

INTERCELLULAR INTERACTIONS PROMOTING NEURAL CIRCUIT FORMATION
AND FUNCTION

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EMILY LAUREN HECKMAN

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DISSERTATION APPROVAL PAGE

Student: Emily Lauren Heckman

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This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Cris Niell	Chairperson
Chris Doe	Advisor
Adam Miller	Core Member
Tory Herman	Core Member
Matt Smear	Institutional Representative

and

Krista Chronister	Vice Provost for Graduate Studies
-------------------	-----------------------------------

Original approval signatures are on file with the University of Oregon Division of Graduate Studies.

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DISSERTATION ABSTRACT

Emily Lauren Heckman

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The function of the nervous system – from perception to behavior - relies on the orderly formation of neural circuits during development. Given that there are billions of neurons in the human brain, and thus billions of potential circuit configurations, it remains enigmatic how cells direct their processes to specific brain regions, identify preferred synaptic partners, and form synaptic connections often with subcellular precision. Moreover, with billions of cells executing such a complex developmental sequence, what safeguards are in place to ensure that the final function of the nervous system is robust to variability in any of these steps? In this dissertation, I describe the establishment of a model system in the larval fruit fly, *Drosophila melanogaster*, that enabled the investigation of these questions in the context of a relatively simple nervous system. Within this model system, I have found that (i) axon guidance cues are sufficient to direct the targeting of axons to different postsynaptic subcellular domains, (ii) a presynaptic neuron is competent to form functional synapses across a range of possible subcellular dendritic domains, (iii) the presence of a presynaptic axon can promote the local elongation of postsynaptic dendrites, and (iv) presynaptic activity levels can negatively regulate postsynaptic dendrite elongation neuron-wide. These simple strategies can be employed to form circuits with consistent cellular memberships, and furthermore, encode flexible cellular responses to variation

in presynaptic targeting and synaptic strengths to ultimately enable robust nervous system function. This dissertation contains previously published and co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Emily Lauren Heckman

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, OR
Lehigh University, Bethlehem, PA

DEGREES AWARDED:

Doctor of Philosophy, Neuroscience, 2022, University of Oregon
Bachelor of Science, Molecular Biology, 2016, Lehigh University

AREAS OF SPECIAL INTEREST:

Cellular & Developmental Neuroscience
Developmental Biology

PROFESSIONAL EXPERIENCE:

Graduate Researcher, University of Oregon, 2017-22
Laboratory of Dr. Chris Q. Doe

Graduate Employee (Teaching Assistant), University of Oregon, 2016-17

Undergraduate Research Assistant, Lehigh University, 2016
Laboratory of Dr. Julie S. Haas

Biosystems Dynamics Summer Research Fellow, Lehigh University, 2015
Laboratory of Dr. Julie S. Haas

Mountaintop Research Fellow, Lehigh University, 2014
Laboratories of Dr. Vassie Ware and Dr. Bryan Berger

GRANTS, AWARDS, AND HONORS:

Department of Biology Diversity, Equity, & Inclusion Grant, submitted on behalf of
Womxn in Neuroscience, Anti-Racism Training, University of Oregon, 2021

Developmental Biology Training Grant, Investigation into mechanisms of synapse
establishment and maintenance, University of Oregon, 2017

Langer-Simon Scholar Award, Homeostatic plasticity of electrical synapses, Lehigh
University, 2015

Poster Symposium Honorable Mention, Genetically minute, but proficient for infection:
Functional explorations into the small genomes of Mycobacterium phage
Butters and Arthrobacter phage Maggie, HHMI, 2014

PUBLICATIONS:

- Heckman, E.L. & Doe, C.Q. (2022). Presynaptic Contact And Activity Opposingly Regulate Postsynaptic Dendrite Outgrowth. *bioRxiv*. <https://doi.org/10.1101/2022.07.27.501752>
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*Consortium of researchers, of which I was a member.
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CHAPTER I

INTRODUCTION

The nervous system controls all behavior, perception, and cognition. To execute these diverse functions, the principal cells of the brain, called neurons, must connect much like wires in a circuit board; neurons extend processes called axons that “plug into” outlets on other neurons called dendrites. In this way, one neuron can transmit chemical or electrical signals to other neurons in the nervous system via a connection called a synapse, and that information is propagated to achieve a desired circuit output.

Specific brain functions generally have dedicated neural circuits. Teleost fish have an escape circuit composed of neurons that sense auditory, visual, and mechanical stimuli to detect potential predators, and transfer that information to spinal cord neurons which in turn coordinate muscle contractions (Kimmel et al., 1990; Nissanov et al., 1990). The male fruit fly, *Drosophila melanogaster*, has a courtship circuit where neurons that sense female pheromones signal to downstream partners regulating the male’s decision to pursue and sing to a female conspecific (Clowney et al., 2015; Seeholzer et al., 2018). Humans have regions of the brain dedicated for speech, memory, visual processing, and decision-making, among other functions (Schoenemann, 2006). Within a species, the circuits that facilitate these behavioral and cognitive functions are generally composed of the same neurons, and the neuron-neuron connections are generally organized in the same pattern.

The human brain contains roughly 80 billion neurons, and even the relatively simple nervous systems of insects and fish contain tens to hundreds of thousands (Azevedo et al., 2009; Friedrich et al., 2013; Jiao et al., 2022; Raji & Potter, 2021). Therefore, a question that has puzzled scientists for over a century is how neurons are capable of reproducibly wiring into functional circuits when the sheer number of neurons confers billions of potential wiring combinations. Simple rules for nervous system development can be hypothesized based on observing neurons and how they are interconnected. A powerful tool for this type of observation is connectomics. Connectomics is the complete description of the neurons and synapses of a nervous system, which typically requires imaging using serial section electron microscopy (EM). The first nervous system to be completely reconstructed was that of the nematode

Caenorhabditis elegans (*C. elegans*) in 1986, and was done completely by hand over the course of 15 years (Emmons, 2015; White et al., 1986).

Today, the generation of connectomes is much faster, aided in large part through computational advances in image registration and automated neuron tracing (Saalfeld et al., 2009; Scheffer et al., 2020; Zheng et al., 2018). Because of this, the field of neural development has been able to increase its rate of observation and hypothesis generation. For example, with much of the larval *Drosophila* brain reconstructed (Ohyama et al., 2015; Schneider-Mizell et al., 2016), researchers were recently able to map known lineally related neurons onto the connectome and look for correlations between cell lineage and circuit connectivity. From there, they found that each lineage generates sensory and motor processing pools of neurons, called hemilineages. Neurons in the same hemilineage that also share similar birth timing also tend to form synapses with the same set of other neurons (Mark et al., 2021). This suggests the testable hypothesis that the temporal patterning of a lineage is necessary for neurons to participate in specific circuits.

Another interesting pattern revealed by connectomics is that neurons not only exhibit a bias for which neurons they form connections with, but also for the *subcellular* location where they form synapses. For example, 72% of *Drosophila* Projection Neurons, which carry odor information to part of the brain called the Lateral Horn, synapse with only one dendritic arbor of local Lateral Horn Neurons (Liu et al., 2022). This type of subcellular synaptic specificity also exists in the mammalian cortex, where different classes of GABAergic neurons synapse onto specific compartments of pyramidal cells (Favuzzi et al., 2019). While the functional impacts of synapsing at a particular subcellular location have been extensively modeled (Liu et al., 2022; Pouille & Scanziani, 2001; Tobin et al., 2017; X.-J. Wang et al., 2004), the developmental mechanisms leading to this precision in circuit wiring are less understood.

Through imaging multiple animals of a species, or even both hemispheres of a single animal's brain, it is clear that while the gross wiring of the brain is highly consistent, other aspects are more variable (Liu et al., 2022; Tobin et al., 2017; Witvliet et al., 2021). The processes that ramify off of an axon or dendrite are never identical; the number of synapses between neurons can vary; and despite some neurons exhibiting a subcellular bias for a particular dendritic arbor, the exact location of synapses on that dendrite is less constrained. In addition to the major question of how brain wiring is established with an exquisite level of specificity, a

second question must be asked of how circuit connectivity and function are achieved when these idiosyncrasies in development exist.

In this dissertation, I will describe the work that I have done to define cellular mechanisms that promote neural circuit establishment and ensure robust circuit wiring. In Chapter II, I will review literature that outlines what is currently known about how developing neurons form synaptic connections with cellular and subcellular specificity, and how these connections are maintained. I will use this review to generate hypotheses about how different strategies may be combined to generate robust circuit connectivity. In Chapter III, I will begin to test these hypotheses by first describing a new model system in the *Drosophila* larva consisting of a pair of synaptically coupled cells, identified through the larval connectome to exhibit a bias for connecting at particular subcellular location. We then test how this bias is generated by targeting the presynaptic axon to alternative neuropil domains. We find that the two cells are competent to form synaptic connections at alternative subcellular locations, which suggests that the native circuit configuration is established using axon guidance cues. In Chapter IV, I will use this same model system to show that multiple safeguards are present to ensure that circuit wiring is robust to variability in presynaptic targeting or synaptic strength. I find that the presynaptic axon can locally promote the elongation of a postsynaptic dendrite, and that presynaptic activity levels can opposingly regulate this elongation to prevent excessive dendrite formation. Together this work reveals a simple set of rules to establish neural circuit connectivity, and also to allow neurons to flexibly respond to variation in presynaptic targeting or synaptic strength.

This dissertation contains previously-published and co-authored material.

CHAPTER II

ESTABLISHMENT AND MAINTENANCE OF NEURAL CIRCUIT ARCHITECTURE

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Author Contributions

ELH compiled the literature. ELH and CQD discussed and wrote the review. ELH made the figures.

Introduction

The steps from when a neuron is born to when it becomes functionally embedded within a circuit have been defined, even if the relevant molecular mechanisms often remain unclear. These steps include extending processes through a complex extracellular environment, terminating in the proper location, identifying the correct partner neurons, and establishing synapses within a specific subcellular domain of those neurons. In this review, we focus on how neurons establish and maintain synapses with the correct cellular and subcellular specificity and refer the reader to other reviews on the preceding steps (Chédotal, 2019; Dickson, 2002; Kolodkin & Tessier-Lavigne, 2011).

How do neurons establish specific wiring patterns?

Full or partial electron microscopy reconstructions of model organism connectomes have provided a reference to identify neurons that are synaptically connected, the number of synapses between connected neurons, and the structural variability of those synaptic connections within and between animals (Gerhard et al., 2017a; Hildebrand et al., 2017; Kasthuri et al., 2015; Scheffer et al., 2020; Schneider-Mizell et al., 2016; Valdes-Aleman et al., 2021; White et al., 1986; Witvliet et al., 2021). From these studies, it is clear that neurons form synapses with only a subset of adjacent neurons. The specificity with which one neuron forms synapses with another neuron is referred to as cellular synaptic specificity (Figure 2.1A). This specificity implies the

existence of partner-derived cues that promote the formation of specific connections and preclude connections with others. Below we summarize key mechanisms that promote the establishment of synaptic specificity.

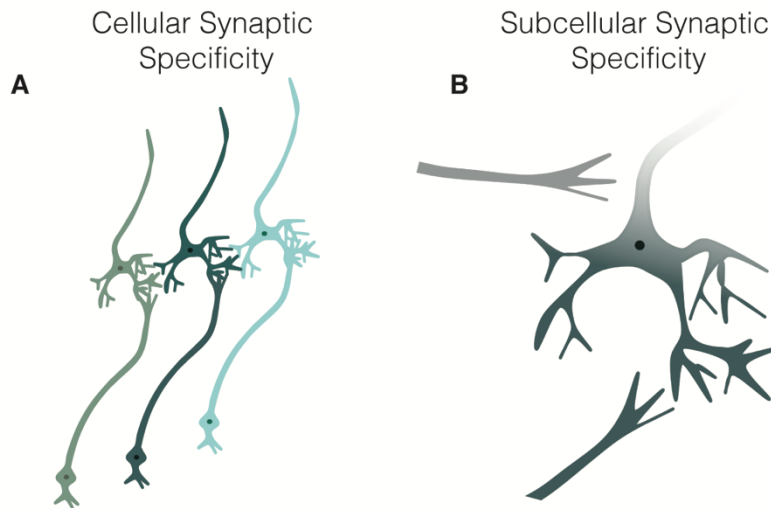


Figure 2.1. Distinction between cellular and subcellular synaptic specificity. (A) Neurons can preferentially form synapses with subsets of other neurons. (B) Neurons can preferentially form synapses at specific subcellular locations.

Cell-surface cues establish synaptic specificity

One hypothesis established ~100 years ago is the chemoaffinity (Langley, 1895; Sperry, 1963), which posits that neurons are endowed with unique molecular tags that enable them to recognize and to be recognized by their appropriate synaptic partners. The hypothesis was published in 1895 by John Langley, who observed that individual spinal cord neurons in the cat innervate specific peripheral organs. After severing the neurons and allowing them to regenerate, the neurons managed to form synapses with their original partners. These findings were later replicated and expanded on by Roger Sperry in the retinotectal system of amphibians. After severing the optic nerve and observing the return of normal vision (Sperry, 1943, 1944), Sperry concluded, “that the cells and fibers of the brain and cord must carry some kind of individual identification tags” (Sperry, 1963).

The chemoaffinity hypothesis put forth by Langley and Sperry generates several predictions about the molecules that could serve as partner-derived cues: (1) the molecules are

expressed at the time of synapse formation; (2) the molecules are expressed in complementary patterns within presynaptic and postsynaptic neurons; (3) non-synaptic partners express less compatible molecules; and (4) removal or ectopic addition of the molecule should result in circuit miswiring (Figure 2.2). Since Langley's and Sperry's original studies, molecules that fit some or all of these criteria have been identified across a variety of model organisms. Many of these molecules are broadly classified as cell adhesion molecules (CAMs). CAMs are transmembrane proteins that have homophilic or heterophilic interactions to promote cell-cell adhesion or cell-cell signaling (de Wit & Ghosh, 2016; Williams et al., 2010).

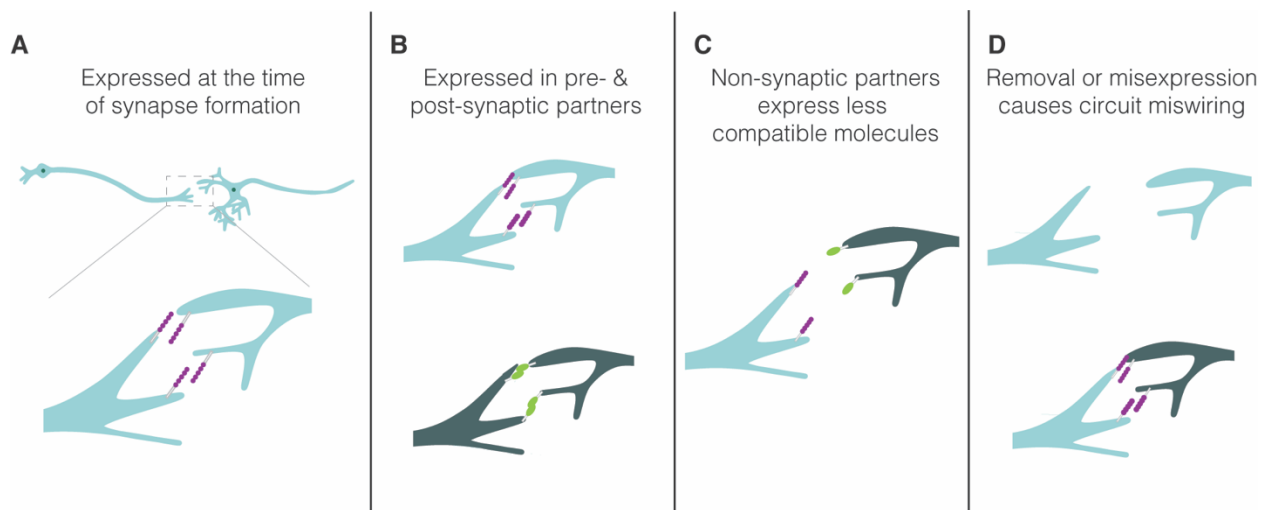


Figure 2.2. Features of proteins that promote synaptic specificity. (A) Molecules that specify connectivity should be at the time of synapse formation. (B) High affinity molecules should be expressed in pre- and postsynaptic neurons. (C) Non-synaptic partners should express molecules with relatively low affinity. (D) Manipulation of molecule expression (removal or misexpression) should result in circuit miswiring.

Recent work in *Drosophila* tested the extent to which cell-surface cues versus location determine synaptic specificity, while also highlighting the power of electron microscopy to assay experimental manipulations of synapse number and connectivity (Valdes-Aleman et al., 2021). The axons of a specific class of larval sensory neuron were mistargeted from a medial to a lateral position of the neuropil through the misexpression of a receptor for a midline-expressed repulsive guidance cue. Valdes-Aleman et al. (2021) asked whether the mistargeted axons would retain synaptic specificity in their ectopic location. The mistargeted neurons and all postsynaptic partners were reconstructed using electron microscopy. Surprisingly, the dendrites of

postsynaptic partner neurons extended into the ectopic territory and successfully located the sensory axons. The mistargeted neurons indeed retained the same cellular synaptic specificity (Valdes-Aleman et al., 2021). These findings provide evidence that partner-derived cues, rather than physical proximity, determines synaptic partner matching in this context.

High-throughput methods have been leveraged to expand the palette of CAMs that promote cellular synaptic specificity. For example, recent work in *Drosophila* has defined an extracellular “interactome” of 202 CAMs, including immunoglobulin (IgSF) proteins, leucine-rich repeat proteins, and fibronectin Type III proteins (Özkan et al., 2013). To test the extracellular binding affinities among these proteins, a novel bait-and-prey assay was developed. After testing over 20,000 pairwise interactions with this assay, 106 unique interactions were identified, of which ~80% had not previously been reported. Among these novel interactions were those between IgSF proteins from two uncharacterized protein families, the Dprs (Defective in Proboscis extension Response) and DIPs (Dpr-Interacting Proteins) ((Carrillo et al., 2015; Nakamura et al., 2002; Özkan et al., 2013). *In vitro*, the extracellular domains of the 21 Dprs and 11 DIPs largely bind in a heterophilic manner. A single Dpr preferentially binds between one and four DIPs, and a single DIP can bind between two and nine Dprs (Carrillo et al., 2015; Cosmanescu et al., 2018; Özkan et al., 2013; Sergeeva et al., 2020). Given the specificity of their extracellular interactions in vitro, it has been hypothesized that Dprs/DIPs act to identify and promote selective interactions between synaptic partners.

These hypotheses have recently been experimentally tested. For example, DIP- β mRNA and protein were found to be uniquely expressed in L4 lamina neurons in the visual system (Tan et al., 2015; Xu et al., 2019). Next, to examine whether DIP- β is necessary for L4 to synapse with its known partner L2 in the proximal lamina, *dip- β* was knocked down in L4 neurons. L4-L2 synapses still formed in the proximal lamina, but there were also ectopic synapses in the distal lamina. This suggested that DIP- β is not required for synapse formation, but instead may have a role in promoting synaptic specificity by limiting the preferred postsynaptic partners of L4. To test if DIPs are sufficient to specify synaptic partnerships, DIP- β or DIP- γ were misexpressed in non-partner lamina neurons. This resulted in novel, aberrant connectivity. Similar results have been observed at the larval neuromuscular (Ashley et al., 2019; Cheng et al., 2019). Therefore, DIP expression is not necessary for synapse formation per se, but rather biases the connectivity preference of a neuron (Xu et al., 2019), leading to synaptic specificity.

The discovery and functional characterization of the DIPs and Dprs highlight how high-throughput techniques have been leveraged to refine our understanding of how cellular synaptic specificity is established. While we chose to focus our attention on the DIPs and Dprs, many other CAMs promote cellular synaptic specificity (Figure 2.3A), through a variety of mechanisms (Sanes & Yamagata, 2009; Sanes & Zipursky, 2020). It is important to note that CAMs can serve different functions (e.g., axon targeting, partner matching, synapse maintenance) depending on the context (Barish et al., 2018). Some of the molecules that promote cellular synaptic specificity may also be required for subcellular synaptic specificity (discussed below) (Figure 2.1B), and vice versa, but in many cases this distinction will require future experiments. Therefore, special care must be given interpreting phenotypes and assigning functions to potential synaptic specificity molecules.

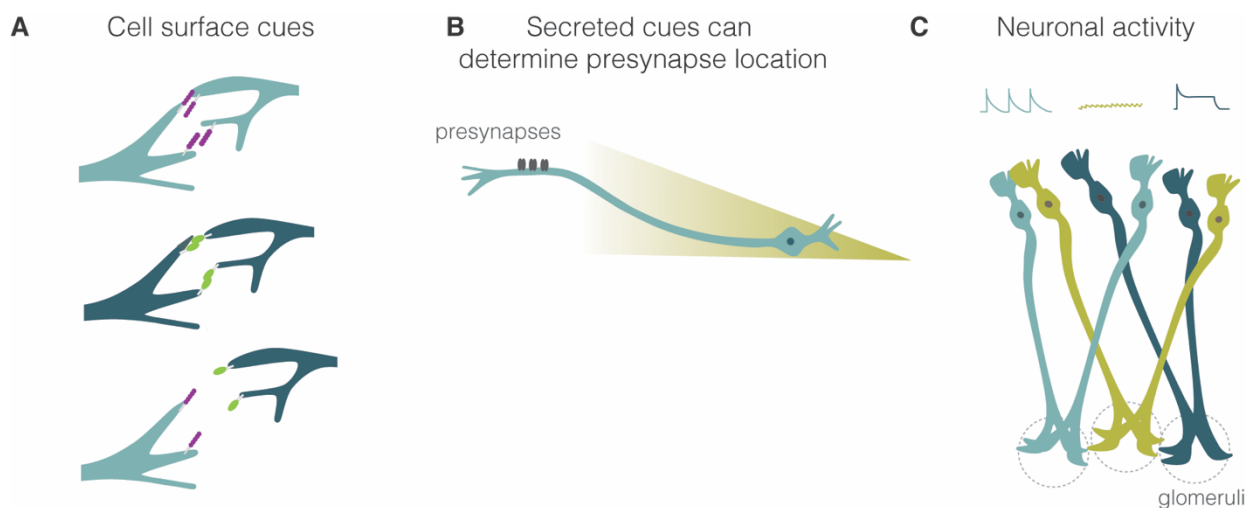


Figure 2.3. Mechanisms that promote the establishment of synaptic specificity. (A) Cell surface cues can promote the proper matching of pre- and postsynaptic partners. (B) Secreted cues can specify the subcellular location of presynapses (adapted from Klassen & Shen, 2007). (C) Patterns of spontaneous neuronal activity influence the organization of olfactory sensory neurons into discrete glomeruli (adapted from Nakashima et al., 2019).

Secreted signaling molecules establish subcellular synaptic specificity

An important component of neural circuit function is the subcellular location of a synapse on a neuron. Functional and modeling studies suggest that subcellular synapse location is critical for neuronal computations, including the timing of action potential generation, dendritic integration, and coincidence detection (Bloss et al., 2016; Hao et al., 2009; Miles et al., 1996;

Pouille et al., 2013; Tobin et al., 2017). In many cases, neurons selectively form synapses at specific subcellular locations along their partners, referred to here as subcellular synaptic specificity (Figure 2.1B). For example, in the mouse cortex and cerebellum, excitatory neurons are innervated at specific locations by different inhibitory interneuron subtypes (Ango et al., 2004; Favuzzi et al., 2019; Huang, 2006; Tai et al., 2019). Despite the functional importance of subcellular synaptic targeting, the precise mechanisms that establish subcellular synaptic specificity are still being uncovered.

Studies in invertebrates have highlighted the importance of secreted signaling molecules in establishing subcellular synaptic specificity (Figure 2.3B,C). Secreted cues direct the local clustering of presynaptic sites at specific subcellular locations in *Caenorhabditis elegans* neurons (Figure 2.3B) (Colón-Ramos et al., 2007; Klassen & Shen, 2007; Poon et al., 2008). For example, the DA9 neuron in *C. elegans* forms *en passant* synapses with dorsal muscles and motor neurons along the anterior-posterior axis in the tail. Just posterior to where it forms these *en passant* synapses, the DA9 axon is devoid of presynaptic sites (Klassen and Shen, 2007). How are synapses confined to one specific region of the axon? Wnt/LIN-44 is secreted from hypodermal cells in the tip of the tail (Herman et al., 1995), and in mutants lacking *wnt/lin-44* or its receptor, *fz/lin-17*, DA9 synapses expanded into the asynaptic region. Both the number and intensity of synaptic puncta remained the same between *wnt/lin-44* mutants and WT animals; the only observed change in *wnt/lin-44* mutants was a posterior shift in synapse position. Using a fluorescently tagged Fz/LIN-17, it was discovered that this receptor normally localizes exclusively to the asynaptic region of the DA9 axon; but in *wnt/lin-44* mutants, Fz/LIN-17 was diffusely distributed along the DA9 axon. To test whether Wnt/LIN-44 is instructive for specifying the location of Fz/ LIN-17 clustering and thus DA9 synapses, Wnt/LIN-44 was ectopically expressed in a more anterior portion of the tail. This resulted in an anterior shift of both Fz/LIN-17 expression and DA9 synapses. These results suggest Wnt/LIN-44 promotes the clustering of Fz/LIN-17, and then Fz/LIN-17 locally inhibits presynapse clustering (Klassen and Shen, 2007). Therefore, the placement of presynaptic sites can be determined through secreted signals, and sculpted by inhibitory cues.

In other model systems, compartmentalized expression of CAMs combines with locally secreted cues to promote subcellular synapse specificity. For example, the Purkinje neurons of the cerebellum receive inputs from inhibitory basket cells at the axon initial segment (AIS)

(Huang, 2006). The presynaptic basket cells express the NRP1 receptor, whereas the postsynaptic Purkinje cells express both the secreted signal SEMA3A and the CAM Neurofascin-186 (NF186). SEMA3A was found to be locally secreted from the Purkinje soma, transducing an attractive signal in the basket cell through the NRP1 receptor (Telley et al., 2016). Additionally, immunofluorescence showed that NF186 is present in a gradient on the Purkinje cell surface: highest at the AIS and lowest at the top of the soma (Ango et al., 2004). The trans-synaptic interaction between Purkinje NF186 and basket cell NRP1 ultimately results in synapse formation at the AIS (Telley et al., 2016). Thus, the targeting of the basket cell axon is initially refined by the locally secreted SEMA3A but is ultimately determined by trans-synaptic CAM interactions (Telley et al., 2016).

Recent RNA-seq approaches continue to expand our knowledge of molecular mechanisms that generate subcellular synaptic specificity. Favuzzi et al. (2019) asked whether distinct molecular programs could explain the observed subcellular targeting preferences among the interneurons that innervate cortical pyramidal neurons. At the time of synapse formation, they performed RNAseq on three populations of interneurons: SST1 cells, PV1 basket cells, and chandelier cells. These three cell types innervate the dendrites, soma, and AIS of pyramidal cells, respectively. Differentially expressed gene subsets were identified for each interneuron subtype. The most differentially enriched gene for each group was further characterized for its role in promoting subcellular synaptic specificity. Cbln4, a member of the C1q secreted protein family, was enriched in SST1 cells; Lgi2, a secreted leucine-rich repeat protein, was enriched in PV1 basket cells; and Fgf13, an intracellular protein, was enriched in chandelier cells. Knockdown of each of these genes in their respective interneuron subtype resulted in a decrease in the number of synapses onto pyramidal cells. This result demonstrated that these genes are required for synapse formation, but do they instruct the ultimate subcellular location of synapses onto pyramidal cells? Indeed, overexpression of Cbln4 in all three interneuron subtypes triggered ectopic synapse formation onto the dendritic domain alone (Favuzzi et al., 2019). This work raises many intriguing questions about how these identified molecules mediate subcellular synaptic specificity. Is there regional heterogeneity in the molecules used by interneurons across the brain to target specific subcellular regions of postsynaptic cells? How do CBLN4 and LGI2, which are both secreted proteins (Kegel et al., 2013; Yuzaki, 2010), signal back to the

interneuron to indicate where to target? What molecules do they interact with on the pyramidal cell to mediate specificity?

Neuronal activity establishes synaptic specificity

Neuronal activity is an important mechanism by which circuitry is established, refined, and maintained. While some circuits can develop in the absence of activity (DiCristo et al., 2004; Hiesinger et al., 2006; Klassen & Shen, 2007), spontaneous (Akin & Zipursky, 2020; Antón-Bolaños et al., 2019; McLaughlin et al., 2003; Wan et al., 2019) and sensory-evoked activity (LeVay et al., 1980; Shatz & Stryker, 1978) helps to organize circuits in other contexts, often within a critical period of development (Ackerman et al., 2021; Jarecki & Keshishian, 1995; LeVay et al., 1980; McLaughlin et al., 2003). Developmental neuronal activity is typically regarded as a process that refines the connectivity of established circuits. While this may be the case in some instances (Changeux & Danchin, 1976; Kapfer et al., 2002; McLaughlin et al., 2003; Valdes-Aleman et al., 2021), evidence has also emerged in recent years that neuronal activity induces patterns of gene expression that instruct the initial organization of circuit connectivity (Inoue et al., 2018; Nakashima et al., 2019; Serizawa et al., 2006).

The link between spontaneous activity and circuit establishment has been well studied in the mouse olfactory bulb, where primary olfactory sensory neurons (OSNs) expressing identical olfactory receptors converge onto the same glomeruli. The segregation of OSN axons into discrete glomeruli is an activity-dependent process (F. Wang et al., 1998; Yu et al., 2004), in which activity in OSNs triggers the expression of axon sorting molecules (Serizawa et al., 2006; Williams et al., 2010; Inoue et al., 2018). However, it was unclear until recently how activity could lead to the expression of different molecules in different sets of OSN axons. Nakashima et al. (2019) found that three subclasses of OSNs exhibited distinct patterns of spontaneous activity (i.e., tonic, short burst, and prolonged burst firing) during development. These activity patterns regulated expression levels of three different CAMs, which in turn promote glomerular organization (Figure 2.3D). The authors distinguished between the Hebbian rule of “neurons that fire together, wire together,” and their proposed model in which neurons that have similar spontaneous firing patterns wire together (Nakashima et al., 2019). As there are ~1000 OSN subtypes in the mouse, it will be important to learn whether each subtype uses similar rules to

establish proper glomerular targeting. In other words, are there ~1000 distinct activity patterns that result in unique combinations of axon sorting molecule expression? Or does axon sorting arise from graded differences in spontaneous activity patterns, and thus expression levels of the identified CAMs?

How are neural circuits maintained?

The continuous function of the nervous system relies on the integrity of the circuits established during development. Indeed, the hallmark of many neurodegenerative diseases is a loss of synapses resulting in the destabilization of axons or dendrites (S. Hong et al., 2016; Lin & Koleske, 2010; Mariano et al., 2018; Sauerbeck et al., 2020; Scheff et al., 2006; Terry et al., 1991). Understanding how the architecture of the nervous system is maintained might therefore aid in the development of therapeutics that prevent or restore synapse loss in disease states. Efforts to characterize the molecular landscape of developing versus mature neurons demonstrate that the regulators of wiring that are present in development are largely downregulated in established circuits (Favuzzi et al., 2019; H. Li et al., 2017; J. Li et al., 2020). Therefore, a distinct set of molecular programs is likely required to maintain circuit architecture. Here, we summarize recent literature that examines how synaptic specificity, synapse location, and synapse stability are maintained.

Maintenance of synaptic specificity during animal growth

Advances in imaging technology and multidimensional image reconstruction have enabled rapid and repeated imaging across biological scales: from single synaptic proteins to whole-animal connectomes. From these advances, we see that the architecture of the nervous system is remarkably stable during postembryonic development. In *Drosophila*, the morphology and connectivity of neurons in a nociceptive circuit are consistent between first and third instar (Gerhard et al., 2017; Schneider-Mizell et al., 2016), during which the size of the neurons and number of synapses between synaptic partners are proportionally scaled to the animal's increased body size. The proportion of synaptic inputs from each presynaptic partner onto a given neuron is also consistent across development, suggesting that there are mechanisms to ensure reliable

circuit output (Gerhard et al., 2017). A similar study was recently undertaken in *C. elegans*. The brains of 8 isogenic animals were imaged by serial-section electron microscopy at successive stages of development, and their connectomes were reconstructed. While considerable nonuniform changes in synapse addition took place during maturation, the shape and positioning of the majority of neurites established at birth remained consistent through adulthood. Roughly 70% of synapses in the adult brain were part of stable connections that maintained proportional strength from birth to adulthood (Witvliet et al., 2021). During such a dynamic process, how are specific connections between neurons maintained? It appears maintenance of synaptic specificity is enabled by both cell-autonomous and nonautonomous mechanisms.

The *Drosophila* larva is a tractable model for studying circuit maintenance during animal growth. From first to third instar, the body surface area increases 100-fold. During this time, neurons increase in size and synapse number while maintaining their overall topology (Gerhard et al., 2017; Parrish et al., 2009). Class IV da (C4da) sensory neurons synapse with A08n interneurons to mediate responses to noxious temperature and touch stimuli (Hu et al., 2017; Hwang et al., 2007; Tracey et al., 2003). Both light and electron microscopy studies of C4da-A08n connections show that the number of C4da synapses onto A08n linearly increases during larval growth; however, the proportion of C4da synapses targeting A08n is constant (Tenedini et al., 2019). Tao kinase was identified in a reverse genetic screen for molecular regulators that maintain cellular synaptic specificity. When knocked down in A08n using RNAi, A08n dendrites had more postsynaptic puncta and formed ectopic synapses with another class of sensory neuron, C3da. Conversely, overactivation of Tao resulted in a reduction in A08n dendritic volume and postsynapse number. Together, these results suggest that the maintenance of synaptic specificity during growth is partially accomplished through the restriction of dendrite overgrowth and synapse addition (Figure 2.4A). Tao likely accomplishes this function through the negative regulation of dendritic cytoskeletal stability; loss of Tao expression was shown elsewhere to elevate the proportion of stable microtubules and levels of F-actin in the dendrite (Hu et al., 2020). Additionally, the human ortholog TaoK2 was able to substitute for *Drosophila* Tao function, suggesting that there is conservation between the fly and human Tao functional protein domains (Hu et al., 2020; Tenedini et al., 2019).

As discussed above, the subcellular placement of synapses is an important feature of circuit function and likewise must be maintained during animal growth. Maintenance of synaptic

placement during growth has been termed “synaptic allometry.” Synaptic allometry requires not only the maintenance of synapse positioning, but also the prevention of ectopic synapse accumulation (Fan et al., 2020). In *C. elegans*, coordinated signaling across multiple cell types underlies presynaptic allometry in a subset of neurons called AIY. The AIY neurons in the worm form synapses with different partners at stereotyped locations along their axons (Colón-Ramos et al., 2007). The locations of these synapses are established in the embryo and maintained in the adult (Colón-Ramos et al., 2007; Shao et al., 2013; White et al., 1986). CIMA-1, part of the SLC17 family of solute transporters, was identified in a forward genetic screen for regulators of AIY synaptic allometry. CIMA-1 was found to be expressed in epidermal cells of the adult. In *cima-1* mutants, AIY presynapse position was normal in the newly hatched larva, but in adults, AIY presynapses were posteriorly displaced and ectopic presynapses formed in an area of the axon typically devoid of synapses (Shao et al., 2013). The ectopic synapses were not opposed to the main AIY postsynaptic partner neuron, RIA. Therefore, CIMA-1 is required for maintaining AIY presynapse subcellular position and potentially restricting inappropriate contacts from larval stages into the adult.

Interestingly, CIMA-1 exerts its effect on synapse maintenance by modifying the position of glial cells. A subset of glial cells (ventral cephalic sheath cells) occupies the space between the AIY synaptic zone and the CIMA-1-expressing epidermis (White et al., 1986). In *cima-1* mutants, glial processes extend aberrantly and contact the AIY asynaptic zone. This aberrant glial positioning correlates with the establishment of ectopic AIY presynapses. To test whether glial cell contact is required for ectopic synapse accumulation in AIY, glial cells were genetically ablated in a *cima-1* mutant background. As a result, AIY presynapses no longer extended into the AIY asynaptic zone. Through a secondary suppressor screen, the FGF receptor EGL-15(5A) was found to be required in epidermal cells for distortion of glial morphology and thus ectopic synapse formation. Only the extracellular adhesive domain of EGL-15(5A) was required to suppress ectopic glial extension and ectopic synapse formation. In *cima-1* mutants, EGL-15(5A) levels were upregulated fivefold, indicating that CIMA-1 normally antagonizes EGL. These results suggest a model in which glia maintain the location of presynapses during growth (Figure 4.4B), and this is enabled by reducing glia-epidermis adhesion. CIMA-1 reduces glia-epidermis adhesion, allowing glia location to be maintained, perhaps by preventing the retrograde extension of glial processes as animal length increases (Breau et al., 2017). As a result of maintaining glial

position, the AIY synaptic pattern is retained (Shao et al., 2013). This study highlights how nonautonomous cues regulate synapse subcellular localization during the postembryonic phase of growth.

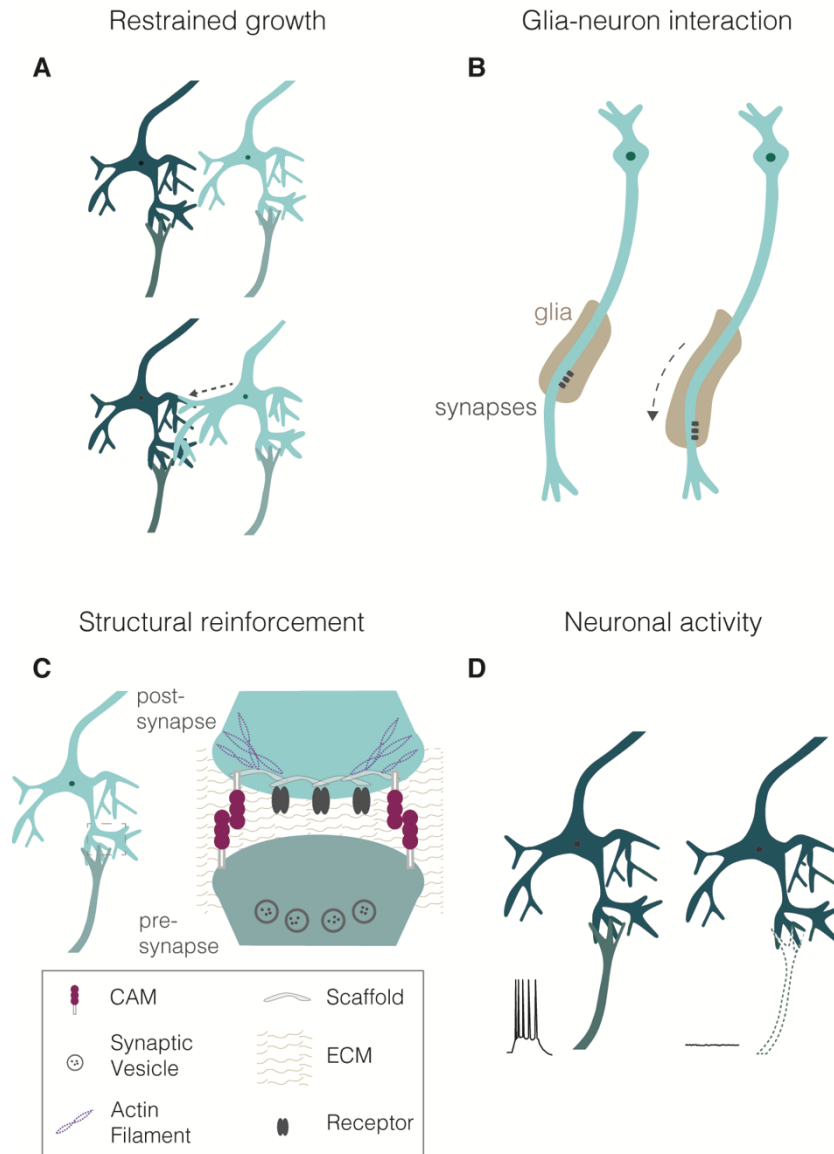


Figure 2.4. Mechanisms that promote neural circuit maintenance. (A) Synaptic partnerships and circuit topology are retained through restricting dendritic overgrowth. (B) Synapse location is maintained through the positioning of glia-neuron contacts (adapted from Shao et al., 2013). (C) Maintenance of synapse stability is facilitated by the cooperation of ECM-CAM-intracellular scaffold-cytoskeletal interactions. (D) Silencing of neuronal activity can result in degeneration or neuron loss.

The mechanisms highlighted in this section restrain ectopic synapse addition. Dendritic expansion is negatively regulated to maintain cellular synaptic specificity, and non-neuronal cells help to affix the location of presynapses from embryonic stages to adulthood. In the following section, we examine how individual synapses are positively reinforced.

Maintenance of synaptic stability

In mammals, dendritic spines are protrusions from the main dendritic shaft, and are the postsynaptic sites of excitatory synapses. Juvenile mice exhibit continual addition and retraction of individual dendritic spines and presynaptic terminals. As these animals enter adulthood, the rate of synapse turnover decreases (Gan et al., 2003; Zuo et al., 2005), with 70% of cortical dendritic spines persisting at least 18 months (Zuo et al., 2005). In humans, the density of synapses is stable until advanced age (Huttenlocher, 1979). Thus, individual synapses may persist in the human brain for decades. Once a synapse is established, how does it persist? The decrease in dendritic spine motility in adult animals coincides with the maturation of the extracellular matrix (ECM) (Frischknecht & Gundelfinger, 2012), a meshwork of glycoproteins and proteoglycans. Studies in the hippocampus and visual cortex have shown that ECM degradation in these areas causes increased spine addition and retraction, formation of filopodial protrusions, and dynamic changes in the size of the spine head (De Vivo et al., 2013; Oray et al., 2004; Orlando et al., 2012). Moreover, when ECM was degraded, Orlando et al. (2012) noted an increase in the clustering and activation of integrins, transmembrane proteins that have established roles in sensing changes to ECM (Park & Goda, 2016). When integrin activation was neutralized with an antagonistic antibody treatment, dendritic spine motility was blocked. Thus, in this system, ECM may inhibit integrin activation to stabilize dendritic spines (Orlando et al., 2012).

Synapse stability is also dependent on cell-cell adhesion to maintain the close apposition of presynaptic and postsynaptic compartments. Although many of the molecules that establish synaptic specificity are downregulated after circuit establishment (H. Li et al., 2017; Favuzzi et al., 2019; J. Li et al., 2020), subsets of CAMs are required to maintain synaptic (Lin & Koleske, 2010; Robbins et al., 2010; Soto et al., 2018). One example is L1CAM, part of the L1 family of IgSF cell surface molecules. L1CAM is required in the mouse cortex to establish and maintain

synapses between presynaptic chandelier cells and postsynaptic pyramidal neurons. As stated earlier, chandelier cells form inhibitory synapses at the AIS of pyramidal neurons. In an RNAi screen for CAM regulators of synapse formation, Tai et al. (2019) found that knockdown of L1CAM in pyramidal neurons during the time of synapse formation greatly reduced the number of chandelier synapses onto pyramidal neurons. They next asked whether L1CAM is required to maintain synapses. Using a tamoxifen-inducible Cre system, they induced expression of *L1CAM* RNAi in adult mice (P28) and assayed for synapse loss 12 d later (P40). They found both the number of synapses and chandelier contacts onto pyramidal cell neurons were reduced (Tai et al., 2019). In *Drosophila*, the L1CAM homolog Neuroglian (Nrg) is also required to establish synaptic contact between neurons in the CNS, and to maintain synapse stability between motor neurons and muscles at the neuromuscular junction (Enneking et al., 2013).

Intracellularly, synapse stability requires the linkage between CAMs and the cytoskeleton. CAMs are linked to the cytoskeleton through a network of scaffolding proteins. For example, L1CAM and Nrg bind intracellularly to the Ankyrin scaffold protein. Ankyrin provides a link between CAMs and the submembranous cytoskeleton (Smith & Penzes, 2018). Accordingly, at the neuromuscular junction of *Drosophila ank2* mutants, microtubule bundles within motor neurons are severely disorganized. CAMs are depleted and no longer stably associated with the synaptic membrane, leading to the retraction of synapses (Enneking et al., 2013; Koch et al., 2008; Pielage et al., 2008). Ankyrins also promote the clustering of CAMs and synaptic machinery at the AIS in vertebrate neurons (Smith and Penzes, 2018), and removal of either Ankyrin or β -spectrin results in a decrease in synaptic inputs onto the AIS (Ango et al., 2004; Tai et al., 2019).

ECM, cell-cell adhesion, intracellular scaffold proteins, and the cytoskeleton provide a rigid support network that allows the perdurance of individual synapses (Figure 2.4C). While continuous brain function requires that circuit integrity be maintained, certain cognitive abilities, such as learning and memory, require that the brain retain the capacity to undergo structural circuit modifications. In the hippocampus, the region of the brain that processes new memories, synapses exhibit a relatively high degree of dynamicity compared with other brain regions (T. Pfeiffer et al., 2018). There is evidence from hippocampus and other brain regions to suggest that processes that directly oppose ECM and CAM stability allow flexible modification of synaptic structures (Frischknecht et al., 2009; Nguyen et al., 2020; Oray et al., 2004;

Robbins et al., 2010). Future work will be necessary to understand the mechanisms underlying the regional heterogeneity of synapse stability, as well as how synapse destabilizing and stabilizing mechanisms are dynamically regulated during memory acquisition and retainment.

Neuronal activity is also important for maintaining synaptic connections (Figure 2.4D). In studies where all neurons were chronically silenced during development, circuitry appeared to develop normally but ultimately resulted in axon degeneration (Verhage et al., 2000). Using a more refined approach, Yu et al. (2004) conditionally silenced a subpopulation of OSNs after olfactory glomeruli were established. In the following days, OSN axons migrated away from their initial glomerular target, and OSN cell numbers subsequently diminished (Yu et al., 2004). In *C. elegans*, axon branching defects have also been observed in sensory-deprived animals late in development (Peckol et al., 1999); thus, activity is possibly an evolutionarily conserved homeostatic mechanism for maintaining circuit organization. Further investigation is needed to determine what molecular programs are downstream of neuronal activity to reinforce existing connections.

Conclusions and future areas of study

Many neurons in the brain exhibit an exquisite precision in the formation of synaptic partnerships, as well as in the subcellular targeting of their synapses. The collective evidence from model systems fueled by improvements in methodologies suggests that cell surface molecules not only promote the matching of synaptic partners but also denote the subcellular placement of synapses. Secreted signaling molecules can refine the targeting of axons to specific subcellular domains of postsynaptic partners, as well as dictate the location of presynaptic sites. Both positive and negative cues sculpt the placement of synapses in certain subcellular compartments and exclude them in others. Finally, neuronal activity can play an instructive role in the establishment and maintenance of synaptic specificity.

There has also been excellent progress in understanding how neural circuit architecture is established and maintained. During the establishment phase, neurons are guided not just to regional domains by axon guidance cues, but also to specific subcellular locations of postsynaptic neurons. Once at their ultimate destination, many neurons are consistently able to synapse with a specific subset of neurons, despite the presence of many additional potential

partners. Since Langley and Sperry first postulated the notion of chemoaffinity tags, studies have identified many cell surface molecules that specify connectivity. Expanding on the chemoaffinity hypothesis, we have seen that secreted cues, interactions between neurons and glia, and neuronal activity are also critical for the precise organization of circuits. After circuit establishment has taken place, there are mechanisms that maintain individual synapses, synapse locations, and cellular synaptic specificity. New players, as well as some that are involved in synapse establishment, provide rigid support to presynaptic and postsynaptic structures.

While our understanding of how the architecture of the nervous system is established and maintained has certainly expanded, there are still a host of questions to be answered. In this review, we chose to focus on mechanisms underlying the wiring of highly deterministic circuits, but there are also examples of circuits with variable and stochastic wiring patterns (Caron et al., 2013; Chou et al., 2010; Linneweber et al., 2020; Witvliet et al., 2021). What are the rules used to wire up these circuits, and how are they different from more deterministic circuits? Importantly, how can we leverage principles of circuit establishment and maintenance to prevent or restore synapse loss in disease? The pathways described in this review could serve as entry points for the development of therapies for this purpose.

Bridge

In this review, I discussed the known mechanisms that guide the formation and maintenance of cellular and subcellular synaptic specificity, focusing on the roles of secreted cues, cell-adhesion molecules, and neural activity. In the following chapter, I will introduce a pair of synaptically coupled cells, where the presynaptic cell synapses specifically with one of dendritic arbors on the postsynaptic cell. My co-authors and I use the reviewed literature in Chapter II to generate and test models for whether secreted or cell-adhesion molecules instruct the establishment of the subcellular synaptic specificity between this pair of neurons.

CHAPTER III

REGULATION OF SUBCELLULAR DENDRITIC SYNAPSE SPECIFICITY BY AXON GUIDANCE CUES

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Author Contributions

ECS, ELH, and CQD conceived of the project. ECS, ELH, and CQD designed experiments. ECS was the major contributor to Figures 2-4 and associated supplemental figures. ELH was the major contributor to Figures 5-7 and the associated supplemental figures. TLW performed data analysis in Figure 6. ECS, ELH, and CQD drafted the original manuscript. All authors reviewed and edited the final manuscript. CQD provided supervision and funding for the project.

Introduction

Nervous system function is determined by the precise connectivity of neurons. From the *Drosophila* larva with 10,000 neurons to the human with 80 billion neurons, all neurons are faced with the challenge of identifying the correct subset of synaptic partners among many potential target neurons. In addition to specificity at a cellular level, neural circuits also exhibit synaptic specificity at the subcellular level (reviewed in Yogev & Shen, 2014). In *Drosophila*, the giant fiber descending neuron targets a specific dendritic domain of the tergotrochanteral motor neuron in a fast jump escape circuit (Godenschwege et al., 2002; Godenschwege & Murphey, 2009). In mammals, cortical pyramidal neurons receive input from martinotti neurons on their distal dendrites and basket neurons on their proximal dendrites (Huang et al., 2007) (Figure 3.1A). The precise targeting of inhibitory neurons to distinct subcellular domains of their target neurons has profound effects on neural processing and circuit function by gating action potential initiation, providing a substrate for plasticity, altering mEPSP amplitude, and modulating dendritic integration (Bloss et al., 2016; Hao et al., 2009; Miles et al., 1996;

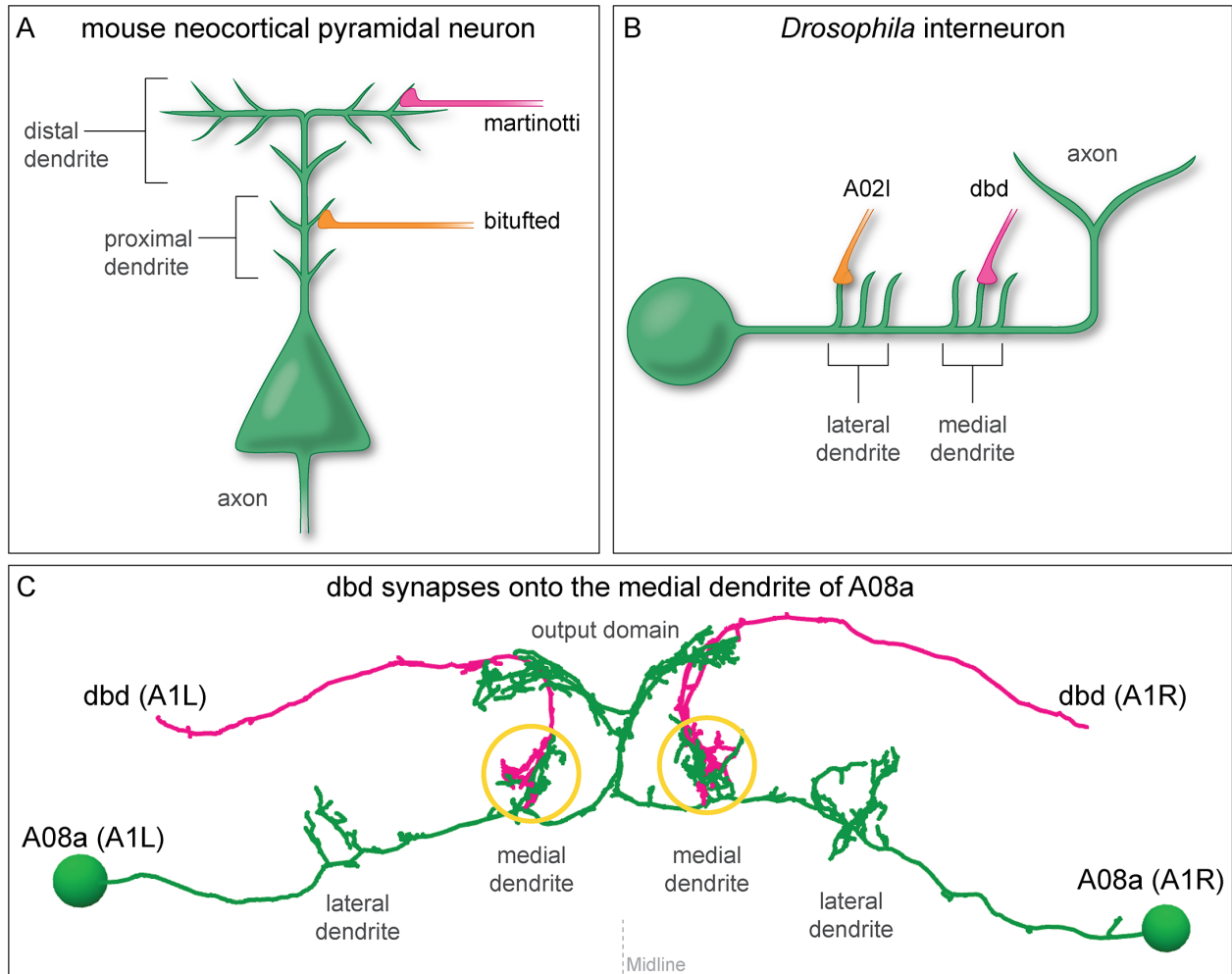


Figure 3.1. Mammalian and insect neurons display subcellular synaptic specificity. (A) Schematic of mouse neocortical pyramidal neuron (green) with a martinotti neuron (magenta) forming synapses onto the distal dendrite and the bitufted neuron (orange) forming synapses onto the proximal dendrite. (B) Schematic of fly A08a neuron (green) with a dbd neuron (magenta) forming synapses onto the lateral dendrite and an A02I neuron (orange) forming synapses onto the medial dendrite. (C) Electron microscopy reconstruction of dbd neurons (magenta) and A08a neurons (green) morphologies in one abdominal (A) segment (A1 left and A1 right) of the *Drosophila* ventral nerve cord (posterior view). dbd forms synapses specifically with the medial dendritic domain, and does not synapse with the lateral dendritic domain or the output domain.

Pouille et al., 2013; Tobin et al., 2017). Although the precise subcellular positioning of synapses is important for proper circuit function, the mechanisms necessary to achieve such specificity are just starting to be explored (Telley et al., 2016).

Two distinct developmental models could explain subcellular synaptic specificity. The first model relies on molecular differences between two subcellular domains to restrict synapse formation to one domain (the ‘labeled arbor’ model). This model is supported by evidence in mouse and *C. elegans* whereby local clustering of cell surface molecules on a postsynaptic neuron dictates synapse position (Ango et al., 2004; Colón-Ramos et al., 2007;

Klassen & Shen, 2007; Mizumoto & Shen, 2013). An alternative mechanism relies on axon guidance cues to restrict pre-synaptic access to one of several acceptable postsynaptic targets (the ‘guidance cue’ model). Guidance cues have a well-characterized role in the gross positioning of axons and (Dickson, 2002; Huang et al., 2007; Keleman & Dickson, 2001; Tessier-Lavigne & Goodman, 1996; Zlatić et al., 2009), but their role in regulating the subcellular position of synapses has yet to be tested.

We sought to test which of these two models generate dendritic subcellular synaptic specificity using a pair of synaptically coupled neurons in the *Drosophila* larval ventral nerve cord (VNC): the dbd sensory neuron and A08a interneuron (Itakura et al., 2015; Schneider-Mizell et al., 2016). A08a has two spatially distinct dendritic arbors, one medial and one lateral, and dbd synapses specifically with the medial dendritic arbor (Figure 3.1B,C). Is this subcellular target choice due to molecular differences between the medial and lateral A08a dendritic arbors? Or are both dendritic arbors competent to accept dbd synaptic input, but axon guidance cues restrict dbd targeting to the medial arbor? Our results support the guidance cue model: we find that when the dbd axon is lateralized in the neuropil by misexpression of the axon guidance receptor Unc-5, it forms functional synapses with the A08a lateral dendritic arbor. Taken together, our data suggest that axon guidance cues establish subcellular synaptic targeting and that there are no molecular differences in the A08a medial and lateral dendritic arbors that restrict dbd synapse formation.

Results

A08a interneuron has two dendritic arbors that receive distinct synaptic input

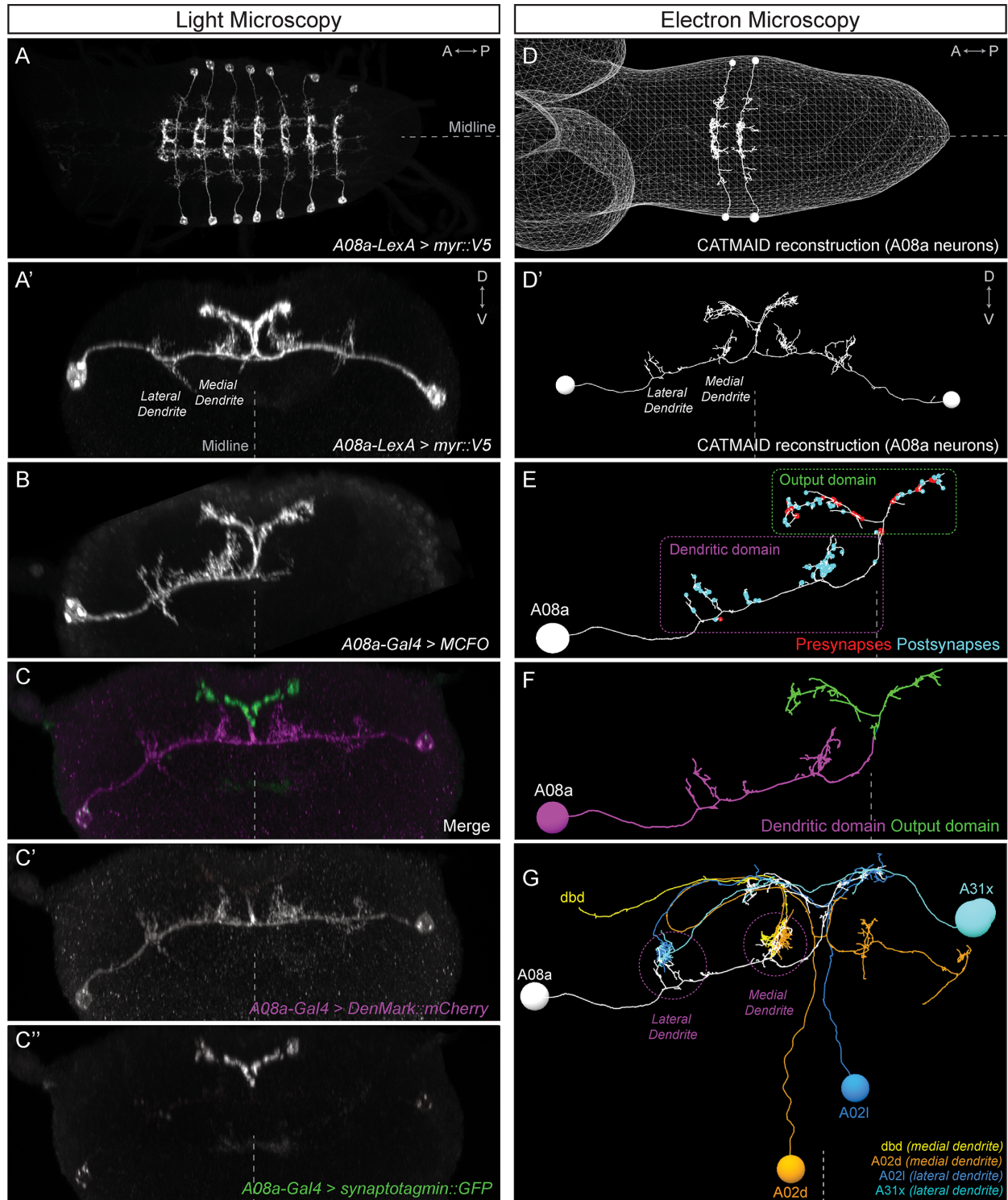
To determine which of our proposed developmental mechanisms regulates subcellular synaptic specificity, we focused on the A08a interneuron as a model system. A08a has spatially distinct medial and lateral dendrites, and receives distinct input to each of these dendrites (Figure 3.2). A08a interneurons can be visualized by light microscopy using the *R26F05(A08a)-LexA* line in larvae (24 ± 4 hr after larval hatching; ALH) in abdominal segments (A) 1–7 (Figure 3.2A–A’,B). By expressing molecular markers, we determined that A08a has a distinct distal axonal (output) domain (mixed pre- and post-synapses) and a more proximal dendritic domain

(predominantly post-synapses). A08a targets the dendritic marker *DenMark::mCherry* (Nicolai et al., 2010) to the dendritic domain which includes two spatially distinct medial and lateral arbors. The A08a output domain forms a characteristic V-shaped projection at the midline, which is specifically labeled by the pre-synaptic marker *Synaptotagmin::GFP* (Zhang et al., 2002) (Figure 3.2C–C’’).

A08a can also be visualized by electron microscopy (EM) in first instar larvae (~5 hr ALH, Figure 3.2D–D’) (Gerhard et al., 2017; Itakura et al., 2015; Schneider-Mizell et al., 2016). The EM reconstruction of A08a has been completed in four hemisegments (A1 left/right, A2 left/right), and in all cases, the A08a neuron has the same arbors as seen in light microscopy: two spatially distinct dendritic arbors that contain only post-synapses, and a V-shaped output domain that contains both pre- and post-synapses (Figure 3.2E). Moreover, the same output and dendritic subcellular compartments as seen with *DenMark::mCherry* and *Synaptotagmin::GFP* can also be detected in the EM reconstructed A08a neuron using the synapse flow centrality algorithm (Schneider-Mizell et al., 2016), which considers path directionality between synaptic input and output locations in the A08a neuron (Figure 3.2F).

Next, we used the EM reconstruction to identify neurons with the most inputs onto A08a. We characterized the four neurons with the most synapses onto A08a dendrites (Table 1), and observed that *dbd* and A02d selectively synapse onto the A08a medial dendrite, whereas A02l and A31x selectively synapse onto the A08a lateral dendrite (Figure 3.2G; Table 1). Moreover, *dbd*-A08a partners have a synapse filling fraction similar to previously described synaptically

Figure 3.2 (next page). The A08a neuron receives arbor-specific synaptic inputs. (A–C’’) Light microscopy (point scanning confocal) imaging of A08a neurons. (A) Dorsal view of the light micrograph (LM) 3D reconstruction of A08a neurons in the larval ventral nerve cord segments A1-7. The A08a neurons are visualized by *26F05(A08a)-LexA > LexAop-myr::smGdP::V5*. Midline, dashed line in all panels. (A’’) Posterior view of the LM 3D reconstruction of paired A08a neurons in segment A1 left/right. (B) Posterior view of a single A08a labeled by MultiColor FlpOut (MCFO), visualized by *A08a-Gal4 > UAS-MCFO*. (C–C’’) *A08a-Gal4* drives expression of *UAS-DenMark::mCherry* (dendrite marker) and *UAS-synaptotagmin::GFP* (presynaptic marker). Note the complementary expression in the dendritic and output domains. (D–G) Electron microscopy (EM) reconstruction of A08a and four synaptic partner neurons. (D) Dorsal view of A08a neurons in segments A1-2. (D’’) Posterior view of A08a neurons in segment A1. (E) A single A08a with presynaptic and postsynaptic sites labeled in red and blue highlight a distinct dendritic domain and a mixed pre- and post-synaptic output domain, respectively. (F) Synapse flow centrality analyses (Schneider-Mizell et al., 2016) shows that A08a has distinct mixed axonal (output) and dendritic compartments. (G) A08a receives dendritic arbor-specific input: *dbd* (yellow) and A02d (orange) synapse specifically on the medial dendrite, whereas A02l (blue) and A31x (cyan) synapse specifically on the lateral dendrite.



connected neurons (Figure 3.2—figure supplement 1) (Gerhard et al., 2017; Stepanyants et al., 2002). A08a also receives synaptic input from additional neurons at its medial and lateral

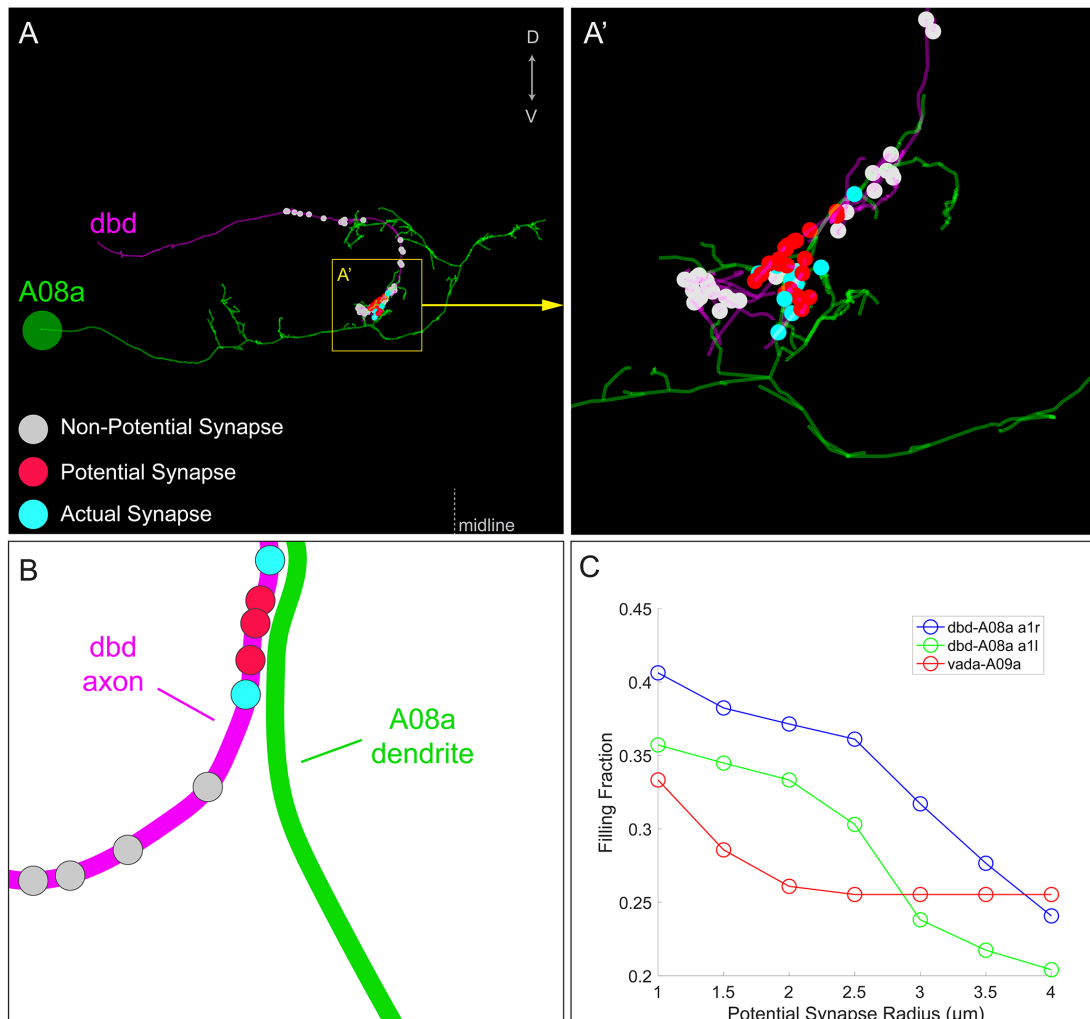


Figure 3.2 – figure supplement 1. Filling fractions between dbd and A08a neurons. (A–A') Posterior view of dbd (magenta) and A08a (green) neurons EM reconstruction in abdominal segment 1, left (A1L). dbd presynapses (enlarged circles) are color coded based on distance from A08a membrane and synaptic connectivity with A08a. Non-potential synapses (gray) indicate dbd presynapses that are more than $2\ \mu\text{m}$ away from the center of A08a dendritic processes (skeleton). Potential synapses (red) indicate dbd presynapses less than $2\ \mu\text{m}$ away from the A08a skeleton that are not connected with A08a. Actual synapses (cyan) indicate dbd presynapses that are synaptically coupled with A08a. Yellow box indicates region enlarged in A'. **(B)** Schematic showing how the synapse types are assigned between dbd (magenta) and A08a (green) neurons in the filling fraction analyses. The filling fraction is the number of actual synapses divided by the sum of actual and potential synapses. The distance threshold which defines non-potential synapses can be changed, allowing the filling fraction to be plotted as a function of distance (shown in C below). **(C)** The filling fraction plotted as a function of distance between dbd presynapses and A08a skeleton. When dbd presynapses are within $2\ \mu\text{m}$ from the A08a skeleton, the percent of synapse formation (filling fraction) is 0.34 (A1L) and 0.38 (A1R). The filling fraction between a different set of neurons, v'ada and A09a, are provided for reference from Gerhard et al. (2017).

dendritic arbors, and these neurons also show a preference for either the medial or lateral dendritic arbor; a different set of neurons has synaptic input on the V-shaped output domain (data not shown). We conclude that the A08a neuron is an ideal model system to investigate the mechanisms generating subcellular synaptic specificity due to (a) Gal4 and LexA lines specifically expressed in A08a, (b) spatially distinct dendritic arbors with highly specific neuronal inputs onto each arbor, and (c) our ability to visualize A08a morphology by both light and electron microscopy. In addition, we have highly specific Gal4 and LexA lines for the dbd sensory neuron, which has specific synaptic input onto the A08a medial arbor (see below).

Table 1. Summary of inputs to A08a medial and lateral dendritic arbors from the first instar larval EM reconstruction. Neurons with ≥ 2 synapses to A08a medial and lateral arbors shown. Neurons with fewer synapses also show specificity for medial or lateral dendritic arbors. Note that dbd has 1-2 synapses with A02l and 9-12 synapses with A02d. Table updated from Sales et al., 2019 to include updated synapse values, neurotransmitter information (Fushiki et al., 2016; Kohsaka et al., 2014; Salvaterra & Kitamoto, 2001), newly annotated partners, and proportion of A08a input contributed by each neuron.

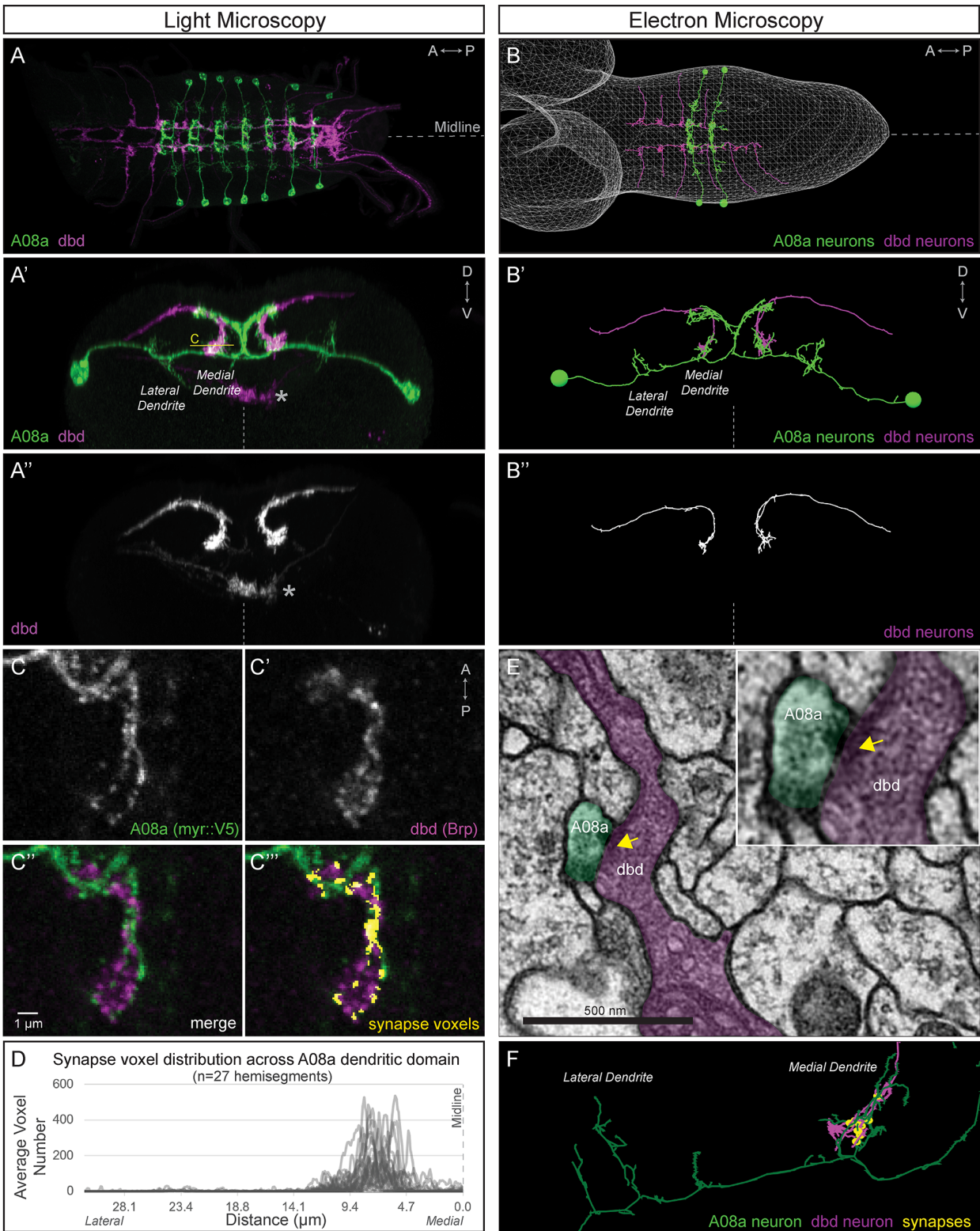
A08a inputs (hemisegment)	Pre-synapse number		Proportion of A08a input	A08a arbor targeted	Neurotransmitter Identity
	Total	with A08a			
dbd (A1L)	419	13	10.4%	medial only	Cholinergic excitatory
dbd (A1R)	408	13	6.5%	medial only	Cholinergic excitatory
vbd (A1L)	349	2	1.6%	lateral only	Cholinergic excitatory
vbd (A1R)	324	10	5%	lateral only	Cholinergic excitatory
A02d (A1L)	293	22	17.6%	medial only	Glutamatergic inhibitory
A02d (A1R)	306	8	4.0%	medial only	Glutamatergic inhibitory
A02l (A1L)	128	12	6.0%	lateral only	Glutamatergic inhibitory
A02l (A1R)	126	4	3.2%	lateral only	Glutamatergic inhibitory
A31x (A1L)	101	9	4.5%	lateral only	GABAergic inhibitory
A31x (A1R)	93	3	2.4%	lateral only	GABAergic inhibitory

Quantifying dbd-A08a synapse voxel position by light microscopy

The EM reconstruction allows precise quantification of synapse number and position between dbd and A08a, but EM is not a high-throughput method for experimental analysis of synaptic contacts. Thus, we developed a light microscopy method for quantifying the position of dbd-A08a putative synapse contacts. We used the genetics described above to label A08a, and additionally used the *165(dbd)-Gal4* line to label the dbd sensory neuron in 24 ± 4 hr ALH larvae. We conclude that dbd and A08a morphology seen in light microscopy precisely matches dbd and A08a morphology seen in the EM reconstruction (Figure 3.3A–B’’).

We next quantified the position of dbd pre-synaptic contacts along the medial-lateral axis of the A08a dendrite. We used *dbd-Gal4* to express the active zone marker Bruchpilot-Short::mStrawberry (Brp-Short-mStraw; Oswald et al., 2010) in the dbd neuron; the truncated Brp protein localizes to presynaptic sites but is not functional for inducing synapse formation, making it an excellent reporter for pre-synapses (Fouquet et al., 2009). In the same larvae, we used the *26F05(A08a)-LexA* line to label the A08a interneuron to express a myristoylated::V5 epitope. The dbd neuron forms synapses with many neurons in addition to A08a, so we considered only the Brp signal in close proximity (<90 nm) to the A08a membrane to define the position of dbd-A08a ‘synapse voxels’ (Figure 3.3C–C’’’). Note that this is not designed to count individual synapse numbers, which are below the resolution limit of standard light microscopy, but rather to measure the position of putative synapses along the medio-lateral axis of the A08a dendrite. Quantifying synapse voxels across the medial-lateral axis of A08a dendrites in wild-type larvae (Figure 3.3D, n = 27 hemisegments, N = 18 animals) mirrors the position of synapses seen by EM (Figure 3.3F). In contrast, we do not observe synapse voxels between the dbd and the A08a output domain, consistent with lack of dbd synapses on the A08a output domain in the EM reconstruction (data not shown). Thus, we have established a light microscopy method for imaging and quantifying the position of dbd presynapses along the A08a dendritic membrane, which is a necessary prerequisite for investigating the mechanisms regulating dbd-A08a subcellular synaptic specificity.

Figure 3.3 (next page). dbd and A08a neurons are synaptic partners by light and electron microscopy analyses. (A) Dorsal view, light microscopy 3D reconstruction showing dbd (magenta) and A08a (green) neurons. A08a is visualized with *A08a-LexA > LexAop-myr::smGdP::V5*. dbd is visualized with *dbd-Gal4 > UAS-myr::smGdP::HA*. Anterior to left; midline, dashed line in all panels. (A’–A’’) Posterior view, light microscopy 3D reconstruction showing dbd and A08a neurons. dbd projects to the A08a medial dendritic arbor but not the A08a lateral dendritic arbor. Apparent colocalization of dbd with the A08a output domain is an artifact of the 3D projection. Asterisk, ventral off-target expression of *dbd-Gal4*. C, focal plane shown in panel C, below. (B–B’’) EM reconstruction of dbd and A08a neurons; B, dorsal view, (A1–A2); B’–B’’, posterior view, (A1). (C–C’’) Single optical section showing a subset of dbd presynapses (magenta, labeled with *dbd-Gal4 > UAS-brp-short-mstraw*) positioned in close proximity to the A08a membrane (green, labeled with *A08a-LexA > LexAop-myr::smGdP::V5*). Voxels containing A08a membrane within 90 nm of voxels containing Brp-mstraw are defined as ‘synapse voxels’ (C’’’, yellow). (D) Quantification of synapse voxel position across A08a dendritic domain shows enrichment on the A08a medial dendritic arbor. (E) Representative chemical synapse between dbd and A08a (arrowhead) in the EM volume. (F) EM reconstruction showing that the dbd neuron (magenta) synapses specifically with the A08a medial but not lateral dendritic arbor (green); synapses, yellow circles.



Lateralized dbd has Brp+ synapse voxels at the A08a lateral dendritic arbor

To determine if the lateral dendritic arbor of A08a is competent to receive input from the dbd neuron, we needed a way to re-direct dbd to a lateral location, giving it the opportunity to interact with the lateral dendrite of A08a. In *Drosophila*, neurons expressing the Netrin receptor Unc-5 or the Slit receptor Robo-2 have a repulsive response to midline-secreted Netrin and Slit ligands, (Keleman & Dickson, 2001; Simpson, Bland, et al., 2000; Simpson, Kidd, et al., 2000; Zlatić et al., 2003). Here, we used *dbd-Gal4* to express either Unc-5 or Robo-2 and found that both receptors could lateralize the dbd axon terminal to varying degrees, with Unc-5 being most effective and Robo-2 having a milder effect (Figure 3.4—figure supplement 1).

Wild-type dbd forms synapse voxels with the A08a medial dendritic arbor (Figure 3.4A–A'',C; Figure 3.4—figure supplement 1B,E). In contrast, overexpression of Unc-5 in dbd can lateralize the dbd axon terminal, positioning dbd adjacent to the A08a lateral dendritic arbor (Figure 3.4B–B'; Figure 3.4—figure supplement 1D,E). These lateralized dbd terminals formed synapse voxels with the lateral dendritic arbor of A08a (Figure 3.4B''). Similarly, overexpression of Robo-2 in dbd resulted in lateralization of the dbd axon terminal; the majority of dbd terminals formed synapse voxels in the intermediate zone between the medial and lateral dendrites (Figure 3.4—figure supplement 1C,E). The close apposition of dbd presynaptic Brp to the A08a dendritic membrane is consistent with, but does not prove, that there is functional connectivity between dbd and A08a at this arbor. Taken together, these results suggest that dbd can form Brp + putative synapses throughout the entire A08a dendritic domain, which is more consistent with the 'guidance cue' model and less consistent with the 'labeled arbor' model.

Lateralized dbd forms functional synapses with the A08a lateral dendritic arbor

Our finding that the lateralized dbd axon terminal localizes Brp + puncta in close apposition to the lateral A08a dendritic arbor suggests that these two neurons are synaptically connected, but falls short of proving functional connectivity. To test for functional connectivity between the lateralized dbd and A08a, we took an optogenetics approach. We used the Gal4/UAS and LexA/LexAop binary expression systems (Brand & Perrimon, 1993; Lai & Lee, 2006) to

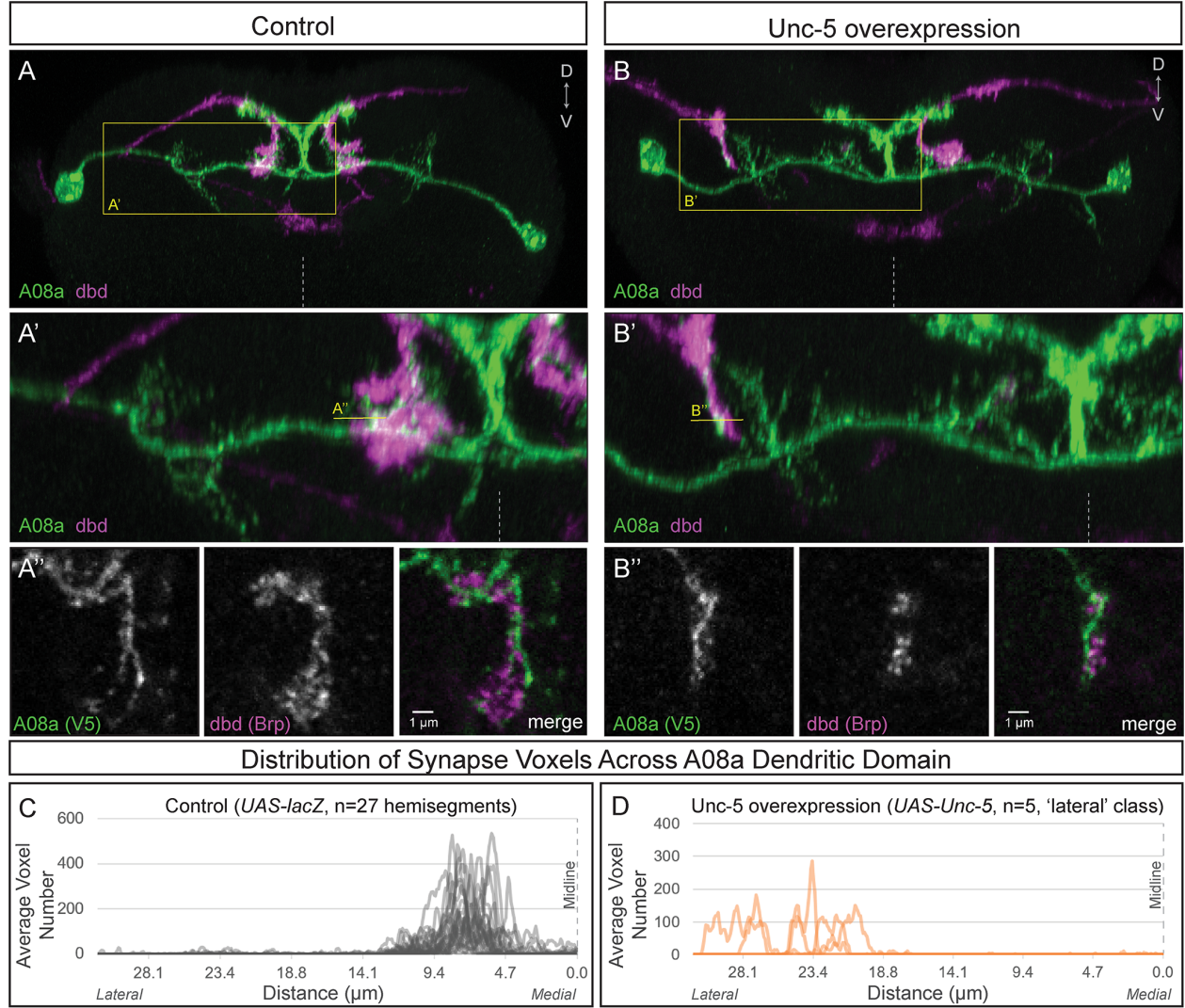


Figure 3.4. Lateralizing dbd results in Brp + putative synapses at the A08a lateral dendritic arbor. (A–A')

In control animals, dbd membrane (magenta, labeled with *dbd-Gal4 > UAS-smGdP::myr::HA*) is positioned in close proximity to the A08a medial dendritic arbor membrane (green, labeled with *A08a-LexA > LexAop-myr::smGdP::V5*). **(A)** Posterior view of one segment; midline, dashed line in all panels; box, area enlarged in **A'**. **(A')** Posterior view of dbd and the A08a medial dendritic arbor; **A''** line, optical section shown in **A''**. **(A'')** Single z-slice shows a subset of dbd presynapses (magenta, labeled with *dbd-Gal4 > UAS-brp-short::mstraw*) in close proximity to the A08a medial dendritic arbor membrane. **(B–B')** Overexpression of Unc-5 in dbd can lateralize the axon terminal of dbd. **B''** line, position of optical section shown in **B''** below. See Figure 4—figure supplement 1E for quantification of lateralization classes. **(B'')** Single z-slice shows a subset of dbd presynapses (magenta, labeled with *dbd-Gal4 > UAS-brp-short::mstraw*) positioned in close proximity to A08a membrane (green, labeled with *A08a-LexA > LexAop-myr::smGdP::V5*). **(C–D)** Quantification of synapse voxel position across the dendritic domain of A08a. **(C)** In control animals, dbd forms synapse voxels on the medial dendritic arbor of A08a; n = 27 hemisegments from 18 animals. Data reproduced from Figure 3D. **(D)** In hemisegments with full lateralization of dbd (as shown in **B'**), dbd forms synapse voxels on the lateral dendritic arbor of A08a; n = 5 hemisegments from five animals. See Figure 4—figure supplement 1E for quantification of lateralization classes.

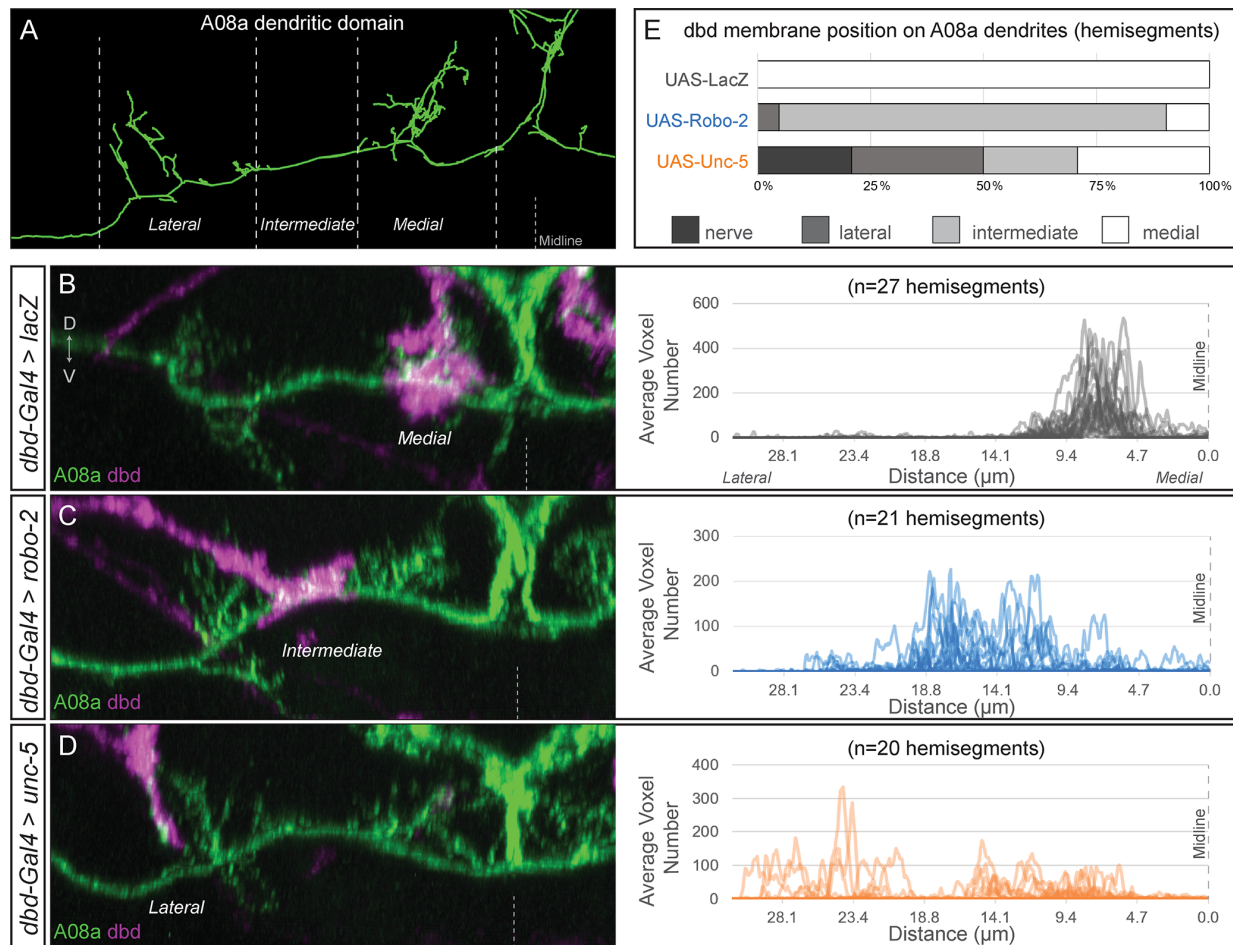
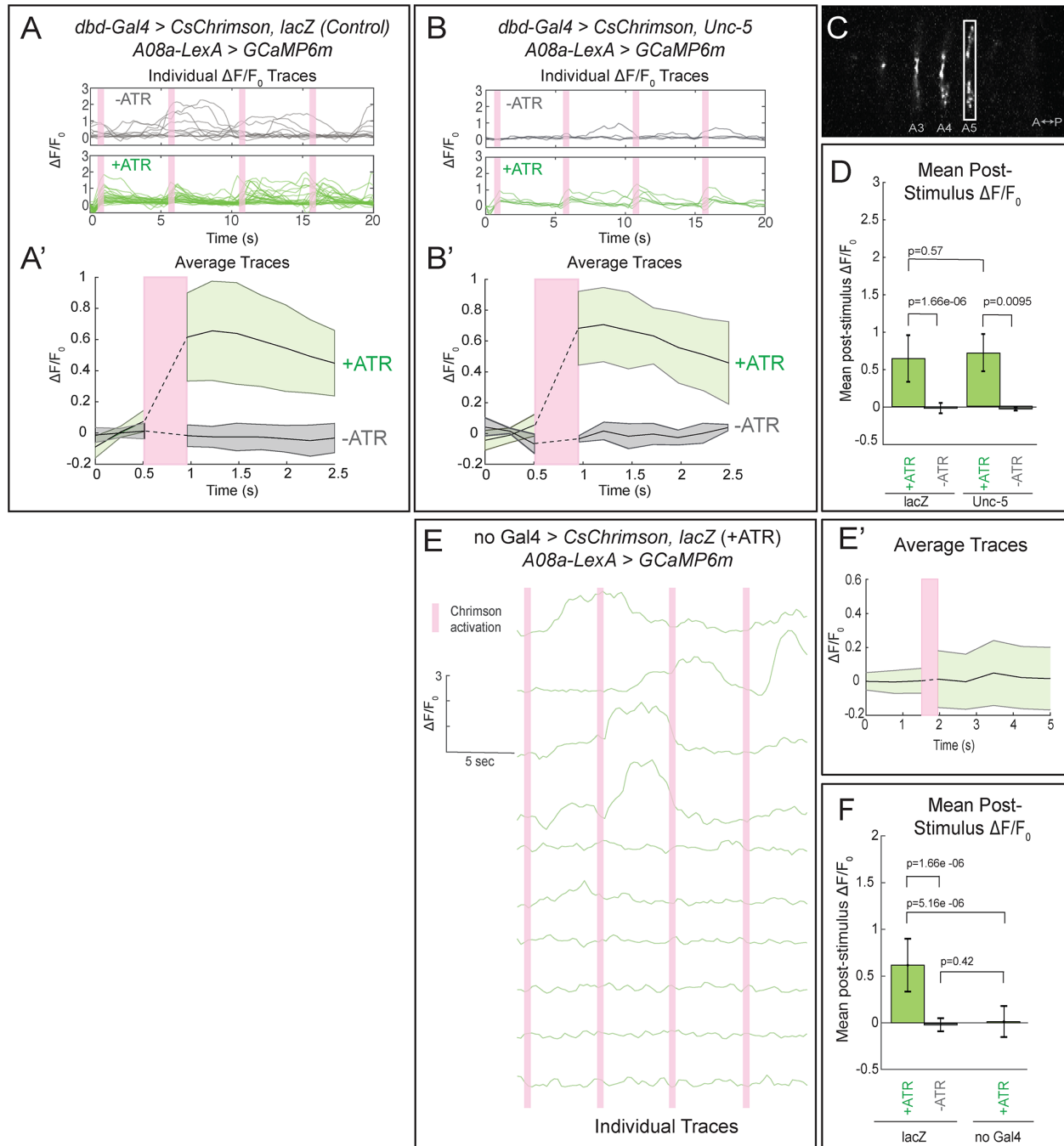


Figure 3.4 – figure supplement 1. dbd axons can be variably lateralized by expression of axon guidance receptors Unc-5 and Robo-2. (A) The A08a neuron in the EM reconstruction can be divided into medial, intermediate, and lateral dendritic domains. (B–D) The dbd neuron membrane (magenta) can target different subcellular domains of A08a (green), posterior view of one hemisegment. (B) Control: dbd contacts the A08a medial dendritic arbor (*dbd-Gal4 > UAS lacZ*). Data reproduced from Figure 3D. (C) Partial lateralization example: dbd contacts the intermediate dendrite domain (*dbd-Gal4 > UAS-robo-2*). (D) Full lateralization example: dbd contacts that lateral A08a dendritic arbor (*dbd-Gal4 > UAS-unc-5*). Data reproduced from Figure 4B',D. Right panels show the distribution of synapse voxels for each genotype. Control, *UAS-lacZ*, n = 27 hemisegments from 18 animals; *UAS-robo-2*, n = 21 hemisegments from 15 animals; *UAS-unc-5*, n = 20 hemisegments from 17 animals. (E) Frequency of dbd membrane lateralization by genotype. dbd will either target mostly the medial, intermediate, or lateral dendritic domains, or not enter the neuropil ('nerve' category). See methods for full genotypes.

express the light-gated cation channel CsChrimson (Chrimson) in dbd, and the calcium indicator GCaMP6m in A08a. For technical feasibility, all optogenetic experiments were done at the third instar larval stage (72 ± 4 hr ALH). Note that the A08a neuron at this stage retains its morphological features, including medial and lateral dendritic arbors plus a V-shaped output domain (Figure 3.5—figure supplement 1).

We first tested for functional connectivity between the wild-type *dbd* and A08a, which had not yet been documented. In wild-type, Chrimson-induced activation of *dbd* resulted in a significant increase in GCaMP6m fluorescence in A08a, but not in the absence of the Chrimson co-factor all-*trans* retinal (ATR) (Figure 3.5A, D), or in the absence of the *dbd-Gal4* transgene (Figure 3.5E, F). We measured GCaMP6m levels in the output domain of A08a, which emitted a larger fluorescence signal compared to the arborizations in the dendritic domain (Figure 3.5C). This is the first experiment showing functional, excitatory connectivity between *dbd* and A08a. Next, we sought to determine whether the putative synapses between the lateralized *dbd* and the A08a lateral dendritic arbor are also functional. Using the same paradigm as in wild-type controls, we find that Chrimson activation of lateralized *dbd* resulted in an increase in GCaMP6m fluorescence in A08a that is statistically indistinguishable from wild-type controls (Figure 3.5B, D). These data are consistent with *dbd* activating A08a equally well using medial arbor connectivity (control) or lateral arbor connectivity (following *Unc-5* expression).

Figure 3.5 (next page). Confocal activation of Chrimson in control and lateralized *dbd* increases A08a GCaMP6m fluorescence. (A–A') In wild-type animals, Chrimson activation of *dbd* neurons results in increased GCaMP6m fluorescence in the A08a output domain. For all figures, +ATR is shown in green, -ATR is shown in gray, and timing of Chrimson activation (500 ms) is represented with a pink bar. (A) A08a GCaMP6m $\Delta F/F_0$ traces from individual A08a pairs resulting from wild-type *dbd* activation. Non-evoked spontaneous activity is present in -ATR control. (A') Average A08a GCaMP6m $\Delta F/F_0$ traces, before and after Chrimson activation of *dbd* neurons. Solid black lines represent the mean $\Delta F/F_0$. Shaded regions represent the standard deviation from the mean. +ATR, $n = 28$ A08a pairs, from 10 animals; -ATR, $n = 11$ A08a pairs, from five animals. (B–B') In animals with fully lateralized *dbd*, Chrimson activation of *dbd* results in increased GCaMP6m fluorescence in A08a axon terminals. (B) A08a GCaMP6m $\Delta F/F_0$ traces from individual A08a pairs resulting from activation of lateralized *dbd*. (B') Average A08a GCaMP6m $\Delta F/F_0$ traces, before and after Chrimson activation of *dbd* neurons. Solid black lines represent the mean $\Delta F/F_0$. Shaded regions represent the standard deviation from the mean. +ATR, $n = 6$ A08a pairs, from five animals; -ATR, $n = 4$ A08a pairs, from three animals. (C) Example ROI used for quantification drawn around A08a axon terminals in segment A5. (D) Quantification of the mean post-stimulus $\Delta F/F_0$ for *lacZ* control and *unc-5*. Error bars represent the standard deviation from the mean. Mean post-stimulus $\Delta F/F_0$: *lacZ* Control +ATR, 0.62 ± 0.28 , $n = 28$ A08a pairs, from 10 animals; *lacZ* Control -ATR, -0.0172 ± 0.07 , $n = 11$ A08a pairs, from five animals; *unc-5* +ATR, 0.68 ± 0.24 , $n = 6$ A08a pairs, from five animals; *unc-5* -ATR, -0.035 ± 0.02 , $n = 4$ A08a pairs, from three animals. (E–E') *dbd-Gal4* is required to produce Chrimson-evoked responses in A08a. A08a expresses GCaMP6m in a genetic background containing *UAS-lacZ* and *20XUAS-CsChrimson*. (E) A08a GCaMP6m $\Delta F/F_0$ traces from individual A08a pairs. (E') Average A08a GCaMP6m $\Delta F/F_0$ traces before and after light stimulus (pink bar). Solid black line represents the mean $\Delta F/F_0$. Shaded region represents the standard deviation from the mean. +ATR is represented in green ($n = 10$ A08a pairs). (F) Quantification of the mean post-stimulus $\Delta F/F_0$ for *lacZ* control +ATR, *lacZ* control -ATR, and no *dbd-gal4* control. Error bars represent the standard deviation from the mean. Mean post-stimulus $\Delta F/F_0$: *lacZ* Control +ATR, 0.62 ± 0.28 , $n = 28$ A08a pairs, from 10 animals (Data reproduced from Figure 6D); *lacZ* control -ATR, -0.0172 ± 0.07 , $n = 11$ A08a pairs, from five animals (Data reproduced from Figure 6D); No *dbd-gal4* Control +ATR, 0.013 ± 0.17 , $n = 10$ A08a pairs, from five animals. Significance between two groups was determined using a Mann-Whitney test.



We observed that the Gal4 line used to express Chrimson in *dbd* also has expression in a subset of ventral neurons (Figure 3.3; Figure 3.5—figure supplement 1), the stimulation of which could have hypothetically contributed to the observed A08a responses. To distinguish the influence of *dbd* neurons and the ventral off-targets on A08a responses, we activated each set of neurons separately via spatially restricted two-photon holographic stimulation (Figure 3.6A,B).

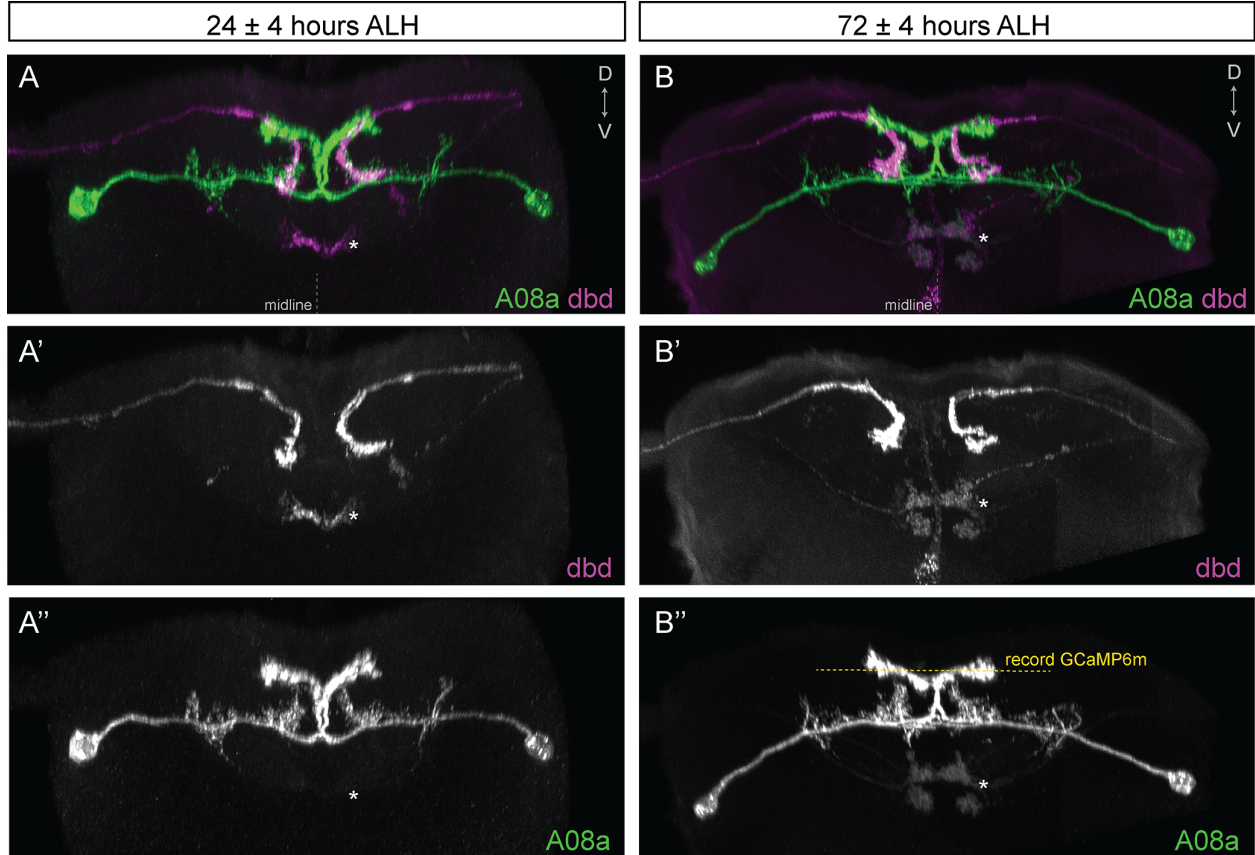


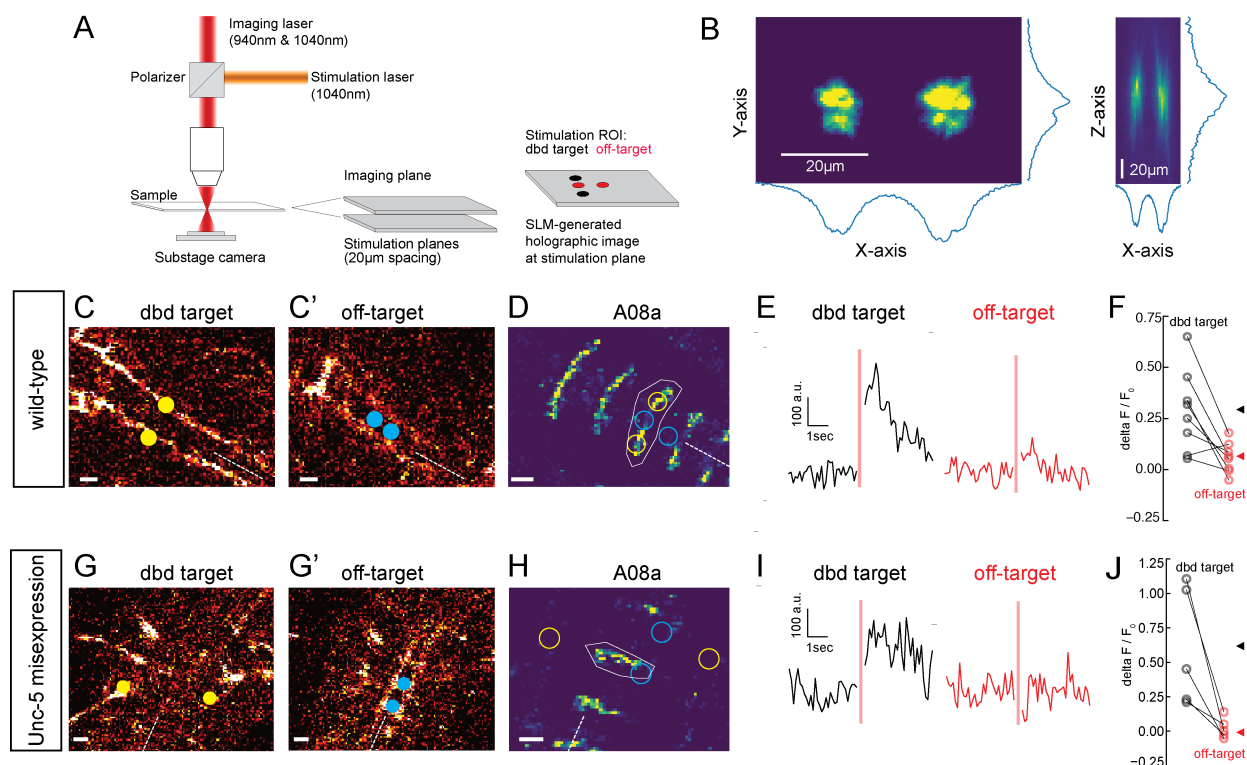
Figure 3.5 – figure supplement 1. dbd and A08a neuronal morphology is similar at 24 hr and 72 hr after larval hatching (ALH). (A–A'') Posterior view of a 3D light microscopy reconstruction in Imaris showing dbd (magenta) and A08a (green) neurons at 24 ± 4 hr ALH. Midline, dashed line in all panels. Asterisk, ventral off-target neurons. (A') The dbd neurons are visualized by *165(dbd)-Gal4 > UAS-myr::smGdP::HA*. (A'') The A08a neurons are visualized by *26F05(A08a)-LexA > LexAop-myr::smGdP::V5*. (B–B'') Posterior view of a 3D light microscopy reconstruction in Imaris showing dbd (magenta) and A08a (green) neurons at 72 ± 4 hr ALH. Yellow dashed line indicates the imaging focal plane used to record GCaMP6m fluorescence changes in A08a neurons. Asterisk, position of ventral off-target neurons. (B') The dbd neurons are visualized by *165(dbd)-Gal4 > UAS-myr::smGdP::HA*. (B'') The A08a neurons are visualized by *26F05(A08a)-LexA > LexAop-myr::smGdP::V5*.

We selected stimulation regions that were specific for each set of neurons. The stimulation regions were targeted to distinct planes and with nonoverlapping cross sections (Figure 3.6C). When we sequentially activated the dbd and off-target neurons within the same larva, we found that A08a had significantly larger GCaMP6m responses following Chromson activation of dbd compared to the off-target neurons (Figure 3.6D-F). Similar results were observed for larvae where *Unc-5* misexpression was used to lateralize the dbd axon (Figure 3.6G-J). We conclude that Chromson activation of dbd neurons drives increased GCaMP6m fluorescence in A08a neurons in both wild-type and *Unc-5* misexpression genotypes.

To determine whether the lateralized dbd provides monosynaptic input to A08a, we performed the same optogenetic experiments in the presence of tetrodotoxin (TTX), a sodium channel blocker that eliminates neuronal action potentials (Narahashi et al., 1964). First, we applied TTX to isolated larval CNS preparations and observed loss of the spontaneous rhythmic neuronal activity characteristic of fictive locomotion (Pulver et al., 2015), confirming that TTX was effective (Figure 3.7A). Next, we assayed the effect of TTX on dbd-A08a connectivity. If dbd-A08a connectivity is monosynaptic, then Chrimson activation of dbd should induce A08a GCaMP6m activity even in the presence of TTX; in contrast, if dbd-A08a connectivity is indirect (e.g. via feedforward excitation) then A08a GCaMP6m activity should be blocked by TTX (summarized in Figure 3.7B) (Petreanu et al., 2009).

We found that TTX does not block dbd-induced A08a activity, in wild-type (Figure 3.7C–C'') or when the dbd axon terminal is lateralized by Unc-5 (Figure 3.7D–D''), showing that the dbd synapses on the lateral dendritic arbor of A08a are functional and monosynaptic.

Figure 3.6 (next page). Two photon activation of dbd, but not off-target neurons, increases A08a GCaMP6m fluorescence. (A) Schematic of two photon microscope used for Ca^{+2} imaging and holographic photostimulation. We used a separate imaging (940 or 1040 nm) and stimulation laser (1040 nm). Holographic photostimulation patterns were constructed with a spatial light modulator (SLM). Stimulation targeted either dbd neurons (yellow circles) or off-target neurons (blue circles), separated on average by 20 μm in the z-axis. (B) XY and XZ profile of fluorescence induced by a holographic stimulation pattern consisting of two 10 μm diameter circles separated center-to-center by 26 μm . Profiles were obtained by moving objective (and therefore stimulation pattern) systematically relative to a fixed slide with a ~ 1 μm fluorescent coating while imaging with a sub-stage camera. Blue lines indicate fluorescence summed across respective axes (arbitrary units). (C–F) Targeting of Chrimson stimulation and Ca^{+2} imaging of A08a neurons in wild-type 72 hr ALH larvae. (C–C') Two photon image (1040 nm) of fluorescent mCherry marker at two imaging planes 20 μm apart. Stimulation ROIs used for targeting dbd (C, yellow dots) and off-target (C', cyan dots) neurons are overlaid. Dashed white line indicates midline. Scale bars, 10 μm . (D) Summed GCaMP6m fluorescence in A08a neurons (940 nm). White polygon depicts spatial region used to quantify fluorescence for traces in E. The stimulation regions shown in C are overlaid (outlines: yellow, dbd; cyan, ventral off-targets). Scale bars, 10 μm . (E) Example Ca^{+2} responses from the wild-type larva shown in C,D. Black trace shows raw A08a fluorescence (arbitrary units) prior to and following 150 ms holographic stimulation of dbd targets. Red trace shows A08a fluorescence in response to ventral off-target stimulation. Stimulation timing depicted with pink rectangle. (F) Mean Ca^{+2} responses ($\Delta F/F_0$) in A08a for each animal to dbd stimulation (black dots) or ventral off-target stimulation (red dots). Triangles are means for each group (dbd, 0.29 ± 0.07 ; off-target, 0.06 ± 0.07). $N = 8$ animals. Scale bars, 10 μm . (G–J) Targeting of Chrimson stimulation and Ca^{+2} imaging of A08a neurons in Unc-5 misexpression larvae at 72 hr ALH. (G–G') Two photon image (1040 nm) of fluorescent mCherry marker at dbd (G) and off-target imaging planes (G'), separated by 20 μm . Stimulation ROIs overlaid (dbd, G, yellow dots; off target, G', cyan dots). (H) Summed GCaMP6m fluorescence in A08a neurons. Stimulation regions and measurement region plotted as in D. (I) Example Ca^{+2} responses from Unc-5 larva shown in G, H. Plotting conventions as in E. Black trace shows raw A08a fluorescence in response to dbd stimulation; red trace is A08a fluorescence in response to off-target stimulation. (J) Mean Ca^{+2} responses ($\Delta F/F_0$) in A08a for each animal to dbd stimulation (black dots) or ventral off-target stimulation (red dots). Triangles are means for each group (dbd, 0.60 ± 0.17 ; off-target, 0.02 ± 0.03). $N = 5$ animals. Scale bars, 10 μm .



Interestingly, A08a GCaMP responses are significantly greater following TTX application in both wild-type and *unc-5* conditions; this may be due to the elimination of feedforward inhibition (see Discussion). We conclude that the lateralized dbd-A08a synapses are monosynaptic and functional. Our data therefore support a model in which axon guidance cues are the major determinants of dbd-A08a subcellular dendritic synaptic specificity.

Discussion

Achieving subcellular synaptic specificity

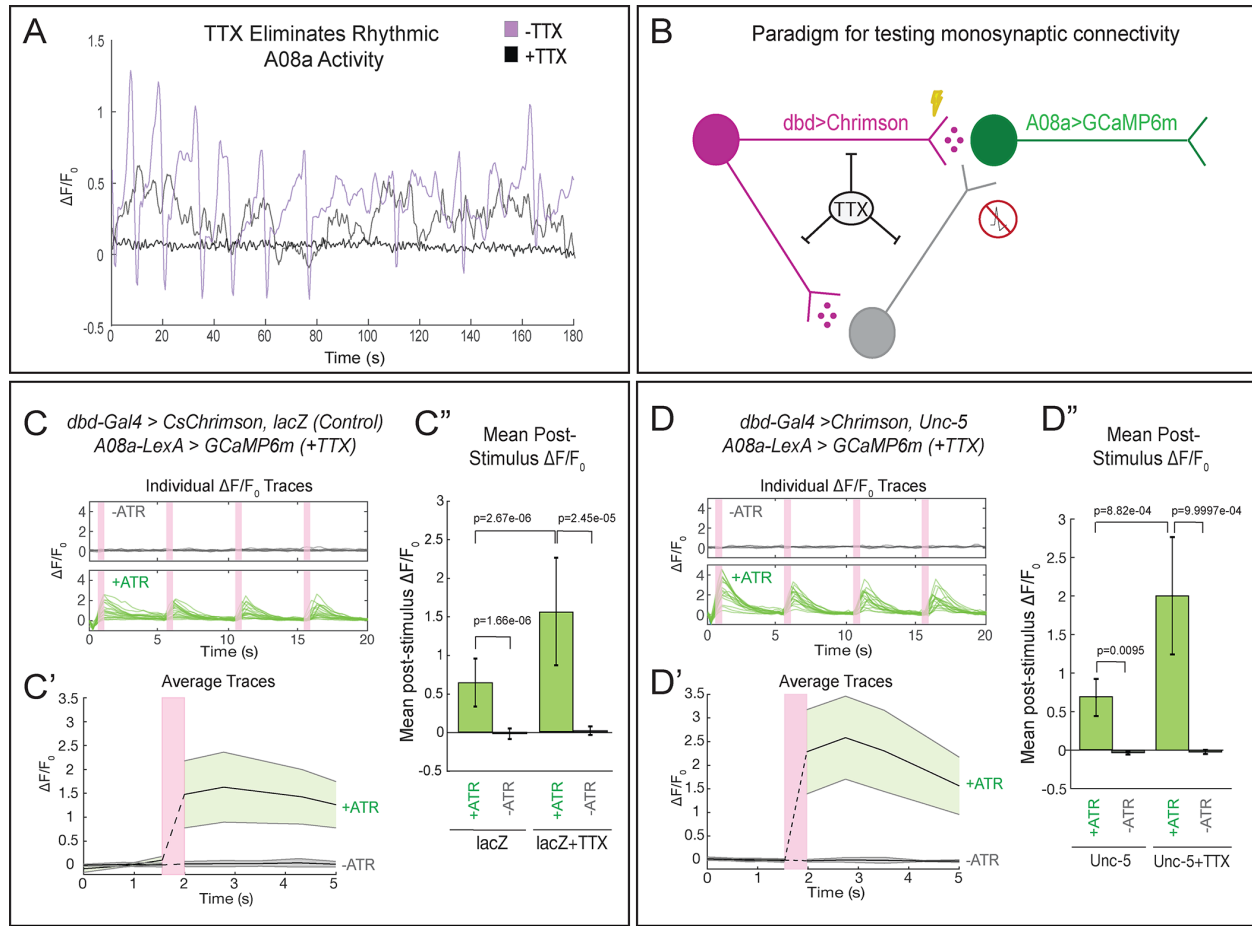
The ability of a presynaptic neuron to form synapses with a specific subcellular domain of its post-synaptic partner is well established in mammals (reviewed in Yogev and Shen, 2014) and has been described previously in *Drosophila*, although not at a mechanistic level. For example, the *Drosophila* giant fiber (GF) descending neuron targets a specific dendritic domain of the tergotrochanteral motor neuron, TTMn (Borgen et al., 2017). The transmembrane Sema-1a protein is required for both GF pathfinding to the motor neuropil, but also for establishing synaptic contact with the TTMn (Godenschwege et al., 2002; Godenschwege & Murphey, 2009).

However, it remains unknown if Sema-1a protein is restricted to the specific dendritic domain of TTMn chosen by the GF, as predicted by the ‘labeled arbor’ model. Similarly, the Jaam1 and Jaam3 interneurons target different domains of their post-synaptic EL neuron partners (Heckscher et al., 2015), but the mechanism is unknown.

Here, we provide evidence that axon guidance cues are the major determinants of subcellular dendritic synaptic specificity between dbd and A08a neurons, and that all regions of the A08a dendrite are competent to receive dbd synaptic inputs. Our findings expand upon the known mechanisms that generate subcellular synapse specificity to include guidance cues that restrict synaptic inputs to one region of a larger dendritic domain that is competent to receive synaptic input. We observed that the dbd axon is positioned close to the A08a output domain but

Figure 3.7 (next page). Lateralized dbd forms direct, monosynaptic connections with the A08a lateral dendrite.

(A) TTX eliminates spontaneous rhythmic neuronal activity in A08a (in which activity is part of an inter-segmental activity wave moving in the anterior or posterior direction representing fictive motor waves; Itakura et al., 2015). Representative traces show the $\Delta F/F_0$ for individual pairs of A08a neurons over the course of 3 minutes in *lacZ* control animals. Purple trace shows A08a $\Delta F/F_0$ without TTX present. Black trace shows A08a $\Delta F/F_0$ in the presence of 3 μ M TTX, in which 20/20 A08a pairs from 8 animals where rhythmic activity was eliminated. In 8/20 of these A08a pairs, non-rhythmic, non-intersegmentally coordinated changes in GCaMP6m fluorescence were observed, exemplified by the gray trace (see Discussion). **(B)** Experiment to test for monosynaptic dbd-A08a connectivity. TTX eliminates action-potential-mediated activity, preventing stimulation of non-Chrimson expressing neurons. Light-activation of Chrimson induces action-potential-independent neurotransmitter release from dbd. If dbd is monosynaptically connected to A08a, increases in A08a GCaMP fluorescence will result. **(C-C’)** Wild-type dbd has excitatory, monosynaptic connection to A08a medial dendritic arbor. **(C)** A08a GCaMP6m $\Delta F/F_0$ traces from individual A08a pairs resulting from wildtype dbd activation in the presence of TTX. **(C’)** Average A08a GCaMP6m $\Delta F/F_0$ traces in the presence of 3 μ M TTX, before and after Chrimson activation of dbd neurons. Solid black lines represent the mean $\Delta F/F_0$. Shaded regions represent the standard deviation from the mean. +ATR, n=20 A08a pairs, from 9 animals; -ATR, n=9 A08a pairs, from 4 animals. **(C’)** Quantification of the mean post-stimulus $\Delta F/F_0$ for *lacZ* control and *lacZ* +TTX animals. Mean post-stimulus $\Delta F/F_0$: *lacZ* Control +ATR, 0.62 ± 0.28 , n=28 A08a pairs, from 10 animals (Data reproduced from Figure 6D); *lacZ* control -ATR, -0.0172 ± 0.07 , n=11 A08a pairs, from 5 animals (Data reproduced from Figure 6D); *lacZ* control +TTX +ATR, 1.48 ± 0.70 , n=20 A08a pairs, from 9 animals; *lacZ* control +TTX -ATR, 0.019 ± 0.055 , n=9 A08a pairs, from 4 animals. **(D-D’)** Lateralized dbd has excitatory, monosynaptic connection to A08a lateral dendritic arbor. **(D)** GCaMP6m $\Delta F/F_0$ traces from A08a pairs after activation of lateralized dbd in the presence of TTX. **(D’)** Average A08a GCaMP6m $\Delta F/F_0$ traces in the presence of 3 μ M TTX, before and after Chrimson activation (pink bar) of dbd neurons. Solid black lines represent the mean $\Delta F/F_0$. Shaded regions represent the standard deviation from the mean. +ATR, n=17 A08a pairs, from 14 animals; -ATR, n=5 A08a pairs, from 4 animals. **(D’)** Quantification of the mean post-stimulus $\Delta F/F_0$ for Unc-5 and Unc-5 +TTX animals. Mean post-stimulus $\Delta F/F_0$: Unc-5 +ATR, 0.68 ± 0.24 , n=6 A08a pairs, from 5 animals (Data reproduced from Figure 6D); Unc-5 -ATR, -0.035 ± 0.02 , n=4 A08a pairs, from 3 animals (Data reproduced from Figure 6D); Unc-5 +TTX +ATR, 2.00 ± 0.76 , n=17 A08a pairs, from 14 animals; Unc-5 +TTX -ATR, 0.023 ± 0.03 , n=5 A08a pairs, from 4 animals. Significance between two groups was determined using a Mann-Whitney test.



never forms presynaptic contacts with this domain, as assayed by light and electron microscopy (data not shown). We speculate that the A08a output domain contains cell surface molecules (CSMs) that locally prevent dbd synapse formation. This is similar to work in *C. elegans* that identified secreted proteins that cluster CSMs to restrict synapse position on the DA9 motor neuron (Klassen and Shen, 2007). Similarly, NF186 expression is confined to the axon initial segment of Purkinje cells and determines the location of basket cell synapses (Ango et al., 2004). These observations suggest that synaptically coupled neurons may utilize both axon guidance cues and arbor-specific molecular cues to achieve subcellular synaptic specificity. We anticipate both ‘labeled arbors’ and ‘guidance cues’ play a role in determining subcellular synaptic specificity – possibly both acting in the same neuron, such as CSMs potentially regulating connectivity between coarse subcellular domains, such as the A08a axon versus dendrite, and

guidance cues refining connectivity within a particular subcellular domain, such as the medial and lateral A08a dendritic domains.

Formation of functional lateralized dbd-A08a synapses

We have shown that the lateralized dbd axon not only makes close Brp contacts with the A08a lateral dendrite, but more importantly also makes functional synapses. Interestingly, there appear to be fewer synapse voxels between the lateralized dbd and A08a than between the medial dbd and A08a, yet functional connectivity is indistinguishable. This may be due to homeostatic mechanisms that increase the efficacy of the lateral dbd-A08a synapses. The fact that the dbd-A08a optogenetic activation occurs even in the presence of TTX, together with the observation of direct dbd-A08a synapses in EM, strongly suggests that dbd and A08a have direct, monosynaptic excitatory connectivity. Interestingly, dbd induced activation of GCaMP6m in A08a is greater in the presence of TTX (in both wild-type and after dbd lateralization), suggesting that dbd may activate an inhibitory feed-forward circuit that is silenced by TTX. A good candidate for such feed-forward inhibition is the A02d neuron, which is an inhibitory neuron that receives input from dbd and has output to A08a (Fushiki et al., 2016; Kohsaka et al., 2014) (Figure 3.2G). In some cases, we detected fluctuations in A08a GCaMP6m activity following TTX application (8/20 A08a pairs; Figure 3.7A); it is unclear if these represent cases of incomplete A08a inactivation, graded Ca^{2+} potentials, or Ca^{2+} release from internal organelles. It is also important to consider that not all insect neurons produce sodium-dependent spikes; therefore, we cannot fully rule out the possibility that the A08a activation we observe in the presence of TTX is due to indirect stimulation from non-spiking interneurons (Pearson & Fournier, 1975; Pippow et al., 2009).

We also note that animals fed ATR (+ATR) have a statistically significant higher baseline level of calcium activity than -ATR controls. This is likely due to our illumination with 488 nm light between 561 nm stimulus pulses (see optogenetic Methods), because 488 nm light was shown previously to weakly activate Chrimson (Klapoetke et al., 2014). It therefore follows that +ATR animals would have a higher baseline level of fluorescence. Importantly, this does not change our interpretation that lateralized dbd neurons form functional synapses with the A08a lateral dendrite.

We have shown that the lateralized dbd maintains synaptic contact with A08a by remapping synaptic connectivity to the lateral arbor of A08a. However, we are unable to determine if dbd still maintains cellular synaptic specificity with its other synaptic partners. In contrast to A08a, other dbd target neurons only have a medial dendritic arbor, such as Jaam-3 (Heckscher et al., 2015). It would be interesting to know how these neurons respond to dbd lateralization; they may extend novel dendrite branches laterally, or may simply lose dbd synaptic inputs. The development of genetic tools to specifically label additional dbd target neurons will be required to understand if cellular synaptic specificity of dbd is maintained upon its remapping in the neuropil.

Functional consequences of subcellular synaptic specificity

In other systems, it is well established that subcellular location of synapses has a profound impact on how a neuron propagates information within a circuit (Bloss et al., 2016; Hao et al., 2009; Miles et al., 1996; Pouille et al., 2013; Tobin et al., 2017). From the *Drosophila* larval EM reconstruction, we show that A08a receives distinct input into its medial and lateral dendritic arbors, which is likely to influence how A08a integrates incoming synaptic activity. dbd is a proprioceptive sensory neuron, and A08a is rhythmically active during fictive motor waves (Itakura et al., 2015). Thus, the proper targeting of dbd and A02d to the medial arbor, and A02l and A31x to the lateral arbor, may be important for processing proprioceptive sensory input during locomotion. Although the *dbd-Gal4* line used in our study has ventral sensory ‘off-target’ expression that precludes a behavioral analysis following dbd lateralization, if this off-target expression could be removed, it is possible that the behavioral consequences of dbd lateralization could be determined using recently developed high-resolution quantitative behavior analysis tools (Almeida de Carvalho et al., 2017; Kabra et al., 2013; Klein et al., n.d.; Risse et al., 2017). Furthermore, future electrophysiological studies could directly test the functional consequences of the subcellular positioning of A08a inputs on neural processing (e.g. dendritic integration, coincidence detection, and noise suppression).

Materials and Methods

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Species (<i>Drosophila melanogaster</i>)	<i>26F05-LexA</i>	BDSC	54702	Expressed in A08a neurons
Species (<i>D. melanogaster</i>)	<i>26F05-Gal4</i>	BDSC	49192	Expressed in A08a neurons
Species (<i>D. melanogaster</i>)	<i>165-Gal4</i>	W. Grueber	N/A	Expressed in dbd neurons
Species (<i>D. melanogaster</i>)	<i>UAS-LacZ</i>	BDSC	8529	Control transgene
Species (<i>D. melanogaster</i>)	<i>UAS-LacZ</i>	BDSC	8530	Control transgene
Species (<i>D. melanogaster</i>)	<i>UAS-unc-5::HA</i>	B. Dickson	N/A	UAS drives unc-5
Species (<i>D. melanogaster</i>)	<i>UAS-robo-2::HA</i>	BDSC	66886	UAS drives robo-2
Species (<i>D. melanogaster</i>)	<i>UAS-bruchpilot(short)-mstrawberry</i>	S. Sigrist	N/A	UAS drives fluorescently labeled truncated bruchpilot
Species (<i>D. melanogaster</i>)	<i>10xUAS-IVS-myr::smGdP::HA, 13xLexAop2-IVS-myr::smGdP::V5</i>	BDSC	64092	UAS drives membrane tags
Species (<i>D. melanogaster</i>)	<i>UAS-MCFO</i>	BDSC	64090	UAS drives multi-colored-flip-out
Species (<i>D. melanogaster</i>)	<i>UAS-DenMark, UAS-syt.eGFP</i>	BDSC	33064	UAS drives DenMark, UAS drives synaptotagmin::GFP
Species (<i>D. melanogaster</i>)	<i>13XLexAop2-IVS-p10-GCaMP6m, 20xUAS-CsChrimson-mCherry</i>	V. Jayaraman	N/A	LexAop drives GCaMP6m, UAS drives Chrimson
Antibody, monoclonal	Mouse anti-V5 tag	Invitrogen, Carlsbad, CA,	Cat. R96025, Lot 1949337	(1:1000)
Antibody, polyclonal	Rabbit anti-mCherry	Novus Biologicals, Littleton, CO	Cat. NBP2-25157, Lot 102816	(1:500)
Antibody, monoclonal	Rat anti-HA tag	Roche Holding, AG, Basel, Switzerland	Cat. 11867423001, Lot 27573500	(1:100, after suggested dilution)
Antibody, monoclonal	Rat anti-OLLAS ^{DyLight-650} conjugated antibody	Novus Biologicals, Littleton, CO	Cat. NBP1-06713C, Lot F-090517c	(1:100)

Antibody, polyclonal	Chicken anti GFP	Aves Labs, Inc, Tigard, OR	Cat. GFP-1020, Lot. GFP697986	(1:1000)
Antibody, polyclonal	Rabbit anti-mCherry	Novus Biologicals, Littleton, CO	Cat. NBP2-25157, Lot 102816	(1:500)
Antibody, secondary	Alexa Fluor® 488 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch, West Grove, PA	Cat. 715-545-151	(1:400)
Antibody, secondary	Rhodamine RedTM-X (RRX) AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch, West Grove, PA	Cat. 711-295-152	(1:400)
Antibody, secondary	Alexa Fluor® 647 AffiniPure Donkey Anti-Rat IgG (H+L)	Jackson ImmunoResearch, West Grove, PA	Cat. 712-605-153	(1:400)
Antibody, secondary	Alexa Fluor® 488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L)	Jackson ImmunoResearch, West Grove, PA	Cat. 703-545-155	(1:400)

Fly stocks

All flies were raised at 25°C on standard cornmeal fly food.

Genotypes	Figure
Females containing <i>10xUAS-IVS-myr::smGdP::HA</i> , <i>13xLexAop2-IVS-myr::smGdP::V5</i> (BDSC# 64092); <i>GMR26F05-LexA</i> (A08a neurons) (BDSC# 54702), <i>UAS-bruchpilot(short)-mstraw</i> ; <i>165-Gal4</i> (dbd neurons) were crossed to males containing <i>UAS-lacZ.Exel</i> (control) (BDSC# 8529)	2A-A'; 3A-A'', 3C-D; 4A-A'', 4C, 4Sup. 1B, E;
Females containing <i>10xUAS-IVS-myr::smGdP::HA</i> , <i>13xLexAop2-IVS-myr::smGdP::V5</i> (BDSC# 64092); <i>GMR26F05-LexA</i> (A08a neurons) (BDSC# 54702), <i>UAS-bruchpilot(short)-mstraw</i> ; <i>165-Gal4</i> (dbd neurons) were crossed to males containing <i>UAS-robo-2::HA</i> (BDSC# 66886)	4Sup 1C, E;
Females containing <i>10xUAS-IVS-myr::smGdP::HA</i> , <i>13xLexAop2-IVS-myr::smGdP::V5</i> (BDSC# 64092); <i>GMR26F05-LexA</i> (A08a neurons) (BDSC# 54702), <i>UAS-bruchpilot(short)-mstraw</i> ; <i>165-Gal4</i> (dbd neurons) were crossed to males containing <i>UAS-unc-5::HA</i>	4B-B'', 4D, 4Sup. 1D-E;

Females containing <i>GMR57C10-FLPL</i> ; <i>10xUAS(FRT.stop)myr::smGdP-OLLAS</i> , <i>10xUAS(FRT.stop)myr::smGdP::HA</i> , <i>10xUAS(FRT.stop)myr::smGdP::V5-THS-10xUAS(FRT.stop)myr::smGdP-FLAG</i> (MCFO) (BDSC# 64090) were crossed to males containing <i>GMR26F05-Gal4</i> (BDSC# 49192)	2B
Females containing <i>GMR26F05-Gal4</i> (BDSC# 49192) were crossed to males containing <i>UAS-DenMark</i> , <i>UAS-syt.eGFP</i> ; <i>In(3L)D</i> , <i>mirr/TM6C</i> , <i>Sb</i> (BDSC# 33064)	2C-C''
Females containing <i>GMR26F05-LexA</i> (BDSC# 54702); <i>165-Gal4</i> were crossed to males containing <i>UAS-lacZ.Exel</i> ; <i>13XLexAop2-IVS-p10-GCaMP6m</i> , <i>20xUAS-CsChrimson-mCherry</i> (control)	6A-A', 6D, 6F, 7A, 7C-C''
Females containing <i>GMR26F05-LexA</i> (BDSC# 54702); <i>165-Gal4</i> , <i>UAS-unc-5::HA</i> were crossed to males containing <i>13XLexAop2-IVS-p10-GCaMP6m</i> , <i>20xUAS-CsChrimson-mCherry</i>	6B-B', 6D, 7D-D''
Females containing <i>UAS-lacZ.Exel</i> ; <i>13XLexAop2-IVS-p10-GCaMP6m</i> , <i>20xUAS-CsChrimson-mCherry</i> were crossed to males containing <i>GMR26F05-LexA</i> (BDSC# 54702) (No Gal4 control)	6E-F

Immunohistochemistry and sample preparation

Larval Preparation

Collection of timed larvae: embryos and larvae were raised at 25°C. Embryos were collected on 3.0% agar apple juice caps with yeast paste for 4 hours and then aged for 21 hours. Embryos were transferred to a fresh 3.0% agar apple juice cap and then aged for 4 hours. Hatched larvae were transferred to standard cornmeal fly food vials and aged until dissection.

Immunohistochemistry

Larval brains were dissected in PBS, mounted on 12mm #1.5 thickness poly-L-lysine coated coverslips (Neuvitro Corporation, Vancouver, WA, Cat# H-12-1.5-PLL) and fixed for 23 minutes in fresh 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA, Cat. 15710) in PBST. Brains were washed in PBST and then blocked with 2.5% normal donkey serum and 2.5% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBST overnight. Brains were incubated in primary antibody for two days at 4°C. The primary was removed and the brains were washed with PBST, then incubated in secondary antibodies overnight at 4°C. The secondary antibody was removed following overnight incubation and the brains were washed in PBST. Brains were dehydrated with an ethanol series

(30%, 50%, 75%, 100%, 100%, 100% ethanol; all v/v, 10 minutes each) (Decon Labs, Inc., King of Prussia, PA, Cat. 2716GEA) then incubated in xylene (Fisher Chemical, Eugene, OR, Cat. X5-1) for 2x 10 minutes. Samples were mounted onto slides containing DPX mountant (Millipore Sigma, Burlington, MA, Cat. 06552) and cured for 3 days then stored at 4°C until imaged.

Light Microscopy

Fixed larval preparations were imaged with a Zeiss LSM 800 laser scanning confocal (Carl Zeiss AG, Oberkochen, Germany) equipped with an Axio Imager.Z2 microscope. A 63x/1.40 NA Oil Plan-Apochromat DIC m27 objective lens and GaAsP photomultiplier tubes were used. Software program used was Zen 2.3 (blue edition) (Carl Zeiss AG, Oberkochen, Germany). For each experiment, all samples were acquired using the same acquisition parameters (see below).

Voxel size	Excitation wavelength (laser power)	Detection wavelength	Pinhole size (AU)	Figure
0.090 x 0.090 x 0.280 μm^3	488 nm (0.13%) 561 nm (0.07%) 640 nm (0.14%)	410-541 nm 541-627 nm 656-700 nm	35 μm for all channels (488 nm: 0.82AU, 561nm: 0.71AU, 647nm: 0.63AU)	3.2A-A', 3.3A-A'', 3.3C-C'', 3.4A-B'', 3.4-Sup. 1B-D
0.067 x 0.067 x 0.280 μm^3	640 nm (0.65%)	656-700 nm	40 μm (0.72AU)	3.2B
0.067 x 0.067 x 0.280 μm^3	488 nm (0.13%) 561 nm (0.25%)	410-540 nm 540-772 nm	43 μm (0.99AU) 38 μm (0.77AU)	3.2C-C''

Image processing and analyses

Quantification of dbd-A08a synapse voxel distribution

The “synapse voxel” image analyses pipeline identifies Brp voxels that are either one voxel away or already overlapping with membrane containing voxels. Since each voxel size is 90nm, then the “synapse voxels” represent the voxels that have Brp less than 90nm away from membrane voxels.

Image processing and analysis was performed using FIJI (ImageJ 1.50d, <https://imagej.net/Fiji>). Stepwise, images were rotated (Image>Transform>Rotate(bicubic)) to align A08a dendrites along the X-axis, then a region of interest was selected in 3D to include

A08a dendrites in one hemi-segment (Rectangular selection>Image>Crop). The Brp and A08a dendrite channels were isolated (Image>Color>Split channels). To quantify the voxels containing A08a dendrite signal within 90nm of voxels containing Brp signal, a mask was manually applied to each channel (Image>Adjust>Threshold). The threshold was assigned to include Brp positive voxels and minimize contribution from background. Because of the inherent variability in pixel intensity between different samples (most likely due to the variability of the Gal4 and LexA systems), we could not assign the same threshold to different samples. We found that manually assigning thresholds was a more accurate method of identifying Brp or membrane containing voxels compared to automatic thresholding methods available in FIJI. Importantly, the Brp and membrane thresholds were assigned separately and prior to quantifying the number of overlapping voxels. The Brp mask channel was dilated one iteration (Process>Binary>Dilate). We assigned the 90nm distance threshold to account for the size of the synaptic cleft (~20nm, measured in EM) and the chromatic aberration between 488nm and 555nm wavelengths used to visualize A08a membrane and dbd presynapses (~70nm, measured in our light microscope). Then image arithmetic was used to identify the voxels that contain intensity in both the masked A08a dendrite and dilated Brp channels (Process>Image Calculator>Operation “AND”). Images were reduced in the z-dimension (Image>Stacks>Z-project>Sum Slices) and a plot profile was obtained to measure the average voxel intensity across the medial-lateral axis of A08a dendrites (Rectangular selection>Analyze>Plot profile). Distance from the midline was calculated by setting a starting point at the midline and then calculating distance along the medio-lateral axis perpendicular to the midline.

Figure preparation

Images in figures were prepared as either 3D projections in Imaris 9.2.0 (Bitplane AG, Zurich, Switzerland) or maximum intensity projections in FIJI (ImageJ 1.50d, <https://imagej.net/Fiji>). Scale bars are given for reference on maximum intensity projections and single z-slice micrographs, but do not necessarily represent actual distances, as the tissue samples undergo changes in size during the tissue clearing protocol. Pixel brightness was adjusted in some images for better visualization; all such adjustments were made uniformly over the entire image.

Scale bars were included in all single focal planes and standard maximum intensity projections. In some cases, figures were “3D projected” images exported from the Imaris software, where the scale bars are assigned to match the scale at the “center” of the 3D projection. In these cases we did not add a scale bar because it would not be accurate for all parts of the image.

Data collection

A power analysis was not performed to determine the appropriate sample size. Many samples were dissected to account for low penetrance of dbd lateralization and to account for damaged samples that were not suitable for image analyses. All sample numbers represent biological replicates. However, we did perform the same experiment on multiple days. We did not exclude any outliers from the data sets. The criteria for excluding samples were as follows. For the fixed tissue preparation, samples with poor dissection quality or poor mounting on slides were excluded as they were unsuitable for the image analyses pipeline. Samples were also excluded if random “off-target” neuron expression interfered with image analysis. For optogenetic experiments, samples were excluded if sample movement in the z-axis precluded accurate quantification of changes in fluorescence. For lateralized dbd optogenetics, brain segments were excluded from analysis if A08a received input from dbd on the medial dendrite. Samples were allocated into groups by genotype; every genotype was treated as an independent group.

Functional connectivity assay and analyses

Newly hatched larvae were aged for 48 ± 4 hours ALH on standard cornmeal fly food at 25°C. At this time, larvae were transferred to apple caps containing wet yeast supplemented with 0.5mM all-*trans* retinal (Sigma-Aldrich, R2500-100MG) and aged at 25°C in the dark. Following another 24 hours (72 ± 4 hours ALH) animals were dissected in HL3.1 saline solution. All dissections were performed in low lighting to prevent premature Chrimson activation. Freshly dissected brains were mounted in HL3.1 saline on 12mm round Poly-L-Lysine coated coverslips. For all Chrimson activation experiments, GCaMP6m signal in postsynaptic A08a axon terminals was imaged using 0.01% power of the 488nm laser with a 40x objective on a Zeiss LSM800 confocal microscope (NA: 1.4; pinhole size: 32 μm (1AU); detection wavelength:

450-550nm, voxel size: $0.782 \times 0.782 \times 1 \mu\text{m}^3$). Chrimson in presynaptic neurons was activated with three pulses of 561 nm laser at 100% power delivered via the same 40x objective using the bleaching function in the ZEN Zeiss software. The total length of the 561nm pulses was about 450msec. After individual recording sessions of *unc-5* expressing samples, Z-stacks of the brain were taken to verify the segments in which A08a exclusively received dbd input onto the lateral dendrite and were therefore permissible for analysis; the few larvae where Chrimson+ off-target neurons were close to A08a neurons were excluded, although due to low signal we can't exclude the possibility of rare or fine contacts. A08a neurons from abdominal segments 3-5 were used for our analyses, as no statistically significant difference in post-stimulus $\Delta F/F_0$ was detected among these neurons.

To quantify $\Delta F/F_0$ traces we used a custom MATLAB script (The MathWorks, Natick, MA). The script first performs rigid registration to correct for movement artifacts during recording, and then allows for ROI selection. ROIs were drawn around A08a axon terminals in individual segments, and ROI size was constant across all experiments (Figure 3.5C). F_0 was set as the average fluorescence of the 3 frames acquired before each 561nm light stimulus. For a single animal, we first average $\Delta F/F_0$ traces for six consecutive 561nm stimuli separated by 20 488nm acquisition frames (4 frames/sec). These 20 frames are enough time to allow GCaMP6m fluorescence to return to baseline. Traces were then averaged across animals to determine the mean $\Delta F/F_0$ for each experimental group. Mean post-stimulus $\Delta F/F_0$ was calculated by first subtracting the mean F_0 from the mean F in the first frame post-stimulus, then dividing the resulting ΔF by the mean F_0 . The resulting number was then divided by the total number of A08a pairs examined in for the experimental group in question.

For demonstrating monosynaptic connectivity between dbd and A08a, brains were dissected and mounted in 3 μM TTX (Abcam, Cambridge, MA, ab120055) diluted in HL3.1. Brains were incubated for 5 minutes in the TTX solution prior to the recording session. To first determine the effectiveness of TTX, spontaneous A08a GCaMP6m activity was recorded over 5 minutes with and without TTX (in *lacZ* control animals). Spontaneous GCaMP6m activity was recorded on an LSM800 with a 40X objective (NA: 1.4; excitation wavelength: 488nm; detection wavelength: 492-555nm; pinhole size: 32 μm (1AU)). Once it was established that TTX eliminates spontaneous rhythmic A08a activity, we dissected fresh brains in TTX and performed

the same Chrimson activation paradigm (using the same bleaching protocol and image acquisition settings) as described above to test monosynaptic connectivity.

Two photon experiments (Figure 3.6)

Images were generated using a galvanometric and resonant scan mirror-based two-photon microscope (VIVO Multiphoton Movable Objective RS +Microscope and Vector resonant galvo scanner, 3i , Denver, CO). A Zeiss W Plan-Apochromat 20x/1.0 NA water dipping objective (apochromatically corrected 480 nm-1300nm) with a working distance of 2.3 mm was used for delivery of excitation and stimulation laser excitation. The imaging system utilizes the Chameleon Discovery dual wavelength laser system (Coherent, Santa Clara, CA) as the pump laser. The pump laser supplies 100 fs pulses at an 80 MHz repetition with an output power of 1.3 W at 940 nm and 3.9 W at 1040 nm. Imaging frames were obtained at a 39.6 Hz, and five frames were averaged per saved image. The scan range was 578 μm x 571 μm , corresponding to a pixel size of 1.47 μm x 1.42 μm . GCaMP6m and mCherry were excited using 940 nm (27 mW) and 1040 nm (200–244 mW) radiation, respectively, while the fluorescence was collected with two fast-gated GaAsP PMTs having filter sets that selectively collect fluorescence between 490 and 560 nm for the green channel and 570 and 640 nm in the red channel.

Sample stimulation was based around a 5 W, 192 fs, 10 MHz laser system for excitation of Chrimson at 1040 nm (FemtoTrain 1040–5, Spectra-Physics, Santa Clara, CA). Excitation was delivered through the objective with a phase-only spatial light modulator (SLM) (Phasor, computer—generated holography system, 3i , Denver, CO) for precise patterned and 3D photomanipulation. Between 21 mW and 66 mW were used in 150 ms stimulation pulses for Chrimson activation. Stimulation ROIs were two 10 μm diameter circles localized over regions of interest guided by two-photon imaging of the mCherry marker. Holographic stimulation allowed for Chrimson activation at arbitrary depths within the sample while continuously monitoring A08a fluorescence in the imaging plane.

For quantification of $\Delta F/F_0$ responses to two-photon activation (Figure 3.6), we computed F_0 as the mean fluorescence over the 20 frames (2.53 s) prior to the 150 ms stimulus. ΔF was computed as the difference between F_0 and the mean fluorescence over the 5 frames following the stimulus (0.63 s).

Statistical analysis

Statistical analyses for optogenetic experiments were performed with MATLAB and R. For analyzing the statistical significance of mean post-stimulus $\Delta F/F_0$, an H-test was used to determine whether the data for each experimental group were normally distributed. Because these data were non-normally distributed, a Mann-Whitney test was performed to determine whether there were statistically significant differences in mean $\Delta F/F_0$ among experimental groups. To analyze potential differences in F_0 among + and - ATR groups we used a Pairwise Wilcox Test to calculate comparisons between each experimental group. This was followed by a Benjamini and Hochberg correction for multiple testing. All code for analysis of optogenetic data in Figures 3.5–7 is deposited at the following GitHub repository https://github.com/timothywarren/elife_larvae_2019 (Warren, 2019; copy archived at https://github.com/elifesciences-publications/elife_larvae_2019).

Bridge

In Chapter III, my colleagues and I characterized a pair of synaptic partners in the *Drosophila* larva, dbd and A08a, that exhibit subcellular synaptic specificity. We established genetic methods that enabled the misrouting of the dbd presynaptic axon and subsequent synapse formation with alternate A08a subcellular domains, including one that is typically devoid of dendritic material. These results demonstrated the robustness of circuit development, as dbd and A08a could recognize and form functional synapses with one another despite dbd occupying an ectopic neuropil domain. In the next chapter, I will examine the cellular mechanisms that enable this observed robustness, and ask if there are cues shared between pre and postsynaptic neurons that can promote synaptic partner matching and ultimately circuit function.

CHAPTER IV

PRESYNAPTIC CONTACT AND ACTIVITY OPPOSINGLY REGULATE POSTSYNAPTIC DENDRITE OUTGROWTH

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Author Contributions

ELH and CQD conceived of the project and designed the experiments. ELH conducted all experiments, analyzed all data, and generated all figures. Both authors reviewed and edited the final manuscript. CQD provided supervision and funding for the project.

Introduction

Neural circuit organization dictates circuit function, influencing behavior, cognition, and perception. While developmental programs in genetically identical animals produce similar final products, variability arises during circuit wiring to produce differences in cellular morphology, synaptic partnerships, and numbers of synapses between partners (Mohr et al., 2004; Chou et al., 2010; Caron et al., 2013; Linneweber et al., 2020; Churgin et al., 2021; Courgeon and Desplan, 2019; Couton et al., 2015; Goodman, 1978; Tobin et al., 2017; Witvliet et al., 2021). Such variability can arise innately due to stochastic processes (e.g. filopodial extension/retraction, lateral signaling, gene expression) (Özel et al., 2015; Troemel et al., 1999; Wernet et al., 2006) or when environmental factors impinge on development (e.g. rearing temperature, sensory experience) (Hubel et al., 1977; Kiral et al., 2021; Shatz & Stryker, 1978). Neural development must be flexible to account for these variations in order to generate robust circuit function.

If the location and strength of synaptic inputs can vary, how do surrounding neurons adapt such that they receive the right type and right amount of input? Dendrites are the major sites of synaptic input onto a neuron. Studies have shown that dendrites alter their morphology in response to varying levels of synaptic input (Ackerman et al., 2021; Takeo et al., 2021; Tripodi et al., 2008), and more recently we showed that dendrites can alter their position when a presynaptic partner is routed to an alternate neuropil location (Valdes-Aleman et al., 2021).

Dendrites are clearly capable of structural plasticity, yet how they appropriately respond to a variable developmental landscape is unclear.

Here we investigate how and when developing dendrites accommodate wiring variation to ensure robust circuit connectivity. Leveraging strategies to manipulate presynaptic activity levels and presynaptic contact with postsynaptic dendrites, we find that there are two opposing mechanisms used to regulate robust partner matching: presynaptic contact promotes dendrite outgrowth locally, while presynaptic activity inhibits elongation of postsynaptic dendrites globally. These two strategies highlight the important role of presynaptic inputs in regulating the placement and subsequent elaboration of postsynaptic dendrites.

Results

dbd-Gal4 labels the dbd sensory neuron prior to formation of presynaptic contacts

To ensure robust circuit function, developing neurons must exhibit specificity and flexibility – specificity in synaptic partner choice, and flexibility to respond to variability in partner neuropil territory. The goal of our study was to determine the cellular mechanisms used by dendrites to compensate for variability in presynaptic axon placement and input strength to facilitate functional connectivity. To study these mechanisms in vivo, we used a model system consisting of the *Drosophila* larval dbd sensory neurons, and their postsynaptic partners, the A08a interneurons. The dbd sensory neurons form segmentally-repeated connections with A08a in the larval abdominal segments. A08a has two distinct dendritic domains: lateral and medial. All major inputs to A08a synapse with a single dendrite, either lateral or medial; the dbd sensory neuron synapses with the medial dendrite (Sales et al., 2019; Schneider-Mizell et al., 2016).

We sought to induce variability in the placement of the dbd axon to determine the extent to which its connectivity with A08a is stringently required at the medial dendrite. To do this we first needed genetic access to dbd prior to its interaction with A08a. We previously used *l65-Gal4* (subsequently referred to as *dbd-Gal4*) to label the dbd sensory neuron (Sales et al., 2019; Valdes-Aleman et al., 2021), but the onset of dbd-Gal4 expression was not determined. Here we use dbd-Gal4 to drive expression of a myr::HA tag, and the 22C10 antibody to label all sensory neurons as a landmark. We observed the first expression of dbd-Gal4 in dbd neurons at

embryonic stage 14. At this time, the axons have just entered the dorsal CNS as immature growth cones (Figure 4.1A-A'), and would not yet have contacted the more ventral neuropil domain occupied by the A08a dendrites. By stage 15, the dbd neurons formed anterior/posterior bilateral branches adjacent to the midline of the neuropil (Figure 4.1B-B'), followed by further elaboration to link adjacent segments in stage 17 (Figure 4.1C-C'). By this time, dbd axon terminals occupy a more ventral region of the neuropil where A08a medial dendrites are formed (Schrader & Merritt, 2000; Zlatić et al., 2003). These patterns of dbd projection into the CNS are schematized in Figure 4.1D.

The A08a interneuron is labeled using a previously characterized LexA (*26F05-LexA*) to express *LexAop-myr::V5* in A08a and its dendritic arbors. This line first labels A08a in the early hours of the first larval instar (Figure 4.1 – figure supplement 1A-A'). By this time, A08a has contacted dbd and has developed its characteristic lateral and medial dendritic arbors. The timing of A08a-LexA expression precludes us from knowing the state of A08a dendrite development at the time of first contact with dbd. However, we conclude that dbd-Gal4 labels the dbd sensory neuron prior to its growth into the A08a neuropil domain, and thus it is an appropriate tool for manipulating dbd prior to the establishment of dbd-A08a synaptic connectivity.

The dbd sensory neuron locally promotes dendrite elongation in the A08a interneuron

In a previous study, we tested the ability of dbd and A08a to compensate for developmentally-induced wiring variation (Sales et al., 2019). We tested for stringent specificity in dbd connectivity with the medial arbor by using genetic methods to target the dbd axon to the lateral neuropil. The dbd axon terminal could be misrouted to the intermediate and lateral neuropils through misexpression of repulsive axon guidance receptors, Robo-2 and Unc-5. dbd could form synapses at both intermediate and lateral dendritic arbors, and the strength of functional connectivity when dbd synapsed with the lateral arbor was indistinguishable from wild-type dbd-A08a connectivity (Sales et al., 2019).

Surprisingly, when dbd was targeted to lateral or intermediate neuropil domains, A08a produced an ectopic dendritic arbor to match the presynaptic contact (Valdes-Aleman et al., 2021). Here we extended and confirmed these findings by showing that the cumulative distribution of A08a dendrite volume corresponds to the location of dbd input (Figure 4.2A-D).

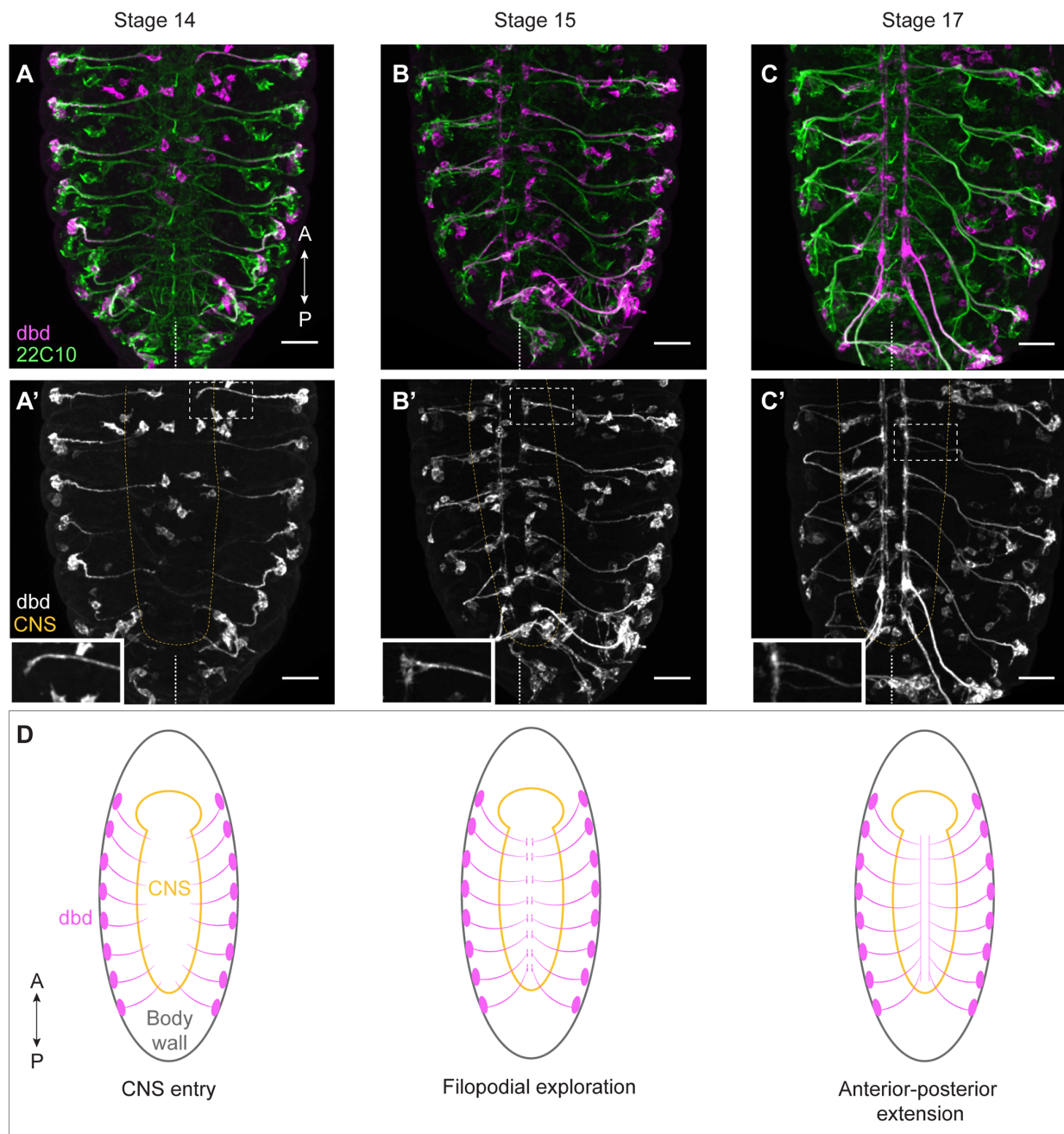


Figure 4.1. Onset of *dbd*-Gal4 expression during *dbd* axogenesis. (A-C) Stage 14, 15, and 17 fixed embryos. Sensory neurons labeled with 22C10 antibody (green) and *dbd*-Gal4 pattern labeled with smGdP-myr::HA (pink). Stage 14, n=4 animals; Stage 15, n=13 animals; Stage 17, n=17 animals. Pink cell bodies not in the body wall are likely part of the gut. A'-C' show just the smGdP-myr::HA channel. Insets show zoomed in view of *dbd* outlined by white dashed box. Yellow dashed line indicates outline of central nervous system (CNS). Midline indicated by white dashed line at the bottom of each image. Scale bars, 20 μm. (D) Illustrations summarizing results in A-C.

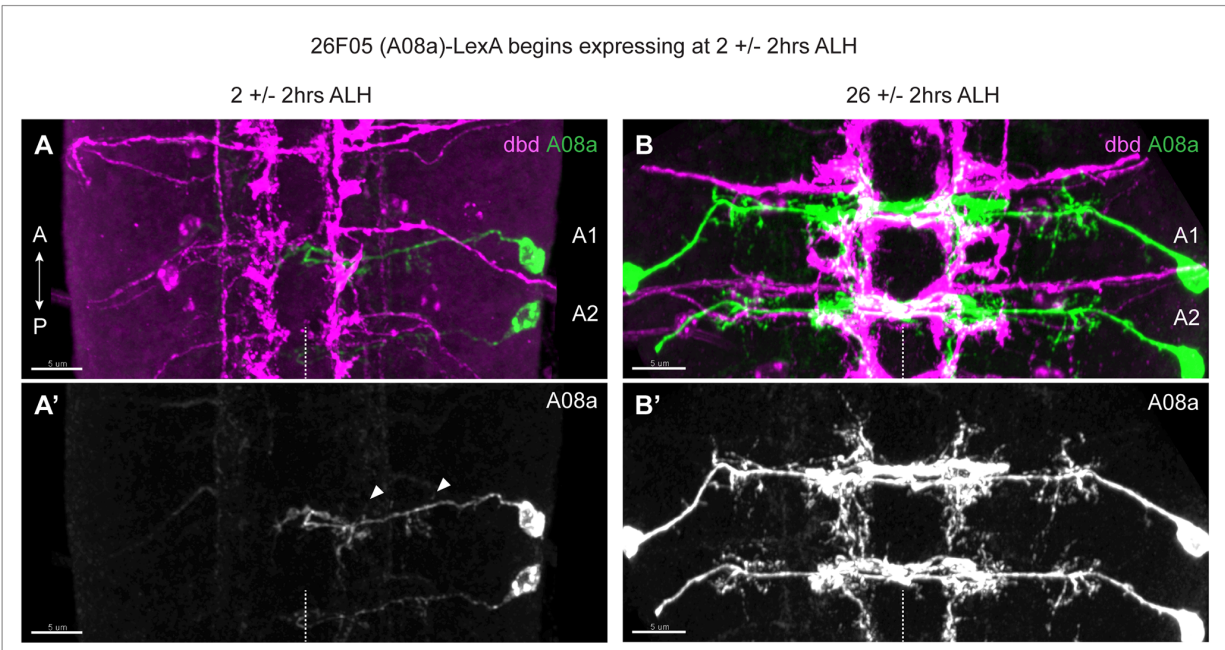


Figure 4.1 – figure supplement 1. Onset of A08a-LexA expression during early larval life. (A) Dorsal view of VNC at 2 +/- 2hrs ALH. Dbd-Gal4 expression pattern in pink, A08a-LexA expression pattern in green. (A') A08a channel alone. Arrow heads indicate the medial and lateral dendrites. In this image, LexA expression is on in A1R, weakly in A2R, and not at all in the opposing A1L and A2L hemisegments. n=7 animals. (B-B') Dorsal view of VNC at 26 +/- 2hrs ALH. A08a-LexA is expressed robustly in all hemisegments at this time. n=7 animals. For all images, scale bar = 5μm; midline indicated by white dotted line; and A1 and A2 label first and second abdominal segments.

The increase in dendrite arbor volume was most striking in the intermediate zone of the A08a dendritic domain where there are few arbors present in wild type (Figure 4.2A-B). Interestingly, when dbd was targeted to intermediate or lateral neuropil regions, the volume of the medial dendrite was decreased (Figure 4.2D).

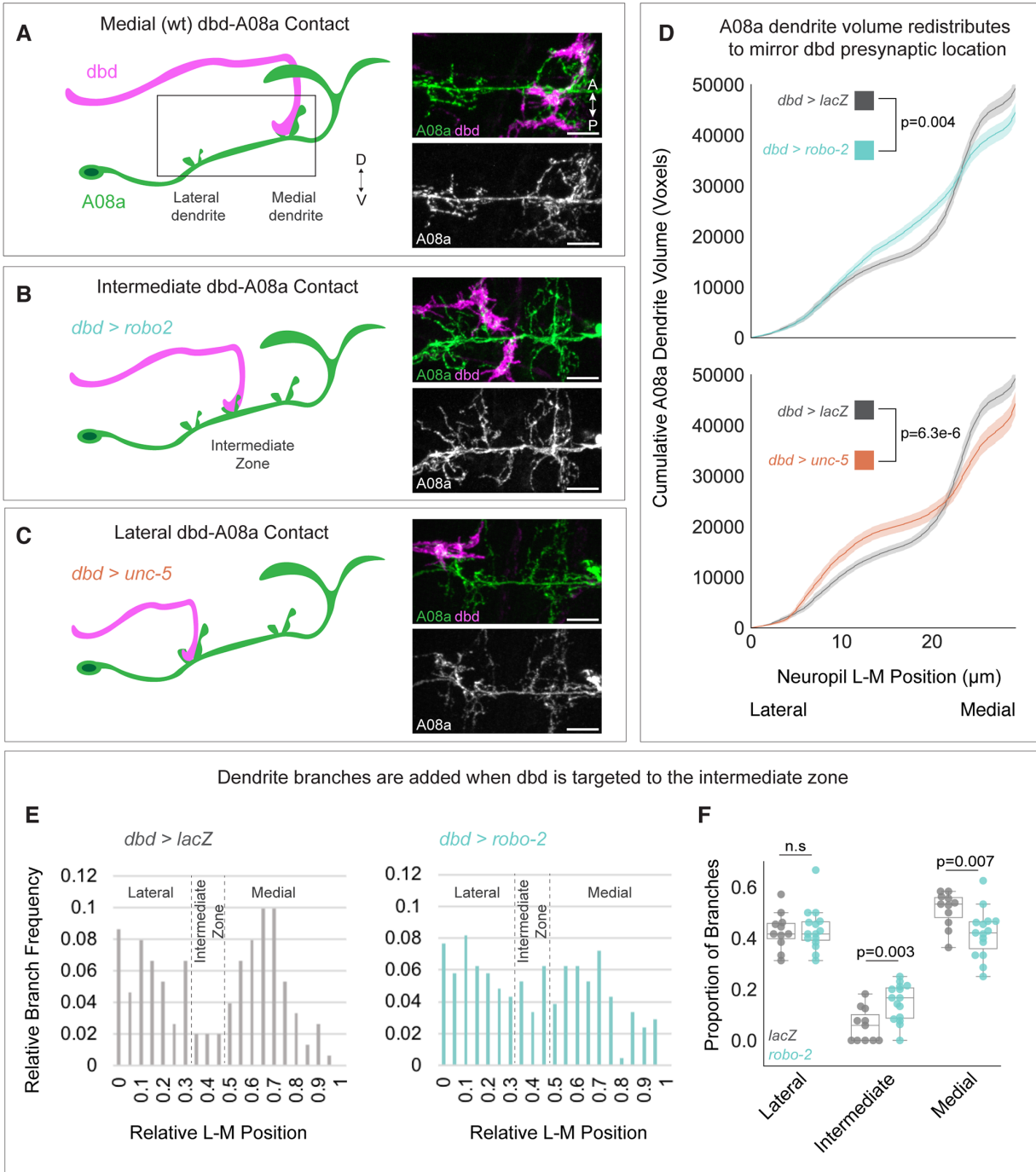
The rearrangement of dendrite volume could be due to novel dendrite outgrowth from the main A08a neurite, or due to elaboration of pre-existing lateral or medial arbors. To distinguish between these possibilities, we plotted the frequency of branch points off of the main A08a neurite across the lateral-medial axis. We found that when dbd is targeted to the intermediate zone, there was an increase in the frequency of dendritic branches off the primary A08a neurite in the intermediate zone, supporting the idea that the observed increase in dendrite volume in the intermediate zone is due to novel dbd-promoted dendrite outgrowth (Figure 4.2E-F). We conclude that presynaptic contact can locally promote postsynaptic dendrite outgrowth, ultimately ensuring robust partner matching.

dbd ablation results in A08a lateral dendrite expansion

Our findings led us to investigate the mechanisms of ectopic A08a dendrite establishment. To test the hypothesis that dbd contact promotes local dendrite outgrowth, we genetically ablated dbd using the *dbd-Gal4* line driving expression of the pro-apoptotic gene *hid*. If dbd promotes medial dendrite outgrowth, we would expect dbd ablation to reduce the A08a medial dendritic arbor.

We confirmed dbd ablation by 22C10 staining, which labels all sensory neurons. Embryonic and larval sensory neuron cell bodies are located in the body wall, and have stereotyped positions. 22C10 marks the dbd neuron cell body, positioned at the base of the dorsal-most cluster of sensory neurons (Figure 4.3 - figure supplement 1A,C) (Ghysen et al., 1986). In addition, we also used the absence of dbd-Gal4 driven *myr::HA* to identify segments where dbd was ablated (Figure 4.3C; Figure 4.3 - figure supplement 1D). *Hid* expression indeed led to a loss of dbd neurons, as detected through the absence of both 22C10⁺ and HA⁺ cell bodies from the body wall (Figure 4.3 - figure supplement 1B,D). After confirming that *Hid* expression eliminated dbd, we next assayed control and dbd ablation larvae at 24 +/- 2hrs after larval hatching (alh) for A08a dendrite length. A08a dendrites were reconstructed using the Imaris

Figure 4.2 (next page). Dendrite development is promoted by presynaptic axons. (A) Left: Illustration of wild-type dbd (pink) and A08a (green). dbd projects to the A08a medial dendrite. Right: A08a dendritic domain (boxed region shown in cartoon). dbd (pink) contacts the medial A08a dendrite (green). Secondary image of A08a channel alone (white) shows two distinct dendritic domains. (B) Left: *Robo2* misexpression leads dbd to project to the A08a intermediate dendritic domain. Right: dbd contacts the intermediate A08a dendritic domain, where there are ectopic dendrites. (C) Left: *Unc-5* misexpression leads dbd to project to the A08a lateral dendrite. Right: dbd contacts the lateral A08a dendrite, where there is ectopic dendritic material. Scale bar, 5µm. Micrographs are from larvae aged 24 +/- 4hrs alh. (D) Cumulative distribution of A08a dendrite volume (voxels) across the lateral-medial axis in conditions where dbd projects to the medial (gray, n=17 cells, 11 animals), intermediate (cyan, n=20 cells, 10 animals), or lateral (orange, n=11 cells, 10 animals) A08a dendrite. Solid line=mean distribution; shaded area=standard error of the mean (SEM). A08a dendrites receiving input from intermediate or lateral dbd neurons have significantly different volume distributions from wild-type dendrites (2-way Kolmogorov-Smirnov test). Note that the control *LacZ* trace is the same for top and bottom panels. (E) Relative Lateral-Medial distribution of A08a dendrite branch points from the main A08a neurite in conditions where dbd projects to the medial (gray, n=11 cells from 7 animals) or intermediate (cyan, n=15 cells from 8 animals) dendritic domain. Lateral, Intermediate, and Medial boundaries are demarcated based on the local minimum of the *LacZ* distribution. (F) Proportion of branches occupying Lateral, Intermediate, and Medial A08a dendritic domains when dbd projects to the medial dendrite (gray, n=11 cells from 7 animals) or intermediate zone (cyan, n=15 cells from 8 animals). Individual points represent single cells. When dbd projects to the intermediate domain, there are more A08a branches in the intermediate domain and fewer in the medial domain. Statistics computed using 2-tailed unpaired t-test with unequal variance.

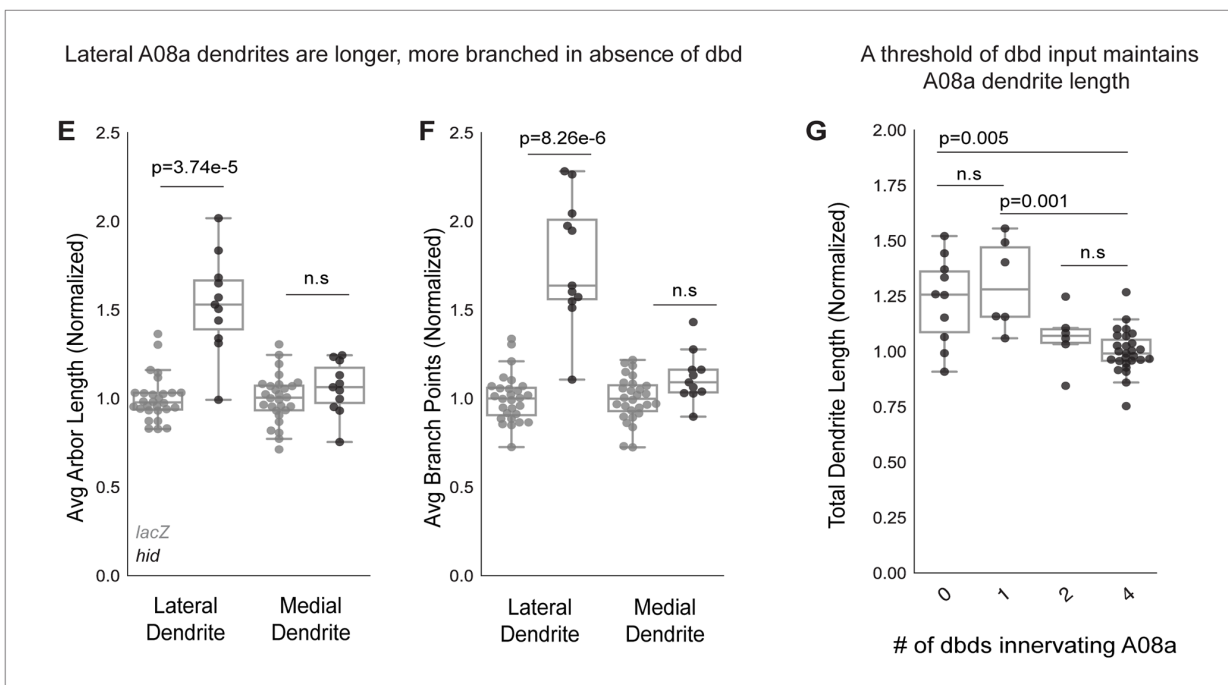
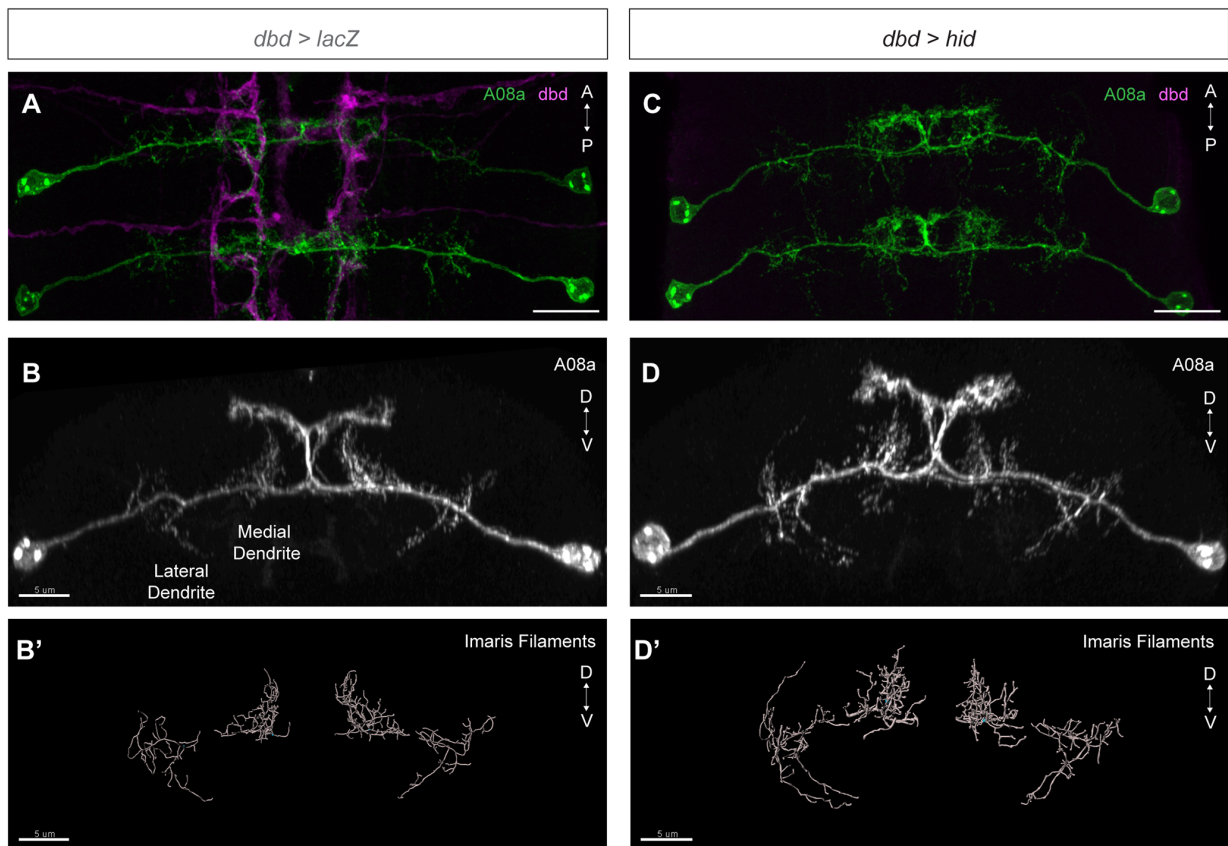


software Filaments tool (Figure 4.3B', D'). As expected, controls showed well-branched medial and lateral dendritic arbors by immunostaining (Figure 4.3A-B) and in the Imaris reconstructions (Figure 4.3B'). In contrast, *dbd* ablation led to qualitatively enlarged lateral dendrites by immunostaining (Figure 4.3C-D) and in the Imaris reconstructions (Figure 4.3D'). Quantification confirmed that complete *dbd* ablation led to A08a lateral dendrites that were longer and more

branched (Figure 4.3E-F). In contrast, there was no significant change in medial dendrite length or branching when dbd was ablated (Figure 4.3E-F).

Hid overexpression resulted in variable numbers of ablated dbd neurons across samples (Figure 4.3 - figure supplement 1B). We could therefore test whether there is a correlation between the number of dbd neurons innervating a segment and the total A08a dendrite length. In wild type, there are four dbd neurons innervating a single VNC segment, two per hemisegment. We found that when 1-2 of the four dbd neurons are ablated, A08a dendrite length is not significantly different than in controls. However, when 3-4 of the four dbd neurons are ablated, A08a lateral dendrite length is significantly increased (Figure 4.3G). This finding suggests that a threshold level rather than a linear summation of dbd input stabilizes A08a dendrite outgrowth. The expansion of lateral A08a dendrite length was surprising, as dbd does not contact the lateral dendrite in wild-type animals (Sales et al., 2019; Schneider-Mizell et al., 2016). The growth in lateral dendrites following dbd ablation revealed that dbd provides negative regulation of A08a dendrite outgrowth, in addition to the positive mechanism revealed by the dbd lateralization experiments. Moreover, the negative mechanism acts throughout the neuron, rather than locally as does the positive mechanism. A likely source of a neuron-wide inhibitor of dendrite outgrowth is neuronal activity, which negatively regulates dendrite arbor size in multiple systems (Ackerman et al., 2021; Shen et al., 2020; Tripodi et al., 2008; Wu & Cline, 1998). We address this possibility in the next section.

Figure 4.3 (next page). dbd ablation causes A08a lateral dendrite expansion. (A) Control VNC at 24 +/- 2hrs alh, dorsal view. A08a neurons (green) are innervated by dbd neurons (magenta). Scale bar, 10µm. **(B)** Control A08a neurons from abdominal segment 1 of (A), posterior view. Scale bar, 5µm. **(B')** Imaris filament reconstruction of A08a dendrites in (B). Scale bar, 5µm. **(C)** dbd ablation VNC at 24 +/- 2hrs alh, dorsal view. A08a neurons (green) lack innervation from dbd neurons (magenta). **(D)** A08a neurons in dbd ablation background from abdominal segment 1 of (C), posterior view. Scale bar, 5µm. **(D')** Imaris filament reconstruction of A08a dendrites in (E). Scale bar, 5µm. **(E)** Average dendrite length of lateral and medial A08a dendritic arbors in LacZ (control, gray, n=28 animals) or Hid-expressing animals (black, n=11 animals). **(F)** Number of dendrite branch points of lateral and medial A08a dendritic arbors in LacZ (control, gray, n=28 animals) or Hid-expressing animals (black, n=11 animals). Values normalized to control mean for either lateral or medial arbor. Circles represent single-animal averages between left and right hemisegments. Values for Hid-expressing animals with 0 remaining dbd neurons innervating A1 segment. **(G)** Total A08a dendrite length when A08a is innervated by 4 (LacZ control, n=28 animals), 2 (n=6 animals), 1 (n=6 animals), or 0 (n=10 animals) dbd neurons. Black circles represent single-animal dendrite length summed across left and right hemisegments. Data are normalized to dendrites innervated by 4 dbd neurons (LacZ control). Statistics computed using 2-tailed unpaired t-test with unequal variance.



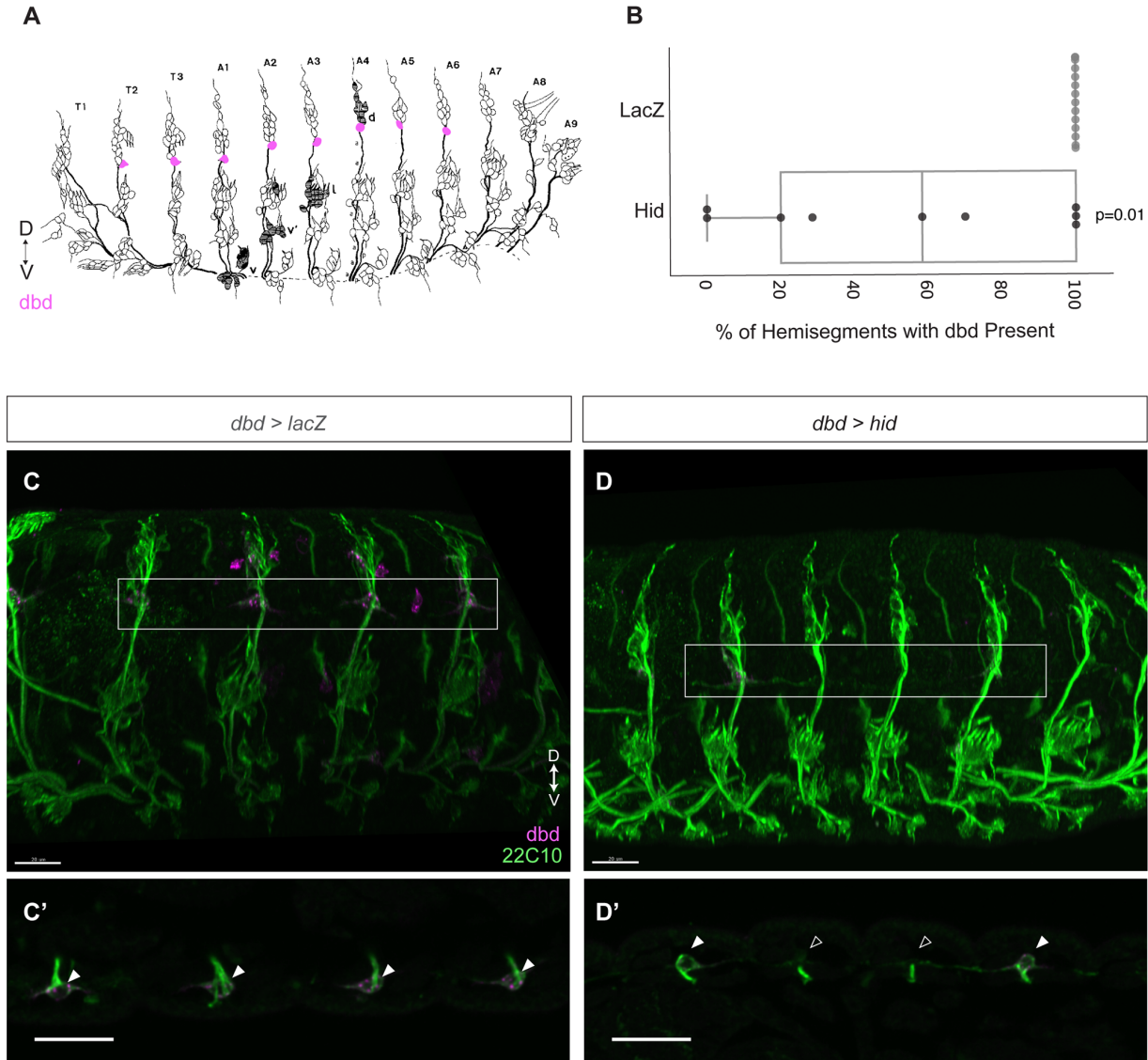


Figure 3 – figure supplement 1. Validation of dbd ablation. (A) Illustration of embryonic sensory neuron anatomy (modified from Ghysen et al., 1986). Sensory neuron anatomy is segmentally repeated, and cell bodies are in stereotyped positions in the dorsal-ventral axis. Dbd cell bodies are highlighted in pink, positioned at the base of the dorsal-most cluster of cell bodies. Anterior is to the left. (B) Percentage of hemisegments containing an intact dbd cell body, identified by 22C10 staining in control (gray) or Hid-expressing (black) embryos. All control embryos had dbd 22C10 labeling in 100% of hemisegments (n=14 embryos). Most Hid-expressing embryos had missing dbd neurons (n=6 embryos). Hid embryos with 100% of dbd neurons intact (n=3 embryos) were likely CyO⁺ instead of Hid⁺ (see Methods). (C-C') Stage 17 control embryo. 22C10 labels all sensory neurons (green), dbd-Gal4>myr::HA labels dbd and other cells (magenta). Scale bar, 20μm (C') Substack of dbd cell bodies (filled arrowheads) boxed in (C). Scale bar, 20μm. (D-D') Stage 17 Hid-expressing embryo. 22C10 labels all sensory neurons (green), dbd-Gal4>smGdP-myr::HA labels dbd and other cells. Scale bar, 20μm (D') Substack of dbd cell bodies boxed in (D). dbd cell bodies are present in the first and last segment (filled arrowhead) and missing in the middle two segments (empty arrowhead), indicative of successful ablation. Scale bar, 20μm.

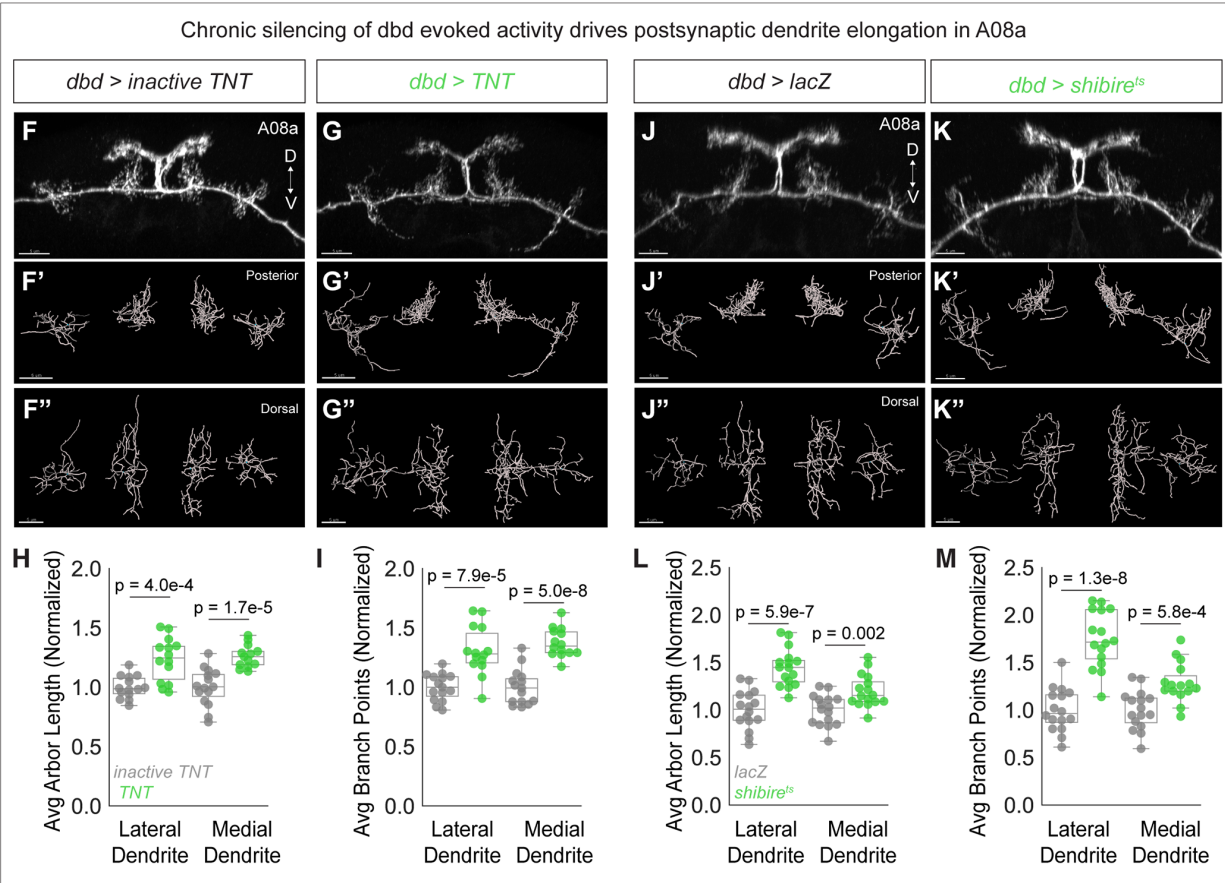
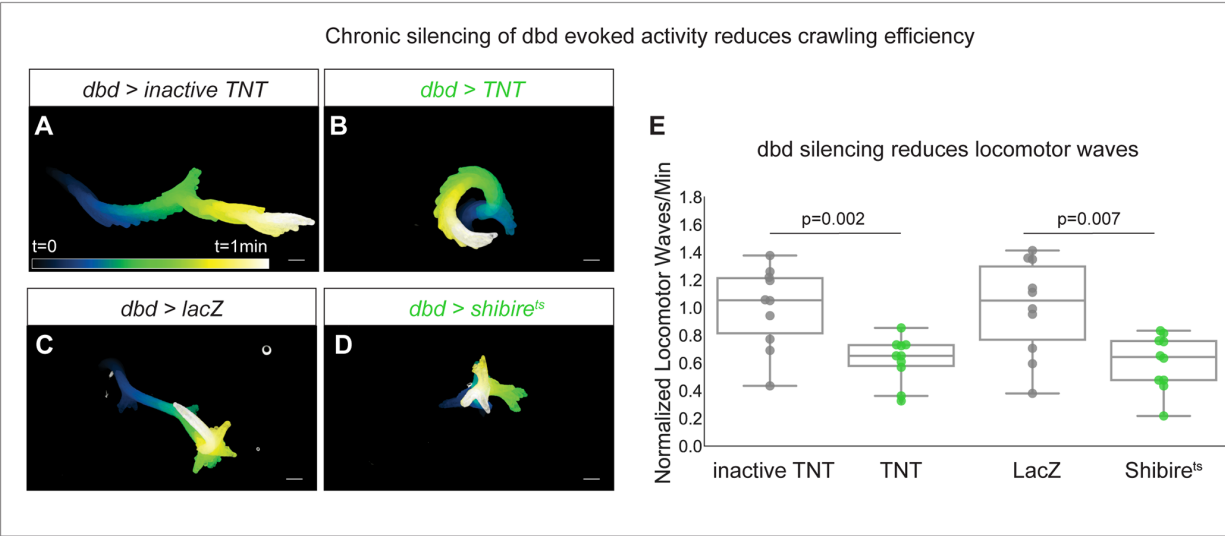
dbd activity globally inhibits A08a dendrite outgrowth

To test if dbd activity influences A08a dendrite size, we used two methods to silence dbd synaptic activity throughout development. The first was dbd-specific expression of either tetanus toxin light chain (TNT) or mutationally inactive TNT as a negative control. TNT cleaves the synaptic vesicle protein synaptobrevin, inhibiting evoked synaptic vesicle release (Sweeney et al., 1995). A previous study showed that dbd silencing led to slowed and uncoordinated larval locomotion (Hughes & Thomas, 2007). We validated that our silencing tools were effectively inhibiting dbd activity by comparing larval crawling behavior between control and dbd-silenced animals. Dbd-silenced animals had fewer waves of crawling activity, consistent with previous results (Figure 4.4A-B, E). Thus, we proceeded to ask if dbd silencing leads to expanded A08a dendrites. We found that constitutive dbd silencing using TNT indeed resulted in longer, more branched lateral and medial A08a dendrites at 26 +/- 2hrs alh (Figure 4.4F-I).

We also used Shibire^{ts} to chronically and specifically silence dbd (Kitamoto, 2001). Shibire^{ts} animals were reared constitutively at 30°C and compared to temperature-matched negative controls expressing LacZ, as developmental temperature was recently shown to impact the extent of neurite branching and synapse formation (Kiral et al., 2021). Silencing of dbd with Shibire^{ts} also impaired larval crawling efficiency (Figure 4.4C-E), and resulted in longer, more

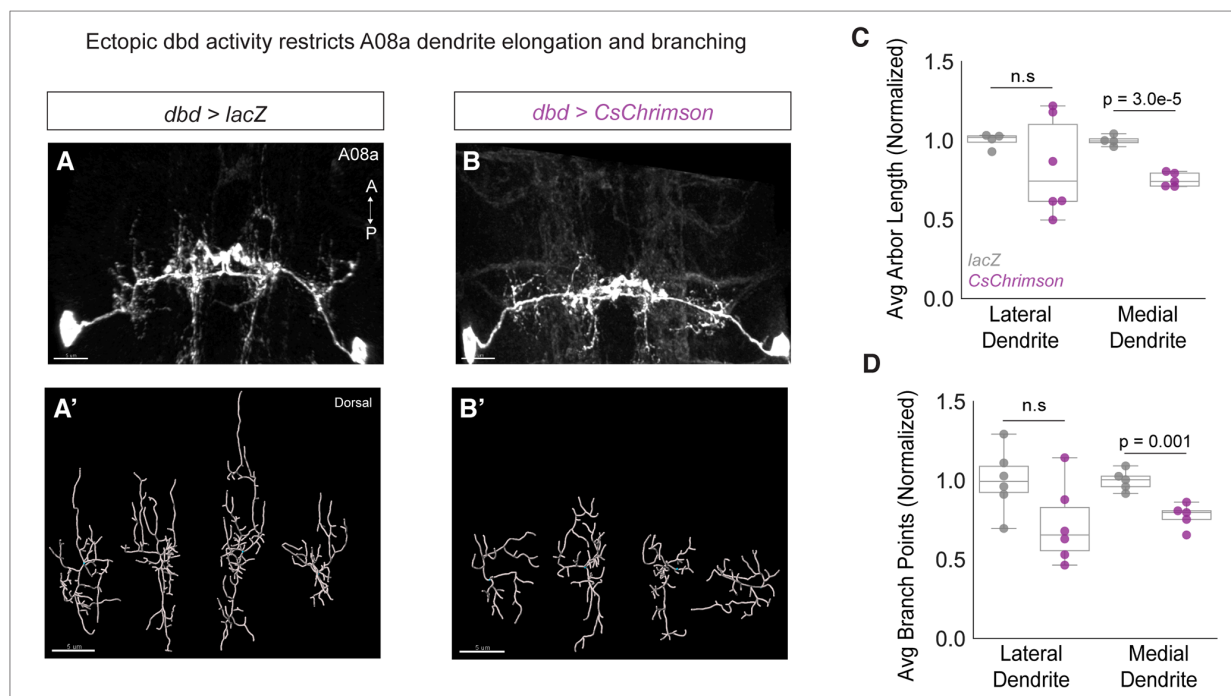
Figure 4.4 (next page). Chronic silencing of dbd activity drives A08a dendrite elongation. (A)

Representative crawling trace of control inactive TNT control larva (26 +/- 2hrs alh). Trace is color-coded by time. Scale bar, 1mm. **(B)** Representative crawling trace of TNT larva (26 +/- 2hrs alh). **(C)** Representative crawling trace of control LacZ-expressing larva (24 +/- 2hrs alh). Trace is color-coded by time. Scale bar, 1mm. **(D)** Representative crawling trace of Shibire^{ts} larva (24 +/- 2hrs alh). **(E)** Number of locomotor waves (forward and reverse) initiated in one minute, normalized to corresponding control. Control animals (gray) initiate more locomotor waves relative to animals with silenced dbd neurons (green). inactive TNT, n= 10animals ; TNT, n= 10animals; LacZ, n=10 animals; Shibire^{ts}, n= 10 animals. Statistics computed using 2-tailed unpaired t-test with unequal variance. Circles represent locomotor waves of single animals. **(F)** Control A08a neurons at 26 +/- 2hrs alh, posterior view. **(F')** Posterior view of Imaris filament reconstruction of A08a dendrites in (F). **(F'')** Dorsal view of (F'). **(G)** A08a neurons receiving input from TNT-expressing dbds at 26 +/- 2hrs alh, posterior view. **(G'-G'')** Imaris Filament reconstructions of dendrites in (B). **(H)** Average dendrite length of lateral and medial A08a dendritic arbors in inactive TNT (control, gray, n=16 animals) or TNT-expressing animals (green, n=19 animals). **(I)** Number of dendrite branch points of lateral and medial A08a dendritic arbors in inactive TNT (control, gray, n=16 animals) or TNT-expressing animals (green, n=19 animals). **(J)** Control A08a neurons at 24 +/- 2hrs alh, posterior view. **(J'-J'')** Imaris Filament reconstructions of dendrites in (J). **(K)** A08a neurons from receiving input from Shibire^{ts}-expressing dbd, posterior view. **(K'-K'')** Imaris Filament reconstructions of dendrites in (K). **(L)** Average dendrite length of lateral and medial A08a dendritic arbors in LacZ (control, gray, n=14 animals) or Shibire^{ts}-expressing animals (green, n=19 animals). **(M)** Number of dendrite branch points of lateral and medial A08a dendritic arbors in LacZ (control, gray, n=14 animals) or Shibire^{ts}-expressing animals (green, n=19 animals).



branched A08a dendrites (Figure 4.4J-M). Presynaptic activity from *dbd* is therefore necessary to prevent excessive postsynaptic dendrite outgrowth in A08a.

If presynaptic activity inhibits A08a dendrite outgrowth, we predicted that elevated levels of dbd activity would result in shorter A08a dendrites. To activate dbd, we expressed the light-sensitive channelrhodopsin CsChrimson using dbd-Gal4 (Klapeetke et al., 2014). Animals were exposed to broad spectrum light throughout development and A08a dendrite length and complexity were assayed at 25 +/- 2hrs alh. Compared to negative controls expressing LacZ, dbd optogenetic activation led to a decrease in overall A08a dendrite length and branching. This effect was most pronounced at the medial dendrite; although some lateral arbors exhibited decreased length and branching, it did not reach statistical significance (Figure 4.5A-D). The decreases in arbor length and branching were likely not due to excitotoxicity, as this method has been previously published in larval motor neurons without inducing excitotoxicity (Ackerman et al, 2021). We conclude that presynaptic activity is necessary and sufficient to restrict A08a dendrite outgrowth.



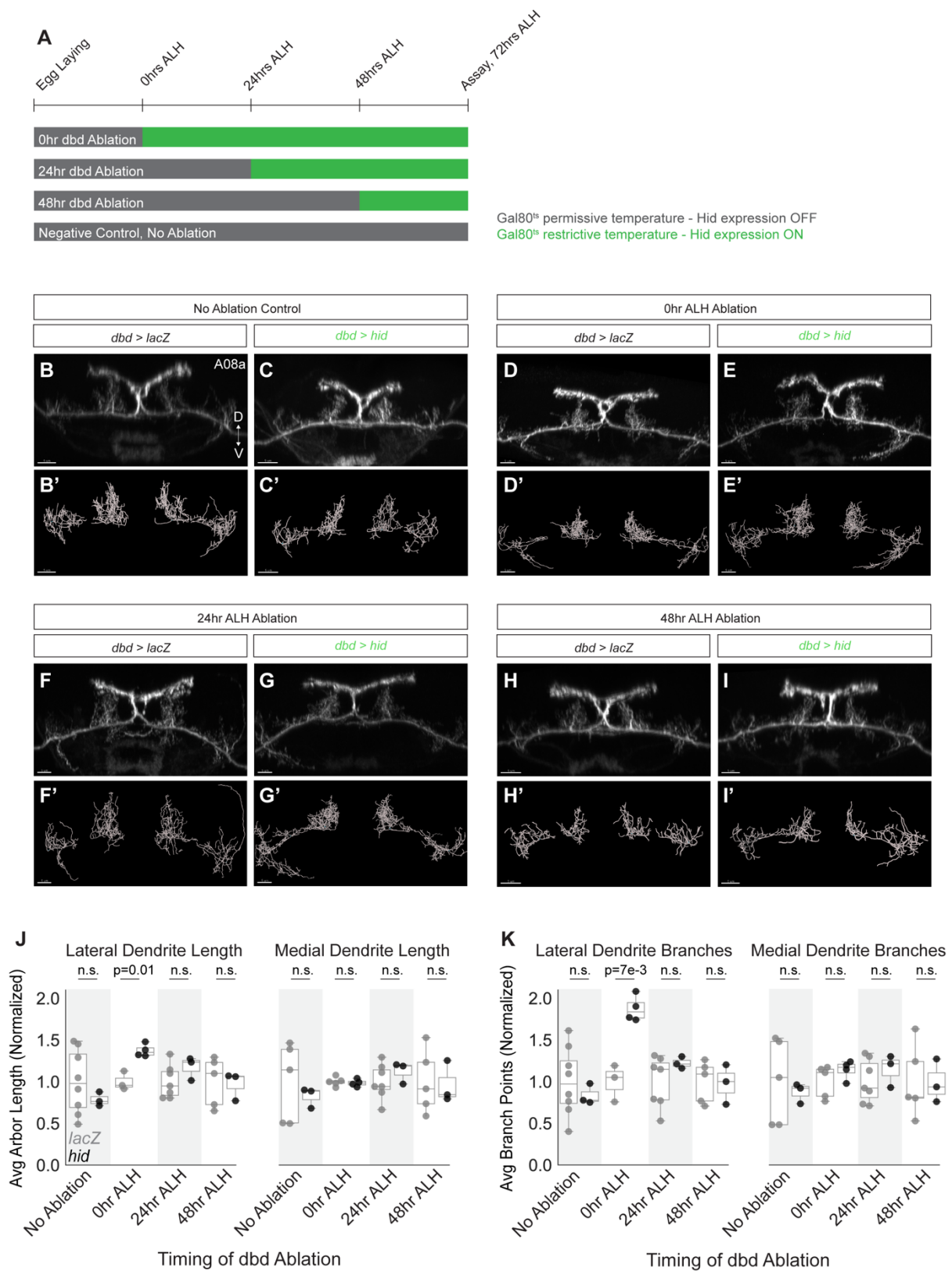
A08a dendrite plasticity is confined to a critical period of development.

Neurons in many animals exhibit transient structural plasticity in early developmental windows that are termed "critical periods" for plasticity (Ackerman et al., 2021; Jarecki & Keshishian, 1995; LeVay et al., 1980; McLaughlin et al., 2003). In *Drosophila* larvae, motor neuron dendrites remain plastic until 8hrs alh, after which astrocytes prevent further dendritic remodeling, at least through 22hrs alh (Ackerman et al., 2021). Larvae also undergo continuous neuronal arbor growth to scale with their increasing body size, which may require some neurons to remain adaptable to a changing cellular environment (Gerhard et al., 2017). We therefore wanted to test whether A08a dendrites remain plastic in later stages of larval life, or if they are subject to the same critical period as motor neurons. To do so, dbd neurons were conditionally ablated by expressing Hid at successive stages of development, and A08a dendrite length and branching were quantified (Figure 4.6A).

We controlled the onset of Hid expression in dbd using temperature-sensitive Gal80 (Gal80^{ts}). Hid expression was induced at 0hrs alh, 24hrs alh, or 48hrs alh (times adjusted to 25°C developmental equivalent). A08a dendrite length was assayed for all experiments at 72hrs alh

Figure 4.6 (next page). A08a dendrite plasticity is confined to a critical period in larval development. (A)

Experimental design. Hid expression was inhibited at 18°C by Gal80^{ts} (gray bars). Hid expression was induced by shifting animals to 30°C (green bars) at the 25°C equivalent of 0hrs alh, 24hrs alh, and 48hrs alh. A08a dendrites were assayed for length and branching at the 25°C equivalent of 72hrs alh. **(B)** Control A08a raised continuously at 18°C. Imaris filament reconstructions of A08a dendrites in B'. **(C)** A08a in hid-expressing animal raised continuously at 18°C. Imaris filament reconstructions of A08a dendrites in (C'). **(D)** A08a in control animal shifted to 30°C at 0hrs alh. Imaris filament reconstructions of A08a dendrites in (D'). **(E)** A08a in hid-expressing animal shifted to 30°C at 0hrs alh. Imaris filament reconstructions of A08a dendrites in (E'). **(F)** A08a in control animal shifted to 30°C at 24hrs alh. Imaris filament reconstructions of A08a dendrites in (F'). **(G)** A08a in hid-expressing animal shifted to 30°C at 24hrs alh. Imaris filament reconstructions of A08a dendrites in (G'). **(H)** A08a in control animal shifted to 30°C at 48hrs alh. Imaris filament reconstructions of A08a dendrites in H'. **(I)** A08a in hid-expressing animal shifted to 30°C at 48hrs alh. Imaris filament reconstructions of A08a dendrites in (I'). **(J)** Average dendrite length of lateral (left) and medial (right) A08a dendritic arbors in LacZ (control, gray) or Hid-expressing animals (black). **(K)** Average number of branch points on lateral (left) and medial (right) A08a dendrites. X-axes, timing of dbd ablation (No ablation control: control n=5-8 animals, Hid n= 3 animals; 0hr alh ablation: control n=3-5 animals, Hid n=4 animals; 24hr alh ablation: control n=7 animals, Hid n=3 animals; 48hr alh ablation: control n=5 animals, Hid n=3 animals). For Hid quantifications, only segments containing 0-1 dbds were analyzed (determined by absence of dbd membrane stain). Scale bars, 5µm. Values for all quantification normalized to control mean for each ablation timepoint. Circles represent single-animal averages between left and right hemisegments. Statistics computed using 2-tailed unpaired t-test with unequal variance.



(Figure 4.6A). If A08a retains the capacity for dendrite plasticity throughout larval life, we would detect increases in dendrite length and branching after ablating dbd at each timepoint. In contrast, compared to no ablation controls (Figure 4.6B,D,F,H,J,K), we found that A08a was competent to expand its lateral dendrites only after dbd was ablated in newly hatched larvae (Figure 4.6D-E', J-K), but not when ablations occurred at 24 or 48hrs alh (Figure 4.6F-K). This result demonstrates that A08a dendrite structural plasticity is confined to an early critical period, perhaps the same critical period as used by the *Drosophila* larval motor system (Ackerman et al., 2021).

Discussion

Presynaptic contact promotes local dendrite outgrowth

We sought to uncover the cellular mechanism by which dendrites respond to variable positioning and input of their synaptic partners. Here and in our previous work, we rerouted the dbd axon terminal to the lateral and intermediate neuropils and found that A08a dendrites mirrored the location of their displaced presynaptic partner (Valdes-Aleman et al., 2021). When dbd was targeted to the A08a intermediate dendritic domain, an area devoid of dendrites in wild-type, ectopic branches were established. At the same time, we observed a decrease in medial dendrite volume and branching when dbd was targeted elsewhere. In these experiments we measured no significant change to dbd-A08a functional connectivity strength (Sales et al., 2019), indicating that these compensations in dendrite length were likely activity-independent and due to contact alone.

Across a variety of model systems, presynaptic contact is correlated with or promotes the local outgrowth of dendrites (Chen et al., 2010; Jacoby & Kimmel, 1982; Kamiyama et al., 2015; Niell et al., 2004; Vaughn, 1989). In classic studies performed on the giant Mauthner (M) cells in zebrafish and axolotl, ablation of sensory afferents resulted in failed M-cell dendrite formation, whereas suprainnervation by these afferents was sufficient to cause overelaboration of the M-cell dendrites (L. A. Goodman & Model, 1988; Kimmel et al., 1977, 1981). Interestingly, when paired with a pharmacological silencing manipulation, suprainnervation of the M-cell still resulted in elongated dendrites, suggesting that contact-based cues are sufficient to drive local

dendrite outgrowth (L. A. Goodman & Model, 1990), matching our results. Our similar results when dbd is mistargeted suggest a conserved mechanism across vertebrates and invertebrates for the local initiation of dendrites by sensory afferents. The ability for an axon to promote local dendrite outgrowth offers a potential strategy for pre- and postsynaptic partner matching that is robust to variable axon positioning.

One outstanding question from our studies is whether the dbd axon induces A08a dendrite formation de novo, or selectively stabilizes nascent dendrites. There are currently no methods for tracking the initial formation of A08a dendrites. The LexA driver used to label A08a is first expressed in early larval life, after first outgrowth of A08a medial and lateral dendrites (Figure 4.1 – figure supplement 1A). At the time of dbd CNS innervation at embryonic stage 14, the morphology of A08a dendrites is unknown. Therefore it is unclear if dbd induces novel dendrite outgrowth from an otherwise bare neurite, or if it stabilizes and promotes continued dendrite elongation from an arbor already beginning to take form. In wild-type animals, A08a dendrites are innervated by multiple neurons (Sales et al., 2019). If dbd is not the first neuron to contact the A08a medial dendritic domain, it is possible that the normal function of the dbd axon is to promote the continuous elongation of the medial dendrite rather than its initial induction. Either possibility would support our finding that presynaptic contact promotes postsynaptic dendrite outgrowth, and clarifying the exact mechanism will be important for identifying the molecular players that support either process.

Presynaptic contact and presynaptic activity have opposing effects on dendrite outgrowth

We further tested the hypothesis that presynaptic contact promotes local dendrite outgrowth by genetically ablating dbd. When dbd was ablated, A08a lateral dendrites were elongated while medial dendrites were unchanged. Direct silencing of dbd caused both lateral and medial dendrites to elongate; this result highlights that dbd also acts as a negative regulator of postsynaptic dendrite outgrowth, and that presynaptic activity can downregulate global dendrite development.

We hypothesize that when dbd is ablated, A08a medial dendrites are unchanged due to the opposing roles of presynaptic activity and contact. Lack of dbd contact (which restricts growth) and lack of dbd activity (which promotes growth) could be working in direct opposition

and thus result in no change in medial arbor size. In contrast, the lateral arbor experiences only the loss of activity, leading to arbor growth. Taken together, our data support a model of dendrite development in which presynaptic contact acts locally to promote dendrite outgrowth, whereas presynaptic activity acts globally across an entire neuron to downregulate dendrite elongation (Figure 4.7). As synapses are added and become functional, activity could act as a negative-feedback mechanism to excessive dendrite elongation.

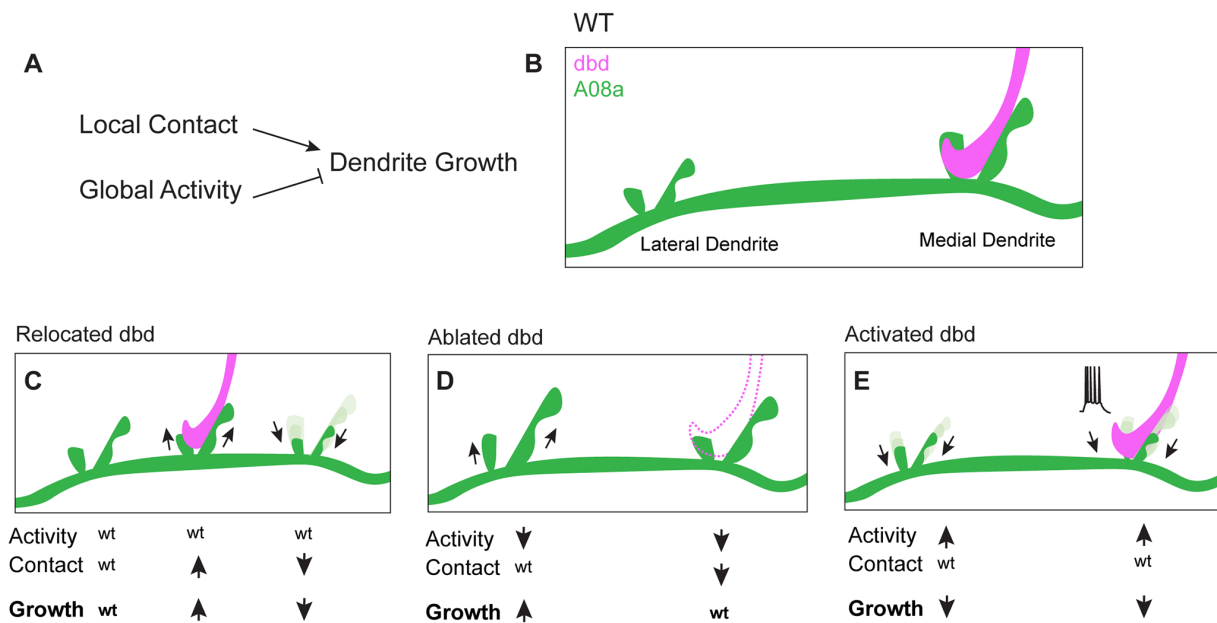


Figure 4.7. Proposed model: Presynaptic activity and contact opposingly regulate dendrite outgrowth.

(A) Presynaptic contact promotes local dendrite outgrowth, while presynaptic activity levels inhibit neuron-wide dendrite outgrowth. (B) Wild type (WT) A08a dendrites. (C) When dbd is mistargeted to intermediate A08a dendritic domain, activity levels are wt. Local outgrowth is promoted at the intermediate dendrite, and inhibited by lack of contact at the medial dendrite. (D) When dbd is ablated, neuron-wide activity levels are decreased. This promotes lateral dendrite outgrowth. Lack of contact at the medial dendrite opposes the activity-dependent drive to elongate so dendrite length remains wt. (E) dbd Activation promotes neuron wide dendrite retraction/premature stabilization.

Homeostatic vs. linear scaling of dendrite growth

Homeostatic structural plasticity is a phenomenon in which neurites adjust their length to counter the effect of too much or too little activity; when activity is excessive, the dendrite shrinks, and when activity is diminished, the dendrite expands. Homeostatic regulation of dendritic arbor size

has been documented in insects (Ackerman et al., 2021; Hoy et al., 1985; Tripodi et al., 2008; Yuan et al., 2011) and vertebrates (Shen et al., 2020; Takeo et al., 2021; Tanvir et al., 2021; Wu & Cline, 1998). We observed a homeostatic relationship between presynaptic activity levels and postsynaptic A08a dendrite length. When synaptic input onto A08a was decreased by silencing evoked dbd activity, A08a dendrites were elongated; when dbd was chronically activated, A08a dendrites were smaller. Compensatory adjustments in dendrite length are likely a strategy to maintain a “set-point” of synaptic input. Such a mechanism would be useful to maintain a constant level of postsynaptic output when the amount of input is variable.

There are also examples in which synaptic activity positively correlates with dendrite elongation, a phenomenon we refer to as “linear scaling.” For example, studies in the *Xenopus* optic tectum showed that rearing tadpoles in the light (increased activity) can increase the rate of dendrite growth, and that growth is inhibited by pharmacologically blocking glutamate receptor activity (Rajan & Cline, 1998; Sin et al., 2002). In *Drosophila*, misexpression of activity-dependent transcription factors and ion channels can also promote dendrite elongation (Hartwig et al., 2008; Timmerman et al., 2013; Vonhoff et al., 2013). It remains an interesting open question why increased activity sometimes reduces dendrite size (homeostatic growth) and sometimes increases dendrite growth (linear scaling).

A critical period for dendrite development in the Drosophila larva

A critical period for *Drosophila* larval motor neuron dendrite homeostasis was recently defined (Ackerman et al., 2021). In this system, dendrite length can be homeostatically modified by levels of activity. These motor dendrites lose the capacity to undergo activity-dependent remodeling at 8hrs alh, early in larval life. The closure of this critical period is governed by astrocytes - their infiltration into the neuropil coincides with critical period closure and their contact with motor dendrites prevents precocious dendrite extension/retraction (Ackerman et al., 2021; Stork et al., 2014).

From 1st to 3rd instar, the *Drosophila* larva increases in body size by two orders of magnitude. The nervous system continues to grow during this time, with individual neurons adding hundreds of microns of overall dendrite length (Gerhard et al., 2017). We wondered if an interneuron such as A08a would be subject to the same critical period described for motor

neuron dendrites, or if dendrite plasticity remains necessary in subsequent stages of larval development to accommodate animal growth. We found that the capacity for A08a dendrites to respond to modified presynaptic input was confined to the first instar of larval development, similar to the critical period for motor dendrite structural plasticity (Ackerman et al., 2021). Ablation of *dbd* in subsequent larval instars did not impact A08a dendrite length. These results suggest that potentially all larval VNC neurons are subject to the same early critical period regulated by astrocytes, and structural plasticity events are not inducible in later stages of development. If presynaptic input is not required to regulate dendrite outgrowth in subsequent stages of larval life, perhaps separate cell-intrinsic (Tenedini et al., 2019; Zwart et al., 2013) or mechanical mechanisms (e.g. stretch or pulling forces) (Balice-Gordon & Lichtman, 1990; Bray, 1984; Tao et al., 2022) are required to allow the scaling of circuits as an organism grows.

Dendrite diversification as a substrate for behavioral evolution

Here and in previous work, we observed that dendrite development is not hard-wired, but is modulated by the location of presynaptic partners and presynaptic activity levels (Sales et al., 2019; Valdes-Aleman et al., 2021). Within a species, subtle variation in neuron morphology that arises in development can diversify behavior. For example, natural variation in *D. melanogaster* Dorsal Cluster Neuron axonal projections directly impacts an animal's ability to orient toward a visual object (Linneweber et al., 2020). Imprecise but constrained morphological development could be a “bet-hedging” strategy to promote the adaptability of an individual species to environmental changes.

Across species, it is intriguing to speculate that species-specific behaviors evolved in part through mechanisms that pattern neurite morphology. For two highly divergent nematode species, *C. elegans* and *P. pacificus*, amphid sensilla neuron number and soma position are highly conserved whereas ciliated dendrite morphology is more diverse. Correspondingly, neurons whose structure is more dissimilar between the species also have more divergent synaptic connections, implying that downstream behaviors would also differ (R. L. Hong et al., 2019). Another study found that in a species of *Drosophila* with evolved attraction to noni fruit, axon branch morphology in an olfactory processing center diverged from that of species that do not exhibit attraction to noni (Auer et al., 2020). For interneurons such as A08a, genomic

changes resulting in alterations to dendrite neuropil position could vary the number and identities of presynaptic partners. It will be interesting to causally test the impact of neurite architecture on behavioral diversification, and specifically the extent to which genes regulating presynaptic axon position, neural activity, or critical period length are nodes of dendritic and ultimately behavioral evolution.

Materials and Methods

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Species (<i>Drosophila melanogaster</i>)	<i>26F05-LexA</i>	BDSC	54702	Expressed in A08a neurons
Species (<i>D. melanogaster</i>)	<i>165-Gal4</i>	W. Grueber	N/A	Expressed in dbd neurons, as well as cIVda neurons
Species (<i>D. melanogaster</i>)	<i>UAS-robo2::HA</i>	BDSC	66886	Expresses HA-tagged Robo2 under UAS control
Species (<i>D. melanogaster</i>)	<i>UAS-unc-5::HA</i>	A. Zarin	N/A	Expresses HA-tagged Unc-5 under UAS control
Species (<i>D. melanogaster</i>)	<i>UAS-hid, tubP-Gal80ts/CyO</i>	M. Freeman	N/A	Expresses Hid under UAS control, Gal80ts negatively regulates Gal4 at 18°C.
Species (<i>D. melanogaster</i>)	<i>UAS-TeTxLC-G2</i>	BDSC	28838	Expresses the light chain of tetanus toxin under UAS control
Species (<i>D. melanogaster</i>)	<i>UAS-inactive TeTxLC</i>	BDSC	28840	Expresses a mutated tetanus toxin light chain gene under UAS control
Species (<i>D. melanogaster</i>)	<i>UAS-shi[ts1].K</i>	BDSC	44222	Expresses temperature-sensitive shi under

				UAS control for inhibiting synaptic transmission at 30°C
Species (<i>D. melanogaster</i>)	<i>UAS-LacZ</i>	BDSC	8529	Control transgene
Species (<i>D. melanogaster</i>)	<i>UAS-LacZ</i>	BDSC	8530	Control transgene
Species (<i>D. melanogaster</i>)	<i>10xUAS-IVS-myr::smGdP::HA, 13xLexAop2-IVS-myr::smGdP::V5</i>	BDSC	64092	Expresses HA membrane tag under UAS control, V5 membrane tag under LexAop control
Species (<i>D. melanogaster</i>)	<i>pUAS-CsChrimson.mVenus (attP2)</i>	V. Jayaraman	N/A	Expresses CsChrimson tagged with mVenus under UAS control
Antibody, monoclonal	Mouse anti-V5 tag	Invitrogen, Carlsbad, CA,	Cat. R96025, Lot 1949337	(1:1000)
Antibody, monoclonal	Rat anti-HA tag	Roche Holding, AG, Basel, Switzerland	Cat. 11867423001, Lot 27573500	(1:100, after suggested dilution)
Antibody, monoclonal	Mouse anti-Futsch (22C10)	DSHB, Iowa City, IA	Concentrate 0.1mL	(1:1000)
Antibody, monoclonal	Rabbit anti-Corazonin	J. Veenstra, Univ Bordeaux	N/A	(1:2000)
Antibody, secondary	Alexa Fluor® 488 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch, West Grove, PA	Cat. 715-545-151	(1:400)

Antibody, secondary	Alexa Fluor® 647 AffiniPure Donkey Anti-Rat IgG (H+L)	Jackson ImmunoRese arch, West Grove, PA	Cat. 712-605- 153	(1:400)
Antibody, secondary	Alexa Fluor® 488 AffiniPure Donkey Anti-Rat IgG (H+L)	Jackson ImmunoRese arch, West Grove, PA	Cat. 712-545- 153	(1:400)
Antibody, secondary	Alexa Fluor® RRX AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoRese arch, West Grove, PA	Cat. 715-295- 151	(1:400)
Antibody, secondary	Alexa Fluor® 647 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoRese arch, West Grove, PA	Cat. 715-605- 151	(1:400)

Fly stocks

Genotypes	Figure
Females containing <i>10xUAS-IVS-myr::smGdP::HA</i> , <i>13xLexAop2-IVS-myr::smGdP::V5</i> (BDSC# 64092); <i>GMR26F05-LexA</i> (A08a neurons) (BDSC# 54702), <i>UAS-bruchpilot (short)-mstraw</i> ; <i>165-Gal4</i> (dbd neurons) were crossed to males containing either (i) <i>UAS-lacZ</i> (BDSC #8529), (ii) <i>UAS-robo2::HA</i> (BDSC #66886), or (iii) <i>UAS-unc-5::HA</i>	(i) Figure 4.2A, D-F (ii) Figure 4.2B, D-F (iii) Figure 4.2C-D
Females containing <i>10xUAS-IVS-myr::smGdP::HA</i> , <i>13xLexAop2-IVS-myr::smGdP::V5</i> (BDSC# 64092); <i>GMR26F05-LexA</i> (A08a neurons) (BDSC# 54702), <i>UAS-bruchpilot (short)-mstraw</i> ; <i>165-Gal4</i> (dbd neurons) were in-crossed to males of the same genotype.	Figure 4.1 A-C'
Females containing <i>10xUAS-IVS-myr::smGdP::HA</i> , <i>13xLexAop2-IVS-myr::smGdP::V5</i> (BDSC# 64092); <i>GMR26F05-LexA</i> (A08a neurons) (BDSC# 54702), <i>UAS-bruchpilot (short)-mstraw</i> ; <i>165-Gal4</i> (dbd neurons) were crossed to males containing either (i) <i>UAS-lacZ.Exel</i> (control) (BDSC# 8529) or (ii) <i>UAS-hid, tubP-Gal80ts/CyO</i> .	(i) Figure 4.3A-B', E-G Figure 4.6B, D, F, H, J-K Figure 4.3 Sup 1B-C'

	(ii) Figure 4.3C-D', E-G Figure 4.6C, E, G, I, J-K Figure 4.3 Sup 1B, D-D'
Females containing <i>10xUAS-IVS-myr::smGdP::HA</i> , <i>13xLexAop2-IVS-myr::smGdP::V5</i> (BDSC# 64092); <i>GMR26F05-LexA</i> (A08a neurons) (BDSC# 54702), <i>UAS-bruchpilot(short)-mstraw</i> ; <i>165-Gal4</i> (dbd neurons) were crossed to males containing either (i) <i>UAS-inactive TeTxLC</i> (BDSC #28840) or (ii) <i>UAS-TeTxLC-G2</i> (BDSC #28838)	(i) Figure 4.4A, E-F', H-I (ii) Figure 4.4B, E, G-I
Females containing <i>10xUAS-IVS-myr::smGdP::HA</i> , <i>13xLexAop2-IVS-myr::smGdP::V5</i> (BDSC# 64092); <i>GMR26F05-LexA</i> (A08a neurons) (BDSC# 54702), <i>UAS-bruchpilot(short)-mstraw</i> ; <i>165-Gal4</i> (dbd neurons) were crossed to males containing either (i) <i>UAS-lacZ</i> (BDSC #8529) or (ii) <i>UAS-shi[ts1].K</i> (BDSC #44222)	(i) Figure 4.4C, E, J- J', L-M (ii) Figure 4.4D-E, K- M
Females containing <i>10xUAS-IVS-myr::smGdP::HA</i> , <i>13xLexAop2-IVS-myr::smGdP::V5</i> (BDSC# 64092); <i>GMR26F05-LexA</i> (A08a neurons) (BDSC# 54702), <i>UAS-bruchpilot(short)-mstraw</i> ; <i>165-Gal4</i> (dbd neurons) were crossed to males containing (i) <i>20xUAS-IVS-CsChrimson.mVenus</i> (attP2) (BDSC# 55136) or (ii) <i>UAS-lacZ</i> (BDSC #8530)	(i) Figure 4.5B-D (ii) Figure 4.5A-A', C-D Figure 4.1 Sup 1

Animal Preparation

Embryo experiments

Dbd-Gal4 Expression (*Figure 4.1*): Embryos were collected overnight for 16hrs on 3.0% agar apple juice caps with yeast paste at 25°C. Embryonic stages were identified post-hoc by analyzing gut morphology. Stage 14, gut is tube shaped. Stage 15, gut is heart shaped. Stage 16, gut is coiled 3 times. Stage 17, gut is coiled 4 times.

Hid Validation (*Figure 4.3 – figure supplement 1*): Embryos were collected 4hrs on 3.0% agar apple juice caps with yeast paste at 25°C and were then aged at 30°C for 11hrs until approximately stage 17.

A08a-LexA Expression (Figure 4.1 – figure supplement 1)

Embryos were collected on 3.0% agar apple juice caps with yeast paste for 4 hours at 25°C. Embryos were then aged for 21 hours. After 21hrs, embryos were transferred to a fresh 3.0% agar apple juice cap and then aged for 4 hours. Half of the hatched larvae were immediately dissected (aged 2 +/- 2hrs ALH). The other half of the larvae were transferred to standard cornmeal fly food dishes and aged an additional 24hrs until dissection at 26 +/- 2hrs alh. Due to stochastic expression of A08a-LexA in newly hatched larvae, samples were stained for Corazonin as a VNC segment landmark. Corazonin labels cells in T2-A6 (Choi et al., 2005).

TNT Experiments (Figure 4.4)

Embryos were collected on 3.0% agar apple juice caps with yeast paste for 4 hours at 25°C. Embryos were then aged for 21 hours. After 21hrs, embryos were transferred to a fresh 3.0% agar apple juice cap and then aged for 4 hours. Hatched larvae were transferred to standard cornmeal fly food dishes and aged until dissection at 26 +/- 2hrs alh.

Shibire^{ts} and Hid experiments (Figure 4.3, Figure 3 – figure supplement 1, and Figure 4.4)

Embryos were collected on 3.0% agar apple juice caps with yeast paste for 4 hours at 25°C. Embryos were then aged for 17 hours at 30°C (Embryos and larvae develop 1.23x faster at 30°C). After 17hrs, embryos were transferred to a fresh 3.0% agar apple juice cap and then aged for 4 hours. Hatched larvae were transferred to standard cornmeal fly food dishes and aged at 30°C until dissection at 24 +/- 2hrs alh.

Chrimson Experiment (Figure 4.5)

All-*trans* retinal (ATR) is a necessary co-factor for CsChrimson. To ensure maternal transfer of ATR to larval progeny, parental crosses were fed yeast paste supplemented with ATR (final concentration 0.5mM; Sigma-Aldrich, R2500-100MG) for 72hrs. ATR yeast was made fresh

daily and kept away from light. Embryos were then collected on 3.0% agar apple juice caps with +ATR yeast paste for 4 hours at 25°C. Embryos and larvae were aged continuously under broad spectrum light, approximately 10cm from the light source (~30,000 lux; measured using Light Meter app for iPhone – Lightray Innovation GmbH). The temperature under the light was 28°C. Embryos age 1.1x faster at 28°C. After 19hrs, embryos were transferred to a fresh 3.0% agar apple juice cap and then aged for 4 hours. Newly hatched larvae were collected after the 4 hours and reared for an additional 23 hours under the light (larvae age 1.03x faster 28°C). Larvae were dissected at 25 +/- 2hrs in low light (<100 lux) to prevent further Chrimson activation.

Critical Period Experiments (Figure 4.6)

Embryos were collected on 3.0% agar apple juice caps with yeast paste for 4 hours at 25°C.

No Ablation Group: Embryos were aged for 42hrs at 18°C (Embryos and larvae develop 2x slower at 18°C). After 42hrs, embryos were transferred to a fresh 3.0% agar apple juice cap and then aged for 4 hours. Hatched larvae were transferred to standard cornmeal fly food dishes and aged at 18°C until dissection at 146 +/- 2hrs alh (25°C equivalent to 72hrs alh, middle of 3rd instar).

1st Instar Ablation Group: Embryos were aged for 42hrs at 18°C. After 42hrs, embryos were transferred to a fresh 3.0% agar apple juice cap and then aged for 4 hours at 30°C. Hatched larvae were transferred to standard cornmeal fly food dishes and aged at 30°C until dissection at 67 +/-2hrs alh (25°C equivalent to 72hrs alh, middle of 3rd instar).

2nd Instar Ablation Group: Embryos were aged for 42hrs at 18°C. After 42hrs, embryos were transferred to a fresh 3.0% agar apple juice cap and then aged for 4 hours. Hatched larvae were transferred to standard cornmeal fly food dishes and aged at 18°C for 48hrs. At this time animals were shifted to 30°C and raised an additional 44hrs until the time of dissection (25°C equivalent to 72hrs alh, middle of 3rd instar).

3rd Instar Ablation Group: Embryos were aged for 42hrs at 18°C. After 42hrs, embryos were transferred to a fresh 3.0% agar apple juice cap and then aged for 4 hours. Hatched larvae were transferred to standard cornmeal fly food dishes and aged at 18°C for 96hrs. At this time animals were shifted to 30°C and raised an additional 22hrs until the time of dissection (25°C equivalent to 72hrs alh, middle of 3rd instar).

No groups could be tested in which animals were reared continuously at 30°C until 3rd instar as all animals expressing dbd>Hid died.

Immunohistochemistry

Larval brain sample preparation

Larval brains were dissected in PBS, mounted on pre-EtOH treated 12mm #1 thickness poly-D-lysine coated coverslips (Neuvitro Corporation, Vancouver, WA, Cat# GG-12-PDL) (primed in 70% EtOH at least one day prior to use). Samples fixed for 23 minutes in fresh 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA, Cat. 15710) in PBST. Samples were washed in 0.3% PBST and then blocked with 2% normal donkey serum and 2% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBST overnight at 4°C or for 1hr at room temperature. Samples incubated in primary antibody for two days at 4°C. The primary was removed, and the samples were washed with 2 quick PBST rinses followed by 3x20min washes in PBST. Samples were then incubated in secondary antibodies overnight at 4°C, shielded from light. The secondary antibody was removed following overnight incubation and the brains were washed in PBST (2 quick rinses, followed by 3x20min washes). Samples were dehydrated with an ethanol series (30%, 50%, 75%, 100% ethanol; all v/v, 10 minutes each) (Decon Labs, Inc., King of Prussia, PA, Cat. 2716GEA) then incubated in xylene (Fisher Chemical, Eugene, OR, Cat. X5-1) for 2x10 minutes. Samples were mounted onto slides containing 2 drops of DPX mountant (Millipore Sigma, Burlington, MA, Cat. 06552) and cured for 1-3 days then stored at 4°C until imaged.

Embryo sample preparation

Embryos were transferred from apple caps into collection baskets and rinsed with dH₂O. Embryos were dechorionated in 100% bleach (Clorox, Oakland, CA) for 3min and 30sec with gentle agitation. Dechorionated embryos were rinsed with dH₂O for 1min. Embryos were fixed 25mins in 2mL Eppendorf tubes containing equal volumes of Heptane (Fisher Chemical,

Eugene, OR, H3505K-4) and 4% PFA diluted in PBS. Fix was removed, and 850uL of Heptane was added to each tube. 650uL of Methanol were then added, and tubes were then subject to vigorous agitation for 1min in a step required for removing the vitelline membrane. Nearly all liquid was removed from the tubes, leaving the embryos. Embryos were rinsed in Methanol (Fisher Chemical, Eugene, OR, Lot# 206197, Cat. A412P-4) twice followed by 2 quick rinses in 0.3% PBST. PBST was removed and embryos were blocked with 2% normal donkey serum and 2 % normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBST for 1hr at room temp. After blocking, embryos were incubated in primary and secondary antibodies and mounted as described above for larval brains.

Light Microscopy

Fixed larval preparations were imaged with a Zeiss LSM 710 or LSM 900 laser scanning confocal (Carl Zeiss AG, Oberkochen, Germany) equipped with an Axio Imager.Z2 microscope. A 63x/ 1.40 NA Oil Plan-Apochromat DIC m27 objective lens and GaAsP photomultiplier tubes were used. Software program used was Zen 2.3 (blue edition) (Carl Zeiss AG, Oberkochen, Germany). For each independent experiment, all samples were acquired using identical acquisition parameters.

Image processing and analysis

Imaris Filament reconstruction and quantification of A08a dendrites (Figures 4.3-6)

Confocal image stacks were loaded into Imaris 9.5.1(Bitplane AG, Zurich, Switzerland). A08a dendrites from A1 and A2 segments were analyzed. A new Imaris Filament object was created for each A08a dendrite (lateral and medial). Briefly, the Filaments tool was selected, and a region of interest (ROI) drawn to encompass the dendrite. The source channel for A08a membrane (488) was selected. An approximation for minimum and maximum dendrite diameters were measured in Slice view, and found to be 0.2 μ m and 1 μ m respectively. These values were used to identify Starting Points and Seed Points for all images. Thresholds for Starting Points and Seed Points were manually adjusted until 1 Starting Point on the main A08a dendritic shaft

remained, and Seed Points labeled A08a dendrite signal without labeling image background. The option to Remove Disconnected Seed Points was selected, with a Smoothing factor of 0.2 μ m. Absolute Intensity Threshold was manually adjusted until all Seed Points were filled. When the Filament was rendered, misidentified structures were selected and manually deleted or adjoined.

The sum length and the sum of all branch points of each dendritic arbor were calculated automatically in Imaris (Statistics > Details > Average Values). The values for left/right lateral dendrites and left/right medial dendrites were averaged for each animal.

For Figure 3G, the length of the A08a Left-Lateral Dendrite, Left-Medial Dendrite, Right-Medial Dendrite, Right-Lateral Dendrite was summed together, as left and right A08a's form recurrent synaptic connections. The values were normalized to the mean total dendrite length of the WT controls (with 4 dbd inputs).

Samples were excluded from analysis if there was damage to the tissue or low signal-to-noise that obstructed the ability to reliably identify dendrite membrane signal.

A08a cumulative dendrite position (Figure 4.2D)

Published data from Sales et al., 2019 and Valdes-Aleman et al., 2021 were used. Larval brains aged 24 +/- 4hrs alh were processed and imaged as described in Sales et al., 2019 and Valdes-Aleman et al., 2021. Briefly, image processing and analysis was performed using FIJI (ImageJ 1.50d, <https://imagej.net/Fiji>). Stepwise, images were rotated (Image > Transform > Rotate(bicubic)) to align dendrites of interest along the x axis, then a standardized ROI was selected in 3D to include the dendrites to analyze in one hemisegment (Rectangular selection > Image > Crop). To identify the voxels that contain dendrite intensity, a mask was manually applied (Image > Adjust > Threshold). The threshold was assigned to include dendrite positive voxels and minimize contribution from background. To quantify the amount of dendrite positive voxels across the medial-lateral axis, images were reduced in the Z-dimension (Image > Stacks > Z-project > Sum Slices) and a plot profile was obtained to measure the average voxel intensity (Rectangular selection > Analyze > Plot profile). The cumulative sum of A08a dendrite voxels was calculated for each individual hemisegment, and a mean voxel distribution was generated from the population data.

Branch distribution (Figure 4.2E-F)

Published data from Sales et al., 2019 and Valdes-Aleman et al., 2021 were used. Hemisegments from A1 and A2 were analyzed. Image analysis was performed in FIJI. Images were rotated to align the dendrites of interest along the x-axis (Image > Transform > Rotate(bicubic)). For each hemisegment analyzed, a rectangular ROI was drawn starting at the midline and ending at the lateral edge of the lateral-most branch point coming from the main A08a neurite. The width of the ROI was logged (in microns). The Cell Counter plugin was used to count the dendrite branches originating from the main A08a neurite (Plugins > Analyze > Cell Counter). The x position of each branch point was measured (in microns) (Cell Counter > Measure). The relative lateral-medial position of each branch point was determined by dividing the branch's x position by ROI width. The relative frequency of branches at a given position was determined by counting the number of branches for that bin, and dividing that value by the total number of branches.

For Figure 2F, Lateral, Intermediate, and Medial domains were determined based on the relative peak branch positions in LacZ controls in Figure 1E. The Lateral domain = <0.35 , Intermediate = ≥ 0.35 and <0.5 , and Medial = >0.5 . The proportion of branches in each domain was determined for each cell, and was then plotted as a single point in Figure 1F.

Figure preparation

Micrographs in figures were prepared as either 3D projections in Imaris 9.5.1 (Bitplane AG, Zurich, Switzerland) or maximum intensity projections in FIJI (ImageJ 1.50d, <https://imagej.net/Fiji>). Scale bars are given for reference on maximum intensity projections but do not necessarily represent actual distances, as the tissue samples undergo changes in size during the tissue clearing protocol. For images exported from the Imaris software, the scale bars are assigned to match the scale at the “center” of the 3D projection. Pixel brightness was adjusted in some images for better visualization; all adjustments were made uniformly over the entire image, and uniformly across corresponding control and experimental images. Larval

crawling traces in Figure 4.3 – figure supplement 1 are temporal projections made in FIJI (Image > Hyperstacks > Temporal Color-Code).

Larval behavior assays

TNT experiments

Newly hatched larvae were aged for 24 hours on standard cornmeal fly food at 25°C. At this time, 5 larvae were gently transferred to a 4x4cm 1.2% agarose (Sigma, Lot# SLCD4639, Cat. A9539-500G) arena. Larvae were spaced apart to prevent collisions during recording. Prior to recording, larvae were acclimated to the arena for 2 minutes. The ambient temperature during recording was 20-22°C. Videos of individual larvae were collected at 5 frames/second for 1 minute.

Shibire experiments

Newly hatched larvae were aged for 22 hours on standard cornmeal fly food at 30°C. At this time, individual larvae were transferred to a 0.5mm thick 1.2% agarose pad positioned on top of a 22x40mm coverslip (Corning, Lot# 14418013, Cat. 2980-224, #1.5 thickness). The larva and coverslip were placed on top of a CherryTemp microfluidic chip (Cherry Biotech, Montreuil, France) with an 18°C surface temperature. Larvae were acclimated to the surface for 2 minutes prior to recording. Videos of individual larvae were collected at 5 frames/second for 1 minute. For all behavior experiments, animals that failed to move during the 1-minute recording were excluded from analysis.

Analysis of larval locomotor behavior

Movie processing and analysis was performed using FIJI (ImageJ 1.50d, <https://imagej.net/Fiji>). Locomotor waves were manually quantified. Forward and backward waves were summed together for each animal. The number of waves for each animal was normalized to the control average of each independent experiment.

Statistical analyses

Statistics were computed using Excel or Python (scipy.stats). All statistical tests used are listed in the figure legends. Statistical outliers were identified and removed by computing the 1st and 3rd quartiles for each group and setting upper and lower thresholds that deviated above or below the interquartile range (IQR) (lower threshold = 1st Quartile – 1.5*IQR; upper threshold = 3rd quartile + 1.5*IQR). P-values are reported in the figures. n.s. = not significant, where $p > 0.05$. Plots were generated using Excel or Seaborn and Matplotlib packages in Python.

CHAPTER V

DISCUSSION

The work in this dissertation began with two observations: (i) neurons can exhibit remarkable dendritic specificity with respect to where they form synapses with other neurons, and (ii) despite this specificity, other aspects of neural circuit architecture such as neurite morphology, synapse number, and the precise location along a dendrite where synapses form are more variable. I therefore wanted to understand how neurons achieve dendritic synapse specificity, especially when the nervous system is crowded with many other potential synaptic partners. I also wanted to know what developmental algorithms are in place that enable a neuron to adapt to variability in presynaptic neurite placement, morphology, and synaptic strength and achieve circuit function.

The establishment of dendritic synaptic specificity

To determine the mechanism by which a neuron comes to selectively synapse at a particular dendritic arbor, we introduced a new model system consisting of the *Drosophila* larval A08a interneuron and dbd sensory neuron. A08a has two spatially distinct dendritic arbors, one medial and one lateral. Every input onto the A08a dendrites either synapses with one dendrite or the other, never both (Table 1). Dbd synapses with the medial dendrite. How does dbd come to synapse with the A08a medial dendrite? We postulated that (i) either the two A08a dendrites are molecularly distinct – expressing different sets of cell adhesion molecules that could bias dbd to synapse with the medial dendrite, or (ii) dbd is guided to the medial neuropil through axon guidance cues and synapses with the medial A08a dendrite because it is more proximal. We could discern between these two hypotheses by misrouting the dbd axon to the lateral A08a dendritic arbor. We reasoned that if the two arbors are molecularly distinct, that dbd would be less likely to form synapses with the lateral arbor when targeted there. However, if the observed dendritic specificity in wild-type is a matter of where dbd is guided, and the A08a dendrites are otherwise molecularly equivalent, then dbd should be able to form synapses when targeted to the lateral A08a dendrite.

We found the latter hypothesis was confirmed: that dbd and A08a were competent to form synapses across the entire A08a dendritic domain, and therefore the wild-type dendritic synapse specificity is likely a product of where in the neuropil the dbd axon is targeted by axon guidance cues. This result is compatible with previous descriptions of wild-type dbd axon guidance, where the axon takes a dorsal route through the developing neuropil before projecting ventrally once it reaches the medial neuropil (Schrader & Merritt, 2000; Zlatić et al., 2003). This dorsal route through the neuropil would likely preclude dbd's ability to ever contact the A08a lateral dendrite. The use of axon guidance cues to establish circuit connectivity offers a layer of robustness to potential variability in neurite position that could arise during development. The presumed lack of cell adhesion molecules specifying precise dbd-A08a connectivity at the medial dendrite and prohibiting dbd-A08a connectivity at the lateral dendrite means that the circuit would still function even if the dbd input is not perfectly aligned with the A08a medial dendrite, as we demonstrated in Chapter III.

Does this mean that all cellular and dendritic synaptic specificity is a product of spatial proximity alone, an idea referred to as “Peters’ Rule” (Rees et al., 2017)? Spatial proximity is an obvious requirement for two neurons to synapse, but it is not sufficient for them to do so. In a parallel study to ours, Valdes-Aleman et al. showed using connectomics that when they misrouted the location of a sensory neuron axon from the medial to the lateral neuropil, it retained most of the same synaptic partners while adding only one new partner (Valdes-Aleman et al., 2021). This result indicates that even with increased access to non-synaptic partners, a neuron still retains its original cellular synaptic specificity. Perhaps axon guidance cues are sufficient to establish subcellular or dendritic synaptic specificity within our dbd-A08a model system, but cellular synaptic specificity is gated through chemoaffinity-based cues.

Linking dendritic synaptic specificity to dendrite establishment

In our work determining the cellular mechanism that promotes dendritic synapse specificity between dbd and A08a, we used the misexpression of repulsive axon-guidance receptors as tools to relocate the dbd axon. The misexpression of one of these receptors, Robo-2, targeted the dbd axon to an intermediate location between the medial and lateral A08a dendrites. In wild-type animals, this intermediate location contains very little dendritic material. We found that when

Robo-2 was used to target dbd to the A08a intermediate dendritic domain that ectopic dendrites branched off the main A08a neurite, matching the location of the presynaptic input. Similar results were observed when the Unc-5 receptor was used to target the dbd axon to the A08a lateral dendrite. During these manipulations, the medial A08a dendrite was also reduced in volume and branching. These results provide evidence that the dbd presynaptic axon can promote the local outgrowth of postsynaptic dendrites. This has interesting implications on the establishment of dendritic synaptic specificity. If a presynaptic axon can establish a postsynaptic dendrite, this would imply that dendritic synaptic specificity can arise simply as a matter of where an axon is targeted rather than there being a choice among potential dendrites. As long as the gross targeting of the axon is reliable, then development can yield the same axon-dendrite connectivity and functional circuit output.

A limitation to the work presented in this dissertation is that the full extent of A08a dendrite development is unknown. The driver used to label A08a is expressed beginning at a point in larval development after the lateral and medial dendrites have started to take form, and after dbd has synapsed with A08a. Drivers that enable earlier access to A08a remain to be discovered, but could potentially be identified through determining the neuron's neuroblast of origin. VNC neuroblasts are specified through the expression of spatially restricted transcription factors (Doe & Technau, 1993), the identities of which could enable the engineering of a new developmental driver for A08a using the Split Gal4 system (Luan et al., 2006; B. D. Pfeiffer et al., 2010). Once a method for early monitoring of A08a dendrite development is established, important questions will become testable, including (i) are A08a dendrites induced by presynaptic partners, or do the dendrites initiate outgrowth themselves and then become stabilized by presynaptic partners? And (ii) what is the temporal relationship between dbd-A08a contact, synapse formation, and subsequent A08a dendrite elongation?

Until such tools are developed, previous work on *Drosophila* motor neuron dendrite development can help generate predictions about the mechanisms and molecules used to potentially facilitate contact-mediated dendrite outgrowth between dbd and A08a. The larval aCC motor neuron in particular is a well-studied model for dendrite development. aCC begins dendritogenesis 13 hours after egg laying (Kamiyama et al., 2015). Kamiyama and colleagues found that the initiation of aCC dendrite outgrowth coincides with when the cell receives presynaptic input from the pioneering interneuron MP1. MP1 expresses the Dscam1 cell

adhesion molecule, which is required to interact with aCC and induce the local outgrowth of dendrites (Kamiyama et al., 2015). Perhaps a similar mechanism underlies the observed ectopic A08a dendrite formation when dbd is targeted to the intermediate zone between lateral and medial dendrites. Dscam1 will be an interesting candidate to test, and additional candidates for molecules that can promote A08a dendrite elongation could be generated in the future through single-cell RNA sequencing of dbd and A08a, and subsequent identification of cognate ligand/receptor pairs (Voss et al., 2022).

It is important to make clear that not all neurons will share in this mechanism of dendrite induction/stabilization. *Drosophila* sensory neurons, for example, do not have presynaptic partners, and therefore their dendrites cannot be induced or stabilized by the axon of another neuron. Rather, cell-intrinsic genetic programs specify the morphology of their dendrites (Corty et al., 2016; Moore et al., 2002). The dendrites of Kenyon Cells, the primary neurons composing the *Drosophila* associative learning center, can form in the absence of most inputs (Elkahlah et al., 2020). Even dendrites on the same neuron can respond differently to the presence of a presynaptic input. Ablation of trigeminal sensory afferents to the zebrafish Mauthner lateral dendrite has no effect on the development of the dendrite (Kimmel et al., 1990), whereas ablation of vestibular afferents stunts its development (Kimmel et al., 1982). These results in the zebrafish demonstrate that presynaptic axons may not all possess a uniform ability to promote local dendrite elongation, making the identification of the molecular cues that promote dendrite elongation all the more intriguing.

A critical period for homeostatic dendrite elongation

Through both ablating dbd and manipulating dbd activity, we found that A08a can alter its dendrite length and branching in a homeostatic manner. Reducing excitatory activity from dbd through ablation or silencing can increase A08a dendrite length neuron-wide, and increasing dbd activity can decrease A08a dendrite length. We propose that the inhibitory action of presynaptic activity on postsynaptic dendrite elongation could be used as a negative feedback mechanism to prevent run-away dendrite elongation promoted by presynaptic contact.

Curious about whether A08a dendrite length could be modified in response to changing levels of presynaptic input throughout larval life, we ablated dbd at successively later stages of

larval development and measured A08a dendrite length. Similar to a recently discovered critical period for *Drosophila* motor dendrite plasticity (Ackerman et al., 2021), we found that A08a dendrites elongated only when dbd was ablated in newly hatched larvae. The critical period for motor dendrite homeostatic plasticity is regulated by the infiltration of astrocytes into the neuropil. Astrocytic processes contact motor neuron dendrites through a Nlg-2/4 and Nr1-1 interaction, reducing the overall dynamicity and ability of dendrites to extend and retract in response to activity (Ackerman et al., 2021). Our critical period results suggest that VNC dendrite plasticity may generally be downregulated by astrocytes; it will be necessary to test this hypothesis, perhaps through dual astrocyte and dbd ablation studies.

Structural homeostatic plasticity is one strategy used to prevent too much or too little excitation, and maintain consistent circuit output. If dendrites are not readily altered in later stages of development to maintain circuit homeostasis, it is possible that circuit homeostasis is regulated during these later stages at the site of the synapse. Scaling of neurotransmitter receptors, ion channels, presynaptic release sites, or quantal content of neurotransmitter vesicles are all mechanisms observed in various invertebrate and vertebrate systems to maintain circuit homeostasis, and may allow a neuron to work within the confines of glial cells that restrict more dramatic dendrite rearrangements (Ackerman et al., 2021; Frank, 2014; Marder & Goaillard, 2006; Turrigiano, 2012).

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