Role of the immunoglobulin superfamily member Basigin in sensory neuron dendrite morphogenesis in *Drosophila*

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ABSTRACT

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Neurons develop highly stereotypic dendritic arbors that influence establishment of proper connections and integration of information they receive to generate an appropriate output. Morphogenesis of dendrites is coordinated by both cell-intrinsic and extrinsic factors. Recent studies have begun to elucidate how interactions between neurons shape dendrite morphogenesis. However, influence of the substrate upon which neurons grow their dendritic arbors in this process is relatively poorly understood. Here I have used the peripheral sensory neurons of the *Drosophila* larva that grow dendrites over epithelial cell substrates to gain insights into how interactions with the substrate may influence dendrite development. In this thesis, I present data showing that Basigin, an immunoglobulin superfamily member, has somatodendritic and axonal localization in sensory neurons, and is enriched at cell borders and beneath class IV dendrites in epithelial cells. Loss of function analyses indicate that Basigin is required both in neurons and epithelial cell substrates for proper morphogenesis of the highly complex dendrites of class IV sensory neurons. Reduced innervation of the dendritic field of *basigin* mutant neurons was observed even at an immature stage, indicating a requirement of Basigin in these neurons for developmental elaboration of dendritic arbors. Structure-function analysis revealed that membrane-tethering of Basigin on the neuronal surface is essential for its function. In addition, a highly conserved tri-basic motif consisting of positively charged residues that may bind cytoskeletal adaptor proteins is required for its function in neurons. Results of genetic interaction analysis suggest that Basigin-mediated regulation of dendrite morphogenesis does not

involve Integrin and matrix metalloproteinases, both of which have been implicated in Basigin function in other cellular contexts. I show that Basigin exhibits genetic interaction with Tropomodulin, an actin-capping protein, suggesting that they function in the same molecular pathway in regulating dendrite development. Taken together, data presented in this thesis support a model in which interaction between Basigin on the surfaces of neurons and epithelial cells regulate the underlying cytoskeleton within dendrites to influence their development. Thus, these results identify a novel molecular pathway that may mediate communication between neurons and their substrates that is essential for proper dendrite morphogenesis

TABLE OF CONTENTS

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Dedication

I dedicate this work to my parents, who valued my education above else, and gracefully endured many hurdles in support of my academic pursuits.

CHAPTER I: INTRODUCTION

More than a century ago, exquisite neuroanatomical drawings by the famous Spanish neuroscientist Ramón y Cajal revealed the remarkable diversity of neuronal morphology in the vertebrate nervous system (Ramón y Cajal, 1911). Scientists have been fascinated by various aspects of such diversity: how does diversity of neuron morphology contribute to nervous system function? How is such diversity created? Innovations in molecular, genomic, imaging, and electrophysiological techniques over the last few decades have provided important insights that begin to answer some of the questions that have intrigued scientists for decades.

Despite the rich body of neuroanatomical data and impeccable predictions of how the nervous system may function by Cajal at the turn of the twentieth century, major insights into the molecular origin and significance of neuronal morphological diversity didn't arise until just a few decades ago. With the development of powerful tools to label and image neurons, and rapid progress in genetics and molecular biology, exciting progresses are now being made in these fronts. A picture that has emerged from examination of neurons in different regions of the nervous system and phylogenetic study of neuron morphology across the animal kingdom is that neurons with similar functions tend to have similar morphological features (MacNeil and Masland, 1998; Ramón y Cajal, 1911; Wässle and Boycott, 1991). This has provided an important clue in characterizing the importance of specific dendritic arbor types to neuronal function.

A large body of evidence indicates that proper morphogenesis of dendrites is crucial for different aspects of nervous system development and function. Contribution of dendrite morphology to

neuronal connectivity was correctly predicted by Cajal who, in addition to proposing that dendrites receive inputs from other neurons and relay them to the soma and the axon, argued that dendritic complexity reflects the numbers of connections received by a neuron. Indeed, dendrites greatly increase the surface area of a neuron for receiving inputs, comprising as much as 97% of the surface area in the case of a cat motor neuron, excluding its axon (Ulfhake and Kellerth, 1981). In addition, dendrites arborize in patterns that allow neurons to sample inputs from specific spatial domains around them (Fiala and Harris, 1999), and this has significant implications for the connectivity of the nervous system as a whole.

Electrophysiological examination combined with mathematical modeling of the contribution of dendrites to neuronal function over the past few decades have revealed that the influence of dendrite structure goes well beyond their roles in establishing connections. Morphology influences chemical and electrical compartmentalization of dendrites and consequently the integration of synaptic inputs along a dendritic tree (Hausser et al., 2000). In fact, increasing evidence show that dendrites can perform elementary computations that are functionally relevant for the computations conducted by neurons and, by extension, the circuits they underlie (London and Hausser, 2005). For example, both computational modeling and experimental results have established that dendrite morphology and the spatial distribution of inputs on them influences co-incidence detection by individual neurons (Agmon-Snir et al., 1998; Mathews et al., 2010; Stuart and Hausser, 2001; Xu et al., 2006) .

Evidence linking dendrite morphology with neuronal function also comes from studies in which dendritic defects caused by mutations of genes required for dendrite morphogenesis lead to pronounced defects in animal behavior (Stewart et al., 2012). It is noteworthy that aberrant dendrite morphology has been observed in rodent models of various psychiatric and

neurodegenerative disorders (Berman et al., 2012; de Anda et al., 2012; Kulkarni and Firestein, 2012; Kvajo et al., 2008; Kvajo et al., 2011; Mukai et al., 2008). For instance, mice bearing mutations in *DISC1*, a strong candidate susceptibility gene for schizophrenia, have dendrites that are misoriented and reduced in length, concomitant with working memory and synaptic plasticity defects (Kvajo et al., 2008). Downregulation of the autism spectrum disorder susceptibility gene TAOK2 impairs basal dendrite development in cortical pyramidal neurons (de Anda et al., 2012). Furthermore, post-mortem examination of the brains of human subjects afflicted with various forms of mental retardation has revealed significant defects in dendrite morphology of neurons (Dierssen and Ramakers, 2006).

Although the significance of dendrite morphological diversity continues to be elucidated, it is clear based on studies over the past several decades that it plays significant roles in neuronal connectivity and function. Studies to date have also established that abnormalities in dendritic arbors constitute the neurobiological defects associated with several psychiatric diseases characterized by altered cognitive function. Therefore, understanding the molecular mechanisms of dendrite morphogenesis is an important goal in neuroscience. Not only can this provide us with a better understanding of how neural circuits develop and function, it can also facilitate the identification of key drug targets for treatment of psychiatric diseases.

In the following sections, I describe the progress made to date in uncovering the molecular regulators of dendrite morphogenesis. Insights described here come primarily from neurons of the mouse central nervous system and the *Drosophila* peripheral nervous system, the two most studied models for dendrite morphogenesis. Since my dissertation work involves the latter system, I introduce them briefly below.

Dendritic arborization neurons of *Drosophila* larvae

The dendritic arborization (da) neurons of the *Drosophila* larval peripheral nervous system are a subclass of multidendritic (md) neurons, which grow multiple branched dendrites along the body wall of the larvae. They can be grouped into 4 major classes based on the size and branching complexity of their dendritic arbors (Fig. 1.1) (Grueber et al., 2003a). Class I neurons have the simplest and smallest arbors, class II neurons have arbors of intermediate size and complexity, class III neurons have larger arbors with numerous actin-rich dynamic filopodial structures and class IV neurons have expansive, space-filling, highly branched arbors (Fig. 1.1). These neurons occur in a segmentally repeated manner and in mirror images across the dorsal midline of the larval body (Fig. 1.1). Within each hemisegment, da neurons occupy highly stereotyped positions, allowing the same neuron to be identified easily from segment to segment and across animals (Grueber et al., 2003b; Orgogozo and Grueber, 2005). This also permits easy correlation of expression of certain genes with specific dendrite morphologies and is hence particularly suitable for taking advantage of gene expression analyses.

The dendrites of da neurons grow along the basement membrane, a specialized layer of ECM secreted by and located immediately next to the basal surface of epidermal cells. The advantages of the da sensory neurons in terms of their easy physical and optical access also extend to the basement membrane upon which their dendrites grow and the substrate epidermal cells. Expression and localization of proteins of interest can be readily assessed in both the epidermis and the neurons. In addition, the relative ease of visualizing their dendritic arbors, aided in part by the availability of genetic markers for specific neuronal subclasses, has allowed high throughput phenotypic screens and detailed dissection of molecular mechanisms of dendrite morphogenesis.

Molecular mechanisms of dendrite morphogenesis

From the very first step of polarization of a neuron to the final step of establishment of a mature dendritic tree, dendrite morphogenesis involves several well-defined steps. Acquisition of the characteristic dendritic arbor of a neuron involves branch outgrowth, addition, stabilization, as well as retraction, with these processes biased towards achieving net growth and elaboration of a dendritic tree. In some cases, exuberant branches are formed during an initial phase, but are lost during a pruning phase before a mature dendritic arbor can form. Additionally, dendrites of some neurons innervate specific areas in their vicinity and exhibit precise guidance and targeting. Work carried out primarily in mice and fruitflies have identified a diverse set of molecular factors involved in all of these steps and begin to provide important insights into the mechanisms underlying the great diversity in dendrite morphology in both invertebrate and vertebrate nervous systems. These factors belong to many functional categories ranging from transcription factors to cell surface receptors, ubiquitin ligases, microRNAs, and cytoskeletal regulators, which reflects the complexity of the molecular mechanisms involved.

Transcription factors

Transcription factors, which are often expressed in cell type-specific manner, constitute a large proportion of the molecular factors that have been found to play important roles in dendrite morphogenesis. For instance, Zfp312 is selectively expressed in layer V and VI cortical pyramidal neurons (Chen et al., 2005) in which it promotes dendrite branching. Elaboration of the dendritic arbors of mice cerebellar granule neurons is promoted by the bHLH transcription factor NeuroD (Gaudilliere et al., 2004), while pruning of their exuberant branches to form mature arbors are mediated by the zinc-finger transcription factor Sp4 (Ramos et al., 2007).

In the fly olfactory system, dendrite projections of second-order projection neurons to specific glomerular targets are instructed by an ensemble of transcription factors including the POU-domain transcription factors ACJ6 and Drifter, the homedomain transcription factor Cut, and several LIM-homeodomain transcription factors (Komiyama et al., 2003; Komiyama and Luo, 2007). In fly larval sensory neurons, transcription factors mostly expressed in graded or on-off fashion constitute combinatorial codes that specify the complexity of their dendritic arbors. Cut is expressed at levels that correlate positively with the complexity of branching patterns of these sensory neurons: neurons with the simplest arbors (class I) lack Cut expression, those with intermediate dendrite complexity (class II) express low levels of Cut, while neurons with more complex arbors (class III and IV) express high levels of Cut (Grueber et al., 2003a). Knot is expressed only in class IV sensory neurons and, together with Cut, promotes the highly elaborate dendritic arbors of those neurons (Hattori et al., 2007; Jinushi-Nakao et al., 2007). Class I neurons on the other hand express the BTB-zinc finger protein Abrupt, which is necessary for them to acquire their simple morphology (Sugimura et al., 2004). Misexpression of Abrupt or Knot, or changing Cut expression levels alters dendrite arborization in a manner that indicates that these transcription factors confer class-specific dendrite features to these sensory neurons (Grueber et al., 2003a; Hattori et al., 2007; Sugimura et al., 2004). An exception to the varying expression levels of transcription factors observed in these neurons is Spineless, a bHLH-PAS transcription factor that is expressed in all neurons and helps diversify the morphologies of these neurons from an intermediate, possibly primordial, arbor complexity (Jan and Jan, 2010; Kim et al., 2006). These transcription factors have conserved vertebrate homologs (Jan and Jan, 2010) that may similarly play roles in neuronal morphogenesis in the vertebrate nervous system, as is

the case for Cux1 and Cux2, homologs of Cut that instruct early dendrite development in mouse layer II-III cortical pyramidal neurons (Cubelos et al., 2010).

A recurring theme in the way transcription factors regulate dendrite morphogenesis is that they confer specific properties to dendritic arbors. In the fly larval nervous system described above, Cut is associated with increase in overall dendritic arbor size and, at very high levels, addition of dynamic actin-rich processes (Grueber et al., 2003a; Jinushi-Nakao et al., 2007). On the other hand, Knot is associated with the elaboration of microtubule-based processes in the highly-arborized dendrites of class IV neurons (Jinushi-Nakao et al., 2007). In the case of the fly olfactory projection neurons, a number of transcription factors are dedicated to instruct their dendrites to project to specific regions of the brain. Interestingly, within this functional specialization, these transcription factors have more specific roles. While some of them, such as Cut, instruct coarse targeting, enabling dendrites to reach the right region of the brain, others such as Drifter and ACJ6 control local glomerular choice within that area (Komiyama and Luo, 2007). Thus, an emerging model of dendrite type specification is that transcription factors function in a piece-meal manner, instructing or, in some cases, repressing specific features, such as targeting, branching complexity, polarity, branch spacing, adhesion, that together produce a dendritic tree that suits the connectivity and functional needs of each neuron. It is also noteworthy that cellular and molecular contexts add a layer of complexity on top of instructions provided by transcription factors, as is the case for Cut which controls very distinct properties of dendrites in the larval sensory neurons and the adult olfactory projection neurons, to achieve tremendous diversity in dendrite morphology across different regions of the nervous system.

Cytoskeletal components and regulators

Actin and microtubule filaments constitute the structural framework of dendrites (Georges et al., 2008). Much of what we know about the role of actin and microtubules in growing, stabilizing and maintaining neuronal processes comes from studies on axons and migrating cells. Interestingly, while some aspects of how they work in dendritic branch elaboration are analogous to their roles in other cellular contexts and compartments, there are some important differences. A key difference is in the organization of microtubules. Microtubules are less densely packed in dendrites than in axons (Georges et al., 2008). Additionally, while microtubules in axons are primarily oriented with their plus ends distal from worms to rodents, microtubules in dendrites are predominantly of mixed polarity in rodents (Burton, 1988; Kollins et al., 2009). In *Drosophila* neurons however, they are organized with minus ends distal in ~90% of the branches and have mixed polarity or plus ends distal in higher order branches that are typically short in length (Ori-McKenney et al., 2012; Stone et al., 2008). Likewise, neurons in worms have a predominantly minus-end distal organization of microtubules (Maniar et al., 2012; Yan et al., 2013). The stereotypic differences in axonal and dendritic microtubules in worms are established at least in part by the kinesin motor UNC-116 (Kinesin-1) such that loss of UNC-116 results in dendrites with axon-like microtubule organization. In addition, loss of the microtubule binding protein UNC-33 (CRMP) and the actin-binding protein UNC-44 (Ankyrin) lead to loss of microtubule polarity in both axons and dendrites, indicating that these molecules are also involved. However, it is unknown at what level they interact with the kinesin motor UNC-116. In flies, loss of the minus-end-directed motor dynein results in marked disruption of dendritic cargo transport and appearance of microtubules with mixed orientation in axons (Zheng et al., 2008). Thus, microtubule motors appear essential in transporting microtubules to respective neuronal compartments and thus establishing their polarized microtubule orientations. Factors contributing

to microtubule organization in dendrites of vertebrate neurons are less understood, but recent findings from worms and flies have paved the way for studying the role of kinesin and dynein motor proteins in this process.

The functional and developmental implications of differences in microtubule organization between axons and dendrites are just beginning to emerge. Based on observations that axons could grow well even in substrates offering weak adhesion while dendrites required strong substrate adhesion, Chamak and Prochiantz proposed that difference in microtubule organization may influence the reliance of dendrites and axons on substrate adhesion for branch formation (Chamak and Prochiantz, 1989). Dendrites and axons of the same neuron often traverse drastically different extracellular milieu, so it is plausible that they should be affected differently by substrate cues. Consistent with this, microtubules are known to play critical roles in modulating substrate adhesion complexes in motile cells (Small and Kaverina, 2003). Recent evidence also point towards a role of polarized microtubule organization on distribution of cellular organelles and protein cargoes. In both flies and worms, disruption of polarized microtubule organization in dendrites and axons, which happens upon loss of specific motor proteins, result in mislocalization of cellular cargoes in both compartments (Maniar et al., 2012; Yan et al., 2013; Zheng et al., 2008). For example, loss of dynein, which results in mixed microtubule organization in axons, dendrite-specific organelles such as golgi outposts and proteins such as ion channels enter the axon in flies (Zheng et al., 2008). Likewise, loss of UNC-33 and UNC-44 in worms, which result in both axons and dendrites acquiring mixed microtubule polarity, causes erroneous protein sorting into dendrites as well as axons (Maniar et al., 2012). The findings discussed here suggest that difference in microtubule organization between axons and dendrites permit the delivery of dendrite-specific proteins while preventing transport of

axon-specific cargoes such as proteins and synaptic vesicles to dendrites. Thus, microtubule organization plays a critical role in establishing the biochemical compartmentalization, and by extension the functional specialization, of dendrites and axons.

Actin filaments are primarily enriched in highly dynamic cellular processes, such as lamellopodia, the leading edge of mobile cells, and filopodia, the exploratory processes at the tip of axonal growth cones. Live-imaging of growing dendritic arbors in fly larvae has revealed that a common mechanism of dendrite elaboration is the extension of dynamic filopodia-like branches that are subsequently stabilized. While the initial outgrowth of nascent branches is primarily actin-driven, stabilization of those branches requires recruitment of microtubules into them. In mature dendrites, although actin is distributed throughout the arbor, it is the sole cytoskeletal component of branches that remain highly dynamic. Thus, while microtubule is mainly associated with branch stabilization, actin is associated with branch dynamics. In vertebrate neurons, dendritic spines are enriched in F-actin, and regulation of the actin cytoskeleton underlies the morphological changes associated with synaptic plasticity in spines.

Given their roles in outgrowth of nascent dendritic branches and in providing structural support to existing branches, it is not surprising that changes in actin and microtubule polymerization, distribution, and dynamics in dendrites underlie the point at which all pathways of dendrite regulation converge. Li and Gao conducted a genetic screen in fly larvae and identified the actin binding protein tropomyosin II to act downstream of the seven-pass transmembrane protein Flamingo in regulating the dendritic field size of class IV da neurons. The RhoGEF Trio, which activates Rac and Rho, promotes dendritic elaboration in da sensory neurons in *Drosophila*. Importantly, Trio is required for the pro-branching effects of the transcription factor Cut, which is expressed at levels proportional to branch complexity in these neurons. Although the pathways

converging on regulation of microtubules is comparatively less known, growing evidence indicate that modifiers of microtubule length and stability play critical roles in dendrite morphogenesis. In the absence of Katanin p60-like 1, a microtubule-severing protein that promotes microtubule polymerization, recruitment of microtubules to nascent dendritic branches cannot occur, resulting in lack of stabilization. A role in elaboration of dendritic branches has also been reported for a different microtubule-severing protein Spastin, which destabilizes microtubules to promote addition of new branches. Interestingly, Spastin acts downstream of the transcription factor Knot, which is specifically expressed in the class IV da sensory neurons.

Selective protein trafficking to dendrites

Specification and development of dendrites pose very complex cell biology problems owing in large part to their size, distance from the soma, and need for molecular distinction from the rest of the cell. Biochemical compartmentalization and the resulting functional specialization of dendrites is aided by asymmetric trafficking of a large number of cellular components including ion channels, receptors, synaptic scaffold proteins, and signaling molecules by the microtubule motors dynein and kinesin. Not surprisingly, a growing number of studies are now unmasking the importance of proper delivery of such cellular components to different parts of dendritic arbors in their morphogenesis. In the class IV sensory neurons of fly larvae, transport of dendritic cargo by dlic (dynein light intermediate chain) and khc (kinesin heavy chain) is important to ensure uniform branch elaboration throughout the dendritic tree. Disruption of the function of either of these proteins causes defect in trafficking of Rab5-endosomes to distal dendrites and marked shift in branching to proximal regions of the arbor (Satoh et al., 2008; Zheng et al., 2008). Likewise, kinesin-dependent transport of EphB receptors by the multi-PDZ domain scaffold protein GRIP1 (glutamate receptor interacting protein 1) is necessary for proper

dendrite growth in mice hippocampal neurons (Hoogenraad et al., 2005). In an example of how decentralization of resources can be useful for meeting the challenges of regulated delivery of molecular components to highly expansive dendritic arbors, cellular organelles such as the Golgi have been found to have 'outposts' in dendrites. In class IV sensory neurons of the fly larva, such golgi outposts are localized along dendrites, often enriched at branchpoints, and contribute to dendrite branch dynamics (Ye et al., 2007). Similarly, Golgi outposts have been observed in hippocampal pyramidal neuron dendrites in mice as well (Horton et al., 2005). While a detailed mechanistic understanding of how dendritic Golgi outposts contribute to dendrite morphogenesis is lacking, the findings of a recent study provide some interesting hints. Ori-McKenney et al. found that dendritic Golgi outposts contain microtubule nucleating factors and serve as acentrosomal sites for local generation of microtubules in dendrites, contributing to dendrite-specific microtubule organization (Ori-McKenney et al., 2012). Additionally, dendritic Golgi outposts have also been proposed to contribute, as part of the neuronal secretory pathway, to the important task of adding plasma membrane to dendrites as they grow (Horton and Ehlers, 2004; Ye et al., 2007). Evidence for the presence of Golgi outposts in dendrites in both flies and mice suggest that local generation of cellular components within dendrites may be an evolutionarily conserved solution to the challenge of distributing cellular components in a regulated manner over a large dendritic arbor.

Signaling pathways

How are the myriad extracellular signals that neurons receive integrated with cell-intrinsic molecular programs to produce the final dendritic arbor shapes of neurons? The signaling pathway consisting of phosphatidylinositide 3-kinases (PI3K), Akt, and mechanistic target of Rapamycin (mToR) lies downstream of receptors activated by neurotrophic factors such as

BDNF. In addition to its conserved role in regulating cell differentiation, size and survival (Franke et al., 2003; Scanga et al., 2000), this pathway has been found to be an important regulator of dendrite morphogenesis in a number of studies. PI3K, Akt and their upstream regulators BDNF and Ras have been shown to regulate development and elaboration of dendrites in an mToR-dependent manner in cultured hippocampal neurons (Jaworski et al., 2005; Kumar et al., 2005). Although involvement of the Akt-dependent signaling pathway in dendrite morphogenesis of hippocampal neuron in an *in vivo* context remains to be validated, signaling via Akt likely constitutes an evolutionarily conserved mechanism of regulating dendritic arbor size and complexity. This idea is supported by recent findings that Akt levels regulate scaling growth of dendritic arbors of class IV da sensory neurons of *Drosophila* larvae (Parrish et al., 2009). In order to ensure complete coverage of their dendritic fields, these neurons must grow in proportion to the growth of the larval body. Expression and activity of Akt at a certain level is required in these neurons for proper elaboration and growth of dendrites as the larva continues to grow in size, while down-regulation of Akt is a necessary step to prevent exuberant and redundant growth into the territories of neighboring homotypic neurons at later stages when the larval body size peaks. Furthermore, Akt activity also influences the regenerative potential of the dendrites of these neurons (Song et al., 2012). Interestingly, modulation of Akt level required for both dendritic scaling and regeneration in these neurons is dependent on the activity of the micro-RNA bantam in the underlying epidermal cells (Parrish et al., 2009; Song et al., 2012). Although the identity of the epidermis-derived extracellular signal that results in modulation of neuronal Akt level is unknown, this finding underscores an underappreciated role of neuronal substrate-derived extrinsic factors in ensuring proper dendritic arbor morphogenesis.

Extrinsic factors

The large number of molecules that have been found to influence dendrite morphogenesis in a cell-autonomous manner suggest a largely cell-intrinsic mechanism of dendrite patterning. However, there is evidence for the role of various extrinsic factors in dendrite morphogenesis as well. The role of presynaptic afferents in dendritogenesis has been examined primarily in vertebrate systems. In cultured rat hippocampal neurons, presence of entorhinal cortex explants significantly enhances formation of dendritic branches in an activity-independent manner (Kossel et al., 1997). Consistent with this *in vitro* finding, entorhinal inputs to rat hippocampal and dentate neurons are necessary for stabilizing their distal dendritic terminals *in vivo* (Nitsch et al., 1992; Nitsch and Frotscher, 1991). In the gerbil lateral superior olive neurons, absence of inhibitory transmission from the cochlea leads to hyperbranching of dendrites, possibly indicating persistence of an immature state (Sanes et al., 1992). Cerebellar Purkinje cells develop aberrant dendrite morphology in both *weaver* and *staggerer* mutant mice, in which granule cells that normally project axons to and synapse with dendrites of Purkinje cells are missing (Bradley and Berry, 1978). Since neurons do grow some dendrites even in the absence of afferent terminals in these instances, it appears that only the final step of fine-tuning of the dendritic arbors involves afferent input.

Various neurotrophic factors are thought to underlie the trophic effects of afferent terminals on dendritic morphology. Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin 3 (NT-3) have been found to influence dendrite morphogenesis of neurons in the mouse visual cortex (McAllister et al., 1995). Fruitflies were thought to lack neurotrophins until recently. Identification of a novel family of neurotrophins comprising of *Drosophila* neurotrophin 1 (DNT1), *Drosophila* neurotrophin 2 (DNT2), and Spätzle has opened doors to

the investigation of whether, as in vertebrates, neurotrophins influence dendrite morphogenesis in flies (Zhu et al., 2008).

In addition to coming in contact with potential presynaptic partners, dendrites may also encounter dendrites of neighboring homotypic or heterotypic neurons, which may influence their field size. Class IV sensory neurons of fly larvae have been a valuable model system for understanding the molecular basis of homotypic repulsion between dendrites of neighboring neurons that delimit their arbor boundaries. Flamingo, a seven-pass transmembrane protein of the cadherin family, is required in these neurons to ensure that dendrite growth doesn't occur beyond the boundaries of neighboring neurons (Kimura et al., 2006). The mammalian Flamingo homolog, Celsr2, is required for maintenance of dendritic arbors of cortical pyramidal neurons and Purkinje cells (Shima et al., 2004). In mouse cortical neuron cultures, up-regulation of Notch activity concomitant with interneuronal contacts results in inhibition of dendrite growth (Sestan et al., 1999). However, how widely this mechanism regulates dendrite field formation remains unclear (Parrish et al., 2007).

The role of extrinsic factors in mediating proper dendrite morphogenesis is further highlighted by the significance of various cell surface receptors, many of which were first discovered for their roles in axon guidance, in mediating specific steps of dendrite morphogenesis such as guidance and targeting. In the fly antennal lobe, dendrites of projection neurons express a gradient of Sema 1A (Semaphorin 1A) that targets them to the appropriate glomeruli. The Robo (Roundabout) receptor in flies for the secreted ligand Slit controls the number of high order branches in class IV larval sensory neurons (Dimitrova et al., 2008). In addition, Robo-Slit signaling promotes dendrite formation in the aCC motor neurons of the fly embryonic nervous system (Furrer et al., 2007). Similarly, RP3 motor neuron dendrites utilize Frazzled, the Netrin

receptor, and Robo for guidance as they navigate in the embryonic fly CNS (Furrer et al., 2003). In vertebrates, the secreted semaphorin Sema 3A promotes dendrite branching in cultured cortical neurons. Interestingly, Sema 3A functions as an attractant for cortical apical dendrites, which is opposite to its repulsive effect on cortical axons (Polleux et al., 2000).

Inter-branch interactions

An important consideration for establishing proper connectivity between neurons is the spatial organization of dendritic branches relative to those emanating from the same neuron or adjacent homotypic or heterotypic neurons. Whereas it can be important for dendrites of neighboring homotypic neurons to maintain strictly non-overlapping territories, dendrites also should be oblivious to branches of heterotypic neurons so that they can grow over the same space. A good example of the former is the class IV da sensory neurons of the fly larva (Grueber et al., 2002) that mediate nociception (Hwang et al., 2007) and UV light sensing (Xiang et al., 2010), and hence achieve non-redundant but complete coverage of their receptive fields. The latter case applies to virtually all neurons, so the mechanisms in place to mediate recognition of non-self homotypic neuronal branches (referred to as 'tiling'), or of sister dendrites arising from the same neuron (referred to as 'self-avoidance') must prevent heterotypic neurons from engaging in repulsive interactions.

Studies in flies and mice have identified Dscam (Hughes et al., 2007; Matthews et al., 2007; Soba et al., 2007) and Pcdh (Lefebvre et al., 2012), respectively, as the cell-surface molecules that mediate self-avoidance and self/non-self discrimination among sister dendrites of the same neuron. Stochastic expression of a number of isoforms of the respective proteins imparts unique

identities to neurons, allowing homophilic recognition, and subsequently repulsion, between sister branches but not between branches of homotypic or heterotypic neurons (Hattori et al., 2009; Lefebvre et al., 2012). The signaling pathways downstream of Dscam and Pcdh mediating dendritic self-avoidance in flies and mice, respectively, remain to be identified. Insect *dscam* and vertebrate *pcdh* do not share sequence homology (Lefebvre et al., 2012), so insects and vertebrates appear to have independently evolved similar molecular mechanisms of mediating dendrite self-avoidance. In vertebrates, Dscam and the related protein Dscam-like 1 (DscamL1) are required for spacing of neurites and cell bodies in the retina (Fuerst et al., 2009; Fuerst et al., 2008), but are thought to do so instead by counterbalancing the action of other adhesion molecules (Grueber and Sagasti, 2010).

Tiling between dendrites of homotypic neurons occurs robustly in the class IV da sensory neurons (Grueber et al., 2002; Grueber and Sagasti, 2010) and the Tricornered (Trc) kinase / Furry (Fry) signaling pathway (Emoto et al., 2004) has been shown to act downstream of an as yet unidentified cell surface receptor in mediating heteroneuronal tiling. However, recent studies revealing new insights into the apico-basal positioning of dendritic branches of these neurons (Han et al., 2012; Kim et al., 2012) suggest that at least some of what was previously considered tiling defects instead represent changes in the three-dimensional positioning of the branches (Han et al., 2012). In light of these findings, previously reported roles of cell-surface molecules such as the Ig superfamily member Turtle (Long et al., 2009) and the cadherin Flamingo (Matsubara et al., 2011) in dendritic self-avoidance in fly neurons need to be re-evaluated.

Hormonal control of dendrite morphology

An important aspect of dendrite morphology is the pruning of branches that occurs either to get rid of supernumerary branches or to develop a different arbor type that is specific to a particular stage in the animal's life cycle. While the former type of pruning occurs in all organisms as part of a developmental program before a mature and stable arbor is formed, the latter type of pruning occurs as part of wholesale remodeling of the nervous system, which has been well-studied in holometabolous insects such as fruitflies and the moth *Mandua*.

Metamorphosis occurs in insects as an exquisite series of changes in virtually all tissues orchestrated in part by a number of hormonal factors including the steroid hormone 20-hydroxyecdysone (ecdysone). In a series of pioneering studies, Truman and colleagues showed that both degradation of dendritic arbors and regrowth of dendrites of persistent neurons during metamorphosis occur under the control of hormones in *Manduca*. In *Drosophila*, several studies have shown that neuronal arbor degradation associated with metamorphosis is under the control of ecdysone. Upregulation of various ecdysone receptor isoforms precedes degradation of dendrites, and loss of those receptors blocks this pruning step in motor neurons, peripheral sensory neurons and gamma neurons in the mushroom body (Lee et al., 2000; Santos et al., 2006; Schubiger et al., 1998) of the fly nervous system.

Hormonal control of dendrite development is however not unique to holometabolous insects. In fact, one of the earliest examples of the effect of hormones on neural development was the delay and reduction in branching of the dendritic tree of Purkinje cells of neonatally hypothyroid rats (Vincent et al., 1982). A more recent study has provided additional supporting evidence for the role of thyroid hormone in dendrite development of mouse Purkinje cells (Heuer and Mason, 2003). Importantly, these cells can grow dendrites in culture media lacking thyroid hormone, but exhibit enhanced dendrite branching upon addition of thyroid hormone via the thyroid hormone

receptor alpha 1 (TRalpha1) (Heuer and Mason, 2003). In addition, a number of recent studies have uncovered stimulatory effects of the sex hormones progesterone and estrogen in Purkinje cell development (Kapfhammer, 2004). Exposure to elevated levels of glucocorticoids has also been shown to decrease dendrite length and complexity of CA3 pyramidal neurons of the mouse hippocampus. Although hormones have been found to influence dendrite morphology in both the developing and adult nervous system of rodents (McAllister, 2000), the general significance of this mode of regulation of dendrite shape is less clear. In insects, hormones such as ecdysone have a 'sledge-hammer' effect, inducing the remodeling of almost all tissues in the animal. In the vertebrate nervous system however, hormones seem to play a more specific and minor role based on the evidence collected so far. It is likely that hormonal control of dendrite growth in vertebrates constitutes a mechanism to adjust neuronal morphology and connectivity in relation to the physiological state of the animal.

The extracellular matrix

Besides various trophic factors and signaling molecules that act in a paracrine manner, the three-dimensional environment of neurons contains a complex amalgam of molecules that make up the extracellular matrix (ECM). The ECM is a dense meshwork consisting largely of polysaccharides, proteoglycans, and other large fibrous proteins that reside in the extracellular spaces in the brain and other tissues. In addition to providing structural support to cells, increasing evidence show that the ECM plays critical regulatory roles in important cellular processes such as migration, differentiation, and guidance.

Being in the right place at the right time is extremely important for neurons given the complex three dimensional structure and intricacy of neuronal connections in the brain. Proper neuronal migration is therefore a critical step in the development of the nervous system. Several lines of evidence indicate that interactions between migrating cells and the ECM regulate neuronal migration. For instance, neural crest cells migrate extensively throughout the developing embryo generating a large number of differentiated cell types in different parts of the brain. The kinds of cells produced depend critically on the migratory path the neural crest cells take. Molecular and genetic manipulations in vertebrate embryos have revealed that neural crest cell migration is controlled by time- and space-dependent changes in the balance between permissive and inhibitory components of the ECM (Perris and Perissinotto, 2000). In addition, recent evidence that physical properties of the ECM also influence differentiation of stem cells into neuronal or glial lineages (Her et al., 2013) hint at a more complex relationship between cells and the ECM that surrounds them than that accepted to date.

Extension of neuronal processes faces similar challenges as migrating cells in many respects. Indeed, outgrowth of axons and cell motility involves similar cellular events and molecules. For example, both processes involve precise spatiotemporal regulation and reorganization of the actin cytoskeleton. In addition, both processes rely on extrinsic cues for navigation. It is perhaps not surprising then that there is evidence of important interactions between growing axons and the ECM, just as is the case with migrating cells.

A well-studied example of ECM-neuron interactions involves the integrin family of cell surface receptors. Integrins are localized to the growing edge of migrating cells and the growth cones of axons and play key roles in cell migration (Vicente-Manzanares et al., 2009) and growth cone advance during axonogenesis (Myers et al., 2011; Vicente-Manzanares et al., 2009). Integrins

bind to laminin in the ECM and to cytoskeletal regulators within cells via their cytoplasmic domains, providing a physical link between the ECM and cellular cytoskeleton (Calderwood and Ginsberg, 2003). However, the role of integrin extends far beyond providing a mechanical anchor; integrin engagement at the tips of growth cones results in massive redistribution of cytoskeletal components such as actin and their regulators and activation of GTPases, indicating that they also have a prominent signaling role (Myers et al., 2011).

A variety of other molecules such as fibronectin, collagen, vinculin, tenascin, and several proteoglycans that make up the ECM have been found to play important roles in neuronal migration and axon guidance (Letourneau et al., 1994). Their expression patterns can vary widely in different parts of the nervous system and can change over the course of development of the brain. Characterizing the precise function of many ECM components and identifying their cognate neuronal receptors however have proven challenging largely because of functional redundancy (Letourneau et al., 1994).

The primary sources of molecules that neurons encounter in their vicinity are neighboring neurons, glia, muscle and epithelial cells depending on the neuron type. A well-understood example of how glial cells influence neuronal morphogenesis is the secretion of soluble axon guidance signals by glia in the fly CNS and the vertebrate spinal cord. Glial cells secrete the repulsive cue Slit which prevents midline crossing by axons that contain the Robo receptor, and the attractive cue Netrin which allows midline crossing of axons expressing the Frazzled/DCC receptor. These interactions are necessary for advancing growth cones to either be able to cross the midline or stay on the same side of the midline as necessary. In the mouse visual system, interactions mediated by Nr-CAM, Plexin A1 and Semaphorin6D between growth cones and radial glial cells at the optic chiasm promote contralateral projections of retinal ganglion cell

axons (Kuwajima et al., 2012). Similarly, Sema3A expressed in mouse limbs interact with Neuropilin-1 receptors in motor neurons projecting into them, enabling them to make correct dorsoventral targeting within the limb (Huber et al., 2005). These examples underscore the critical role of extracellular factors such as guidance molecules on axon guidance and targeting.

Although the contribution of extracellular signals in the form of small molecules secreted by non-neuronal cells or resident components of the ECM to neuronal migration and axon guidance is clearly known to be important, whether dendrite morphogenesis is dependent on similar level of support and coordination with such signals is less clear.

Examining how dendrite development may be coordinated or regulated by substrate cells and molecules is an important question for many reasons. The size of the nervous system changes drastically as a neuron elaborates its dendritic arbors. Although the initial shape of the arbor may be established at an early stage, neurons continue to grow in most cases as the size of the animal increases (Parrish et al., 2009). Additionally, the molecular composition of the extracellular space also changes as an animal grows. In vertebrates, a developmentally programmed switch in the composition of ECM from embryonic and early postnatal form to a mature adult form occurs starting about 2 weeks after birth (Zimmermann and Dours-Zimmermann, 2008). Well-coordinated regulation of dendrite morphogenesis likely occurs to ensure that dendritic arbors change or do not change as needed to maintain proper function. Failure of dendrites to keep up with such changes can lead to severe defects in neural connectivity and neural circuit function. In addition, growing evidence suggest that transition from a juvenile ECM composition to mature ECM in vertebrates contributes to restrictions in plasticity and regeneration in the adult nervous system (Zimmermann and Dours-Zimmermann, 2008). Furthermore, ECM components such as phosphocan and lectican have been reported to inhibit axonal growth *in vitro (Zimmermann and*

Dours-Zimmermann, 2008). Understanding how the ECM regulates the capacity of neuronal processes to grow and regrow is of clear clinical significance in the context of brain injury or neurodegenerative disorders.

Examination of the role of neuron-substrate interactions on dendrite morphogenesis lags far behind the scrutiny of cell-intrinsic mechanisms of dendrite patterning. However, growing evidence already show that such interactions are important. In addition to the roles played by Slit and Netrin secreted by glia in dendrite targeting described above, important roles of integrin-mediated signaling in dendrite maintenance have been documented in both mice (Marrs et al., 2006; Warren et al., 2012) and flies (Kim et al., 2012). Increase in dendritic field size of class IV sensory neurons as the fly larva grows in size is dependent on signaling between these neurons and the epidermal substrate cells (Parrish et al., 2009). Furthermore, reshaping of the dendrites of these neurons into adult-specific arbors during metamorphosis involves matrix metalloproteinase (MMP)-dependent remodeling of the basement membrane, a specialized ECM layer upon which these dendrites grow, by epidermal cells (Yasunaga et al., 2010). Although these findings provide clear evidence of the importance of neuronal substrates for dendrite morphogenesis, the nature of interactions between neurons and substrate cells or molecules remains poorly known. In particular, several questions remain to be addressed. How does the substrate influence morphogenesis of dendrites to ensure appropriate coverage of the receptive field of the neuron? Do neurons receive information about changes in the substrate directly from substrate cells, such as epithelial cells in the above example, or via the ECM? What are the molecular mediators of dendrite-substrate interactions? While not all of these answers may be universally applicable to all brain regions or all neurons, understanding them will greatly help us understand how neurons

are able to form and maintain highly intricate circuits as the nervous system grows and changes with development.

To further understand the role of neuron-substrate interactions in dendrite morphogenesis, this thesis examines the role of the immunoglobulin superfamily member Basigin in dendrite morphogenesis in detail. Here I introduce our current understanding of the biological processes Basigin is involved in and the mechanism of its action.

Introduction to Basigin

In 1982, mouse tumor cells were reported to release a soluble factor that stimulated collagenase production by the rabbit fibroblasts they were co-cultured with (Biswas, 1982). An antibody against the stimulatory factor was later raised and a 58 kDa glycoprotein was isolated from the plasma membrane of human lung cancer cells (Ellis et al., 1989). The stimulatory factor was aptly named tumor collagenase stimulating factor (TCSF) (Ellis et al., 1989). After three decades, this remains the most well-characterized function of the protein, which was later renamed extracellular matrix metalloproteinase inducer (EMMPRIN) and is now universally referred to as Basigin.

Expression pattern of Basigin

Basigin is widely expressed in a variety of tissues, including the brain, in vertebrates. Its expression is high on immune cells such as activated T- and B-lymphocytes, dendritic cells, monocytes and macrophages, as well as the heart, placenta, testes and the thyroid (Kanyenda et

al., 2011). Basigin is strongly enriched in neurons in many brain areas including the hippocampus, cortex, retina, cerebellum and amygdala (Fan et al., 1998). While the dynamics of Basigin expression in normal developmental processes has not been carefully examined, several studies have reported highly elevated expression of Basigin in tumor cells associated with many types of cancer (Weidle et al., 2010). Immunohistochemical studies have shown that enrichment of Basigin in cancer occurs at the periphery of invasive tumor clusters (Gabison et al., 2005). This localization of upregulated Basigin is consistent with the proposed contribution of Basigin to tumor cell invasiveness by regulating ECM breakdown. Furthermore, based on its significant up-regulation and function in tumor cell invasiveness, Basigin has been proposed as a biomarker for cancer detection and prognosis (Li et al., 2009; Zheng et al., 2006).

In flies, Basigin is expressed in apposing membranes during dorsal closure at embryonic stages and in the larval NMJ, where it is localized to the periactive zones of presynaptic boutons (Besse et al., 2007). Although direct expression data are not available, loss of function experiments suggest that Basigin is also expressed in photoreceptors in flies.

Structure of Basigin

Basigin is an integral membrane glycoprotein of the immunoglobulin superfamily. It contains extracellular immunoglobulin (Ig) domains and a short cytoplasmic tail. Basigin is well-conserved from invertebrates to vertebrates. Flies have 2 Basigin isoforms that differ in protein length but share the same domain structure. Mice have 2 Basigin isoforms, Basigin-1 and Basigin-2. Basigin-2 is the most common and broadly expressed isoform and contains 2 extracellular Ig-domains. Basigin-1 is longer with 3 extracellular Ig-domains and its expression

is limited to the retina. Humans also have a longer 3 Ig-domain containing isoform named Basigin-1 and a shorter isoform with 2 Ig domains named Basigin-2. In addition, two other isoforms, Basigin-3 and Basigin-4, have also been reported in humans. Human and mouse Basigin are highly homologous with 80% similarity and 58% identity in their amino acid sequences (Miyauchi et al., 1991). Mouse Basigin shares 34% similarity and 26% identity in amino acids with fly Basigin. The transmembrane and juxtamembrane domains of fly Basigin are highly similar to those of mouse Basigin with 80% identity (Curtin et al., 2005), suggesting that these regions are important for Basigin function. Despite the low conservation of amino acid residues in the extracellular region between mouse and fly Basigin (20% identity and 28% similarity (Curtin et al., 2005)), they are structurally similar with a terminal signal sequence and two Ig domains. The highly conserved transmembrane domain of Basigin contains a charged Proline residue, suggesting that Basigin may form complexes with other proteins at the plasma membrane.

Additionally, the juxtamembrane region of Basigin in all three organisms contains a conserved positively-charged tri-basic KRR motif. Mutational analysis of this motif has indicated that it is essential for Basigin function. Occurrence of KRR motifs in fly proteins is poorly known, but several vertebrate proteins have been reported to contain this motif. In the potassium channels TASK-1 and TASK-3, this motif acts as a retention signal, controlling cell-surface levels of the channels (Zuzarte et al., 2009). A KRR motif is also present in vertebrate CD43, the glycoprotein antigen expressed on the surface of T-cells, and mutation of this motif has been found to result in loss of binding to Moesin, a member of the ezrin-radixin-moesin (ERM) family of cytoskeletal regulators (Yonemura et al., 1998).

Known functions of Basigin

Growing evidence indicate that Basigin is a multifunctional protein with important roles in various physiological and pathological processes. Basigin has a wide range of binding partners and although the full extent of the physiological significance of these physical interactions is unknown, it has become increasingly evident that Basigin plays different roles in different cellular contexts.

In the fly eye, Basigin is required for proper nuclear localization of photoreceptors as well as formation of contacts between glial feet, called decapitate projections, and photoreceptors (Curtin et al., 2007). Basigin plays an important role in regulating synapse size and organizing vesicles within presynaptic terminals in the fly larval neuromuscular junction. Organization of cellular cytoskeleton may underlie at least a part of the mechanism by which Basigin mediates these functions. In the larval NMJ, loss of Basigin results in changes in actin organization, as revealed by altered distribution of the actin binding protein Spectrin. This link between Basigin and actin is corroborated by in vitro studies involving *Drosophila* S2 cells, in which overexpression of Basigin results in flattening of cells and increased adherence to the substrate.

In mice, Basigin is expressed in both male and female reproductive tracts. Knockout of Basigin results in decreased fertility in females, owing to failure of implantation of the developing embryo resulting possibly from defective feto-maternal interaction (Igakura et al., 1998). In males, Basigin is required for migration of spermatogonia and spermatocytes in the testes (Igakura et al., 1998). Basigin and matrix metalloproteinase (MMP)-2 are co-localized on the surface of these migratory cells, and interfering with Basigin function results in decreased MMP-2 expression and activity (Chen et al., 2012). This suggests that Basigin may facilitate germ cell

migration by promoting MMP-2 levels and/or activity. Basigin has also been proposed to be a part of the checkpoint mechanism for detecting and killing defective germ cells via caspase-dependent apoptosis in mice testes (Chen et al., 2012).

In addition to its role in inducing MMP expression, Basigin is also involved in interactions with other proteins at the cell surface. Proper targeting of monocarboxylate transporters (MCT) to cellular surfaces is dependent on Basigin in a variety of murine tissues (Kirk et al., 2000; Philp et al., 2003) including the retina, where lactate is produced as a metabolite. Basigin null mice have pronounced defects in MCT expression and targeting in the retinal pigment epithelium (RPE) (Philp et al., 2003).

Basigin also interacts physically with Integrins. Biochemical evidence exist for physical association of Basigin with two integrin heterodimers, $\alpha^3\beta_1$ and $\alpha^6\beta_1$, although the functional significance of such associations in vertebrates is unclear (Berditchevski et al., 1997). In flies, Basigin and Integrins co-localize in the retina *in vivo* and in S2 cells *in vitro* (Curtin et al., 2005). In the former case, genetic interaction between Basigin and Integrin ensures proper nuclear localization within photoreceptors. Dominant genetic interactions between Basigin and Integrin have also been reported in the extraembryonic membrane interactions required for dorsal closure in *Drosophila* embryo. Specifically, Integrin mutant embryos exhibit a mild dorsal closure defect that is abrogated by 50% reduction in Basigin (Reed et al., 2004).

The role of Basigin in cancer progression has been investigated in detail for more than two decades. Basigin is up-regulated in various types of cancer, which leads to increased MMP secretion from both cancerous cells and the surrounding stromal cells (Nabeshima et al., 2006). MMPs break down components of the ECM, allowing cancer cells to migrate. Thus, up-

regulation of Basigin contributes to the invasiveness of cancer cells. Comparison of various cancer cell types with different constitutive levels of cell surface Basigin revealed that those with high constitutive Basigin expression exhibit higher invasiveness, anchorage-independent growth and tumorigenicity compared to those with lower cell surface Basigin levels (Dai et al., 2013). Interestingly, cell-surface Basigin levels also correlate with membrane localization of a variety of cell surface molecules including CD44, EGFR, the ABC-family of drug transporters, and the monocarboxylate transporter MCT4 (Dai et al., 2013). It is noteworthy that this function of Basigin appears to be a re-tooling by cancerous cells of the developmentally programmed role of Basigin in facilitating cell migration during the early stages of germ cell development described above.

In a retrospective clinical study involving breast cancer patients, the level of Basigin expression was found to be positively correlated with tumor recurrence/metastasis and negatively correlated with patient survival. This has led Basigin to be proposed as a biomarker for cancer prognosis (Li et al., 2009) and to be tested as a target of anti-cancer therapy (Xu et al., 2007).

Basigin as a ligand and a receptor

How does Basigin on the surface of cancerous cells induce MMP expression in surrounding stromal cells? Does Basigin act as a ligand for a surface receptor in stromal cells? Considering the capacity of various cell adhesion molecules of the Ig superfamily for homophilic interactions, one possibility is that Basigin cancerous cell surface and the surrounding stromal cells may engage in homophilic binding. Indeed various lines of evidence support this idea.

In a large-scale protein interaction mapping project in which co-affinity purification was carried out in combination with tandem mass-spectrometry to identify the physical interacting partners of various *Drosophila* proteins (Guruharsha et al., 2012), Basigin was found to physically interact with itself. Affinity-tagged Basigin-G pulled down Basigin-D, whereas, in a separate pull-down, Basigin-D pulled down Basigin-E. It is unclear based on this data alone whether the physical interaction is direct or indirect, and whether the observed interaction is simply a result of Basigin oligomerization occurring within a cell. Strong evidence for the capacity of Basigin to engage in homophilic interaction in *trans* comes from overexpression studies involving various cell lines. Expression of Basigin-GFP in *Drosophila* S2 cells causes them to aggregate, which doesn't occur in cells expressing GFP alone (Besse et al., 2007). Consistent with this, expression of full-length Basigin in chinese hamster ovary (COS) cells causes them to adhere to immobilized Basigin-Fc proteins consisting of the 2 extracellular domains of human Basigin fused to the Fc region of mouse IgG2a (Sun and Hemler, 2001). Furthermore, Belton and colleagues (Belton et al., 2008) found that a recombinant Basigin peptide comprising of only the extracellular Ig domains was able to bind full-length Basigin-2 on the surface of uterine fibroblasts *in vitro*. Importantly, they also found that this interaction causes increased MMP levels and activation of the extracellular signal regulated kinase 1/2 (ERK1/2) pathway in fibroblasts, validating the functional significance of homophilic binding of Basigin at cell surfaces (Belton et al., 2008). Taken together, these findings strongly suggest that induction of MMPs by Basigin is mediated at least in part by homophilic interactions between Basigin that results in activation of the ERK1/2 signaling pathway. Homophilic interaction between Basigin on closely apposed cellular surfaces may also underlie Basigin function in other known roles of Basigin that don't involve MMP production, such as regulation of NMJ size (Besse et al., 2007)

and interactions between glia and photoreceptors (Curtin et al., 2007) in *Drosophila*. In the former example, Basigin has been found to be required in both the presynaptic and postsynaptic cells for proper NMJ morphogenesis (Besse et al., 2007), which lends further support to this model.

Various lines of evidence indicate that Basigin is capable of interacting with itself (Igakura et al., 1998). The human-specific Basigin isoform Basigin-3 interacts physically with the internalized ligand-receptor complex of Basigin-2 (Liao et al., 2011). Interestingly, overexpression of Basigin-3 inhibits cell proliferation, MMP induction and cell invasion in hepatocellular carcinoma (HPCC) cells *in vitro* and *in vivo* (Liao et al., 2011). Although it has been suggested that this inhibitory function is achieved by antagonizing the function of Basigin-2, which normally promotes these cellular events, it remains to be tested whether physical association between Basigin-2 and Basigin-3 is involved in this role.

In addition, Basigin has been shown to act as a receptor for Cyclophilin A via heparans, resulting in activation of the ERK signaling pathway, in CHO cells (Yurchenko et al., 2002). Cyclophilin A is a potent chemoattractant for immune cells such as neutrophils, eosinophils and T cells (Yurchenko et al., 2002).

Thus, studies so far indicate that Basigin is a multi-functional protein with the capacity to mediate cell-cell interactions, influence ECM remodeling, and mediate signaling from the extracellular environment to the cytoplasm in different biological contexts. Our understanding of the function of Basigin in flies lags far behind that in vertebrates, but a growing number of studies implicate Basigin in cellular cytoskeletal regulation in flies.

Figure 1.1: Dendritic arborization (da) neurons of the Drosophila larval peripheral nervous system

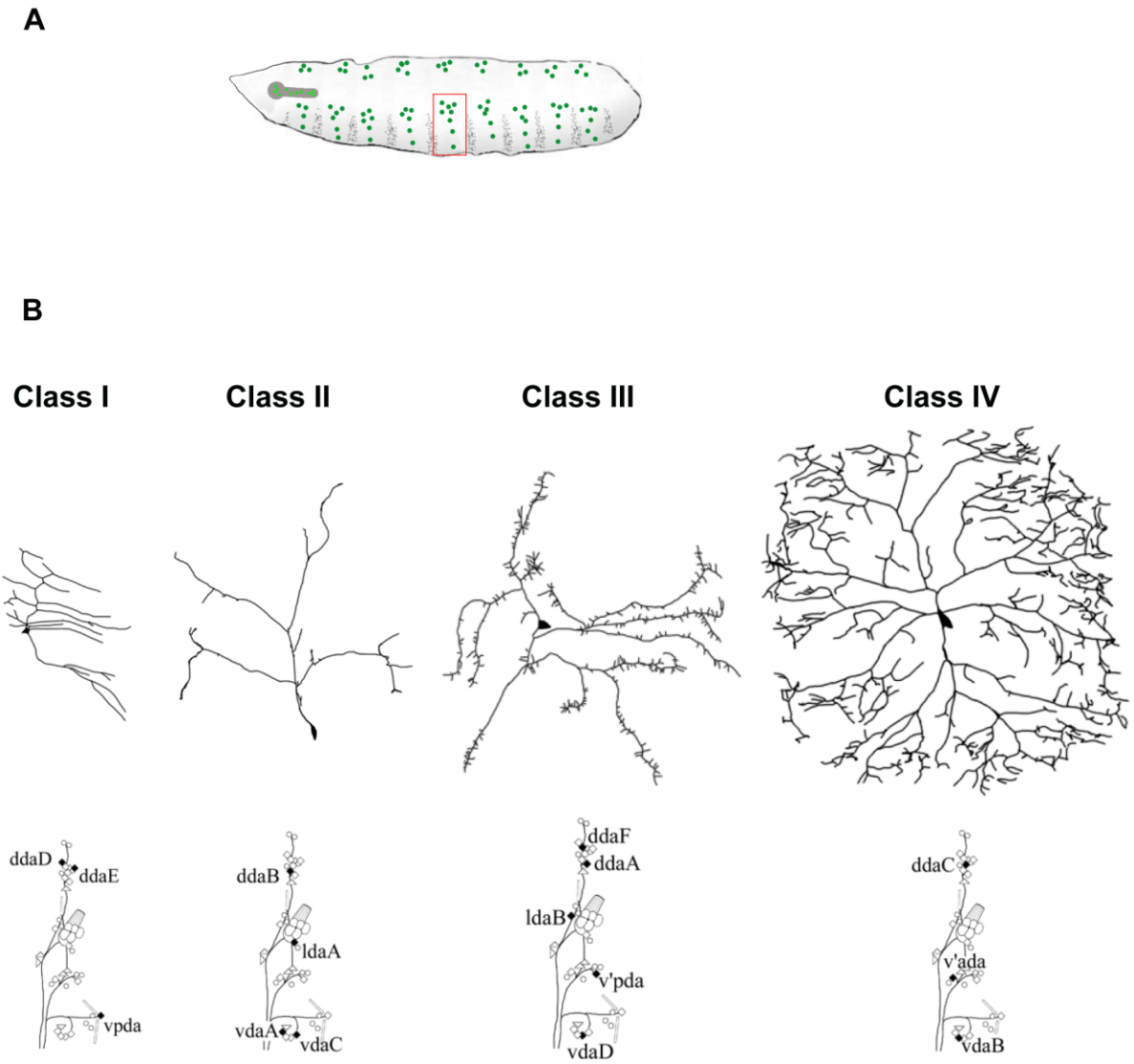


Figure 1.1: Dendritic arborization (da) neurons of the *Drosophila* larval peripheral nervous system

(A) Dendritic arborization neurons of the *Drosophila* larva innervate the larval body wall and occupy stereotyped positions (green dots) on each side of the midline within each segment.

(B) Neurons in a hemi-segment (red box in A) can be grouped into four classes based on the complexity of their dendritic arbors. Note the striking difference in branch complexity between class I and class IV neurons. Schematic diagram below each image of a neuron indicates the position of neurons of that class (filled shapes) within a larval abdominal segment. Open shapes indicate other sensory neyrons in the same hemi-segment. Images not shown to scale.

Images in B were adapted with permission from Grueber et al., (2003).

Figure 1.2: Structure of Basigin and its conservation from flies to humans

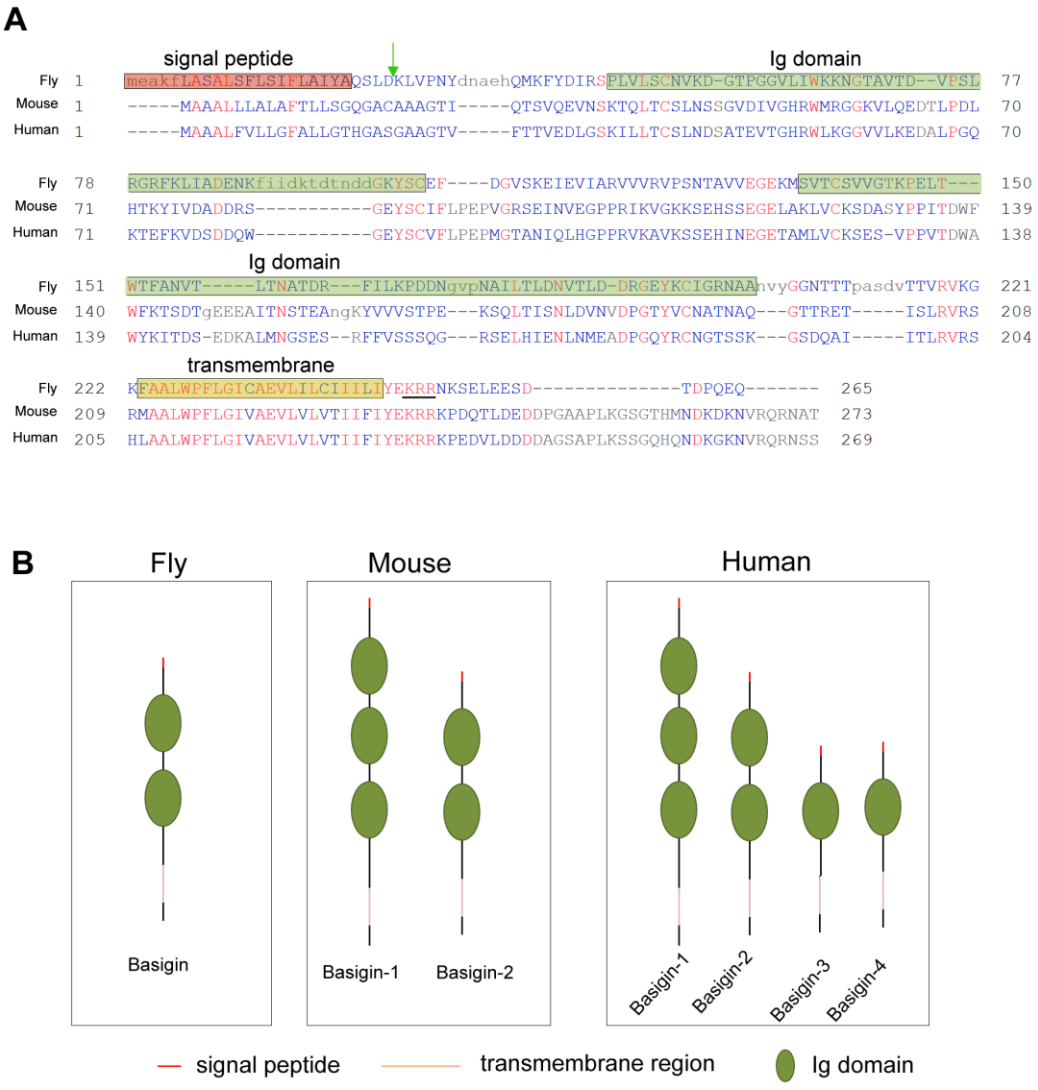


Figure 1.2: Structure of Basigin and its conservation from flies to humans

(A) Basigin is a cell-surface molecule with extracellular Ig domains, a short cytoplasmic tail and an N-terminal signal peptide sequence. Although Basigin in mice and humans share the same domain organization, sequence homology is high only in the transmembrane and intracellular juxtamembrane regions. The latter region contains a tri-basic positively charged KRR motif (underlined). Green arrow indicates the site of insertion of GFP in the Bsg::GFP protein trap fly line used to visualize Basigin expression and localization in this thesis. Red letters indicate conserved identity in all 3 species.

(B) Existing evidence indicate fly Basigin has 2 Ig domains, while mouse has a longer Basigin isoform with 3 Ig domains in addition to a 2 Ig domain isoform. Humans contain the same two Basigin isoforms as mouse Basigin and, in addition, two other isoforms with only 1 Ig domain each.

CHAPTER II: AN EXPRESSION PATTERN-BASED APPROACH FOR IDENTIFYING NOVEL REGULATORS OF DENDRITE MORPHOGENESIS[1]

Abstract

Our knowledge of the mechanisms by which neurons develop their stereotypic dendritic arbors has been greatly enhanced over the last two decades, owing in large part to a combination of advanced techniques permitting labeling and visualization of individual neurons with classical genetic approaches of mutagenesis and high throughput screens. The dendritic arborization (da) neurons of the *Drosophila* larval peripheral nervous system (PNS) have emerged as a particularly powerful model system for detailed dissection of the molecular mechanisms that underlie the diversity of dendrite morphologies that is characteristic of all nervous systems. Here, I have taken advantage of the ability to easily visualize gene expression and correlate such data with dendrite arbor complexity in this model system to conduct an expression pattern-based screen of candidate regulators of dendrite morphogenesis. Expression data was combined with available information on putative molecular function and evolutionary conservation of genes to select candidates for a secondary screen of function and relationship with known regulators of dendrite morphogenesis. Two candidates with varying expression levels among da neurons were found to be regulated by the transcriptional repressor Cut. One of them is the microtubule-binding protein Jupiter, which I found to control dendritic branch tortuosity. Localization of several septate junction resident proteins beneath some dendrites of da neurons provided novel insights into development of their arbors in three dimensions. Preliminary loss of function data on several genes suggested possible functional roles in regulating dendrite morphogenesis. One

[1] A part of work described in this chapter (i.e., screening of a subset of the protein trap lines) was carried out in collaboration with Megan Corty, Cate Jensen, Jennifer Lee, Ben Matthews, and Justina Tam.

of the candidates was Basigin, an immunoglobulin superfamily member, which was followed up on in detail. An expression pattern-based screen is thus an effective approach for identifying novel regulators of dendrite morphogenesis.

Introduction

The *Drosophila* nervous system is an excellent model system for investigating the molecular genetic regulation of dendrite morphogenesis mainly for the ability to conduct sophisticated genetic manipulations and to develop molecular markers that allow specific genetic labeling of neurons. While early forays into the examination of neuronal morphology in vertebrate systems were primarily anatomical, the last decade has seen significant contribution to our understanding of the molecular mechanisms governing dendrite morphogenesis from studies on the *Drosophila* nervous system, as discussed in detail in the previous chapter. Novel insights on dendrite morphogenesis in flies have come from several forward genetic screens on da neurons of the fly PNS and neurons in the mushroom bodies of the fly CNS (Grueber et al., 2007; Medina et al., 2006; Reuter et al., 2003). Screens involving the da neurons were conducted in genetically marked backgrounds to identify regulators of specific aspects of dendrite morphogenesis. For example, Grueber et al. conducted an EMS mutagenesis screen in animals in which class IV neurons were labeled with GFP in order to identify regulators of dendrite-specific features as well as co-regulators of dendritic and axonal morphology (Grueber et al., 2007). Medina et al. conducted a forward genetic screen in actin::GFP flies to specifically isolate mutations affecting the actin cytoskeleton of dendrites (Medina et al., 2006).

In addition, reverse genetic screens in flies have also provided useful insights into development of dendrites. Notably, a gain of function screen in peripheral da neurons and central motor neurons revealed mechanisms that are both distinct and common to the fly PNS and CNS (Ou et al., 2008). Furthermore, a genome wide screen for transcriptional regulators of dendrite morphogenesis has also been carried out in da neurons using RNA interference (RNAi) (Parrish et al., 2006).

Although these approaches are powerful and have been effective in uncovering novel regulators of dendrite morphogenesis, they have several drawbacks. For example, EMS mutagenesis is highly effective for generating a large number of mutants for phenotypic screening, but subsequent follow-up to map mutations is often highly laborious and resource-intensive, making it difficult to take full advantage of the mutants generated in the process. RNAi screens offer the advantage of knowing the gene beforehand, but are limited by the efficacy of the RNAi process. Non-specific knockdown and incomplete knockdown of target genes are both legitimate concerns when using RNAi.

Here, we have undertaken a different approach of using expression patterns and subcellular localization of proteins as a starting point to hone in on candidate regulators of dendrite morphogenesis in the fly larval PNS. Previous studies have shown that finding out the expression patterns of genes of interest, or using expression patterns of unknown genes as hints of their possible function can be a useful strategy for understanding diversity in neuronal morphology. For example, observations of differing expression levels of the homeodomain transcription factor Cut among the sensory neurons of fly embryos hinted at its role in dendrite morphology specification and subsequently led to the uncovering of its critical role in dendrite patterning in the fly PNS (Grueber et al., 2003a). In fact, high-throughput screens aimed at identifying

regulators of important biological processes based on expression patterns of genes is widely applicable in biology. This approach has been used to study genetic regulators of various physiological processes in mice, including lung morphogenesis (Herriges et al., 2012), specification and maturation of neurons in the urinogenital tract (Wiese et al., 2012), and specification of vertebrate glial lineages (Fu et al., 2009).

A limiting factor in conducting large scale expression based screen is the availability of tools for assessing expression. In both antibody- and *in situ* hybridization-based approaches, reagents for visualizing protein or mRNA expression need to be generated one by one, which can be very costly in terms of time and resources. Tagging endogenously produced proteins with fluorescent markers in a genetically tractable model such as *Drosophila* represents an excellent alternative to these approaches, as reagent generation and subsequent screen for expression can both be done in a high-throughput manner. In recent years, several groups have generated transgenic GFP trap flies, in which a GFP-coding exon containing splice acceptor and donor sites is randomly inserted within an intron of a gene, which results in the protein product of the gene being tagged with GFP (Fig. 2.1). Endogenous proteins tagged in this manner show normal localization and expression level in the majority of cases (Buszczak et al., 2007; Clyne et al., 2003; Quinones-Coello et al., 2007), making this a particularly useful approach for screening the expression and localization patterns of a large number of genes in a wide variety of developmental stages and cellular contexts. This approach has also been successfully used to perform a more specific screen for proteins enriched at and required for proper development of the *Drosophila* NMJ (Besse et al., 2007). We took advantage of this availability of endogenously GFP-tagged transgenic lines for our expression pattern-based screen.

Methods

Larval dissection

Wandering third instar larvae were picked using tweezers or scooped out from vials containing molasses-based fly food and placed in a glass dish. Larvae were washed several times with PBS (phosphate buffered saline) with the help of a paint brush to get rid of fly food stuck to their cuticle. They were then placed in a dissection dish with a clear sylgard base. Enough PBS was added to the dish to keep the larvae immersed in PBS during dissection. Larvae were pinned on the sylgard base and filleted open as described in Shrestha et al. (Kim et al., 2012). A maximum of 6 larvae was dissected in each dish to minimize time spent before fixation. The dissected larvae were fixed in 4% paraformaldehyde for 18 minutes at room temperature with gentle shaking. Then, larvae were rinsed thrice with PBS, unpinned, and washed further in PBS with 0.3% (v/v) Triton X-100 (PBS-TX) for 30 minutes in 5 mL snap-cap plastic tubes placed in a rotator. After rinsing, larvae were first incubated for 1 hour in 5% normal donkey serum (NDS) in PBS-TX at 4 °C, then overnight in 250 µL PBS-TX containing primary antibodies at 4 °C. Larvae were rinsed in PBS-TX every 20 minutes for 2 hours. Secondary antibody incubations were carried out overnight at 4 °C. Larvae were washed in PBS-TX every 20 minutes for 2 hours, then mounted in poly-L-lysine coated coverslips. In preparation for mounting, larvae were dehydrated sequentially in 30%, 50%, 70%, 90%, 100% and 100% ethanol for 5 minutes each. Coverslips containing the larvae were then immersed in xylene twice for 10 minutes each and placed on glass slides using DPX (Electron Microscopy Sciences, Cat. #13510) as the mountant. Slides were allowed to air dry overnight at room temperature before image acquisition.

Loss of function analysis

Requirement of candidate genes for dendrite morphogenesis of da neurons was assessed by two approaches depending on reagent availability. If a null allele was not available, an RNAi transgene against the gene was expressed in da neurons using the pan-sensory neuron driver *109(2)80-GAL4*. Genes for which a null allele was available were studied by the MARCM (mosaic analysis with a repressible cell marker) approach as described previously (Lee and Luo, 1999; Shrestha and Grueber, 2011). Briefly, flies of the appropriate genotype were crossed and allowed to lay eggs on a grape agar plate for 3 hours. The embryos were kept at 25 ºC for 3.5 hours, then heat-shocked as follows: 38 ºC for 30 minutes, room temperature for 30 minutes, and 38 ºC for 45 minutes. The embryos were kept at 25 ºC until the late third instar stage, at which point they were screened for presence of mosaic clones expressing GFP in the larval PNS using a fluorescence microscope. Larvae containing such mosaic clones were then dissected, fixed and immunostained as described above.

Antibodies

The following primary antibodies were used: anti-GFP (1:1000), anti-HRP (1:250), anti-coracle (1:50), anti-cut (1:20). Appropriate secondary antibodies were used at 1:250 dilution.

Fly stocks

GFP trap lines with ID beginning with CA, CB, or CC were made by the laboratory of A. Spradling at the Carnegie Institute for Science (Buszczak et al., 2007) and provided to us by B. Ohlstein (Columbia University). All other GFP trap lines were obtained from the laboratory of L. Cooley (Quinones-Coello et al., 2007) at Yale University. Fly stocks for loss of function analysis

were obtained from the Bloomington Stock Center. Null allele for *bsg* (*bsg*$^{\delta 265}$) was provided to us by Dr. Kathryn Curtin at the University of Arkansas (Curtin et al., 2005).

Analysis

Dendrite morphology was analyzed using Neurolucida and Neurolucida Explorer (MBF Bioscience). Fluorescence intensity was analyzed using Image J. Statistical analysis was done using the R software package (R Core Team, 2012) and the online statistical tool VassarStats (http://www.vassarstats.net/). All data are presented as mean ± standard error of mean (SEM).

Results

An expression pattern-based primary screen

In order to identify novel candidate regulators of dendrite morphogenesis, we took an expression pattern-based approach by systematically cataloguing the expression pattern of a large number of genes in fly larvae (Fig. 2.1). Specifically, we obtained 262 GFP trap lines and documented the expression pattern of 258 unique genes in the fly larval PNS and the epidermal cells that make up or secrete the substrata that PNS dendrites grow on. A total of 125 (48%) genes were found to have some form of expression in the PNS. Of these, 48 (38%) are expressed specifically in the PNS without any epidermal cell expression while 77 are expressed in the epidermis as well. The 125 genes found to be expressed in the PNS have varying subcellular localization: 12 localize strictly to the nuclei, 59 are only in the soma, while 45 have somatodendritic localization. A total of 27 proteins of the 45 with somatodendritic localization also show epidermal expression.

While the expression and localization patterns of some genes were very unique, many fell into obvious and interesting categories. For example, a subset of genes was found to be expressed at levels either directly or inversely proportional to dendrite branching complexity in da neurons. Another subset was observed to localize to cell borders and along da neuron dendrites in the epidermis in addition to being expressed in da neurons. A group of genes known to be part of the cellular translational machinery was found to have somatodendritic localization. These types of expression patterns are interesting for their functional implications and are discussed below in further detail.

Genes with varying expression levels in da neurons

Several genes were found to be expressed at levels that varied either directly or inversely with the complexity of the dendritic arbors. For example, Tsp42Ee, a protein involved in organizing molecular complexes at cell surfaces, is expressed at low levels in class I neurons, which have the simplest dendritic arbor and at progressively higher levels in neurons with more complex arbors (Fig. 2.2). On the other hand, expression of *vesicular acetylcholine transferase* (*vAChT*) was found to vary inversely with dendrite complexity: it is expressed at low levels in the complex class IV neurons and at the highest levels in the simplest class I neurons (Fig. 2.2). Likewise, expression of *mustard* (*mtd*) was also observed to be high in class I neurons and progressively lower in neurons with increasing branching complexity (Fig. 2.2). CAP, which binds to the cytoskeletal adapter molecule Vinculin, is enriched in class I neurons and is weakly expressed in other da neurons (Fig 2.2).

Besides these, a subset of genes was found to be expressed in an all-or-none manner among the various classes of sensory neurons. For example, Jupiter, a microtubule-binding protein, is

expressed specifically in class I neurons (Fig. 2.2), suggesting the possibility that it may be part of a molecular program for specifying class I-specific dendritic arbors. Notably, several transcription factors with class-specific expression levels have previously been described to play important roles in generating the diversity of dendrite morphology among da sensory neurons (Fig. 2.2) (Corty et al., 2009). Thus, varying expression levels of the genes described above hint at possible genetic regulation by these transcription factors and involvement in dendrite morphogenesis of da neurons.

Dendritic localization of genes implicated in translational regulation

Several genes implicated in translational regulation were found to be enriched in da neurons and, interestingly, localized to their dendrites in addition to the cell body. Such genes include the elongation factor 2B (Ef2B) (Fig. 2.3), belle (bel), RpL13A, and elongation initiation factor 4E (eiF4E). A growing body of evidence indicates that local translation of proteins in dendrites is closely associated with modification of neuronal structure and function. Dendritically synthesized proteins have been implicated in a variety of processes that are relevant for dendrite structure such as actin dynamics, receptor trafficking, and modulation of the ECM (Bramham and Wells, 2007). In addition, RNA-binding proteins are one of the main regulators of mRNA-specific local translation in dendrites (Bramham and Wells, 2007). Our observation of somatodendritic localization of several proteins predicted to bind RNA and be a part of the protein synthesis machinery in da neurons suggests that local protein synthesis may play important roles in the morphogenesis of their dendrites. Furthermore, these observations also suggest that da neurons may be a good model system to investigate the role of dendritic local translation in dendrite morphogenesis.

Strong localization of proteins along dendrites

While the vast majority of proteins localized to dendrites, including those involved in translational regulation as described above, were detected there at very low levels, some were found to be highly enriched in da neuron dendrites. For example, Charybde, the fly homolog of the human pro-apoptotic gene RTP801, was found to be localized uniformly and at very high levels in da neuron dendrites (Fig. 2.3). In contrast, ArgK showed punctate localization along dendrites with strong enrichment at dendritic branch points and terminals (Fig. 2.3). ArgK is an ATP/guanidine phosphotransferase that provides ATP to various ATP-dependent cellular processes. Although ArgK has been previously reported to have highly localized subcellular distribution, its localization in neuronal dendrites has not been reported to date. Interestingly, it has also been found to be concentrated in growth cones and tips of filopodia in neurons undergoing axonogenesis (Wang et al., 1998). Based on the observation that the amount of ArgK localization varied widely even among growth cones of the same neuron, it has been proposed to participate in the selective growth of specific growth cones (Wang et al., 1998). Since the roles of ArgK and Charybde in dendrite morphogenesis are unknown, these localization data may provide helpful starting points in understanding their possible roles in dendrite development and function.

In addition, a number of proteins implicated in translational regulation, such as elongation factor 2b (Ef2b), were found to have strong dendritic localization (Fig 2.3). Although the role of local translation in axon and dendrite development is known, it has not been described to date in da neurons. Our expression data suggests that dendritic local translation may be very pronounced in da neurons and that these neurons may be an excellent system for detailed molecular analysis of how local translation in dendrites influences dendrite morphogenesis.

Epidermal enrichment of proteins along da neuron dendrites

Strikingly, a number of proteins were found to be expressed in da neurons as well as the underlying epidermal cells, where they are localized to cell borders as well as the region directly beneath some da neuron dendrites (Fig. 2.4). While some of these proteins are known septate junction (SJ) resident proteins (e.g., Scribbled, Neuroglian, Neurexin-IV), our screen uncovered several proteins that have never been reported to be a part of but have identical localization patterns as those of components of SJs (e.g., Basigin, Syndecan, l(2)08717, CG15926, CG3036, tropomodulin) (Fig 2.4). Labeling with antibodies against the known septate junction protein Coracle revealed similar enrichment of Coracle along the dendrites of da neurons. In fact, GFP-tagged proteins that fell into this category were found to co-localize perfectly with Coracle, indicating that molecular complexes, which we refer to here as dendrite-junction complexes (DJC), comprising of resident septate junction proteins form along some da neuron dendrites.

The majority of dendrites of da neurons reside on the basement membrane, which lies next to the basal surface of the epidermal cell. Ongoing research in our laboratory had indicated that, unlike the SJ proteins described above, basal hemi-adherens junction components such as integrin show uniform epidermal enrichment along da neuron dendrites. We therefore reasoned that dendritic segments with underyling epidermal enrichment of SJ proteins may be seated more apically along the apico-basal plane of the epidermis. Indeed, surface labeling for the pan-neuronal marker HRP in the absence of detergents revealed that dendritic segments with Coracle enrichment were partially blocked, possibly by the epidermal membrane, for antibody access as revealed by weaker HRP immunostaining along them. Since the DJC proteins we identified co-localize with Coracle, this result suggests that epidermal enrichment of those proteins beneath dendrites mark regions of dendrites that are embedded deeper into the epidermal cell layer. It is

likely that these proteins form molecular complexes at sites where the dendrites come in close contact with the epidermal membrane.

Secondary screen for candidate regulators of dendrite morphogenesis

Combining information on the expression patterns of many genes with their putative molecular function based on bioinformatic data available from Flybase allowed us to create a shortlist of putative regulators of dendrite morphogenesis for our secondary screen for function. Since a number of genes were identified to be expressed at varying levels in da neurons, we assessed whether they are regulated by the transcriptional repressor Cut, which has been shown in previous studies to act as a master regulator specifying dendrite complexity of da neurons. In addition, we obtained mutants and transgenic RNAi lines for a total of 12 genes to assess whether they have functional roles in dendrite morphogenesis of these neurons.

Regulation by the transcriptional repressor Cut

As indicated above, Mtd is expressed at high levels in class I neurons, which are Cut-negative and have the simplest dendritic arbors of all da neurons. Mtd expression levels are progressively higher and precisely inverse of the expression levels of Cut in class II, IV and III neurons (Fig. 2.2). *mtd* encodes a protein with peptidoglycan-binding lysine domains and is involved in innate immune response in flies. Its expression and function in neurons has not been described to date. In order to assess whether *mtd* expression is regulated by Cut in da neurons, we examined changes in Mtd::GFP levels in class I neurons in which Cut was ectopically expressed using the pan-sensory driver PO163-GAL4 (Fig. 2.5). Interestingly, expression of Cut led to significant downregulation of *mtd* expression, as indicated by reduction in Mtd::GFP levels in ddaD and

ddaE, both of which are class I neurons that innervate the dorsal body wall of the fly larva (Fig. 2.5).

Unlike Mtd which had different expression levels, Jupiter was observed to be expressed specifically in class I neurons. Jupiter is a microtubule-binding protein with no clear vertebrate homologs and contains the microtubule binding motif PGGG, which is also found in the well-studied microtubule binding protein Tau (Karpova et al., 2006). Dendrites of class I da neurons have abundant microtubules as revealed by staining with the microtubule-binding protein Futsch, and are also very stable compared to those of class III and class IV dendrites. Interestingly, Jupiter has been shown to bind preferentially to stabilized microtubules *in vitro* (Karpova et al., 2006). The specific expression of Jupiter in class I neurons thus suggests that class I dendrites may have more stable microtubules than those of other classes. Additionally, transcriptional repression of microtubule-binding proteins such as Jupiter by Cut may constitute part of the molecular program by which other classes of neurons are able to develop more complex and dynamic dendritic arbors. In order to test whether *jupiter* expression is regulated by Cut, I misexpressed Cut in class I neurons using the pan-sensory driver PO163-GAL4 in a Jupiter::GFP background (Fig. 2.6). Indeed, ectopic Cut expression significantly downregulated Jupiter::GFP levels in the class I neuron ddaE, suggesting that Jupiter expression is regulated by Cut (Fig. 2.6). However, a trend towards downregulation but no significant change in Jupiter::GFP level was observed in a different class I neuron, ddaD (Fig. 2.6). This may reflect either difference in the sensitivity of Jupiter levels to Cut in different class I neurons, or differences in degrees of ectopic Cut expression achieved using the PO163-GAL4 driver.

In order to further assess the functional role of Jupiter in dendrite morphogenesis of class I neurons, I knocked down Jupiter by driving expression of Jupiter RNAi transgenes in these

neurons using the 221-GAL4 driver. No significant difference in the length and branching of class I dendrites was observed (Fig. 2.6). This may be because of redundant function of other microtubule binding proteins such as Futsch in these neurons. When both Jupiter and Futsch were knocked down at the same time, the length and number of branches of class I neurons remained unaffected. However, branches of class I neurons, particularly shorter ones near the cell body, appeared more curved and sinuous compared to those of control neurons (Fig. 2.6). Quantitative analysis of branch tortuosity (measured as the ratio of the length of the branch and the shortest distance between its endings) of class I dendrites revealed that, indeed, knockdown of both Jupiter and Futsch resulted in branches with significantly higher tortuosity. Importantly, knocking down Futsch alone did not cause this phenotype (Fig. 2.6). This suggested that Jupiter and Futsch act together to regulate the tortuosity of dendrites in class I neurons. It is noteworthy that higher tortuosity is typically observed in the dendrites of the more complex class IV da neurons, which grow space-filling dendritic arbors, and in the filopodia of class III neurons. These results suggest that Jupiter may be a part of the molecular program that restricts branch tortuosity in class I neurons, and repression of Jupiter by high Cut expression may be a mechanism by which class III and IV neurons are able to develop branches with increased tortuosity.

Loss or knockdown of candidate genes

As part of our secondary screen, we assayed the functional requirement of several other candidate genes in dendrite morphogenesis of da neurons. Genes for which null mutants were available were screened using the MARCM technique, and the rest were screened using transgenic RNAi lines. As shown in Figure 2.7, some genes were found to play no roles in

dendrite morphogenesis while others were found to be required for proper morphogenesis of class IV dendrites. For example, in contrast to the well-spaced and evenly branched arbors of class IV neurons, *shep* mutant class IV neurons grew fewer dendrites while *bsg* mutant neurons grew arbors with highly disorganized branches. Knockdown of CAP in class IV neurons also led to reduction of dendrite branching and uneven innervation of the dendritic field (Fig. 2.7).

With these results in hand, I decided to focus on Basigin for further investigation based on its expression pattern, domain structure, and strong conservation from flies to humans.

Discussion

In this chapter, I have described the results of our expression-pattern based screen for candidate regulators of dendrite morphogenesis in the fly larval PNS. A wide variety of expression patterns were observed for genes that were selected in an unbiased manner for screening. Secondary screen for transcriptional regulation and loss of function phenotypes suggested possible roles of several candidate genes in dendrite morphogenesis of da neurons. One candidate gene was followed up and its role in dendrite morphogenesis was characterized in detail, which is described in subsequent chapters. Our findings also revealed new insights into the growth of class IV da neuron dendrites, which were previously thought to be highly planar, in three dimensions. In addition, data from our screen should provide useful entry points for examining the functional roles of a number of other genes in dendrite morphogenesis.

Expression-pattern based strategy as an effective tool for finding candidates

Development of genetic tools to label specific subpopulations of neurons in the fly nervous system slightly over a decade ago paved the way for highly effective forward genetic screens for regulators of dendrite morphogenesis (Grueber et al., 2007; Medina et al., 2006; Reuter et al., 2003). Early studies following this approach identified several molecules that began to reveal the molecular mechanisms controlling dendrite morphogenesis. However, the time and resources required to map mutations were often very high. In fact, of the large number of mutants with dendrite arborization phenotypes identified in such screens, only a small subset have gone on to be mapped and studied in detail primarily because of these constraints. This has led investigators to take advantage of recent innovations in bioinformatics to take candidate-based reverse genetic approaches. One example is the use of RNAi to knock down genes of interest in a high-throughput manner, which offers the advantage of instantly knowing the identity, structure, and putative molecular function of any gene (Parrish et al., 2006). The caveat of this approach however is that RNAi is not consistently effective across all genes, and can sometimes have non-specific effects, so one is limited by the effectiveness of the RNAi technique in avoiding false negatives and false positives. In addition, finding out the cell types in which the gene is required often calls for follow-up experiments.

Screening based on the expression pattern of genes combines the strengths of both approaches while allowing the investigator to propose testable hypotheses beyond loss of function phenotypes, such as transcriptional regulation, level dependent effects and subcellular sites of action. We took advantage of GFP trap lines generated by laboratories at the Carnegie Institute and Yale University (Buszczak et al., 2007; Quinones-Coello et al., 2007) to visualize the expression and localization patterns of 259 unique genes. Our screen revealed several notable expression patterns that can be informative not only in selecting them in candidate-based LOF

analyses but also in interpreting any LOF phenotypes associated with the gene. In addition to well-studied genes such as *bazooka*, *myosin heavy chain*, and *neuroglian*, which were found to be expressed and localized as expected, our screen has also identified interesting expression patterns of many genes, such as *mustard*, *belle*, *CAP*, that have not been studied before in the nervous system of the fly. I expect that these data will provide a useful starting point for investigators interested in the role of these genes in neuronal morphogenesis in flies.

The approach we took for expression-based screen has a few caveats that are related to limitations of the GFP-trapping technique itself. Firstly, insertion of the GFP exon at an intron doesn't guarantee that it is spliced with all protein isoforms transcribed from a given locus. Therefore, the observed expression pattern of any given gene may be that of a subset of its splice isoforms. Secondly, insertion of the 6xHIS-tagged GFP peptide within a protein may disrupt its functional domains, which may cause mislocalization and/or decrease in protein levels due to poor stability of the dysfunctional protein. The observed expression pattern and levels in these cases may be very different from the native expression pattern of the endogenous untagged protein. For example, the GFP trap line for the homedomain transcription factor Cut contained no specific expression anywhere in the larval PNS (data not shown) even though antibodies against Cut show highly specific nuclear localization and differences in expression levels in the sensory neurons. In addition, some cases of mislocalization of GFP-tagged proteins have also been reported. Buszczak et al. found that in the GFP trap line for CG15015, the GFP coding exon disrupts its FES/Cip4 domain, which is required in its mammalian homolog Cip4 to bind to Cdc42 and regulate the actin cytoskeleton (Buszczak et al., 2007). GFP-tagged CG15015 proteins in this line localize to the nucleus, whereas proteins derived from N- and C-terminally tagged transgenes of the same gene localize to the cytoplasm in *Drosophila* S2 cells. However,

only 4 out of a sample of 107 GFP trap lines sampled by Buszczak et al. showed mislocalization of tagged proteins, suggesting that this occurs at a fairly low frequency (Buszczak et al., 2007). Nevertheless, independent validation with antibodies should be done where available to confirm the expression and localization patterns revealed by the GFP trap lines. In addition, multiple protein trap lines with insertion of the fluorescent reporter at different sites within the same gene are available in some cases. If antibodies are not available, comparison of expression patterns among such lines, as I did for tropomodulin, can also lend confidence to the expression observed in any single line.

GFP trap lines can be a useful resource for visualizing various important developmental processes such as cell division. Having GFP tags on endogenous proteins of interest can provide a rare view of events such as chromosomal segregation and organelle movement. This is true of neuronal morphogenesis as well. The catalog of expression and localization patterns of proteins in the larval PNS can be a useful resource in visualizing cellular processes influencing dendrite or axon development. For example, the highly punctate distribution of Arginine Kinase (Fig. 2.3) in the dendrites of sensory neurons can potentially be tracked to monitor energy demands in different regions of the dendrites as they grow. Likewise, levels of local protein translation in dendrites under various conditions may be inferred by using one of several GFP trap lines of genes involved in translational regulation (Fig. 2.3). Finally, growth of dendrites in relation to that of the substrate epidermal cells in growing larvae may be monitored by using GFP trap lines in which the tagged protein localizes beneath dendrites in epidermal cells.

Since our screen was unbiased in the selection of trap lines, our dataset consists of genes that belong to a wide range of molecular classes, from transcription factors to kinases, cytoskeletal regulators, cell surface proteins and components of the protein translation machinery. This body

of data can thus be a helpful resource for studying transcriptional regulation, as I did for regulation of *mtd* and *jupiter* by Cut. Many other proteins showed neuron-type specific expression levels and it may be worthwhile to investigate whether they are also regulated by one or more of the several transcription factors that have been shown to constitute a combinatorial code regulating dendrite morphogenesis of the sensory neurons. This can be a good entrypoint for efforts to uncover at a molecular level how specific transcription factor code in each neuron type contributes to the specific morphological features of the neuron. Furthermore, studies on protein co-localization and mislocalization could also benefit from this work. As an example, a growing body of evidence indicates that protein cargoes are sorted in a highly regulated manner to serve the functional and morphological specializations of dendrites and axons (Horton et al., 2005; Ye et al., 2007). Some of the protein trap lines could be used to visualize how localization of dendritic or axonal proteins is altered upon knockdown of genes of interest. Finally, our screen revealed the dendritic localization of a number of RNA-binding proteins implicated in translational regulation, which may be informative not only for future studies on the mechanisms regulating local translation but also for investigating how regulation of local translation shapes dendrite morphogenesis.

The strategy of conducting an expression pattern-based screen to arrive at candidate regulators of dendrite morphogenesis is a powerful one, considering the insights it has lent us in understanding the roles of Basigin, which is the focus of this dissertation, and scalloped (Corty MM et al., unpublished) in dendrite morphogenesis, as well as the growth of class IV dendritic arbors, which were previously thought to be highly planar, in three dimensions (Kim et al., 2012). Future studies taking advantage of the interesting expression patterns we observed for many other genes will further extend the validity of our approach. One limitation of the approach we

used is that availability of reagents such as mutants and RNAi or overexpression lines necessary for functional characterization of genes of interest can vary widely depending on how well the gene has been studied previously. If no mutants are available, for instance, generation of one can be done in a reasonable time frame of several months, but this limitation can still greatly reduce the number of lines with interesting expression patterns that can be used in a secondary screen for function. Several large scale projects for genome-wide mutagenesis (Choi et al., 2009; St Johnston, 2002) or generation of RNAi lines (Dietzl et al., 2007; Ni et al., 2009) have been carried out recently in flies by various groups around the world and this should continue to be a useful resource for following up on genes showing interesting expression patterns. Furthermore, Perrimon and colleagues (Neumuller et al., 2012) have recently developed highly specific shRNA transgenes directed against GFP for RNAi-mediated knockdown of GFP-tagged transcripts in GFP-trap lines. These transgenic lines can be useful in conducting a parallel screen for phenotypes using the same GFP-trap lines used for screening expression.

mustard and jupiter may be regulated by cut

We found a number of genes to be expressed at levels that correlate either directly or inversely with dendritic arbor complexity in da neurons. These expression patterns are highly interesting in light of the fact that several transcription factors are known to be expressed similarly at cell-type specific levels and to confer specific properties to dendritic arbors. Our observations led us to hypothesize a regulatory relationship between the transcriptional repressor Cut and two genes we screened, namely *mtd* and *jupiter*. Interestingly, we found that ectopic expression of Cut led to downregulation of both *mtd* and *Jupiter* in class I neurons, which are normally Cut-negative.

Mtd is a protein containing peptidoglycan-binding domains and its role in neuronal function or morphogenesis has never been described to date. Preliminary analysis using a deficiency line covering the *mtd* locus did not reveal an obvious role for Mtd in dendrite morphogenesis of da neurons. A mutant allele of Mtd has now been characterized, which should facilitate a more thorough analysis of its role, especially in the context of its regulation by Cut.

Jupiter is a microtubule-binding protein that binds preferentially to stabilized microtubules *in vitro* (Karpova et al., 2006). The observation that it is expressed specifically in class I neurons suggested the possibility that it may underlie some morphological properties specific to class I dendrites. Indeed, preliminary results from our secondary screen showed that together with Futsch, a microtubule-binding protein, Jupiter regulates the tortuosity of class I dendrites. High tortuosity is normally associated with the terminal branches of class IV neurons, which are highly dynamic and devoid of microtubules. In contrast, class I neurons are more stable and are enriched with microtubule bundles, as revealed by antibodies against Futsch. Our preliminary findings suggest that Jupiter and Futsch may be part of the molecular machinery in place in class I neurons to make them 'stiff' and 'straight', and thus less tortuous and less dynamic. Although our results need to be validated using a null allele of *jupiter*, it is interesting to speculate that development of a class-IV specific arbor in the presence of high levels of Cut may involve repressing molecules such as Jupiter that contribute to such 'stiffness'. A prediction of this model would be that overexpressing Jupiter in class IV neurons should decrease branch tortuosity. In addition, co-expressing both Cut and Jupiter in class I neurons should affect the tortuosity of the supernumerary branches that grow when Cut is expressed in these neurons. Future studies should test these predictions. Preliminary results showed that overexpression of Jupiter using the class IV-specific driver ppk-GAL4 caused failure of class IV neurons to

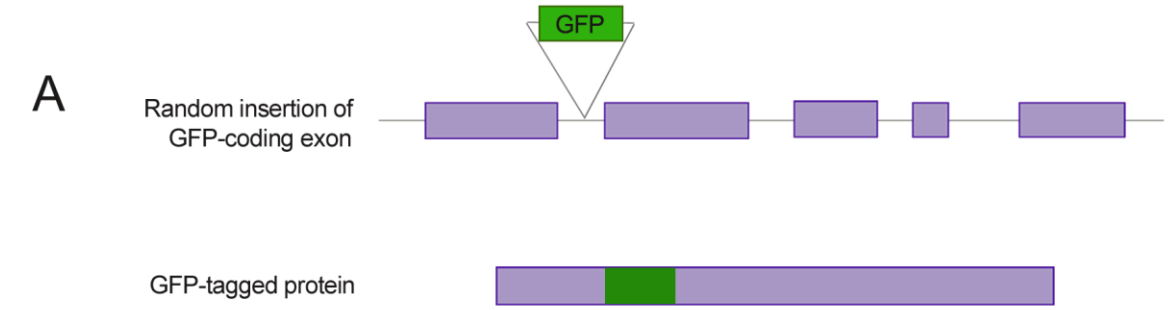
completely cover their dendritic fields at the dorsal midline without any overt effects on branch tortuosity. One explanation for this phenotype is that the molecular context is very different from that of class I neurons for it to be able to simply alter the tortuosity of class IV branches. Even in class I neurons, Futsch appears to be at least partly redundant with Jupiter in regulating branch tortuosity. Although Futsch is normally expressed in class IV neurons, it is not localized to the terminal branches. Hence, the lack of change in tortuosity of class IV branches upon Jupiter overexpression may be due to lack of overlap in Futsch and Jupiter localization.

Epidermal enrichment of septate junction proteins along da neuron dendrites

Our screen uncovered enrichment of septate junction proteins and several others in epidermal cells beneath some segments of da neuron dendrites, which hinted at the possibility that these segments were positioned more apical than the rest of the dendritic arbor. Consistent with this idea, surface labeling with antibodies against HRP in the absence of detergents revealed weaker labeling along segments of dendrites with underlying enrichment of coracle, a septate junction protein (Kim et al., 2012). Class IV dendrites were previously thought to grow over a largely 2-dimensional plane. Our observations of enrichment of SJ proteins underneath some dendrites led us to consider growth of class IV dendritic branches away from the 2-dimensional plane, the details of which have been published as part of a separate report (Kim et al., 2012). It is noteworthy that the developmental time course of such embedding of class IV dendrites in the epidermis is unknown, and these GFP-trap lines can serve as excellent tools for addressing this *in vivo* in growing embryos and larvae. Furthermore, since our screen has identified various molecular components of the DJC around enclosed dendrites, our findings can provide good starting points for probing the importance of DJC enrichment on dendrite morphogenesis.

Our screen has also identified several molecules that have previously never been described as a septate junction protein. These include Basigin, CG3036, l(2)08717, and CG15296. Such information about the localization and putative function of these proteins can be very useful in formulating testable hypotheses about their function in the epidermis.

Figure 2.1: Protein trap strategy and outline of the expression pattern-based screen for candidate regulators of dendrite morphogenesis



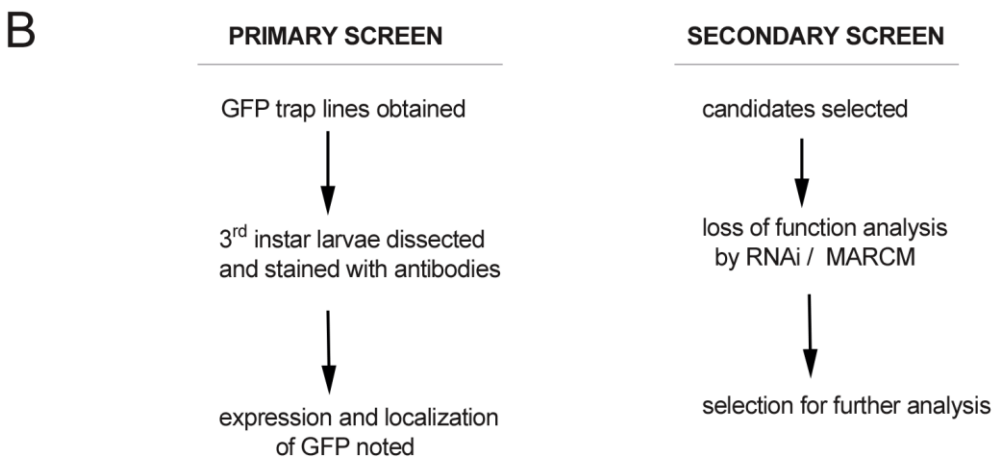


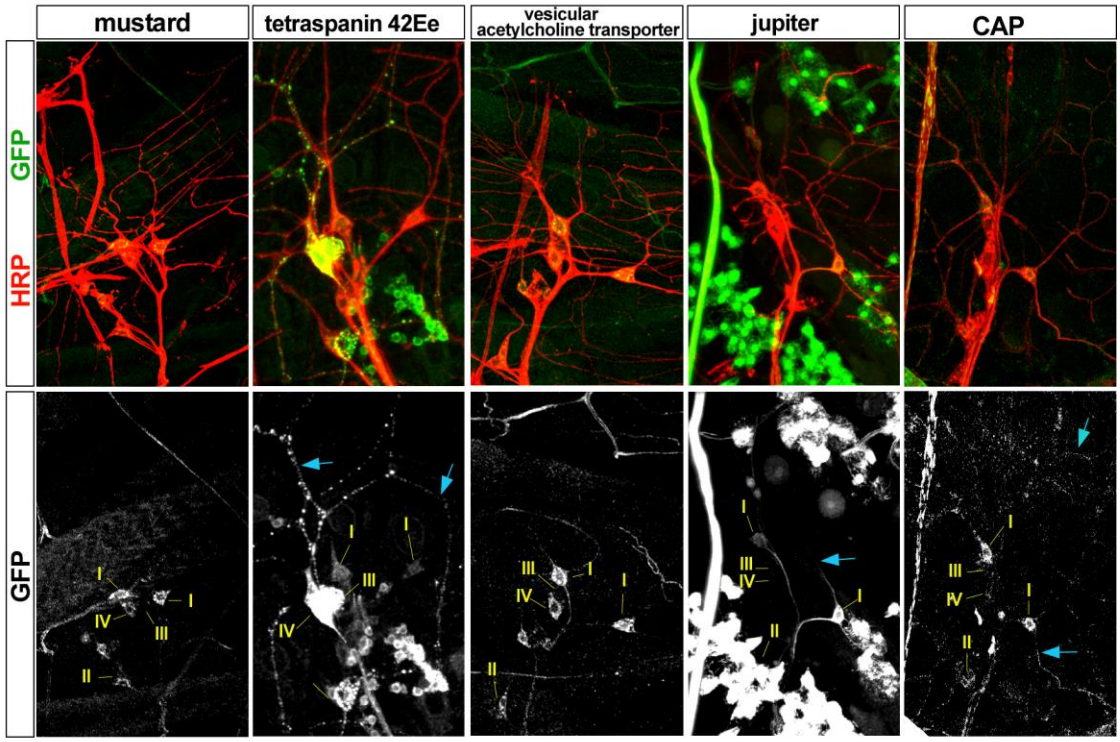
Figure 2.1: Protein trap strategy and outline of the expression pattern-based screen for candidate regulators of dendrite morphogenesis

(A) Each protein trap line consisted of a random in-frame insertion of a GFP exon without start and stop codons in the intronic region of a gene. Splicing of the GFP exon with other exons of the gene resulted in a GFP-tagged protein product.

(B) Outline of the expression pattern-based screen.

Figure 2.2: Several genes were found to be expressed at varying levels in da neurons

A



B

Gene	da neurons class I	II	III	IV
cut		+	++++	+++
knot				+
abrupt	+			
mustard	+++	++		+
tsp42Ee	+	++	++++	++++
vAchT	+++	++		+
jupiter	+			
CAP	++	+		+

Figure 2.2: Several genes were found to be expressed at varying levels in da neurons

(A) Examples of genes expressed at different levels in different classes of da neurons. Neuronal cell bodies and processes are labeled with the pan-neuronal marker HRP. Numbers in the lower panel indicate cell bodies of the respective classes of da neurons.

(B) Comparison of the expression levels of genes shown in A with those of transcription factors known to regulate dendrite morphogenesis in da neurons. Absence of + indicates lack of detectable expression.

Figure 2.3: A subset of genes were localized to da neuron dendrites

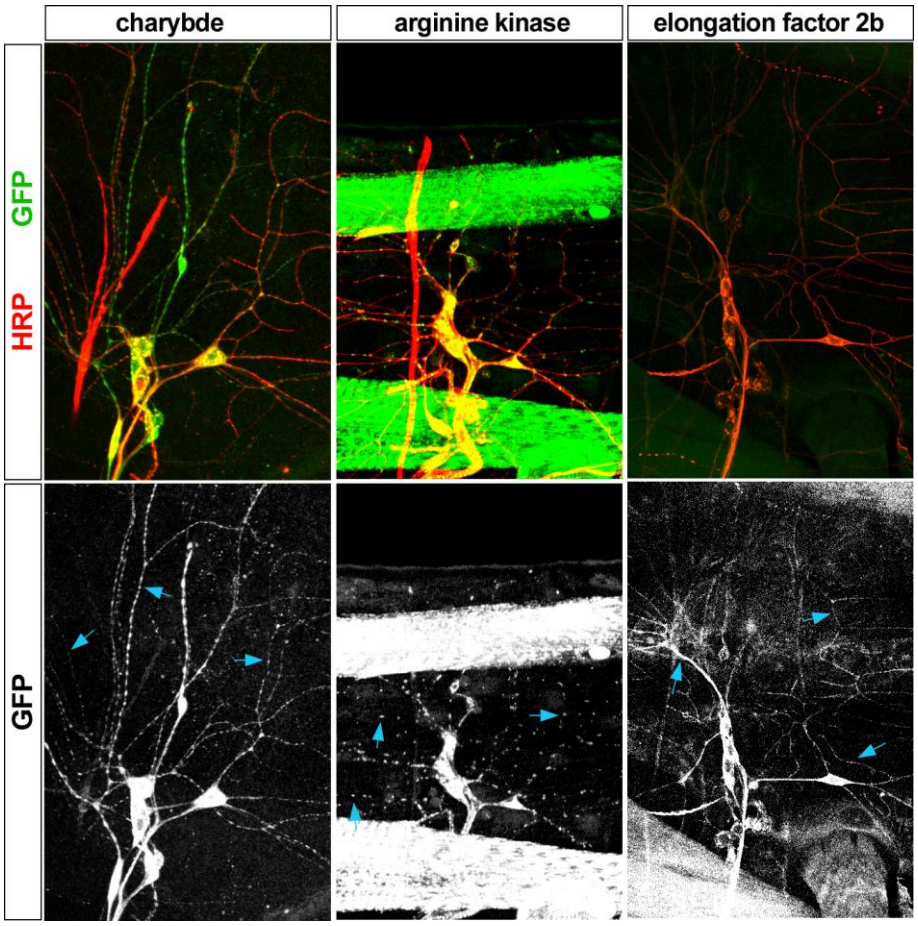


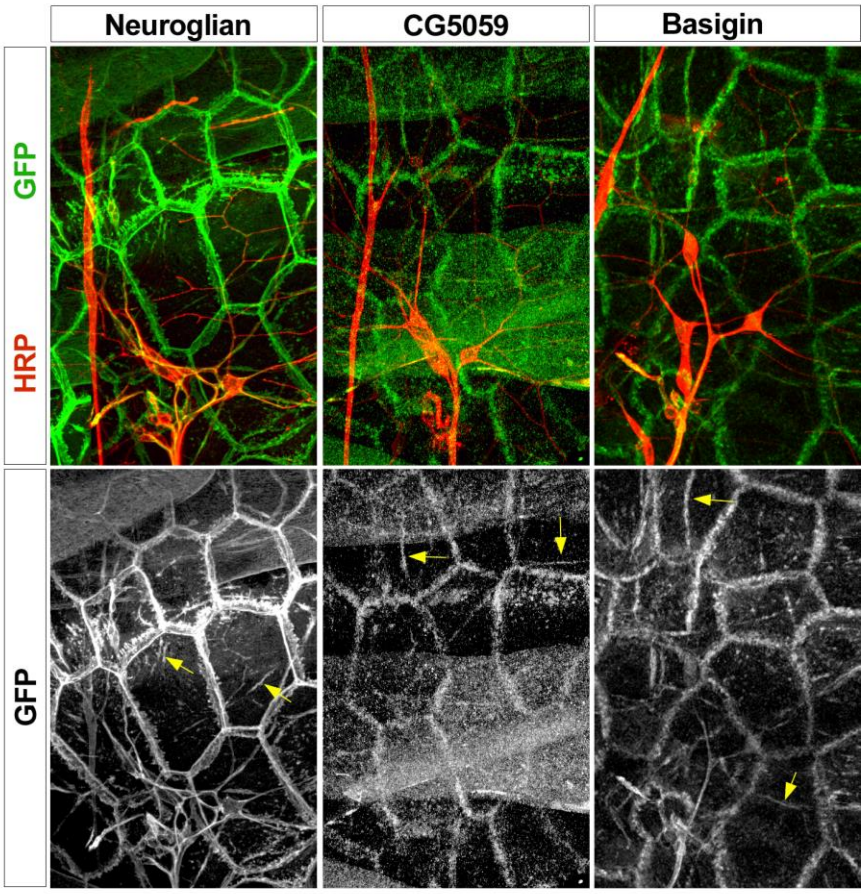
Figure 2.3: A subset of genes were localized to da neuron dendrites

Charybde had the strongest dendritic localization of all the lines we screened. Arginine kinase localization was highly punctate in dendrites of da neurons. Elongation factor 2B showed strong somatodendritic and axonal localization.

Blue arrows indicate dendritic localization.

Figure 2.4: Several proteins were found to be expressed in neurons as well as epithelial cells

A



B

Protein	Known septate junction protein?
Basigin	No
CG15296	No
CG3036	No
L(2)08717	No
Neurexin-IV	Yes
Neuroglian	Yes
Scribbled	Yes
Syndecan	No
Tropomodulin	No

Figure 2.4: Several proteins were found to be expressed in neurons as well as epithelial cells

(A) A group of genes were found to be expressed in both neurons and the epidermis, with localization to dendrites in neurons, and to cell borders and beneath dendrites in the epidermis. Expression and localization pattern of Neuroglian, CG5059 and Basigin are shown as examples. Yellow arrows indicate localization along the dendrites in the epidermis.

(B) List of proteins with expression and localization patterns as those shown in (A). Several proteins with previously unknown functions were found to share localization with septate junction proteins.

Figure 2.5: mustard expression is regulated by cut in class I neurons

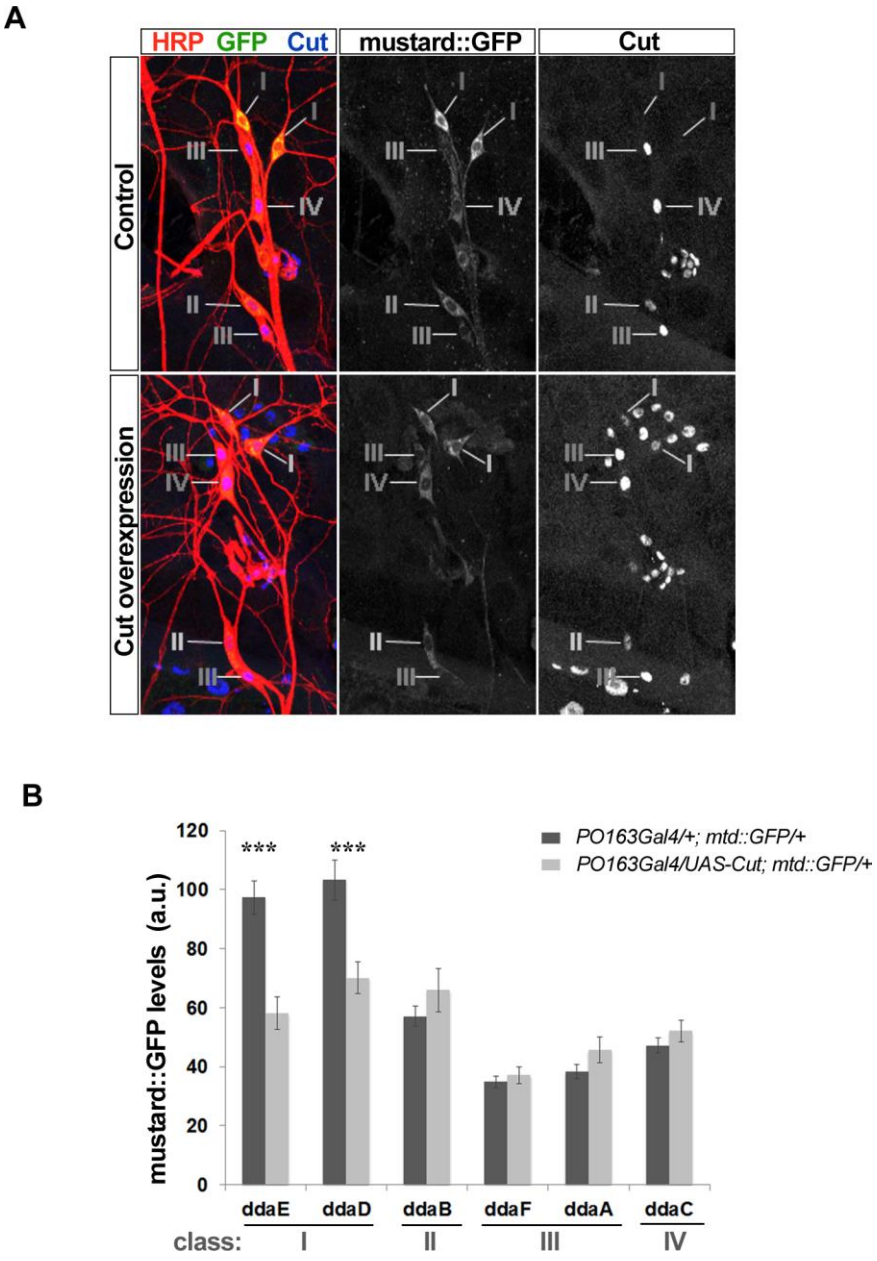


Figure 2.5: *mustard* expression is regulated by *cut* in class I neurons

(A) Mustard levels are high in class I da neurons and progressively lower in class II, III and IV neurons (top panel) in wild-type animals. Misexpression of Cut using the pan-sensory driver *PO163-GAL4*, verified by Cut antibody staining, led to decrease in Mustard levels (lower panel).

(B) Mustard::GFP levels were quantified by comparing GFP fluorescence intensity across animals processed together for immunostaining. Downregulation of Mustard::GFP level was observed in both of the class I neurons (ddaD, ddaE) examined but not in other neurons. ddaE: Control (97.29±5.75 a.u.), UAS-Cut (58.19±5.49 a.u.). ddaD: Control (103.25±6.77 a.u.), UAS-cut (70.10±5.37 a.u.), N≥15 neurons in each group. a.u.: arbitrary units of fluorescence, ***$p<0.001$. $p>0.05$ and N≥12 neurons for all other groups.

Figure 2.6: Jupiter is regulated by Cut and restricts branch tortuosity together with Futsch in class I da neurons.

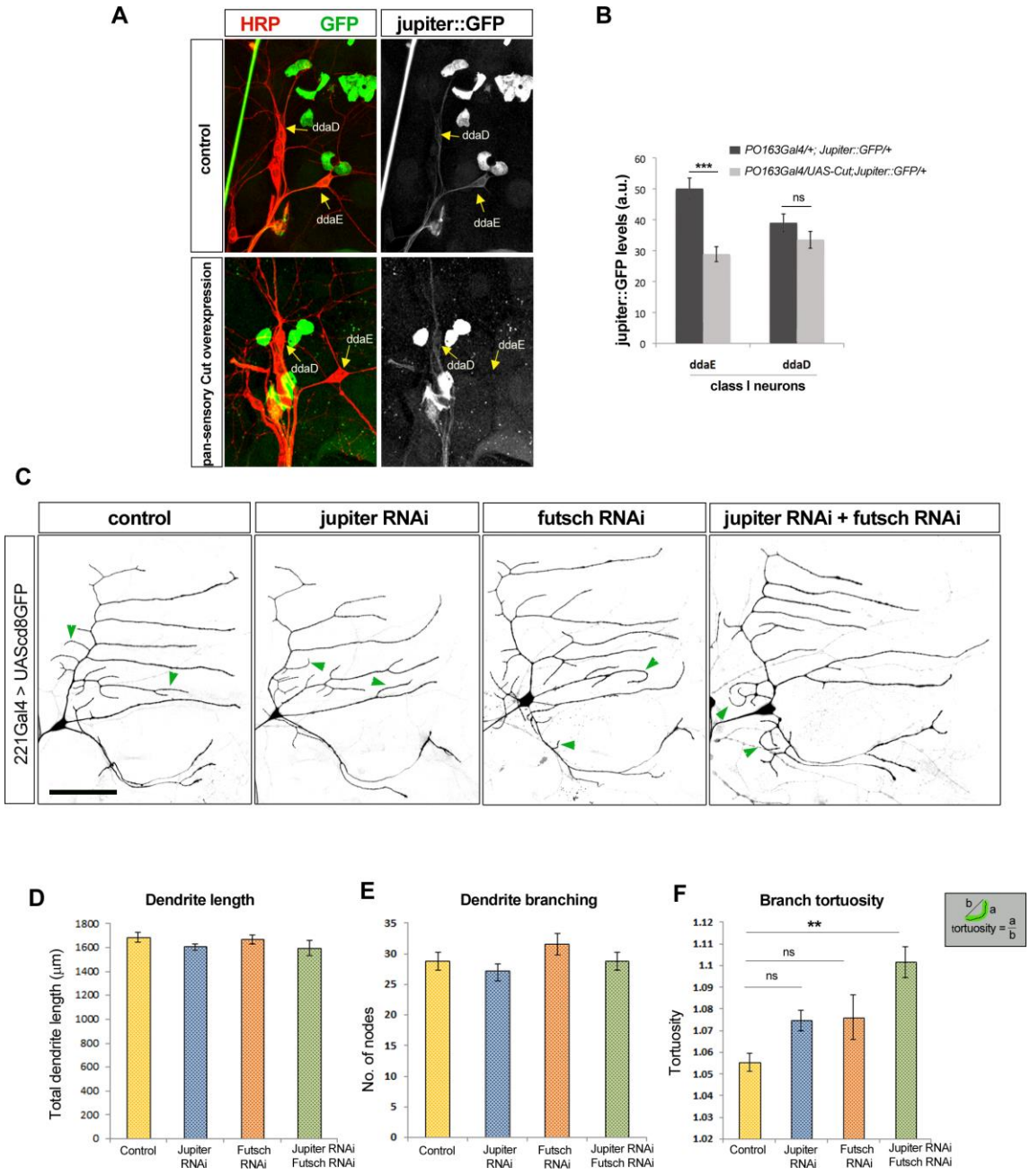


Figure 2.6: Jupiter is regulated by Cut and restricts branch tortuosity together with Futsch in class I da neurons.

(A) Jupiter is expressed only in class I da neurons, which are Cut-negative. Misexpression of Cut using the pan-sensory driver *PO163-GAL4* led to decrease in Jupiter levels. Yellow arrows indicate cell bodies of class I neurons.

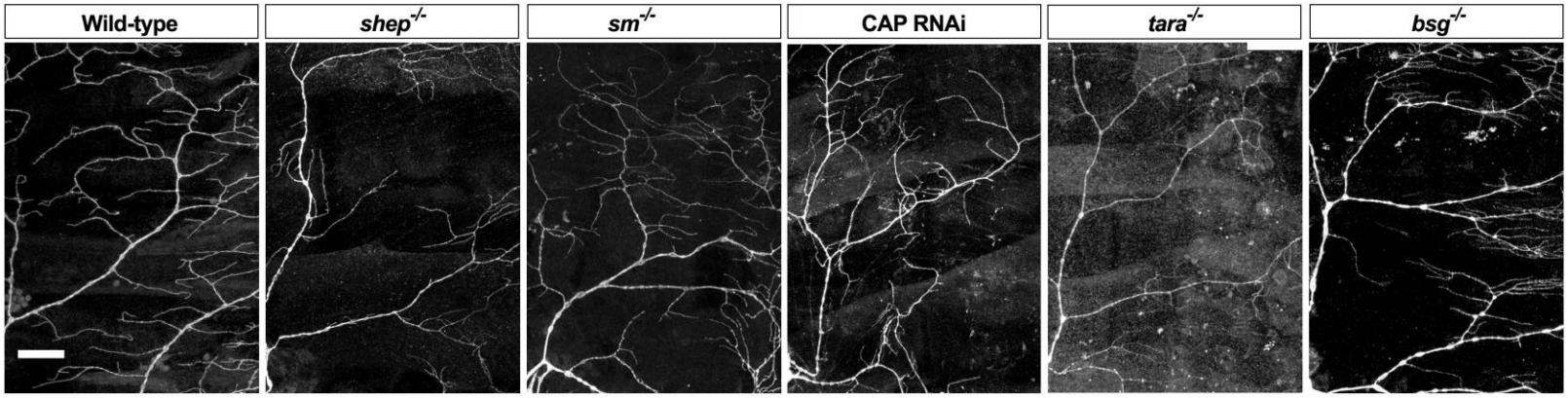
(B) Jupiter::GFP level was quantified by measuring GFP fluorescence in animals stained together with GFP antibody. ddaE: Control (50.04±3.45 a.u.), UAS-Cut (28.85±2.42 a.u.); ddaD: Control (38.97±2.89 a.u.), UAS-Cut (33.49±2.73 a.u.). N ≥ 10 neurons in each group.

Knockdown of Jupiter in class I neurons using the driver *221-GAL4* (C) did not affect dendrite length (D) and branching (E) but caused a trend towards increase in branch tortuosity (F). Knockdown of Jupiter as well as Futsch, a microtubule-binding protein normally expressed in all sensory neurons, led to a statistically significant increase in branch tortuosity in class I neurons (F). Control (1.05±.01), Futsch RNAi+Jupiter RNAi (1.10±0.01). N≥9 neurons in each group. One-Way Anova, F=6.95, p=0.0009. **Tukey HSD Test (control vs. Jupiter RNAi+Futsch RNAi) p<0.01.

Branch tortuosity was measured as the ratio of the actual length of the branch to that of a straight line between its two ends (inset in F). Green arrowheads in (C) indicate tortuous branches.

Figure 2.7: A secondary loss of function screen identified several candidate regulators of dendrite morphogenesis

A



B

	gene	reagent	phenotype
1)	argK	null	no phenotype
2)	bsg	null	disorganized branching (class IV dendrites
3)	CAP	RNAi	disorganized (class IV) or reduced (class I) branching
4)	eip63Ee	null	no phenotype
5)	ftz-f1	null	no phenotype
6)	jupiter	RNAi	class I branches more tortuous; regulated by Cut
7)	kis	RNAi	reduced coverage (class IV), increased dendritic field (class I)
8)	mtd	deficiency	no phenotype; regulated by Cut
9)	shep	null	reduced and disorganized branches
10)	smooth	null	no phenotype
11)	tara	null	reduced coverage (class IV)
12)	tsp42Ee	RNAi	some loss of dendritic coverage (class IV)

Figure 2.7: A secondary loss of function screen identified several candidate regulators of dendrite morphogenesis

(A) Images showing the results of loss of function analysis of various candidate genes by the MARCM approach, except for CAP, which was analyzed by RNAi. Each panel shows the dorsal posterior quadrant of wild-type or mutant class IV neuron as indicated. Dendrite morphogenesis defects were observed upon loss or knock down of *shep*, *CAP*, *tara*, and *bsg*. Scale bar, 25 μm.

(B) Summary of secondary loss of function screen. 'null' indicates that a null allele was used to assess dendrite phenotypes by the MARCM approach. RNAi lines were used for *CAP*, *jupiter*, *kismet*, and *tsp42Ee*. Requirement of *mtd* for dendrite development was assessed using a line with deletion spanning the *mtd* locus.

CHAPTER III: Role of Basigin in dendrite morphogenesis

Abstract

Our protein trap screen uncovered that the Ig superfamily member Basigin is expressed in da neurons as well as their substrate epidermal cells in the *Drosophila* larval peripheral nervous system, which we verified using antibodies against Basigin. Basigin is also enriched in neurons in the vertebrate brain, but its role in neuronal morphogenesis has not been described to date in any model system. Taking our expression data together with previously described roles of Basigin in matrix metalloproteinase induction and organization of cellular cytoskeleton, we hypothesized that Basigin mediates dendrite-substrate interactions important for dendrite morphogenesis in da neurons. Loss of function analysis by the MARCM approach showed that Basigin promotes elaboration of dendritic arbors during development and thus contributes to adequate coverage of the dendritic field of class IV neurons. Interestingly, RNAi-mediated knockdown of Basigin in the epidermis also resulted in defects in class IV dendrite morphogenesis without any overt defects in epithelial cell size and localization of several cell junction proteins. These effects are specific to class IV neurons, as class I neurons were found to be unaffected by loss of Basigin in both cases. Basigin has been shown previously to be capable of homophilic binding and has been implicated in mediating intercellular interactions. Taken together, our findings support a model in which Basigin on the surfaces of epithelial cells and dendrites engage in homophilic interactions and likely mediate transfer of growth-promoting signal from the epidermis to neurons. Our findings have important implications for non-cell-autonomous regulation of neuronal morphology and provide new insights into the role of Basigin in neuronal morphogenesis and function.

Introduction

A large body of evidence indicates that a complex interplay of cell-intrinsic factors spanning a wide range of functional categories shape the dendritic arbors of neurons (Corty et al., 2009; Jan and Jan, 2010). However, neurons must also interact with their extracellular environment not only to target dendrites to specific regions in their vicinity or to find the right presynaptic partners, but also to adjust arbor morphogenesis based on changes that occur in the size, molecular composition and other physical attributes of their surroundings. A growing number of studies have shed light on the mechanisms by which interactions between neurons that are synaptic partners or homotypic neighbors influence morphogenesis of dendrites. For example, evidence exists for modulation of dendrite development by presynaptic neurons (Cline and Haas, 2008; Kohara et al., 2003; Tripodi et al., 2008) as well as for repulsive interactions that define dendritic boundaries between neurons (Grueber et al., 2002; Grueber and Sagasti, 2010). Additionally, effect of glial cells on morphogenesis of dendrites has been demonstrated in both invertebrate and vertebrate models (Buard et al., 2010; Lein et al., 2002; McAllister, 2000; Procko and Shaham, 2010). These examples underscore the critical contribution of extrinsic factors in conferring stereotyped shapes to neuronal dendritic arbors. However, our understanding of the contribution of neuronal interactions with molecules in the substrate and with non-neuronal cells on dendrite morphogenesis is relatively poor. Evidence from studies on neurogenesis and migrating neurons indicates that Integrin is an important mediator of interactions between neurons and the ECM (Schmid and Anton, 2003; Tate et al., 2004). However, loss of Integrin has been found to result primarily in defects in maintenance of dendrites once they are formed (Kerrisk et al., 2013; Kim et al., 2012), so it is unclear how interactions that may occur during development of dendrites, when critical signaling to neurons

may be necessary to regulate the extent of dendrite coverage or the size of dendritic field, is mediated. A recent study has shown that coordination between increase in dendritic field size and growth of the underlying epidermis is mediated via the microRNA *bantam* in epithelial cells and *akt* in neurons in the *Drosophila* larval PNS (Parrish et al., 2009). However, the molecular mechanism by which communication between epithelial cells and neurons is accomplished is unknown. Molecular interactions between neurons and substrate cells may constitute an important mechanism by which neurons are able to take various environmental variables into account during morphogenesis of dendritic arbors.

In the previous chapter, I described our discovery of neuronal and epidermal expression of several proteins that may be directly relevant for addressing this gap in our knowledge of neuron-substrate interaction. Among the molecules we identified, the immunoglobulin superfamily member Basigin is a strong candidate mediator of such interactions based on its expression, structure, evolutionary conservation, and putative molecular function. We found that Basigin is localized to dendrites in da neurons and also shows intermittent localization beneath dendrites along the epidermis. In addition, Basigin has been implicated in cell-cell interactions, modulation of the ECM, and organization of cellular cytoskeleton in various biological contexts (reviewed in Chapter I). In this chapter, I describe experiments I carried out to determine the role of Basigin in morphogenesis of da neuron dendrites. My data reveal cell-autonomous and non-cell-autonomous requirement of Basigin in dendrite morphogenesis of class IV neurons, but not class I neurons, and suggest that Basigin may mediate communication between epithelial cells and neurons that is important for promoting growth of class IV dendrites to ensure appropriate coverage of the larval body wall.

Methods

Loss of function analysis

Loss of function analysis of Basigin was done by two approaches. First, requirement of Basigin in da sensory neurons was assessed by creating *basigin* mutant clones in an otherwise heterozygous animal using the MARCM technique as described in chapter II. Briefly, flies of the appropriate genotype (1) w^{1118}*, hsflp, elav-GAL4, UAS-mcd8::GFP; FRT40A, GAL80*, 2) w^{1118}*; FRT40A, FRT42D / CyO-GFP*$_{nuclear}$, and 3) w^{1118}*;* $bsg^{\delta 265}$*, FRT40A / CyO-GFP*$_{nuclear}$) were crossed and put in a collection chamber. On the day of embryo collection, flies were allowed to lay eggs on a grape agar plate with fresh yeast paste for 3 hours at 25 °C. The embryos were kept at 25 °C for an additional 3.5 hours before they were heat-shocked as follows: 30 min at 38 °C, 30 min at room temperature, and 45 min at 38 °C. The embryos were then kept in a humidified chamber at 25 °C. Larvae containing GFP-positive mosaic clones in the larval PNS were selected under a fluorescence microscope at the appropriate age (72 hrs or 96 hrs after egg-laying), then dissected and immunostained as described in chapter II.

Second, requirement of Basigin in the epidermis was investigated by expressing RNAi transgenes against *basigin* (Vienna Drosophila RNAi Center, stock # 105293) (Dietzl et al., 2007) specifically in epidermal cells using the 871-GAL4 driver. Larvae were selected, dissected and immunostained as described previously.

Antibodies

The following primary antibodies were used: α-GFP (1:500), α-HRP (1:250), α-Coracle (1:50), α-dsRed (1:250), α-βPS Integrin (1:50), and α-dECad (1:20). Respective secondary antibodies were used at 1:250 dilution. All primary and secondary antibody incubations were done

overnight at 4 °C and immunostained animals were washed in PBS containing 0.03% Triton X-100 (Sigma) at room temperature.

Image acquisition and analysis

Images of neurons were acquired using a confocal microscope and processed using the LSM 510 software package (Carl Zeiss) and Adobe Photoshop (Adobe Systems Incorporated). Morphometric data on neurons was obtained by first tracing all dendrites using Neurolucida (MBF Biosciences) and processing the traced images using Neurolucida Explorer (MBF Biosciences). Analysis of coverage density (CD) of dendritic arbors was done by overlaying a grid of squares over the dendritic field (Appendix 2). The size of the square used varied in proportion to the size of dendritic field and was calculated using the formula: $square\ area = \sqrt{(dendritic\ field\ area}$). The number of squares that contained dendrite was counted and coverage density was calculated as the ratio of the number of such squares over the total number of squares covering the dendritic field (Appendix 2)

$$(CD = \frac{\#\ of\ squares\ containing\ dendrites}{total\ \#\ of\ squares\ covering\ the\ dendritic\ field}).$$

Statistical analysis

Data were analyzed using the R statistical software package (R Core Team, 2012). Normality of data within each group was evaluated using the Shapiro-Wilk test. Student's T-test and Wilcoxon Rank Sum test were used for parametric and non-parametric comparison, respectively, as appropriate of various groups. Data are presented as mean ± standard error of mean (SEM).

Results

Basigin is expressed in the larval PNS, epidermis, NMJ and the CNS

We examined the expression and localization patterns of GFP-tagged endogenous Basigin protein (Basigin::GFP) in 3rd instar larvae by staining with anti-GFP antibodies. Basigin::GFP was found to be highly enriched at epidermal cell borders and the NMJ (Fig. 3.1 A, B). Expression in the CNS was found to be high in some regions (e.g., photoreceptors) but relatively weak in most areas (Fig. 3.1 A). Basigin was also found to be expressed in all classes of da neurons with localization in the cell bodies, dendrites and axons. Furthermore, we observed intermittent enrichment of Basigin beneath some dendrites along the epidermis (Fig. 3.1 A). Given the similarity of this subcellular localization to that of the septate junction proteins described in Chapter II, we sought to examine whether dendritic segments with epidermal Basigin enrichment were also positioned more apically towards the cuticle. We stained Bsg::GFP animals with both GFP and Coracle. We found that Bsg::GFP localization at these regions of intermittent epidermal enrichment along dendrites overlapped completely with that of Coracle (Fig. 3.1 A). To further confirm that these regions are embedded within epidermal cells, as suggested by co-enrichment of Coracle, we conducted surface labeling with anti-HRP in the absence of detergents. Anti-HRP labeling at sites of epidermal Bsg enrichment beneath dendrites was weaker than at sites without underlying epidermal enrichment (data not shown), suggesting that the former sites are indeed embedded within epidermal cells.

Although localization of Basigin to the growing outer edge of tumor cells and to apposing surfaces of cells has been reported before (Curtin et al., 2005; Le Floch et al., 2011), localization to epidermal cell borders has not been described to date in any model system. Adding a tag as

large as the GFP molecule inside a protein may prevent it from being localized properly either due to disruption of a critical localization signal or problems with protein folding. To examine the possibility that enrichment of Basigin in the epidermis along its borders and beneath da neuron dendrites may be a result of Bsg::GFP mislocalization, we stained wild-type larvae with Basigin antibody. Consistent with our Bsg::GFP localization data, we found that endogenous untagged Basigin also localized to epidermal cell borders and underneath some da neuron dendrites (Fig. 3.1 D). However, this localization pattern was seen only when Basigin antibody was applied prior to fixation of animals with paraformaldehyde. Immunostaining larvae with the standard protocol of first fixing, then rinsing and incubating in primary antibodies did not show this localization pattern (Fig. 3.1 C). This may be because the epidermis expresses a different Basigin isoform compared to neurons and the epidermal basigin may undergo conformational changes upon fixation, making it harder for the antibody probes to access the right epitope. Using the antibody with our standard immunostaining protocol, we were able to confirm enrichment of Basigin in the NMJ and notable dendritic localization of Basigin in all da neurons (Fig. 3.1 C). Because epidermal Basigin was not labeled under this condition, it afforded us a clearer view of the dendritic localization of Basigin.

Taken together, these data indicate that Basigin is expressed in da neurons with localization to dendrites, cell bodies and axons. In addition, Basigin is enriched in the epidermis along cell borders and intermittently around dendrites that are embedded in the epidermal cell, as revealed by overlap with Coracle and surface labeling with anti-HRP.

Basigin is required for proper morphogenesis of class IV da neuron dendrites

Based on the expression patterns of Basigin described above and evidence suggesting its role in cytoskeletal organization of cells and ECM remodeling in different biological contexts, we hypothesized that Basigin is required for proper morphogenesis of da neuron dendrites. We used an embryonic lethal allele of Basigin ($bsg^{\delta 265}$) generated and described in a previous study (Curtin et al., 2005) to create mosaic null clones of da neurons by the MARCM approach (Lee and Luo, 1999). Since enrichment of epidermal Basigin was mostly observed under dendrites of class IV neurons, we focused first on analyzing the role of Basigin in morphogenesis of class IV dendrites.

Class IV neurons normally develop highly elaborate dendritic arbors, resulting in complete coverage of the dendritic field they innervate. Loss of Basigin led to reduction of dendrite branching and consequent decrease in coverage of the dendritic field (Fig. 3.2). Unlike in wild-type neurons, large gaps devoid of dendrites were observed within the dendritic field of mutant neurons (Fig. 3.2 B). Indeed, quantitative morphometric analysis of dendritic arbors revealed that mutant class IV neurons had 26% reduction in total dendrite length and 24% decrease in the number of branches (nodes) compared to wild-type neurons (Fig. 3.2 C). As a result of these defects, mutant neurons innervate their dendritic fields 14% less than wild-type neurons (Fig. 3.2 C). These results show that Basigin has an important role in promoting proper morphogenesis of the elaborate dendritic arbors of class IV neurons.

Dendrite morphogenesis of class I neurons are unaffected by loss of Basigin

Expression data obtained from both the Bsg::GFP line and antibody staining of wild-type animals indicate that Basigin is expressed in all da neurons (Fig. 3.1 A). Therefore, we next sought to examine whether requirement of Basigin for dendrite morphogenesis also extends to other da neurons. Contrary to class IV dendrites, dendrites of class I neurons are very simple, with far fewer dendritic branches that cover a much smaller dendritic field. Class I neurons differ significantly from class IV neurons in the way their branches are elaborated and in their cytoskeletal composition. Hence, we decided to assess the requirement of Basigin in class I neurons for development of its dendrites. Mutant clones of class I neurons were generated using the MARCM approach as we did for class IV neurons. Surprisingly, dendrites of class I neurons were unaffected by loss of Basigin. Mutant neurons developed dendritic arbors that had the same asymmetry and simplicity in branching that is characteristic of wild-type class I neurons (Fig. 3.3 A). Quantitative analysis showed that both the number of branches and total dendrite length in mutant neurons were statistically identical to those of wild-type class I neurons (Fig. 3.3 B). Thus, our data reveal a requirement of Basigin for dendrite morphogenesis of the complex space-filling dendrites of class IV da neurons, and not of the relatively simple arbors of class I neurons.

Dendrite morphogenesis defects in Basigin mutant class IV neurons arise during development

Dendrites of da neurons first appear approximately 14 hours after egg-laying (AEL) (Grueber et al., 2003b) and continue to grow as the larva increases in size from the 1st through 3rd instar stages (Parrish et al., 2009). In order to achieve complete coverage of the larval body wall, class IV dendrites need to not only grow peripherally to maintain tiling with neighboring neurons as the animal grows, but also continue to add branches within the dendritic tree to maintain

appropriate coverage as their substrate (epidermal cell) expands in size (Parrish et al., 2009). In addition, branches also have to be maintained to prevent their loss as the larva grows. Reduction in dendrite branching at the late third instar stage described above may thus result from defects in developmental elaboration of dendrites at intermediate states, or defects in branch maintenance at the third instar stage.

In order to distinguish between these two possibilities, we next sought to compare the dendritic trees of wild-type and mutant class IV neurons at an earlier developmental stage. The null allele $bsg^{\delta 265}$ is homozygous lethal and the majority of homozygous animals die as embryos. About 10% of them hatch and become first instar larvae but are very sickly and die soon afterwards (Curtin et al., 2007). Therefore, we decided to use the same MARCM approach of creating mosaic null clones of sensory neurons we did above to assess dendrite morphology at intermediate stages. We chose the end of 2nd instar stage (72 hrs AEL) for this analysis because class IV neurons grow significantly from this stage to the late third instar stage (96 hrs AEL), and they are labeled sufficiently strongly with GFP compared to the beginning of 2nd instar stage (48 hrs AEL) for reliable assessment of dendrite morphology. In addition, examination of mutant class IV neuronal clones at much earlier stages may be confounded by perdurance of the Basigin protein in the mosaic clones.

We found that mutant class IV neurons had defective dendrite branching even at the late 2nd instar stage (Fig. 3.4). Significant decrease in dendrite length and number of branches were observed in the mutant neurons compared to wild-type neurons (Fig. 3.4 B). This suggests that Basigin is required for developmental elaboration of dendritic arbors of class IV neurons. Although our data does not necessarily rule out a contribution of maintenance defects at late

stages, our results suggest a strong contribution of defects in addition of branches as the animal grows in size.

One interpretation of this result is that branch elaboration is stalled at an intermediate stage in mutant neurons, resulting in neurons with fewer dendrites and poorer coverage of the dendritic field at the late 3rd instar stage. If this is the case, dendrite branching at the late 2nd and late 3rd instar stages should be comparable in the mutants, while branching should increase commensurate with expansion of epidermal cells between these two stages in wild-type neurons. Parrish et al. (2009) conducted a careful time-course analysis of increase in epidermal cell size throughout larval development. Their data show a significant increase in epithelial cell area between 2nd and 3rd instar stages (Parrish et al., 2009). In the data that we obtained, comparison of dendrite branching between late 2nd and late 3rd instar stages in wild-type neurons showed ~30% increase in branching (Fig. 3.4 C). Interestingly, about 42% increase in dendrite branching between these stages is observed in mutant neurons, indicating that dendrite growth is not stalled at a premature stage (Fig. 3.4 C). Thus, Basigin mutant neurons carry out scaling growth of their dendritic arbors with increase in the body size of the larvae, just as wild-type neurons do. It is noteworthy that increase in dendrite branching between the late 2nd and late 3rd instar stages is slightly higher in mutant neurons than in wild-type neurons. Correspondingly, the extent of reduction in branching in mutant neurons relative to wild-type neurons at the late 3rd third instar stage (~27%) is lower than that at the late 2nd instar stage (~34%).

Thus, our data show that reduction of dendritic branching and coverage in Basigin mutant class IV neurons arises early during development. While our experiments do not directly address whether defects in branch maintenance at late stages contribute to the observed reduction in dendrite branching and coverage in mutant neurons, our results indicate a strong contribution of

defect in developmental elaboration at least up to the late 2nd instar stage. Interestingly, our results also suggest compensatory over-branching between the late 3rd instar and late 2nd instar stages which narrows the gap between the extent of branching in wild-type and mutant neurons by the late 3rd instar stage. Future studies with more detailed time-course analysis and comparison of dendrite turnover at early vs. late stages should address this.

Knock down of epidermal Basigin specifically affects class IV dendrite morphogenesis

An intriguing aspect of the expression pattern of Basigin is that in addition to being expressed in da neurons, it is also expressed in the underlying epithelial cells. Electron micrographs of the larval body wall have revealed that da neuron dendrites grow largely along the basal surface of the underlying epidermis, sandwiched between the basement membrane, a specialized ECM layer, and the basal epidermal membrane (Han et al., 2012; Kim et al., 2012). In addition, a subset of dendrites invaginates into the epidermis and remains surrounded by epidermal membranes (Han et al., 2012; Kim et al., 2012). In both contexts, the dendritic surface is exposed directly to the epidermal membrane, which may permit molecular interaction between them. Expression of Basigin in the epidermis as well as da neurons may thus reflect direct intercellular interaction mediated by Basigin. Alternatively, Basigin on the epidermal cells may influence the composition or breakdown of the basement membrane, to which a large majority of da neuron dendrites are exposed. In either scenario, epidermal Basigin may have significant influence on the development of da neuron dendrites.

In order to directly examine whether epidermal Basigin is required for dendrite morphogenesis of da neurons, we knocked down Basigin specifically in the epidermis using the RNAi approach.

Expression of RNAi transgenes against *basigin* was driven using an epidermis-specific GAL4 line (871-GAL4) while class IV neurons were labeled with GFP under the control of the *ppk* promoter (Fig. 3.5). Interestingly, knock down of Basigin specifically in the epidermis resulted in severe defects in morphogenesis of class IV dendrites. Quantitative analysis revealed significant reduction (10%) in dendritic field coverage of class IV neurons in animals in which *basigin* was knocked down in the epidermis compared to those in control animals (Fig. 3.5). This phenotype is reminiscent of and comparable in magnitude to the decrease in dendritic field coverage observed in $bsg^{-/-}$ class IV neurons (14%) described earlier (Fig. 3.2).

Our experiments had revealed a requirement of Basigin for proper dendrite morphogenesis in class IV neurons but not in class I neurons. Therefore, we next asked the question whether epidermal requirement of Basigin for proper dendrite development is applicable to class I neurons. Morphology of class I neurons were compared between animals in which Basigin was knocked down in the epidermis and control animals (Fig. 3.6). Interestingly, we did not find any significant change in the total length and number of branches of class I dendrites when expression of Basigin in the epidermis was reduced (Fig. 3.6 B). Thus, Basigin in the epidermis influences morphogenesis of class IV neurons, but not class I neurons.

Considering the strong enrichment of Basigin in epidermal cell borders, one explanation for the observed dendrite phenotypes of class IV neurons upon knockdown of epidermal Basigin is that the effects on dendrites are secondary to changes that occur in epidermal cell size, shape or overall health. To find out if this is the case, we knocked down Basigin specifically in the epidermis as described above and compared the size of epidermal cell in these animals with that in control animals. We did not find any significant difference in average epidermal cell size (Fig. 3.7). Likewise, no defect was found in the localization of epidermal proteins such as

Coracle, dE-cadherin and βPS integrin (Fig. 3.7 A, B). Notably, localization of βPS integrin along dendrites of da neurons was also unaffected (yellow arrows in Fig. 3.7 B). Thus, our results indicate a requirement for epidermal Basigin without any overt effects on the epidermis itself. Our findings do not necessarily rule out any role for Basigin in the epidermis, however. It is important to note that for technical reasons, we had to use the RNAi approach to knock down Basigin in the epidermis. This approach doesn't result in complete knock down of a gene's function, so it is possible that complete loss of Basigin from the epidermis would cause severe defects in epidermal cells that we are unable to see via the RNAi approach.

Nevertheless, combined with our neuronal loss of function experiments, these results show that Basigin is required both cell-autonomously and non-cell-autonomously for proper morphogenesis of class IV da neuron dendrites.

Knock down of Basigin in both epidermis and neurons leads to dendrite morphogenesis defects

So far, I have described results of experiments in which Basigin was removed or knocked down only in the neuron or epidermis. We next examined the effect of knocking down Basigin in both epithelial cells and neurons at the same time.

We drove expression of an RNAi transgene against *basigin* in both neurons and epithelial cells using the driver *arm-GAL4* (Fig. 3.8). This GAL4 line has been described in a previous study to drive expression only in the epidermis (Parrish et al., 2009). However, we found that it also drives expression in da neurons, as revealed by GFP expression in both neurons and the epidermis in *arm-GAL4, UAS-cd8GFP* larvae, thus making it suitable for our experiment (data not shown).

We observed that, similar to previous results, knocking down Basigin in both neurons and epidermis caused statistically significant decrease (19%) in dendrite coverage of class IV neurons (Fig. 3.8). One interpretation of this result, taken together with loss of function data described before, is that Basigin is expressed in neurons and the substrate epidermal cells at levels that provide requisite level of signaling between the epidermis and dendrites. Decrease in Basigin level in either cell or both cells reduces this signaling, causing neuronal dendrite morphogenesis defects. Our observed similarity in the level of reduction of dendritic coverage relative to the respective controls when Basigin was knocked down or lost from neurons alone (14%), epidermis alone (10%), or from both neurons and epidermis (19%), supports this idea.

Discussion

Despite evidence indicating expression of Basigin in neurons in the vertebrate nervous system, our understanding of the function of Basigin in neurons remains largely unknown. Existing literature indicate that at very high levels, such as that which occurs in tumor cells in vertebrates, Basigin is capable of inducing the secretion of matrix metalloproteinases, which mediate ECM breakdown. Studies in insect cell lines and the fly NMJ have shown that Basigin modulates intracellular cytoskeleton organization. In addition, Basigin has been shown to interact with cell surface molecules with known roles in neuronal morphogenesis, such as integrins, and is capable of homophilic binding. Taken together, these data suggest that Basigin may be involved in neuronal morphogenesis, which hasn't been investigated to date in any model system.

In this chapter, I have described the expression pattern of Basigin, an Ig superfamily member, in fly larvae as revealed by endogenously GFP-tagged Basigin and validated using an anti-Basigin

antibody. I have also described the experiments we conducted to investigate its role in dendrite morphogenesis of da neurons. Briefly, we found Basigin to be expressed in and localized to the cell bodies, dendrites and axons of all da neurons. In addition, Basigin is also expressed in the underlying epidermal cells. Loss of function analysis and RNAi mediated knock down of Basigin show that Basigin is required in both the neurons and the epidermis for proper morphogenesis of dendrites of class IV neurons. Interestingly, dendrites of class I neurons are not affected by loss of Basigin. Our results reveal a potentially neuron-type specific requirement of Basigin for proper dendrite morphogenesis and suggest differences among da neurons in their interactions with the ECM and substrate epidermal cells.

Basigin is expressed in the larval PNS, epidermis and the CNS

Basigin has been reported previously to be expressed in apposing membranes of early embryonic fly tissues (Curtin et al., 2005) and the fly NMJ (Besse et al., 2007). In addition, expression of Basigin in the the fly eye can be inferred based on loss of function phenotypes (Curtin et al., 2005). Here, we have found that Basigin is expressed in neurons in the fly larval PNS and throughout the CNS with particularly strong expression in the photoreceptors and strong localization at the presynaptic endings of motor neurons. Interestingly, Basigin is also expressed highly in the epidermis. Dendrites of da neurons grow in close physical contact with the epidermis as well as the ECM they secrete. Thus, expression of Basigin on both the dendrites and the epidermis has important implications for a potential role in mediating extrinsic regulation of dendrite morphogenesis. More specifically, when taken together with the fact that it is a cell surface molecule with extracellular Ig domains, the expression data suggests that Basigin may mediate intercellular interaction between the epidermis and dendrites. In addition, considering

the known capacity of Basigin to influence the ECM by inducing MMP secretion, Basigin may also regulate dendrite morphogenesis via changes in the ECM around da neurons.

GFP-tagged proteins can mislocalize if the site of GFP insertion contains a localization signal or if the GFP peptide alters the secondary structure of the protein. I found that the protein trap line (CA06978) we used contains GFP insertion immediately next to the signal peptide sequence at the N-terminus and well before the Ig domains. Insertion of GFP at this site is unlikely to disrupt Basigin function, as Besse et al. found that a Basigin overexpression construct containing GFP tag at the same site[2] can rescue lethality and NMJ morphogenesis defects in trans-heterozygotes of two hypomorphic lines (Besse et al., 2007). Nevertheless, we verified using anti-Basigin antibody in wild-type animals that the expression and localization patterns of Basigin are indeed faithfully reported by GFP-tagged Basigin in the protein trap lines.

Cell-autonomous and non-cell-autonomous functions of Basigin in class IV dendrite morphogenesis

Our loss of function and knock down experiments show that Basigin is required both cell-autonomously and non-cell-autonomously for class IV dendrite morphogenesis. Although several other molecules with Ig domains (e.g., Turtle, Dscam) have been reported to influence dendrite morphogenesis of da neurons, none have been shown to be required in the epidermis as well. Thus, our findings are the first to date of requirement of a molecule both in the neuron as well as the substrate epidermal cells for dendrite development in da neurons.

[2] The GFP exon in the Bsg::GFP line we examined (CA06978, Flybase ID: FBti0099720) has been reported to be inserted at position 2L:8104393(+) in the *Drosophila* genome (Flybase version FB2013_03, released May 7, 2013). This site lies in the intron between exons 10 and 11 in the *Basigin* locus. The amino acid residue 'D' at position 25 in the short isoform of Basigin spans these two exons, so it is unclear whether the GFP tag lies immediately before or after this residue in this Bsg::GFP line. For the purposes of this thesis report, I have chosen to interpret that the GFP peptide comes after the 'D' residue in the tagged protein. Besse et al. (2007) also reported that GFP tags in the Bsg::GFP line they used as well as the GFP-tagged Basigin overexpression construct they made lie immediately after the amino acid residue 'D' at position 25.

Notably, the dendrite defects we observed upon knockdown of epidermal Basigin occurred in the absence of overt morphological defects in the epithelial cells. For instance, no significant difference was observed in the size of epithelial cells. Likewise, localization of several different types of epithelial cell junction proteins, including Coracle, dE-Cadherin, and βPS-integrin were observed to be identical to those in control animals. These observations suggest that more subtle changes either at the level of direct signaling by Basigin or in the ECM may lead to dendrite morphogenesis defects of class IV neurons.

Consistent with this idea, one explanation for our results is that Basigin on the epidermal surface and dendrites engage in homophilic interaction. Evidence supporting the ability of fly Basigin for homophilic interaction comes from a study in which GFP-tagged Basigin was overexpressed in *Drosophila* S2 cells (Besse et al., 2007). While expression of GFP alone didn't alter the growth of S2 cells in culture, expression of GFP-tagged Basigin led to significant cell aggregation. Furthermore, application of peptides consisting only of the extracellular domain of Basigin to human uterine fibroblast cell cultures revealed the capacity of the truncated peptide to bind full-length Basigin on the surface of the fibroblasts (Belton et al., 2008). Yu et al. (2008) examined the crystal structure of the soluble extracellular portion of human Basigin and found that it forms homo-oligomers, which they also verified by polyacrylamide gel electrophoresis (PAGE). Taken together, these findings suggest that the capacity of Basigin for homophilic binding via its extracellular Ig domains is conserved from flies to humans and may be a critical part of its function.

One possible requirement for homophilic binding of Basigin between dendrites and epidermal cells is that the two cell surfaces have to lie in close proximity of each other. Indeed, electron micrographs of the larval body wall have shown that da neuron dendrites lie close to the

epidermal cell surface. While a subset of class IV dendrites remain embedded in epidermal cells, where the dendritic surface is surrounded by and in close contact with epidermal membranes, others remain in contact with the basal surface of the epidermis on one side and the basement membrane in the other (Kim et al., 2012). Such close physical association of the dendritic surface with epidermal cell surface is consistent with the idea of homophilic interaction in *trans* between the two cell surfaces. While we favor the regions of dendrites enclosed by the epidermis being the primary sites of such homophilic interactions, we do not rule out the possibility that such interactions may occur, albeit at a lower level, in non-enclosed dendrites that lie at the ECM-epidermis interface.

One way to determine if epidermal Basigin and neuronal Basigin do indeed bind homophilically would be to drive expression of Basigin with different tags in neurons and the epidermis and conduct an immunoprecipitation assay to check if an antibody against one tag pulls down proteins with the other tag. This would require very specific expression of the two tagged Basigin variants in the neurons and the epidermis. Our current knowledge of neuron-specific and epidermis-specific enhancers (e.g., *ppk*-GAL4 and 871-GAL4, respectively) in combination with the availability of two different binary inducible systems—GAL4/UAS and LexA/Op—should allow this experiment to be done in flies. As a complement to this approach, the split-GFP system, which is based on the expression of two fragments of the GFP protein that emits GFP signal only when the fragments are in close proximity of each other, can also be utilized. This approach would involve using two different binary inducible systems and specific enhancers as described above to drive two GFP fragments specifically in the epidermis and neurons. Observation of GFP signal along da neuron dendrites would strongly support our model of homophilic Basigin binding at the dendrite-epidermis interface. Although initial efforts at using

the split-GFP system to assess protein interactions *in vivo* suffered from the problem of instability and increased susceptibility of tagged proteins to aggregation, recent advancements in the development of split-superpositive GFP variants that are resistant to aggregation (Blakeley et al., 2012) offer a promising solution to the limitation of this approach.

Requirement of Basigin for dendrite morphogenesis in class IV neurons, but not class I neurons

Data obtained using the Bsg::GFP protein trap line as well as α-Basigin antibodies indicate that Basigin is expressed in and localized to dendrites of all da neurons. However, loss of function analysis indicated that Basigin has functional roles in dendrite morphogenesis of class IV neurons but not of class I neurons. This result has important implications for how Basigin might mediate its role in dendrite morphogenesis and for differences among different da neurons in how and whether growth of their dendrites is coordinated with changes in their substrates.

Given the requirement of Basigin in both epithelial and neuronal cell surfaces, and enrichment of Basigin at sites of dendritic enclosure, one likely mechanism, as discussed above, is that Basigin mediates homophilic interaction at sites where class IV dendrites remain in close physical association with epithelial cell surface. Dendrites that invaginate into the epidermis and are surrounded by epithelial membranes are primarily those of class IV neurons (Kim et al., 2012). Therefore, class IV dendrites are better positioned for such interactions than class I neurons. Our observation that class IV dendrites were selectively affected upon loss of Basigin in neurons or epithelial cells thus lends support to our model of homophilic interaction between Basigin on the surfaces of epithelial cells and neurons.

In addition, the cytoskeletal structure of class IV and class I neurons are very different. While the former contains a large number of dynamic actin-rich branches, the latter grows dendrites with near-complete microtubule enrichment and high stability. Interestingly, previous studies have reported changes in the actin cytoskeleton of insect cell lines and fly NMJ upon overexpression and loss of Basigin, respectively. Therefore, the actin cytoskeleton appears to be one of the primary targets of modulation by Basigin. Given the high proportion of microtubule-negative and actin-rich processes in class IV neurons relative to class I neurons, and the selective effect of Basigin knock down on class IV dendrite morphogenesis, it is possible that the mechanism by which Basigin contributes to dendrite morphogenesis is by promoting the elaboration of actin-rich dendritic processes. Comparing the proportion of actin-rich branches in wild-type and Basigin mutant class IV neurons would be one way of determining if this is indeed the case. Likewise, time-lapse studies of actin-dynamics would also provide insights into how dendritic actin is affected upon knockdown of Basigin in the neuron itself or in the epidermis.

Influence of Basigin on developmental elaboration of dendritic arbors

Examination of dendrite morphology of class IV neurons at late 2nd instar stage revealed that Basigin is required for developmental elaboration of class IV dendritic branches. Although a contribution of late-stage defects in branch maintenance cannot be ruled out, our findings point to significant early defects in dendrite elaboration that must occur as the substrate beneath the dendrites continue to expand with growth of the animal. Given the model of homophilic interaction between Basigin on epithelial and dendritic surfaces proposed above, we speculate that this interaction underlies signaling that coordinates growth of dendrites with that of substrate epithelial cells.

A previous study has shown that the epidermis 'informs' class IV da neurons to maintain a level of growth (called scaling growth) necessary to maintain appropriate dendritic field size as the animal expands via pathways that are regulated by the microRNA *bantam* in epithelial cells and the signaling pathway associated with Akt/PI3K in neurons (Parrish et al., 2009). The molecular 'conveyor' of such scaling information from epithelial cells to neurons is unknown. We speculate that a molecule like Basigin could mediate such a role. Our results however do not support the idea that Basigin itself could mediate the kind of scaling growth described by Parrish et al. (2009). Firstly, loss of function phenotypes for Basigin and scaling growth defects caused by disruption of *bantam* in epithelial cells or of *akt* in neurons are different. Whereas we observed fewer branches and decreased coverage of the dendritic field of class IV neurons upon loss of Basigin, Parrish et al. (2009) reported increase in dendritic field size upon knock down of *bantam* levels in the epidermis. In their model of dendrite scaling, Parrish et al. (2009) proposed an inherent capacity for rapid growth in class IV neurons that is suppressed by scaling factors such as *bantam* in the epidermis to ensure dendrites do not grow larger than the territories they need to innervate at any given animal size. In contrast, our observations support a model in which Basigin conveys a positive signal that promotes elaboration of dendritic branches from the epidermis to neurons. It is likely, however, that Basigin-mediated positive signal and *bantam*-mediated repressive signal are part of a large molecular program that coordinates changes in size of the substrate epidermal cells with growth and elaboration of class IV dendritic arbors.

It is not clear from our data whether the effect of Basigin activation or engagement is local or global in terms of its effect on dendrite morphogenesis. Class IV dendrites are heterogeneous in their cytoskeletal properties—while proximal dendrites are highly enriched in microtubules, as revealed by staining with the microtubule binding protein Futsch, distal higher order branches

lack microtubules and are thought to primarily contain actin filaments. Consistent with this, high degree of branch dynamics has been observed in the terminal branches of class IV arbors. Although parts of class IV dendrites that are enclosed in the epidermis and are likely the major sites of Basigin interaction are scattered throughout the arbor, they occur less in the terminal branches than in the proximal branches. However, loss of Basigin primarily affects higher order branches, with the lower-order branches that form the primary scaffold of the arbor relatively unaffected. Considering these information, we speculate that the effect of Basigin-mediated signaling from the epidermis to the neuron is global, i.e., it contributes to branch elaboration throughout the arbor. The lack of effect on microtubule-rich parts of the dendritic arbor despite a global effect of Basigin loss can be explained by selective targeting of molecules involved in actin regulation by Basigin-mediated signaling. This idea is consistent with previous studies showing changes in the actin cytoskeleton of insect cell lines (Curtin et al., 2005) and fly NMJ (Besse et al., 2007) upon manipulation of Basigin levels.

Experimental validation of a global effect within class IV neurons of Basigin-mediated signaling from the epidermis will require spatially restricted knockdown of Basigin either in the epidermis or in class IV neurons. Achieving this in neurons is not possible with the tools currently available to us. For spatially restricted knock down in the epidermis, one can take advantage of the heterogeneity of epidermal cells to drive expression of RNAi transgene against *basigin* in subsets of epidermal cells. For instance, *engrailed* is expressed in a narrow strip of epidermal cells over which class IV dendrites grow. Selective dendrite defects over cells expressing *engrailed* in animals expressing RNAi transgene against Basigin under the control of *engailed-GAL4* would support local effect of Basigin knockdown. In contrast, appearance of dendrite defects that

extend beyond the *engrailed*-expressing epidermal cells would support a model of global effect of Basigin signaling within class IV neurons.

Conclusion

Our study shows that Basigin, an Ig-superfamily member, is expressed in da neurons and the epidermal substrate over which their dendrites grow. Loss of function experiments in neurons and RNAi-mediated knockdown in the epidermis indicate that Basigin has both cell-autonomous and non-cell-autonomous roles in regulating morphogenesis of the dendrites of class IV neurons, but not of those of the morphologically simple class I neurons.

Developing dendritic arbors that uniformly innervate their receptive field is critical for the nociceptive and light-sensing function of class IV neurons. A challenge for these neurons in achieving this is that their substrate changes drastically in size over the lifetime of the neuron. Our observation that Basigin is required in the epidermis for class IV dendrite morphogenesis offers an important new insight into the role of the substrate in the functionally critical task of establishing appropriate level of coverage of class IV dendrites over them. Furthermore, our data showing that Basigin is also required in the class IV neurons for proper coverage of the dendritic field supports a model in which Basigin in epithelial cells and neurons interact directly in *trans*, mediating a positive signal from the substrate to neurons to elaborate their dendritic arbors. Broadly speaking, our findings may provide important insights into how the size and complexity of dendritic arbors are coordinated with changes in the substrate composition and size in other parts of the fly nervous system as well. Although the effect of afferent innervation on dendrite branching has been studied in various contexts in vertebrates, coordination of neuronal

morphology with their surrounding substrates and the overall size of the nervous system remains poorly studied. Given the conservation of Basigin from flies to vertebrates and the enrichment of Basigin in neurons in the vertebrate nervous system, our findings may also provide a useful entrypoint for examining the molecular basis of coordination between substrate changes and dendrite growth in vertebrate neurons.

Figure 3.1: Characterization of Basigin expression in Drosophila larvae

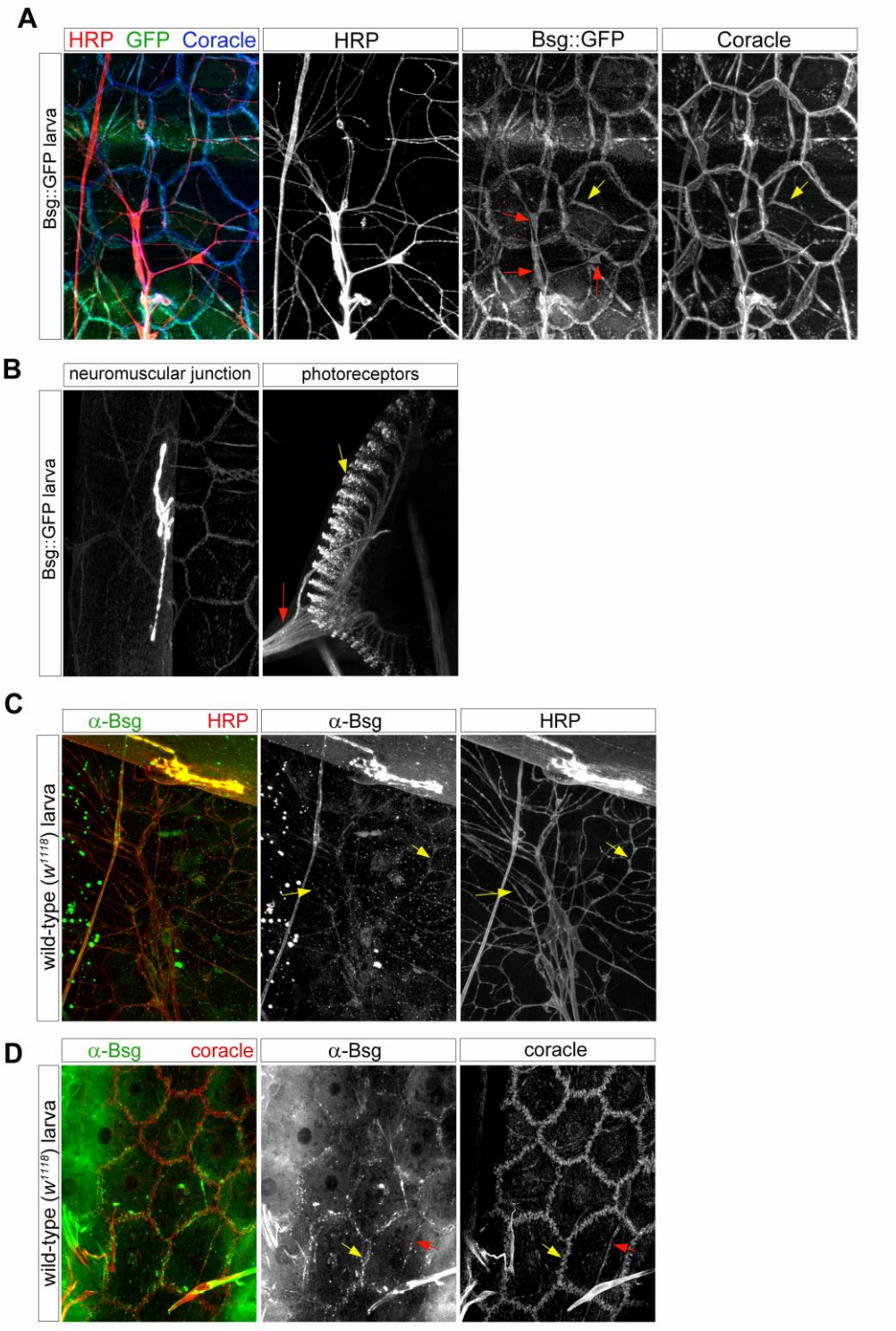


Figure 3.1: Characterization of Basigin expression in *Drosophila* larvae

(A) Basigin is expressed in both neurons and epithelial cells as revealed by GFP fluorescence in the Bsg::GFP transgenic line. Cell bodies and dendrites of da sensory neurons are labeled with HRP. In neurons, Basigin is localized to soma (red arrows), dendrites and axons. Epidermal Basigin is localized to cell borders and enriched intermittently along da neuron dendrites. Localization of epidermal Basigin along dendrites overlaps with that of the septate junction protein Coracle (yellow arrows).

(B) Consistent with previous reports, Basigin was found to be strongly enriched at the neuromuscular junction. Strong enrichment was also observed in photoreceptors (yellow arrow) and their axons (red arrow) in the larval CNS.

(C) Epidermal expression and localization of Basigin were verified by using α-Basigin antibody on wild-type larvae. As with the Bsg::GFP line, antibody staining revealed expression and dendritic localization (yellow arrows) of Basigin in da sensory neurons as well as strong enrichment in the neuromuscular junction. Immunostaining of these animals were done following fixation in 4% paraformaldehyde and epidermal expression was not observed under this condition.

(D) Incubation of filleted larvae with α-Basigin antibody before fixation, followed by fixation and subsequent staining with secondary antibodies revealed epidermal expression of Basigin. Localization of Basigin to cell borders (yellow arrow) and faint enrichment along dendrites red arrow) were observed. Basigin localization in the epidermis overlapped with that of Coracle, but was often significantly weaker and discontinuous, perhaps due to sub-optimal immunostaining conditions.

Figure 3.2: Basigin is required for proper morphogenesis of class IV da neuron dendrites

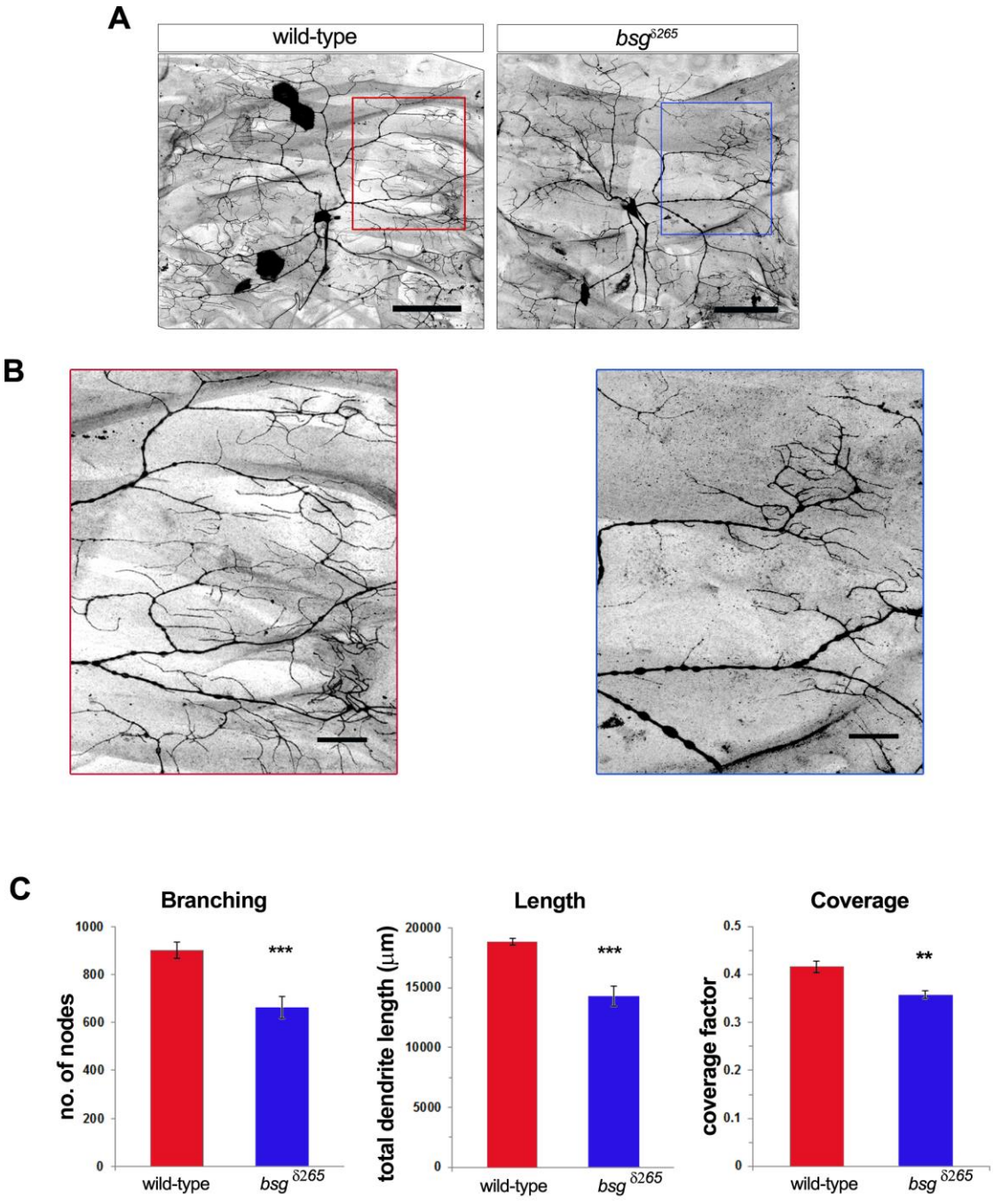


Figure 3.2: Basigin is required for proper morphogenesis of class IV da neuron dendrites

(A) Wild-type class IV neurons grow highly elaborate space-filling dendritic arbors. Basigin-null class IV neurons (*bsg*$^{\delta 265}$) created by the MARCM technique exhibit severe defects in dendrite morphogenesis. Scale bar, 100 μm.

(B) Magnified images of the regions marked in (A) highlight differences in dendrite arborization and dendritic field innervation in wild-type and *bsg*$^{\delta 265}$ neurons. Scale bar, 25 μm.

(C) Quantitative analysis of dendritic arbors revealed significant reduction in dendrite branching, length and coverage of dendritic field upon loss of Basigin. ***P < 0.001, **P < 0.01.

No. of nodes
wild-type: 902.67 ± 33.65, N=6
bsg$^{\delta 265}$: 661.56 ± 45.33, N=9, P <0.001

Total dendrite length
wild-type: 18845.85 ± 306.72 μm, N=6
bsg$^{\delta 265}$: 14296.58 ± 828.09 μm, N=9, P < 0.001

Coverage factor
wild-type: 0.42 ± 0.012, N=6
bsg$^{\delta 265}$: 0.36 ± 0.01, N=9, P < 0.01

Figure 3.3: Dendrites of class I da neurons are not affected by loss of Basigin

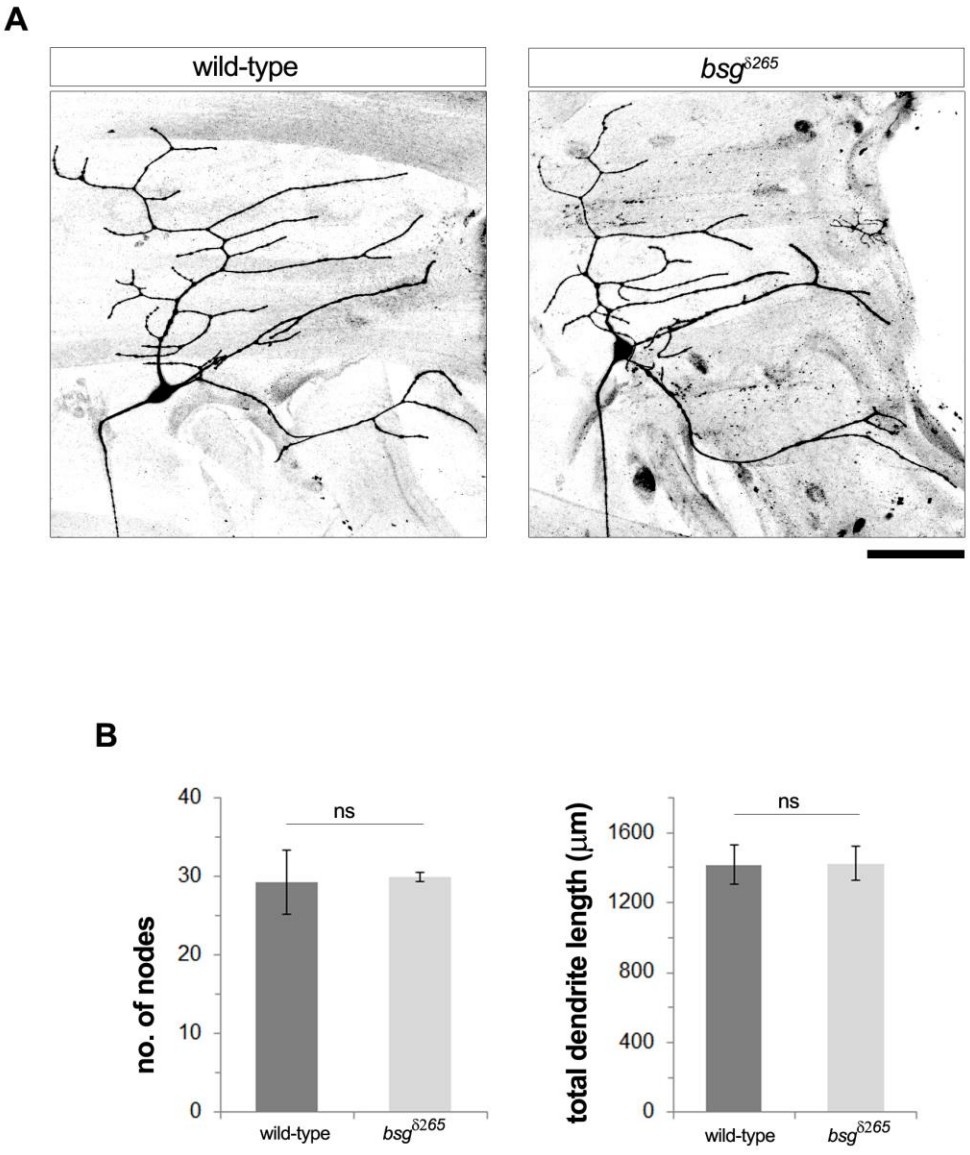


Figure 3.3: Dendrites of class I da neurons are not affected by loss of Basigin

(A) Basigin-null (*bsg*$^{\delta 265}$) class I neurons develop dendritic arbors that are similar to those of wild-type neurons. Images shown are those of mosaic clones created by the MARCM approach. Scale bar, 50 micron.

(B) No difference was found in the no. of nodes and total dendrite length of *bsg*$^{\delta 265}$ and wild-type class I neurons. 'ns' indicates not significant.

No. of nodes
wild-type: 29.33 ± 4.08, N=3
bsg$^{\delta 265}$: 30 ± 0.58, N=3, P > 0.05

Total dendrite length
wild-type: 1417.67 ± 111.62 μm, N=3
bsg$^{\delta 265}$: 1425.03 ± 95.32 μm, N=3, P > 0.05

Figure 3.4: *basigin* mutant neurons exhibit dendrite morphogenesis defects at 2nd instar stage

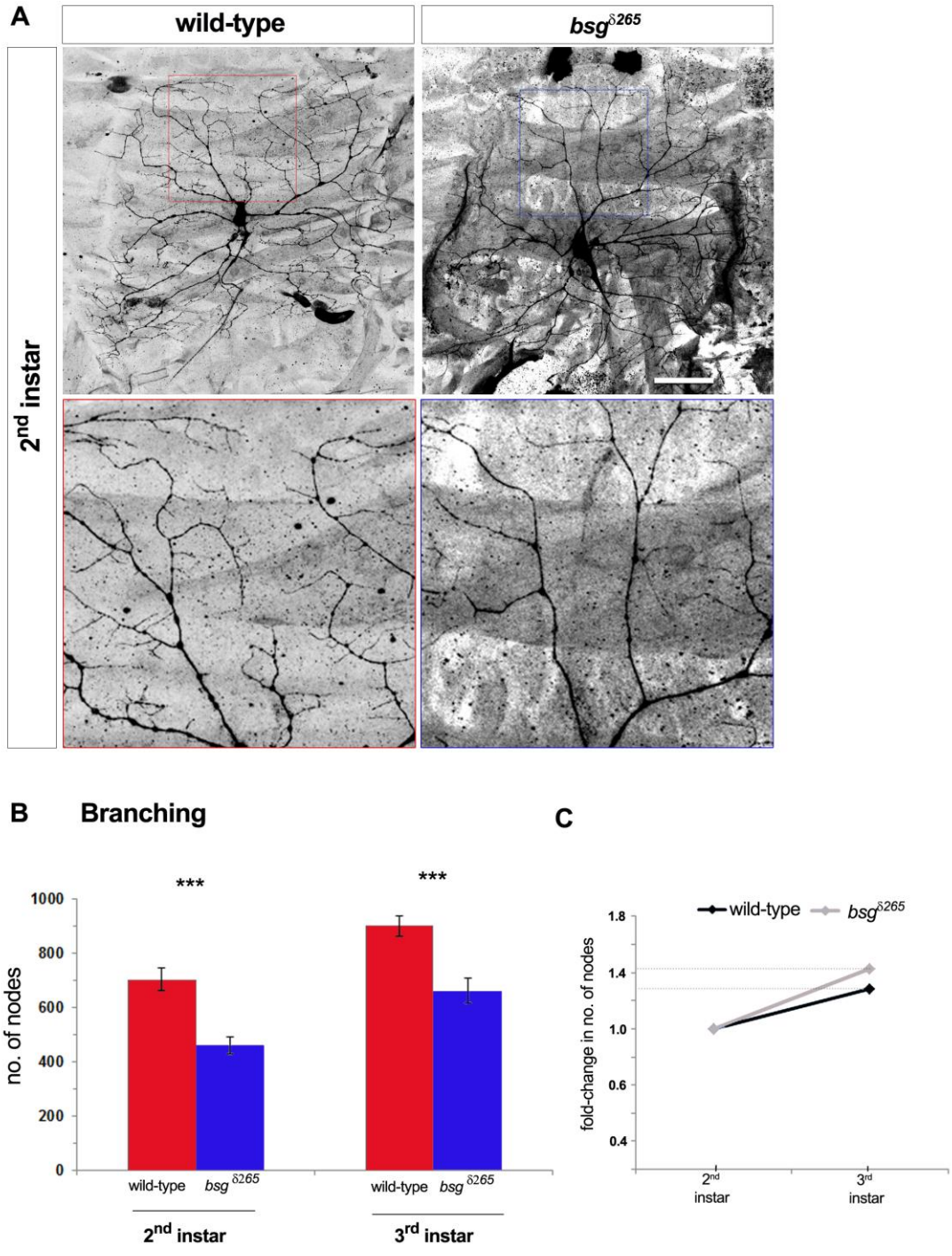


Figure 3.4: *basigin* mutant neurons exhibit dendrite morphogenesis defects at 2nd instar stage

(A) *basigin* mutant class IV neurons exhibit reduced branching of dendrites compared to wild-type neurons at 2nd instar. Bottom panel shows magnified view of regions outlined in the top panel. Scale bar, 50 μm for images in the top panel.

(B) Quantitative analysis revealed significant reduction in the no. of nodes in $bsg^{\delta 265}$ neurons at 2nd instar. ***P < 0.001. Dendrite branching at the 3rd instar stage (from Figure 3.2) is presented for comparison.

(C) Increase in dendrite branching from 2nd instar (normalized to '1' within each genotype) to 3rd instar.

No. of nodes at 2nd instar
wild-type: 704.0 ± 40.41, N=3
$bsg^{\delta 265}$: 464.75 ± 30.85, N=3, P < 0.001.

Fold-change in branching from 2nd instar to 3rd instar
wild-type: 1.28
$bsg^{\delta 265}$: 1.42

Figure 3.5: Knockdown of epidermal Basigin causes defects in class IV dendrite morphogenesis

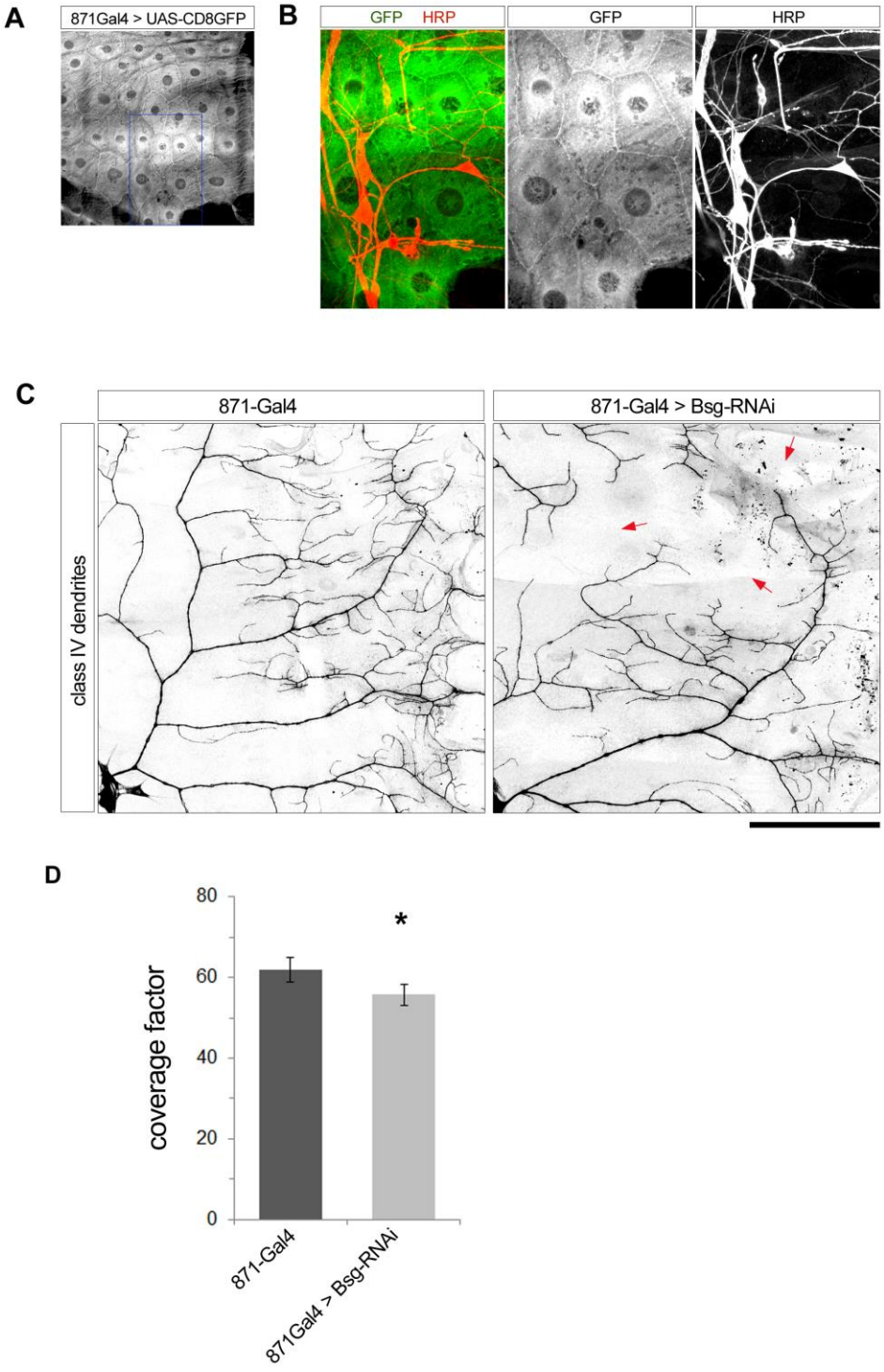


Figure 3.5: Knockdown of epidermal Basigin causes defects in class IV dendrite morphogenesis

(A) In order to validate epidermis-specific expression, expression of *UAS-CD8GFP* was driven with the *871-GAL4* driver. GFP signal was seen in the epidermis but not in neurons.

(B) Magnified view of the region outlined in (A). HRP labels all da sensory neurons and their processes. No GFP was observed in the cell bodies, dendrites and axons of neurons.

(C) RNAi transgene against *basigin* was expressed in the epidermis under the control of *871-GAL4* driver and class IV neurons were visualized by GFP expressed under the control of *ppk* promoter. Aberrant dendrite growth with large gaps in dendritic fields (red arrows) was observed in class IV neurons upon knockdown of epidermal Basigin. Scale bar = 100 μm.

(D) Quantitative analysis showed that dendritic coverage was reduced by 10% in animals in which Basigin was knocked down in the epidermis compared to control animals. * indicates $P < 0.05$.

Coverage factor:
Control (*871-GAL4 / ppkcd4GFP*): 61.81±3.06, N = 16
Basigin-RNAi (*UAS-bsgRNAi / bsg*$^{\delta 265}$*; 871-GAL4 / ppkcd4GFP*): 55.75 ± 2.64, N = 15

Figure 3.6: Knockdown of epidermal Basigin does not affect morphogenesis of class I dendrites

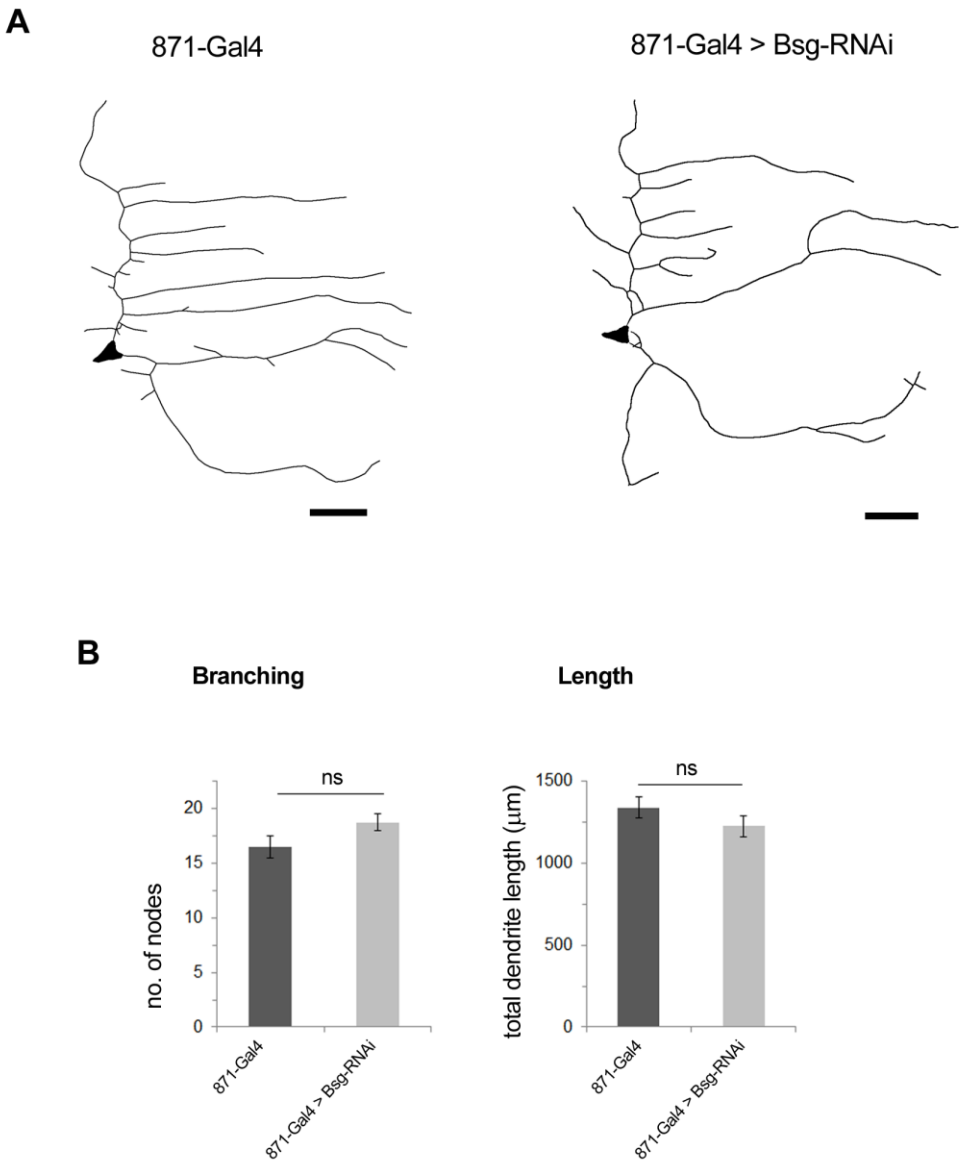


Figure 3.6: Knockdown of epidermal Basigin does not affect morphogenesis of class I dendrites

(A) Knockdown of Basigin specifically in the epidermis had no effect on dendrite growth of ddaE, a class I neuron. Dendrites of class I neurons were visualized by staining with anti-HRP, which labels all sensory neurons and their processes. Images shown are traces of neuronal arbor drawn using the software Neurolucida (MBF Biosciences). Scale bar = 25 μm.

(B) Quantitative analysis confirmed that knockdown of epidermal Basigin had no effect on dendrite branching and length. 'ns' indicates not significant.

No. of Nodes
Control (*871-GAL4 / ppkcd4GFP*): 16.5 ± 0.98, N=8
Bsg-RNAi (*UAS-bsgRNAi / bsg$^{\delta 265}$; 871-GAL4 / ppkcd4GFP*): 18.75 ± 0.77, N=8, P > 0.05

Total dendrite length
Control (*871-GAL4 / ppkcd4GFP*): 1337.78 ± 64.28 μm, N = 8
Bsg-RNAi (*UAS-bsgRNAi / bsg$^{\delta 265}$; 871-GAL4 / ppkcd4GFP*): 1225.58 ± 62.52 μm, N = 8, P > 0.05

Figure 3.7: Knockdown of Basigin in the epidermis does not affect epidermal cell size and localization of epidermal junction proteins.

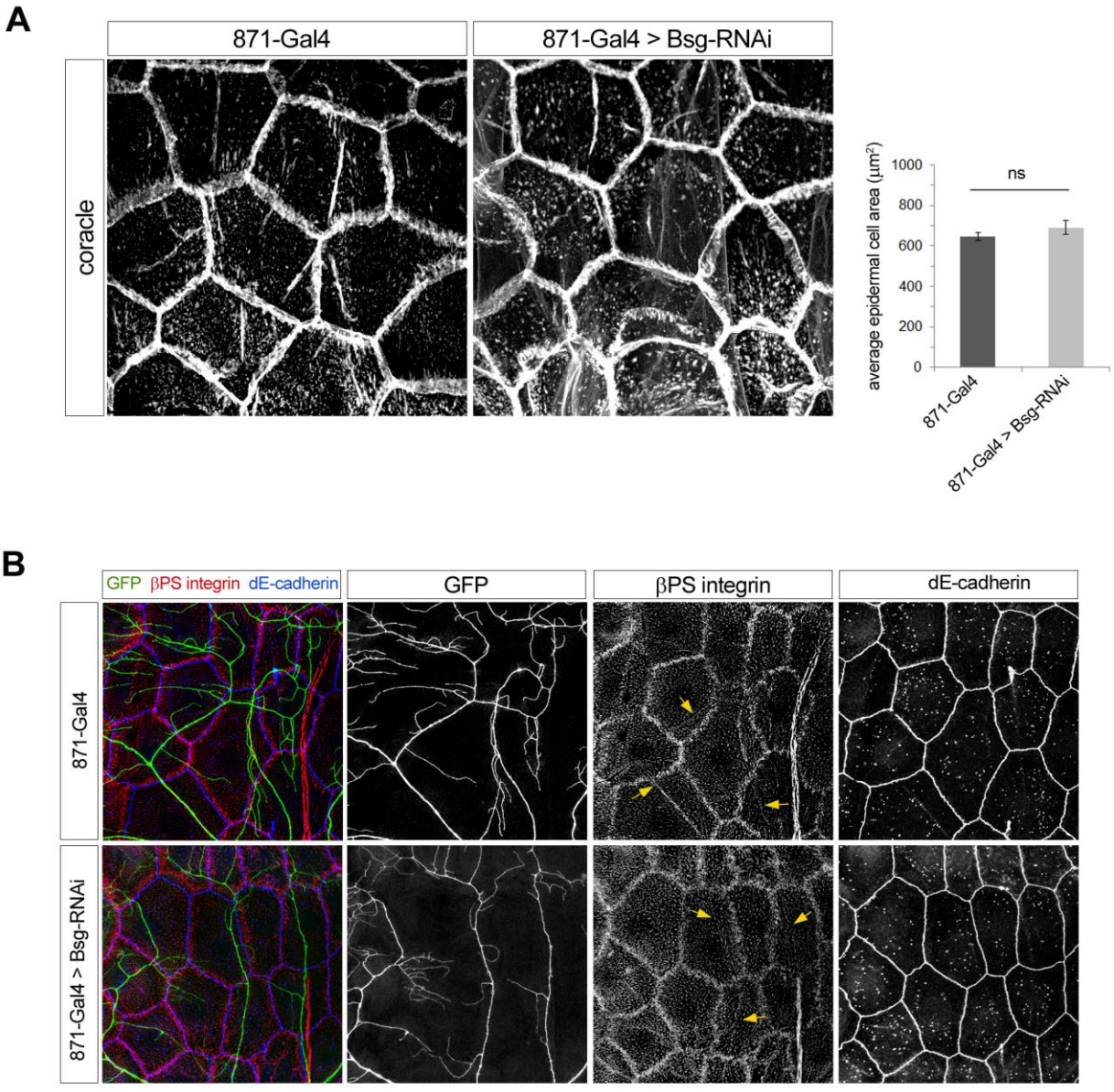


Figure 3.7: Knockdown of Basigin in the epidermis does not affect epidermal cell size and localization of epidermal junction proteins.

(A) Epidermal cells exhibit no changes in size upon knockdown of Basigin. α-Coracle staining, which labels cell borders, was utilized to compare average cell size between control animals and animals in which Basigin was knocked down specifically in the epidermis. Notably, localization of Coracle also appeared unaffected. 'ns' indicates not significant.

Average epidermal cell area:
Control (*871-GAL4 / ppkcd4GFP*): 646.36 ± 19.14 μm^2, N = 12
Bsg-RNAi (*UAS-bsgRNAi / bsg$^{\delta 265}$; 871-GAL4 / ppkcd4GFP*): 691.16 ± 33.70 μm^2, N = 15, P > 0.05

(B) Localization of other epidermal junction proteins was unaffected: dE-cadherin, an adherens junction protein, and βPS integrin, a hemi-adherens junction protein. Arrows indicate epidermal enrichment of βPS integrin along dendrites in both control and epidermal knockdown animals.

Figure 3.8: Knocking down Basigin in both neurons and the epidermis caused defects in class IV dendrite morphogenesis.

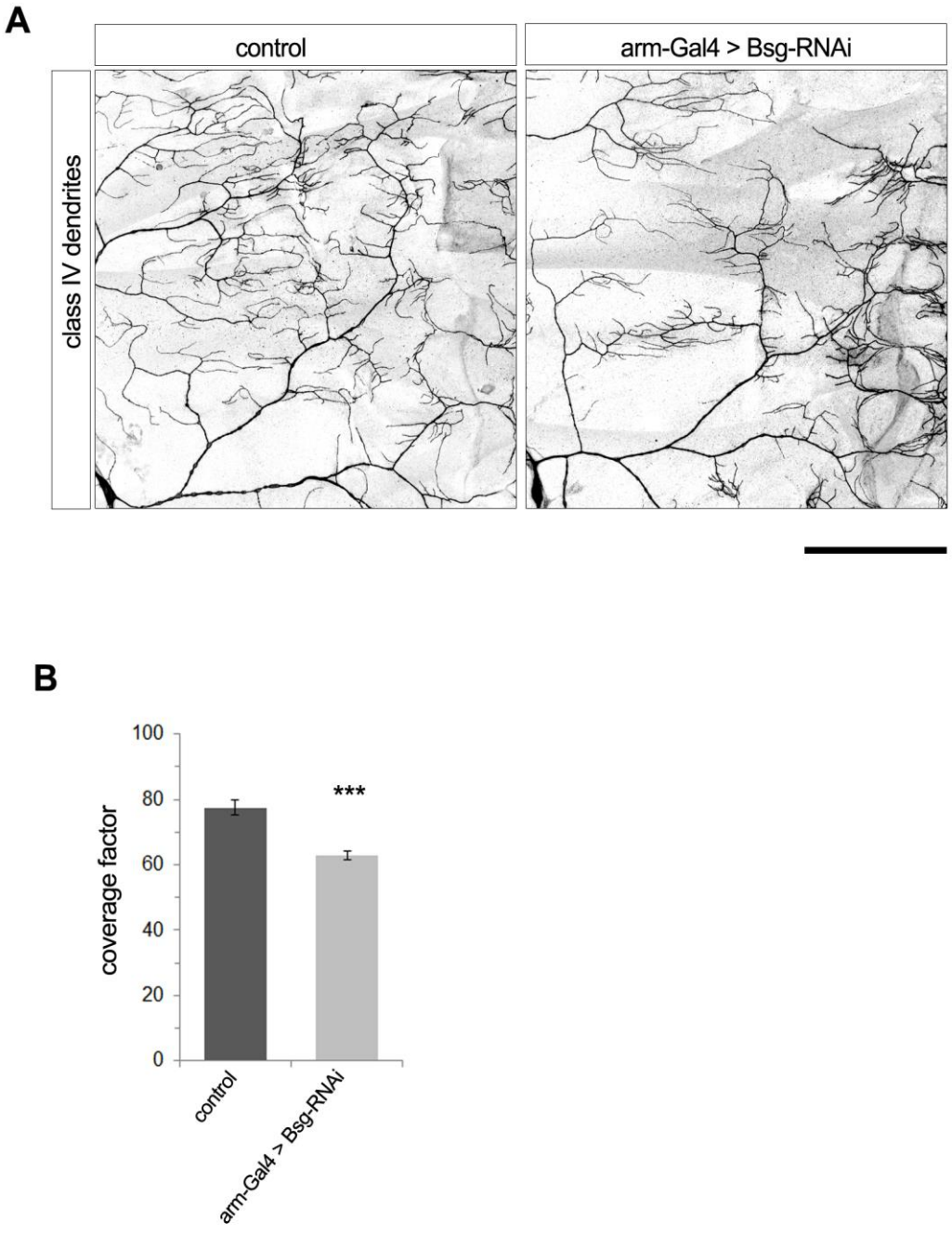


Figure 3.8: Knocking down Basigin in both neurons and the epidermis caused defects in class IV dendrite morphogenesis.

(A) Defect in dendrite morphogenesis of class IV neurons was observed upon knockdown of Basigin in both neurons and the epidermis using the *arm-GAL4* driver. Scale bar, 100 μm.

(B) Quantitative analysis revealed significant decrease in dendritic field coverage upon knockdown of Basigin in both neurons and the epidermis. *** $P < 0.001$

Coverage factor:
Control (*UAS-bsgRNAi / ppkcd4Tomato*): 77.4 ± 2.27, N = 3
Bsg-RNAi (*UAS-bsgRNAi / +; arm-GAL4 / ppkcd4Tomato*): 62.85 ± 1.42, N = 6, $P > 0.001$

Chapter IV: Mechanism of Basigin function in dendrite morphogenesis

Abstract

Data presented in the previous chapter revealed both cell-autonomous and non-cell-autonomous roles of Basigin in dendrite morphogenesis of class IV neurons. Here, I describe the experiments I conducted to elucidate the mechanism by which Basigin mediates these roles. First, I compared the abilities of wild-type Basigin with its truncated and mutated variants to rescue dendrite defects observed in *basigin* mutant neurons. My results show that expression of the Basigin extracellular domains alone in neurons is not sufficient to rescue the dendrite defect, suggesting a critical requirement of membrane-tethering of neuronal Basigin for its role in dendrite development. In addition, we found that the highly conserved KRR motif, which is a putative binding site for cytoskeletal adapter proteins, in the cytoplasmic tail of Basigin is required for its function. Second, we conducted a genetic interaction assay to identify molecular interactors of Basigin. Our findings suggest that the role of Basigin in dendrite morphogenesis does not involve the known molecular interactors Integrin and matrix metalloproteinases, but may be mediated by Tropomodulin, an actin-capping protein. These findings are consistent with previous studies implicating Basigin in organization of cellular cytoskeleton and lend support to a model in which Basigin on the neuronal surface acts as a receptor involved in coordinating growth of dendrites with that of the substrate epithelial cells by regulating the actin cytoskeleton of dendrites. Lastly, we identified structural differences between the long and short isoforms of Basigin that suggest functional differences and have important implications for how Basigin may mediate communication between epithelial cells and neurons.

Introduction

Our findings (presented in Chapter III) that Basigin is required both in neurons and their substrates for dendrite morphogenesis raise interesting questions about its potential mechanism of action. Basigin was originally identified as a secreted molecule in lung carcinoma cells in mice that could stimulate collagenase activity of fibroblasts in co-cultures (Biswas, 1982). Several functions of Basigin in normal tissues have been described since then, but its ability to induce secretion of MMPs from stromal cells surrounding tumors, in which it is highly upregulated, remains its best-characterized function. Consistent with the original experiment that led to the discovery of Basigin, recent studies have shown that Basigin is secreted in micro-vesicles by tumor cells, and application of soluble Basigin to stromal cell cultures can induce the secretion of MMPs (Gabison et al., 2005). This raises an obvious but important question- what is the Basigin receptor? To address this, Belton et al. used a soluble Basigin-2 fragment consisting only of its extracellular Ig domains to conduct an affinity purification assay combined with MALDI/MS-MS sequencing to determine protein identity (Belton et al., 2008). They found that the soluble Basigin fragment interacted with full-length Basigin-2 on fibroblast cell surface, and the complex internalized and subsequently interacted with Basigin-3 within the cell. Importantly, binding of the soluble Basigin fragment with Basigin-2 led to activation of the ERK1/2 signaling pathway, and subsequently, to upregulation of MMP expression. Findings of this study provided the first strong evidence in support of the capacity of Basigin to bind homophilically in *trans* in a functionally relevant manner and to function as a signaling molecule.

Compared to our knowledge of how Basigin functions in cancerous tissues, mechanistic insight into the function of Basigin in normal tissues is relatively poor. Analysis of Basigin null mice

has revealed roles for Basigin in embryo implantation, spermatogenesis, and brain development (Igakura et al., 1998; Ochrietor et al., 2001). Embryo implantation in the rodent uterus is known to be regulated in part by MMPs and their inhibitor, tissue inhibitors of MMPs (TIMPs) (Chen et al., 2009). However, evidence for the role of Basigin in embryo implantation being mediated via regulation of MMPs is merely correlative— *in vivo* examination of changes in expression of MMPs in the uterus of Basigin null animals has not been conducted. As such, the extent to which the function of Basigin in normal tissues involves regulation of MMPs remains unknown.

A few lines of evidence indicate that Basigin acts in some contexts by interacting with other transmembrane proteins. In the case of monocarboxylate transporter 1 (MCT1), which mediates lactate shuttling, *cis*-interaction with Basigin is important for its localization to the cell surface in the murine retina and spermatozoa (Mannowetz et al., 2012; Philp et al., 2003). Basigin-MCT1 interaction occurs via the highly conserved transmembrane domain of Basigin (Finch et al., 2009). Notably, Basigin contains a charged glutamic acid residue that is conserved from flies to humans in the middle of its transmembrane domain and is thought to mediate interactions with other proteins at the plasma membrane (Muramatsu and Miyauchi, 2003).

Evidence from studies on the human fibrosarcoma cell line HT1080 shows that Basigin also associates physically with $\alpha_3\beta_1$ and $\alpha_6\beta_1$ intgerins. The functional significance of Basigin-integrin association is unclear and is unlikely to be related to the well-characterized role of integrins in mediating adhesion and signaling at focal adhesion sites, as Basigin was found to co-localize with integrins at cell-cell contacts (Curtin et al., 2005), but not at focal adhesion sites of cultured HT1080 cells (Berditchevski et al., 1997).

Although Basigin has not been studied extensively in flies, structure function analyses and genetic interaction studies have provided some mechanistic insights into its roles in various developmental contexts. In the fly NMJ, where loss of Basigin leads to abnormal synaptic development and function via defects in organization of cortical actin, the KRR motif in the intracellular domain of Basigin is critical for its function (Besse et al., 2007). In addition, expression of the extracellular region of Basigin either by itself or tethered to the transmembrane domain of a different protein is not sufficient to rescue NMJ phenotypes caused by loss of Basigin. In the fly eye, where loss of Basigin causes defects in nuclear placement within photoreceptors and malformed glial projections into photoreceptor terminals, mutations in the transmembrane domain and the Ig domain closest to it were found to disrupt the ability of Basigin to rescue these phenotypes (Munro et al., 2010). Interestingly, while the extracellular region by itself did not rescue these phenotypes as in the case of NMJ defects, the intracellular KRR motif was found to be dispensable for both photoreceptor nuclear placement and glial projection development (Munro et al., 2010). Thus, the mechanism of Basigin function in different cellular contexts is likely different within flies. In addition, MMP mutant flies do not show any of the eye phenotypes observed in Basigin mutants (Curtin et al., 2005), suggesting that the function of Basigin in regulating organelle placement within photoreceptors and in mediating neuron-glia interactions is unlikely to involve MMPs.

Basigin expression is highly enriched in extra-embryonic tissues in *Drosophila* embryos. Live-imaging of Basigin and Integrin mutant embryos has revealed that Basigin interacts functionally with βPS-integrins in mediating dorsal closure (Reed et al., 2004), a complex morphogenetic process in which interactions between membranes of the extra-embryonic tissues establish epidermal continuity on the dorsal surface of embryos. Furthermore, Basigin and βPS-integrin

double mutant fly eyes show nuclear placement defects more severe than those of Basigin single mutants, suggesting a functional interaction between them (Curtin et al., 2005). Consistent with these findings, cytoskeletal and cell shape changes observed upon overexpression of Basigin in *Drosophila* S2 cells were found to be mediated via integrins, as application of GRGDS, a competitive inhibitor of integrin, blocked those changes (Curtin et al., 2005). Surprisingly, no evidence of genetic interaction between Basigin and βPS-integrin was found at the fly NMJ (Besse et al., 2007).

Thus, studies conducted so far indicate that Basigin mediates cell-cell interactions in flies and a likely general mechanism of Basigin function in flies involves regulation of cellular cytoskeleton, which may be mediated via Integrin in some contexts. In this chapter, I describe the experiments we carried out to understand the mechanism by which Basigin acts to regulate dendrite morphogenesis. We used a combination of structure-function analysis and genetic interaction experiments to identify the domains that are involved and its putative interacting partners. Our findings reveal similarities between the mechanisms of Basigin function in dendrites and at the NMJ.

Methods

Rescue experiments

Rescue experiments were conducted using various wild-type and mutated or truncated Basigin variants by the MARCM approach (Lee and Luo, 1999). Flies of the following genotypes were used: 1) w^{1118}, *hsflp, elav-GAL4, UAS-mcd8::GFP; FRT40A, GAL80*, 2) w^{1118}*; FRT40A, FRT42D / CyO-GFP*$_{nuclear}$, and 3) w^{1118}*;* $bsg^{\delta 265}$*, FRT40A / CyO-GFP*$_{nuclear}$*; UAS-Basigin*^{XX} */ TM6B* (where XX indicates one of 3 Basigin variants: full-length (FL), extracellular domain alone (extra) or full-length protein with mutations in the KRR motif (KRR>NGG)). All experimental procedures for generation of MARCM clones were identical to those described in chapters II and III.

Antibodies

The following primary antibodies were used: α-GFP (1:500), α-HRP (1:250), α-Coracle (1:50), and α-dsRed (1:250). Respective secondary antibodies were used at 1:250 dilution. All primary and secondary antibody incubations were done overnight at 4 °C and immunostained animals were washed in PBS containing 0.03% Triton X-100 (Sigma) at room temperature.

Quantitative analysis

Quantitative analysis of dendrite morphology in all rescue experiments was done by tracing dendrites using the software Neurolucida (MBF Bioscience). Measurement of coverage factor

was done as described in Chapter II and Appendix 2 by dividing the dendritic field into grids and counting the number of boxes containing dendrites. Our analysis focused on the dorsal posterior quadrant of the class IV neuron (ddaC) in the dorsal cluster of third instar larvae.

Statistical analysis was done using the R software package (R Core Team, 2012) and the web-based statistical computation tool VassarStats (http://www.vassarstats.net/). All data are presented as mean ± standard error of the mean (SEM).

Results

The results of our loss of function experiments indicate an important role for Basigin in dendrite morphogenesis of class IV neurons. Our observation that Basigin is required both cell-autonomously and non-cell-autonomously for dendrite morphogenesis suggested a possible mechanism by which it may act on dendrites. Findings from structure-function analyses conducted in flies offered important insights into potential mechanisms and served as a good starting point to investigate how Basigin is involved in dendrite morphogenesis of class IV neurons. In addition, we took advantage of information from previous interaction studies in both flies and mice to conduct a genetic interaction screen to identify molecules Basigin may interact with in promoting class IV dendrite development.

Extracellular domain of Basigin is not sufficient for Basigin function in dendrites

As described before, studies on various forms of cancer in vertebrates suggest that one mode of action of Basigin is that tumor cells release microvesicles containing Basigin, which then induces MMP secretion from surrounding cells (Millimaggi et al., 2007). Consistent with this, the extracellular domains alone of Basigin have been found to be sufficient for induction of MMP expression in fibroblasts in culture (Belton et al., 2008).

In the larval PNS, class IV dendrites grow in close association with the basement membrane, a specialized layer of ECM, as well as the epidermis. If Basigin on the dendritic surface takes part in locally modifying the ECM around it by acting as a ligand for ECM or epidermal cell surface molecules, which in return affects dendrite development, expression of the extracellular portion alone should be as effective as expression of the full-length Basigin protein in promoting

dendrite development. We tested this prediction using the MARCM approach to make *bsg*$^{-/-}$ mutant clones that also express either full-length wild-type Basigin or its mutated variants. This approach allowed us to restore truncated Basigin consisting of only the extracellular region in mutant cells without affecting Basigin expression in non-mutant cells. We then compared the ability of the truncated Basigin with that of full-length wild-type Basigin to rescue dendrite defects in *bsg*$^{-/-}$ neurons (Fig. 4.1). Expression of truncated Basigin consisting only of its extracellular domains in an insect cell line has previously been found to result in secretion of the protein, which validates the suitability of our approach to test the above prediction (Munro et al., 2010).

We found that while full-length Basigin was able to rescue the dendrite defects observed in *bsg*$^{-/-}$ class IV neurons, the truncated protein was not (Fig. 4.1). Dendrite branching in mutant neurons expressing full-length Basigin was statistically identical to those of wild-type neurons. On the other hand, mutant neurons expressing truncated Basigin exhibited dendrite branching at a level comparable to those of *bsg*$^{-/-}$ neurons and significantly lower than those of wild-type neurons and of mutant neurons expressing full-length Basigin (Fig. 4.1 B,C) .

These results indicate that the extracellular region of Basigin is not sufficient for Basigin function in dendrites and do not support a model in which Basigin in dendrites engage in 'inside-out' signaling by acting as a ligand for molecules in the ECM or the epidermis. Furthermore, our data suggest that membrane-tethering of Basigin is essential in dendrites. This led us to focus on specific conserved motifs in full-length Basigin.

The juxtamembrane KRR motif of Basigin is important for its function in dendrite morphogenesis

Basigin has a short intracellular segment consisting only of 20 amino acid residues. This region does not contain any conserved domains except for a positively-charged motif comprised of Lysine-Arginine-Arginine (KRR) residues immediately next to the transmembrane segment (described in Chapter I). This motif is conserved from flies to humans, suggesting that it is critical for Basigin function. Structure-function analyses in flies have shown that the KRR motif is essential for Basigin function in NMJ development but dispensable in the eye for photoreceptor nuclear placement and glia-photoreceptor interactions (Besse et al., 2007; Munro et al., 2010). Given evidence from vertebrate studies suggesting it as a binding site for cytoskeletal regulators, we hypothesized that the KRR motif of Basigin is important for its role in promoting proper arbor morphogenesis of class IV neurons.

We compared the ability of mutated Basigin, in which the juxtamembrane KRR residues were replaced with 3 uncharged residues Asparagine-Glycine-Glycine (NGG), with that of the full-length wild-type Basigin to rescue $bsg^{-/-}$ phenotypes in class IV neurons (Fig. 4.1). We found that the mutated Basigin protein (KRR→ NGG) was unable to rescue the branching defects, with the number of nodes in mutant neurons expressing the mutated Basigin protein statistically lower than that of wild-type neurons and similar to that of $bsg^{-/-}$ class IV neurons (Fig. 4.1, B,C). This shows that the evolutionarily conserved KRR motif in Basigin is important for its function in dendrite morphogenesis, and suggests that Basigin may function as a receptor in neurons with its intracellular activities mediated via interactions with its highly conserved KRR motif.

To verify that the lack of rescue of dendrite phenotype does not arise from instability of the mutant protein or defect in its subcellular localization, we expressed GFP-tagged variants of wild-type Basigin and mutant Basigin with KRR→NGG substitution specifically in class IV neurons under the control of *ppk-GAL4* driver (Fig. 4.1 D). Although we detected lower level of

mutated Basigin compared to full-length Basigin in class IV neurons, gross subcellular localization of the mutated protein was unaffected with obviously detectable signal in dendrites of class IV neurons (Fig. 4.1 D). Since levels of endogenous Basigin, as revealed by both α-Basigin antibodies and the Basigin::GFP transgenic line (Chapter III, Figure 3.1), are also very low in dendrites, relatively lower levels of the mutated Basigin compared to wild-type Basigin observed in this experiment is unlikely to account for the lack of rescue of dendrite phenotypes by the mutated protein. Thus, the results of our rescue analysis indicate that the KRR motif of Basigin is an important mediator of its function in dendrites.

Basigin does not interact functionally with Integrin in mediating dendrite morphogenesis

Previous studies in vertebrates and flies have indicated that Basigin and integrins associate physically and interact functionally in different cellular contexts (Curtin et al., 2005; Muramatsu and Miyauchi, 2003). The presence of a charged glutamic acid residue in the middle of the transmembrane region of Basigin is also consistent with possible interactions with other transmembrane proteins such as integrins (Munro et al., 2010). In addition, integrins have been shown to be important for dendrite maintenance in the mouse retina and the fly larval PNS (Kerrisk et al., 2013; Kim et al., 2012). We therefore conducted a genetic interaction assay to determine whether Basigin and Integrin are likely to function together in dendrite morphogenesis.

Morphogenesis of class IV dendrites was compared among animals heterozygous for the $bsg^{\delta 265}$ allele, those heterozygous for the mys^{1} allele (*mys* encodes the *Drosophila* βPS integrin protein), and those doubly heterozygous (referred to hereafter as trans-heterozygous) for both $bsg^{\delta 265}$ and

mys^{1} alleles. All neurons were labeled with CD4::GFP expressed under the control of the class IV-specific *ppk* enhancer (Fig. 4.2 A). Neurons that were singly heterozygous for the *bsg*$^{\delta 265}$ and *mys*^{1} alleles developed dendritic arbors that were comparable in coverage to those of wild-type neurons. Neurons trans-heterozygous for *bsg*$^{\delta 265}$ and *mys*^{1} exhibited dendrite coverage that did not differ statistically from those of animals singly heterozygous for either allele (Fig. 4.2 B). This suggested to us that the role of Basigin in dendrite morphogenesis of class IV neurons is not mediated via Integrin. Taken together with recent evidence for the role of Integrin in dendrite morphogenesis of class IV neurons (Kim et al., 2012), our findings suggest that Basigin and Integrin function independent of each other.

Role of Basigin in dendrite morphogenesis is not mediated via MMPs

Next, we examined whether Basigin acts via MMPs in regulating dendrite morphogenesis of class IV neurons. MMPs play an important role in degradation of severed dendrites during dendrite remodeling of da neurons at the end of larval stage (Kuo et al., 2005). In the adult fly PNS, MMPs are required for local degradation of the basement membrane, which facilitates reshaping of class IV neuron dendrites into their adult-specific morphology (Yasunaga et al., 2010). However, role of MMPs in development of the larval PNS has not been addressed to date. Given the well-characterized ability of Basigin in tumor cells to induce MMP secretion, we speculated that Basigin may promote class IV dendrite development via regulation of MMPs and, consequently, remodeling of the ECM surrounding dendrites.

We carried out a trans-heterozygous interaction experiment as described above to examine whether Basigin and MMPs exhibit functional interaction. The fly genome contains two MMPs, namely Mmp1 and Mmp2. In order to account for possible redundancy and compensation

between the two MMPs, we used flies that are null for both Mmp1 and Mmp2 (*mmp2*^{W307}*mmp1*^{Q112}) (Miller et al., 2008). Flies singly heterozygous for *mmp2*^{W307}*mmp1*^{Q112} were able to grow arbors with dendritic field coverage comparable to those of flies singly heterozygous for Basigin (Fig. 4.3). Flies trans-heterozygous for Mmp1 and Mmp2, and Bsg (*mmp2W307 mmp1Q112 / bsg*$^{\delta 265}$*)* grew dendritic arbors with statistically similar coverage factor compared to those of singly heterozygous animals (Fig. 4.3). Thus, our results suggest that MMPs do not mediate the function of Basigin in dendrite morphogenesis of class IV neurons. Our results do not necessarily rule out a developmental role for MMPs in regulating dendrite morphogenesis of da neurons. Careful analyses of dendrite morphogenesis in homozygous *mmp2*^{W307}*mmp1*^{Q112} animals would be required to address that. It is noteworthy that while homozygous *bsg*$^{\delta 265}$ animals die as embryos, the majority of embryos mutant for Mmp1 or Mmp2 survive through the embryonic stage, with a significant number lasting until the third instar larval stage (Page-McCaw et al., 2003). Consistent with our findings, this further suggests that the function of Basigin in early developmental stages of fly embryos and larvae are unlikely to be mediated via MMPs.

A screen for functional interaction with proteins that co-immunoprecipitate with Basigin

Since two genes that have been implicated to interact with Basigin in other physiological contexts were found to not exhibit functional interaction with Basigin, we looked further for other putative molecular interactors. To identify candidate interacting partners of Basigin, we took advantage of publicly available data generated by the *Drosophila* Protein Interaction Mapping Project (Guruharsha et al., 2011) (Fig. 4.4). They utilized co-affinity purification

combined with mass spectrometric analysis to identify proteins that physically associate with almost 5,000 individual FLAG-HA epitope-tagged *Drosophila* proteins. Two of the proteins they used as baits in their screen were Bsg-PD and Bsg-PG, the short and long isoforms of Basigin, respectively. As shown in Figure 4.4, several proteins that either interact with or regulate the cellular cytoskeleton (e.g., RhoGEF3, Tmod, Sw) were amongst those that co-immunoprecipitated with Basigin. This finding is consistent with previously proposed roles of Basigin in organizing cellular cytoskeleton in *Drosophila* cells. In order to determine whether any of these proteins might mediate the function of Basigin in dendrite morphogenesis of class IV neurons, we utilized two approaches depending on reagent availability (Fig. 4.4 B): 1) if a null allele for the corresponding gene was available, a trans-heterozygous screen, as described in the preceding sections, was conducted to determine genetic interaction, 2) if no null allele was available, the effect of knocking down the gene by RNAi on class IV dendrites was assessed. The results of our experiments are summarized in Figure 4.4 B. RNAi-mediated knockdown of *arf79F*, *CG15098*, *CG6937*, *pde8*, *rhoGEF3*, and *trap1* did not reveal dendritic phenotypes that resembled those of $bsg^{-/-}$ neurons, suggesting that these genes are unlikely to interact with Basigin in dendrite morphogenesis.

We next examined Tmod (Tropomodulin), an actin-binding protein that was found to physically associate with Bsg-PG, the long isoform of Basigin. Tmod binds to the minus ends of actin filaments and regulates their length in muscle and other cells (Gokhin and Fowler, 2011). Notably, our protein trap screen had also identified Tmod as being expressed in da neurons as well as the epidermis, and like Basigin, being enriched intermittently along dendrites beneath da neuron dendrites (Fig. 4.5 A). This similarity in localization and expression between Basigin and Tmod, combined with previous studies implicating Basigin in actin cytoskeleton organization,

suggested that Tmod was a good candidate for mediating the effect of Basigin on class IV dendrite morphogenesis.

We tested this hypothesis by examining dendrite development in animals trans-heterozygous for Tmod and Bsg alleles. Animals singly heterozygous for $tmod^{00848}$, a null allele of *tmod*, had class IV dendrites with dendrite coverage similar to that of control animals and $bsg^{\delta 265}$ single-het animals (Fig. 4.5 B). By contrast, animals that were trans-heterozygous for $tmod^{00848}$ and $bsg^{\delta 265}$ exhibited statistically significant decrease in dendrite coverage compared to the $tmod^{00848}$ and $bsg^{\delta 265}$ single-hets as well as control animals (Fig. 4.5 C). Consistent with a failure to appropriately innervate its receptive field, large gaps were seen in the dendritic arbors of class IV neurons (outlined with red dots in Figure 4.5 B) in trans-heterozygote animals. This evidence of genetic interaction between *bsg* and *tmod* suggested to us that they function in the same pathway in promoting dendrite morphogenesis of class IV neurons. Taken together with published data showing that they physically associate with each other (Guruharsha et al., 2011), and our finding that a motif that putatively mediates interaction of Basigin with cytoskeletal regulators is essential for its function in class IV dendrite morphogenesis (Fig. 4.1), this result lends support to a model in which Basigin on the dendrite surface regulates dendritic actin cytoskeleton to coordinate its development with that of the epidermal substrate.

The long isoform of Basigin is closely related but structurally distinct from the short isoform

Although the *basigin* locus in the fly genome has been known to encode two distinct Basigin isoforms, one with a longer extracellular region than the other, studies to date have described both proteins to have the same domain structure with an N-terminal signal peptide, two

extracellular Ig domains, a single-pass transmembrane region, and a short cytoplasmic tail. However, data retrieved from the DPIM database revealed that the molecular complex associated with the two isoforms are clearly distinct with no overlaps (Fig. 4.4). This suggested the possibility that the long and short isoforms of Basigin may be functionally distinct with differences in their protein structure.

Since most of the difference between the two isoforms lie in the extracellular region, I performed bioinformatic analysis on protein sequence that is unique to the extracellular region of the long Basigin isoform (Bsg-G) using the NCBI Conserved Domain Search (Marchler-Bauer and Bryant, 2004; Marchler-Bauer et al., 2013; NCBI, 2013b). Interestingly, I found that the sequence unique to Bsg-G in the extracellular region contains a previously unrecognized Ig-domain, making full-length Bsg-G a 3-Ig domain-containing protein (Fig. 4.6, Appendix 3). Previous studies might have missed identifying the third Ig domain in Bsg-G because it has a slightly higher chance of being predicted by chance occurrence than the 2 previously identified Ig domains. Expect value (E-value), which provides a measure of the chance of false positive prediction, is higher for the Ig domain I identified (0.02 vs. 2.21e-03 and 1.02e-06 for the other two common Ig domains; Appendix 3). However, since the E-value for the newly identified Ig domain is well below 1, above which a prediction is generally considered a false-positive hit (NCBI, 2013a), it is strongly possible that we have identified a bonafide third Ig domain in Bsg-G.

The transmembrane domains in both isoforms of Basigin are identical. The juxtamembrane KRR motif which we found to be required for Basigin function in dendrite morphogenesis are also found in both isoforms (indicated by colored dots in Figure 4.6 A). However, the C-terminal end of Bsg-G contains 8 more amino acids than the short isoform of Basigin. The terminal 10

residues of Bsg-G (Fig. 4.6) are part of a putative nuclear localization signal (NLS) based on predictions made by the cNLS Mapper program (Kosugi et al., 2009). Consistent with this prediction, the KKKRR motif contained within those 10 residues has been shown in mice to act as a functional NLS via importin-alpha2 binding (Yang et al., 2010). Although Basigin has never been implicated before in signaling to the nucleus, our finding raises the possibility that this may indeed occur in flies. In addition, the extracellular region unique to Bsg-G contains several short poly-Q repeats (underlined in Fig. 4.6 A), which may confer different aggregation properties to Bsg-G compared to the short Basigin isoform.

Mice and humans each contain a Basigin isoform with 3 Ig domains (Basigin-1). However, Bsg-G in flies is substantially longer than Basigin-1 (648 vs. 389 amino acids) in mice, and the region encoding the newly identified Ig domain in Bsg-G does not have any sequence homology with Basigin-1. In addition, Basigin-1 does not have the putative NLS we found in Bsg-G. Thus, Bsg-G is unlikely to be the ortholog of Basigin-1.

Thus, we found that the long isoform of Basigin is structurally related but distinct in several aspects from the short Basigin isoform. This is the first characterization of the differences between the long and short isoforms of Basigin and raises important questions about the mechanisms by which Basigin mediates inter-cellular interactions that should be addressed in future studies.

Discussion

In this chapter, I have described the work carried out to understand the mechanism underlying the cell-autonomous and non-cell-autonomous roles of Basigin in regulating dendrite morphogenesis. We conducted a structure-function analysis to assay the ability of truncated and mutated forms of Basigin to rescue the dendrite morphogenesis defects caused by loss of Basigin in neurons. Our results show that membrane-tethering and the highly conserved juxtamembrane KRR motif in the cytoplasmic tail of Basigin are critical for its function in dendrites. Genetic interaction assays revealed that the function of Basigin in dendrite morphogenesis is unlikely to be mediated via Integrins and MMPs, which have been implicated in Basigin-mediated regulation of cellular cytoskeleton and ECM modification, respectively, in previous studies. Interestingly, we found that Basigin and Tropomodulin, an actin-capping protein, exhibit genetic interaction in regulating dendrite morphogenesis, suggesting that they function in the same pathway. Taken together with published data showing that Basigin and Tropomodulin physically associate with each other and our observations that they have similar localization patterns in the epidermis and dendrites, our finding supports a model in which Basigin on the surface of neurons organizes the underlying actin cytoskeleton via the cytoskeletal regulator Tropomodulin.

Rescue experiments and genetic interaction analysis with MMPs argue against an 'inside-out' signaling model of Basigin function in dendrite morphogenesis

Vertebrate studies have shown that Basigin is released in microvesicles by tumor cells, which then induces secretion of MMPs and angiogenic factors (Belton et al., 2008; Millimaggi et al., 2007). Secretion of Basigin has not yet been reported in an *in vivo* context in *Drosophila*, but

expression of the extracellular domains alone in insect cells have been found to result in its secretion from those cells (Munro et al., 2010). One explanation for our findings of requirement of Basigin in both neurons and epidermis is that neuronal Basigin, in secreted or membrane-tethered form, acts as a ligand for epidermal Basigin, which results in modulation of release of ECM components from the epidermis. This model of 'inside-out' signaling predicts that expression of truncated Basigin consisting of the extracellular domains in neurons should be able to restore the Basigin loss of function phenotypes of class IV neurons. However, our rescue experiments revealed that this is not the case—expression of the extracellular region alone was not able to restore dendrite coverage defects as was done by expression of full-length Basigin. Our data argues against the 'inside-out' signaling model and suggests that Basigin on the surface of neurons may be at the receiving end of signaling that occurs between the epidermis and neurons. This interpretation makes the assumption that truncated Basigin with the extracellular domains alone is capable of binding with receptors in the surrounding and thus restoring the signaling lost upon knockout of Basigin from neurons. Although Basigin is poorly conserved at the sequence level over the Ig domains, the domain organization is well-conserved from flies to humans, and the previously demonstrated ability of soluble human Basigin consisting of only the extracellular domains to stimulate MMP expression in fibroblast cultures supports our assumption.

In addition, my rescue experiments show that the KRR motif of Basigin is critical for its function in dendrite morphogenesis and support the model that dendritic Basigin acts as a receptor for signaling that is mediated via its intracellular cytoplasmic interactions. KRR motifs have been found in a few vertebrate proteins and studies relating to those proteins have provided clues to its function. For example, in the potassium channels TASK-1 and TASK-3, this motif acts as a

retention signal, controlling cell-surface levels of the channels (Zuzarte et al., 2009). A KRR motif is also present in vertebrate CD43, the glycoprotein antigen expressed on the surface of T-cells, and mutation of this motif has been found to result in loss of binding to Moesin, a member of the ezrin-radixin-moesin (ERM) family of cytoskeletal regulators (Yonemura et al., 1998). Interestingly, Basigin has also been implicated in organization of cellular cytoskeleton in insect cell lines (Curtin et al., 2005) and the fly NMJ (Besse et al., 2007). In the latter case, replacement of the charged KRR residues with uncharged residues renders the Basigin protein incapable of rescuing NMJ defects (Besse et al., 2007).

An alternate interpretation of the lack of rescue by the KRR→NGG mutated version of Basigin is that the mutation may have somehow affected the ability of the Ig domains of Basigin to mediate extracellular interactions. However, this is unlikely to be the case because, like full-length wild-type Basigin, Basigin with KRR→NGG mutation has been reported to be capable of inducing cell aggregation in *Drosophila* S2 cells (Besse et al., 2007). In addition, a similar mutation of the KRR motif (KRR→MGG) was able to fully rescue the photoreceptor nuclear placement and glia-neuron interaction defects in the fly eye while mutations in the Ig domains that putatively block extracellular interactions were unable to rescue those defects (Munro et al., 2010). These results suggest that mutation in the KRR motif does not influence the extracellular activities of Basigin and the observed inability of mutated Basigin to restore dendrite defects likely reflects the importance of this motif in mediating intracellular interactions relevant for dendrite development in neurons.

Evidence from a previous study shows that the KRR motif in CD43, a vertebrate cell-surface protein with extracellular Ig domains, mediates interactions with Moesin, which cross-links cell-surface proteins with actin cytoskeleton (Tong et al., 2004). Therefore, the simplest explanation

of our results is that Basigin on the dendritic surface engages in organization of the underlying actin cytoskeleton via interactions with cytoskeletal cross-linking proteins, such as those of the Ezrin-Radixin-Moesin (ERM) family. Future studies examining genetic interaction between *basigin* and members of the ERM family in flies as well as changes in the localization of those proteins in *basigin* mutant neurons could provide further mechanistic insights into Basigin function.

basigin exhibits genetic interaction with tmod in regulating class IV dendrite morphogenesis

Several lines of evidence indicate that *basigin* interacts with *tmod* to promote dendrite morphogenesis. Tmod has similar localization pattern as Basigin in the larval PNS and epidermis, as indicated by our protein trap screen. Guruharsha et al. (2011) found that Tmod co-immunoprecipitates with Basigin as part of the same complex. In our study, we found that *tmod* and *basigin* interact genetically in the regulation of class IV dendrite morphogenesis. Taken together, these findings strongly suggest that Tmod mediates, at least in part, the role of Basigin in dendrite morphogenesis. Although our genetic interaction assay doesn't address where this interaction occurs, we favor the possibility that Basigin and Tmod interact in dendrites to regulate the dendritic actin cytoskeleton. One way to determine if this is the case would be to examine if overexpression of wild-type Basigin only in class IV neurons can rescue the phenotype observed in animals that are trans-heterozygous for $tmod^{00848}$ and $bsg^{\delta 265}$ in all cells. Full restoration of dendrite coverage to wild-type levels in this experiment would support our model. In addition, comparison of dendrite phenotypes in *basigin* and *tmod* single-mutant

neurons (in wild-type background) with those of double-mutant neurons would further indicate whether the interaction does indeed occur in neurons.

As a molecule that binds directly to the minus end of actin and prevents it from depolymerizing, Tmod is unlikely to bind directly with a transmembrane protein such as Basigin. We speculate that the physical association detected in the co-immunoprecipitation assay conducted by Gurharsha et al. (2011) likely reflects indirect binding via other molecules in the complex. Proteins of the ERM family are known to act as bridges between transmembrane proteins and cellular cytoskeleton. Our finding that a putative binding site for ERM proteins in Basigin is required for its function in dendrite development raises the possibility that such proteins may link Basigin with actin regulators such as Tmod. Whether the role of Tmod depends on interactions with the KRR motif in Basigin can be determined by comparing the ability of KRR→NGG mutated version of Basigin with that of wild-type Basigin to restore the dendrite phenotypes in $tmod^{00848}$ / $bsg^{\delta 265}$ trans-heterozygotes. Thus, our findings provide a useful entry-point for detailed molecular analysis of how Basigin influences dendrite development at the level of dendritic cytoskeleton organization.

The short and long isoforms of Basigin are structurally different

Hints from protein interaction data available through the DPIM database prompted a careful analysis of the secondary structure of Basigin, which resulted in the identification of a key structural distinction between the short and long isoforms of Basigin. The presence of an additional Ig domain in Bsg-G, making it a 3-Ig domain protein, has not been reported to date in flies.

Understanding how the two isoforms differ functionally may be informative for understanding signaling between the epidermis and neurons. Based on the site of GFP exon insertion, both the long and short isoforms of Basigin are tagged with GFP in the Bsg::GFP line described in chapter II. In addition, the antibody used to verify Bsg::GFP expression pattern was made using a shared peptide fragment and should detect both isoforms. Hence, it is not yet known whether the two isoforms differ in their expression and localization patterns. One possible scenario is that the two isoforms have cell-type specific expression and interact in *trans* to mediate inter-cell signaling. Since the isoform used in our rescue experiments was the short Basigin isoform, our observation that it is sufficient to restore the dendrite coverage of mutant class IV neurons to almost wild-type levels indicate either that it is normally expressed in neurons or can effectively substitute for the expressed isoforms. It will be interesting to examine whether the 3-Ig form of Basigin can rescue mutant phenotypes, or whether the two forms are functionally distinct.

Conclusion

Taken together with evidence for cell-autonomous and non-cell autonomous requirement of Basigin for dendrite morphogenesis of da neurons in the previous chapter, data presented in this chapter lend support to a model in which Basigin on the surface of neurons functions as a receptor and organizes neuronal cytoskeleton via cytoplasmic interactions mediated at least in part by its highly conserved KRR motif. Although I do not provide direct evidence of physical interaction between epidermal and neuronal Basigin, I favor this interpretation based on existing literature that show capacity of Basigin for homophilic binding and mediating cell-cell interaction.

Although the epidermis has been shown previously to influence dendrite morphogenesis in the larval PNS, the molecular mechanism by which communication between epithelial cells and neurons occurs is unknown. Additionally, a direct molecular pathway mediating interaction between neurons and substrate cells that is relevant for dendrite morphogenesis is not known in other biological contexts. Thus, data presented in this thesis provides a novel molecular pathway, comprising of Basigin-mediated signaling, that may underlie communication between substrate cells and neurons that is important for proper dendrite morphogenesis. In addition, our findings reveal a novel role for Basigin in regulating dendrite morphogenesis of neurons. Given that Basigin is conserved from flies to humans, and Basigin is expressed strongly in neurons in the mouse brain, our findings open doors to investigation of Basigin in neuronal morphogenesis in vertebrates in addition to bringing attention to the role of neuron-substrate interaction in shaping dendrite morphogenesis.

Figure 4.1: Membrane-tethering and the intracellular KRR motif of Basigin are critical for its function in dendrites

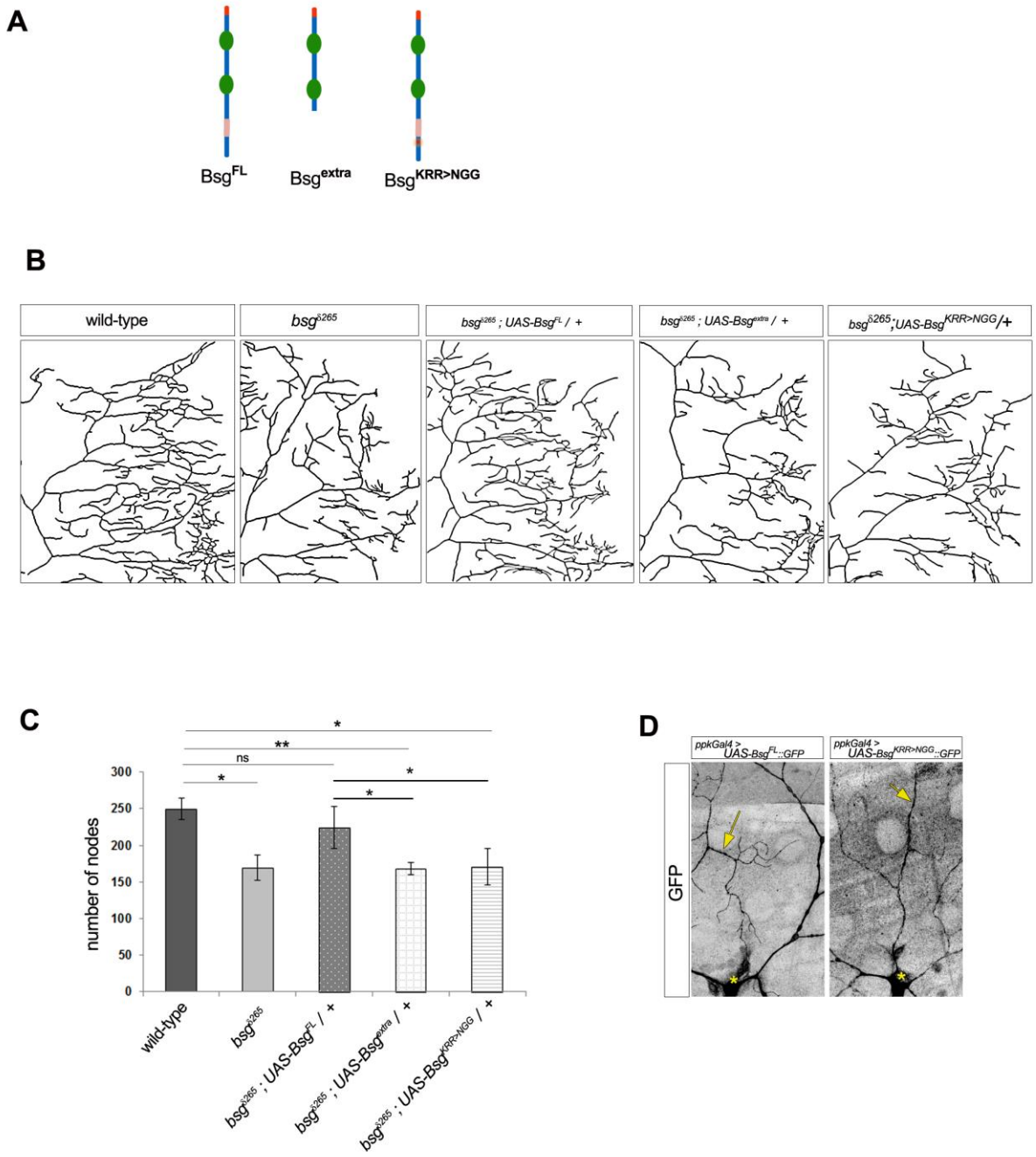


Figure 4.1: Membrane-tethering and the intracellular KRR motif of Basigin are critical for its function in dendrites

(A) Schematic showing the domains deleted or mutated in the constructs used for structure-function analysis. Bsg^{FL} indicates full-length wild-type protein, Bsg^{extra} indicates truncated Basigin consisting only of its extracellular domains, and $Bsg^{KRR>NGG}$ indicates mutated Basigin with the juxtamembrane positively charged KRR residues mutated to NGG.

(B) Results of rescue analysis by MARCM. Images show dorsal posterior quadrants of class IV dendritic arbors. Full-length wild-type Basigin rescued dendrite defects to a level statistically similar to that of wild-type neurons. Truncated Basigin without its transmembrane and cytoplasmic domains, and the full-length variant with KRR>NGG mutation were both unable to rescue the dendrite defects of $bsg^{\delta 265}$ neurons.

(C) Quantification of the number of nodes contained in the dorsal posterior quadrant of each neuron confirmed the inability of the truncated and mutated variants to rescue decreased branching of $bsg^{\delta 265}$ neurons. * $P < 0.05$, ** $P < 0.01$, 'ns' indicates not significant.

No. of nodes

wild-type: 249.67 ± 14.93, N = 6
$bsg^{\delta 265}$: 179.33 ± 18.85, N = 9
$bsg^{\delta 265}$; *UAS-*Bsg^{FL} / +: 224.25 ± 28.25, N = 4
$bsg^{\delta 265}$; *UAS-*Bsg^{extra} / +: 171.0 ± 8.13, N = 5
$bsg^{\delta 265}$; *UAS-*$Bsg^{KRR>NGG}$ / +: 168.56 ± 25.04, N = 9

(D) GFP-tagged wild-type Basigin and Basigin with KRR>NGG mutation expressed with the *ppk-GAL4* driver both localize to dendrites of class IV neurons. Dendritic localization of the mutated protein is weaker than that of the wild-type protein. Arrows indicate localization of GFP-tagged proteins to class IV dendrites. ⁎ indicates cell body of class IV neuron.

Figure 4.2: Function of Basigin in class IV dendrite morphogenesis does not involve Integrin

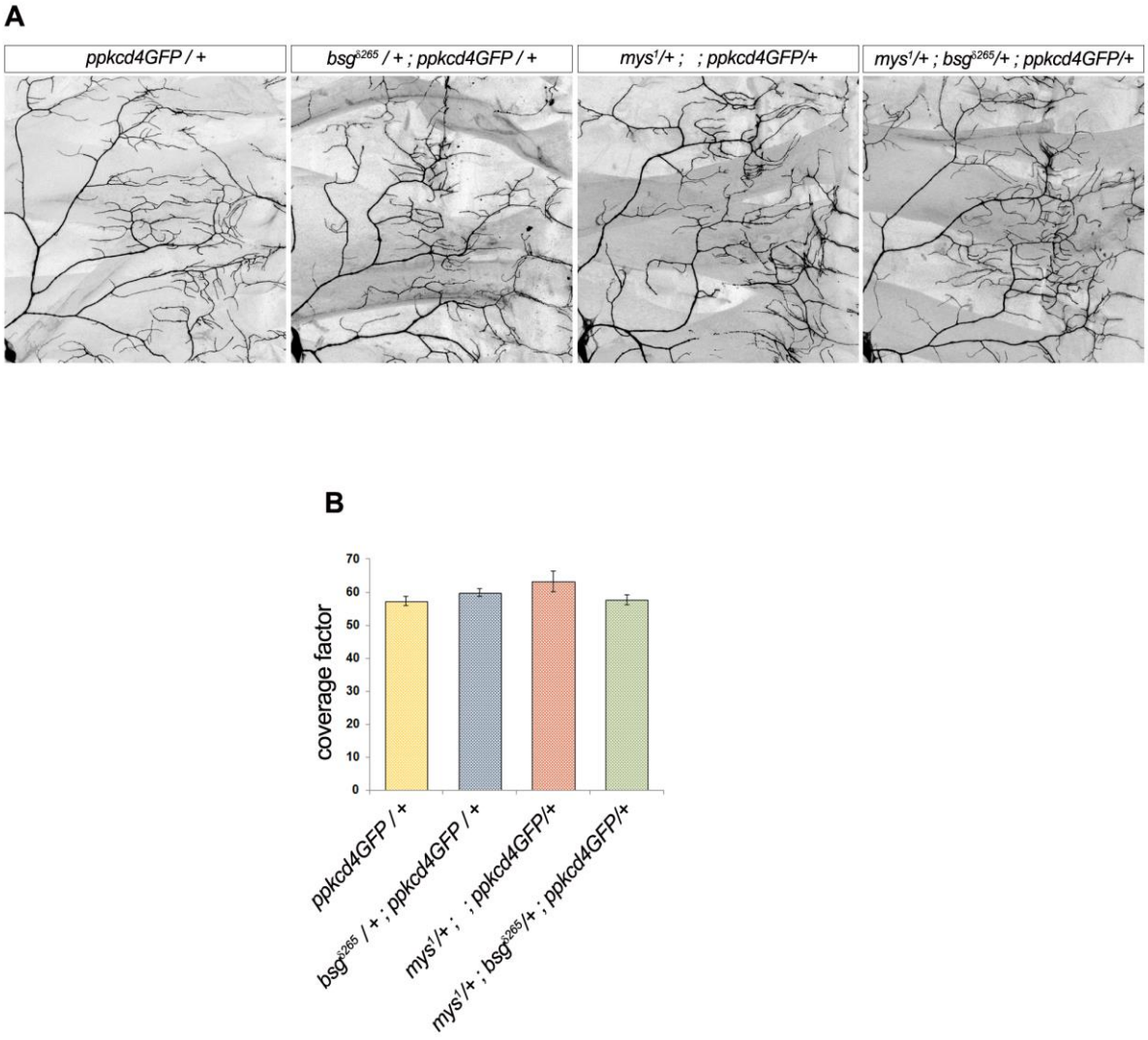


Figure 4.2: Function of Basigin in class IV dendrite morphogenesis does not involve Integrin

(A) Results of trans-heterozygous analysis of *bsg*$^{\delta 265}$ and *mys*^{1}. Neither the single-het nor the double-het animals exhibit dendrite defects, suggesting that *basigin* and *myspheroid* (which encodes βPS integrin) do not interact functionally in regulating dendrite development.

(B) Quantitative analysis of dendrite morphogenesis in control, single-het and trans-het animals reveal no statistically significant difference in dendritic field coverage of class IV neurons.

Coverage factor

Control (*ppkcd4GFP* / +): 57.3 ± 1.46, N = 13

Basigin-het (*bsg*$^{\delta 265}$ / +*; ppkcd4GFP* / +): 59.84 ± 1.22, N = 17

Integrin-het (*mys*^{1} / +*; ; ppkcd4GFP* / +): 63.3 ± 3.1, N = 3

Basigin-Integrin trans-het (*mys*^{1} / +*; bsg*$^{\delta 265}$/ +*; ppkcd4GFP* / +): 57.65 ± 1.53, N = 10

P > 0.05 for comparisons across all groups.

Figure 4.3: Role of Basigin in class IV dendrite morphogenesis is not mediated via MMPs

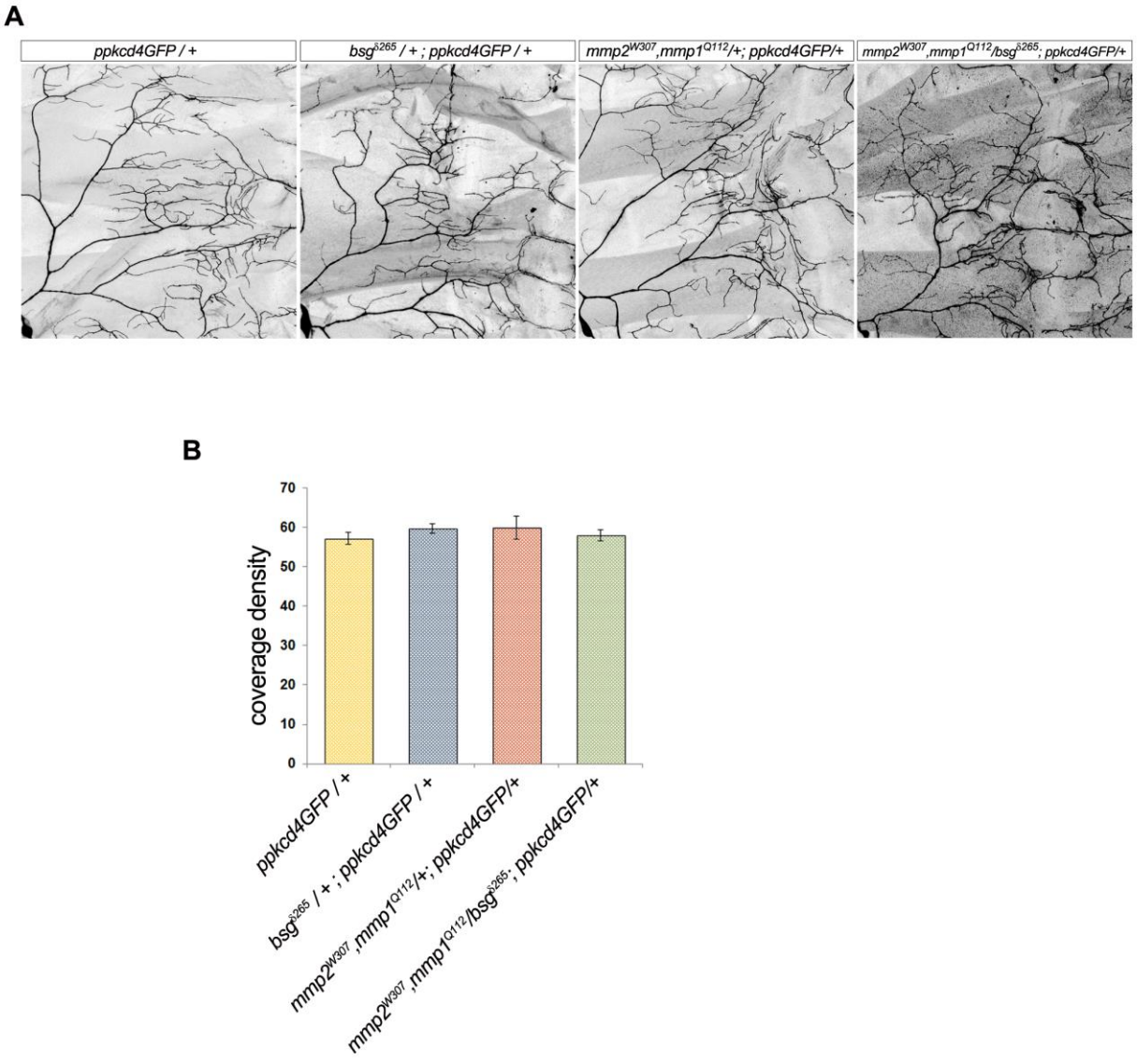


Figure 4.3: Role of Basigin in class IV dendrite morphogenesis is not mediated via MMPs

(A) Class IV neurons in single-hets of *bsg*$^{\delta 265}$ and *mmp2*^{W307}*mmp1*^{Q112}, and *bsg*$^{\delta 265}$/ *mmp2*^{W307}*mmp1*^{Q112} trans-heterozygotes have similar dendrite morphology as control animals. This suggests that *basigin* and MMPs do not interact functionally in regulating dendrite morphogenesis of these neurons.

(B) Quantitative analysis of dendrite morphogenesis in control, single-het and trans-het animals reveal no statistically significant difference in dendritic field coverage of class IV neurons.

Coverage factor
Control (*ppkcd4GFP* / +): 57.3 ± 1.46, N = 13
Basigin-het (*bsg*$^{\delta 265}$ / +; *ppkcd4GFP* / +): 59.84 ± 1.22, N = 17
MMP-het (*mmp2*^{W307}*mmp1*^{Q112} / +; *ppkcd4GFP* / +): 60.01 ± 2.86, N = 7
Basigin-MMP trans-het (*bsg*$^{\delta 265}$/ *mmp2*^{W307}*mmp1*^{Q112}; *ppkcd4GFP* / +): 58.0 ± 1.34, N = 6

P > 0.05 for comparisons across all groups.

Figure 4.4: A number of putative interactors of Basigin were identified using published data from the *Drosophila* protein interaction mapping project (DPIM)

A **Proteins that physically interact with Bsg-D / Bsg-E (short-isoform) in *Drosophila***

Protein	Molecular Process	Biological process
Arf79F	GTPase activity; GTP binding	regulation of cell shape; cell adhesion; ARF protein signal transduction
CG15098	unknown	unknown
CG3817	unknown	neurogenesis
RhoGEF3	signal transducer activity; Rho guanyl-nucleotide exchange factor activity	melanotic encapsulation of foreign target
CG6937	mRNA binding	neurogenesis
Gp210	unknown	olfactory learning; learning or memory
Vha68-2	ATP binding; proton-transporting ATPase activity, rotational mechanism.	endosomal lumen acidification; imaginal disc growth.
Bsg-PD		
Bsg-PE		

Proteins that physically interact with Bsg-G (long-isoform) in *Drosophila*

Protein	Molecular Process	Biological Process
Pde8	3',5'-cyclic-AMP phosphodiesterase activity	mesoderm development
Rps15Ab	structural constituent of ribosome	mitotic spindle organization
Sw (dic)	dynein light chain binding	centrosome localization; cytoplasmic transport
Tmod	actin binding	imaginal disc-derived wing morphogenesis.
Trap1	ATPase activity	protein folding; response to stress
Bsg-PD		
Bsg-PG		

Source: Protein interaction data was obtained from the Drosophila Protein Interaction Mapping Project (https://interfly.med.harvard.edu/) (Guruharsha et al., 2011). Retrieved on April 10, 2013.

Annotation of involvement in indicated Molecular Process and Biological Process were obtained from Flybase (http://flybase.org), version FB2013_02, released March 8, 2013. Retrieved on April 10, 2013.

B **Results of screen to identify candidate molecular interactors of Basigin**

Gene	Approach	Result (class IV neurons)
arf79F	RNAi	no phenotype
CG15098	RNAi	no phenotype
CG6937	RNAi	no phenotype
pde8	RNAi	no phenotype
rhoGEF3	RNAi	no phenotype
sw	Null allele / transhet assay	both single hets and transhets show dendrite defects
tmod	Null allele / transhet assay	transheterozygous interaction detected
trap1	RNAi	no phenotype
vha68-2	Null allele and deficiency line / transhet assay	mild transhet phenotype with null allele but not with deficiency

Figure 4.4: A number of putative interactors of Basigin were identified using published data from the *Drosophila* protein interaction mapping project (DPIM)

(A) List of proteins that co-immunoprecipitated with the short and long isoforms of Basigin in *Drosophila*. Molecular and biological processes that the interacting proteins are implicated in are also indicated. Several proteins have known or putative roles in cytoskeletal regulation. Notably, Bsg-D, a short isoform, co-immunoprecipitates with Bsg-G, the long Basigin isoform in flies.

(B) Results of a screen carried out to determine if the function of Basigin in dendrite morphogenesis is mediated via any of the interacting partners listed in (A). Either RNAi or trans-heterozygous experiment was conducted depending on fly reagent availability. Genetic interaction was detected between *basigin* and *tropomodulin*.

Figure 4.5: Tropomodulin (Tmod) and Basigin have similar expression and localization patterns, and exhibit functional interaction in regulating dendrite morphogenesis

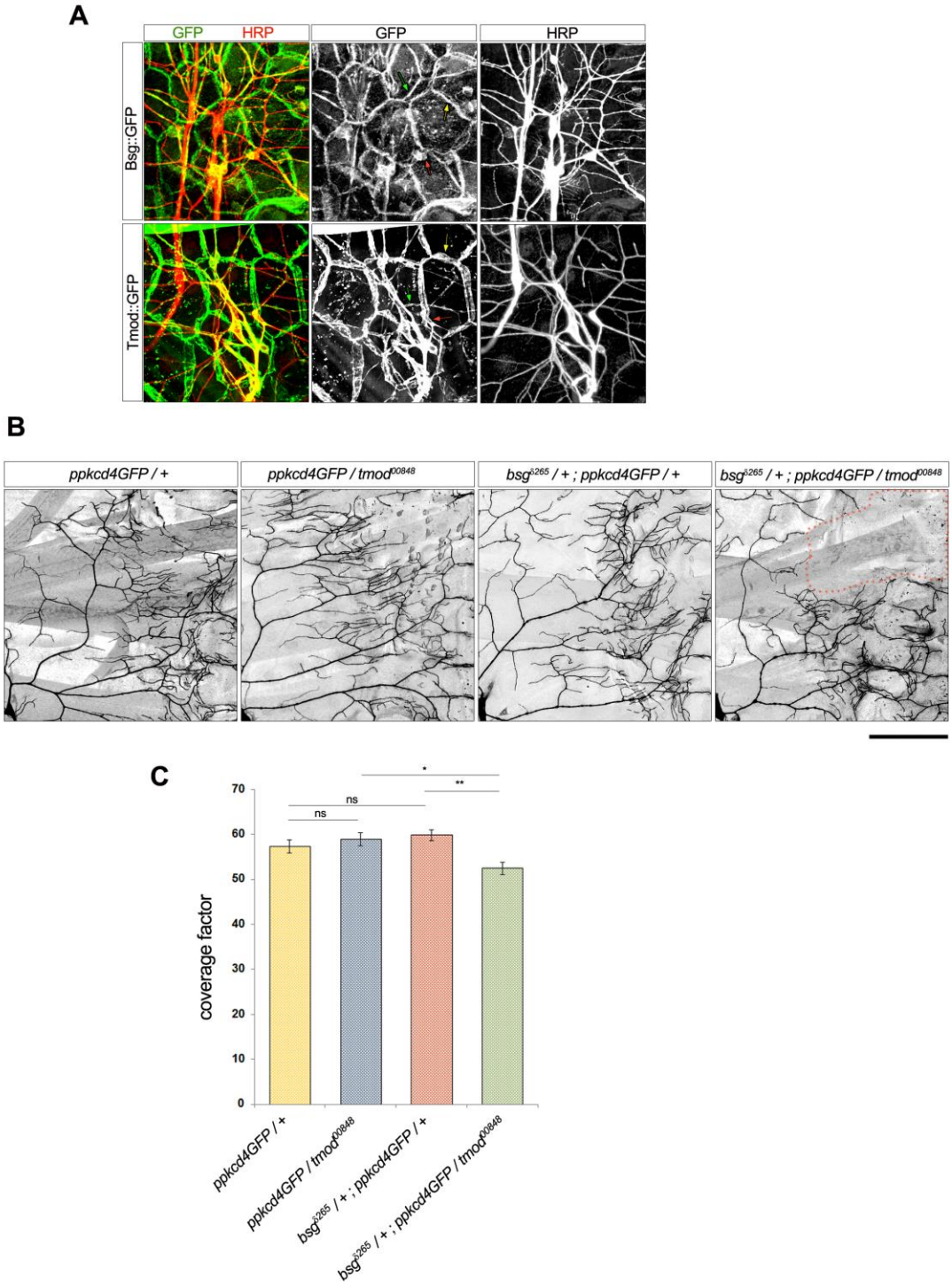


Figure 4.5: Tropomodulin (Tmod) and Basigin have similar expression and localization patterns, and exhibit functional interaction in regulating dendrite morphogenesis

(A) Like Basigin, Tmod is enriched at cell borders (yellow arrows) and beneath dendrites (green arrows) in epidermal cells. In addition, Tmod is also expressed in sensory neurons (red arrows). HRP staining shows all sensory neurons and their processes.

(B) Class IV neurons in single-hets of $bsg^{\delta 265}$ and $tmod^{00848}$ have similar dendrite morphology as control animals. However, $bsg^{\delta 265}/tmod^{00848}$ trans-heterozygotes exhibit striking defects in dendrite morphogenesis characterized by large gaps in the dendritic fields of class IV neurons (e.g., area enclosed by red dotted line). Scale bar, 100 μm.

(C) Quantitative analysis of dendritic field coverage of class IV neurons in control, single-het and trans-het animals. * $P < 0.05$, ** $P < 0.01$, 'ns' indicates not significant.

Coverage factor
Control (*ppkcd4GFP / +*): 57.3 ± 1.46, N = 13
Tmod-het (*ppkcd4GFP / tmod*^{00848}): 58.9 ± 1.48, N = 12
Basigin-het ($bsg^{\delta 265}$ */ +; ppkcd4GFP / +*): 59.84 ± 1.22, N = 17
Basigin-Tmod trans-het ($bsg^{\delta 265}$*/ +; ppkcd4GFP / tmod*^{00848}): 52.47 ± 1.38, N = 10

Figure 4.6: The long isoform of Basigin is structurally related but distinct from the short isoform

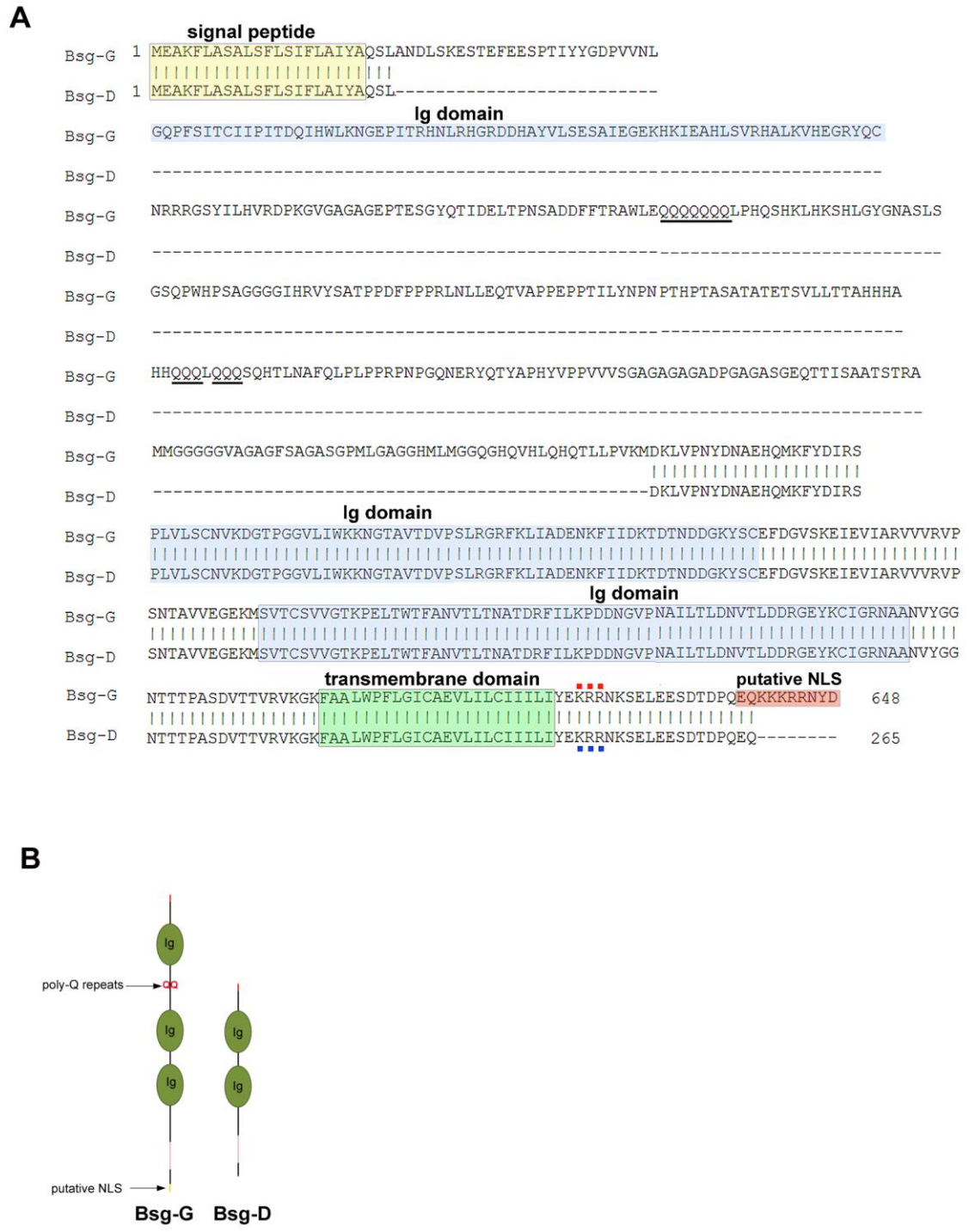


Figure 4.6: The long isoform of Basigin is structurally related but distinct from the short isoform

(A) Comparison of amino acid residues between the short (Bsg-D) and long (Bsg-G) isoforms of Basigin. In contrast to Bsg-D, Bsg-G contains 3 Ig domains in its extracellular region. In addition, multiple small polyglutamine (underlined) repeats are present in the extracellular region unique to Bsg-G. Sequences over the N-terminal signal peptide, the 2 membrane-proximal Ig domains, and the transmembrane domain are identical. All residues present in the cytoplasmic region of Bsg-D, including the KRR motif (marked with ***), are also present in Bsg-G. However, Bsg-G contains 8 extra amino acids that encode a putative nuclear localization signal (NLS). Further details of bioinformatic analysis of Bsg-G structure are presented in Appendix 3 and Appendix 4.

(B) Schematic showing the newly identified structural differences between the long and short isoforms of Basigin.

Chapter V: Conclusions and Future Directions

Development of the stereotypic dendritic arbors of neurons is orchestrated by a combination of cell-intrinsic and extrinsic factors. Recent studies have provided important new insights into how interactions between neurons shape their dendritic arbors. However, our understanding of the influence of interactions between neurons and the substrates upon which dendritic arbors grow is relatively poor. Such interactions can be important to ensure that dendritic arbors undergo requisite changes in accordance with alterations in the size, molecular environment and other physical attributes of their substrates. Work presented in this thesis identifies a molecular pathway that may underlie communication between sensory neurons of the *Drosophila* larval PNS and their substrate epithelial cells necessary to ensure proper dendrite morphogenesis of those neurons.

In an expression pattern-based screen for candidate regulators of dendrite morphogenesis, we identified Basigin as a cell-surface molecule of the Immunoglobulin superfamily expressed in both neurons and the epithelial cells they grow over. Previous studies have implicated Basigin in cell-cell interaction, organization of cellular cytoskeleton, and ECM remodeling via MMPs. Our study identifies a novel role of Basigin in cell-autonomous and non-cell-autonomous regulation of morphogenesis of the highly complex dendrites of class IV da sensory neurons. Further, we show that membrane-tethering of Basigin and a highly conserved KRR motif in its cytoplasmic tail are essential for its function in neurons. Results of our genetic interaction analysis suggest that Basigin-mediated regulation of dendrite morphogenesis does not involve interactions with Integrin and MMPs, both of which are known to interact with Basigin in other contexts, but instead involves Tropomodulin, an actin-capping protein. Taking our findings together with

existing evidence for the capacity of Basigin for homophilic interaction and organization of cellular cytoskeleton, we propose a model in which Basigin on the surfaces of epithelial cells and neurons engage in homophilic interaction that results in regulation of the dendritic cytoskeleton (Fig. 5.1). Further, the intracellular KRR motif may mediate cytoplasmic interactions of dendritic Basigin with cytoskeletal adapters and, indirectly, with regulators of actin, such as Tropmodulin (Fig. 5.1). Thus, our findings uncover a novel molecular pathway that may mediate epithelial cell-to-neuron communication for proper morphogenesis of dendritic arbors.

Establishing appropriate coverage of their dendritic fields is important for neurons to ensure adequate sampling of synaptic or sensory stimuli. Interactions between neurons and their substrates may be important for neurons to achieve this. A previous study has shown that functions of the microRNA *bantam* in epithelial cells and *akt* in da neurons constitute a scaling mechanism by which dendritic fields of class IV neurons are restricted to match the rate of increase in epidermal cell size. However, how information from the epithelial cell is communicated to the neurons at a molecular level is unknown. Data presented in this thesis show that Basigin is required in both neurons and epithelial cells for proper dendrite morphogenesis and may underlie communication between epithelial cells and da neurons that is directly relevant for regulation of dendrite morphogenesis. This is the first example of a molecular pathway that may mediate signaling between the epithelial cells and neurons in the *Drosophila* larval PNS and has broad implications for molecular dissection of neuron-substrate interactions in other systems.

Basigin is well-conserved from flies to humans and is expressed strongly in neurons in the vertebrate brain. The specific motifs in *Drosophila* Basigin that we found to be critical for its function in neurons—namely, the transmembrane domain and the intracellular KRR motif—exhibit the highest degree of conservation over the entire protein from flies to mice and humans,

suggesting not only the critical importance of these motifs but also that some functions of Basigin itself may be conserved from invertebrates to vertebrates. Further understanding how Basigin mediates interactions between neurons and their substrate in the cellular contexts described here may thus provide important molecular insights into such interactions that may occur during development of the vertebrate nervous system as well.

Utility of an expression pattern-based approach for identifying novel regulators of dendrite morphogenesis

Examination of the role of neuronal and epithelial Basigin on dendrite morphogenesis was prompted by data obtained as part of our expression pattern-based screen. In addition to Basigin, preliminary loss of function analysis following our screen identified several other molecules with interesting expression patterns and putative roles in dendrite morphogenesis. Considering the diversity of molecular classes represented in our list of candidate regulators of dendrite development, following up on one or more of these lines may provide important new insights into morphogenesis of da neuron dendrites. Additionally, our catalog of the expression and localization patterns of molecules ranging from transcription factors to translational regulators and cytoskeletal proteins may serve as a useful reference for future studies, such those seeking to identify transcriptional targets or to study the role of dendritic local translation on dendrite morphogenesis.

Our results also suggest that this approach may be broadly applicable to other parts of the nervous system. One limitation of our approach is that lack of availability of reagents, such as those necessary for gain of function and loss of function analyses, can greatly reduce the number

of candidates with interesting expression patterns that can be followed up on. However, ongoing efforts in the wider scientific community to create mutants and RNAi lines in a high-throughput manner may significantly improve the usability of data generated by taking this kind of approach in the near future.

Basigin as a mediator of epithelial cell-to-neuron communication

One explanation for the non-cell-autonomous and cell-autonomous roles of Basigin identified in this study is that Basigin on the surfaces of epithelial cells and neurons bind via extracellular Ig domains. In this model of Basigin function, Basigin on the neuronal surface serves as a receptor and transduces the signal from epidermis via its cytoplasmic interactions within dendrites. Although I cannot rule out the possibility that epithelial Basigin also organizes the underlying actin cytoskeleton, I favor the above interpretation based on the results of our structure-function analysis in neurons. A question this raises is how the 'signal' flows in any given direction when both interacting molecules are identical. For instance, if the epithelial cell and neuron both express Bsg-D, which is a short Basigin isoform, additional mechanisms need to be in place to confer directionality of signaling. Presence of a *cis*-binding partner on either side may accomplish this, but given our evidence suggesting that Basigin does not interact with Integrin, its most likely *cis*-binding partner, in regulating dendrite morphogenesis, this is unlikely. An alternate explanation I favor is that neurons and epithelial cells express distinct Basigin isoforms. Taking into consideration my finding that Bsg-G, the long isoform, is structurally distinct in both extracellular and intracellular regions from the short isoform, this model explains a mechanism by which neuronal Basigin may be distinguished from epithelial Basigin.

Our data does not provide a clear idea of which isoform may be expressed by neurons and this question should be addressed in future studies. Tmod co-immunoprecipitates with Bsg-G, suggesting that Bsg-G may be expressed in neurons. However, it is important to note that the co-immunoprecipitation experiments that produced this data was conducted on S2R+ cells, a derivative of the popular *Drososphila* S2 cell line with a macrophage-like lineage (Guruharsha et al., 2011). Therefore, lack of co-immunoprecipitation between Bsg-D, the short isoform, and Tmod in published data does not necessarily rule out physical interaction between Bsg-D and Tmod in neurons. On the other hand, we found in our rescue experiments that the short isoform was able to rescue the mutant dendrite phenotype of class IV neurons and this ability is dependent on its KRR motif, which is a putative binding site for cytoskeletal adaptor proteins. Therefore, one possibility is that the short isoform of Basigin is the neuron-specific isoform that interacts with Bsg-G, the long isoform, on epithelial cell surface.

Experimental validation of this model of epithelial cell-to-neuron communication can be done by two sets of experiments. First, cell sorting techniques may be used to obtain pure populations of epithelial cells and neurons, and mRNA transcripts of both isoforms may be detected by qRT-PCR. Second, binding between neuronal and epidermal Basigin may be verified by expressing the two isoforms with different epitope tags in the respective cell types and conducting a pull-down assay.

We propose the above model as the most straightforward explanation of our results, but cannot rule out interaction of dendritic Basigin with other molecules on the epithelial cell surface or in the ECM. Our knowledge of binding partners of Basigin is not adequate for us to take non-homophilic binding of Basigin into account in our model. There is evidence for the role of Basigin as a receptor for extracellular cyclophilin A-mediated signaling in vertebrates.

Therefore, investigating whether *ninaA*, the *Drosophila* cyclophilin, exhibits genetic interaction with *basigin* and whether the NinaA protein shows localization along dendrites of da neurons would begin to provide insights to the contribution of non-homophilic interactions of Basigin on dendrite morphogenesis.

Neuronal Basigin as a regulator of actin-based dendrite growth

Our findings that the KRR motif in Basigin is required for its function in neurons and that *basigin* and *tropomodulin* exhibit genetic interaction suggest a role for neuronal Basigin in regulating the actin cytoskeleton of dendrites. Tropomodulin is involved in regulating the length of actin filaments by capping their pointed ends. Previous studies have shown that actin organization is important for neuronal morphogenesis, but how actin filament length is related to dendrite elaboration is largely unclear. Our findings and the model we have proposed also raise questions about how signaling from the epidermis may accomplish coordination of dendrite morphogenesis with substrate growth by regulating the dendritic actin cytoskeleton.

A possible answer to the latter question may lie in the point that higher order branches of class IV neurons, which contribute to the space-filling property of their dendritic arbors, primarily contain actin. Organization of dendritic actin may thus be a means for the epithelial cell substrate to organize dendrite growth to maintain appropriate substrate innervation as the animal grows in size. Along these lines, it is possible that defects in actin reorganization arising from mislocalization of stabilizing factors such as Tropomodulin leads to aberrant elaboration of such higher order branches, which explains the *basigin* loss of function we observed. Thus, it would be of great to interest to examine whether Tropomodulin is mislocalized or localized at reduced

levels in dendrites of *bsg*$^{-/-}$ neurons. Furthermore, expression of Moe::GFP, which is commonly used as a marker for actin dynamics, in *bsg*$^{-/-}$ neurons would permit visualization of changes in actin filament growth and movement that may contribute to defect in developmental elaboration of dendritic arbors.

Regulation of actin stability via interaction with an actin binding protein by a cell-surface molecule, as we have proposed here, is not without precedence. Li and Gao (2003) found that Tropomyosin II, an actin filament-stabilizing protein, is required for restricting dendritic field sizes of class IV neurons in the embryonic PNS and that it interacts genetically with *flamingo,* which encodes a cell surface molecule with extracellular cadherin repeats. However, the phenotypes we observed in *bsg*$^{-/-}$ neurons and *tmod*^{00848} / *bsg*$^{\delta 265}$ trans-heterozygotes are very different from those observed by Li and Gao in *tropomyosin II* mutants, suggesting that Basigin-mediated effects on the actin cytoskeleton may involve mechanisms other than just change in the stability of actin filaments. Activation of Basigin in cultured human fibroblasts has been found to result in activation of the extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated signaling pathway. It would be interesting to examine if changes in activity of the ERK1/2 signaling pathway or other growth pathways occur in *bsg*$^{-/-}$ neurons. Any positive findings on this would lend further support to our model that neuron Basigin functions as a receptor with a signaling function mediated via its cytoplasmic domain.

Possible roles of Basigin in energetics and oxidative stress

In the vertebrate eye, Basigin plays an important role in transporting monocarboxylate transporter 1 (MCT1) to the cell surface (Clamp et al., 2004). In fact, it has been proposed that Basigin and MCT1 form a lactate shuttle complex that is necessary for proper function and

survival of photoreceptors in the eye (Ochrietor and Linser, 2004). A similar finding has also been reported in murine spermatozoa (Mannowetz et al., 2012).

Basigin is also expressed at high levels in the photoreceptors in the *Drosophila* larval eye (Figure 2.1). Although interaction of Basigin with a lactate transporter has not yet been reported in *Drosophila,* Basigin may have similar functions of supplying energy via interactions with a lactate transporter in these photoreceptors. Interestingly, class IV neurons of the larval PNS, which were the focus of our study, have also been shown to function as extra-ocular photoreceptors, allowing larvae to sense light over their entire body and avoid danger (Xiang et al., 2010). Therefore, it is interesting to speculate that Basigin may also pair with a lactate transporter to import lactate (or equivalent energy currency) into class IV neurons. Although these neurons use distinct photo-transduction machinery compared to ocular photoreceptors, high energetic demand may be a common property of photoreceptors in general. In addition, MCTs are also involved in alleviating cellular oxidative stress (Whitaker-Menezes et al., 2011), so another possibility is that Basigin may aid in managing oxidative stress in class IV neurons. Thus, a radically different interpretation of the data we obtained in this study is that the dendrite morphogenesis defects in $bsg^{-/-}$ neurons are caused by unmet energetic needs, or high levels of oxidative stress in those neurons. Given our data showing requirement of its KRR motif and genetic interaction with Tropomodulin, Basigin may be in a position to directly translate status of energy supply and oxidative stress to cytoskeletal changes in dendrites. Considering the large size of their dendritic arbors, it is plausible that class IV neurons have evolved local energy-shuttling mechanisms comprising of Basigin and as yet unidentified ancillary proteins within their expansive dendritic arbors. Available evidence for physical association between Basigin and proton-transporting ATPases such as Vha68-2 and Trap1 (Fig. 4.4) (Guruharsha et al., 2011)

support this idea. Electron micrographs of the larval body wall showing close physical association of class IV dendrites with epithelial cells (Kim et al., 2012) discussed throughout this thesis is also consistent with this hypothetical model.

In addition, energy consumption in *Drosophila* ocular photoreceptors is known to change in different lighting conditions (Okawa et al., 2008). Thus, it would be interesting to examine the extent of dendrite defects in $bsg^{-/-}$ neurons under conditions of differing energetic demands by rearing animals in the dark or on a regular light-dark cycle. In addition, distribution of mitochondria may be different in $bsg^{-/-}$ neurons compared to wild-type neurons if Basigin is indeed involved in energy homeostasis in dendrites. Likewise, possible role of elevated oxidative stress in dendrite morphogenesis defects observed in $bsg^{-/-}$ neurons could be examined by testing whether inhibition of oxidative stress responders, such as components of JNK and fos signaling, in $bsg^{-/-}$ neurons rescues the dendrite defects.

Regardless of the outcome of these experiments, the suggestions for future directions described here underscore the multi-functional nature of Basigin that is being elucidated by multiple approaches in vertebrates and flies. As an example, regulation of MMP expression in stromal cells is widely accepted as the mechanism by which Basigin contributes to invasiveness of tumors. However, a recent study reported that Basigin also controls the energetics of tumors via MCT1/MCT4 (Le Floch et al., 2011). Although the function of Basigin in neurons in a developmental context may be different from its role in tumors, this example points to a need for caution in interpreting the results of knocking out a multi-functional protein such as Basigin.

Possible role of Basigin in neuronal morphogenesis in vertebrates

As described before, Basigin is conserved from flies to mice and humans, and is expressed in neurons in the mouse brain (Fig. 5.2 A). Given the findings of our study and previous characterization of its role in cytoskeletal organization, cell-cell interactions, and regulation of MMPs in other studies, it will be very interesting to determine whether Basigin is required for proper neuronal morphogenesis in vertebrate neurons as well. Consistent with this prediction, brains of *basigin*$^{-/-}$ mice have been reported to display gross morphogenetic defects, particularly in the retina, and impaired olfactory learning and short-term memory.

We conducted a preliminary analysis of the role of Basigin in dendrite morphogenesis of vertebrate neurons by comparing the dendrites of hippocampal pyramidal neurons in a postnatal day 18 *basigin*$^{-/-}$ mouse brain and its age-matched control. As shown in Figure 5.2, we observed striking disorganization of dendrites in the *basigin*$^{-/-}$ mouse brain, revealed by α-calbindin staining, relative to that of the control mouse. Staining with an antibody against neurofilament revealed differences in morphology of axons traversing the region containing these dendrites, suggesting possible role of Basigin in axon development as well (Fig. 5.2 B). Following up on this preliminary result to characterize defects in dendrite morphogenesis of neurons in the *basigin*$^{-/-}$ mouse brain would be an important next step in understanding the conserved role of Basigin in neuronal morphogenesis.

Figure 5.1: A working model of mechanism of Basigin-mediated regulation of dendrite morphogenesis

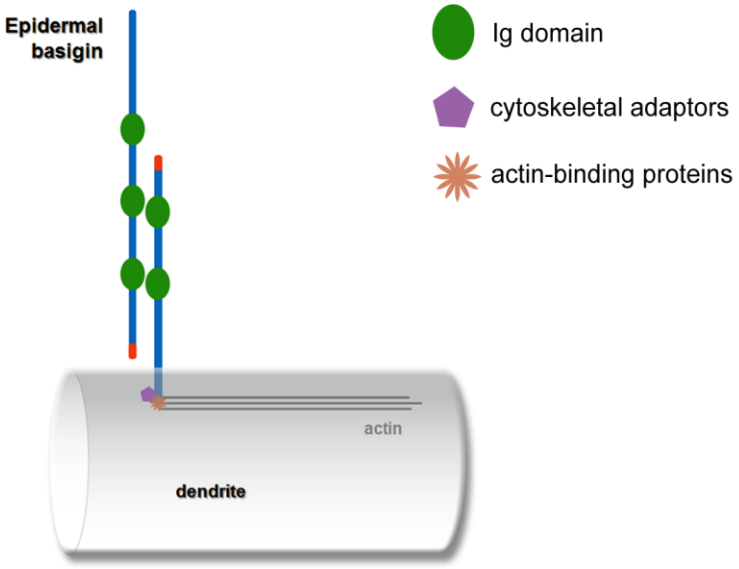


Figure 5.1: A working model of mechanism of Basigin-mediated regulation of dendrite morphogenesis

A working model of the putative mechanism by which Basigin mediates morphogenesis of class IV dendrites is presented. Basigin on the surfaces of epithelial cells and neurons interact homophilically. Through its interaction with cytoskeletal adaptor proteins, which cross-link cell-surface molecules with cellular cytoskeleton (e.g., proteins of the ERM family), dendritic Basigin then organizes actin cytoskeleton within dendrites. In this model, Basigin mediates epithelial-cell-to-neuron communication that is important for morphogenesis of neuronal dendrites. Although we favor tissue-specific expression of Basigin isoforms as shown in the figure, *trans* interactions between identical isoforms would also be consistent with this model.

Figure 5.2: Preliminary data reveal abnormal dendrite morphology of hippocampal neurons in the *Basigin*$^{-/-}$ mouse brain

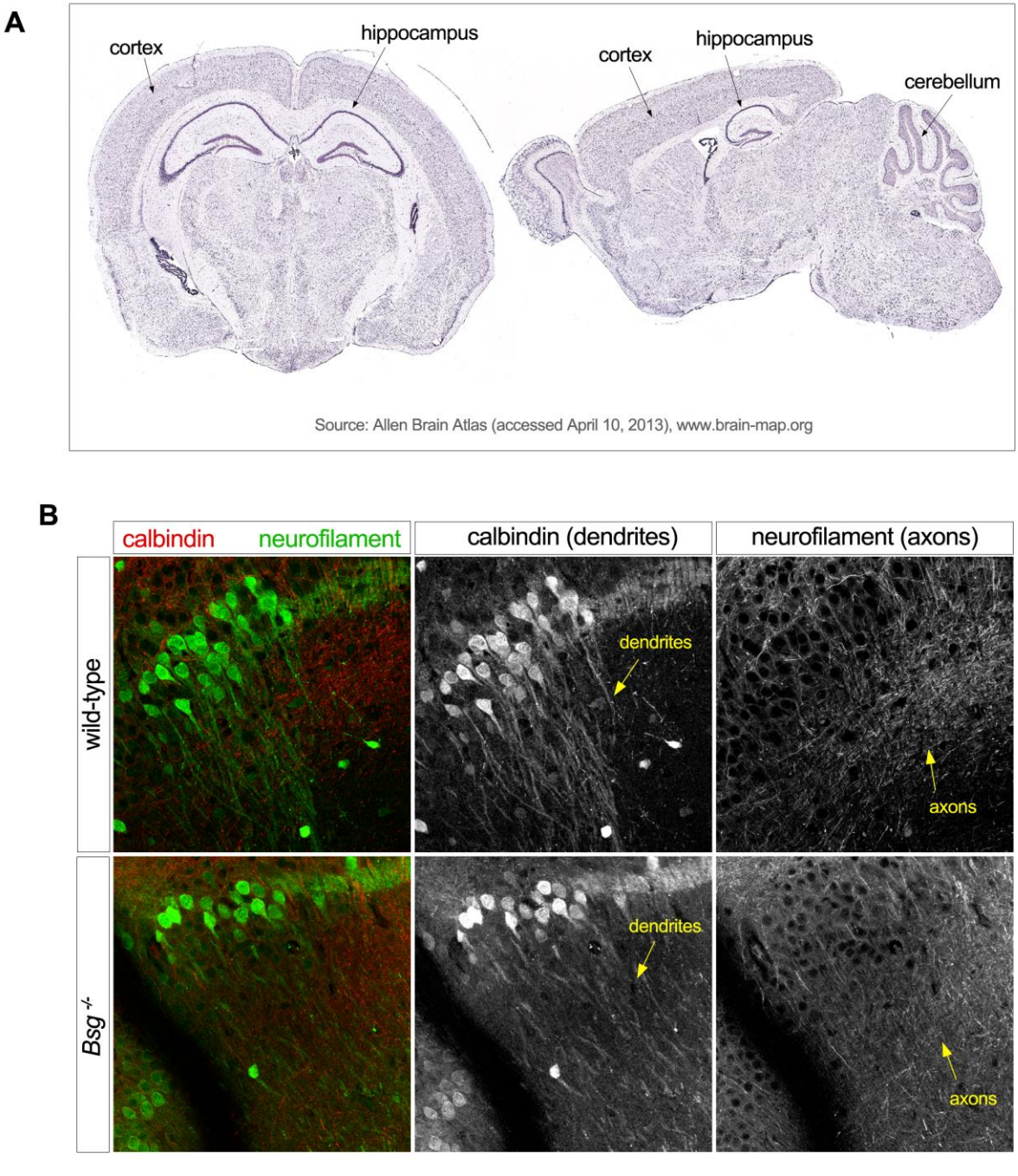


Figure 5.2: Preliminary data reveal abnormal dendrite morphology of hippocampal neurons in the *Basigin*$^{-/-}$ mouse brain

(A) *In situ* hybridization data of the mouse brain (56 days old, Male, C57BL/6J) show expression of Basigin in neurons in the cortex, hippocampus and cerebellum. Coronal (left) and sagittal (right) sections are shown. Images were obtained from the Allen Brain Atlas (accessed on April 10, 2013), www.brain-map.org

(B) Sections of postnatal day 18 wild-type and *Basigin*$^{-/-}$ mouse brains stained with α-calbindin and α-neurofilament antibodies, to label dendrites and axons, respectively, following standard immunohistochemistry protocols. Images show CA2 pyramidal neurons of the hippocampus. Dendrites in the *Basigin*$^{-/-}$ mouse brain (N=1) appear more disheveled and fragmented compared to those in the wild-type mouse brain (N=1). Similarly, axons traversing the dendritic layer appear less dense and more fragmented in the *Basigin*$^{-/-}$ mouse brain.

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Appendix 1

Detailed result of the expression pattern-based screen described in Chapter II.

The following symbols and abbreviations have been used in the table below:

+, expression

c1, class I neurons

c4, class IV neurons

CNS, central nervous system

djc, dendrite junction complex

e, epidermis

eb, epidermal cell border

hv, homozygous viable

id, imaginal discs

m, muscles

NA, not available

NMJ, neuromuscular junction

PNS, peripheral nervous system

uc, ubiquitous cytoplasmic

un, ubiquitous nuclear

Gene	Line ID	Insertion site	Chr	Viability	PNS nucleus	PNS soma	PNS dendrite	epidermis	Comments
14-3-3epsilon	CA06506	14068962	3R	lethal					un
26-29-p	CA06735	13987313	3L	hv		+	+		CNS,id
Acon	CC00758	21169155	2L	lethal		+			id, m
Actn	CC01961	1927332	X	hv					m
Aldh-III	ZCL2060	3361543	2R	hv					un
Alh	CC01367	2935653	3R	hv					un
apt	CC01392	19468319	2R	hv					CNS
arg	CB03579	417662	X	hv	+	+			CNS,m
Argk	CB05492	9051633	3L	lethal	+	+	+		
Argk	CB03789	9056078	3L	lethal		+	+		m, punctate in dendrites
Asph	ZCL1605	11998586	2R			+			m, CNS, higher in lateral chordotonal organs and c1
Atpalpha	CC00319	16783418	3R	lethal				+	eb
baz	CC01941	17072582	X	hv				+	m,eb
bel	CC00869	4485350	3R	hv		+	+	+	CNS, uc
betaTub56D	CC02069	15338563	2R	lethal		+		+	m
bl	YD0788	16478112	2R	hv					un, NMJ
Bsg	CA06978	8104393	2L	hv		+	+	+	eb, NMJ, djc
Btk29A	YD0649	8276990	2L	lethal		+		+	NMJ, m
bun	CB03431	12482943	2L	hv					un
Cam	CC00814	8149270	2R	lethal					un
CAP	CA06924	6190378	2R	hv		+	+	+	eb,m
Cat	CC00907	18815951	3L	lethal					non-specific
ced-6	CA07185	5331863	2R	hv					NMJ
cg	CC01469	10063184	2R	hv					un
CG10383	YB0066	18694243	2L	hv					NMJ, midline glia
CG10576	YD0846	5758328	3L	hv					none
CG10677	G00108	NA				+		+	uc
CG10686	G00140	12508921	3L			+		+	uc
CG10724	CA07499	13406231	3L	hv		+		+	uc
CG10927	ZCL2856	14500808	2R						un, nucleoli, aberrant dendrites
CG10990	G00093	13641443	X			+		+	CNS, m
CG1104	ZCL1943	2901123	3R			+		+	uc
CG11138	CA06844	12472570	X	hv					un
CG11255	CB04917	13015302	3L	hv		+		+	CNS
CG11266	CC01391	7033157	2L	hv					un
CG12094	YD0050	10022298	X	hv					non-specific
CG12163	CC00625	1076935	3R	hv		+	+		m
CG12785	CC06135	12098103	3R	lethal					m
CG13624	YD0205	20395356	3R	sl				+	m, midline glia
CG1440	CA07287	8331699	X	hv					un
CG14648	CA06610	229231	3R	hv				+	
CG14656	CA06996	624015	3R	lethal	+	+		+	perinuclear and nuclear
CG15926	CB04063	12365107	X	hv		+	+	+	eb, djc
CG1600	CB03410	3416244	2R	hv				+	m
CG17273	CC01294	16654986	3R	lethal		+	+	+	m,CNS
CG17618	YB0120DE	18553292	3R			+			CNS
CG17646	CB02833	1737431	2L	hv				+	

Gene	Line ID	Insertion site	Chr	Viability	PNS nucleus	PNS soma	PNS dendrite	epidermis	Comments
CG1888	CB02075	5434043	2R	hv					un
CG1910	CC01491	27573862	3R	hv					un
CG3036	CA06801	4894157	2L	hv		+	+	+	eb
CG31012	CA06686	26640622	3R	hv					m,CNS
CG31529	YD1073	630672	3R	lethal		+		+	CNS
CG31782	YD0213	16707239	2L	hv		+		+	strong at chordotonal attachment sites
CG32062	CC00511	10515608	3L	hv		+			m, CNS
CG32479	CA06614	882041	3L	lethal		+		+	CNS,m
CG32486	C00904	3060297	3L	hv					un
CG32549	G00102	18299348	X		+	+		+	m, nuclear in some PNS and CNS
CG32560	CA06772	17632469	X	hv		+			
CG33070	YD0758	6986121	X	hv		+		+	CNS, NMJ, m
CG33936	CC01586	5176796	3R	lethal					none
CG3810	CA07694	1802294	X	hv					none
CG3921	YD1046	3799438	2L	lethal		+			CNS, NMJ, m
CG3939	CA07562	3069871	X	hv					cells along trachea
CG4743	YD1015	21505270	3R	lethal					CNS, oenocytes
CG5059	CA06926	20509742	3L	hv		+	+	+	uc, eb
CG5174	CA07176	14309292	2R	hv		+		+	eb, CNS,m
CG5392	BA00207	15001227	3L			+			CNS,m,NMJ
CG6084	G00112	NA	3L		+			+	m, CNS
CG6330	CB03223	22779020	3R	lethal		+	+		un
CG6416	CC00858	8627040	3L	lethal					m
CG6424	CC00677	13604099	2R	hv		+	+		m
CG6783	CA06960	7392313	3R	hv					m,CNS
CG6852	YD0130	18862808	3L	hv		+	+	+	NMJ, m
CG6854	CA07332	15099355	3L	lethal				+	m
CG6930	CA06556	7597410	3R	hv					un
CG6945	CC00864	15088263	3L	lethal					none
CG7185	CC00645	8308089	3L	hv					un
CG7201	YB0115	8284218	3L	hv					un
CG7484	CB04101	17647286	3L	hv					un
CG7986	YC0069DE	8180070	3L						un
CG8036	YB0146	4496705	3R	lethal		+			
CG8209	CB02086	7969693	3L	hv					un
CG8213	BA00169	4863309	2R						NMJ
CG8291	YD0259	11809438	2R	hv					CNS midline, m
CG8443	CA06604	12075198	2R	hv				+	m
CG8552	CA07352	8159580	2L	hv				+	m, possible ER localization
CG8583	CA06603	7354388	3L	hv		+		+	uc
CG8920	C00825	16208199	2R	hv					none
CG9331	CB04962	20824559	2L	hv	+				varying levels in PNS
CG9772	CB02188	163496	3R	hv					un
CG9796	CC00817	9226998	3R	hv		+		+	m, possible ER localization
charybde	G00378	12106602	3L			+		+	CNS, NMJ, m
Crc	CA06507	5456158	3R	hv		+		+	CNS, id
crp	CB03073	16280297	2L	hv	+				
CycB	CC01846	18694008	2R	hv				+	id, glia

Gene	Line ID	Insertion site	Chr	Viability	PNS nucleus	PNS soma	PNS dendrite	epidermis	Comments
D1	YB0011	5065738	3R	hv					un, nucleoli
Dek	CC00921	12743940	2R	hv					un
Dek	CA06616	12744777	2R	hv					un
desat1	CC01694	8269738	3R	hv					uc
dlg1	CC01936	11286274	X	hv		+	+	+	eb, NMJ
DLP	CA06573	6480860	2L	hv	+	+			only soma in c4
dnc	G00219	3182884	X		+			+	CNS, m
Doa	CB03889	24717483	3R	hv		+	+		m, CNS
dom	BA00164	17211471	2R						un
Dp	CA06594	9111298	2R	hv					un
drl	CC00251	19190343	2L	hv					none
Ef2b	CC01924	21681694	2L	lethal		+	+	+	
eff	CC01915	10565092	3R	lethal				+	m, CNS
eIF-2beta	CC06208	12519527	3L	lethal					
eIF3-S9	CB04769	13423919	2R	hv		+			m, CNS, strongest c1
eIF-4a	CB03721	5982474	2L	lethal					un
eIF-4E	CC00392	9395078	3L	hv				+	m
eIF-5A	BA00155	19945688	2R					+	m
eIF-5C	BA00280	1425192	3R						none
Eip63E	CA06742	3569399	3L	hv	+	+	+	+	eb, m, CNS
eRF1	CB03931	20342600	3L	lethal					
Fas3	G00258	18333873	2L			+	+	+	CNS, eb
Fer1HCH	CA06503	26212791	3R	lethal				+	m
Fer2LCH	CA07607	26215006	3R	lethal					m, CNS
Fim	CC01493	17185787	X	hv		+		+	
Fkbp13	CA07340	17385064	2R	hv					m, CNS
for	YC0045LE	3633137	2L	l				+	NMJ, CNS, m, dendrite phenotype
Fpps	CB04937	7194698	2R	hv					uc
Fs(2)Ket	CA07301	20735659	2L	hv	+	+	+	+	un
ftz-f1	YB0007	18764815	3L	l					un
Gdi	CA07108	9493967	2L	lethal		+	+	+	strong at muscle attachment
gish	CB02804	12106314	3R	hv		+	+		
GlcAT-S	CA07168	9616795	2L	hv					none
Gli	CB02989	15762784	2L	hv		+	+		NMJ, CNS
G-oalpha47A	CA06658	6331783	2R	semi-lethal		+	+		glia,
gp210	CC00195	1647884	2R	hv		+			m
Gpdh	YC0027	5944738	2L						CNS, m
HDAC4	CA07134	13172850	X	hv		+			m, CNS, glia
heph	CC00664	27763272	3R	lethal					m
His2Av	CC00358	22693293	3R	lethal					un, NMJ
homer	CB02121	6722933	2L	hv		+			un
how	CA07414	17881934	3R	lethal					un
Hrb98DE	CC01563	24425969	3R	hv					un
Hs6st	YD0159	15778130	3R	hv					CNS
Imp	CB04573	10702676	X	hv		+	+	+	perinuclear
Indy	CC00377	18833638	3L	hv				+	
inx7	CB04539	6892590	X	hv					un
jumu	CC00294	6182245	3R	hv	+				CNS, id

Gene	Line ID	Insertion site	Chr	Viability	PNS nucleus	PNS soma	PNS dendrite	epidermis	Comments
Jupiter	ZCL2129	7430657	3R			+	+		only in Cut-negative neurons
kay	CC01156	25608214	3R	hv					un
kek1	CB02190	12822834	2L	hv				+	id, NMJ
kis	CC01466	220632	2L	hv		+	+	+	CNS, m
kis	CC00801	221924	2L	hv					un
L	G00255	10369386	2R		+			+	CNS, m, eb
l(1)G0084	CC01368	19525602	X	hv					un
l(1)G0168	CC00492	15393000	X	hv		+			m
l(1)G0320	CA06684	9447368	X	hv		+		+	
l(2)08717	CA06962	14688632	2R	hv		+	+	+	eb, djc
l(2)k01209	YB0218	13308856	2R	hv	+			+	un, high in c1
l(3)02640	CA07460	1336285	3L	lethal	+				different levels
l(3)82Fd	CA07520	1123169	3R	lethal		+			different levels, high in c1
LamC	CB04957	10462132	2R	lethal					un
larp	CC06230	24152038	3R	hv		+		+	
Lasp	YC0059	16693136	3L	l		+			high in CNS midline and chordotonal organ
Lsd-2	CA07051	14969607	X	hv		+	+		m
M6	CA06602	21502447	3L	hv		+	+	+	NOS-like pattern, CNS, id, m
Map205	CC00109	27891553	3R	hv		+	+		m
Mdh	CB04968	22978192	3R	hv					m
me31B	CB05282	10240237	2L	hv					un
Men	CC06325	8545125	3R	hv		+		+	CNS, m
Mhc	YD0783	16768672	2L	l					m
Mi-2	CA06598	19901556	3L	lethal					un
Mio	YD1036	21307825	2L	l		+		+	CNS, NMJ, m,
mip120	P02006	9395080	2R						un
Mob1	CB04396	11546502	3L	hv					un
mod(mdg4)	CA07012	17200382	3R	hv					m
mub	CC01995	21909824	3L	hv	+			+	soma of epidermis
NetB	BA00253	14596460	X	hv				+	m
neur	YD1143	4859203	3R	l		+	+	+	CNS, m, dendrites of c1 only
NFAT	CA07788	13534781	X	hv					un
Nlp	CC01224	25831370	3R	hv		+	+	+	m
Nmdmc	CB02647	4873684	3R	hv		+	+		+
Nrg	G00305	8443451	X			+	+	+	CNS, eb, peripheral glia, djc
nrv2	ZCL2903	6792118	2L			+		+	CNS, eb, neuron soma edges, peripheral glia
Nrx-IV	CA06597	12141797	3L	hv		+	+	+	glia, eb, djc
oaf	ZCL3245	2494476	2L						peripheral glia, CNS, m
Oda	CB03751	8060372	2R	hv					un
osa	CC00445	13529472	3R	lethal					un
Pabp2	CC00380	4019736	2R	hv					un, faint c4
Past1	CB02132	8523601	3R	hv					un, NMJ
Pde8	CA07101	19554469	2R	hv				+	NMJ, eb
Pdi	CA06526	15134669	3L	lethal		+		+	uc
Pdp1	CB02246	7847944	3L	hv					un
Picot	CA07474	12548787	2R	lethal					un
Pkn	CC01654	5157995	2R	hv		+		+	CNS, id

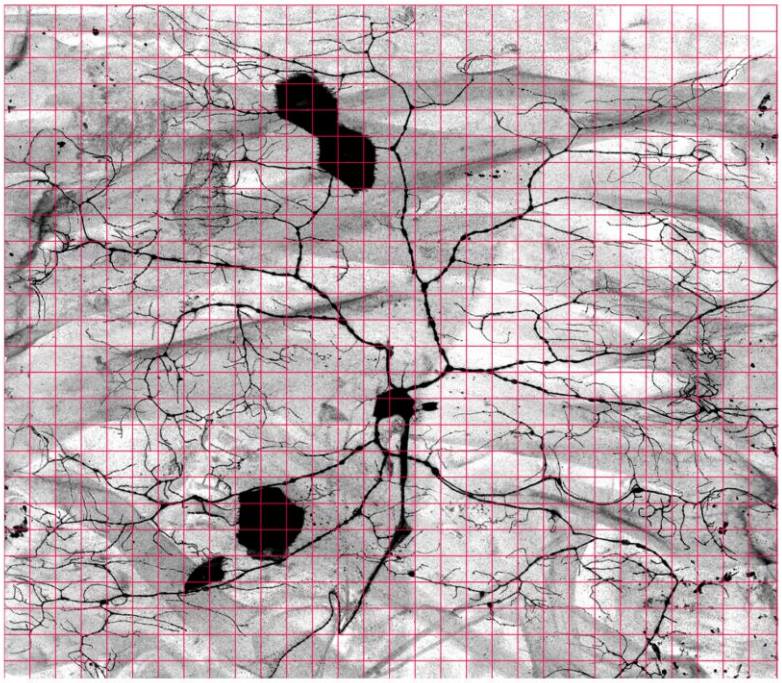
Gene	Line ID	Insertion site	Chr	Viability	PNS nucleus	PNS soma	PNS dendrite	epidermis	Comments
Pli	CB03040	19712573	3R	hv					un
psq	CC01645	NA	2R	lethal					m
Ptp10D	CC06344	11538719	X	hv				+	m, perinuclear in epidermis
pUf68	CA06961	1501291	3L	hv					un, faint c4
pum	CC00479	4983771	3R	lethal		+			CNS, m
Rab11	CA07717	16937950	3R	lethal		+		+	m,CNS
Rab2	CA07465	2584592	2R	lethal					un, no PNS
Rho1	ZCL1957	11991871	2R			+	+	+	CNS, eb, m
Rm62	CB02119	1833559	3R	Mfsterile	+	+	+		varying nuclear/soma
RpL13A	CC01920	1449810	3R	hv		+	+		NMJ, m
Rtnl1	CA06523	5000772	2L	hv		+		+	uc
Rtnl1	CA06547	4997815	2L	hv		+		+	uc
S6k	CC01583	5802962	3L	hv		+		+	
Sap-r	CA07241	26714539	3R	hv		+			
sar1	CA07674	18184952	3R	lethal		+	+	+	m, e, NMJ, CNS
scrib	CA07683	22393784	3R	hv			+	+	djc, eb, glia
sd	CA07575	15712098	X	hv	+				un,CNS, strongest in c3
Sdc	CC00871	17362946	2R	hv		+	+	+	CNS,eb, djc
Sec61alpha	CC00735	6479544	2L	lethal		+	+	+	m, NMJ, e, CNS
Sema-2a	CA06989	12411885	2R	hv				+	chordotonal organ attachment sites
sgg	CA06683	2536739	X	hv		+		+	m, CNS
Sh3beta	CC01823	7361764	3L	lethal		+		+	uc
shot	YD0728	9753390	2R	l					none, dendrite phenotype
Sin	CC01921	21024944	3L	lethal		+	+		NMJ
sls	CA06744	2107593	3L	hv					m
sm	CC00233	15504045	2R	hv		+	+		perinuclear
smi21F	CA07211	1119094	2L	hv					none
sno	CC01032	13104608	X	hv					un
snRNP69D	CB02932	12727708	3L	lethal	+	+			un, m, id, CNS
sop	CB02294	9897503	2L	lethal		+		+	perinuclear
spdo	YC0019	26389435	3R	hv		+	+	+	m, CNS, djc
SPoCk	CA06644	22766451	3L	hv					CNS, m, e
Spt6	CA07692	6162464	X	hv					un
sqd	CB02655	9470746	3R	hv		+	+		id, NMJ
sqd	YB0060	9471747	3R	hv					un, dendrite phenotype
stg	YD0246	25079922	3R					+	CNS, id (mitotically active cells)
stwl	CA07249	14402679	3L	lethal				+	m, nuclear
Su(var)2-10	CC02013	5004591	2R	hv					un
Surf4	CC01684	11171291	3R	lethal				+	CNS
sws	CC01711	7861288	X	hv					un
Sxl	CB05562	6986034	X	semi-lethal	+	+		+	non-specific
tara	YD0165	12068799	3R	l		+		+	CNS, m
TER94	CB04973	5877016	2R	hv		+		+	m, CNS
Tm1	CC00578	11117364	3R	lethal					m
tmod	CC00416	26389239	3R	hv					NMJ
tmod	CA07346	26400579	3R	lethal		+	+	+	eb, m, NMJ, id
Tpr2	YB0112	16470708	2L	hv		+		+	CNS, m, eb
tra2	CC01925	10491357	2R	hv					un

Gene	Line ID	Insertion site	Chr	Viability	PNS nucleus	PNS soma	PNS dendrite	epidermis	Comments
tral	CA06517	12508905	3L	lethal					un
trol	ZCL1700	2412207	X						ecm
trxr-1	CA06750	8137659	X	hv					m
Tsp42Ee	CC01420	2903327	2R	hv		+			m, CNS, varying levels in PNS
Tsp96F	CC01830	21707093	3R	lethal					m, CNS
Tudor-SN	CC00737	262842	3L	hv		+		+	uc
tun	CC00482	11681495	2R	hv					m
twin	CA06641	20044629	3R	hv		+		+	
Uev1A	CA07496	5358821	3L	lethal		+		+	muscle attachment site, c4 nuclear
VAChT	CA06666	14538229	3R	lethal		+	+		CNS, stronger in Cut-negative neurons
Vha13	CA07644	15469816	3R	lethal					gut
Vha16	CA06708	2518996	2R	lethal				+	perinculear
Vha26	CC01380	1417892	3R	lethal		+			m
Vha55	CA07634	8452738	3R	lethal					trachea
vib	CB05330	15045962	3R	lethal				+	m
vkg	CC00791	5019005	2L	hv		+	+	+	djc
vsg	CA07004	9707771	3L	hv		+			ecm
xl6	CB03248	6918786	2L	hv	+			+	weaker in c4
zip	CC01626	20896845	2R	lethal					un
Zn72D	CA07703	16102655	3L	hv	+				cytoplasmic in c4

Appendix 2

Determination of dendritic coverage factor (CF)

Coverage factor (CF) was determined by overlaying a grid on the dendritic arbor and counting the number of squares containing dendrites relative to the total number of squares over the dendritic field. Grid size varied with total dendritic field area as indicated below.



$$square\ area = \sqrt{dendritic\ field\ area}$$

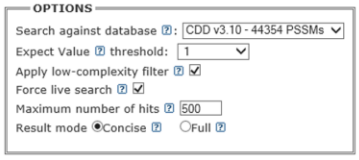
$$Coverage\ factor\ (CF) = \frac{\#\ of\ squares\ containing\ dendrites}{total\ \#\ of\ squares\ covering\ the\ dendritic\ field}$$

Appendix 3

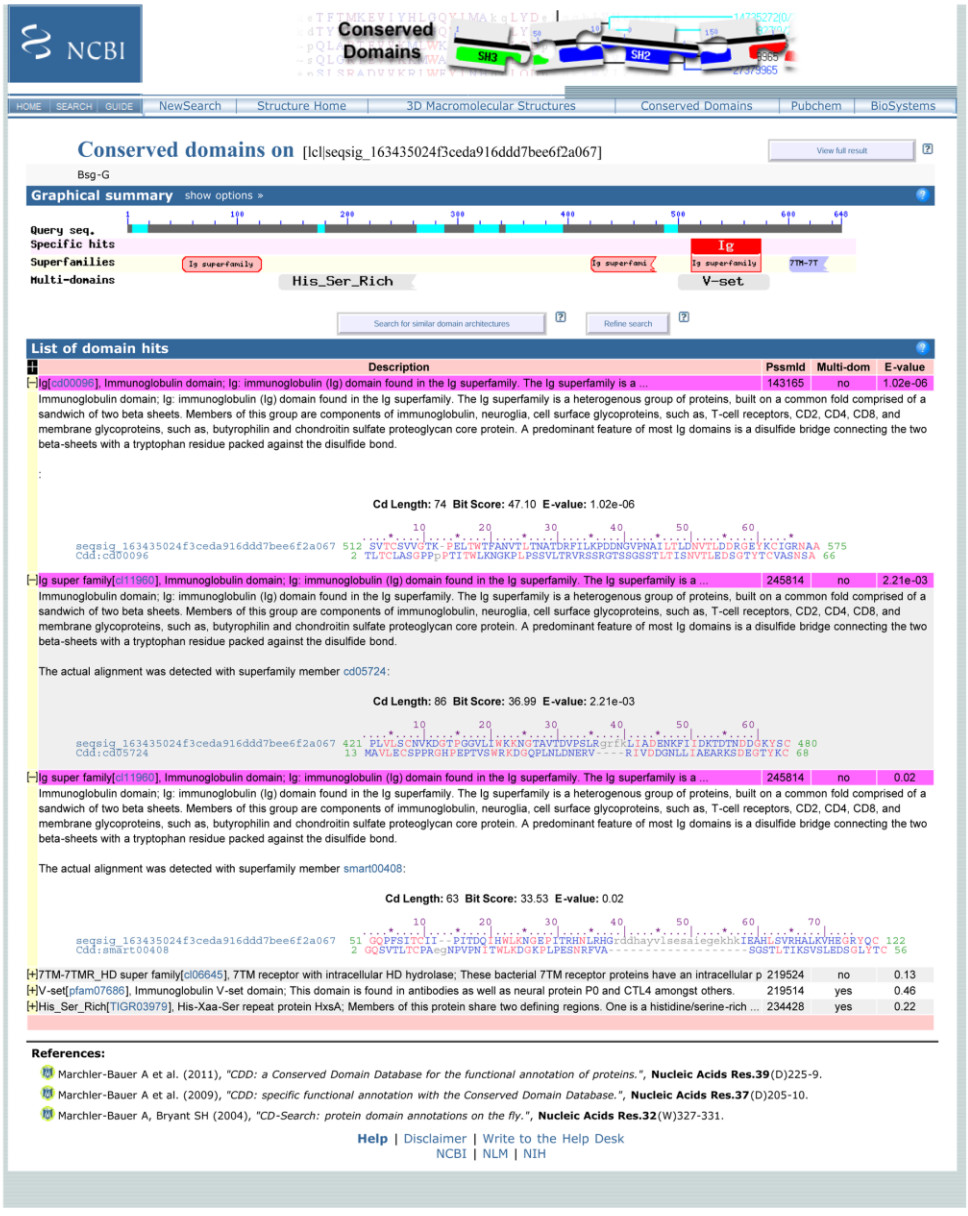
Conserved domains in the long isoform of Basigin (Bsg-G)

Conserved domains in Bsg-G were searched using the NCBI Conserved Domain Search tool available at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

Search options were set as shown to the right.



NCBI Conserved Domain Search

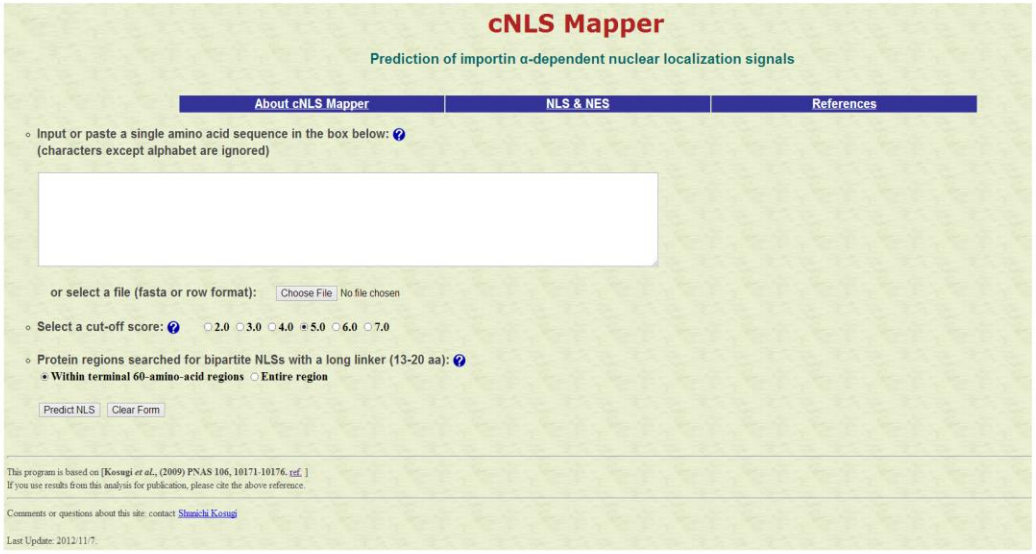


http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi[4/20/2013 10:40:56 AM]

Appendix 4

C-terminus of Bsg-G contains a putative nuclear localization signal

A web-based nuclear localization signal prediction tool was used as shown below.
http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi (accessed on 2 March, 2013)



cNLS Mapper

Prediction of importin α-dependent nuclear localization signals

About cNLS Mapper | NLS & NES | References

- Input or paste a single amino acid sequence in the box below:
  (characters except alphabet are ignored)

or select a file (fasta or row format): Choose File No file chosen

- Select a cut-off score: 2.0 3.0 4.0 5.0 6.0 7.0
- Protein regions searched for bipartite NLSs with a long linker (13-20 aa):
  Within terminal 60-amino-acid regions Entire region

Predict NLS Clear Form

This program is based on [Kosugi *et al.*, (2009) PNAS 106, 10171-10176, ref.]
If you use results from this analysis for publication, please cite the above reference.

Comments or questions about this site: contact Shunichi Kosugi

Last Update: 2012/11/7.

Result generated by the cNLS Mapper program:

cNLS Mapper Result

Predicted NLSs in query sequence

```
MEAKFLASALSFLSIFLAIYAQSLANDLSKESTEFEESPTIYYGDPVVNL 50
GQPFSITCIIPITDQIHWLKNGEPITRHNLRHGRDDHAYVLSESAIEGEK 100
HKIEAHLSVRHALKVHEGRYQCNRRRGSYILHVRDPKGVGAGAGEPTESG 150
YQTIDELTPNSADDFFTRAWLEQQQQQQQLPHQSHKLHKSHLGYGNASLS 200
GSQPWHPSAGGGGIHRVYSATPPDFPPPRLNLLEQTVAPPEPPTILYNPN 250
PTHPTASATATETSVLLTTAHHHAHHQQQLQQQSQHTLNAFQLPLPPRPN 300
PGQNERYQTYAPHYVPPVVVSGAGAGAGADPGAGASGEQTTISAATSTRA 350
MMGGGGGVAGAGFSAGASGPMLGAGGHMLMGGQGHQVHLQHQTLLPVKMD 400
KLVPNYDNAEHQMKFYDIRSPLVLSCNVKDGTPGGVLIWKKNGTAVTDVP 450
SLRGRFKLIADENKFIIDKTDTNDDGKYSCEFDGVSKEIEVIARVVVRVP 500
SNTAVVEGEKMSVTCSVVGTKPELTWTFANVTLTNATDRFILKPDDNGVP 550
NAILTLDNVTLDDRGEYKCIGRNAANVYGGNTTTPASDVTTVRVKGKFAA 600
LWPFLGICAEVLILCIIILIYEKRRNKSELEESDTDPQEQKKKRRNYD   648
```

Predicted monopartite NLS		
Pos.	Sequence	Score
639	EQKKKRRNYD	6

Predicted bipartite NLS		
Pos.	Sequence	Score
621	YEKRRNKSELEESDTDPQEQKKKRRN	7.1
621	YEKRRNKSELEESDTDPQEQKKKRRN	7.7
621	YEKRRNKSELEESDTDPQEQKKKRRNYD	8.4
620	IYEKRRNKSELEESDTDPQEQKKKRRNYD	5.6
622	EKRRNKSELEESDTDPQEQKKKRRNYD	5.2