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Methods for Exploring the Genetic Control of Sensory Neuron Dendrite Morphogenesis in *Drosophila*

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INTRODUCTION

Dendrite morphological diversity helps to define the properties of neural circuits by influencing circuit organization and information processing. Dendrite development has been shown to be driven by a combination of cell-intrinsic and -extrinsic factors. However, much remains to be discovered about the cellular and molecular mechanisms that give rise to unique yet highly stereotyped dendrite arbors of diverse neuronal types. Work in the last decade has established the *Drosophila* system as an excellent model for studies of dendrite morphogenesis. Work has been performed primarily in three systems: the dendritic arborization sensory neurons, the adult olfactory system, and the embryonic and larval motor system. Each of these systems offers a manageable number of neurons that are easily accessible, both optically and physically, and can be genetically manipulated to address a broad range of questions regarding dendrite development and patterning. This article provides a brief overview of the organization of these systems together with aspects of dendrite development that have been elucidated in recent years and methods for studying dendrite morphogenesis in embryonic and larval sensory neurons.

RELATED INFORMATION

Protocols are available for Analysis of Dendrite Development in *Drosophila* Embryos<prot5658> (Shrestha and Grueber 2011a) and Generation and Staining of MARCM Clones in *Drosophila*<prot5659> (Shrestha and Grueber 2011b).

BACKGROUND

Neurons are highly polarized cells that consist of dendrites, which are specialized to receive and process inputs, and axons, which are specialized to transfer information to other neurons or nonneuronal cells. In drawings of Golgi-stained neurons first published more than a century ago, Santiago Ramón y Cajal illustrated the critical differences in morphology between dendrites and axons as well as the tremendous diversity in dendritic arborization patterns (Ramón y Cajal 1995). Addressing how diversity in dendritic morphology arises during development was prohibitively difficult in *Drosophila* until the last decade or so. Two major technical achievements occurred that opened the door to detailed genetic analysis of dendrite patterning, and these continue to be critical methodologies common to nearly all ensuing studies. First, live imaging strategies were established for carrying out large-scale forward genetic screens for genes regulating dendrite development in embryonic stages (Gao et al. 1999). This screening strategy and subsequent screens using analogous methodology have led to the identification of hundreds of new candidate loci affecting dendrite morphology (Gao et al. 1999; Parrish et al. 2006; Grueber et al. 2007; Zheng et al. 2008). Second, the mosaic analysis with a repressible cell marker (MARCM) approach was developed, which permits resolution of dendrites at a single-cell level and genetic manipulation of individual neurons to assess gene function during neuronal morphogenesis

(Lee and Luo 1999; Wu and Luo 2006). This system has led to rapid progress in understanding the cell-autonomous basis of dendritic morphological development. In recent years, scientists have begun to identify the factors that determine the complexities and shapes of particular dendritic arbors, thus piecing together the molecular codes that generate different arbor types. Below, we provide brief introductions to the different neuronal systems being studied in *Drosophila*, as we discuss key transcription factors, cell adhesion molecules, and signaling proteins that are known to be important for dendrite morphogenesis in those neurons.

MAJOR SYSTEMS IN WHICH DENDRITE MORPHOGENESIS IS STUDIED IN *DROSOPHILA*

Multidendritic Neurons of the Larval Peripheral Nervous System

Forward genetic screens for dendrite morphology mutants were first performed using the multidendritic (md) group of neurons that lie just beneath the larval body wall. The key experimental advantage of this group of neurons is their accessibility for immunostaining, imaging, and genetic manipulation. The md neurons comprise about half of the 45 sensory neurons in each abdominal hemisegment of *Drosophila* embryos and larvae, the other major types being the external sensory (es) neurons and chordotonal (ch) neurons. Each es and ch neuron has a single unbranched dendrite, whereas md neurons have more highly branched arborizations. The md neurons have been categorized into three subtypes based on their morphology and substrate: The most numerous are the dendritic arborization (da) neurons, but, in addition, there are bipolar dendrite (bd) neurons and tracheal dendrite (td) neurons (Bodmer et al. 1987). The 15 da neurons in each hemisegment are further divided into classes I–IV based on the increasing complexities of their dendritic arbors (Grueber et al. 2002). The diversity in their dendrite arborization patterns and their accessible position along the body wall make the da neurons of the larval peripheral nervous system (PNS) an excellent system for studying the control of dendrite branching and territory formation (Gao et al. 1999; Sweeney et al. 2002; Grueber et al. 2003b; Orgogozo and Grueber 2005; Corty et al. 2009).

Olfactory Projection Neurons

The mechanisms of dendrite development have been studied extensively in the context of olfactory circuit formation. Olfactory information received by olfactory receptor neurons (ORNs) in antennae and maxillary palps is relayed to projection neurons (PNs) at the antennal lobe (AL). At the AL, ORN axons synapse with PN dendrites at approximately 50 discrete glomeruli. There are three major lineages arranged around the AL that produce adult PNs: the anterodorsal (adPNs), lateral (IPNs), and ventral (vPNs) lineages. For information to be transferred properly at the AL, PNs from these lineages must project dendrites to distinct and stereotyped glomeruli. Developmental studies indicate that dendrites project to protoglomeruli before ORN axon arrival, suggesting an important role for dendrite targeting in AL wiring (Jefferis et al. 2004). Dendrite targeting has been shown to depend on three major mechanisms: (1) graded cues that determine general dendrite positioning in the AL (Komiyama et al. 2007); (2) intrinsic combinatorial codes of transcription factors that impact precise glomerular targeting (Komiyama et al. 2003; Komiyama and Luo 2007); and (3) dendrite–dendrite interactions that determine glomerular coverage (Zhu and Luo 2004; Zhu et al. 2006).

Motor Neurons

The *Drosophila* embryonic motor system is comprised of a segmentally repeated array of 36 motor neurons per half-segment of the central nervous system (CNS) (Landgraf and Thor

2006). Motor neuron dendrites are situated in the dorsal region of the neuropil segregated from the sensory afferents that project to more ventral regions. Pairwise retrograde labeling of motor neurons revealed that the dendrites of motor neurons are arranged in a “myotopic map,” in which dendritic territories represent centrally the location of cognate axon terminals in the peripheral muscle field (Landgraf et al. 2003). Different types of motor neurons show distinct responses to midline cues. Some dendritic arbors remain ipsilateral, some project to the contralateral neuropil, and others have both ipsilateral and contralateral arborizations. The motor system has thus provided important insights into how dendritic targeting is regulated by midline-derived guidance signals.

MOLECULAR CONTROL OF DENDRITE MORPHOGENESIS

Dendrite Branching Diversity

Genetic screens and studies of candidate genes have begun to reveal how dendrites develop characteristic branching morphologies. Sensory neurons generate either single-dendrite or multiple-dendrite morphologies in the PNS. The alternate dendrite morphologies appear to be specified by the gene *hamlet* (*ham*), which encodes a zinc-finger protein that is required for a single-dendrite fate (Moore et al. 2002). Loss-of-function analysis using the MARCM system and gain-of-function approaches have identified a group of transcription factors, including Cut, Abrupt, Spineless, and Knot, that in turn help to specify class-specific branching complexities in DA neurons (Grueber et al. 2003a; Li et al. 2004; Sugimura et al. 2004; Kim et al. 2006; Hattori et al. 2007; Jinushi-Nakao et al. 2007; Crozatier and Vincent 2008). The factors that are regulated by these various transcriptional regulators are largely unknown.

The microtubule cytoskeleton plays a critical role in regulating dendritic branch patterning. Dendritic microtubules are either mixed plus-end distal and minus-end distal (in vertebrates) or primarily minus-end distal (several types of neurons in *Drosophila*) (Sharp et al. 1997; Stone et al. 2008). Forward genetic screens have identified components of the minus-end-directed microtubule motor dynein as having a critical role in the patterning of branches along DA neuron dendrite arbors (Satoh et al. 2008; Zheng et al. 2008). Dynein appears to deliver cargoes to distal dendrites that are necessary for dendrite branching, including fractions of a satellite secretory system, the Golgi outposts (Ye et al. 2007), and endosomes (Satoh et al. 2008).

Specifying Dendritic Targeting

The territories of dendrites define the region from which they can receive input; thus an important focus of current research in the field is to identify how dendritic territories are specified. This problem can be separated into two major questions: First, how are dendrites targeted to particular regions of the nervous system and, second, once dendritic targeting is specified, how is dendrite growth limited so that branches grow into appropriate territories?

Significant progress in this area has been made in *Drosophila* motor and olfactory systems. In the olfactory system, combinatorial expression of different transcription factors appears to provide an instructive code for diverse dendritic targeting by mediating both general and glomerular-specific targeting (Komiya et al. 2003, 2007). Both PN dendrite and motor dendrite targeting also require guidance cues that have been shown to be important for axonal targeting, including semaphorins, Slit/Robo and Netrin/Frazzled (Komiya et al. 2007). Although Semaphorin proteins typically act as ligands for plexin receptors in *Drosophila*, the use of MARCM to verify cell-autonomous requirements suggests that Semaphorin-1a acts in this case as a receptor (the identity of the presumptive ligand is as yet unknown) (Komiya et al. 2007). The midline guidance cues Slit and Netrin act as repulsive and attractive cues,

respectively, for axons in the CNS (for review, see Garbe and Bashaw 2004), and play analogous roles in the guidance of motor dendrites (Furrer et al. 2003, 2007).

Self-Avoidance and Tiling

Two principles of dendritic territory patterning that have been studied in flies are self-avoidance and tiling. Self-avoidance refers to the capacity of sister dendrites to repel each other and ensure nonredundant territory coverage. Tiling is an analogous phenomenon that occurs between different cells within the same functional class to achieve complete, but nonredundant, coverage of a receptive area. Clones lacking functional Dscam (Down syndrome cell adhesion molecule) transmembrane receptors show disrupted self-avoidance (Hughes et al. 2007; Matthews et al. 2007; Soba et al. 2007). Self-avoidance is also controlled in at least some da neurons by the Tricornered (Trc) kinase and Furry (Fry). *trc* and *fry* mutant neurons show self-crossing phenotypes, likely because of a defective like-repels-like turning response. Trc signaling similarly impacts tiling of dendritic arbors (Emoto et al. 2004).

Dendrite Remodeling

At the end of the larval stage, the nervous system of *Drosophila* undergoes tremendous changes to coincide with the different life history and remodeled body of the adult fly. Some larval-specific neurons perish, whereas others persist to become functional adult neurons after substantial remodeling of dendritic arbors. The widespread anatomical changes that occur during insect metamorphosis are orchestrated by two hormones, juvenile hormone (JH) and ecdysone. Metamorphic remodeling is observed among the larval da neurons, and their proximity to the body surface has allowed high-resolution analysis of the cellular and molecular events associated with dendritic pruning and growth (Williams and Truman 2004; Kuo et al. 2005; Williams and Truman 2005a,b; Kuo et al. 2006; Williams et al. 2006). Interestingly, this process involves selective loss of dendrites but little or no change in the axonal arbors. Williams and Truman followed single MARCM clones from larval to adult stages using two-photon microscopy to study morphological alterations in identified remodeling arbors (Williams and Truman 2004). The transition to adult forms involves removal of larval branches by local degeneration and branch retraction. Regrowth of the adult arbor involves laying down of the adult scaffold, which is accomplished by dynamic branch additions and retractions, and then filling in of the stabilized scaffold with higher-order branches. Ecdysone and JH are required, respectively, for pruning of dendritic branches by local degeneration and for controlling the balance between extension and retraction programs at different phases of arbor rebuilding (Williams and Truman 2004, 2005a).

Metamorphic remodeling of dendrites has been studied in several different neuronal types, including mushroom body neurons and olfactory projection neurons (Zheng et al. 2003; Marin et al. 2005). In the mushroom body, a genetic screen has identified the ecdysone receptor B1 isoform (EcR-B1) (Zheng et al. 2003) as critical for remodeling. The transforming growth factor β (TGF- β) type-I receptor Baboon is required for initiation of EcR-B1 expression and dendrite remodeling (Zheng et al. 2003; Marin et al. 2005).

METHODS FOR STUDYING DENDRITIC MORPHOGENESIS

The identification of molecules important for dendrite development has been aided greatly by recent advances in imaging and manipulation of single neurons in *Drosophila*. There are numerous techniques that are applicable to the study of dendrite biology. Many genetic studies described for the da neuron system analyze phenotypes in mutant embryos using transgenic markers of subsets of sensory neurons and also use mosaic analysis of loss-of-

function phenotypes. Protocols are available which present our implementation of embryonic (Analysis of Dendrite Development in *Drosophila* Embryos [Shrestha and Grueber 2011a]) and larval (Generation and Staining of MARCM Clones in *Drosophila*) techniques for studying wild-type and mutant da neuron dendritic arbors. In studies of da neuron dendritic development that use these methodologies, dendrites are imaged by confocal microscopy in embryonic and larval stages. The advantage of studies of embryonic stages is the relative ease of screening candidate genes and the ability to examine large numbers of neurons for phenotypes. Mosaic systems for examining mutant phenotypes are advantageous for studies of larval stages because of the ability to discern cell-autonomous phenotypes at very high resolution. However, there is a considerable time investment required for examining each individual gene of interest. These protocols should allow interested researchers to use the da neurons as models for studying genetic and molecular determinants of dendrite patterning. We also provide information that should help in determining the best experimental approach for a particular line of research.

QUANTIFICATION OF DENDRITIC ARBOR MORPHOLOGY

Quantification of dendritic arbor morphology is a critical step for understanding the role of a particular gene in dendrite morphogenesis. Some of the more commonly used morphometric parameters include total branch length, branch number, branch order, branch interval, and field size, which together provide a good starting point for any analysis of da neuron branching (Table 1). Sholl analysis is a simple, but powerful technique for determining the spatial geometry of a dendritic arbor. Parameters such as number and order of branches intersecting or enclosed between evenly spaced concentric circles/spheres centered at the soma are measured to quantify differences in spatial distribution of dendrite branching (e.g., see Libersat and Duch 2002; Uylings and van Pelt 2002; Zheng et al. 2008). Standard Sholl analysis can be adapted to reveal information about dendrite orientation by grouping morphometric parameters at each sphere or circle into cones or quadrants, respectively.

Several software packages have tools that allow measurements of these and numerous other parameters. Different software packages suit different budgets and needs. Programs such as ImageJ or Fiji offer powerful and platform-independent image analysis. Both ImageJ and Fiji are free for download and offer a large library of plug-ins for expansion, including NeuronJ for tracing and morphometric analysis, and the Simple Neurite Tracer plug-in for semiautomated tracing in Fiji. NeuroLucida is a package commercially available from MBF Bioscience, Inc., which offers a large number of tools for reconstruction, mapping, and morphometric analysis of dendrites. Tracing and full reconstruction of dendritic arbors in NeuroLucida is followed by quantitative analysis with NeuroLucida Explorer, which is included with the NeuroLucida package. Additionally, Amira software has an interactive automated three-dimensional tracing function that can be used effectively for reconstruction and analysis of some da neurons. The largest time investment in quantitative analysis is the phase of manual arbor reconstruction (Fig. 1). However, tools for automated and semiautomated reconstruction are likely to continue to improve in the coming years. Three-dimensional analysis of arbor geometry can also provide insights into the volume occupied by dendritic arbors, the spatial relationships between arbors, and relationships between arbors and other neurons or tissue. For example, dendritic growth of different genetically manipulated motor neurons has been assessed using volumetric measurements (Hartwig et al. 2008). There are several software packages available to support three-dimensional analysis of confocal images, including Amira, Volocity, MetaMorph, and Fiji. We recommend trials of these and other software packages to determine which one(s) best fit your specific or general needs.

Image analysis software does not usually include tools for basic (or sophisticated) statistical analyses. Quantitative results can be exported to software with such a capability, including R, which is an open source environment for data analysis that allows simple and advanced statistical operations, or analyzed with commercially available statistical programs such as Stata (StataCorp LP), SPSS (SPSS, Inc.), and JMP (SAS Institute, Inc.).

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FIGURE 1.

(A) A MARCM clone of ddaC, a class IV da neuron, was imaged on a confocal microscope. Four high-resolution images of different parts of the dendritic arbor were captured and merged using Adobe Photoshop to produce this image of the entire dendritic field. The arrowhead indicates the axon. (B) The neuron in A was traced in Neurolucida by hand using a drawing tablet.

Table 1

Publications that illustrate and exemplify the methods for quantification of different aspects of dendrite morphology

Dendrite property	Quantitative measure	Publication(s)
Branching	Branch order, number, length	Grueber et al. 2003a
Branch organization	Sholl analysis	Hartwig et al. 2008; Zheng et al. 2008
Dynamics	Extension, retraction	Parrish et al. 2007
Maintenance	Branch points, branch length, extension, retraction	Emoto et al. 2006; Parrish et al. 2007
Remodeling	Extension, retraction, organization	Williams and Truman 2004; Kuo et al. 2006
Self-avoidance	Branch (tip) overlap	Hughes et al. 2007; Matthews et al. 2007; Soba et al. 2007
Targeting	Dendritic distribution	Komiyama and Luo 2007
Tiling/territory	Territory size, overlap	Grueber et al. 2002; Emoto et al. 2006