Dendrite Patterning as a Model for Self-Organizing Systems

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Dendrite patterning as a model for self-organizing systems

A dissertation presented
by
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to
The Division of Medical Sciences

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Dendrite patterning as a model for self-organizing systems

Abstract

Biological systems self-organize into complex well-ordered structures and can evolve new patterns when perturbed. To identify principles underlying self-organization, I turned to the model organism *C. elegans* for its simple, highly reproducible anatomy and powerful genetics. In particular, I studied the sensory neuron PVD, which extends a highly elaborate dendrite arbor that covers the body wall, and the amphid sense organ, which consists of a bundle of 12 dendrites arranged in a highly reproducible order.

First, I asked how the patterning of the PVD dendrite was affected when I introduced additional copies of this neuron. I found that the ectopic PVDs extended smaller arbors that filled the body wall without overlapping, a pattern called dendrite tiling that is not normally seen in *C. elegans*. I found that dendrite tiling arose as a byproduct of molecular pathways normally used for dendrite self-avoidance. This example shows how even a simple nervous system can re-purpose existing pathways to generate well-ordered structures in a way that could explain the evolution of new patterns.

Next, I asked how the sensory dendrites of the amphid are organized into a bundle. Each amphid neuron extends an unbranched dendrite to the nose, together with the processes of two glial cells. Previous work suggested that the dendrites are bundled together in a reproducible order. I developed a method to visualize and quantify dendrite order in wild-type animals. Then, I used a candidate genetic screen to identify three CAMs that alter dendrite order. Loss of CDH-
4/Fat-like cadherin causes a complete randomization of dendrite order. In contrast, loss of PTP-3/LAR or SAX-7/L1CAM surprisingly causes dendrites to take on a new, reproducible order. Further, misexpressing SAX-7 also leads to a new dendrite order. Altogether, my results suggest that differential expression of CAMs can organize dendrites in a way that is stereotyped across a wild-type population, yet that can easily give rise to novel patterns.

These examples demonstrate how a simple nervous system makes use of self-organizing principles to generate ordered structures without sacrificing its evolvability.
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CHAPTER 1

Introduction
How biological systems are organized into ordered, patterned structures is a question that has spurred the curiosity of even the earliest of philosophers. As early as the first century, scholars put forth hypotheses as to how developing animals are “organized” into adults. Pliny the Elder (23–79 AD), a commander of the Roman Empire, author, and natural philosopher, describes in *Naturalis historiae* his hypothesis that bears are born as shapeless white balls of flesh with claws, and are gradually licked into the shape of a bear by their mothers (Plinii Secundi, 77 AD). This hypothesis has long since been discredited – indeed, George Henry Lewes, an English philosopher, remarked that “Pliny... makes the statement, and for untrustworthiness of statement he cannot easily be surpassed” (Lewes, 1864) – but, the question remains. It is quite remarkable that biological systems are, in essence, composed of simple macromolecules, which assemble into cells to form tissues. This process can in turn generate structures as complex as the human brain, which consists of over 100 billion neurons and non-neuronal cells with distinct shapes, positions, and functions and can form precise connections to one another (Herculano-Houzel, 2009). How do complex biological patterns form?

From first principles, one might envision a top-down, deterministic approach whereby the shape, position, and connections of each biological part is specified, like a blueprint for a building or a wiring diagram for an electrical circuit. In fact, this idea in the form of preformationism was widely accepted for centuries up to the early 19th century, popularized in 1694 by Nicolas Hartsoeker, who put forth the theory that the sperm of humans and other animals was a homunculus (Hartsoeker, 1694). Setting aside the obvious flaws of preformationism, a top-down blueprint approach to building organized biological structures might theoretically be possible for simple organisms, but it is laughable to imagine a blueprint
that would specify the shape, position, and connection of every cell in the brain and also accommodate external environmental inputs and changes over time. Even the *C. elegans* cell lineage, commonly described to be highly deterministic, must cope with high variability in cell division timing and cell positioning during the very early stages of embryogenesis (Schnabel, 1997). Thus, it is unlikely that structures as complex as the brain are organized using a strictly deterministic approach.

Because it is highly unlikely that a true blueprint exists, the most likely alternative to explain the formation of complex structures and patterns is a bottom-up, rules-based approach, where biological components obey rules that result in their self-organization and thus the emergence of a well-ordered pattern. In this thesis, I use the *C. elegans* nervous system – in particular, the development of sensory dendrites – as a model for understanding the rules that facilitate the formation of ordered cell patterns and structures. The introduction is divided into two sections. First, I will review the principles and major themes of biological pattern formation, with special attention to principles that apply to the development of the nervous system, especially dendrites. Then, I will introduce *C. elegans* as a model system and describe principles of organization and principles of development in the *C. elegans* nervous system, as well as how they relate to other systems.

I. **Rules and principles of biological pattern formation**

How might cells self-organize into ordered biological structures during morphogenesis? First, biological components need to be able to communicate with their neighbors and *vice versa*. Models for this communication and feedback have been described in at least two forms – in
cellular automata, where cells follow a set of rules applicable to themselves and their neighbors, and in reaction-diffusion systems, where two interacting ingredients form positive and negative feedback loops to create stable patterns. Related to reaction-diffusion systems are cellular and mechanical ways of generating periodic patterns, which will also be discussed. Second, biological components need a way to selectively stick together to self-organize into multicellular structures – generic glue is not sufficient. Differential adhesion provides a method for that. Another important concept in self-organization is the use of oscillators, which provides spatial-temporal control to collective behaviors such as swarming behavior and synchronized flashing in fireflies. However, oscillators will not be covered in this introduction because it is less applicable for morphogenesis and the formation of biological patterns. For a comprehensive review of principles of self-organization, please see reviews by Karsenti and Saetzler et al. (Karsenti, 2008; Saetzler et al., 2011).

**I.1 Cellular automata can generate stable patterns**

Cellular automata are discrete dynamical systems that were first invented as computer models by mathematician John von Neumann in the 1950s, and popularized in the 1970s by British mathematician John Conway with Conway’s Game of Life (Von Neumann, 1951; Gardner, 1970). A cellular automaton can be characterized by a grid of cells of a finite number of dimensions where each cell has a defined number of neighbors and number of possible states. For example, Figure 1.1A shows a two-dimensional $8 \times 8$ square grid where each cell that is not on the edge of the grid has 4 neighbors and two possible states (each cell can be either white or black). The grid evolves through a series of time steps, such that at each time point, the state of each cell is assigned based on a list of rules that takes into consideration the states of its
Figure 1.1. Description of a basic cellular automaton

(A) This figure shows a two-dimensional, $8 \times 8$ square grid where each cell can have one of two states at any time point. At each time point, the state of each cell is updated according to a set of rules that govern the state of that cell and the states of its neighbors. (B) Wolfram’s Rule 30 and the shell pattern of the cone snail *Conus textile*. Adapted from (Fieguth, 2016).
neighbors. Thus, with a series of locally applicable rules, surfaces can self-organize into stable or bi-stable intricate and elaborate patterns.

Patterns from cellular automata have been shown to resemble coloration patterns on seashells. In particular, the shell pattern of the cone snail *Conus textile* bears a striking resemblance to the pattern generated by mathematician Stephen Wolfram’s Rule 30 (Figure 1.1B) (Fieguth, 2016; Wolfram, 1983), and suggests the possibility that cellular automata offer strategies that can give rise to a diverse set of complex biological patterns.

I.2. Turing instabilities can form periodic patterns

In 1952, Alan Turing, most famous for his work as a computer scientist, proposed a theoretical model to explain how an embryo that consists of a symmetrical ball of cells can develop into an organism with distinct features (Turing, 1990). Twenty years later, Gierer and Meinhardt independently suggested a very similar system for self-organization (Gierer and Meinhardt, 1972). Both the proposed models, which are also known as reaction-diffusion systems, suggest that stationary chemical patterns can result from two ingredients – an activator that can make more of itself through positive feedback, and an inhibitor that negatively regulates the activator. Importantly, the activator is a short-range activator and the inhibitor is a long-range inhibitor, such that the inhibitor has a faster rate of diffusion than the activator. The inherent instability in the process of diffusion can rapidly amplify small changes in the system under some conditions, and in practical terms, this means that the activator is gathered in patches and the inhibitor prevents neighboring patches of activator from growing too close. Intriguingly,
This activator-inhibitor behavior was also proposed for predator-prey relationships (Segel and Jackson, 1972).

This reaction-diffusion model is interesting because it can generate many different types of periodic patterns found in biology such as spots and stripes of mammals and fish, patterns on bird feathers, and the reticulated pattern of giraffes (Kondo and Asal, 1995; Kondo and Miura, 2010; Prum and Williamson, 2002; Richardson et al., 1990; Yamaguchi and Yoshimoto, 2007). In addition to animal print patterns, reaction-diffusion systems have also been found for many other types of patterning such as digit patterning during limb development (Badugu et al., 2012; Raspopovic et al., 2014; Sheth et al., 2012), branching during lung morphogenesis (Hagiwara et al., 2015; Menshykau et al., 2012; Xu et al., 2017), and hair follicle spacing (Mooney and Nagorcka, 1985; Sick et al., 2006). In general, the challenge has been to find corresponding molecules that implement the reaction-diffusion systems. However, in some instances, the molecular correlates have been found – hair follicle spacing in mice involves Wnt as the activator and Dkk2 and Dkk4 as the inhibitors (Sick et al., 2006), and digit patterning involves Bmp and Wnt signaling pathways together with Sox9 (Raspopovic et al., 2014). Finally, Economou et al. also showed that fibroblast growth factor (FGF) and Sonic hedgehog (Shh) are required as an activator-inhibitor pair to form regularly spaced ridges of the mouth (Economou et al., 2012).

Although there is a heavy focus on Turing’s reaction-diffusion systems for generating periodic patterns, there are other ways as well – mechanical instability and cell-cell interactions can also produce periodic patterns that vary in spacing depending on tissue properties (Harris et
al., 1984; Oster et al., 1983; Vaughan et al., 2013). For example, Shyer et al. showed that mechanical forces can pattern villi in the human and chick guts (Shyer et al., 2013). Additionally, studies have shown in zebrafish that the periodic, striped pattern on zebrafish skin is formed by cell-cell interactions between the pigment cells (Nakamasu et al., 2009; Yamaguchi and Yoshimoto, 2007). Altogether, these studies show that reaction-diffusion models, as well as models incorporating mechanical forces and cell-cell interactions, can generate periodic patterns.

Cellular automata and reaction-diffusion systems are distinct models for pattern generation. Although they seem similar in that both involve local interactions to generate patterns, mathematically, they are completely different. A cellular automaton is a discrete solution to pattern generation – each cell can have a limited number of states – whereas a reaction-diffusion system is a diffusion process and thus generates a continuous model. Given that these are two distinct models, is it possible to generate one from the other? A very interesting study from Manukyan et al. showed that a continuous reaction-diffusion model can output discrete processes to generate the colored pattern of scales on the skin of a southwestern European lizard (*Timon lepidus*) (Manukyan et al., 2017). Juvenile lizards are brown with about sixty white ocelli (Figure 1.2A). This pattern changes into a stable green-and-black labyrinthine pattern as the animals grow into adults where individual scales continue to flicker between green and black over time (Figure 1.2B). Manukyan et al. showed that this skin pattern change from juveniles to adults is due to a cellular automaton process, where the color of each scale depends on neighboring scales. Finally, they were able to link reaction-diffusion dynamics that describe interactions among pigment cells to output a cellular automaton by adding a variable that takes into consideration the variation of skin thickness between scales. Not only was this the first
Figure 1.2. *Timon lepidus* skin patterns in juveniles and adults

(A) Juvenile ocellated lizards are brown with roughly 60 white ocelli surrounded by a darker brown ring. Adapted from Manukyan *et al.* (Manukyan et al., 2017). (B) Adult ocellated lizards have a green-and-black labyrinthine skin pattern. Photo by Bernard DuPont (DuPont, 2014).
demonstration of the use of cellular automata in vivo, but it also demonstrated a model by which the cellular automaton could have been derived using reaction-diffusion dynamics.

I.3. Differential adhesion can organize structures

“Were the various types of cells to lose their stickiness for one another and for the supporting extracellular white fibers, reticuli, etc., our bodies would at once disintegrate and flow off into the ground in a mixed stream of ectodermal, muscle, mesenchyme, endothelial, liver, pancreatic, and many other types of cells.”

(Lewis, 1922)

As Warren Lewis, pioneer in cell biology, pointed out in 1922, cell-cell adhesion is fundamentally important for organismal survival (Lewis, 1922). Importantly, the idea of differential adhesion, where cells have a selective affinity for specific cells or cell types, provides a strategy by which cells can organize into complex structures. One of the earliest demonstrations of differential adhesion showed that mixing mechanically dissociated cells from two species of sponges resulted in a species-specific segregation to produce two different organisms, each composed of cells from a single species (Wilson, 1907). Johannes Holtfreter further demonstrated this phenomenon in 1939. He mixed ectoderm, mesoderm, and endoderm derived from amphibian embryonic cells and found that each cell type displayed a positive affinity for each other (Holtfreter, 1939; Townes and Holtfreter, 1955). It was not until 1964 that Malcolm Steinberg proposed a mechanism by which cells could exploit differential adhesion to form organized biological structures. Steinberg’s Differential Adhesion Hypothesis postulated that different cell types mixed together sort out thermodynamically – cells exchange weaker contacts for stronger ones to achieve maximal adhesion between cell surfaces (Steinberg, 1964).
Given a cell adhesion molecule or group of cell adhesion molecules expressed on the surface of a group of cells, how might differential adhesion work to create order between cells? There are two general strategies. One strategy to create differential adhesion between cells is by changing the relative expression levels of a single adhesion molecule. Indeed, Steinberg showed that this “expression levels” strategy was a possibility when he expressed the surface adhesion molecule P-cadherin strongly in a group of cells and weakly in a second group of cells of the same type, and found that, when these two groups were mixed together, the cells would sort out based on differential levels of the P-cadherin (Figure 1.3A) (Steinberg and Takeichi, 1994).

Additionally, three studies have shown that differential expression of a single cell adhesion molecule can organize cellular structures \textit{in vivo}. In two studies published simultaneously, Godt and Tepass and González-Reyes and St Johnston showed in \textit{Drosophila} that the oocyte is always located posteriorly among sixteen germline cells because the oocyte and its surrounding follicle cells express the highest levels of E-cadherin (Godt and Tepass, 1998; González-Reyes and Johnston, 1998). In the nervous system, differential adhesion has also been shown to organize cartridges in the \textit{Drosophila} visual system (Figure 1.3B). Cartridges make up the compound eye in \textit{Drosophila} and are composed of two lamina cell dendrites surrounded by six photoreceptor axons. Schwabe \textit{et al.} recently showed that the differential expression of N-cadherin determines the relative positions of the neurites in the cartridge, with the cells with the highest expression of N-cadherin – the lamina cells – in the middle (Schwabe \textit{et al.}, 2014). Thus, \textit{in vitro} and \textit{in vivo} studies have shown that (a) cells expressing different levels of a single cell adhesion molecule sort out by adhesive strength, and (b) ordered structures can be organized by differential expression of a single adhesion molecule.
Figure 1.3. Differential adhesion by differential levels of a single adhesion molecule

(A) Steinberg and Takeichi mixed 2 populations of the same cell type – one expressing low levels of P-cadherin, and the other expressing high levels of P-cadherin. Over time, the mixed population sorted out such that cells expressing low P-cadherin surrounded cells expressing high P-cadherin. (Steinberg and Takeichi, 1994) 

(B) Arrangement of R axons and L dendrites of a single cartridge in the Drosophila visual system. R axons on the periphery express lower levels of NCAM (neural cell adhesion molecule) compared to the L dendrites, which are in the center of the cartridge. (Schwabe et al., 2014)
Figure 1.3. (Continued) Differential adhesion by differential levels of a single adhesion molecule
Another version of the differential adhesion hypothesis involves cells expressing different types of cell adhesion molecules that selectively adhere to each other in a “lock and key” code (Figure 1.4A). Paul Weiss raised this idea in 1941 in the context of the nervous system, when he was trying to understand how motor and sensory axons sort out into motor and sensory nerve bundles:

“We know from anatomical and physiological experiences that motor and sensory fibers are constitutionally different. This differential might very well predispose them for different contact affinities…guided by surface affinities, sensory and motor fibers would apply themselves each to the corresponding type.

The fact of surface affinities among identical kinds of nerve fibers seems to be well supported. For what else can it mean when the neurologist describes the fibers which mediate special sensations, such as pain, proprioception, cutaneous sensation, and so forth, as running in their intraspinal course as separate fiber bundles? Or the fact, that even in a peripheral nerve the sensory and the motor fibers hold pretty much to themselves, although imbedded in a common nerve tract?

…”The intimate fusion or adhesion between tissues of different origin presupposes a compatibility between contact surfaces which thus far has not found sufficient elucidation.” (Weiss, 1941)

Indeed, the importance of this “lock and key” code of adhesion molecules was demonstrated in a series of papers about axon bundles from Corey Goodman’s lab in the 1980s. Ablation experiments in the A/P longitudinal axon pathway in the grasshopper indicated that groups of axons fasciculate tightly with one another within a bundle (Figure 1.4B) (Bastiani et al., 1984). A and P axons pioneer the A/P longitudinal pathway, which the follower neuron G then climbs on. The G axon and growth cone displays a preference for the P axon: if the A and P neurons are ablated before the G axon extends, the G growth cone behaves abnormally, branches randomly and displays no affinity for other axon bundles around it. If only A is ablated, G fasciculates with the P axon and extends an axon like normal. However, if P is ablated, G does not fasciculate with the A axon or any other bundles around it. This behavior of the G axon is...
Figure 1.4. Differential adhesion by a lock-and-key code of cell adhesion molecules

(A) Under a simplified lock-and-key model of differential adhesion, cells can only adhere to other cells that express common cell adhesion molecules (in this figure, homophilic binding is assumed for visualization purposes). (B) Ablation experiments in the grasshopper longitudinal pathway suggest that growing axons selectively fasciculate with specific axons in the axon bundle. In this case, the G axon selectively fasciculates with P -- G does not extend an axon into the bundle in the absence of the P axon (Bastiani et al., 1984). (C) FasII controls selective fasciculation in Drosophila – loss of FasII causes axons to defasciculated while overexpression FasII causes overfasciculation (Lin et al., 1994).
Figure 1.4. (Continued) Differential adhesion by a lock-and-key code of cell adhesion molecules
intriguing as it suggests that, in this case, fasciculation reflects an absolute “lock and key” code of adhesion molecules, rather than a hierarchical set of graded affinities.

The Goodman lab went on to show in *Drosophila* that one molecule that mediates this specificity is fasciclin II (Fas II), the *Drosophila* homolog of NCAM (Lin et al., 1994). Fas II is expressed on a subset of axons in different bundles, and is required for the tight fasciculation of select axons in the longitudinal pathway. *fasII* mutants cause axons to defasciculate from their longitudinal pathways, and overexpression of Fas II rescues the defasciculation phenotype and can even cause neighboring axon bundles to fuse together abnormally (Figure 1.4C). These findings demonstrate the importance of adhesion molecules in specific patterns of fasciculation and, in the words of Paul Weiss, “thus, it may become necessary to concede to nerve fibers the attribute not only of being just adhesive, but of selective adhesiveness” (Weiss, 1941). In a broader context, these results also suggest that a lock-and-key code of adhesion molecules can also form ordered structures in biological systems.

I.4. Advantages of a rules-based system: robustness and evolvability

A rules-based system to generating patterns and complex biological structures is advantageous because it allows systems to be robust and adaptable in the face of changing external conditions. For example, a rules-based patterning mechanism for cone snail shells where the pattern depends solely on neighboring pigment cells will robustly produce similar patterns from cone snail to cone snail regardless of the size, shape, or curvature of the shell. Additionally, a rules-based system also allows biological systems to be adaptable such that
perturbations to the system do not break the system but instead allows the biological system to take on a new pattern.

In some sense, robustness and adaptability is a bit of a paradox – an isolated biological process cannot both be robust and adaptable to changes at the same time. An interesting study by Wagner (2008) resolves this paradox by studying RNA genotypes and their secondary structure phenotypes to suggest that this paradox can be resolved when defined in the context of a genotype or a phenotype – genotypic robustness is associated with low evolvability, but phenotypic robustness promotes evolvability (Wagner, 2008). Regardless, both robustness and evolvability are critical for evolutionary progression – robustness allows mutations to accumulate while still producing the same pattern, and evolvability allows a biological system to take on different outcomes and build new structures.

For example, a study in *C. elegans* showed that 96% of deleterious point mutations were not detectable in fitness assays, suggesting that *C. elegans* as an organism is remarkably robust (Davies et al., 1999). Yet, it is possible that an accumulation of these cryptic mutations could result in novel genetic and developmental functions that have been adapted over evolution to present themselves in higher organisms.

**II. Principles of organization in the *C. elegans* nervous system**

To understand how well ordered biological structures and patterns are derived, I will look at the *C. elegans* nervous system, and in particular, dendrites. Although my research focuses on dendrites, there are also many highly organized structures in the *C. elegans* nervous system with
interesting stories. In this section, I will first discuss these organizational features of the C. elegans nervous system (Section II.1). Next, I will discuss general strategies by which these well-ordered structures are developed (Section II.2) and maintained (Section II.3).

II.1. The C. elegans nervous system is well-organized

We turn to the nematode C. elegans as a model system for its advantages in imaging, reproducible anatomy, and genetics. The nervous system in adults consists of 302 neurons of 118 distinct neuron classes and 50 neurally-derived glia plus 6 glia-like cells from a mesodermal lineage (Hobert, 2010; Oikonomou and Shaham, 2011; Ward et al., 1975). Oddly, 301 of these neurons are not required for viability, which makes C. elegans an excellent organism to study nervous system function because damaging neurons will not cause lethality (Avery and Horvitz, 1989). The position of each neuronal cell body, morphology of each neuron, and location of its synaptic targets is highly stereotyped (White et al., 1986). Thus, the simplicity of the nervous system combined with the genetic tools available in this model organism make C. elegans a powerful model organism to understand organizing principles of biological structures.

Additionally, extensive serial electron micrograph (EM) reconstructions of adult and juvenile animals have revealed a number of intriguing organizational features and patterns in the nervous system. Many of these features are highly conserved and found in other biological systems, but I will focus on those in C. elegans below. The five features I will describe are: (1) neurons are organized into clusters (Section II.1.1), neurites are organized into bundles (Section II.1.2), neurons exhibit diverse morphologies (Section II.1.3), mechanisms of spacing in neurites (Section II.1.4), and asymmetry in the nervous system (Section II.1.5).
II.1. Neurons are organized in clusters

Similar to other invertebrates, many neural cells in *C. elegans* are clustered into well-defined ganglia (Chitwood and Chitwood, 1950; Hall et al., 2006; White et al., 1986). The largest ganglion is the anterior ganglion, which is located near the nerve ring in the head and is composed of neurons and sheath and socket glial cells in the head. The second largest ganglion is the tail ganglion. The ganglia are not organized by function, but adhesion molecules actively maintain the positions of cell bodies within ganglia (Sasakura et al., 2005).

Why are neurons placed the way they are? The idea of wiring economy, first proposed by Santiago Ramón y Cajal, suggests that neurons are positioned in a way that minimizes wiring cost (Santiago, 1899). This neuron placement not only has to minimize the total length of neuron connections, but also has to solve the problem of volume exclusion – optimal configurations for neuron placement results in the placement of multiple neurons on top of one another, which is unrealistic because each neuron takes up volume. Interestingly, a number of computational studies have also shown that neuron placement for all of *C. elegans*, the cartridges in the *Drosophila* visual system, as well as across different species and brain areas, is optimized to minimize wiring cost (Chen et al., 2006; Rivera-Alba et al., 2011; Rivera-Alba et al., 2014).

II.1.2. Axons and dendrites are organized into bundles

In *C. elegans*, most neurons in the body direct an axon ventrally to fasciculate with the ventral nerve cord (VNC). The VNC consists of two longitudinal axon pathways, one on each of the left and right sides, closely apposed to the midline, that run along the entire length of the
animal (White et al., 1976). There are other nerve cords in the body, such as the dorsal and lateral nerve cords, where additional axon and dendrite processes fasciculate. In the head, the nerve ring, situated posterior to the anterior ganglion, contains a large number of axonal processes coming from neurons both from the head and the body that synapse onto each other (White et al., 1986). Anterior to the nerve ring, there are 4 bilaterally symmetric bundles of dendrites: the amphid bundle as well as the dorsal, ventral, and lateral bundles (Figure 1.5) (Ward et al., 1975). Within these bundles, each neuron extends a single dendrite into the bundle that ends at the nose tip. Most of these dendrites have a ciliated ending, but some dendrites, like URX, do not (Doroquez et al., 2014).

EM reconstructions have shown that the order of axons in the VNC, the nerve ring, and dendrites in the amphid bundle are stereotyped, but not in a way that one might expect (White et al., 1976). For example, the DD motor neurons, which extend axons into the VNC, are born much earlier than other neurons that send axons into the VNC, such as the post-embryonic VD motor neurons. Thus, the prediction is that DD motor neurons should be found near the middle of the VNC. However, that is not the case – their axons are located at the periphery of the VNC, hinting at the possibility that the VNC might be organized by function, or by synaptic partners, rather than simply being like a wire coil with earlier-born axons occupying the center and newer axons added along the outside (White et al., 1976).
Figure 1.5. Dendrite bundles in the *C. elegans* head

The *C. elegans* head neurons form four bilaterally symmetric bundles of dendrites that each extend to the nose. Amphid bundles are in red, dorsal bundles in green, lateral bundles in blue, and ventral bundles in purple. Adapted from Ward *et al.* (Ward et al., 1975).
II.1.3. *C. elegans* neurons exhibit diverse morphologies specialized for their function

Although most neurons in *C. elegans* extend very simple processes containing a single unbranched axon and a single unbranched dendrite, some neurons and ciliated structures have unique morphologies. For example, PVDs are a pair of sensory neurons in the body that each extends a gigantic dendrite arbor covering half the body of the animal (Figure 1.6A) (Oren-Suissa et al., 2010). FLPs (pronounced “flap”) are a pair of sensory neurons that are morphologically similar to but functionally distinct from PVD, except instead of extending a dendrite arbor that covers half the body, FLPs cover the head (Figure 1.6A) (Albeg et al., 2011; Smith et al., 2010). Recent work has also found that PVD arbors are guided by stripes of SAX-7/L1 which complexes with MNR-1/menorin in the skin, the leucine-rich repeat receptor DMA-1 on the dendrite surface, and LECT-2/Chondromodulin II, secreted from body wall muscles (Dong et al., 2013; Díaz-Balzac et al., 2016; Salzberg et al., 2013; Zou et al., 2016).

Another interesting behavior is the ability of certain neurons to produce different shapes at different developmental stages. The ILs are a six-fold symmetric class of neurons that each extend a single, unbranched dendrite ending in a sensory cilium that is exposed to the external environment (see Figure 1.6B for cilia morphology). Interestingly, under stressful environmental conditions, when *C. elegans* undergoes an alternate larval stage called dauer, two of the six ILs remodel to extend highly branched, highly organized dendrite arbors that are morphologically similar to that of FLP dendrite arbors (Figure 1.6A) (Schroeder et al., 2013). This remodeled arbor is incompletely retracted when animals exit the dauer stage, and work from Schroeder *et al.* showed that this process is regulated by the serine protease KPC-1/furin, which also regulates FLP and PVD dendrite morphology (Schroeder et al., 2013).
Figure 1.6. Unique neuron and cilia morphologies in C. elegans

(A) FLP neuron (gray) and PVD neuron (purple) dendrite arbors. (B) Cilia morphologies of sensory dendrites. Adapted from WormAtlas (Altun and Crocker, 2010).
Additionally, there are two classes of dendrites that can be separated by the way they interact with glial cells (Doroquez et al., 2014; Ward et al., 1975). One class is the dendrites that extend their ciliated endings to the nose tip and are ensheathed by the sheath and socket glial cells at the tip (Figure 1.7). Dendrites in this class include all the amphid neurons in the amphid bundle, as well as the CEP neurons in the dorsal and ventral bundles, as well as the OL and IL neurons that reside in the dorsal, ventral, and lateral bundles. Another class of dendrites includes dendrites that are intimately associated with glia but are not ensheathed by them. Sensory dendrites in the head in this class include URX, URY, BAG, and FLP. These two classes of dendrites are interesting because Heiman and Shaham as well as work by my colleagues have shown that these two classes of dendrites use distinct mechanisms to extend their dendrites during development (Heiman and Shaham, 2009).

Finally, the cilia in a variety of sensory neurons have diverse morphologies (Figure 1.6B). There are two interesting points here. First, EM reconstructions of the amphid cilia, which are tightly fasciculated in a bundle, have shown that the cilia are ordered within the bundle (Doroquez et al., 2014; Perkins et al., 1986; Ward et al., 1975). Second, BAG and FLP are special in that part of the BAG and FLP dendrites wrap around part of the socket glial cell that it is associated with, the IL socket (Doroquez et al., 2014; Perkins et al., 1986; Ward et al., 1975; Ware et al., 1975). Altogether, these diverse neuronal morphologies are important and likely reflect the diversity of sensory function in the nervous system of *C. elegans.*
Figure 1.7. Glial-ensheathed and glial-associated neurons in *C. elegans*

There are two types classes of sensory dendrites in the head – glial-ensheathed dendrites (red) and glial associated dendrites (orange). Figure adapted from Lamkin and Heiman (Lamkin and Heiman, 2017).
II.1.4. Mechanisms of spacing in axons and dendrites

In addition to dendrite and cilia morphologies, neurites in *C. elegans* also have interesting spacing and positioning properties. For example, FLP and PVD extend highly branched, highly organized dendrite arbors which have dendrites that exhibit self-avoidance – that is, sister dendrites from the same arbor avoid each other, mediated by UNC-6/Netrin and its receptors UNC-40/DCC and UNC-5 (Smith et al., 2010; Smith et al., 2012). In Chapter 2, I show how this self-avoidance property of PVD dendrites is also responsible for a novel tiling pattern in *C. elegans* when ectopic PVD neurons are added to the animal (Yip and Heiman, 2016).

The positioning of synapses can also be ordered. White *et al.* demonstrated this with the DA neurons, which are a class of 9 motor neurons that extend their axons into the dorsal nerve cord to form synapses onto dorsal muscles. In the nerve cord, although their axons overlap with one another, synapses made by one motor neuron do not overlap with the synapses made by other motor neurons, a phenomenon Mizumo and Shen coined “synaptic tiling” (Mizumoto and Shen, 2013; White *et al.*, 1976). Using DA8 and DA9 motor neurons, Mizumoto and Shen found that PLX-1/Plexin and Semaphorins are required for synaptic tiling – PLX-1 is found at the synapse-free segments of the axons and inhibits formation of synapses (Mizumoto and Shen, 2013).

II.1.5. The nervous system is not always symmetric

While most of *C. elegans* is bilaterally symmetrical, there are some interesting features of the nervous system that are not. These deviations from bilateral symmetry are interesting in the
context of understanding how organized structures are made because it suggests that there are mechanisms that actively break symmetry to produce an organized nervous system.

- **Bundles can be asymmetric**: Interestingly, the VNC in *C. elegans* is highly asymmetric—most axons run in the right VNC with over 90% of axonal processes in the right VNC (Durbin, 1987). A study by Hutter et al. showed that *ast-4* and *ast-7* are required for setting up this asymmetry, although their exact functions are unknown (Hutter et al., 2005).

- **Neurons can be asymmetric**: Intriguingly, the amphid neurons AWC and ASE, responsible for sensing odor and taste respectively, are left-right asymmetric. AWCL (AWC left) and AWCR (AWC right) are bilaterally symmetric when measured by functional and morphological criteria, but oddly have an asymmetrical pattern of expression of the G-protein-coupled receptor, *str-2*, in that only one of the two AWC neurons will stochastically express *str-2* (Troemel et al., 1999). Even more oddly, this asymmetry is required for proper neuronal function (Wes and Bargmann, 2001). ASEL and ASER are also morphologically symmetrical but have differential patterns of expression—ASEL expresses *gcy-6* and *gcy-7* while ASER expresses *gcy-5*—with functional consequences (Hobert et al., 1999; Pierce-Shimomura et al., 2001; Yu et al., 1997). Etchberger et al. found that the terminal selector transcription factor CHE-1, as well as a series of transcription factors and miRNAs, modulated by a set of cis-regulatory elements, are required for specifying this asymmetry (Chang et al., 2003). Additionally, in the body, PVD neurons have an interesting asymmetry—PVDL and PVDR differ in the number of secondary branches, a process that is attributed to motor neuron commissures in the animal (O'Brien et al., 2017).
• **Cell lineages can be asymmetric:** There are interesting asymmetries in the *C. elegans* cell lineage. Most left-right neuron pairs are derived from bilateral symmetric lineages, however, that is not true for about a third of the neuron pairs (Sulston et al., 1983). For example, AWBL and AWBR are morphologically and functionally similar, but are not derived in a bilaterally symmetric fashion.

• **Cell migration can be asymmetric:** Q cells are a pair of neuroblasts (QR and QL) that undergo further divisions that make three neuronal classes, AVM/PVM, AWR/PQR, and SDQL/SDQR (Sulston and Horvitz, 1977). QR and QL are born bilaterally symmetric, but QL migrates posteriorly while QR migrates anteriorly, they divide, and the daughter cells continue migrating along the anterior-posterior axis. Further work suggests that Q cell migration is controlled by the homeobox gene *mab-5* (Salser and Kenyon, 1992), depends on the dosage of EGL-20/Wingless (Harris et al., 1996; Maloof et al., 1999; Whangbo and Kenyon, 1999), and is also affected in *unc-40/DCC* mutants (Honigberg and Kenyon, 2000).

### II.2. Principles of nervous system development in *C. elegans* and other organisms

For the nervous system to function properly, it needs to be wired appropriately – neurons need receive input and send output to the correct cells. However, achieving precision in wiring is a difficult problem not only because there are lots of neurons (over 100 billion in humans (Herculano-Houzel, 2009)) – and thus an exponential number of potential targets – but the functions, shapes, and positions of neurons can also change over the lifetime of an animal.
To overcome these challenges, the nervous system takes a step-wise approach to wiring the brain – first by getting axons to approximately the right location (long-range guidance), and then refining axonal connections to obtain precise wiring (short-range precision). For long-range guidance, mechanisms that are important include guidepost cells (Section II.2.1), axon guidance mechanisms (Section II.2.2), retrograde extension (Section II.2.3), and selective fasciculation (Section II.2.4). The mechanisms for short-range precision I will discuss are achieving synaptic specificity through adhesion molecules (Section II.2.5) and pruning for refining synaptic connections (Section II.2.6).

**Strategies for long-range guidance**

**II.2.1. Guidepost cells provide intermediate targets for growing pioneer axons**

Guidepost cells are discrete cells or populations of cells that serve as intermediate target stepping-stones for growing pioneer axons, which are axons that are the first to extend processes in a region. In general, ablating pioneer axons is highly disruptive for growth and targeting of follower axons and can lead to disorganized axon bundles (Durbin, 1987; Hutter, 2003; Lin et al., 1995; Ren et al., 1999). Guidepost cells were first discovered in the developing limb of grasshopper embryos (Bate, 1976), and have been shown to be immature neurons or glia, including astrocytes and microglia, that contact the growth cone filopodia of growing axons (Squarzoni et al., 2015). For example, Bergmann glia provide a scaffold for growing stellate axons to contact Purkinje cell dendrites (Ango et al., 2008). Guidepost cells act on pioneer axons by secreting attractive or repulsive guidance cues or adhesion molecules such as Netrin, which attract axons towards the guideposts and Slits, which help axons grow away from guideposts once they make contact (Chao et al., 2009).
In *C. elegans*, PVT, located in the preanal ganglion, is an interneuron that serves as a guidepost for axons entering the VNCL from the posterior end, secretes Netrin, and is required for maintaining axon fasciculation to the VNCL (Figure 1.8) (Aurelio et al., 2002; Durbin, 1987; Ren et al., 1999). Ablating PVT causes follower axons to take multiple routes to enter the VNC (Antebi et al., 1997; Aurelio et al., 2002; Ren et al., 1999; Wadsworth et al., 1996). Another *C. elegans* guidepost neuron is AVG, which pioneers VNCR, and guides axons entering the VNC from the anterior end (Figure 1.8). Like PVT, ablating AVG causes VNC disorganization (Durbin, 1987).

II.2.2. Axon growth is mediated by chemical cues, mechanical forces, and self-organizing polarization

The predominant model for axon guidance is that chemoattractive and chemorepulsive cues secreted by guidepost cells form gradients that attract or repel axons. The last 25 years have revealed many conserved short- and long-range guidance molecules along with their molecular mechanisms involved in axon guidance such as Netrin and Netrin receptors, Slits, Robo, ephrins, Eph receptors, Semaphorins, Plexins (Dickson, 2002; Kolodkin and Tessier-Lavigne, 2011; Tessier-Lavigne and Goodman, 1996). Actin-rich growth cones at the tip of growing axons use filopodia to detect guidance cues and can grow towards or retract from these cues, thus guiding growing axons to their target regions (Tamariz and Varela-Echavarria, 2015).

Another contributing factor for axon guidance that is important but much less appreciated is how mechanical forces affect axon growth and guidance. Indeed, *in vitro* studies have revealed how substrate stiffness affects neurite extension, branching, and growth (Balgude
Figure 1.8. Positions of AVG and PVT neurons

PVT and AVG are respectively anterior and posterior guidepost cells for axons growing into the VNC. Ablating either neuron causes VNC disorganization.
et al., 2001; Flanagan et al., 2002; Georges et al., 2006; Jiang et al., 2010; Koch et al., 2012; Kostic et al., 2007; Weiss P, 1934). A recent study by Koser et al. showed that retinal ganglion axons in *Xenopus* grow towards softer tissue *in vivo*; manipulating substrate stiffness or neuron mechanosensitivity resulted in axon guidance defects suggesting that *in vivo* mechanical forces are important for growing axons (Koser et al., 2016).

Finally, axon guidance can be influenced by a self-organizing mechanism within the growing tip of the axon that orients outgrowth activity to a single site (Chisholm et al., 2016). Evidence for this comes a study that showed that the Netrin receptor UNC-40/DCC can intrinsically be polarized to one site within the neuron, and that the location where UNC-40/DCC polarizes is a stochastic process. The presence of an UNC-6/Netrin gradient increases the probability that this self-organizing polarization happens at a specific site (which would be closest to the source of the UNC-6/Netrin gradient) (Xu et al., 2009). Thus, this model assumes that axons grow using a random walk model (e.g., for a growing axon, at any point in time, there is some probability that the axon grows in any direction), but the presence of chemical cues increases the probability of outgrowth machinery self-organizing at a location in the axon tip that is closest to the chemical cue (assuming a chemoattractant), thus biasing the probability of outgrowth in a specific direction (Chisholm et al., 2016; Kulkarni et al., 2013).

**II.2.3. Neuronal processes can also grow by retrograde extension**

Although the canonical mode of neurite extension and elongation involves active outgrowth at the tip of the axon or dendrite, neuronal processes can also extend by a process called retrograde extension, whereby the tip of the neurite is anchored in place and the cell body
moves away, elaborating a neuronal process behind. In *C. elegans*, the amphid sense organ extends its dendrites by retrograde extension, and requires the extracellular proteins DEX-1 and DYF-7 to anchor the dendrite tips (Heiman and Shaham, 2009). A recent paper showed that axons in the zebrafish olfactory placode extend using a similar mechanism of retrograde extension, and also showed that cell body movement away from the axons are passive, likely triggered by neighboring cell movements squeezing the cell bodies away from the axons (Breau et al., 2017). Additionally, work in zebrafish lateral line system has shown that axons can be towed by their target cells as another mechanism for axon growth (Gilmour et al., 2004). Another “fishy” example comes from the development of the M2 axon in *C. elegans*, where the M2 axon elongates because the axon tip is attached to another cell (likely the sister cell of M2, M3) that migrates away from M2, a process described by Pilon as the “fishing line paradigm” (Pilon and Mörck, 2005; Pilon, 2008).

II.2.4. Neurites selectively fasciculate with other neurites to form bundles

For neurites to reach distant targets, they selectively fasciculate into axon and dendrite bundles, an organizational feature that I discussed in Sections I.3 and II.1.2 and will further discuss in Chapter 3. An interesting question about selective fasciculation is whether neurite bundles are functional or merely vestiges of development, a question that will be discussed in the discussion section of Chapter 3.
Strategies for short-range precision

II.2.5. Synapse specificity through adhesion molecules

Another way to achieve wiring specificity is through the homophilic binding of adhesion molecules found on the pre- and post-synaptic targets. There are hints of this in early studies of Dscam in the olfactory system of Drosophila, where overexpressing Dscam in projection neurons caused a positional change of projection neuron dendrites, and corresponded with a similar change of position for ORN axons as well, suggesting some synapse matching mechanism independent of glomeruli (Zhu et al., 2006). Subsequent studies have identified cell surface molecules that are required for synaptic partner matching, such as Teneurin and Toll proteins in Drosophila olfactory system (Hong et al., 2012; Ward et al., 2015), Sidekick 2 in the mammalian retina (Krishnaswamy et al., 2015), and Dprs and their interacting partners DIPs in the Drosophila visual system (Tan et al., 2015). However, a comprehensive understanding of synaptic partner matching is still lacking as there has yet to be a system described that fully explains how each axon matches up with its post-synaptic partner.

II.2.6. Pruning to refine connections

During development, the nervous system refines connections to ensure that circuits function properly, a process called synaptic pruning. Generally, cells use three strategies to accomplish this. The first method involves apoptosis, which is responsible for removing the majority of excess projections in the developing nervous system (Cowan et al., 1984; Yuan et al., 2003). The second method involves synapse disassembly or pruning. Synapse disassembly removes only small subpopulations of synapses and has been studied most at the neuromuscular junction (Eaton and Davis, 2003; Goda and Davis, 2003; Lichtman and Balice-Gordon, 1990);
Additionally, there is a body of literature showing that microglia can phagocytose synapses as a method of refining connectivity (Hong and Stevens, 2016). Interestingly, this is an activity-dependent process that depends on complement proteins and receptors that are seen in the peripheral immune system (Hong and Stevens, 2016). Finally, there is also process elimination where axon branches are pruned. Glia have been shown to be important for axon and dendrite pruning (Low and Cheng, 2006). In general, pruning is activity dependent and highly competitive – in the neuromuscular junction, axons providing stronger inputs are retained while axons providing weak inputs are pruned away (Colman et al., 1997; Walsh and Lichtman, 2003).

Even in the simple nervous system of *C. elegans*, there is evidence of pruning to refine synaptic connections. Kage *et al.* showed that neurites of AIM interneurons are pruned in larvae, a process that is controlled by the transcription factor MBR-1 (Kage et al., 2005). Another study showed sex-specific synaptic pruning of neurons that were shared between males and hermaphrodites – synapses between PHB and AVA and PHB and AVG are found in juveniles, but only the PHB-AVG synapses remain in adult males, while PHB-AVA synapses remain in adult hermaphrodites (Oren-Suissa et al., 2016). Altogether, these studies suggest that refining connections is an important part of building proper neural circuits for a functional nervous system.

II.3. **Maintaining order in the nervous system**

In addition to establishment, nervous system architecture also requires maintenance throughout the life of the animal. This process involves maintaining the positions of cell bodies
and axons, the shapes of dendrite arbors, and the positions of synapses. Most of the molecules required for these processes have been elucidated in *C. elegans* and come in the form of secreted and transmembrane adhesion molecules, extracellular matrix components, kinases, and molecules involved in axon guidance.

The adhesion molecule SAX-7/L1CAM has been shown to be important for maintaining neuron cell body positions as well as axon and dendrite fasciculation (Pocock et al., 2008; Sasakura et al., 2005; Wang et al., 2005). Interestingly, there are two isoforms of SAX-7, a long and a short isoform, which have opposing functions – SAX-7S has an adhesive function while SAX-7L is anti-adhesive (Bénard et al., 2012). Further studies have also shown that *lon-1* may suppress maintenance phenotypes of *sax-7* (Blanchette, 2016), and that SAX-7 physically associates with DYS-1/dystrophin to maintain axon positions (Zhou and Chen, 2011). Other molecules involved in maintaining axon and neuron cell body positioning are the DGN-1/dystroglycan, which genetically interacts with the giant, secreted immunoglobulin superfamily molecule DIG-1, ANC-1 (Bénard et al., 2006; Johnson and Kramer, 2012), UNC-116/KIF5/Kinesin-1 Heavy Chain which acts with UNC-6/Netrin and UNC-40/DCC (Barsi-Rhyne et al., 2013), the secreted extracellular matrix protein SPON-1/F-spondin family (Woo et al., 2008), and the FGF receptor and tyrosine kinase EGL-15 (Bülow et al., 2004). Additionally, there is a class of two-immunoglobulin domain proteins in *C. elegans* called ZIG proteins (‘zwei Ig’) that are required for maintaining axon position in the VNC (Aurelio et al., 2002; Bénard et al., 2009). The ZIGs are interesting because a large number of them are expressed post-embryonically in a neuron called PVT, which extends its axon in the VNC. Ablation of PVT, or
loss of zig-3 or zig-4 causes maintenance defects in a number of VNC axons (Aurelio et al., 2002; Bénard et al., 2009).

Studies in Drosophila and mice have also shown the requirement of kinases in maintaining dendrite arbors. In Drosophila, genes required in the hippo pathway are required for maintenance of dendrite tiling (Emoto et al., 2006). Additionally, loss of Abelson (Abl) and Arg tyrosine kinases reduces dendrite arbor size due to ineffective dendrite branch maintenance (Moresco et al., 2005).

Another interesting aspect of nervous system maintenance is how synapses are maintained. Neuron-glia interactions play key roles in maintaining synaptic stability --- a study by Nishida and Okabe using hippocampal cultures showed that dendrite protrusions are more likely to become spines if contacted with astrocytes (Nishida and Okabe, 2007). In C. elegans, CEP sheath glia, which send processes that wrap the nerve ring, are required for maintaining synaptic connectivity (Shao et al., 2013). Additionally, a recent study by Cherra and Jin showed that zig-10 is required for maintaining synaptic density of cholinergic motor neurons; loss of zig-10 increases synapse density and exacerbates convulsion behavior (Cherra and Jin, 2016). Altogether, these studies show that a host of adhesion molecules, kinases, extracellular matrix components, and glia are required for nervous system maintenance.

III. Summary of dissertation

In this dissertation, I use molecular biology and genetics to shed light on some of the rules that build a brain. In Chapter 2, I use the C. elegans PVD neuron to understand how
existing rules in biological systems can generate new patterns when applied in a novel context.

As mentioned in the introduction, PVD neurons are a pair of bilaterally symmetric touch-sensitive cells that each extends a dendrite arbor covering the entire right or left body of the animal. A key feature of PVD dendrite arbors is that the dendrites exhibit self-avoidance, a patterning mechanism that mediates the repulsion of sister dendrites of the same neuron, ensuring that the dendrites do not overlap. When I genetically duplicated PVD such that the number of PVDs per side increased from one to five, I found that each PVD dendrite arbor shrank to one-fifth of the size of a wild-type arbor and did not overlap with neighboring PVD arbors. This behavior is known as dendrite tiling, which is a patterning mechanism found in Drosophila and vertebrates but not in C. elegans. Upon further investigation, I found that this dendrite tiling behavior arose as a byproduct of self-avoidance molecular pathways, suggesting that complex cellular patterns can emerge in a simple nervous system merely by repurposing existing pathways used for other functions. Thus, a simple self-avoidance mechanism allows C. elegans to accommodate changes in neuron number, and represents one way by which dendrite tiling as a patterning mechanism could have evolved.

In Chapter 3, I ask how well-organized structures are made by studying how dendrites are ordered in the amphid sense organ. Classical EM studies had suggested that the relative position of each dendrite in this bundle is stereotyped. To understand the rules required for this order, I developed a fluorescent imaging-based approach to visualize and quantify dendrite order and showed that dendrite order is stereotyped. Armed with the hypothesis that dendrite order is determined by a combinatorial code of cell adhesion molecules (CAMs) expressed in different but partially overlapping sets of amphid neurons, I conducted a candidate screen for CAMs
required for dendrite order and found cdh-4/FAT-like cadherin, sax-7/L1CAM and ptp-3/LAR to be required for fasciculation. I found that loss of cdh-4 causes dendrites to become randomly ordered, suggesting that cdh-4 might act as generic glue that keeps the bundle intact. But surprisingly, loss of either sax-7 or ptp-3 caused dendrites to arrange in a new order, consistent with the idea of a combinatorial code. Additionally, my data suggest that sax-7 and ptp-3 may be expressed in partially overlapping subsets of amphid neurons. Finally, to determine whether SAX-7 acts permissively or instructively to specify dendrite order, I misexpressed SAX-7 in all amphid neurons in wild type and found that amphid dendrites took on a new order, suggesting that SAX-7 plays an instructive role in establishing order. Altogether my results suggest that CAMs can organize dendrites in a way that is robust and replicable. These features might help to explain how other nervous systems, including our own, generate such precise order without sacrificing their evolvability.

Finally, in **Chapter 4**, I conclude by summarizing Chapters 2 and 3 as well as and discussing some future directions for my work.
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CHAPTER 2

Duplication of a single neuron in *C. elegans*

reveals a pathway for dendrite tiling

by mutual repulsion

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Z.C.Y. and M.G.H conceived the ideas and designed the experiments. Z.C.Y. performed the experiments, analyzed the data, and made the figures. Z.C.Y. and M.G.H. wrote the manuscript.
SUMMARY

Simple cell-cell interactions can give rise to complex cellular patterns. For example, neurons of the same type can interact to create a complex patchwork of non-overlapping dendrite arbors, a pattern known as dendrite tiling. Dendrite tiling often involves mutual repulsion between neighboring neurons. While dendrite tiling is found across nervous systems, the nematode *C. elegans* has a relatively simple nervous system with few opportunities for tiling. Here, we show that genetic duplication of a single neuron, PVD, is sufficient to create dendrite tiling among the resulting ectopic neurons. We use laser ablation to show that this tiling is mediated by mutual repulsion between neighbors. Furthermore, we find that tiling requires a repulsion signal (UNC-6/Netrin and its receptors UNC-40/DCC and UNC-5) that normally patterns the PVD dendrite arbor. These results demonstrate that an apparently complex cellular pattern can emerge in a simple nervous system merely by increasing neuron number.
INTRODUCTION

Neurons are complex machines that rely on their diverse geometries for proper function. Their precisely sculpted shapes include intricate patterns of dendrite arborization. Two key mechanisms of dendrite patterning are self-avoidance and tiling. Dendrite self-avoidance is mediated by repulsion between sister dendrites of a single neuron, ensuring that they splay apart to cover a large area (Cameron and Rao, 2010; Grueber and Sagasti, 2010). Self-avoidance requires adhesion molecules from large diverse families—thousands of spliced isoforms of Dscam1 in Drosophila, and dozens of combinations of protocadherin proteins in the mammalian retina and cerebellum—such that each neuron recognizes only itself and not its neighbors (Grueber and Sagasti, 2010; Hughes et al., 2007; Lefebvre et al., 2012; Matthews et al., 2007; Soba et al., 2007; Zipursky and Grueber, 2013). In contrast, dendrite tiling refers to the constrained growth of dendrite arbors from neighboring neurons such that they occupy distinct territories with minimal overlap. Tiling arises from combinations of mechanisms that regulate cell body spacing and dendrite arbor size. Here, we will focus on the role of mutual repulsion in tiling.

Notably, the molecules that have been implicated in tiling by mutual repulsion do not exhibit the diversity of isoforms characteristic of dendrite self-avoidance molecules—these include cadherin in Drosophila mechanosensory dendrites (Gao et al., 2000; Grueber et al., 2002), as well as Dscam2 and Plexin-Semaphorin in tiling-like interactions between axonal or synaptic regions respectively (Cameron and Rao, 2010; Millard and Zipursky, 2008; Mizumoto and Shen, 2013). Yet, despite their molecular differences, both self-avoidance and tiling can involve mutual repulsion between dendrites, raising the question whether a single pathway could, in
principle, mediate both. Indeed, pioneering studies showed that self-avoidance molecules can be experimentally engineered to mediate tiling by mutual repulsion. Forced expression of a single version of Dscam1 in Drosophila da neurons or protocadherin in mouse retinal starburst amacrine cells caused the neurons to aberrantly begin to tile, presumably by rendering them unable to distinguish self from nonself (Hughes et al., 2007; Lefebvre et al., 2012; Matthews et al., 2007; Soba et al., 2007). These results suggest a model in which tiling can emerge merely as a secondary byproduct of self-avoidance: each neuron “tries” to use self-recognition and repulsion to prevent its own dendrites from crossing but, because its neighbors also use the same molecules, it cannot distinguish self from nonself, with the result that neighbors end up tiling. It is important to note, however, that additional tiling mechanisms are already present in Drosophila and mouse, where these experiments were performed.

A strong prediction of the emergent tiling model is that it should be possible to create tiling by mutual repulsion in an organism where it does not normally exist, by using the endogenous mutual repulsion pathways that normally mediate dendrite self-avoidance. We reasoned that we could test this prediction by taking advantage of the highly simplified nervous system of C. elegans. Notably, several phenomena related to tiling have been described in this system, but are not known to involve mutual repulsion. The mechanosensory neurons ALM and PLM extend sensory processes into non-overlapping domains in the anterior and posterior portions of the animal, respectively, reminiscent of dendrite tiling (Gallegos and Bargmann, 2004). However, this differs in key respects from tiling of dendrite arbors by mutual repulsion: (1) the neurites are not branched and do not form arbors; (2) the neurites do not approach one another, as ALM lies in the dorsal portion of the body and PLM lies ventrally; and (3) it does not involve mutual
repulsion, as laser ablation or genetic removal of ALM does not lead to a change in the PLM neurite territory (Gallegos and Bargmann, 2004). Another related phenomenon is synaptic tiling, in which the neurites themselves do not change shape or size but the positioning of their synaptic fields shows mutual antagonism (Mizumoto and Shen, 2013). In fact, most C. elegans neurons extend non-branched dendrites, and only a few (PVD, FLP, IL2) can form branched arbors that exhibit self-avoidance. Intriguingly, PVD and FLP have been shown to form non-overlapping arbors, but it was not known whether these arise by mutual repulsion (Albeg et al., 2011; Smith et al., 2010). In some cell fate mutants, another neuron is converted to a PVD-like identity, and rare mosaic animals were used to show that this PVD-like neuron can form a dendrite arbor that does not overlap with that of FLP in the head or the endogenous PVD in the body (Smith et al., 2013). These results suggested that PVD neurons, in particular, might be capable of undergoing dendrite tiling by mutual repulsion.

We therefore focused on the PVD neurons, a pair of bilaterally symmetric touch-sensitive cells that each extends a dendrite arbor covering the entire body of the animal on its left or right side, positioned just under the skin (Figure 2.1A). Primary dendrites originating from each PVD cell body extend along the anterior-posterior axis, running along the lateral nerve cord and terminating posterior to the head at a structure called the nerve ring (Smith et al., 2010). Secondary, tertiary, and quaternary dendrites branch in orthogonal arrays from the primary dendrite to form repeating units of menorah-like structures that cover the body wall (Oren-Suissa et al., 2010). The tertiary dendrites exhibit self-avoidance, which is mediated by the guidance molecule UNC-6/Netrin and its cell-surface receptors UNC-40/DCC and UNC-5 (Smith et al., 2010; Smith et al., 2012). Because there is only one PVD neuron on each side of the animal,
Figure 2.1. *lin-22* mutants generate multiple PVD neurons.

(A) Wild-type animal (head is to the left) visualized by PVD::kaede. Arrowheads indicate the anterior and posterior extents of the PVD arbor. Bottom: tracing of PVD dendrites. (B) *lin-22* (*n*372) individual expressing PVD::kaede following photoconversion of PVD5. Red arrowheads indicate extent of the PVD5 arbor. *: cell body of a head neuron, OLL, which also expresses the PVD marker. (C) Quantification of the anterior dendrite extent of PVD, in wild-type (blue lines; *n* = 12) and PVD5 in *lin-22* (red lines; *n* = 19) animals. *P* = 5E-13 by Student's t-test. Gray lines, population average. (D) Anterior and posterior dendrite extents of PVD1 (*n* = 3), PVD2 (*n* = 6), PVD3 (*n* = 6) and PVD4 (*n* = 9) dendrite arbors in *lin-22*. Circles represent PVD cell body positions. x-axis values in (C) and (D) represent normalized distance units; dendrite extent measurements are normalized to the distance from the PVD5 cell body to the nerve ring (see Methods). Scale bars, 50 µm.
Figure 2.1. (Continued) *lin-22* mutants generate multiple PVD neurons.
PVD neurons do not normally have other PVD neighbors with which to tile. Thus, we asked what would happen to the PVD dendrite arbors if we increased the number of PVD neurons on each side of the body.
EXPERIMENTAL PROCEDURES

Strains were constructed in the N2 background and cultured under standard conditions (Brenner, 1974; Stiernagle, 2006). In addition to the wild-type strain N2, the mutations, transgenes, and strains used in this study are described in Appendix 1. For CHB1374, only animals lacking the hT2 balancer were analyzed.

Microscopy and Image Processing

Paneled image stacks were collected on a DeltaVision Core imaging system (Applied Precision) with a UApO 40×/1.35 NA oil-immersion objective and a Photometrics CoolSnap HQ2 camera (Roper Scientific). 1-day adults were mounted on an agarose pad with 10-50 mM sodium azide or 10 mM levamisole. For optical cell marking, photoconversion was performed by targeting 1-2 50 ms pulses of a 406 nm laser at 10% power at a PVD cell body. The animals were immediately recovered from the slides to standard Nematode Growth Medium (NGM) plates containing OP50 and incubated for ≥ 2 h to permit diffusion of photoconverted material. Animals were again mounted on slides and imaged in yellow (excitation (EX) 513 nm/emission (EM) 559 nm) and red channels (EX 575 nm/EM 632 nm).

Deconvolution and analysis of images were performed with Softworx (Applied Precision) and ImageJ (National Institutes of Health, Bethesda, MD). Maximum brightness projections were obtained using contiguous optical sections. Individual panels of maximum brightness projections were stitched together using the Stitch tool in Softworx. Projections were adjusted for brightness, contrast, and were pseudo-colored in Photoshop (Adobe). Because the quaternary PVD dendrites are much thinner than the PVD cell body, and thus harder to visualize, nonlinear
gamma settings were applied to the entire image using the Curves adjustment tool in Photoshop. Merged color images were assembled using the Screen layer mode in Photoshop.

PVD dendrite arbors were manually reconstructed using the Pencil Tool in Illustrator using a stitched maximum-brightness image projection of the entire animal. Figures were assembled using Photoshop CS5.1 and Illustrator CS5.1.

**Laser ablations**

PVD ablations in *lin-22* animals (Figures 2.4 and 2.5A): *lin-22* animals in the late second larval stage (L2) or third larval stage (L3) expressing the PVD-specific marker *ser-2prom3::kaede* (*PVD::kaede, CHB422*) were anesthetized with 10 mM sodium azide and mounted on agarose pads between a slide and a coverslip. Ablations were timed to target newly born PVD neurons in which dendrite extension was not yet observed. PVD neurons were identified by *PVD::kaede* under a 100× oil objective using a Zeiss Axioplan microscope. PVDs were killed with a pulsed nitrogen laser (Laser Science, Inc. VSL-337) focused through a dye cell containing coumarin (5mM in methanol), which shifted the wavelength of the laser from 337nm to 435nm. The PVD-ablated animals were recovered and then imaged two or more days later, as 1-, 3-, and 5-day adults, using photoconversion as described where necessary (see Figure 2.5A).

FLP and PVD ablations in wild-type animals (Figures 2.6 and 2.7): FLP neurons were ablated in animals expressing *PVD::mCherry* and *mec-3::GFP* (CHB1226). Since FLP neurons are born during embryogenesis – much earlier than PVD neurons, which are born in late L2 – we
ablated FLP in first larval stage (L1) or early L2 animals, prior to PVD neuron birth (assayed by the lack of expression of PVD::mCherry). For PVD neuron ablations, we laser ablated newly born PVD neurons in CHB1226, about 30 h after plating starvation-synchronized L1 animals. FLP- and PVD-ablated animals were imaged as 1-day and 3-day adults in the green channel (EX 475 nm/EM 525 nm) for FLP and red channel (EX 575 nm/EM 632 nm) for PVD.

**Quantification of PVD and FLP dendrite arbors**

Dendrite arbor extent was quantified using the segmented line tool in ImageJ using stitched maximum brightness projections. Dendrite extent was measured as the distance from the labeled PVD cell body to its most anterior or posterior dendrite branch, normalized to the distance between the *lin-22 PVD5* cell body or wild-type PVD and the nerve ring. As a reference, the mean distance between the *lin-22 PVD5* (or wild-type PVD) cell body and the nerve ring in Day 1 adults across all genotypes in this paper (n = 202) was 475.4 ± 7.0 µm (mean ± SEM). Thus, x-axis values are arbitrary normalized distance units. PVD1-4 cell body positions labeled on the schematized animal immediately below the x-axes represent the average PVD positions of *lin-22* (*n*372) animals. We used Student’s t-test to determine *P*-values.

For FLP dendrite arbor measurements (Figure 2.6B), dendrite extent was measured as the distance from the FLP cell body to its most posterior dendrite branch, normalized to the distance between the FLP cell body and the nerve ring. The PVD and FLP cell body positions in Figure 2.6B labeled on the schematized animal immediately below the x-axes represent the average distance between PVD and nerve ring in the unablated animal, normalized to the distance between the FLP cell body and the nerve ring.
Quantification of photoconverted fluorescence intensity along the primary dendrite

Wild-type 1-day adult animals expressing PVD::kaede were imaged in yellow (EX 513 nm/EM 559 nm) and red channels (EX 575 nm/EM 632 nm) before, 1 min and 120 min after kaede photoconversion in the PVD cell body (Figure 2.2). To quantify the fold difference between red and yellow (R/Y) fluorescence intensities, summed projections through the optical stack were used to measure the fluorescence intensities along the length of the primary dendrite. After subtracting the background noise, which was determined for individual image panels by measuring the mean intensity value in a 20 × 20 µm area, the R/Y fluorescence intensity ratio was calculated along the primary dendrite. The R/Y data was smoothed using a central moving average of data points contained within a 15 µm interval.

Quantification of quaternary dendrite branch density

To quantify dendrite branch density in Figure 2.3D, we counted the number of quaternary dendrites located in an 80 µm region immediately posterior to the PVD5 cell body in wild-type and lin-22 1-day adult animals. Only dendrite branches on one side of each animal (either dorsolateral or ventrolateral) were included. For clarity of presentation, data points with identical values in Figure 2.3D were nudged to prevent them falling exactly on top of each other.

Time-lapse imaging of PVD dendrite growth

Time-lapse imaging of PVD dendrite growth was conducted using a modified version of the protocol described in (Smith et al., 2010). The L3-stage individual (CHB2163) was first immobilized using 13 mM levamisole + 0.05% tricaine in M9 buffer, and then mounted onto an
agarose pad containing the same concentration of levamisole and tricaine. Image stacks (0.75 µm/step) were collected every 2.5 min on the DeltaVision Core imaging system using a PlanApo 60×/1.42 NA oil-immersion objective. Figure 2.5B panels were created using maximum-brightness projections of the image stack at the indicated timepoints.

**Statistical analysis**

We used Student’s t-test to compare statistical significance of two independent groups. *P* < 0.05 was considered significant. All data are shown as mean ± SEM.
RESULTS

Increasing the number of PVD neurons leads to spatially restricted dendrite arbors

We generated four additional PVD neurons using the cell lineage mutant *lin-22*. *lin-22* encodes a transcription factor that directs the lateral epidermal seam cells V1-V4 to generate hypodermal cells (hyp), while the V5 seam cell produces PVD as well as other cells (Horvitz et al., 1983; Wrischnik and Kenyon, 1997). Loss of LIN-22 causes V1-V4 to adopt the lineage of the V5 seam cell; *lin-22* mutants recently were shown to generate four pairs of PVDs (PVD1-PVD4) in addition to the normal PVD pair (PVD5) (Liang et al., 2015). Consistent with these observations, in the course of a genetic screen we serendipitously isolated three new *lin-22* alleles (*hmn23*, *hmn44*, and *hmn59*) as displaying extra PVDs; here, we use the reference allele *n372* throughout this work.

Because PVD1-5 all express the same markers, we could not resolve individual dendrite arbors using GFP. Therefore, we developed an optical cell marking strategy in which we expressed the photoconvertible fluorescent protein kaede (PVD::kaede) in all PVDs and then photoconverted a single PVD (Ando et al., 2002). Because PVD dendrites are very thin, we were concerned that the photoconverted material might not efficiently fill the entire dendrite arbor. Therefore, as a control, we photoconverted PVD in wild-type animals, determined the time necessary to label the entire arbor, and conducted all experiments under these conditions (Figure 2.2).

Using this method in *lin-22* animals, we found that PVD5 forms a spatially restricted arbor that terminates near the neighboring PVD cell body (Figure 2.1B). The dendrites often appeared
Figure 2.2. Photoconverted kaede labels the wild-type PVD primary dendrite within 2 h.

(A) Wild-type animal expressing PVD::kaede imaged before photoconversion (top), or 1 min (middle) or 120 min (bottom) after photoconversion of the PVD cell body. Stitched montages of maximum-intensity projections. The red-to-yellow (R/Y) fluorescence intensity ratio is shown in Figure 2.2B, Animal 1. Scale bars, 50 µm. (B) Quantification of fold difference of R/Y fluorescence intensity along the PVD primary dendrite before photoconversion (orange), or 1 min (blue) or 120 min (red) after photoconversion in four representative animals.
Figure 2.2. (Continued) Photoconverted kaede labels the wild-type PVD primary dendrite within 2 h.
to be packed more densely (Figure 2.3; average number of quaternary dendrites in a sampled region ± SEM = 11.2 ± 0.4, wild type; 18.8 ± 1.7, *lin-22*). We also observed quaternary dendrites occasionally interdigitating with dendrites from neighboring PVDs (Figures 2.3B and 2.3C, orange brackets), reminiscent of the way dendrites interdigitate while maintaining uniform interdendrite spacing in regions of overlap between retinal ganglion cells (Dacey, 1989; Wässle et al., 1983). We observed some examples of fasciculation between neighboring dendrites, typically involving primary dendrite branches, although instances involving quaternary branches were also identified (Figure 2.3, arrowheads). All of these observations are consistent with the idea that each PVD neuron is attempting to elaborate a normally-sized arbor in a restricted area. Due to these complexities, we decided to quantify the linear anterior-posterior extent of each arbor as a simple, robust proxy for tiling. We measured the distance from a PVD cell body to its most distant dendrite along the anterior-posterior axis, and normalized these measurements to the distance from the PVD cell body to the nerve ring (NR) to account for differences in body size. We found that PVD5 dendrites terminate far short of their normal end point at the nerve ring (*P* < 0.0001) (Figure 2.1C). PVD1-4 dendrite arbors behave similarly, with each arbor terminating near the neighboring PVD cell body and only partially overlapping with adjacent arbors (Figure 2.1D), thus dividing up the body into a series of distinct territories.

**PVD dendrite territories form by mutual repulsion**

We hypothesized that the reduced arbor size reflects mutual repulsion between PVDs. To test this hypothesis, we laser ablated PVD1-4 soon after their birth and measured the extent of the mature PVD5 arbor (Figures 2.4A and 2.4C). Under these conditions, PVD5 arbors extended farther than in non-ablated controls (*P* = 0.0004), showing that arbor size is indeed determined
Figure 2.3. *lin-22* PVDs extend dense dendrite branches that interdigitate and fasciculate with dendrites emanating from neighboring PVDs.

*lin-22* PVDs extend long tertiary branches containing a high density of quaternary dendrites (A, blue brackets) compared to wild-type PVD (Figure 2.1A). *lin-22* PVD dendrites also interdigitate with dendrites from neighboring PVDs (B and C, orange brackets), and, in rare instances, quaternary branches fasciculate with neighboring PVD dendrites (C, arrowheads). (B) Magnification of dendrite arbors in Figure 2.1B (from the main text) showing a high density of quaternary dendrites. (D) Quantification of the number of PVD quaternary dendrites in an 80 µm region (“Region A”, as indicated in Figure 2.3B) located immediately posterior to the PVD5 cell body in wild type and *lin-22* animals expressing PVD::kaede. Scale bars, 50 µm.
Figure 2.3. (Continued) lin-22 PVDs extend dense dendrite branches that interdigitate and fasciculate with dendrites emanating from neighboring PVDs.
Figure 2.4. Restricted PVD dendrite arbors reflect mutual repulsion.

(A) lin-22 animals expressing PVD::kaede following ablation of PVD1-4. Orange ovals indicate approximate locations of ablated cell bodies. Scale bar, 50 µm. (B) lin-22 animals expressing PVD::kaede following ablation of PVD2-4 and photoconversion of PVD5. Orange ovals indicate approximate locations of ablated cell bodies. Scale bar, 50 µm. (C) Quantification of the anterior dendrite extent of PVD (wild type; blue lines) or PVD5 (lin-22; red lines), following PVD1-4 ablation (red lines, bottom row) or controls (red lines, middle row). Gray lines, population average. (D) Quantification of the dendrite extent of PVD5 (red) and unablated PVDs (green) (n = 5, individuals are shown in Figures 2.4B). Circles represent cell body positions. x-axis values in (C) and (D) as in Figure 2.1.
Figure 2.4. (Continued) Restricted PVD dendrite arbors reflect mutual repulsion.
by mutual repulsion between neighbors. 14/14 PVD5 arbors terminated beyond the normal position of the PVD4 cell body, and 2/14 arbors extended to the nerve ring (Figures 2.4A and 2.4C). This extent of growth is probably an underestimate – as shown in Figure 2.4A, higher-order PVD5 dendrite branches between the ablated cell bodies were sometimes absent, possibly reflecting either a decrease in dendrite growth rate due to effects of the lin-22 mutation or tissue damage caused by ablation that delayed or inhibited dendrite growth. Indeed, we found that the dendrite extent of PVD5 continued to increase beyond the time point used for our measurements (Figure 2.5A). In a second experiment designed to test for the role of repulsion, we laser ablated two or three PVDs in lin-22 animals and distinguished between the remaining PVDs by photoconverting PVD5 (Figures 2.4B and 2.4D). In all five cases, the non-ablated PVDs extended their dendrites to completely fill the body wall, yet did not overlap with one another (Figures 2.4B and 2.4D). Consistent with the notion that PVD dendrite arbors tile by mutual repulsion, we conducted time-lapse imaging experiments of growing PVD dendrites in lin-22 and observed contact-and-repulsion events between dendrites that appear to originate from neighboring PVDs (Figure 2.5B). Together, these experiments show that the spatially restricted PVD territories observed in lin-22 reflect tiling by repulsion.

**Dendrite tiling in PVD uses the same molecules as self-avoidance**

How might PVD tiling arise? We considered two hypotheses. First, it is possible that a mechanism for tiling by mutual repulsion between neighbors is used in the development of the wild-type PVD and can be co-opted for PVD-PVD tiling. FLP is a touch-sensitive neuron in the head that elaborates a dendrite arbor similar to that of PVD in the body (Figure 2.6A) (Smith et al., 2010). FLP and PVD arbors occupy distinct territories and do not overlap (Albeg et al.,
Figure 2.5. PVD dendrites undergo contact-mediated repulsion in lin-22 animals.

(A) PVD5 dendrites continue growing during adulthood after ablation of PVD1-4. PVD5 dendrite extent was measured in PVD1-4-ablated lin-22 animals (n = 5) as 1-day adults (red, corresponding to the time point used in Figure 2C), 3-day adults (orange), and 5-day adults (blue). x-axis values as in Figure 2.1. (B) Time-lapse imaging of a contact-and-repulsion event between PVD dendrites in a single L3 lin-22 animal (CHB2163) at timepoints indicated, visualized with PVD::mCherry. Asterisks indicate PVD cell bodies. Red arrows indicate the site of contact and repulsion between dendrites. Scale bars, 10 µm.
Figure 2.5. (Continued) PVD dendrites undergo contact-mediated repulsion in lin-22 animals.
Figure 2.6. FLP and PVD establish dendrite arbors using a shared boundary.

(A) Schematic representation of FLP (red) and PVD (blue) neurons and their dendrite territories (shaded areas).

(B) Quantification of PVD primary dendrite length in wild-type and FLP-ablated animals bearing FLP::GFP (mec-3pro:GFP) and PVD::mCherry (CHB1226). \( P = 0.5 \) by Student’s t-test.

(C) Quantification of FLP posterior dendrite length in wild-type and PVD-ablated animals bearing FLP::GFP (mec-3pro:GFP) and PVD::mCherry (CHB1226). \( P = 0.09 \) by Student’s t-test.

x-axis values in (B) and (C) as in Figure 2.1.
The mechanism underlying this pattern has not been identified; in particular, it is not known whether it requires nonself recognition or mutual repulsion. Similarly, the bilaterally symmetric PVDs (PVDL and PVDR) occupy distinct territories on the left and right sides of the body and do not overlap. We reasoned that if FLP-PVD or PVDL-PVDR tile by mutual repulsion, then the molecules they use could also mediate tiling of ectopic PVDs.

To test this hypothesis, we performed laser ablation of FLP and asked whether PVD dendrites extended into the FLP dendrite territory. FLP ablation was performed in wild-type first larval stage (L1) animals before PVD neurons are born, and the resulting PVD dendrite arbors were examined three days later when the animals reached adulthood (1-day adults) and again two days later (3-day adults). Conversely, we also ablated the PVD neuron in wild-type second larval stage (L2) animals, shortly after PVD neurons are born, and examined the FLP dendrite arbor in 1-day and 3-day adults. In all cases, we did not observe PVD primary dendrites extending into the head when FLP was ablated (n = 7) (Figures 2.6B and 2.7) or any change in FLP dendrite arbor size when PVD was ablated (n = 7) (Figures 2.6C and 2.7). These results strongly suggest that the non-overlapping FLP and PVD territories reflect the presence of an anatomical or molecular barrier, such as a ridge of tissue or the localized presence of non-permissive growth signals. An alternative possibility is that dendrite extension was prevented by tissue damage caused by the laser ablation; however, at least in the case of the PVD ablations this is unlikely, as the ablated cell body is ~500 µm from the FLP dendrites. Importantly, when we ablated PVD on one side of wild-type animals (Figure 2.7), or PVD1-4 in lin-22 mutants (Figure 2.4), we did not observe invasion of PVD dendrites from the contralateral side,
Figure 2.7. FLP and PVD do not tile by repulsion.

(A - C) Representative images of FLP::GFP in wild type (CHB1226) of the following: Day 1 adult with PVD intact (A and A'); Day 1 adult with PVD ablated (B and B'), and the same animal 2 days later (C and C'). Arrowheads indicate posterior dendrite extent of FLP, and double arrowheads indicate sensory processes belonging to other neurons. (D - F)

Representative images of PVD::mCherry in wild type (CHB1226) of the following: Day 1 adult with FLP intact (D and D'); Day 1 adult with FLP ablated (E and E'), and the same animal 2 days later (F and F'). Asterisks indicate OLL neuron cell bodies. Labeled axon belongs to OLL.

(A – F) Stitched montages of maximum-intensity projections. Scale bars, 100 µm. (A’ – F’) Scale bars, 20 µm.
suggesting that PVDL and PVDR respect a barrier at the midline (data not shown). Thus, we observed no evidence for tiling via nonself recognition and repulsion in wild-type animals.

Second, we considered the hypothesis that the PVD self-avoidance molecules (UNC-6/Netrin and its receptors DCC/UNC-40 and UNC-5 (Smith et al., 2012)) could result in the emergence of tiling when extra neurons are introduced. To test this idea, we generated lin-22; unc-6 mutants using either of two alleles of unc-6 (e78 and ev400), with the prediction that loss of UNC-6 would abolish dendrite tiling and result in overlapping PVD dendrite territories. Indeed, loss of UNC-6 disrupted PVD tiling ($P = 0.0008$ and $0.004$ for e78 and ev400 respectively) (Figures 2.8 and 2.9A), as did the simultaneous loss of both Netrin receptors UNC-40 and UNC-5 ($P = 0.002$). In contrast, while loss of either UNC-40 or UNC-5 alone has been shown to disrupt self-avoidance (Smith et al., 2012), these single mutants showed negligible or mild tiling defects ($P = 0.3$ and $0.06$ respectively), suggesting that these receptors act redundantly in the case of tiling (Figures 2.8B and 2.9). Taken together, our results strongly suggest that endogenous self-avoidance molecules can create dendrite tiling by mutual repulsion in an organism where it does not normally exist.
Figure 2.8. UNC-6/Netrin signaling is required for dendrite tiling.

(A) lin-22 (n372); unc-6 (e78) individual expressing PVD::kaede following photoconversion of PVD5. Labels and scale bar as in Figure 2.1. (B) Quantification of the anterior dendrite extent of PVD5 in genotypes indicated, normalized to average dendrite extent in corresponding lin-22 (+) controls (red lines). Gray lines, population average. x-axis values as in Figure 2.1. P-values by Student's t-test.
Figure 2.9. Multiple alleles of UNC-6/Netrin signaling show that Netrin signaling is required for dendrite tiling.

(A) Quantification of PVD5 anterior dendrite extents in two unc-6 alleles (ev400 and e78), unc-40 (e271), and unc-5 (e152), and unc-40 (e271); unc-5 (e152) double mutants (red and blue lines). x-axis values as in Figure 2.1. P-values (by Student's t-test) normalized to lin-22 (+) animals (blue lines). (B) Dendrite tiling is normal in unc-40; lin-22 mutants (P = 0.3 by Student's t-test, normalized to unc-40 animals). (C) Dendrite tiling is partially normal in unc-5; lin-22 mutants (P = 0.06 by Student's t-test, normalized to unc-5 animals). (D) Dendrite tiling is disrupted in unc-40; unc-5; lin-22 mutants (P = 0.002 by Student's t-test, normalized to unc-40; unc-5 double mutant animals). Green and red arrowheads indicate the anterior and posterior extents of the PVD1-4 and PVD5 arbors, respectively. (B – D) Stitched montages of maximum-intensity projections. Scale bars, 50 μm.
Figure 2.9. (Continued) Multiple alleles of UNC-6/Netrin signaling show that Netrin signaling is required for dendrite tiling.
DISCUSSION

Dendrite tiling can emerge as a byproduct of self-avoidance

Here, we show that repulsive signaling pathways that normally pattern the dendrite arbor of a single neuron can give rise to dendrite tiling when the number of neurons is increased. These results are consistent with previous observations in which another neuron was converted to the PVD fate and, in rare genetic mosaics, was shown to form a dendrite arbor that did not overlap with that of the endogenous PVD (Smith et al., 2013). Our results are surprising because the development of an apparently complex neuronal pattern like tiling might have been expected to require the evolution of new, dedicated molecular pathways. That is, natural selection would have favored animals bearing gene variants that promoted dendrite tiling. In contrast, our results support a possible alternative model in which dendrite tiling was not directly subject to natural selection, but rather arose as a byproduct of mutual repulsion pathways that evolved to mediate dendrite self-avoidance.

This model resembles Gould and Lewontin’s classical concept of ‘exaptation’, in which a structural feature emerges as a secondary byproduct of another — their architectural metaphor involved the spandrels that form when an arch is built beneath a domed ceiling (Gould and Lewontin, 1979). Importantly, the idea that tiling is a byproduct of self-avoidance does not mean it lacks functional importance in the nervous system. Indeed, a central tenet of exaptation is that these new byproduct structures are exceptionally well poised to impart new functionality. Classic examples include feathers, which are hypothesized to have evolved as insulators and only later contributed to flight, and bones, which are hypothesized to have evolved as phosphate storage organs and only later contributed to mechanical support (Gould and Vrba, 1982).
Similarly, tiling could have arisen as an evolutionary "spandrel" but, once present, provided an important functional contribution to neuronal circuits by allowing better spatial resolution of incoming signals. Interestingly, in systems where tiling was detrimental, there would have been selective pressure to reduce tiling without affecting self-avoidance — strategies for this might have included the extensive splicing of Dscam1, which occurs only in a sublineage of arthropods, and the use of the expanded protocadherin gene cluster in mammals (Armitage et al., 2012; Chen and Maniatis, 2013).

Netrin receptors act in dendrite repulsion across species

The use of Netrin signaling in *C. elegans* seems, on its face, to be different from the more well-established dendrite repulsion signaling mediated by Dscam1. However, it is worth noting that Dscam itself is a Netrin receptor – both the *Drosophila* and mammalian forms of Dscam bind Netrin with affinities comparable to that of its canonical receptor UNC-40/DCC (Andrews et al., 2008; Ly et al., 2008) — and DSCAM can physically interact with UNC5C/UNC-5 to transduce Netrin-mediated repulsion signals (Purohit et al., 2012). Intriguingly, Slit/Robo signaling — which often acts in conjunction with Netrin signaling during axon guidance — was recently shown to act as a dendrite repulsion signal in the mammalian cerebellum (Gibson et al., 2014). It is therefore interesting to speculate whether dendrite repulsion pathways in *C. elegans*, *Drosophila*, and mammals all share a common origin with pathways used in axon guidance.

Non-overlapping dendrite arbors can be established by mutual repulsion or by barriers

Finally, our results suggest the existence of at least two distinct mechanisms that prevent overlap between dendrite arbors. First, tiling can be established by mutual repulsion, as occurs
among the ectopic PVDs. Interestingly, although our study and others show that mutual repulsion alone can explain tiling (Hughes et al., 2007; Lefebvre et al., 2012; Matthews et al., 2007; Soba et al., 2007), observations in the retina suggest that tiling is not always so simple. The dendrite arbors of some retinal ganglion cells exhibit mutual repulsion at their edges — first, they overlap less than if they were oriented randomly (Wässle et al., 1981); second, in regions where they do overlap, dendrite branches interdigitate rather than crossing (Dacey, 1989); and third, when neurons are killed, the remaining arbors reorient towards the vacated territory (Eysel et al., 1985) — yet genetically ablating most of these cells does not lead to expanded growth of the remaining dendrite arbors, as a simple mutual repulsion model would predict (Eysel et al., 1985). Rather, some other mechanism seems to constrain the overall growth of their dendrite arbors. Conversely, the dendrite arbors of retinal horizontal and bipolar cells exhibit extensive overlap between neighbors — suggesting an absence of mutual repulsion — yet these arbors nevertheless expand or reduce in size when cell number is decreased or increased, respectively, implying some other mechanism that coordinates the dendritic territory of a neuron with that of its neighbors (Lee et al., 2011; Poché et al., 2008). These experiments indicate that, in the retina, mutual repulsion alone is neither necessary nor sufficient to determine the extent of overlap between neighboring dendrite arbors, but may have been combined with additional mechanisms that contribute to more complex developmental interactions among dendrites.

Second, tiling can be established by a barrier, as seems to occur between PVD and FLP. Importantly, recent studies show that local anatomical cues from epithelia or muscles can play an important role in shaping dendrite arbors, including imposing restrictions on the size of a dendrite arbor (Dong et al., 2013; Liang et al., 2015; Parrish et al., 2009; Salzberg et al., 2013).
The presence of anatomical cues that do not require nonself recognition and repulsion may explain the seemingly paradoxical result that some classes of Dscam1 mutant neurons continue to tile normally despite failing to self-avoid: what has been grouped together as “tiling” may in fact be a mixture of repulsion-mediated effects, which are a secondary byproduct of self-avoidance, and restricted growth due to local anatomical cues, which provide an entirely independent way to ensure non-overlapping arbors.
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REFERENCES


CHAPTER 3

Ordered arrangement of dendrites

within the *C. elegans* amphid bundle

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Z.C.Y. and M.G.H conceived the ideas and designed the experiments. Z.C.Y. performed the experiments, analyzed the data, and made the figures. Z.C.Y. and M.G.H. wrote the manuscript.
SUMMARY

Biological systems self-organize into complex well-ordered structures and can evolve new patterns when perturbed. To identify principles underlying self-organization, we turned to *C. elegans* for its simple anatomy and powerful genetics. In particular, we studied the amphid sense organ, which consists of a bundle of 12 dendrites arranged in a highly reproducible order, and asked how the dendrites are organized into a bundle. We developed a method to visualize and quantify dendrite order in wild-type animals. Then, we used a candidate genetic screen to identify three CAMs that alter dendrite order. Loss of CDH-4/Fat-like cadherin causes a complete randomization of dendrite order. In contrast, loss of PTP-3/LAR or SAX-7/L1CAM surprisingly causes dendrites to take on a new, reproducible order. Further, misexpressing SAX-7 also leads to a new dendrite order. Altogether, our results suggest that differential expression of CAMs can organize dendrites in a way that is stereotyped across a wild-type population, yet that can easily give rise to novel patterns.
INTRODUCTION

Biological systems reflect a remarkable hierarchy of structural organization – proteins assemble into nanometer-scale machines, that in turn build cells, that are then organized and patterned to form tissues and organs, ultimately generating structures as complex as the human brain. How do these pieces come together in predictable ways? From first principles, one can imagine a top-down deterministic approach where the precise shape, position, and connections of each biological part are specified, similar to an electrical wiring diagram or an architectural blueprint. While this approach is reasonable for small systems, it breaks down quickly in the face of the size and complexity we encounter in biology. For example, the human brain consists of over 100 billion neurons and glial cells (Herculano-Houzel, 2009), all of which are precisely connected to form a functioning organ. It is hard to imagine a top-down program that can reliably produce such similar brains across a population of individuals.

A powerful alternative strategy for organization is a bottom-up, rules-based approach which, indeed, has been widely considered in studies of morphogenesis and pattern formation. For example, Alan Turing’s reaction-diffusion mechanism, which consists of an activator molecule that can make more of itself, an inhibitor molecule that inhibits production of the activator, and a mechanism for diffusing these two molecules (Turing, 1990), has been shown to create a diverse array of biological patterns found in fish stripes (Kondo and Asal, 1995), in seashells (Hans, 1995), and in fur and skin coloration such as those of giraffes and lizards (Manukyan et al., 2017; Walter et al., 1998). Another example of a rule that governs biological pattern formation is differential adhesion, where biological parts sort themselves based on adhesivity to create elaborate structures and patterns. This concept was first demonstrated in 1955 by Townes and
Holtfreter, where they dissociated and then mixed different germ layers of embryonic amphibian cells, and found that these cells aggregated and then sorted out into different layers according to their cell type (Townes and Holtfreter, 1955). Differential adhesion has also been shown to organize bundles of axons in the Drosophila visual system (Schwabe et al., 2014). In these examples, well-ordered patterns emerge from a series of local interactions without needing to follow central blueprint. These bottom-up strategies may also be more "evolvable", as changes to the starting conditions do not lead to a breakdown in the system but instead give rise to novel, well-ordered patterns. For example, we recently showed how a novel pattern can emerge in the nervous system of C. elegans simply by altering cell number (Yip and Heiman, 2016).

To identify rules that help to organize cells into other kinds of ordered structures, we decided to study a simple, stereotyped structure in the C. elegans nervous system called the amphid sense organ. The amphid contains 12 sensory neurons and two glial cells. Each neuron extends a single unbranched dendrite that terminates at the nose tip in a sensory cilium that senses environmental stimuli (Ward et al., 1975). Together, these dendrites and the associated glial processes fasciculate to form one of four bilaterally symmetric bundles that constitute the sensory structures of the head. These structures were the first portion of the C. elegans nervous system to be reconstructed by electron microscopy (Ward et al., 1975). Based on analysis of four animals, Ward and colleagues noted that amphid dendrites appeared to be ordered consistently within the bundle relative to one another, commenting that "Although individual worms are not precise replicas of each other down to the finest details they are remarkably exact copies." These observations are also consistent with recent EM (Doroquez et al., 2014). This
constitutes a remarkable degree of order, yet the problem of how it arises has not been pursued, largely because of the painstaking methods required to examine it.

In addition to the evidence from these EM studies, we chose to study dendrite order in the amphid for three reasons. First, the amphid bundle is a relatively simple and well-isolated system compared to other bundles of processes in the nervous system, containing only 12 unique and identifiable dendrites with no gap junctions or synapses between them (Ward et al., 1975). Second, the system allows us to easily distinguish defects in fasciculation versus guidance. Amphid dendrites do not grow outwards to the nose from a stationary cell body, but instead form by anchoring to the embryonic nose tip while the cell bodies migrate away together, a phenomenon known as retrograde extension (Heiman and Shaham, 2009). Thus, amphid dendrite fasciculation can be studied independently of outgrowth and guidance. Finally, promoters with single-cell-specificity are readily available for all amphid neurons, allowing us to easily visualize and genetically manipulate any single amphid neuron in live animals.
EXPERIMENTAL PROCEDURES

Strains and maintenance

Strains were constructed in the N2 background and cultured under standard conditions (Brenner, 1974; Stiernagle, 2006). In addition to the wild-type strain N2, the mutations, transgenes, and strains used in this study are described in Appendix 2. Unless otherwise specified, all animals were imaged in the L4 stage, corresponding with ~48h after bleach synchronization.

cdh-4 strain construction

To look at dendrite order in cdh-4 mutant animals, we crossed cdh-4(rh310) animals into a strain expressing a three-neuron marker. Because cdh-4 is located on the same chromosome as one of our integrated fluorescent markers (hmns23, which is AWA::YFP), we created a strain (CHB2770) carrying AWA::YFP on an extrachromosomal array (hmns1486) and crossed that array into a strain containing hmnIs17 (AFD::CFP, ASE::mCherry) for all cdh-4 analyses. For the permutation test comparing cdh-4 to wild type dendrite orders, we used CHB2646 instead of CHB1963 as the control.

Time point analyses

Animals were bleach-synchronized (20% bleach, 25% 1M NaOH in dH2O) and then plated onto agar plates containing food and cultured under standard conditions. We imaged animals at three different time points: second larval stage (L2-stage; 24 hours after plating), fourth larval stage (L4-stage; 48 hours after plating), and 1-day adult (72 hours after plating).
Microscopy

Image stacks were collected on a DeltaVision Core imaging system (Applied Precision) either with a UApo 40×/1.35 NA oil-immersion objective or a PlanApo 60×/1.42 NA oil-immersion objective and a Photometrics CoolSnap HQ2 camera (Roper Scientific). Animals were mounted on an agarose pad with 20-40mM sodium azide and imaged in yellow (excitation [EX] 513 nm/emission [EM] 559 nm), red (EX 575 nm/EM 632 nm), blue (EX 438 nm/EM 470 nm), and/or green (EX 475 nm/EM 525 nm) channels.

Deconvolution and analysis of images were performed with Softworx (Applied Precision), ImageJ (NIH, Bethesda, MD). Maximum brightness projections were obtained using contiguous optical sections.

Notes on image processing

Projections were adjusted for brightness, contrast, and were pseudo-colored in Photoshop (Adobe). Merged color images were assembled using the Screen layer mode in Photoshop. Figures were assembled using Photoshop CS5.1 and Illustrator CS5.1.

Image analysis

Image analysis for each animal was done in three parts. First, we imaged three amphid neurons using our three-neuron marker (ASE::mCherry, AWA::YFP, AFD::CFP). We then input this image stack into a custom-made Matlab script that detected the 3D coordinates of each dendrite and generated csv files containing the pairwise distances between each dendrite (e.g., AWA-ASE distance, AWA-AFD distance, and AFD-ASE distance) at every point along the
dendrite bundle (see Section I: generating 3D coordinates and pairwise distances). Second, we manually inspected and corrected the computer-generated dendrite traces (see Section II: manual inspection and selection of dendrite traces). Finally, we pooled animals belonging to the same population and wrote scripts in Python to generate figures to visualize the data as well as conduct statistical tests to compare between populations (see Section III: data visualization, resampling methods, and statistical analysis). Scripts for all the analysis in this paper are available for download at https://github.com/zcandiceyip.

Section I: Generating 3D coordinates and pairwise distances

The first step in our image-processing pipeline was to obtain 3D coordinates for the ASE, AWA, and AFD dendrites in each animal so we can determine the pairwise distances between the three dendrites at every point along the dendrite bundle. To do this, we wrote a script in Matlab that takes an image stack containing three amphid dendrites imaged in three different channels as input and returns several files. One file contains pairwise distances between the three dendrites along the length of the dendrite bundle starting at the dendrite tip. Another set of files contains maximum brightness projections of the images with the dendrite trace superimposed on top of the projection. Here, we will describe how the pairwise distances between dendrites are generated.

As mentioned above, our script takes as input an image stack containing three amphid neurons imaged in three different channels. For each amphid neuron, we manually select the start and end points of the dendrite by clicking on 2D projections of that image generated in Matlab. We consistently chose the starting point to be the ciliated ending at the tip of the
dendrite and the ending point to be the cell body of that amphid neuron. Next, for each dendrite, we use Dijkstra’s algorithm to find the brightest path between the start and end points. Dijkstra’s algorithm finds the shortest path between two nodes, where the objective function is to minimize the distance between the two nodes. In this case, the two nodes are the user-defined start- and endpoints, and distance is defined by the inverse of the intensity of each pixel between the start and end points. Thus, the brightest pixels between the start- and end-points represent the shortest path. Since the brightest pixels in our image stack between the start- and end-points correspond to the dendrite itself, Dijkstra’s algorithm yields the 3D coordinates of the dendrite, thus generating a computerized trace of that dendrite.

After applying Dijkstra’s algorithm to get computerized traces of the three amphid dendrites of an animal, the next step is to generate a centroid line that runs in the middle of the three of the dendrites. To do this, we first cropped the start and endpoints of the three dendrites so that the tips of the three dendrites are close to one another. We did this by averaging the coordinates of the three dendrite tips on each end, selecting the dendrite that gave the shortest distance between the average dendrite tip and the opposite end of that dendrite, and found dendrite points on the other two dendrites that were closest to the starting point of the shortest dendrite. Those two dendrite points became the new starting points for the two longer dendrites. This process was repeated for the other side of the dendrite bundle. Next, we generated new start and end points of the dendrite bundle by averaging the coordinates of the three cropped dendrites on each end, and found a centroid line by averaging the coordinates of all the points along the three dendrite traces. Finally, we used that centroid line to determine a series of planes that intersect each of the three dendrite traces once by walking along the average trace pixel by pixel, and used
Dijkstra’s algorithm to find the shortest path between the centroid line to each of the three traces.

These planes, or cross-sections, yield triangles where each vertex of the triangle is a point along one of the three dendrite traces and the sides of the triangle give the pairwise distances between amphid dendrites at a single cross-section. We then calculate each of these pairwise distances by finding the Euclidean distance between two vertices, and save them in a csv file. In addition, we obtained 2D projections of the computerized traces of each dendrite and superimposed them onto maximum brightness projections of each image stack to confirm that the dendrite trace followed the actual dendrite in the image.

Section II: Manual inspection and selection of dendrite traces

To confirm that the computer-generated dendrite traces and resulting pairwise distances for each animal were accurate, we manually inspected maximum brightness projections of each image stack projected in XY- and YZ-planes and superimposed the computer-generated dendrite trace onto the projections. If the computer-generated dendrite trace did not follow the dendrite in the maximum brightness projections, we excluded that animal in further analyses.

In general, the computer-generated dendrite traces followed the dendrites with high fidelity. However, the start- and end-points of the computer-generated traces were usually inaccurate, as the chosen start- and end-points were selected a few microns away outside the actual start- and end-points of the dendrite. To solve these inaccuracies and standardize the start- and end-points, we used the XY-projections to count the number of pixels that were traced inaccurately at the proximal and distal dendrite ends, and deleted that number of rows in the corresponding csv file.
This technique ensured that we were looking at dendrite tracings that, across all animals, begin at the dendrite tip (excluding the cilium) and end at the nerve ring.

To correct for variations in animal size within a population, we measured the length of an anatomical feature of the head of each animal (distance from the distal pharyngeal bulb to the nose tip) and used that distance to normalize the length of the dendrite traces. Because we are making point-by-point comparisons along the dendrite bundle across animals, we further segmented each dendrite bundle into bins such that the dendrite bundle for each animal was segmented into an equal number of bins (n = 100 bins), and took the mean pairwise distance within each bin for further analysis. For example, if an animal had pairwise distance measurements for 300 positions along the dendrite bundle, we segmented the bundle into 100 bins, where each bin contains 3 positions worth of distance measurements, and the pairwise distance measurement for that bin would be the mean of the three positions’ measurements.

Section III: Data visualization, resampling methods, and statistical analysis

**Maximum pairwise distance plots**: For each animal, we plotted the maximum pairwise distance at each point along the dendrite bundle. We defined dendrite bundles to be defasciculated (in pink) if the maximum pairwise distances for an animal were significantly larger than the mean maximum pairwise distance, and confirmed the defasciculation by visually inspecting maximum brightness projections of the dendrite bundle. Animals with defasciculated dendrite bundles were excluded in misfasciculation analyses because we define misfasciculation as an intact dendrite bundle with a different order of dendrites.
**Heatmap and summary plots:** Each column in the heatmap represents the middle dendrite points (ASE in red, AWA in yellow, AFD in blue) for each animal. To create summary plots, we calculated and plotted three fractions – the counts of ASE, AFD, and AWA as a fraction of the total for every point along the dendrite bundle. For populations with ordered dendrite bundles, one of those three fractions should be close to 1 while the other two fractions should be close to 0, whereas populations with highly disordered bundles have all three fractions closer to 0.33.

**Statistical analyses:** We used two approaches to test whether the dendrite order for a population is significantly different from random. First, we used a chi-squared test to test for the independence of two populations (genotype v. random). We chose the chi-squared test because our data is categorical (dendrite points are either AWA, ASE, or AFD), and the expected frequency count is ≥ 5. We calculated the chi-squared values (chi-squared test statistic:

\[
\sum \frac{(observed - expected)^2}{expected}
\]

where the observed values are the middle dendrite counts of ASE, AWA, and AFD for a given genotype and expected values is \(n/3\), where \(n\) is the number of dendrite bundles analyzed for the given genotype. We plotted the \(p\)-values using a line plot (\(x\)-axis, \(p\)-value; \(y\)-axis, position along the dendrite bundle; see Figure 3.3A).

The second approach we took was to conduct a permutation test with Fisher’s exact test (2 × 3) as the test statistic. In this case, the null hypothesis is that the two populations (genotype v. random) are drawn from the same distribution. We created the random population by drawing middle dendrite values (ASE, AWA, or AFD) from a uniform distribution for an equal number of animals in the population we were comparing against. Next, we calculated \(p\)-values by
comparing the counts of AWA, ASE, and AFD of both populations using Fisher’s exact test as the test statistic. Smaller $p$-values indicate that the two populations are different. Then, for 500 iterations, we merged the two populations of middle dendrite values, randomly split the mixed populations into two equally-sized groups, and calculated $p$-values comparing the two populations using Fisher’s exact test. This approach yields 501 $p$-values (1 true $p$-value + 500 $p$-values from resampling) for each point along the length of the dendrite bundle. Finally, we determined the percentile rank of the true $p$-value and plotted that rank using a blue log-scale color bar. Darker blues on the color bar indicate that the dendrite order of one genotype is different from random (Figure 3.1, blue bars).

To compare mutant and wild type dendrite orders, we also used permutation tests with Fisher’s exact test as described above, but compared mutant to wild type populations instead (Figure 3.2, red bars). Darker red on the color bar indicate that the two populations are different.

**Switchpoint swarmplot (Figure 3.3C)**

To create this swarmplot, we measured three different lengths in wild-type L4 animals. First, we measured the distance from the switchpoint to the nosetip. To do this, we defined the switchpoint to be the point where the middle dendrite changes from one dendrite to another for more than one bin (for definition of a bin, see Section II under “Image analysis” methods section). We then measured the distance between the nosetip to that defined switchpoint. Second, we measured the distance from the nosetip to the dendrite tip of AUA. Finally, we measured the length of the amphid socket that is fasciculated with the amphid; we used the amphid dendrite AFD (blue) as a proxy for the amphid bundle. We created the figure using the
swarmplot function in the Python Seaborn package. To determine the difference between populations, we used a two-sample Kolmogorov-Smirnov test because the measurements for the switchpoint were not normally distributed.

**Candidate screen of cell adhesion molecules**

For each mutant, we used DiO (Sigma, D4292) to dyefill 6/12 amphid neurons and scored 50 L4 animals for amphid defasciculation phenotypes using a fluorescence dissecting microscope (Altun et al., 2002).

**Expression pattern analysis**

To determine the identity of the amphid neurons that express *sax-7* or *ptp-3* we used DiI (Sigma, 468495) to dye-fill L1 animals expressing a nuclear-localized mCherry (NLS-mCherry-NLS) under the control of *sax-7* or *ptp-3* promoters (CHB1687 and CHB1840 respectively). We then collected image stacks for these animals in the red and green channels as L1-stage animals. Because the identity and positions of the dye-filled amphid neuron cell bodies are known for L1 animals (Sulston et al., 1983), we used the position of the dye-filled amphid cell bodies to infer the positions of the cell bodies of the other amphid neurons. We then used this method to obtain the identity of the amphid neurons expressing nuclear-localized mCherry.
RESULTS

Development of a semi-automated method to quantify dendrite order

To study the cellular and molecular mechanisms underlying dendrite order in the amphid, we first sought to develop methods to measure, quantify, and compare dendrite order in populations of animals. Ideally, we would label each of the 12 amphid dendrites with a unique fluorescent marker, examine cross sections of the dendrite bundle, and determine how dendrites are ordered across individuals. However, we lacked the technical ability to generate 12 differently-colored cell-specific markers and, even if we could uniquely mark each neuron, quantifying the degree of stereotypy of 12 dendrites is a mathematically complex problem. Instead, we decided to simplify the problem by labeling only three dendrites and examining their relative order as a proxy for overall bundle order. We reasoned that, if the overall bundle is well-ordered, then any three dendrites would be similarly well-ordered. Our method can be broken down into three parts: imaging, quantification, and statistical analysis (Figure 3.1).

First, we generated a strain to label three amphid neurons in different colors (AWA:YFP, AFD:CFP, ASE:mCherry) by combining two separately integrated transgenes (one with AWA:YFP; another with AFD:CFP and ASE:mCherry). We used this YFP + CFP/mCherry approach because we consistently observed recombination artifacts when CFP and YFP were introduced on the same transgene. When we imaged this strain, we found that, using traditional widefield deconvolution microscopy, we were able to separately discern the three dendrites despite each dendrite being only about 0.5µm in diameter, close to our effective resolution limit (Figure 3.1A).
Figure 3.1. Imaging pipeline for quantifying dendrite order

Our imaging pipeline can be broken down into three parts: imaging (A), quantification (B), and statistics (C). (A) Imaging: We express ASE::mCherry, AWA::YFP, and AFD::CFP and image the animal. (B) Quantification: We extract 3D coordinates of each dendrite trace. For each point along the dendrite bundle, we determine the dendrite in the middle and plot the color of that dendrite point. We can visualize a population by assembling all the columns together. (C) Statistics: We conduct permutation testing with Fisher’s $3 \times 2$ exact test as a test statistic, and plot the rank of the true comparison on a log scale as a color bar.
Figure 3.1. (Continued) Imaging pipeline for quantifying dendrite order
Next, we sought to quantify the order of dendrites within individual animals. To do this, we used a custom MATLAB script that extracts the x-, y-, and z-coordinates of the three dendrites, calculates a centroid line that runs between the three dendrites, and, for each point along the centroid, finds the point on each of the three dendrite traces that is closest to the centroid (Figure 3.1B, i and ii). These three dendrite points define a single cross section (Figure 3.1Bii). Thus, independent of any curvature or rotation in the head of a given animal, this approach identifies a series of cross-sections along the length of the dendrite bundle that intersect the three dendrites exactly once. Each cross section contains a triangle consisting of one ASE, AFD, and AWA dendrite point. We calculated the pairwise distances between these points (Figure 3.1Bii, AWA-ASE = $d_1$, AWA-AFD = $d_2$, and AFD-ASE = $d_3$).

We used these three pairwise distances to quantify dendrite order in two ways. First, we used the longest pairwise distance as a proxy for bundle width, allowing us to measure how tightly or loosely the dendrites are bundled. To do this, we simply plotted the value of the longest pairwise distance at every point along the bundle (Figure 3.1Biii, blue plot). For example, for the animal shown in Figure 3.1, all of the longest pairwise distances are less than 2 µm, suggesting that the dendrites in this animal are tightly bundled together (Figure 3.1Biii, blue plot). Second, we created a categorical variable that describes relative dendrite order by identifying the dendrite "in the middle" of the others at every point along the bundle. A dendrite is considered to be in the middle if it is opposite the longest side of the triangle, i.e. the longest pairwise distance. For example, if the longest pairwise distance is between AWA (Figure 3.1Bii, yellow) and ASE (Figure 3.1Bii, red), then the dendrite in the middle at that point along the bundle is AFD (Figure 3.1Bii, blue). Then, to visualize dendrite order along the length of the bundle in a single animal,
we simply plot the color of the middle dendrite at each position along the bundle in a column. For example, for the animal in Figure 3.1, the dendrite in the middle is AFD (blue) near the nose tip and then switches to ASE (red) closer to the cell bodies. To visualize dendrite order within a population, we generate a "population plot" by arranging these individual columns side-by-side (Figure 3.1Biii).

Finally, we employed a statistical approach based on permutation tests to quantitatively compare the observed dendrite order in a population to a simulated random order (Figure 3.1C, see Methods.). We also used this approach to compare dendrite order between wild-type and mutant populations. In both cases, our null hypothesis is that the two populations are drawn from the same distribution and any differences between them are due to sampling error. First, the observed and test populations are compared using a test statistic (we used Fisher's exact test, see Methods) to yield a nominal $p$-value. These populations are then computationally merged and re-sampled to create mock populations, and the test statistic is recalculated. Repeating this permutation process (500 iterations, in our case) gives a representative set of $p$-values for populations that have the same composition as our true samples but, by definition, differ only due to sampling error. This approach yields 501 $p$-values (1 true $p$-value + 500 $p$-values from resampling) for each point along the length of the dendrite bundle. The percentile rank of the true $p$-value at each point is plotted using a log-scale color bar (Figure 3.1Cii). Darker colors indicate a low rank, meaning that the observed dendrite order is significantly different from the test distribution.
To summarize, we have developed a robust and semi-automated pipeline to detect, quantify, and compare dendrite order, which now allows us to determine dendrite order in a wild-type population and to ask whether it is altered by various manipulations.

**Amphid dendrites are fasciculated and ordered**

To determine wild-type dendrite order, we imaged animals expressing CFP, YFP, and mCherry in AFD, AWA, and ASE respectively at three time points following synchronization of animals at the first larval stage (L1) (24h, early larval stage (L2/3); 48h, late larval stage (L4); 72h, one-day adult). We found that dendrites are tightly fasciculated throughout larval growth (Figure 3.2A) (average longest pairwise distance ± standard deviation: 24h, 0.88 ± 0.09µm; 48h, 1.11 ± 0.22µm; 72h, 1.51 ± 0.25µm). The approximate doubling in bundle width may reflect an increased diameter of each dendrite and thus the entire bundle, and roughly corresponds to the overall growth of the head during these stages.

Next, we looked at dendrite order across the population (Figure 3.2B and 3.2C). We visualized dendrite order using population plots as described above (Figure 3.2C) as well as summary plots that represent the fraction of animals with the ASE, AWA, or AFD dendrite in the middle at every point along the bundle (Figure 3.2B). The summary plot provides a compact way to visualize how well-ordered dendrites are in a population. If all of the lines converge at 0.33 then it indicates that dendrite order is random for that population, whereas if any of the lines approach 1 or 0 then it suggests that dendrites are arranged in a consistent, non-random order.

We found that amphid dendrites are well ordered, especially in younger animals (Figures
Figure 3.2. Wild-type dendrite bundles are fasciculated and ordered

(A) Maximum pairwise distances for early stage larvae (24h post bleach synchronization; L2/L3-stage), late stage larvae (48h post bleach synchronization, L4-stage), and early adults (72h post bleach synchronization; one-day adults). (B) Summary plots and color bars for 24h, 48h, and 72h time points. (C) Heat maps showing dendrite order for 24h, 48h, and 72h time points.
To quantify the degree of order, we compared our observed samples to a simulated random sample using a permutation test (Figure 3.2B, blue bars) as well as a chi-squared test (Figure 3.3A, see Methods). Interestingly, although dendrite order is stereotyped, it is not uniform along the length of the bundle. Instead, it exhibits a switch point, which itself is stereotyped (Figures 3.2B and 3.2C). Close to the nose tip, AFD (blue) is predominantly in the middle but, following the switch point, ASE (red) takes over as the middle dendrite for the rest of the length of the bundle. AWA (yellow) is rarely in the middle, and only for short segments (Figures 3.2B and 3.2C). This order is maintained throughout larval development, although it becomes increasingly "noisy" with age. We further confirmed these results by showing that a different set of three amphid dendrites (AWA, YFP; AWC, CFP; ASG, mCherry) are also well-ordered and exhibit a similar switch point (Figure 3.3B).

The cause of the switch point is unclear. Similar discontinuities were observed in other bundles by classical EM, and it was noted that the "abrupt changes in neighbourhood exhibited by some neurons" might arise from mechanical obstacles in the local environment or from changes in the composition of the bundle (White et al., 1986). Indeed, examination of classical EM sections reveals changes in the overall shape of the bundle along its length, switching from a cylinder to a sheet as it is pressed against the basement membrane of the pharynx. The composition of the bundle also changes along its length. The neuron AUA extends a dendrite in the posterior portion of the bundle that does not reach the nose, while the amphid socket glial cell extends a process that enters the bundle and extends along only the anterior portion of the bundle. We measured the positions of the AUA dendrite ending and the length the amphid socket glial cell is fasciculated with the amphid bundle, and found the position of the switch point does not
Figure 3.3. Other measurements pertaining to wild-type dendrite order

(A) Dendrite order measured by chi-squared test (see Experimental Procedures). y-axis is distance along the nose. x-axis is p-values from 0 to 0.06. (B) Dendrite bundle width and dendrite order using a different set of three neurons: AWA::YFP in green, AWC::CFP in purple, ASG::mCherry in red. (C) A swarmplot of wild-type L4 animal data showing distance from the dendrite tips to the switch point (left column), the distance from the nose tip to the end of AUA (middle column), and the length of the amphid sheath (AMso) that is fasciculated with the amphid bundle (right column).
Figure 3.3. (Continued) Other measurements pertaining to wild-type dendrite order
correlate with either the AUA ending or the entry of the socket glial cell into the bundle (Figure 3.3C).

To summarize, we used our imaging pipeline to show that amphid dendrites are well-ordered in young animals and that this order is maintained – albeit imperfectly – during larval growth, despite an approximately two-fold increase in the length and width of the bundle and despite ongoing bending movements caused by locomotion of the animal.

**Amphid cilia and sheath glia are not required for dendrite order**

Our data show that amphid dendrites exhibit the most consistent order in the distal region of the dendrite bundle, closest to the nose. This region is especially interesting because it is rich in cell biological features, including dendritic cilia used to detect signals from the environment and cell-cell junctions between each dendrite and the amphid sheath glial cell. By contrast, the remainder of the dendrites is comparatively featureless, lacking gap junctions, synapses, or any other cell biological specializations. We therefore wanted to test whether cilia or interactions with the sheath glial cell contribute to amphid dendrite order (Figures 3.4A and 3.5).

We first looked at amphid cilia as a potential source of dendrite order. Early EM reconstructions showed that the order of amphid cilia is also stereotyped, albeit different from that of amphid dendrites (Ward et al., 1975). To test the hypothesis that amphid cilia are required for dendrite order, we crossed our markers into a mutant lacking the RFX transcription factor DAF-19 required to activate the genetic program for ciliogenesis (Figures 3.4A and 3.5). Because *daf-19* mutants constitutively enter a non-reproductive developmental state called dau...
Figure 3.4. Amphid cilia and sheath glia are not required for dendrite order

(A) Schematic of wild-type amphid bundle, glial cells, and nose tip (inset). (B-D) Dendrite bundle width and dendrite order in mutants that lack cilia (B), have divergent cilia (C), and no sheath glia (D). Color bars: blue color bars test whether genotype is significantly different from random – darker blues indicate well-ordered and lighter blues indicate that dendrite order is random. Red color bars test whether genotype is significantly different from wild type – darker reds indicate that the genotype is very different from wild type and lighter reds indicate that the genotype is similar to wild type.
Figure 3.4. (Continued) Amphid cilia and sheath glia are not required for dendrite order
Figure 3.5. Dendrite orders for amphid cilia mutants and amphid sheath glia ablation are not perturbed

Dendrite order heatmaps for cilia mutants *daf-12; daf-19* (left), *mec-8* (middle), and genetic glia ablation (right).
we also introduced a mutation in \textit{daf-12}, which encodes a receptor required for dauer entry. \textit{daf-19}; \textit{daf-12} animals lack cilia but do not enter dauer, allowing the strain to be maintained as a homozygous stock. We found that lack of cilia had no effect on fasciculation or dendrite order (Figures 3.4B and 3.5). To statistically quantify this observation, we ran permutation tests comparing \textit{daf-19}; \textit{daf-12} with simulated random distributions (blue) or with our observed wild-type population (red). Darker blues indicate that \textit{daf-19} dendrite order is non-random, while the absence of darker reds indicates that \textit{daf-19} dendrite order does not differ from wild type. These tests are consistent with the qualitative impression that dendrite order is unaffected. As cilia are also required for normal neuronal activity, this result also implies that dendrite order does not depend on normal patterns of activity.

To further test the role of dendrite endings in establishing dendrite order, we examined mutants lacking the splicing factor MEC-8. Whereas wild-type dendrite endings enter the amphid sheath glial cell in a stereotyped order and converge into a central channel, in \textit{mec-8} mutants the amphid dendrite endings are disorganized and diverge into separate, isolated channels in the sheath glial cell (Perkins et al., 1986). Despite this disorganization, we found that overall dendrite fasciculation and order along the length of the amphid bundle remain normal (Figures 3.4A, 3.4C, and 3.5). Altogether, our results show that proper arrangement of amphid cilia is not required for dendrite order.

We next tested whether interactions with the amphid glial cell are required for dendrite order. Amphid dendrites form cell junctions with the sheath glial cell, and the sheath glial cell secretes factors that promote the normal development and function of the dendrite sensory endings. To
test the possibility that the sheath glial cell might impose order on the dendrites, we genetically ablated sheath glia using diphtheria toxin expressed under the control of a late embryonic-stage amphid sheath promoter (Figures 3.4A and 3.5). We were not able to ablate the sheath glia in early embryonic-stages, as that causes amphid dendrites to fail to extend (Bacaj et al., 2008). Our strain also carried a fluorescent marker for the amphid sheath glial cell, allowing us to identify and exclude rare animals in which ablation failed. In glia-ablated animals, we found dendrite fasciculation and order to be unchanged from wild type, suggesting that sheath glia are not required to maintain dendrite order (Figures 3.4D and 3.5). Together, our data suggest that the main cell biological features of amphid dendrites – their cilia and cell-cell junctions – are not required for dendrite fasciculation or order.

A candidate screen of cell adhesion molecules reveals that cdh-4, sax-7, and ptp-3 are required for dendrite fasciculation

Next, we considered the possibility that cell adhesion molecules (CAMs) along the length of the dendrites might be required for fasciculation and dendrite order. Previous studies in C. elegans and other organisms have shown that cell adhesion molecules such as SAX-7/L1CAM and the large extracellular matrix molecule DIG-1 are required for dendrite and axon fasciculation (Burket et al., 2006; Bénard et al., 2006; Sasakura et al., 2005). However, only a handful of studies have explored how CAMs order axons or dendrites. We wanted to identify other CAMs that affect amphid dendrite fasciculation and order. Since it is technically unfeasible to conduct a large-scale forward genetic screen for dendrite order defects using our imaging pipeline, we decided to conduct a candidate screen looking for mutants that had amphid defasciculation defects, with the hypothesis that mutants with defasciculation phenotypes would
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| *dig-1(n1321)* | 24                     | • A giant (13,100 amino acids) secreted immunoglobulin superfamily protein with Ig domains, FNIII domains, EGF domains, and others.  
• Non-cell autonomously affects neurite fasciculation  
• (Burket et al., 2006; Bénard et al., 2006) |
| *ptp-3(mu256)* | 14                     | • Leukocyte Common Antigen Related (LAR) ortholog; a receptor tyrosine phosphatase  
• Axon guidance defects in *C. elegans* (Ackley et al., 2005)  
• (Ch'ng et al., 2003) |
| *sax-7(ky146)* | 12                     | • Human L1CAM ortholog  
• Affects amphid dendrite fasciculation (Sasakura et al., 2005)  
• Enriched in AFD and/or AWB (Colosimo et al., 2004)  
• (Zallen et al., 1999) |
| *vab-1(dx31)*  | 10                     | • Only Eph receptor in *C. elegans*  
• Many gross morphological defects, including axon outgrowth defects (Mohamed and Chin-Sang, 2006)  
• (George et al., 1998) |
| *sax-3(ky123)* | 8                      | • Ortholog of Robo  
• Many axon and dendrite phenotypes, including axon guidance defects  
• (Zallen et al., 1999) |
| *cdh-4(rh310)* | 6                      | • Fat-like cadherin  
• Affects axon fasciculation in *C. elegans*  
• Enriched in AFD and/or AWB (Colosimo et al., 2004)  
• (Schmitz et al., 2008) |
| *nrx-1(wy778)* | 2                      | • Ortholog of neurexins, contains extracellular laminin and EGF domains  
• Enriched in AFD and/or AWB (Colosimo et al., 2004)  
• (Maro et al., 2015) |
| *unc-40(e271)* | 2                      | • Netrin receptor homolog  
• Enriched in AFD and/or AWB (Colosimo et al., 2004)  
• (Hedgecock et al., 1990) |
| *dma-1(wy686)* | 0                      | • A cell surface receptor composing of leucine-rich repeats  
• Enriched in AFD and/or AWB (Colosimo et al., 2004)  
• Known interactor of *sax-7*  
• (Liu and Shen, 2011) |
| *ptp-3(ok244)* | 0                      | • See *ptp-3(mu256)*  
• *C. elegans* Gene Knockout Consortium |
| *igcm-1(ok711)*| 0                      | • 6 Ig domains, 1 FNIII domains, transmembrane domain, PDZ binding motif  
• Enriched in AFD and/or AWB (Colosimo et al., 2004)  
• *C. elegans* Gene Knockout Consortium |
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| **plx-2(ev773)** | 0 | - One of two *C. elegans* plexins  
- Enriched in AFD and/or AWB (Colosimo et al., 2004)  
- (Ikegami et al., 2004) |
| **nlr-1(tm2050)** | 0 | - *Caspr* ortholog  
- Enriched in AFD and/or AWB (Colosimo et al., 2004)  
- *C. elegans* Gene Knockout Consortium |
| **mnr-1(wy758)** | 0 | - A member of Fam151 proteins  
- Known interactor of sax-7  
- (Dong et al., 2013; Salzberg et al., 2013) |
| **ptp-4(gk715362)** | 0 | - Enriched in AFD and/or AWB (Colosimo et al., 2004)  
- (Thompson et al., 2013) |
| **syg-1(ky652)** | 0 | - Homolog of IrreC (*Drosophila*)/NEPH1, 2, and 3 in humans  
- Enriched in AFD and/or AWB (Colosimo et al., 2004)  
- (Shen and Bargmann, 2003) |
| **fmi-1(rh308)** | 0 | - Cadherin-like protein with 6 EGP and 2 laminin G domains, a GPS cleavage site and a seven-pass transmembrane domain  
- Enriched in AFD and/or AWB (Colosimo et al., 2004)  
- (Steimel et al., 2010) |
| **casy-1(ok739)** | 0 | - Ortholog of calsytenins/alcadeins, encodes a type I transmembrane protein with 2 cadherin domains and an LG/LNS domain  
- After we tested *casy-1* for defasciculation, Kim and Emmons reported in 2017 that it is required for axon fasciculation in the male tail (Kim and Emmons, 2017)  
- *C. elegans* Gene Knockout Consortium |
| **scd-2(ok565)** | 0 | - Homolog of the anaplastic lymphoma kinase receptor tyrosine kinase  
- Enriched in AFD and/or AWB (Colosimo et al., 2004)  
- *C. elegans* Gene Knockout Consortium |
| **rig-3(ok2156)** | 0 | - Immunoglobulin superfamily protein with a GPI anchor, 2 Ig domains, and 1 FNIII domain  
- Enriched in AFD and/or AWB (Colosimo et al., 2004)  
- *C. elegans* Gene Knockout Consortium |
| **dgn-1(cg121)** | 0 | - Dystroglycan, ortholog of human DG  
- Enriched in AFD and/or AWB (Colosimo et al., 2004)  
- (Johnson et al., 2006) |
have a higher probability of losing dendrite order.

To this end, we generated a list of 23 CAMs that were strong candidates to affect amphid dendrite fasciculation. We chose these CAMs either because their expression is highly enriched in amphid neurons or because they interact with known CAMs affecting neurite fasciculation in other areas of the *C. elegans* nervous system or in other organisms (Table 3.1). Next, we screened through these mutants for dendrite fasciculation defects in late larval stage animals (L4) by using a dye-filling method. Briefly, animals are soaked in (concentration) of the lipophilic fluorescent dye DiI for 45 minutes, which for unknown reasons leads to bright and highly specific labeling of six amphid neurons, thus providing a fast and marker-independent method to visualize the overall structure of the amphid bundle. As a positive control, *dig-1* mutants exhibited readily apparent defasciculation defects in this assay (24% of animals, Table 3.1).

Using this approach, we identified seven additional CAM mutants that caused defasciculation defects (*ptp-3, sax-7, vab-1, sax-3, cdh-4, nrx-1, and unc-40;* Table 3.1). Interestingly, even the most pronounced defects among these are weakly penetrant (< 20%) suggesting that a redundant system of CAMs control dendrite fasciculation. Loss of *sax-7* had been observed to cause "loosening" of amphid dendrites in some animals, along with other defects, and this aspect of its phenotype had not been pursued (Sasakura et al., 2005). For the rest of this study, we will focus on three mutants – *cdh-4 sax-7/L1CAM, and ptp-3/LAR* – in which the morphology of the amphid neurons is grossly normal.
Amphid dendrite are randomly ordered in \textit{cdh-4} mutants

From our candidate screen, we found \textit{cdh-4} to be required for dendrite fasciculation. \textit{cdh-4} encodes a Fat-like cadherin characterized by the large number of cadherin repeats in its extracellular domain (Figure 3.6A). In \textit{C. elegans}, \textit{cdh-4} has been implicated in axon fasciculation in the dorsal and ventral nerve cords, cell migration, and hypodermis and pharynx development (Schmitz et al., 2007; Schmitz et al., 2008). For this study we used the \textit{rh310} allele, which introduces a premature stop codon in the extracellular domain (Schmitz et al., 2008).

Since only a small number of \textit{cdh-4} mutant animals had dendrite fasciculation defects (6%), we wanted to ask whether dendrites are still ordered in \textit{cdh-4} mutants that had normally fasciculated dendrite bundles. To do this, we crossed our markers into \textit{cdh-4} animals. Consistent with our dye-filling results (Table 3.1), we observed defasciculated amphid dendrites in only 1/21 animals examined (this animal is shown in Figure 3.6B; the plot of its longest pairwise distances along the bundle is shown in light red in Figure 3.6C). However, in the remaining 20/21 animals, we found that dendrite order was lost despite the bundle remaining intact and well-fasciculated (Figures 3.6D and 3.7), with the ASE, AFD, and AWA dendrites occupying the middle position with approximately equal frequency along the entire length of the bundle. Permutation tests confirmed that dendrite order in this population is not significantly different from random (light shading in blue color bar) and is significantly different from wild type (dark shading in red color bar). Our results show that, although \textit{cdh-4} mutants exhibit only mild and low-penetrant defects in fasciculation, they show nearly complete loss of dendrite order within the bundle, suggesting CDH-4 plays an essential role in specifying dendrite order despite having a more redundant role in overall fasciculation.
Figure 3.6. *cdh-4* mutants exhibit a loss of dendrite order

(A) Protein structure for CDH-4. TM, transmembrane domain; EGF-like, epidermal growth factor-like domain. (B) L4-stage animal with three neuron marker showing defasciculated dendrites. (C) Maximum pairwise distance plot for *cdh-4* mutants (L4-stage). (D) Heatmap, summary plot, and statistical tests for *cdh-4*. 
Figure 3.7. Wild-type control for *cdh-4* shows an ordered bundle

Maximum pairwise distance, heatmap, and summary plots for CHB2646 (control for *cdh-4* data).
**Amphid dendrites take on a new order in sax-7/L1CAM and ptp-3/LAR mutants**

We next examined how *ptp-3* and *sax-7* contribute to amphid dendrite fasciculation and order. *ptp-3* encodes a receptor-like protein tyrosine phosphatase that is part of the leukocyte antigen related (LAR) family of protein tyrosine phosphatases. Protein tyrosine phosphatases play important roles in nervous system development, including in the development and maintenance of synapses, axon guidance and defasciculation in other systems (Clandinin et al., 2001; Dunah et al., 2005; Krueger et al., 1996; Maurel-Zaffran et al., 2001; Wills et al., 1999). In *C. elegans*, *ptp-3* is important for axon guidance and synapse formation as well as embryonic morphogenesis (Ackley et al., 2005; Harrington et al., 2002). *sax-7* encodes a homolog of the human L1 cell adhesion molecule that is part of a group of highly conserved CAMs in the immunoglobulin superfamily, characterized by immunoglobulin (Ig domains) and fibronectin III (FNIII) repeats in the extracellular domain. L1 is involved in many developmental processes in the nervous system, including axon guidance, fasciculation, myelination, synapse formation maintenance, and maintenance of the nervous system (Chen and Zhou, 2010), and is disrupted in a human neurological disorder called L1 syndrome (Jouet et al., 1994; Rosenthal et al., 1992; Van Camp et al., 1993; Fransen et al., 1995). In *C. elegans*, *sax-7* affects axon guidance, fasciculation of neuronal processes, and maintenance of neuronal cell body positions (Kim and Emmons, 2017; Sasakura et al., 2005).

In *C. elegans*, there are two protein isoforms of *ptp-3*, PTP-3A and PTP-3B. PTP-3A consists of three Ig domains and eight FNIII repeats in the ectodomain, a transmembrane region and two protein tyrosine phosphatase domains on the intracellular side, whereas PTP-3B has a
smaller ectodomain containing the last four FNIII repeats (Figure 3.8A). We examined two alleles of \textit{ptp-3}: \textit{mu256}, a point mutation that causes a frameshift leading to a premature stop in both \textit{ptp-3} isoforms; and \textit{ok244}, a deletion that disrupts only \textit{ptp-3a} (Figure 3.8A). For \textit{SAX-7}, there are also two protein isoforms – short (\textit{SAX-7S}) and long (\textit{SAX-7L}) – that differ in the number of Ig domains (\textit{SAX-7S}, 4 Ig domains; \textit{SAX-7L}, 6 Ig domains) (Figure 3.8B). We examined two alleles of \textit{sax-7}, both of which affect both isoforms. The reference allele \textit{ky146} is a point mutation in the third FNIII repeat that leads to a premature stop codon, and \textit{eq1} is a deletion that removes the fifth FNIII repeat, the transmembrane domain, the cytoplasmic tail, as well as part of the 3’ untranslated region (Figure 3.8B) (Wang et al., 2005; Zallen et al., 1999). We used \textit{ky146} for most of our analyses, and confirmed our results using the \textit{eq1} allele.

To examine dendrite defects, we crossed our markers to each mutant. As expected from our dye-filling assays, \textit{ptp-3(mu256)} and \textit{sax-7(ky146)} exhibit amphid defasciculation defects with low penetrance (Figures 3.8C and 3.8G). In both mutants, defects become progressively more severe throughout larval development (Figures 3.8D, 3.8H, 3.9B, and 3.9C). This progressive phenotype is consistent with the idea that \textit{sax-7} is required for the maintenance of nervous system architecture (Sasakura et al., 2005). Fasciculation defects were not observed in \textit{ptp-3(ok244)}, suggesting that PTP-3B is sufficient for fasciculation (Figure 3.9A).

To examine dendrite order, we examined only dendrite bundles that remained tightly fasciculated. In contrast to what we observed with \textit{cdh-4}, in \textit{ptp-3(mu256)} or \textit{sax-7} young larvae we found the dendrites had a non-random order and, surprisingly, took on a new order that was different from wild type (Figures 3.8E and 3.8I). In \textit{ptp-3(mu256)}, ASE (red) is predominantly
Figure 3.8. Loss of either *ptp-3* or *sax-7* generates a new order of dendrites

(A-B) Protein structures for PTP-3 (A) and SAX-7S (B). (C) Image of *ptp-3(mu256)* mutant animal with defasciculated dendrites. (D-E) Time point imaging (24h-, 48h-, and 72h-post bleach synchronization) with maximum pairwise distance plots (D) and summary plots (E) for *ptp-3* animals. (F) L4-animals expressing nuclear mCherry (red) under a *ptp-3b* promoter with dye-filled amphid neurons in green. (G) Image of *sax-7(ky146)* mutant animal with defasciculated dendrites. (H-I) Time point imaging (24h-, 48h-, and 72h-post bleach synchronization) with maximum pairwise distance plots (H) and summary plots (I) for *ptp-3* animals. (J) L4-animals expressing nuclear mCherry (red) under a *sax-7* promoter with dye-filled amphid neurons in green.
Figure 3.8. (Continued) Loss of either *ptp-3* or *sax-7* generates a new order of dendrites.
Figure 3.9. *ptp-3* and *sax-7* time point analysis, other alleles, and interactors

(A) Maximum pairwise distance, heat map, summary plot, and statistical tests for *ptp-3(ok244)*.

(B-C) Time point imaging (24h-, 48h-, and 72h-post bleach synchronized animals) for *ptp-3(mu256)* (B) and *sax-7(ky146)* (C). (D) Maximum pairwise distance, heat map, summary plot, and statistical tests for *ptp-3(mu256); sax-7(ky146)* L4 animals. (E-H) Maximum pairwise distance, heat map, summary plot, and statistical tests for *dma-1* (E), *clr-1* (F), *igcm-1* (G), and *sax-7(eq1)* (H).
Figure 3.9. (Continued) *ptp-3* and *sax-7* time point analysis, other alleles, and interactors.
in the middle throughout the length of the bundle. In sax-7, both ASE and AWA (red and yellow) are frequently observed in the middle of the dendrite bundle but AFD (blue) is not. This order progressively deteriorates over time, such that dendrite bundles of 1-day sax-7 adults are random (Figure 3.8I). Loss of PTP-3 and SAX-7 together (ptp-3(mu256); sax-7(ky146)) leads to enhanced defects in both defasciculation and dendrite order, consistent with these molecules acting partly redundantly (Figure 3.9D). Dendrite order is not affected by loss of PTP-3A alone (ptp-3(ok244), Figure 3.9A) or by loss of adhesion molecules that interact genetically or physically with SAX-7 in other contexts (DMA-1, CLR-1, IGCM-1, RIG-6), suggesting these defects are specific to PTP-3B and SAX-7 (Figures 3.9E-G). As a further specificity control, we observed similar defects with sax-7(eq1) (Figure 3.9H).

Together, our results suggest that PTP-3 and SAX-7 are required, first, to establish the correct order during early development and, second, to maintain dendrite order throughout larval growth. The observation that loss of a single CAM can generate a new dendrite order suggests that dendrites might normally "choose" from among several potential neighbors based on differential adhesion, and that these affinities are altered when a given CAM is removed.

Differential adhesion among dendrites might reflect CAMs being expressed at different levels in distinct amphid neurons. For example, an elegant study of Drosophila retinal axons showed that they fasciculate in a stereotyped configuration based on individual neurons expressing low or high levels of N-cadherin. Therefore, we examined the expression patterns of ptp-3b and sax-7 by fusing promoter regions upstream of their coding regions (7kb, ptp-3b; 3kb, sax-7) to a nuclear-localized mCherry. We imaged early larval stage (L1) animals and used the
six dye-filling neurons, stained in green, as landmarks to assist in cell identification. We found that *ptp-3b* is expressed at low but detectable levels in many cells, including amphid neurons, with highest expression in AWB and ASE (Figure 3.8F). We found that *sax-7* is expressed throughout the nervous system and, within the amphid, is consistently expressed in AWC, ASE, and ASK (Figure 3.8J). While it is interesting that both CAMs are expressed in one of the three neurons we used for imaging (ASE), it is important to note that changes in dendrite order likely reflect an overall rearrangement of the bundle. The differential expression we observe is consistent with a differential adhesion model.

To summarize, we found that loss of either *sax-7* or *ptp-3* causes amphid dendrites to take on a new order of dendrites that is distinct from wild type. In both *sax-7* and *ptp-3* mutants, this order became increasingly random over time, and correlates with increased defasciculation. We also found that *sax-7* and *ptp-3* were expressed in a subset of amphid neurons, suggesting that these adhesion molecules are not uniformly expressed across all amphid neurons. Taken together, our data suggests that *sax-7* and *ptp-3* are required for ordering dendrites within the amphid bundle, such that loss of either CAM causes a weakly-penetrant defasciculation phenotype and a highly-penetrant change in dendrite order.

**Misexpression of SAX-7 can reorder amphid dendrites**

Finally, we reasoned that if dendrite order reflects differential adhesion among dendrites, then experimentally misexpressing a CAM might alter dendrite order. To test our hypothesis, we misexpressed SAX-7 in all amphid neurons using a pan-amphid promoter (*osm-6pro*) in wild-type animals. We used SAX-7 because, in our hands, PTP-3 appears to undergo post-
transcriptional regulation that makes it difficult to manipulate. We hypothesized that if SAX-7 acts permissively, then SAX-7 misexpression would not affect dendrite order. Conversely, if SAX-7 acts instructively, then misexpression of SAX-7 might randomize dendrite order or confer a new dendrite order. Indeed, upon misexpression of SAX-7, we found that amphid dendrites took on a new order (Figure 3.10), distinct from that observed in wild-type, sax-7, or ptp-3(mu256) animals. These results suggest that SAX-7 may play an instructive role in specifying amphid dendrite order, possibly by contributing to patterns of differential adhesion among the dendrites.
Figure 3.10. Misexpression of SAX-7 in all amphid neurons generates a new dendrite order

Maximum pairwise distance, heat map, summary plot, and statistical tests for CHB2407 (osm-6pro::SAX-7S).
DISCUSSION

In developmental neurobiology, there is a significant body of research that seeks to understand the mechanisms behind axon and dendrite guidance pathfinding. Indeed, landmark findings over the last 30 years have elucidated a number of guidance cues and their receptors such as Netrins, Ephrins, and Slits, and Robos (Dickson, 2002; Kolodkin and Tessier-Lavigne, 2011; Tessier-Lavigne and Goodman, 1996). A subset of this work involves understanding the molecular mechanisms by which neurites fasciculate with and defasciculated from nerve bundles (for a review, see Van Vactor, 1998). For example, Luxey et al. showed that Eph:ephrin-B1 signaling controls axon fasciculation in sensory and motor axons in the limb (Luxey et al., 2013).

A smaller subset of this research is focused understanding how neurites choose which bundle to fasciculate with. For example, classical axon guidance studies in grasshoppers by Goodman and colleagues showed that when a certain growing axon (G) enters an axon bundle, it selectively adheres to a defined “partner” axon (P). If the P axon is ablated, the G axon does not adhere to other axons in this bundle, or to axons in any nearby neighboring bundles (Bastiani et al., 1984; Lin et al., 1994; Raper et al., 1984). However, to our knowledge, only one study has taken a mechanistic approach towards understanding how axons and dendrites are ordered within a bundle. In this study, Schwabe et al. studied the cartridges of the Drosophila visual system, where there is an invariant organization in which neurites from lamina cells (L) are surrounded by photoreceptor (R) axons. They found that this organization reflects differential expression levels of N-cadherin between the L and R cells, with the higher-expressing L neurites forming an adhesive core surrounded by the lower-expressing R axons (Schwabe et al., 2014).
The lack of attention towards this question is not due to the lack of ordered bundles in nervous systems – studies of axons projecting from the retina, as well as the olfactory bulb, have provided evidence that these bundles have a defined arrangement, in some cases according to topographic or dorsal-ventral patterns and in other cases reflecting the chronology of axon outgrowth (Chan and Chung, 1999; Walsh and Guillery, 1984). Additionally, in *C. elegans*, the axons in the nerve ring and the ventral nerve cord also form ordered bundles (White et al., 1976). A likely explanation is that technical limitations have made it hard to study neurite order within a bundle. In most cases it has only been possible to assess large populations of axons at once. Thus, except in special cases like the grasshopper, where individual axons can be recognized, it remains impossible to know whether a given neuronal process selectively adheres to specific partners within a bundle.

In this study, we took advantage of the simple nervous system of *C. elegans* to develop a method to study dendrite order within a bundle. We found that amphid dendrites are ordered within the amphid dendrite bundle, and this order is maintained fairly steadily over time. Neither amphid sheath glia nor cilia are required for this dendrite order, suggesting that dendrite order is not conferred by structures near the nose tip. We found 3 CAMs that control amphid dendrite order in two different ways -- loss of FAT-like cadherin, CDH-4 causes a loss of dendrite order, while interestingly, loss of either SAX-7/L1CAM or PTP-3/LAR causes dendrites to take on a new order of dendrites. This new order deteriorates over time, suggesting that *sax-7* and *ptp-3* play roles in development and maintenance of dendrite order. Finally, we found that SAX-7S acts instructively to specify dendrite order. Taken together, our results suggest that the
differential adhesion between dendrites, specified by an unknown number of adhesion molecules, control dendrite order within the amphid.

**Functional consequences of ordered bundles**

Why might neurite bundles be ordered? From first principles, one might expect a neurite bundle to be ordered according to age of the neurite, such that the oldest neurite, typically the pioneer axon or dendrite, to be in the middle of the bundle, and the youngest neurite would be expected to be on the periphery. However, that is not always the case – in *C. elegans*, the VD motor neurons, which are born post-embryonically and extend axons along the ventral nerve cord after the nerve cord is established, insinuate its axons deep into the ventral nerve cord and bundle closely with the DD motor neurons, a highly similar set of GABAergic motoneurons that are born embryonically and lie at the center of the ventral nerve cord (White et al., 1976). This result is interesting because it suggests that (1) neurite order within a bundle is not a passive process, and (2) neurite processes may be ordered within a bundle according to similarity or function.

As a prime example of how structure is a prerequisite for function, bundles sometimes reflect the anatomical sorting of neurons into functional categories. For example, individual cranial nerves arise by bundling of axons dedicated to smell, vision, oculomotor control, or other functions. Similarly, selective adhesion among motor neurons during development results in the segregation of "fast" and "slow" motor axons into anatomically distinct bundles (Milner et al., 1998). Additionally, in the mammalian cortex, dendrites arising from neurons in different cortical layers come together to form microcolumns (Fleischhauer et al., 1972; Fleischhauer,
1974; Peters and Walsh, 1972). Altogether, these examples suggest that ordered bundles are important for creating a functional nervous system.

One intriguing hypothesis for the existence of dendrite bundles is the possibility that they influence behavior in the mature nervous system. For example, dendrite bundles of gonadotropin-releasing hormone neurons are important for synchronization of hormone release onto common synaptic inputs (Campbell et al., 2009). More recently, Carlson and colleagues have published the very exciting finding that non-synaptic inhibition between dendrites of Drosophila olfactory receptor neurons (ORNs) in a sensillum can influence behavior (Su et al., 2012). An olfactory sensillum in Drosophila consists of a bundle of dendrites belonging to a few ORNs. Su et al. found that when sustained firing of one ORN (ab3A) is combined with a short pulse of a second odorant activating its neighbor (ab3B), ab3B fires and inhibits the tonic firing of ab3A. They attributed this lateral inhibition to non-synaptic inhibition, as blocking gap junctions or synaptic activity of all ORNs had no effect on lateral inhibition, suggesting that ORNs in a sensillum are ephaptically coupled; that is, ORNs within a sensillum can affect the activity of their neighbors through non-synaptic mechanisms. Most importantly, they showed that this lateral inhibition could affect behavioral responses. These results indicate that dendritic bundles may not be a mere vestigial structure from development and suggest that the position of each dendrite relative to its neighbors can affect its activity, an intriguing possibility that we imagine could be extended to the amphid sense organ as well.
Simple rules can generate complex patterns

Our work also points to the possibility that one way by which ordered structures in biological systems emerge is that cells are organized in a way that optimizes for adhesive strength between them, such that, in the absence of one adhesion molecule, the system reorders itself to maximize adhesion, thus producing a new order of the same cells. In this context, it is interesting to reconsider how the switch in wild-type amphid dendrite order may have occurred (Figure 3.2). As described by White et al. in 1986, “it seems likely that mechanical disturbances have mixed the processes, introducing them to novel neighbours. Some of these neighbours may have high adhesive affinities for the newly introduced processes and act to guide and establish the processes in their new territory. Such a notion carries the implication that specific neighbourhoods are not uniquely attractive for a particular process, but rather that there may be several neighbourhoods in which a process could equally well reside, the one selected being dependent on the initial placement of the process in the bundle” (White et al., 1986).

Evolutionarily, this “optimize adhesion” rule provides a general, reusable strategy to create a diverse set of complex structures given a limited set of adhesion molecules. Indeed, amphid sensilla in other nematode species such as Acrobeles complexus, Strongyloides stercoralis, and Haemonchus contortus are also ordered, albeit in different configurations compared to C. elegans, and it is interesting to speculate whether similar combinations of CAMs also determine amphid dendrite order in those other species (Bumbarger et al., 2009).

This leads to a second question: what is the minimum number of distinct cell adhesion molecules that are required to fully specify amphid dendrite order? Of course there are many
factors about each adhesion molecule that would influence the number, such as the expression level, adhesive strength, and whether the adhesion molecules adhere using homophilic or heterophilic mechanisms, but assuming all adhesion molecules act equally and bind homophilically, and assuming that the dendrites are positioned in a way that maximizes adhesion, this should be a solvable problem. If we further simplify the system and assume that the 12 amphid neurons are arranged in a $4 \times 3$ configuration and are 2D structures like cells on a plate, then we know that the upper bound on the minimum number of distinct adhesion molecules that are required is 17 (Figure 3.11). This hints at the possibility that perhaps there are other adhesion molecules that govern amphid dendrite order besides SAX-7, PTP-3, and CDH-4, and would also explain why the penetrance of defasciculation of amphid dendrites in individual CAM mutants is low. Altogether, our results contribute to an understanding of how adhesion molecules can create ordered structures in the nervous system.
Figure 3.11. Number of adhesion molecules required to specify dendrite order

Schematic cross-section of amphid dendrite bundle with distinct adhesion molecules schematized with dashes. The upper bound on the minimum number of adhesion molecules to specify a $4 \times 3$ bundle of dendrites is 17.
ACKNOWLEDGMENTS

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REFERENCES


CHAPTER 4

Conclusions and future directions
Simple rules can generate complex patterns

In this dissertation, I studied two dendrite structures of the *C. elegans* nervous system to show how local rules that govern cell-cell interactions can generate complex patterns and well-ordered structures. In Chapter 2, I found that a simple “avoid yourself” rule can give rise to novel patterns for the animal in a different context. In Chapter 3, I found that two classes of adhesion molecules control dendrite order – one class that results in loss of dendrite order when removed, and another class that results in a new order of dendrites when removed. My results point towards the existence of a set of adhesion molecules that are differentially expressed among amphid neurons that controls dendrite order, and raise the possibility that new patterns can be made by the addition or removal of a single class of adhesion molecule.

In both projects, one can imagine these rules resulting in novel structures in other systems. Dendrite tiling by mutual repulsion does occur in larger nervous systems such as in the mammalian retina and between *Drosophila* mechanosensory neurons, and it is interesting to speculate whether tiling arose as a necessary consequence of duplicating neurons with self-avoidance properties (Amthor and Oyster, 1995; Blackshaw et al., 1982; Dacey, 1993; Grueber et al., 2002; Grueber et al., 2003; Jan and Jan, 2003; Vaney, 1994; Wässle et al., 1981). Additionally, selective fasciculation and ordered bundles of neurites also occur in other systems. In the case of amphid sense organs in other nematodes, it is interesting to consider the possibility that similar sets of adhesion molecules also control amphid dendrite order in other species as well.
Future directions for dendrite tiling project

Are ectopic PVD neurons functional? One question that would be interesting to explore is whether the ectopic PVD neurons in the lin-22 mutant are functional. PVD is normally responsible for sensing harsh touch and cold temperatures (Chatzigeorgiou et al., 2010; Way and Chalfie, 1989). It would be interesting to understand whether (1) the ectopic PVDs are functional, and (2) ectopic PVDs increase the animal’s sensitivity to harsh touch and cold temperatures. Related to this project would also be to understand how PVD dendrites communicate environmental stimulus to the cell body given that the cell body is not placed in the middle of the dendrite arbor. The PVD cell body is located closer towards the posterior end of the animal but the dendrites extend all the way to the nerve ring. From first principles, cable theory would tell us that stimulus towards the anterior end of the dendrite arbor would not be communicated to the cell body as strongly as stimulus close to the cell body, or towards the posterior end of the dendrite arbor would. These questions are important because provide a mechanism for sensory systems to become more sensitive.

Are there nematode species with multiple PVDs? It would be very interesting to understand whether other nematode species have multiple PVD neurons that arose through a lin-22 (or similar) mutation, and whether those species have PVD dendrite arbors that tile with one another, controlled by Netrin signaling. Although it is somewhat of an unrealistic project, it is an important question because it helps us understand how realistic it might have been for a cell duplication event to generate a stable, novel pattern in other organisms.
**Future directions for dendrite fasciculation project**

**How does dendrite order change with the addition or deletion of neurons in the amphid bundle?**  This is an important question because it helps us validate the idea that amphid dendrites use a model of differential adhesion to generate order within the bundle. Unfortunately with our current method of measuring dendrite order this project is a bit technically unfeasible for two reasons. First, we would need to influence the birth or deletion of the neurons we wish to add or delete early during embryonic development, before dendrites are elaborated, and we do not easily have that level of technical control. Second, with our current methods of measuring dendrite order (looking at three dendrites), it may be difficult to detect a change.

**How do amphid dendrites get ordered during development?**  This is an important question because it helps us understand whether the order of dendrites was derived during amphid neuron birth and dendrite extension or whether there are adhesion molecules that are actively maintaining order during development. It would also be interesting to understand whether dendrite order changes between embryogenesis and larvae, and whether we can understand how the switch point we see in dendrite order was formed.

**What is the upper bound on the minimum number of cell adhesion molecules that are required to specify amphid dendrite order?**  This computational modeling experiment was described in the discussion section of Chapter 3. It is a very interesting question with the prediction that adding or removing adhesion molecules would lead to a new order of dendrites. If we were able to execute this experiment, it would greatly contribute to our model, as we would be able to show that our results agree both from experimental and computational methods.
Can we create order in vitro using SAX-7 and PTP-3? It would be interesting to recreate a model of differential adhesion using SAX-7 and PTP-3 with a cell line with cells that do not usually stick together, such as S2 cells. This experiment is important because it again contributes evidence to a model that SAX-7 and PTP-3 participate in a model where differential expression of adhesion molecules within amphid neurons can create ordered structures.

What is the function of ordered dendrite bundles? This was also discussed in the discussion section of Chapter 3. There are two questions here. First, does activity in one amphid neuron affect the activity of neighboring amphid neurons? If so, how does misfasciculation or defasciculation affect this activity? We have created strains to answer these questions, where the general idea is to conduct calcium recordings of all the amphid neurons while chemically activating a single amphid neuron. We have created a strain expressing an integrated nuclear GCaMP6 in all the amphid neurons. In this strain, we expressed exogenous TRPV1, the human capsaicin receptor, in single amphid neurons, and have also conducted behavioral experiments to show that the TRPV1 is functional and is activated in the presence of capsaicin (Tobin et al., 2002). The next step is to record calcium signals from all the amphid neurons, while activating a single one with capsaicin, to see whether neighboring neurons are also activated. If these experiments, currently in the hands of a collaborator, prove to be successful, then we will look in our fasciculation mutants to see how loss of dendrite order changes this activity. This is an important question because it helps us understand why amphid dendrites are ordered in a particular way.
Taken together, my work in this dissertation opens many new questions that would be interesting to pursue in the future.
REFERENCES


APPENDIX 1 – SUPPLEMENTAL TABLES FOR CHAPTER 2

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APPENDIX 2 – SUPPLEMENTAL TABLES FOR CHAPTER 3

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### Extrachromosomal:

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REFERENCES


