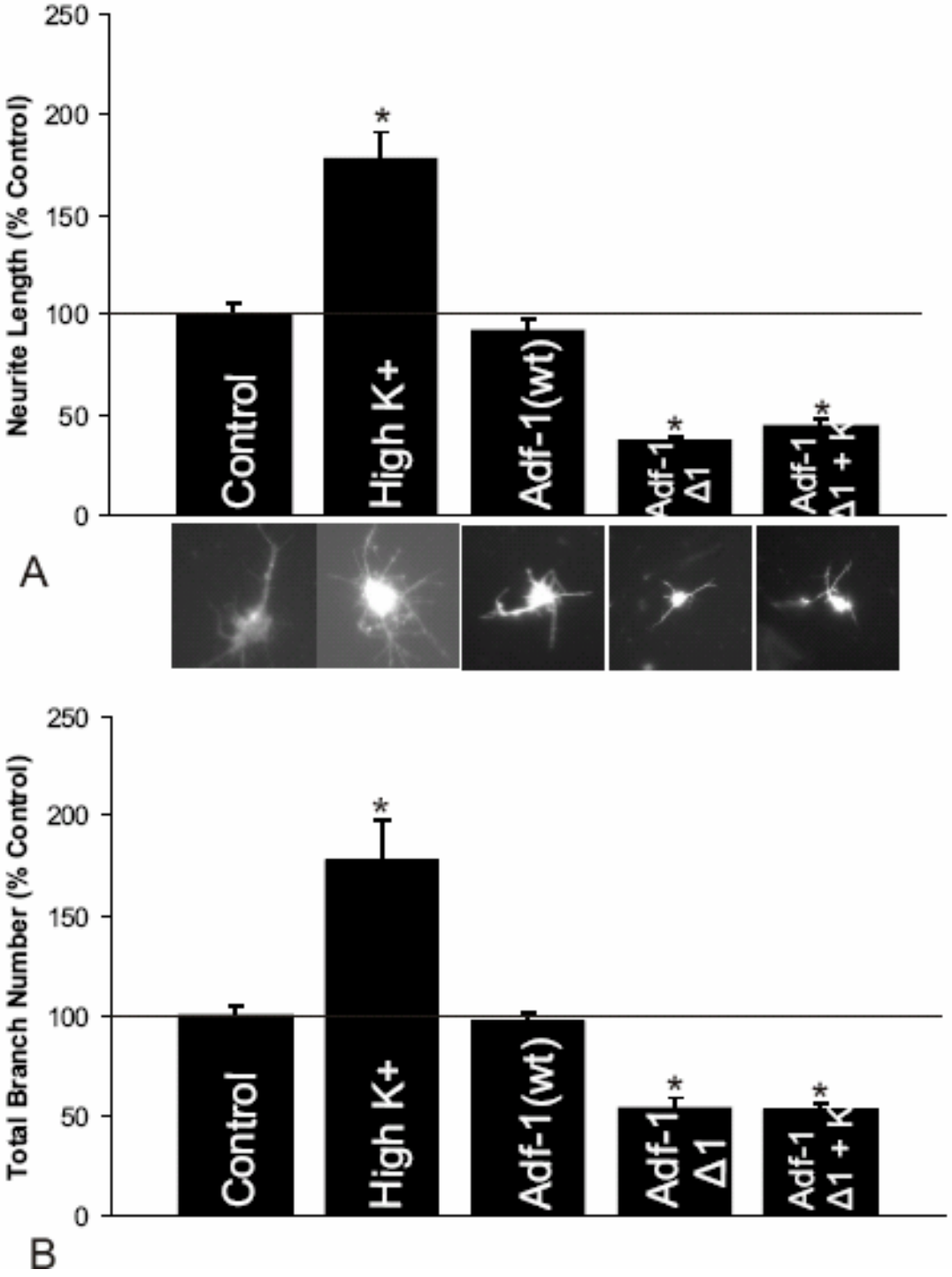

Neurite Length (% Control)
250
200
150
100
50
0
*
Control
High K+
CREB2a
CREB2b
CREB2b +
A
Total Branch Number (% Control)
250
200
150
100
50
0
*
Control
High K+
CREB2a
CREB2b
CREB2b +
B

Figure 7


Neurite Length (% Control)
250
200
150
100
50
0
Control
High K+
Adf-1(wt)
Adf-1 Δ1
Adf-1 Δ1 + K
*
A
Total Branch Number (% Control)
250
200
150
100
50
0
Control
High K+
Adf-1(wt)
Adf-1 Δ1
Adf-1 Δ1 + K
*
B

6.7 Figure Legends

Figure 1

A schematic of the expression pattern of various GAL4 lines that were tested. The w;RN2-GAL4,UAS-CD8-GFP;UAS-flp,act<<GAL4 line is used for all of the *in vivo* experiments. The isolated RP2 neuron containing segments were selected for imaging. Only segments corresponding to abdominal segments 3-6 were used since the size of the neuropil varies by segment. The C380GAL4,UAS-CD8-GFP;;chaGAL80 line was used for all of the *in vitro* experiments to boost the number of cells for analysis in the cultures.

Figure 2

A transgenic scheme to generate somatic mosaics in the ventral ganglion (A). Somatic mosaics showing mCD8-GFP labeling of RP2 and/or aCC neurons in the ventral ganglion, counterstained with anti-elav (B). RP2 (C) and aCC (D) dendritic architecture. Peripheral innervation of RP2 (E and F) and peripheral innervation of aCC (G and H), both are counterstained with anti-HRP to show the whole nerve. 3D reconstruction of dendrites and volume measurement using AMIRA (I).

Figure 3

Depolarization induced motor neuron dendritic outgrowth. Manipulation of external K^+ induced neurite outgrowth *in vitro* as described in Chapter 4 (A). Expression of EKI enhances dendritic growth *in vivo* as was observed *in vitro* (see Chapter 4). K^+ channel manipulation through inactivation of *ether-a-go-go* (eag) and *shaker* (sh) voltage gated K^+ channels with a double dominant negative construct results in an increase in the

dendritic field of RP2 (B). Depolarization induced growth *in vitro* is dependent on protein synthesis as the translational inhibitor, cycloheximide, blocks depolarization induced growth (C). Protein synthesis is also necessary for the basal level of neurite outgrowth observed in controls.

Figure 4

Fos is involved in both basal and depolarization induced dendritic outgrowth *in vivo* (A) and *in vitro* (B). A fos dominant negative protein, fbz, prevents the normal and depolarized (High K^+ in vitro, EKI in vivo) dendritic growth and branching *in vitro* (C). Overexpression of fos and jun (which dimerize to create the functional transcription factor AP-1) is sufficient to induce dendritic/neurite outgrowth. Jun, unlike fos, is only found to block depolarization induced outgrowth (D) and branching (E) *in vitro*. A jun dominant negative, jbz, does not block basal levels of neurite outgrowth, but does mute depolarization induced outgrowth.

Figure 5

Treatment of motor neuron cultures with constant high K^+ directly induces fos by 4 hrs after exposure (A) that declines by 20 hrs after exposure. The levels of fos are detected as a red nuclear signal (see green arrow) in GFP expressing cells (not shown) by immunocytochemistry for depolarized (C) control (B) neurons.

Figure 6

CREB is necessary for depolarization induced neurite outgrowth *in vitro*. Expression of the *Drosophila* CREB inactivator CREB2b, blocks depolarization induced

neurite growth (A) and branching (B). CREB2b, however, does not block the basal level of neurite growth compared to control growth and over-expression of the CREB activator CREB2a does not influence neurite growth parameters (note that the control and high K^+ data are the same as in Figure 4).

Figure 7

Adf-1 is necessary for neurite growth *in vitro*. Interference of Adf-1 activity with the dominant negative Adf1Δ1 results in reduced neurite growth and prevents depolarization induced outgrowth (A) and branching (B). Over-expression of Adf-1 alone is not sufficient to cause neurite outgrowth compared to controls (note that the control and high K^+ data are the same as in Figure 4).

CHAPTER 7 CONCLUSIONS

7.1 Brief Summary of Findings

This dissertation explored the mechanisms of depolarization induced motor neuron dendritic growth and branching. The generation of a motor neuron specific GAL4 line and the development of several assays to measure neurite growth in culture, identify dendrites in culture, and design a protocol to induce dendritic growth by both depolarizing levels of K^+ in the culture medium and transgenically manipulating the levels of functioning K^+ channel expression were done to fulfill this goal. An acceleration of dendritic growth takes place when cells are depolarized, so that they reach maximal growth faster than controls. Certain factors, including voltage sensitive $I_{Ca}{}^{+2}$ and the transcription factor CREB are necessary for depolarization induced acceleration of motor neuron dendritic growth. Other factors, including protein synthesis, the transcription factors AP-1 and Adf-1, are involved in the basal level of neurite growth in vitro as well as depolarization induced outgrowth. Fos is directly upregulated downstream of high K^+ stimulation, so this directly links it to the accelerated growth that depolarization induced.

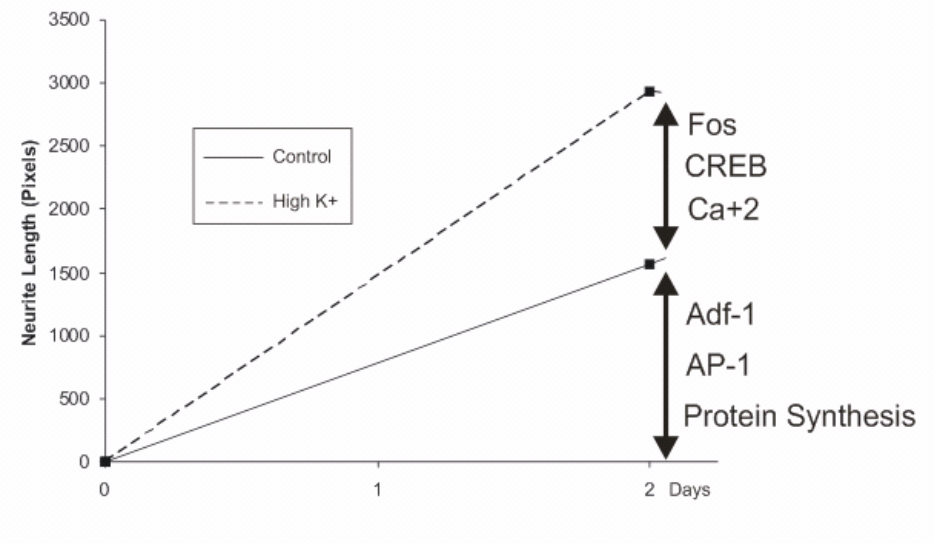


Figure 1 Diagram summarizing the major findings of this dissertation. Basal levels of outgrowth require protein synthesis, AP-1 and Adf-1 while depolarization induced growth requires CREB, I_{Ca}^{+2}, and fos.

Depolarization induced dendritic growth *in vitro* had a strikingly similar phenotype when observed *in vivo*. The growth of RP2 motor neuron dendrites *in vivo* was also consistent with the AP-1 findings *in vitro*. Dendritic outgrowth occurred when the neurons were transgenically manipulated and fos expression was necessary for this depolarization induced growth. Also, AP-1 over-expression alone was sufficient to cause dendritic outgrowth. This provides evidence that the study of mechanisms involved in dendritic growth in culture represent *bona fide* mechanisms of dendritic growth in the intact animal, despite the lack of descending inputs and synaptic targets under culture conditions.

7.2 Future Directions and Applications

These results opened up many intriguing questions. Since Ca^{+2} is involved in depolarization induced growth, it is likely that it is activating downstream kinases upstream of transcription factor activation. There is evidence in other culture systems that Ca^{+2} dependent depolarization induced dendritic growth leads to activation of MAPK

(Wu *et al*., 2001), CAMK (Gaudilliere *et al*., 2004), or both cascades (Redmond *et al*., 2002; Vaillant *et al*., 2002). These cascades are also involved in plasticity at the Drosophila neuromuscular junction (Koh *et al*., 2002; Park *et al*., 2002), so it will be worthwhile to investigate these cascades with respect to dendritic growth.

It would also be informative to link the I_{Ca}^{+2} necessary depolarization induced growth with putative kinase activation. It is possible that MAPK, CAMK, or a combination of both kinases is involved. Both CREB and AP-1 have been linked to the CAMK and MAPK cascades (Redmond *et al*, 2002; Kockel *et al*., 2001). This would contribute to a complete picture of signal cascades activated during depolarization induced dendritic growth.

There is evidence of crosstalk between pathways involved in dendritic plasticity, which could be explored with this system. For example, motor neuron remodeling takes place during metamorphosis (as described in Chapter 2), with the dendrites undergoing regression during the first 8hrs after puparium formation (see Figure 2a-g). Some of these neurons are destined to die (see Figure 2h) while others will be signaled by ecdysone to persist to innervate adult muscles (see Figure 2i). While extensive work has been done to explore the steroid hormone regulation during metamorphosis, this system could be used to explore possible crosstalk between ecdysone mediated dendritic growth and pathways involved in depolarization induced growth. For instance, there is evidence that AP-1 is necessary for ecdysone induced cell death in other Drosophila tissues during

early metamorphosis (Lehmann *et al*., 2002). Does ecdysone lead to an increase in fos expression again during late metamorphosis to induce dendritic growth just as

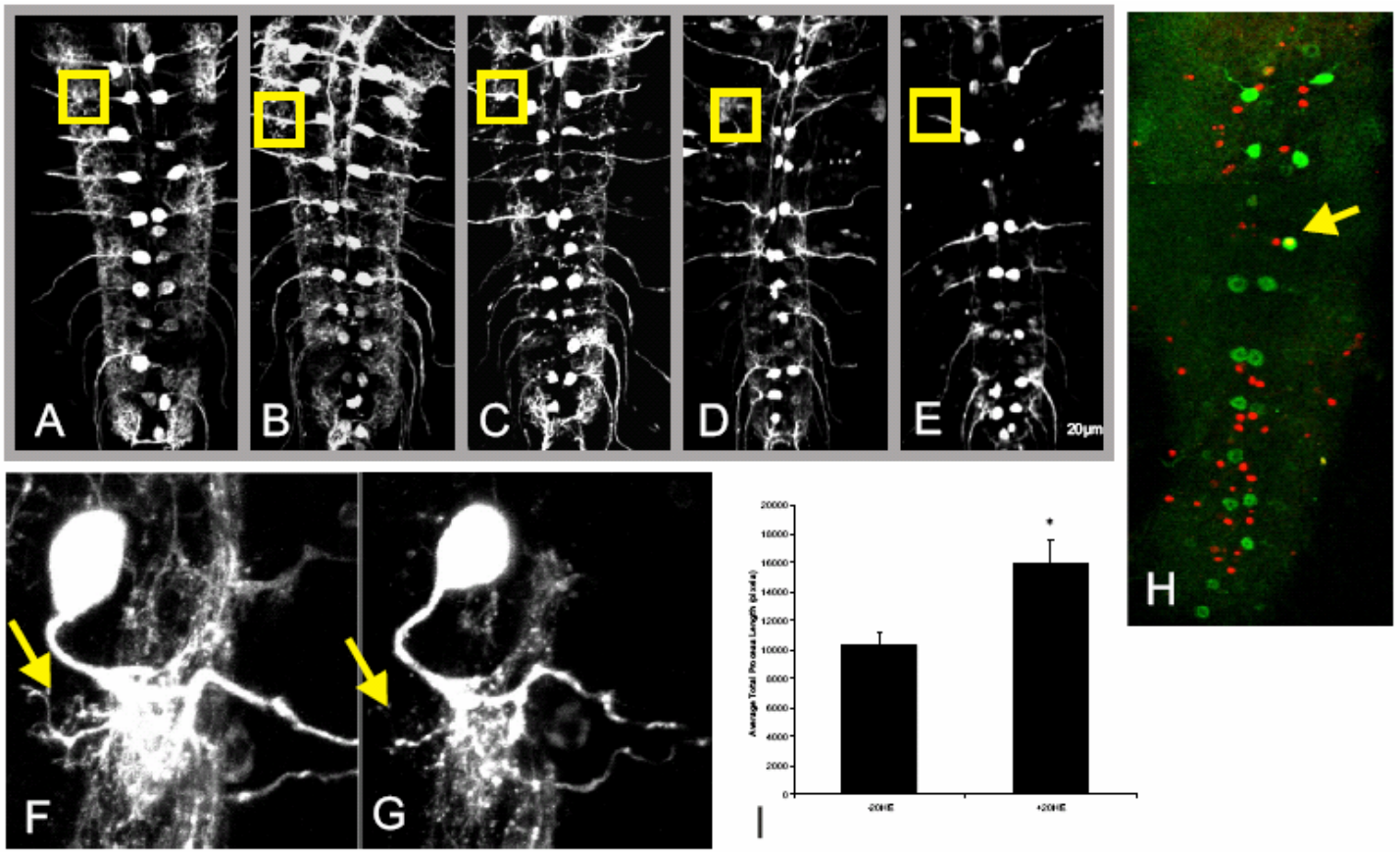


Figure 2 *Drosophila* thoracic abdominal ganglion 0 (A), 2 (B), 4 (C) 6 (D) and 8 (E) hrs after the initiation of metamorphosis. Live time-lapse imaging demonstrates the regression of fine dendritic processes from 0 (F) to 3.5 hrs (G) after the onset of metamorphosis. TUNEL staining labels a dying motor neuron 6-7hrs after the onset of metamorphosis (H). Pupal neurons *in vitro* increase total neurite length in response to exposure to the steroid hormone ecdysone (I).

depolarization does? It is possible that parallel pathways exist in order to reach a similar endpoint.

There are also many candidate proteins in *Drosophila* that are involved in dendritic growth and plasticity of other neuron types or that play a role in plasticity at the neuromuscular junction. A recent microarray study revealed many genes that regulate *Drosophila* sensory neuron morphology (Parrish *et al*, 2006). Proteins at the neuromuscular junction including cAMP dependent pathways, CaMK dependent signal cascades, and cell adhesion molecules, all could be investigated for their role in motor neuron dendritic growth (Koh *et al*., 2002).

Many of the *Drosophila* genes involved in dendritic growth and plasticity are also involved in learning and memory in flies. Behavioral screens in fruit flies are a common assay to detect genes involved in learning. One extensively used behavioral screen is the adult Pavlovian olfactory learning assay where flies are exposed to two odors; one of the odors paired with a foot shock. Wild-type flies then given the option of both odors will choose the non-shock odor over 90% of the time (Tully and Quinn, 1985). This associative behavior requires specific gene expression. Overall, 24 genes, including CREB (cAMP responsive element binding protein), have been identified as necessary for associative olfactory learning either directly or indirectly in loss of function mutant flies (Dubnau and Tully, 1998).

The circuit involved in olfactory learning in *Drosophila* involves olfactory receptor neurons that project to glomeruli in the antennal lobes, which in turn project to

mushroom bodies and surrounding areas. Mushroom bodies are the olfactory learning center in flies. They are composed of 2,500 Kenyon cells in each cephalic region whose cell bodies reside in the dorsal posterior fly brain. Each Kenyon cell has an axon which projects dendrites ventrally in an area called the calyx, where they receive inputs from several regions, including the antennal lobes (Yasuyama *et al*., 2002). There are 3 Kenyon cell types, divided based on the projection of their axon, and each group has a characteristic and distinct non-overlapping dendritic pattern (see Figure 3). Mushroom bodies are essential for olfactory associative learning, as animals with ablations to

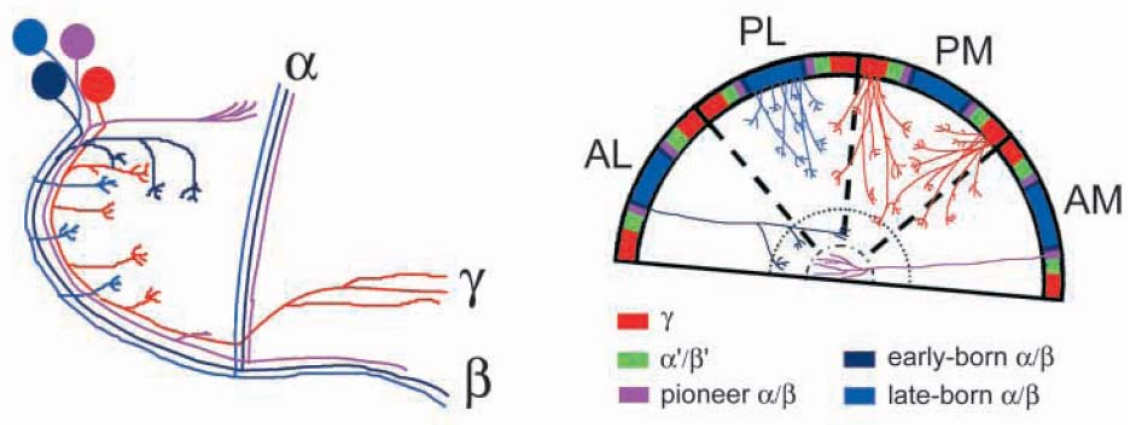


Figure 3 Characterization of Kenyon cell dendrites of adult *Drosophila* mushroom bodies. The dendrites form a dendrodendritic map that can be recognized by their morphology within the anterior lateral (AL), posterior lateral (PL), posterior medial (PM), and anterior medial (AM) regions of the fly brain (Zhu et al., 2003).

this region are able to sense and respond to olfactory stimuli, but cannot form associative behaviors (de Belle and Heisenberg, 1994). How are dendrites involved in memory formation in flies?

Can changes in dendritic morphology alter behavior? Although the genetic regulation of Kenyon cell dendrite development has recently been investigated, fewer studies link genetic learning defects with dendritic deficits. *Baboon* (TGF- β) is needed for proper development of mushroom body neurons in the adult (Zhu *et al*., 2003). Furthermore, Cul3, a cullin protein that functions as a scaffold protein of ubiquitin ligase complexes, is necessary for proper dendritic elaboration (Zhu *et al.*, 2005). Whether or not these mutants disrupt learning as well as dendrite morphology is not known. To date, the olfactory learning studies have not made correlations between the influence a protein has on both the structure and behavioral function of *Drosophila* dendrites. One mutant in the gene known as *Mushroom body miniature*, results in reduced mushroom bodies (including the Kenyon cell dendrites) and also presents olfactory learning deficits (Raabe *et al*., 2004). Although this study does not focus on dendrite specific abnormalities, it is a start of the investigation involving the many genes known to disrupt learning in *Drosophila* and how they may affect the morphology of dendrites and vice versa. If the approaches for neuron specific identification described in this dissertation were used in Kenyon cells, this approach could reveal the influence that these learning related genes have on dendrites. Such information would contribute to the study of dendritic defects found in learning disorders involved in mental retardation.

Finally, this dendritic analysis could be utilized to gain knowledge about the mechanisms of human disease involving dendritic pathology. 60% of known human disease genes have *Drosophila* homologues (Schneider, 2000; Reiter *et al*., 2001) with several involving mental retardation specifically (see table below). Using the *Drosophila*

transgenic system in combination with measurements of dendrites *in vitro*, *Drosophila* homologues to human mutations or expression of the human protein itself within a *Drosophila* neuron could give insights into mechanisms dictating not only plasticity and growth regulatory pathways in mammalian neurons, but also mechanisms related to dendritic dysfunction and abnormality.

Table 1. Drosophila Mental Retardation Genes[a]

Human Gene (OMIM #)	Clinical Disorder (OMIM #)	Fly Gene	Fly Phenotype Affects	References
FLNA (300017)	Periventricular heterotopia (300049)	*cheerio*	LT memory (classical associative learning)	Dubnau et al. [2003]
FMR1 (309550)	Fragile X syndrome (309550)	*dfmrl*	ST memory (courtship conditioning)	McBride et al. [2005]
GNASI (139320)	Albright hereditary osteodystrophy (103580)	*G protein sα60A*	Learning (classical associative)	Connolly et al. [1996]
NFI (162200)	Neurofibromatosis 1[b] (162200)	*Neurofibromin 1*	Learning and ST memory (classical)	Guo et al. [2000]
RSK2 (300075)	Coffin-Lowry s.; MRX19 (303600)	*rsk (S6KII)*	Learning (associative: operant and classical)	Putz et al. [2004]

[a]s., syndrome; LT, long-term; ST, short-term.
[b]The cognitive deficits of *NFI* patients may result in part from interactions between *NFI* and adjacent genes [Venturin et al. 2004].

Table 1 A list of the known mental retardation related fly genes that have human homologues (Restifo, 2005).

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