JACK-OF-ALL-TRADES, THE ROLE OF ASTROCYTES IN CIRCUIT FORMATION AND PLASTICITY

by

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A THESIS

Presented to the Department of Biological Science
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June, 2020

An Abstract of the Thesis of

Nelson A. Perez-Catalan for the degree of Bachelor of Science

in the Department of Biology to be taken June of 2020

Title: **Jack-of-all-trades, The Role of Astrocytes in Circuit Formation and Plasticity**

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Thesis Abstract

Neurons are electrically excitable cells that transmit information throughout the nervous system with high speed and accuracy. This is largely facilitated by their specialized morphology, with dendrites receiving diverse information, to axons propagating the message to specific neighboring neuronal partners. During development, neuronal networks undergo rapid changes, ranging from short-term changes on the order of milliseconds, to long-term modifications in neural architecture that could last as long as the lifetime of the organism. This 'plasticity' ensures that neuronal networks, or circuits, undergo constant checks during development, while also facilitating a degree of adaptability that acts as the basis for learning and memory. The mechanisms that the nervous system employs to establish the correct connections and regulate plasticity remain a poorly understood topic in neuroscience. Research in both mammals and invertebrates, including *Drosophila*, have defined that glial cells are capable of instructing neurons to find partners to form synapses, a specialized chemical junction between two neurons where electrical signals propagate. More specifically, studies in astrocytes, the most abundant glial cell subtype in the central nervous system, have demonstrated that while neurogenesis precedes astrogenesis in the cortex, neuronal synapses only begin to form after astrocytes have been generated (explored in Chapter 1). Astrocyte development is crucial for circuit formation in the nervous system, and their dysfunction can lead to neurodevelopmental, neurodegenerative, neuroimmune, and neoplastic diseases, such as ALS and Alzheimer's. This thesis explores a subset of the mechanisms employed by the nervous system to regulate circuit plasticity and circuit establishment during development, with a specific focus on astroglia.

My first goal was to characterize plasticity within a model neural circuit during development. In the second chapter of this thesis, I use the highly specialized genetic toolkit available for *Drosophila* to characterize the structural dynamics of motor neuron dendrites during development *in vivo* by utilizing fluorescence microscopy. By manipulating neuronal activity in my model motor neurons, I show that the presence of stable microtubule populations within dendrites is directly correlated with structurally stable arbors. Furthermore, overexpression of the cell adhesion molecule Neurexin in motor neurons led to the increased stability of microtubule populations within dendritic arbors. Finally, I demonstrate that astrocytes are required to restrict motor dendrite plasticity to newly hatched larva. Interestingly, astrocytes robustly express Neuroligins, which are binding partners for Neurexin, suggesting that astrocyte-secreted proteins are capable of directly regulating neuronal morphology and plasticity.

Previous studies *in vitro* have shown that in addition to regulating circuit plasticity, astrocyte-derived secreted and cell surface molecules (CSMs) can modify synaptogenesis during circuit development. In a separate line of questioning, I explore the role of astrocyte-secreted and cell surface proteins in the formation of excitatory cholinergic synapses *in vivo* (described in Chapter 3). Specifically, I took part in a reverse genetic screen to knock down astrocyte-derived proteins using commercially available RNAi lines. Concurrently, we labeled both neuronal membranes and their pre-synaptic sites (Brp+) using Synaptic Tagging with Recombination (STaR) to assess non-cell autonomous changes in synapse number. We performed two parallel screens, the first labeled individual dorsal bipolar dendritic (Dbd) sensory neurons. The second targeted neurons that generate synapses localized in the mushroom body, a memory and

learning center in the Drosophila brain. Excitingly, the major astrocyte-secreted molecules that induce synapse formation (e.g. TGF-β) or inhibit synapse development (e.g. SPARC) in vertebrates are conserved in fly, and we identified fourteen novel genes (of 245 tested) required in astrocytes for synaptogenesis.

In sum, this work further characterizes dendritic dynamics during a critical period in *Drosophila* development. My data shows that altered neuronal activity in aCC/RP2 motor neurons within a critical period of motor circuit plasticity causes significant dendritic remodeling within minutes, and that astrocytes are required for proper critical period closure. Further, I demonstrate that the ablation of astrocytes post-critical period induces abnormal period of heightened plasticity. Finally, this work provides direct evidence of the key regulatory function of astrocytes in synaptogenesis, and their role in regulating global synapse formation in the central nervous system.

Acknowledgements

This thesis is dedicated to my wife, Holly. Just like astrocytes, your support and your efforts have always steered me to function correctly. Thank you for always being there for me.

I would also like to acknowledge my mentor SD Ackerman. Thank you for teaching me that a scientist always goes beyond the bench and the desk. You are a terrific mentor, and I have no doubts that you will also be a fantastic PI.

To the folk of the Doe lab. Each of your stories and teachings have impacted my personality in tremendous ways. You are a special group encompassing all sorts of values and backgrounds. Thank you for making me feel at home, and helping me realize that my accent is also part of my story.

Finally, I would like to extend my gratitude to the ICSP program at the UO, and the wonderful staff of the CHC. You made my hopes for higher education go from dream to reality.

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Introduction / Chapter 1: Molecular and cellular mechanisms of neural circuit development

Neuronal development

The nervous system is one of the earliest organ systems to begin forming during development, and the last to be completed after birth[1]. The developmental mechanisms that guide nervous system formation are complex, and genetic or physical insults during this temporal window may have critical consequences to cognitive function and behavior into adulthood[2,3,4]. Some forms of epilepsy, for example, are thought to arise from failed developmental programs that regulate neuronal activity, causing the person to become hypersensitive to environmental triggers into adulthood[5]. Accordingly, understanding the basic mechanisms that instruct and regulate neural development is of pivotal medical importance.

The nervous system is an intricate network composed of two primary subtypes of cells, neurons and glia, the latter remaining poorly understudied until recently (see below) [6]. At the center stage of the nervous system, neurons have evolved to transmit electrochemical signals with high speed and accuracy[7]. There are three basic components to neuronal architecture across animal species: soma, axons, and dendrites[8] (Figure 1a). The soma, also called cell body, is a bulbous structure that contains the nuclear DNA of the neuron[9]. The phrase "neurons talk to each other" derives from the transmission of an action potential (electrical signal) from the axon of one neuron to the dendrite of another[10]. Axons are output units composed of highly specialized projections which conduct electrical impulses away from the soma. Dendrites are fine,

multibranched extensions, capable of receiving input from thousands of neighboring cells[11]. When the axon of one neuron comes into close proximity to the dendrite of another during development, they could associate through a synapse. A synapse is a 20-40 nanometers wide conduct capable of passing chemical (neurotransmitter) signals, axon to dendrite, following action potential propagation through the pre-synaptic neuron[12-13] (Figure 1b). In humans, gestational week 34 marks entry into the peak period of synaptogenesis, during which almost 40 000 new synapses are formed every second, a process that continues well into early postnatal life[13]. Not all synapses are created equal; the wide variety of neurotransmitters, such as GABA (inhibitory) and cholinergic (excitatory), allow circuits to carry out complex behavioral tasks, such as engaging and relaxing subsets of muscles during speech generation[14].

Although the basic neuronal structure (dendrite, soma, axon) is conserved across chordates, genetic variation within an organism allows for the generation of a large diversity of neuron types, each with a unique role and function[15] (Figure 2). For example, a simple motor circuit could be composed of excitatory pre-motor neurons that initiate a signal, inhibitory interneurons that regulate the strength of the message, and subsequent activation of several motor neurons that innervate muscle filaments to initiate locomotion[16] (Figure 3a). Regardless of circuit complexity, excitatory and inhibitory components function together (E/I balance) in neuronal networks to achieve the appropriate level of activity needed for stereotyped motor behaviors[18], from bipedal locomotion in humans to wing flapping in flies[5, 17].

Neural circuit development

A major goal of developmental neuroscience is to understand how neural circuits are formed, *e.g.* how do neurons find their appropriate locations in the nervous system, and how do axons/dendrites subsequently extend, find appropriate partners, and synapse to create a functional network. Here I will expound on synaptogenesis and circuit refinement, which are the primary focus of this thesis (Chapters 2-3). Behaviors such as locomotion require synaptic connections to be precisely tuned to engage muscles with precision, and functionally integrate with other circuits to respond to environmental cues during navigation[18]. During synaptogenesis, neurons begin to receive input from heterogeneous groups of neurons, including cells equipped with varying morphologies and neurotransmitter identities[19] (Figure 3b). The timing of synaptogenesis differs across circuits (*e.g.* sensory versus motor), which forces nervous systems to continually integrate new circuits as they develop, suggesting that global regulators of circuit formation exist to coordinate circuit integration across long developmental stretches[20]. Once synapses are established, circuits undergo a long process of refinement and modification, which includes strengthening of functional synapses and elimination of unnecessary connections, to ensure robust circuit function.[21,22]

Developmental studies using both vertebrate and invertebrate animal models have uncovered a large number of molecular regulators of circuit development[24-23]. Recently, the importance of cell surface molecules (CSMs) in circuit development has come to light. As CSMs are physically attentive to both cell intrinsic cascades and signaling from neighboring cells, they're poised to instruct both synaptogenesis and

circuit refinement[24]. For example, the CSMs Neurexins/Neuroligins (receptor/ligand pair) have been shown to regulate the proper number, distribution, and function of synaptic connections during development, and are generally considered to be synaptic organizers. Both Neurexins and Neuroligins act trans-synaptically by organizing neurotransmitters, synaptic receptors, and synapse excitability[25]. For example, overexpression of full-length or truncated versions of Neuroligins in neurons can increase synapse protein accumulation at the terminals, leading to abnormal activity recordings in excitatory and inhibitory postsynaptic currents[26]. Moreover, *in vitro* studies showed that blocking synaptic activity between neuronal partners reduced the ability of Neurexin/Neuroligin to perform their "synaptogenic" role, suggesting that they stabilize and/or strengthen existing synapses in an activity dependent manner[27]. How Neurexins/Neuroligins are regulated in response to changes in activity is an unresolved question, but their stabilization role in so many aspects of synapse and circuit development has gained a lot of attention in the medical community[28]. Indeed, recent studies on patients with familial Autism have linked disease predisposition with abnormal mutations on Neurexin-1β, suggesting that Neurexin signaling may be a viable therapeutic target for diseases such as Autism [29].

Neuronal plasticity regulates circuit formation and function

During development, the organization and function of circuits can change rapidly to facilitate the rise of stereotyped behavior. These adaptive changes, also known as plasticity, are driven by patterns of neuronal activity generated by both external sensory experience and internal sources[30]. For example, the production of speech in humans requires the integration of auditory, somatosensory, and motor

circuits, all of which develop at different times [31]. Accordingly, the circuits underlying individual sensory modalities required for human speech experience activity-dependent feedback in specialized waves called "critical periods" to integrate connections across sensory systems over time (multisensory integration), tuning the circuit into an intricate network capable of producing speech behavior[32]. Critical periods of plasticity are abundant during early development and are capable of enacting circuit changes that are remarkably long lasting. During critical periods, modified activity across a synapse can alter neuronal architecture (*homeostatic structural plasticity*), which can also affect their ability to synapse with neighboring neurons[22]. Moreover, studies on epilepsy, a disease marked by hyperexcitable neurons, propose that the neuropathy arises from an improper closure of critical periods of circuit plasticity as a result of unresponsive "off-switches" extending far into adulthood, underscoring the importance of precise critical period closure [9, 33].

Microtubules, the cytoskeleton that shapes neuronal morphogenesis

Both circuit development and circuit plasticity require precise changes to neuronal morphology. These changes are governed by the careful coordination of the neuronal cytoskeleton, comprised of microtubules (MTs), actin, and intermediate filament networks[34]. In contrast to other cytoskeletal filaments, which are composed of a variety of different fibrous proteins, MTs are composed of a single type of globular protein, called tubulin[35]. MTs are dynamic structures that undergo continual assembly and disassembly within the cell[14]. Neurons depend on the highly dynamic MT cytoskeleton for many different processes during early embryonic development including cell division and migration, intracellular trafficking and signal transduction,

as well as proper axon guidance and dendrite arborization [36]. Moreover, in mature neurons, MTs continue to maintain the structure of axons and dendrites, and serve as tracks for intracellular trafficking, allowing motor proteins to deliver specific cargoes within the cell [12]. For some time, it was thought that dendritic spines were devoid of MTs, and that actin was the main regulator of spine morphology and dynamics associated with synaptic plasticity. However, within the last decade, the use of new visualization techniques revealed that MT are present at the tips of dendritic filopodia, and may play a large role in synaptic development and synaptic plasticity [37]. Further, many neurodevelopmental disorders including Autism, a putative critical period disease have been associated to mutations in MT stability [38, 39,40]. The link between regulatory signals at the synapse level during activity-induced plasticity and implementation of changes to cytoskeletal proteins such as MTs to reshape neuronal structural stability remains poorly defined.

Neuron-glia networks power the nervous system

Neuronal morphology is not only influenced by intrinsic changes to cytoskeletal networks, but by the interaction between neurons and an equally numerous cell population in the brain called glia. To ensure proper network function, the nervous system requires highly specialized maintenance provided by glial cells, who collectively instruct, support, and guide neuronal activity beginning early on in circuit development formation[41]. Glial cells were first identified by the 19th century's leading neuroscientists including Rudolf Virchow, Santiago Ramón y Cajal and Pío del Río-Hortega. At that time, glia were thought to solely function as "Nervenkitt" (the German word for nerve glue). This is also reflected in the name "glial cell" derived from the

ancient Greek word “glía” meaning “glue” in English[43]. Although they were discovered over a century ago, glia were only attributed a passive support role to neurons, largely due to being electrically inactive and therefore thought to be unimportant to circuit function.

With technological improvements over the past couple of decades, we now appreciate that glia function as master regulators of nervous system development, controlling numerous aspects of synaptogenesis, plasticity, and disease [42]. Evidence suggests that astrocytes, a subtype of glia, are integral and functional elements of synapses, capable of responding to circuit activity and regulating synaptic strength [43]. Indeed, astrocytes have been described to be actively involved in the processing, transfer, and storage of synaptic information. Reports suggest that a single human astrocytes is capable of supporting up to 2 million synapses[44]. Astrocytes, therefore, have the potential to modulate inter-neuronal communication and locally integrate information from an amazingly large number of synapses, providing exceptional computational power. These functions of astrocytes challenge the neuro-centric notion that brain function results exclusively from neuronal activity, and promotes the idea that the effectiveness of the nervous system actually arises from neuron–glia networks.

Astrocytes are key players of neural circuit assembly and support

As aforementioned, research over the past two decades describe astrocytic roles in a range of brain functions far beyond basic metabolic support[45]. Astrocytes ensheathe multiple synapses by extending thousands of processes across a large radius. Astrocytic processes are highly dynamic, and their ability to shapeshift (extend/retract) may further expand their impact on the circuit network if each process is capable of supporting more

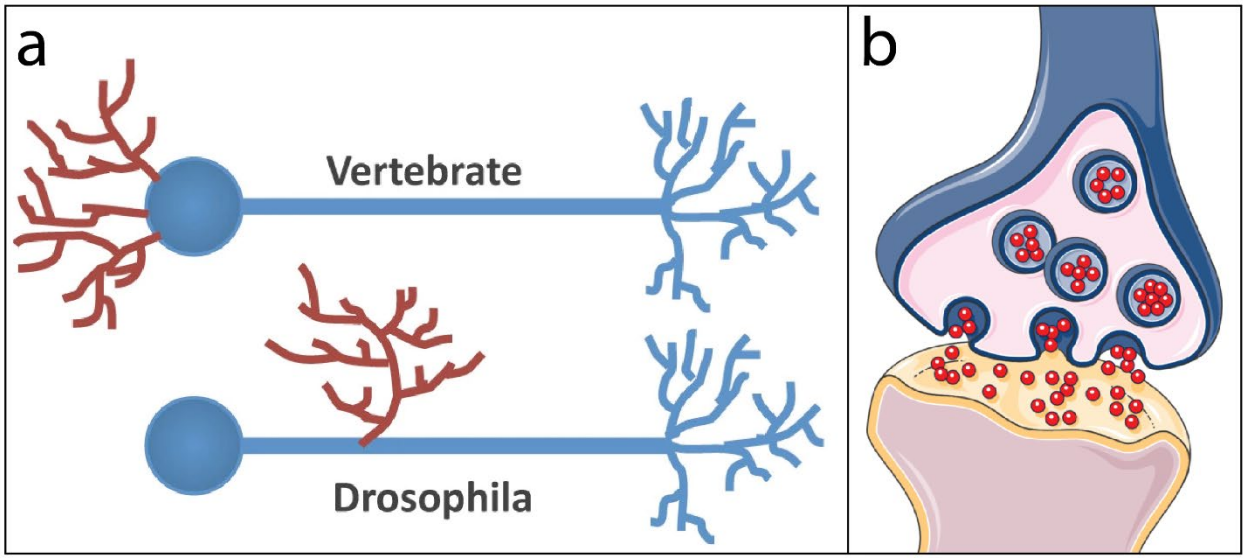
than one synapse at a time [46]. At the synapse, astrocytes express highly specialized proteins that support neuron function, including metabolite transport, neurotransmitter recycling, and modulation of synaptic strength. Through such networks, astrocytes can also buffer ions including potassium and sodium, as well as neurotransmitters such as acetylcholine (ACh) at cholinergic synapses[47]. Further, astrocytes are connected to each other through gap junctions that support intercellular communication, connecting the synapses they infiltrate to a large network of glia modulating circuit communication and nutrient availability[48]. Accordingly, neurons are highly dependent on astrocytic functions at the synapse, and ablation of astrocytes alone causes neural dysfunction and premature death[49].

Project Summary

In this thesis, I will use *Drosophila* larvae as a model system to interrogate the role of astrocytes in circuit plasticity during development. In chapter 2, I show that stable microtubule populations are crucial in stabilizing motor neuron dendritic arbors, and that Neurexin-1 signaling increases MT stability. Concomitantly, I demonstrate that astrocytes are essential for closing a critical period of motor neuron plasticity, likely via astrocyte Neuroligin to motor neuron Neurexin signaling to stabilize dendritic MTs. In chapter 3, I describe astrocytic morphology as a function of synaptogenesis, and the progression of astrocyte-synapse ensheathment during development. Finally, I describe my contribution to a genetic screen aimed at defining the mechanisms used by astrocytes to regulate synaptogenesis, and thus circuit development, supporting the critical role of glial cells in instructing and maintaining neural circuits. In sum, this thesis furthers our understanding of astrocyte dynamics *in vivo* in a developmental

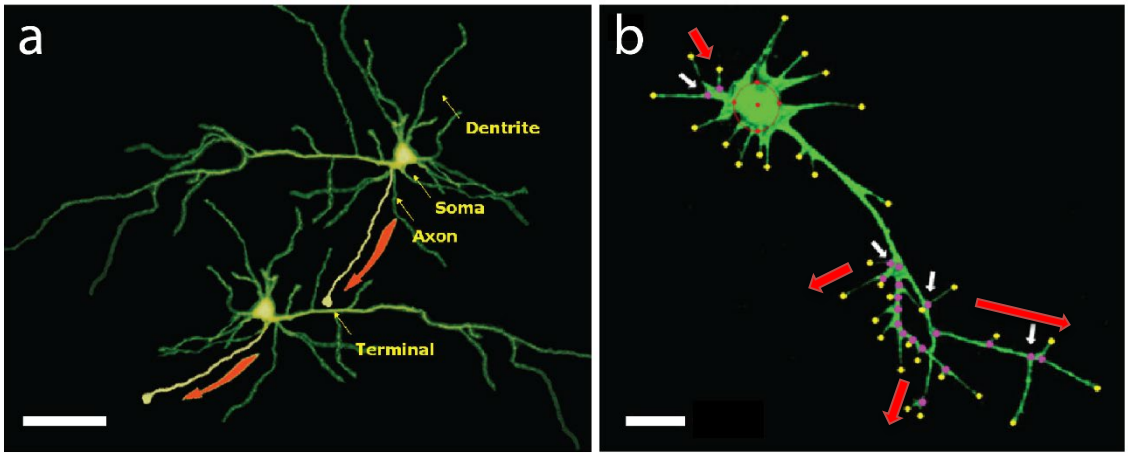
context, and may prove advantageous for the development of medical therapies to address phenotypes in neurodevelopmental disorders and progressive neuropathies.

Chapter 1 Figures and Legends



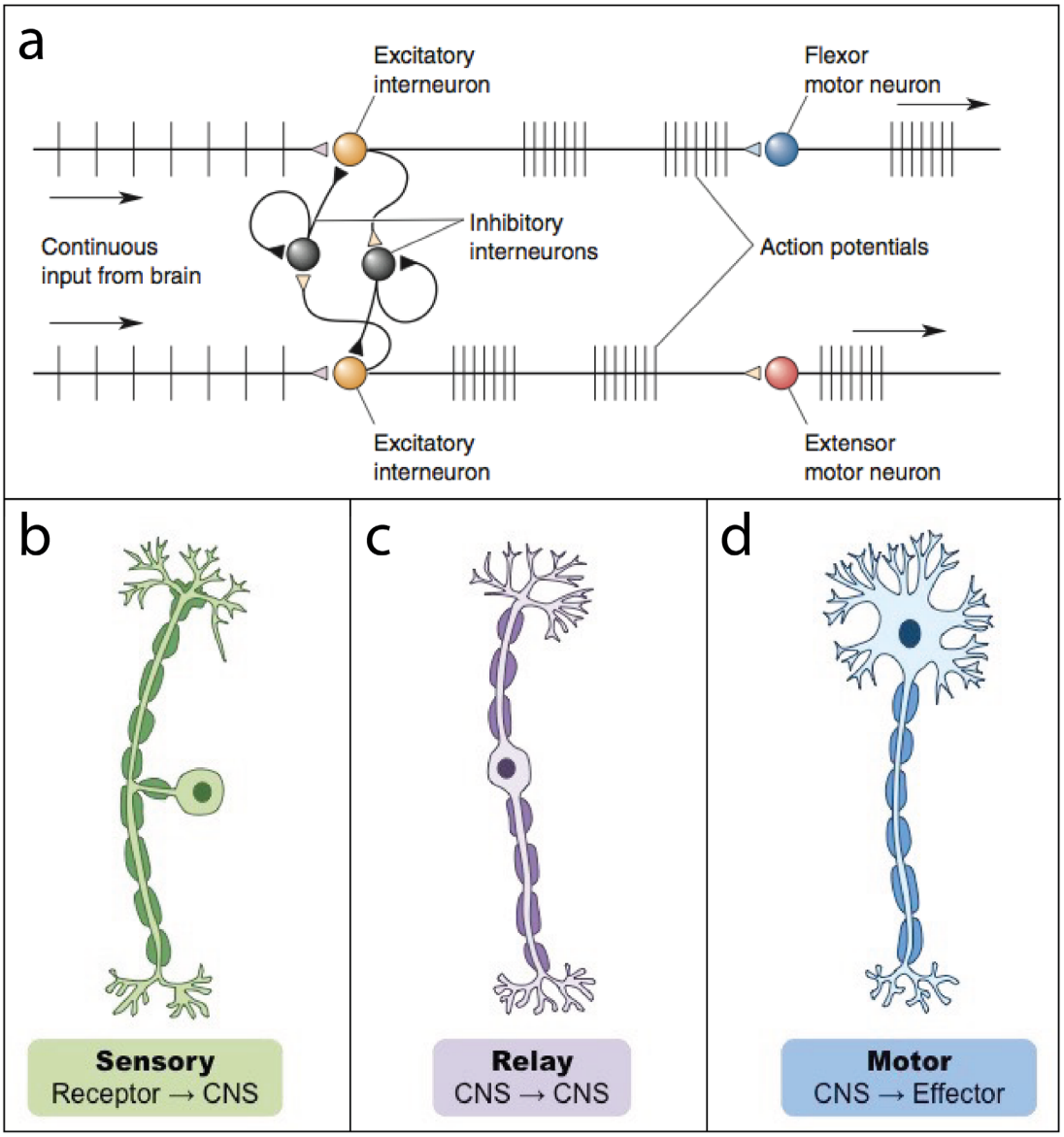
Ch1 Figure 1. Neurons are highly specialized, electricity-conducting cells.

(**a**) A schematic representing the morphological differences between vertebrate and fly neurons. Dendrites in red. Neuronal soma and axon in blue. (**b**) A schematic of a synapse between two neuronal partners. Presynaptic terminal (axon) in blue. Postsynaptic terminal (dendrite) in yellow. Neurotransmitters are shown in red. Adapted from Spindler 2018[8].



Ch1 Figure 2. Neuronal morphology dictates its function.

(**a**) A 3D projection of cultured human interneurons. Direction of electrical transmission shown in red. (**b**) A 3D projection of a multi-axon Drosophila sensory neuron. Yellow dots show terminal points. Magenta dots show axon branching points. Red arrows show direction of transmission. Adapted from Stouffer 2016[39].



Ch1 Figure 3. Circuits are composed of diverse neuronal types.

(**a**) A schematic depicting a simple motor circuit. Electric input comes from the brain onto excitatory interneurons (yellow). Inhibitory neurons regulate the strength of the output (black). Motor neurons innervate the muscle filaments to produce movement (blue and red). Strength and interval of message across the circuit shown as vertical lines. (**b-d**) A schematic showing the different morphologies of the neurons participating in the circuit shown in "a". Adapted from Boldog (2018)[6]

Chapter 2: Astrocytes regulate a critical period of motor dendrite dynamicity

Abstract

Neural circuit development is achieved through carefully regulated mechanisms that instruct the correct development, maintenance or pruning to achieve the correct number of synapses between neuronal partners. One mechanism, known as circuit plasticity, can change the structure of neurons and their synaptic affinity according to levels of activity within the circuit. Heightened circuit plasticity windows occur almost exclusively during development. These 'critical period's of remodeling can drastically modify circuits in an activity-dependent manner, by modifying synaptogenesis, dendrite stability, and network function. Despite their crucial role in neurodevelopment, we still do not know how critical period are mechanistically and temporally regulated. Here we take advantage of *Drosophila* genetics to describe and manipulate a critical period of motor circuit development. We observed that dendritic arbor remodeling is heightened between 0h to 4h after larval hatching. Moreover, our data shows that microtubules stability increases after the critical period, driving a reduction of dendrite structural plasticity. Finally, we show that astrocytes, a subtype of glial cell, close the critical period of motor neuron plasticity by stabilizing microtubules via Neurexin/Neuroligin signaling.

Introduction

During development, the organization and activity of neural circuits are adaptively modified to be able to generate stereotyped behavior[50]. These adaptations are achieved through a fundamental property of neurons: their ability to respond to changing patterns of neural activity and modify the strength and efficacy of synaptic transmission through a diverse set of mechanisms collectively referred to as plasticity. Together, these activity-dependent changes are capable of reorganizing the structure, functions, and connections of a neuronal circuit in order to arrive to a functionally stable state, also known as homeostasis[51].

One of the earliest known forms of plasticity was described by Donald Hebb's theory of cognition in 1940[52]. Hebb proposed that neurons were organized so that a circuit could be self-excited, allowing circuit activity to be sustained and rapidly re-implemented. Most importantly, Hebbian plasticity proposes that two synaptic partners can increase the strength of their connection linearly with the increase of electrical activity between them, also known as "neurons that fire together wire together."[53-54]. Hebbian plasticity occurs on a synaptic scale rather than a global scale, where an increase in presynaptic strength increases the probability of a further increase in postsynaptic gain.

In addition to Hebbian, or synaptic plasticity, the brain also employs large-scale changes to the structural organization of synapses through a different type of plasticity known as *homeostatic structural plasticity*[55]. Homeostatic structural plasticity responds to the electrical activity passing through a circuit to counterbalance Hebbian plasticity, and is capable of readjusting circuits by different magnitudes of spatial and functional

organization (local and long-range)[56]. For example, early morphological studies in hippocampal slices from adult rat showed that dendrites became more spiny in slices with blocked synaptic transmission, shedding light into mechanisms that regulate dendritic extension as a function of pre-synaptic activity[57]. Similarly, hyperpolarization of membrane potentials in presynaptic motor neurons at the *Drosophila* neuromuscular junction caused terminal regression from the postsynaptic partner (muscle), demonstrating that neuronal morphology is directly regulated by synaptic activity[58].

Although synaptic plasticity can occur throughout an organism's lifetime, startling large-scale remodeling events almost exclusively occur during development. These highly specialized temporal windows of heightened plasticity (both Hebbian and homeostatic), also known as critical periods, are essential for fine-tuning nascent circuits on a stimuli-dependent basis. Typically, critical periods open with the onset of sensory experience and close after a defined period of refinement, during which neural circuitry is modified to better respond to the sensory environment[59]. For example, studies of the critical period in olfactory neurons in *Drosophila* showed that circuit refinement is eliminated if upstream sensory neurons are desensitized to odor stimuli[60].

Disruption of critical periods can alter the developmental trajectory of a circuit, and may result in a high risk to develop irreversible neuropathies[61,62,63]. For example, one of the best defined critical periods of plasticity is the ocular dominance plasticity window in mice. When adolescent mice are subjected to monocular deprivation, GABAergic (inhibitory) synapses onto cortical pyramidal neurons in the visual cortex are aberrantly strengthened, causing irreversible visual damage to one eye due to inappropriate visual activity during the remodeling window[64]. Serious

neurodevelopmental conditions arise from improper regulation of critical periods, including Alzheimer's, ALS, motor seizures, and Autism, yet, the mechanisms that drive critical period duration are poorly defined[65].

Previous work on critical periods has focused primarily on vertebrates, but there are excellent examples of critical periods in invertebrate models[66,67,68]. In this study, I use the well-characterized *Drosophila* motor neurons aCC and RP2, known to be excitatory glutamatergic neurons, to study activity-dependent dendritic remodeling during a critical period of structural plasticity[69]. Moreover, I describe a novel role for astrocytes, a prominent glial cell type, as drivers of critical period closure and regulators of stable microtubule populations in motor dendrites. Finally, I propose a mechanism for astrocyte-mediated critical period closure, which involves juxtracrine (contact-mediated) signaling by the cell adhesion protein pair Neurexin and Neuroligin to stabilize dendritic microtubules.

Results

Activation of aCC and RP2 motor neurons reduces dendritic arbor size within 12 minutes

Plasticity is well characterized at the neuromuscular junction[70,71,72]. In contrast, temporal dynamics of motor circuit plasticity in the central nervous system (CNS) has been poorly defined *in vivo*, mostly due to the complexity of manipulating and recording developmental windows within the intact brain[73,74,75].Recent work in the Doe lab has defined that *Drosophila* motor neurons change their dendritic architecture in response to neuronal activity[76], but the rapidity of these phenotypes could not be assessed in fixed tissues (Figure 1). I took advantage of the powerful *Drosophila* optogenetic toolkit to manipulate developing motor circuits in an activity-dependent manner. By employing the use of the *RN2-gal4* system to drive expression of the channelrhodopsin *UAS-CsChrimson::mVenus* in aCC and RP2 motor neurons, I was able to visualize activity-induced remodeling 0 hours (h) after larval hatching (ALH) and used confocal microscopy to generate 4D stacks of dendrites every 45 sec for a total of 15 min (Figure 2b-c). Each hemisegment of the larval brain contains a single RP2 dendritic arbor, projecting away from the midline, as well as a single aCC neuron located medially that merges their dendrites with the RP2 arbor (Figure 2a). Activation using the channelrhodopsin dramatically increased the excitation levels of the neuron, leading to a homeostatic decrease in dendritic volume (Figure 2a-c).

To quantify the kinetics of individual dendritic filopodia in control and activating conditions, I reconstructed and analyzed dendritic length over time using the image analysis software “Imaris”. Specifically, I manually reconstructed individual

filopodial membranes using the "Filaments" function over the acquisition period and compared dendrite length between controls and experimentals (Figure 2b'-c'').

Activation led to significant filament retraction ($p<0.05$, one-way ANOVA) starting at 12 minutes, which kept progressively retracting through the end of the acquisition period. This data supports that activity-dependent remodeling in dendrites of aCC and RP2 neurons occurs rapidly, on a scale of minutes, and is capable of changing the kinetics of individual filopodial processes from extending to retracting.

Microtubule populations retract prior to dendritic collapse

I next wanted to understand how dendritic remodeling was achieved on a minutes time scale, when structural remodeling takes hours to days to achieve in mammals[77]. Studies have shown that structural support molecules are present in developing filopodia, and extending/retracting dendrites probably requires mobilizing cytoskeletal proteins to drive motility of the membrane[78]. Activation of RP2/aCC was achieved by *Chrimson::mVenus*, which carries a membrane-bound fluorescent tag, alongside co-expression of *Cherry::zeus*, which labels stable microtubules. 4D stacks were collected every 10 seconds, for a period of 10 minutes (Figure 3a-b). Quantification was conducted using the Imaris "Surface" function to obtain the sum of fluorescent intensity for both membrane and microtubule channels. Individual filopodia were analyzed over a period of 40 seconds (4 timeframes), which is much quicker than the experiments above taken at 45 second per stack (Figure 2). I observed that retraction of the stable microtubule population preceded retraction of the membrane with a ten second average difference (Two-way ANOVA, $p<0.01$) (Figure 3b). The correlation

between destabilization of microtubule populations and collapsing filopodia suggests that microtubule stability is predictive of dendrite retraction.

Dendrites lose dynamicity over critical period closure

Supporting data from the lab demonstrated that activity-induced remodeling of motor neuron dendrites occurs during a critical period of plasticity that closes by 8 h ALH[76]. I sought to further characterize dendrite dynamics during this critical period of remodeling to determine if wildtype dendrites become more stable during critical period closure. I conducted live imaging of RP2/aCC motor arbors carrying a fluorescent membrane tag (*myr::GFP*), across multiple time-points of larval development (0h, 4h, 8h, 22h ALH) (Figure 4a-c'). Indeed, the total dendritic displacement (measured in µm) between 0h ALH and 22h ALH decreased significantly ($p<0.001$), and most notably between 8h and 22h (Figure 4d). In an effort to further characterize dynamicity during this critical period, I quantified extension and retraction events of individual filopodia across the aforementioned timepoints. Dynamicity decreases between 4h and 8h ALH (0h *vs.* 8h ALH, $p<0.05$), with a marked increase in stability at 22h ALH (0h *vs.* 22h ALH, $p<0.001$) (Figure 4e). To tease out whether arbors showed an overall trend in growth direction (extending or retracting) across the critical period, I quantified the average length of extension/retraction events. Extension events were highest at 0h ALH, and significantly decreased between 4h and 8h ALH (0h *vs.* 8h ALH, $p<0.05$). Interestingly, significant changes to retraction length is not appreciated until 22h ALH (0h *vs.* 22h ALH, $p<0.01$) (Figure 4f). Together, these results support the existence of a critical period of dendrite remodeling in RP2/aCC, which extends from 0h until 8H ALH, reaching seemingly stable arbors by 22h post-hatching (Figure 4g-h).

Stable microtubule populations increase as the critical period closes

I have shown that dendrite stability increases as the critical period closes (Figure 4a-f), and that microtubule stability is predictive of dendrite retraction (Figure 3). I next sought to characterize whether the levels of stable microtubules increase as the critical period closes. To test this hypothesis, I conducted live imaging of RP2/aCC dendritic arbors carrying a fluorescent membrane tag (*myr::GFP*), alongside the co-expression of *Cherry::zeus*, which labels stable microtubules (Figure 5a). Assessment was done across multiple time-points of larval development (0h, 4h, 8h, 22h ALH), which includes the critical period between 0h to 8h ALH. Indeed, stable microtubule populations significantly increased between 0h and 8h ALH (0h v/s 8h ALH, $p<0.01$), and between 8h to 22h ALH (8h v/s 22h ALH, $p<0.01$) (Figure 5e). These findings support a model where the loss of dendritic dynamicity is achieved by the stabilization of microtubule populations to support closure of the critical period (Figure 5f-g).

Astrocytes close the critical period of RP2/aCC remodeling

It has been previously established that astrocytes are indispensable for synaptogenesis, synapse pruning, and neuronal support[79]. Astrocyte processes invade the neuropil during late Drosophila embryogenesis, alongside initial synaptogenesis[46]. Therefore, I hypothesized that they play an active role in regulating the critical period of activity-induced remodeling. To that end, I monitored dendrite dynamics (*RN2-gal4,UAS-myr::GFP)*, combined with programmed ablation of astrocytes (*lexAop-rpr*) under the transcriptional control of *alrm-LexA* (pan-astrocyte driver). Due to the pivotal role of astrocytes in proper circuit development, ablation animals were unhealthy at later larval stages. I compared dendrite dynamicity between

controls and ablation larvae at 22h ALH, when ablation larvae are still indistinguishable from controls. The total dendritic displacement (measured in μm) increased significantly in the ablation condition ($p<0.001$) (Figure 4d, dashed line). Similarly, overall dynamicity was pronouncedly increased in *lexAop-rpr* animals ($p<0.001$, psi letters), and were statistically indistinguishable from wildtype animals at 8h ALH ($p>0.05$) (Figure 4e, dashed line). Filopodial extension events differed significantly between controls and ablation animals at 22h ALH ($p>0.01$), again, more comparable to wildtype animals at 8h ALH ($p>0.05$) (Figure 4f, dashed line). These data provide substantial support for a novel role for astrocytes in closing a critical period of dendrite remodeling (Figure 4g-i).

Nrx-1 stabilizes dendritic arbors to close the critical period

How is microtubule stability increased across critical period closure? Recent reports suggest that Neurexin signaling increases axonal microtubule stability in motor neurons[81]. Moreover, interactions between Neurexins and Neuroligins (a receptor-ligand pair) have recently been documented between motor dendrites and astrocytes, respectively[82,76]. Together, these reports suggest a mechanism for critical period closure whereby astrocyte Neuroligins increase microtubule stability through Neurexin signaling. To test this hypothesis, I employed the use of Cherry::Zeus alongside constitutive overexpression (OE) of the Neurexin-1 (Nrx-1) gene in aCC and RP2 and evaluated both dendrite and microtubule volume at 4h ALH, when the critical period is still open (Figure 5a,b). Fixed preparations tagging stable microtubule populations demonstrate that Nrx-1 OE increases stable microtubule volume in dendritic arbors ($p<0.001$) (Figure 5c). Concomitantly, Nrx-1 OE drives larger dendritic arbors when

compared to controls ($p<0.01$) (Figure 5d), though this effect is less pronounced than the increase in relative microtubule volume. Live imaging using the same paradigm revealed a significant decrease of dendritic dynamicity in Nrx-1 OE, suggesting that the Nrx-1 OE stabilizes dendritic arbors even during the critical period ($p<0.05$) (Figure 5e-g). Indeed, complimentary work from the lab demonstrated that Nrx-1 OE is sufficient to close the critical period early[76]. Together, these data support a model in which astrocytes drive the closure of a critical period of heightened structural remodeling in larval motor neurons through Neuroligin to Neurexin signaling to stabilize dendritic microtubules.

Discussion

Synaptic connectivity has the capacity to rapidly change as a result of plasticity during critical periods[83]. This activity-dependent plasticity is a balancing act, where the interplay between Hebbian and homeostatic structural plasticity mechanisms in dendrites and axons drives the correct circuit tuning to achieve stereotyped behavior[84]. For example, monocular deprivation during the critical period of plasticity in the visual cortex initially causes reduced firing rates (Hebbian), but rebounds to wildtype over extended periods of sensory deprivation (Homeostatic)[85]. Failure to correctly integrate mechanisms of synaptic modification can lead to dysfunctionally high/low network activity which is detrimental to circuit function[86]. Defects in critical period timing are thought to contribute to diseases such as familial Amyotrophic lateral sclerosis, Autism Spectrum Disorders, and Epilepsy[87], making their characterization of significant medical relevance.

This study characterizes a critical period of remodeling during development of larval MNs involved in locomotion. Ectopic neuronal activation for 12 or more minutes during the first 4 hours of larval life induced remarkably rapid remodeling of dendritic filopodia. Comparatively, critical periods of plasticity in murine cortical somatosensory cells have been reported to occur between 2-7 days postnatally[88], and in the order of months to years in human visual circuit development[89]. The relatively short timeline of this critical period (8 hours) is in accordance with the short development time of fruit flies relative to mice or humans, considering that *Drosophila* embryos hatch at twenty-one hours after egg-laying (25°C), and that the establishment of larval motor circuits occurs 4h-prior to hatching[90].

Remodeling windows depend on the ability of dendrites and axons to rapidly extend/retract by modifying their cytoskeleton[91]. Indeed, labeling dendritic MTs demonstrated that MT collapse preceded filopodial membrane retraction during the motor circuit critical period. Moreover, MT populations stabilized across development, in correlation with decreased dendritic dynamicity between 8h to 22h ALH. Studies have shown that altered MT stability in neurons leads to overlapping neuropathies to those displayed by critical period disruption[5,92], suggesting that therapies that affect MTs may be efficacious in treating critical period disorders.

How does microtubule stabilization occur to close the critical period? A recent report showed that mutations in presynaptic Neurexins alters microtubule stabilization in axons, an effect that is partially rescued by postsynaptic overexpression of BMP ligands[93]. I observed that Nrx-1 OE animals assayed at 4h ALH exhibited decreased microtubule dynamicity, and other work in the Doe lab showed that this is sufficient to precociously close the critical period[76] . Astrocytes begin to ensheathe dendrites during circuit development[30], and I showed that loss of astrocytes increased dendrite dynamicity, supporting the hypothesis that astrocytes close this critical period of remodeling[76]. Interestingly, a recent report showed that juxtracrine Neuroligin/Neurexin signaling (Nrx/Nlg) helps regulate astrocyte morphogenesis, an event that occurs in tune with the expansion of activity in circuits[94]. Given this report, it will be important to test whether astrocytic Neuroligins signal back through MN Neurexins to stabilize dendritic MTs for critical period closure. Finally, because mutations Neurexins and Neuroligins are linked to critical period disorders including Autism[43], it is of immense medical

interest to identify the linker proteins between microtubules and Neurexins to establish a more complete cellular mechanism.

Methods

Fly husbandry

Flies were reared at 25°C on cornmeal fly food.

Genetics (in order of appearance)

1. RN2-gal4[95]
2. 10XUAS-myr::GFP (BDSC# 32198)
3. 20XUAS-CsChrimson::mVenus (BDSC# 55136)
4. alrm-lexA[30]
5. lexAop-rpr[96]
6. *UAS-cherry::zeus*[97]
7. UAS-SAM.dCas9.GS05146 (BDSC# 82741)
8. 13lexAop-CsChrimson::tdTomato (courtesy of Vivek Jayaraman, Janelia Research Campus)

Animal Collection

Collection of specimens for optogenetic manipulation: Fly crosses were kept at 25 º C in collection bottles capped with 3.0% agar apple juice carrying a mixture of yeast paste and 0.5mM all-*trans* retinal (+ATR) (Sigma-Aldrich, R2500-100MG). Adult flies were supplied with a yeast diet (+ATR) for 72h in advance of collections to ensure maternal transfer of ATR to embryos. Embryonic collections were done at 25º C for 1.5 hours in fresh caps carrying the yeast + ATR mixture. All collections and aging were performed at 25º C in the dark to prevent optogenetic activation prior to the experiment. *Drosophila* embryos take 21 hours after egg laying to hatching at 25º C in a humid

chamber[98]. The developmental timepoints of remodeling experiments were calculated after the 21-hour incubation, and reported as hours after larval hatching (ALH) (e.g. 0h, 4h, 8h, 22h ALH). For animals manipulated at 4h, 8h, and 22h ALH, larvae were transferred post-collection at 0h ALH onto fresh apple caps carrying a mixture of yeast paste (+ATR), and aged until dissection and subsequent 15' optogenetic activation via confocal imaging. All brains were dissected in low-light conditions to prevent activation (<100 lx) and imaged immediately after confocal laser exposure.

Collection of non-light sensitive specimens: Fly crosses were kept at 25 º C in collection bottles capped with 3.0% agar apple juice carrying plain yeast paste. Embryonic collections were done for 1.5 hours as above. Embryos were allowed to age at 25º C through hatching. The developmental timepoints of dendritic assessment were calculated after the 21-hour incubation at 25º C in a humid chamber, and reported as hours after larval hatching (ALH) (e.g. 0h, 4h, 8h, 22h ALH). All brains were dissected immediately after reaching their target developmental time.

Time-lapse Confocal Microscopy

Fictive preparations (isolated CNS) were prepared in a hemolymph-like solution (HL3.1); brain lobes and ventral nerve chord were dissected undamaged. Mounting was performed on a 12mm #1 thickness poly-D-lysine coated round coverslips (Corning® BioCoat™, 354085), a single 18mmx18mmx0.16mm cover glass (Fisher, 12-542B) was used to stabilize the brain by slightly pressing the glass onto the brain lobes to prevent drifting, a drop of HL3.1was used to seal the stage. Light-sensitive samples were mounted under low light conditions (<100 lx) to prevent non-experimental optogenetic activation. Imaging was performed using a Zeiss LSM 800 laser scanning confocal

fitted with a 63x/1.40 NA Oil Plan-Apochromat DIC m27 objective lens and GaAsP photomultiplier tubes. Time-lapse movies of Chrimson experiments and controls for Figures 2 and 4 were generated by scanning a z-stack of 25 µm of two VNC hemisegments (allowing for drift in Z) with 1 µm step size every 45 seconds, for a total duration of 15 minutes. A separate full spectrum lightbulb was placed over the imaging stage to ensure even optogenetic activation for all samples. Timecourse experiments to assess microtubule volume over time (Figure 5) were obtained by generating a 25 µm z-stack with a 0.5 µm step size for a duration of 15 minutes, alongside the Zen Blue Auto-Focus module with a 20 µm scanning range every 5 stacks to avoid sample drift. For microtubule retraction quantification using *Chrimson::mVenus* and *UAS-cherry::zeus* (Figure 3), imaging was achieved by acquiring a z-stack of 21 µm (allowing for drift in Z) with 0.3 µm step size, with only one dendritic arbor in the field of view. Continuous scans were obtained every 10 seconds, for 10 minutes.

Figure Preparation

Images were prepared by taking a snapshot of 3D projections in Imaris 9.2.0 (Bitplane AG) or 3D projections in FIJI (ImageJ 1.52h) and assembled using Adobe Illustrator. Schematics were drawn in Adobe Illustrator.

Time-lapse Analysis

Filament reconstruction: I used the Imaris built-in "Fiji 3D registration" plugin to correct for the 3D drift caused by live imaging. The image was then exported to Fiji for bleaching correction using the 'histogram matching' function. Data was reimported to Imaris for quantitative analysis. Membrane/Microtubule reconstructions (filaments

function) were done by automatic filament detection of the first timepoint (starting position: base of cell body; largest diameter filament: 2 μm; seed points: ~.15 μm; thresholds depend on fluorescence intensity). Individual dendrites were manually reconstructed for each subsequent timepoint using the automatic detection as a landmark (filament drawing tool; 'cone' reconstruction was set to .3 μm). Statistics for each dendrite were obtained by recording the length of one process across the duration of the movie.

Filament dynamicity: To assess dynamicity of a process, I defined an extension/retraction "event" as a filament length difference of 0.50 μm when compared to the previous timepoint. "Motile dendrite" was defined as at least one filopodial event over the 15-min experiment. To determine motility, I assigned a value of 1 to the occurrence of an "event", and a value of 0 for stable filaments at each time frame. In Chrimson experiments, dendrite lengths were normalized to values of t=0 to assess retraction over time. Lengths were compartmentalized to 10 normally distributed values using MATLAB (Mathworks) to minimize variations in brain size across developmental timepoints and process length between WT and Chrimson-activation. Both conditions were plotted against one another as a function of time.

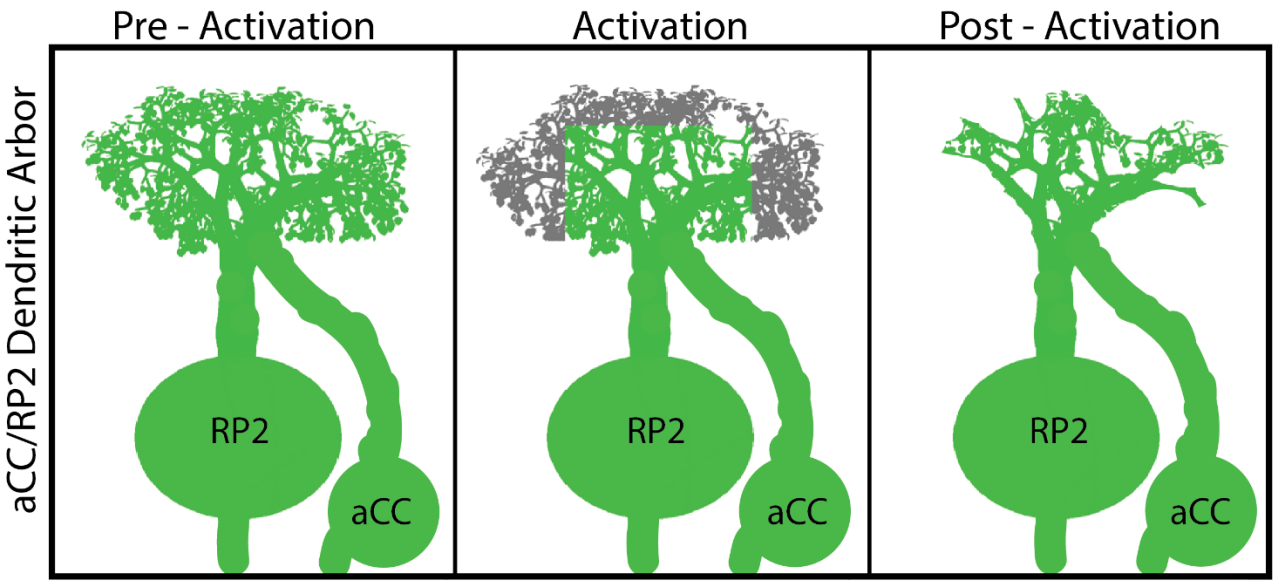
Microtubule retraction: Rapidly collapsing dendritic filopodia of RP2 motor neurons were identified using Imaris 3D viewer. A retraction event was defined as a ≥0.50 μm change in filopodial length within a period of 40 seconds. The lower boundary of the ROI was created in the rapidly retracting process using the final filopodial length as a landmark at t=40 seconds. The upper boundary of the ROI spanned from the landmark

to the most distal length of the filopodial process 40 seconds prior. Channel intensity was calculated using the "intensity sum" variable within Imaris' "surface" function.

Statistical analyses

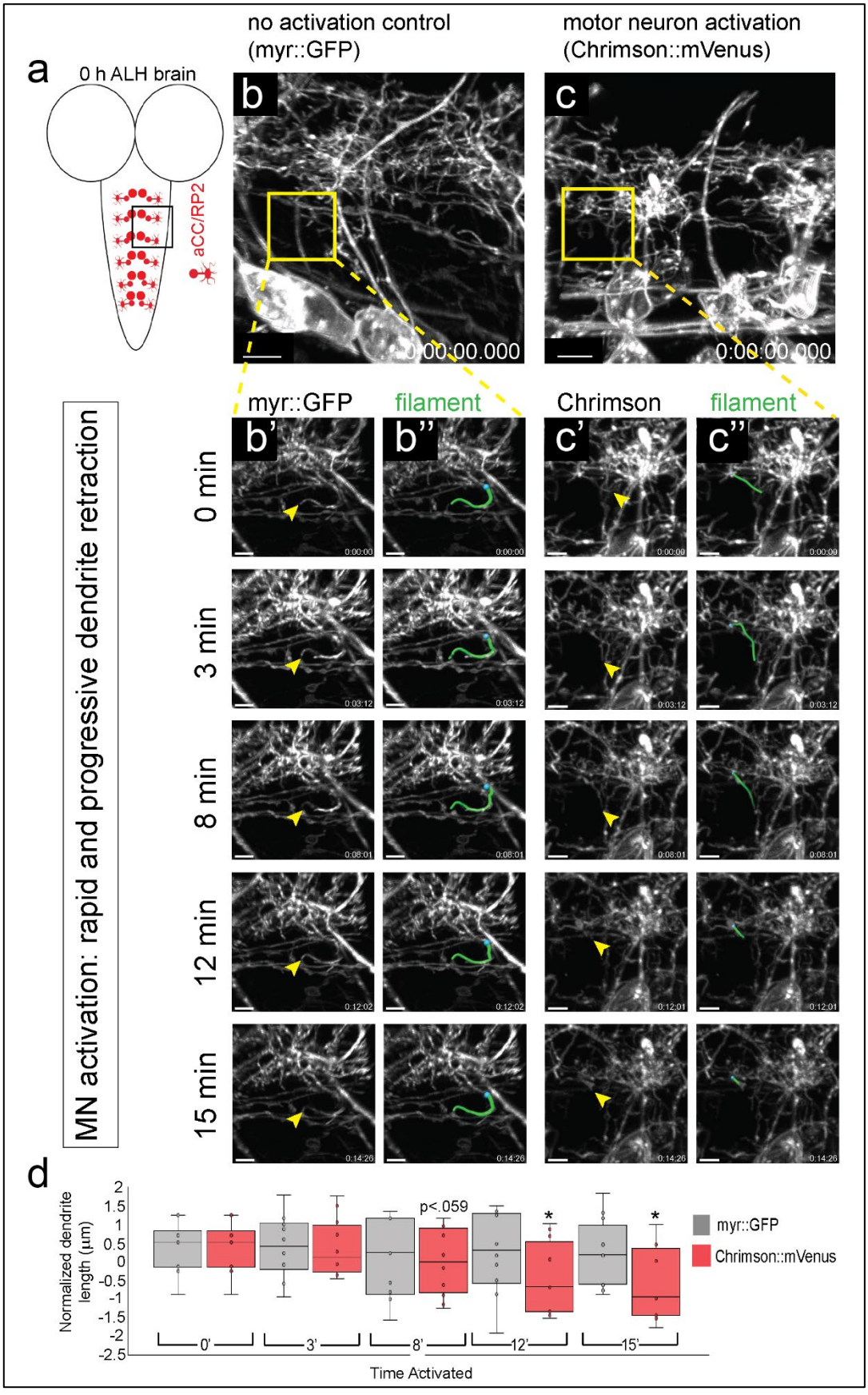
Significance analyses were performed using Microsoft Excel, MATLAB (MathWorks), and Prism (GraphPad). One-way ANOVA was used unless noted otherwise. Alpha values were set to 0.05 to define the level of significance. Significance: *, $p<.05$; **, $p<.01$; ***, $p<.001$; ****, $p<.0001$, NS= not significant. Figure legends and figure labels contain sample size, statistical test employed, and variance results.

Chapter 2 Figures and Legends



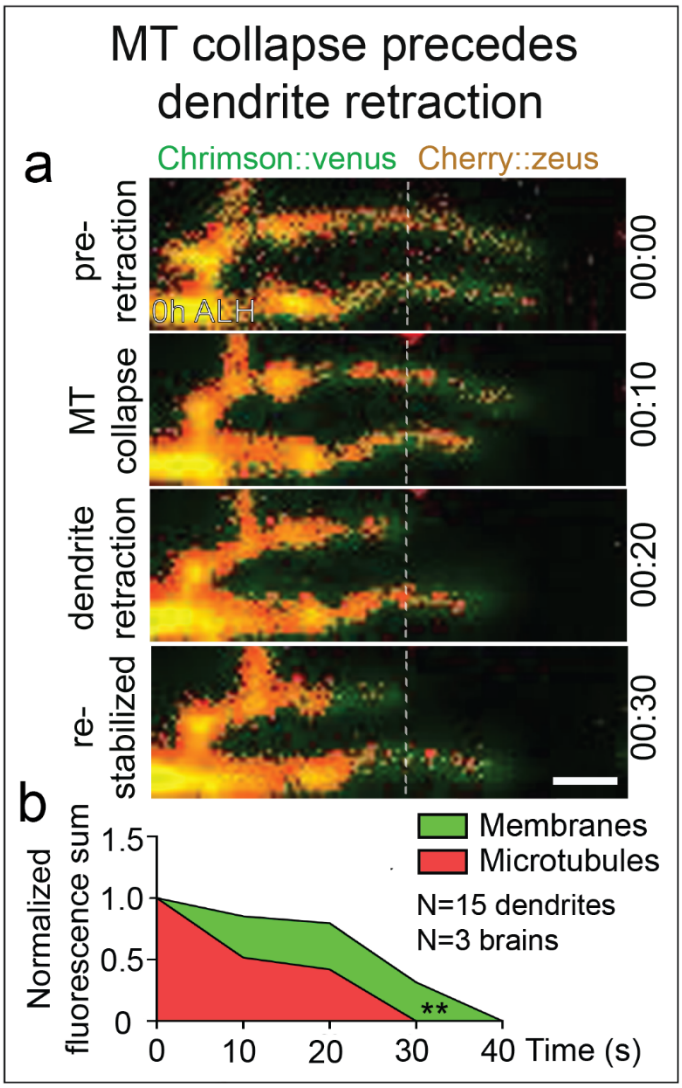
Ch2 Figure 1. Manipulation of developing motor circuits in an activity-dependent manner.

A schematic representing the dendritic arbor of aCC/RP2 neurons. **Left**: Each hemisegment of the larval brain contains an aCC and RP2 dendritic arbor, projecting away from the midline (bottom of the image). **Center**: Activation using a channelrhodopsin (*RN2-gal4, UAS-CsChrimson::mVenus)* causes ectopic neuronal activation to drive dendrite retraction. **Right**: Sustained activation progressively decreases dendritic volume.


a
0 h ALH brain
aCC/RP2
no activation control
(myr::GFP)
motor neuron activation
(Chrimson::mVenus)
b
c
0:00:00.000
0:00:00.000
myr::GFP
filament
Chrimson
filament
b'
b''
c'
c''
0 min
3 min
8 min
12 min
15 min
MN activation: rapid and progressive dendrite retraction
d
Normalized dendrite length (µm)
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
p<.059
*
*
0'
3'
8'
12'
15'
Time Activated
myr::GFP
Chrimson::mVenus

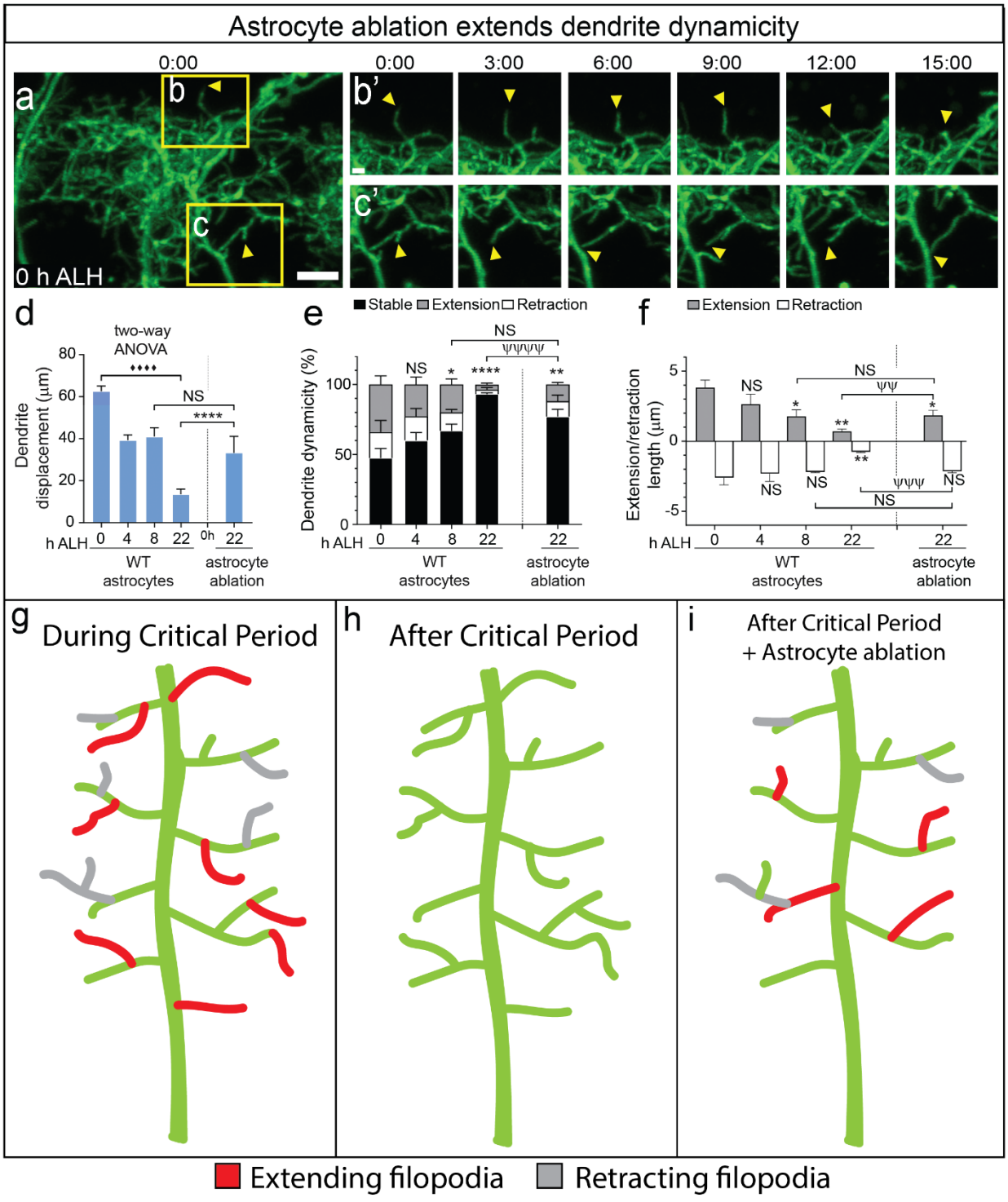
Ch2 Figure 2. Activation of aCC and RP2 motor neurons reduces dendritic volume within 12 minutes.

(**a**) Schematic representing a *Drosophila* brain and VNC at 0h ALH. Motor neurons aCC/RP2 are shown in red. Box marks the two hemisegments imaged per experiment. (**b**) 3D projection of a fictive preparation (isolated CNS) at 0h ALH (*RN2-gal4; UAS-myr::GFP;* + ATR). Yellow box shows region of interest (below) used for dendritic filament reconstruction. Scale bar, 5 μm. (**b'-b''**) Region of interest for manual filopodial reconstruction over the15-minute acquisition period. (**b'**) myr::GFP signal alone. Yellow arrowhead marks the distal end of a dendritic process (**b''**) Imaris filament reconstruction. Scale bars, 1 μm. (**c**) 3D projection of a fictive brain preparation at 0h ALH for Chrimson-activation (*RN2-gal4; UAS-Chrimson::mVenus;* + ATR) prior to Chrimson activation. Yellow box highlights region used for dendrite reconstruction. Scale bar, 4 μm. (**c'-c''**) Region of interest for manual filopodial reconstruction over the15-minute acquisition period. Chrimson::mVenus signal alone. (**c'**) Yellow arrowhead marks the tip of a reconstructed filament (**c''**) Imaris filament in green. Scale bars, 1 μm. (**d**) Normalized dendrite length across time in myr::GFP controls v/s Chrimson-activation (N=10 processes each for N=4 brains per condition. Processes binned by length into 10 categories equally distributed categories to reduce variation by brain size). Controls remained stable over the 15-minute acquisition period, whereas activating with Chrimson results in progressive retraction motor dendrites. Statistics assessed by one-way ANOVA. Here and below, *, $p<.05$; **, $p<.01$; ***, $p<.001$; ****, $p<.0001$.



Ch2 Figure 3. Microtubule collapse precedes dendrite retraction.

(**a**) Timelapse of aCC/RP2 dendrites at 0h ALH carrying Chrimson::mVenus (green) and Cherry::zeus tagging stable microtubules (Orange. Heatmap generated in ImageJ). Dashed line: retraction landmark (see methods). (**b**) Fluorescence intensity of membranes *vs.* microtubules, Two-way ANOVA.


Astrocyte ablation extends dendrite dynamicity
0:00
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3:00
6:00
9:00
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a
b
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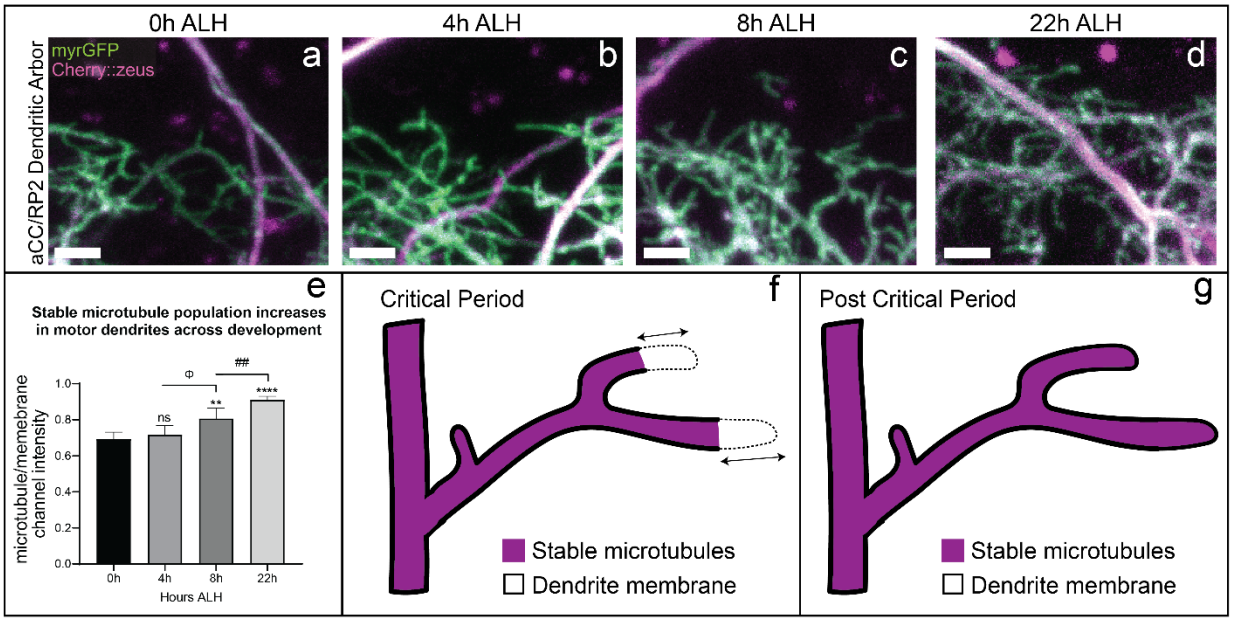
0 h ALH
d
two-way ANOVA
Dendrite displacement (µm)
h ALH
WT astrocytes
astrocyte ablation
e
Stable
Extension
Retraction
Dendrite dynamicity (%)
f
Extension
Retraction
Extension/retraction length (µm)
g
During Critical Period
h
After Critical Period
i
After Critical Period + Astrocyte ablation
Extending filopodia
Retracting filopodia

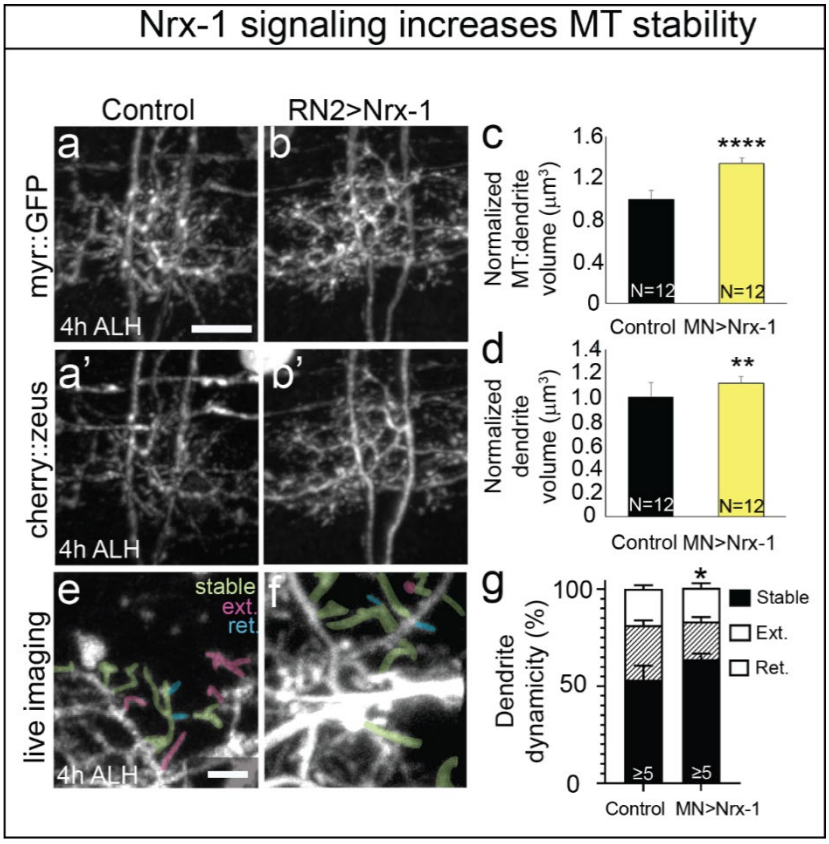
Ch2 Figure 4. Astrocytes close the critical period.

(**a-f**) Astrocyte ablation extends the critical period and dendrite dynamicity **(a-c')** aCC/RP2 dendrites in one hemisegment at 0h ALH. (**a**) 3D projection, of one hemisegment of aCC/RP2 dendrites at time 0. Scale bar: 5 μm. (**b'-c'**) Yellow boxes regions followed across movie. Dynamic dendrite filopodia (yellow arrowheads) imaged every 45" for 15'. Scale bar, 1 μm. Genotypes: *RN2-gal4,UAS-myr::GFP* (control), *RN2-gal4,UAS-myr::GFP; alrm-lexA,lexAop-rpr* (ablation). (**d-f**) Quantification. N=50 dendrites from 5 brains per timepoint. ψ: comparisons between astrocyte ablation and controls (Fisher's exact tests). (**g**) Schematic depicting dynamicity during the critical period. (**h**) Schematic showing dendrite stability after 8h ALH. (**i**) Schematic depicting prolonging of dynamicity post-critical period following astrocyte ablation.



Ch2 Figure 5. Loss of dendrite dynamicity post-critical period occurs in parallel with the stabilization of microtubules

(**a-d**) 3D projection of a fictive preparation (isolated CNS) of aCC/RP2 dendrites tagged for stable microtubule populations (magenta) and dendritic membranes (green) across multiple developmental points, including the critical period (0h-8h ALH). (*RN2-gal4; UAS-Cherry::zeus, UAS-myr::GFP).* Scale bar, 2 µm. (**e**) Quantification of the fluorescence intensity ratio between microtubules and membranes. The end of the critical period (8h ALH) is marked by a significant increase of stable microtubules in dendritic arbors of aCC/RP2 compared to 0h ALH (starts above bars). Statistical comparisons between non-0h ALH timepoints showed as ϕ and #. N=6 arbors per timepoint. (**f-g**) Schematic showing that the stability of dendritic arbors post-critical period arises as a product of persistent invasion of stable microtubule populations into the tips of membrane filopodia.



Ch2 Figure 6. Nrx-1 OE induces MT stability during the critical period

(**a-b**) Dendritic (myr::GFP) distribution of (**a'-b'**) microtubules (Cherry::Zeus) in (**a-a'**) controls and (**b-b'**) overexpression of Nrx-1 in MNs at 4 h ALH. Genotypes: (**a-a'**) *RN2-gal4,UAS-myr::GFP,UAS-Cherry::Zeus,UAS-redstingerNLS* (**b-b'**) *RN2-gal4,UAS-myr::GFP, UAS-Cherry::Zeus,UAS-Nrx-1*. (**c-d**) Quantification of dendrite volume or microtubule to dendrite volume. (**e-f**) Live imaging of microtubules (Cherry::Zeus+) in aCC/RP2 (**e**) control or (**f**) Nrx-1 OE. Genotypes: (**e**) *RN2-gal4,UAS-myr::GFP,UAS-Cherry::Zeus*, (**f**) *RN2-gal4, UAS-Cherry::Zeus,UAS-Nrx-1*. Coloring: stable MT (green), extending (pink), or retracting (blue). (**g**) Quantification showed that Nrx-1 OE caused a significant decrease in dendrite dynamicity following by Fisher's Exact Test.

Chapter 3: Neurons depend on astrocytes to maintain cholinergic synapses

Abstract

Astrocytes, the most abundant cell type in the human brain, have been shown to be master regulators of circuit formation and homeostasis. Recent reports show that astrocytes closely associate with neuronal synapses during development, forming the tripartite synapse. Further, previous studies *in vitro* have shown that astrocyte-derived cell surface molecules (CSMs) and secreted proteins can modify synaptogenesis during circuit development. Here we take advantage of Synaptic Tagging with Recombination (STaR) tools to assess non-cell autonomous changes in synapse numbers that arise as a product of astrocyte manipulations. We observed that astrocytes drive the correct number of cholinergic synapses in two cell types, Kenyon cells of the mushroom body and dorsal bipolar dendritic neurons. Moreover, we screened through 245 genes conserved between vertebrate and fly astrocytes, and found fourteen novel genes required for synaptogenesis. This work provides evidence that astrocytes regulate cholinergic synaptogenesis *in vivo*.

Introduction

Our brains are composed of a network of billions of neurons and glia which accurately support trillions of synapses. During development, the correct formation of neural circuits is highly dependent on the careful orchestration of mechanisms regulating structural and functional integration of neurons[99]. Synaptogenesis is a complex process that involves multiple signaling molecules that influence not only synaptic specificity, but also synaptic strength and stability[100] . Some of molecular regulators of synaptogenesis are able to communicate at a long distance (*e.g. NGF and SPARC*) [101] to drive dendrite and axon targeting and subsequent synaptogenesis[102-103]. In contrast, many pro-synaptogenic proteins act locally at the junction between growing axons and dendrites. The cell surface molecule (CSM) family of proteins has been extensively described as having a critical early role in synapse docking and maturation. Trans-synaptic adhesion molecules can regulate differentiation of nascent synapses, and recruit the machinery necessary for electrochemical transmission[104]. This was first demonstrated for Neuroligins, postsynaptic membrane proteins that bind presynaptic Neurexins[105,106]. For example, studies conducted at the *Drosophila* neuromuscular junction report that deletions of Neurexin alone is sufficient to decrease the number and efficacy of synaptic boutons in the presynaptic terminal[107]. Although scientists have uncovered the identity of some of the signaling molecules that regulate synaptogenesis, our knowledge of how they interact temporally and spatially to form the trillions of synapses in the human brain is far from complete. Understanding these processes on the molecular level not only provides insights into a fundamental problem of cellular

neuroscience, but is of profound clinical importance. Mutations in synaptogenic genes have been linked to neuropathies such as Autism, Alzheimer's and ALS[108-19].

Recent reports identify astrocytes, a prominent glial cell type, as important regulators of circuit development. Astrocytes infiltrate the neuropil 5 days prenatally in mice, and in the final stage of *Drosophila* embryogenesis, extending primary branches from the soma that gradually divide into finer and finer processes to generate a dense network that ensheaths virtually all CNS synapses[108,109,110]. Neurons require astrocytes to survive[98]; thus, the role of astrocytes in neurodevelopment, including synapse formation and function, was not assessed until recently[112,113]. A pioneering study using cultured glutamatergic retinal ganglion cells grown in the presence or absence of astrocytes showed that astrocytes are necessary to establish normal synapse number. Moreover, adding astrocytes to the glia-deprived culture rescued synapse numbers and increased synapse strength[114]; thus, astrocytes promote synaptogenesis *in vitro*. Further, while neurogenesis precedes astrogenesis in the cortex, neuronal synapses only begin to form after astrocytes have been generated, concurrent with neuronal branching and process elaboration[115]. More recently, studies have supported the critical role of astrocytes in synapse formation *in vivo*, and have identified a handful of glia-derived secreted and CSMs that regulate the number of structural synapses (*e.g.* thrombospondins [TSPs], secreted protein acidic rich in cysteine [SPARC], and TGF-β)[116]. Unfortunately, our knowledge of these CSMs is largely restricted to astrocyte-regulated glutamatergic synapse development[18], and is likely not a good representation of all astrocyte-derived signals that instruct synapse formation in different neuronal types.

In addition to glia-mediated synapse formation, astrocytes have also been shown to regulate synapse elimination. Astrocytes express several phagocytic receptors, such as MEGF10 (*Drosophila* Draper), that engulf weaker synapses in a model of synaptic competition. Additionally, elimination of MEGF10 in mouse causes ectopic synapse numbers and excess functional synapses in the dorsal lateral geniculate nucleus[117]. These findings, alongside others, demonstrate that glia are critical for stabilizing CNS synapses, and that CNS synapse number can be profoundly regulated by non-neuronal signals. To that end, we sought out to define the astrocyte-derived regulators of synaptogenesis with a focus on a relatively understudied synapse subtype: excitatory cholinergic synapses.

Here, we take advantage of *Drosophila's* powerful genetic toolkit to correlate astrocytic development and synaptogenesis. Moreover, we developed a pipeline to reliably quantify cholinergic presynaptic puncta from two CNS neuro subtypes: the Mushroom Body (MB), the insect learning and memory center[118], and the dorsal bipolar dendritic sensory neuron (Dbd), a well-documented sensory unit involved in locomotion and peripheral sensing[119]. We show that astrocytes regulate synapse formation for both MB and Dbd neurons, and identify several novel glia-secreted molecules that instruct synaptogenesis during development.

Results

Astrocytes support a large number of synapses in the developing fly brain

Despite their critical roles in circuit function, astrocytes are still largely understudied. The advancement of imaging techniques has allowed us to visibly appreciate how astrocytes participate in the conformation of the tripartite synapse (Figure 1a)[46]. Moreover, genetic tools have allowed us to uncover some of their active roles, such as gliotransmitter release, ion buffering, and neurotransmitter recycling (Figure 1b). In this study, we used *Drosophila* to first characterize astrocyte-synapse association across development, and then to define the mechanisms used by astrocytes to regulate synaptogenesis. In the *Drosophila* neuropil, a synaptically dense region of the central nervous system (CNS), astrocyte cell bodies settle over the neuropil surface and begins to extend their processes inwards (Figure 1c). Astrocyte processes invade the neuropil during late *Drosophila* embryogenesis[109], concurrent with initial synaptogenesis[120]

Characterization of astrocytic morphology and territory expansion across development

From late embryogenesis through the last larval stage, *Drosophila* larvae increase their body surface over 50-fold, while their brain expands by a 100-fold magnitude[121]. During that expansion, synapse number within the neuropil increases dramatically. In contrast, the number of astrocytes per hemisegment (N=6) remains constant from embryogenesis throughout larval life, necessitating a huge expansion in astrocytic membrane to support new synapses as the brain grows[122]. To characterize astrocyte-synapse association across development, we developed transgenic animals

carrying temperature-sensitive Multi Color Flip Out (MCFO) tags driven by the astrocyte-specific *alrm-gal4* to label individual astrocytes, and used immunofluorescence to label neuropil synapses (Brp+) (Figure 2a). Then, I characterized three representative timepoints of 4h, 24h, and 72h After Larval Hatching (first, second, and third larval instar, respectively). Astrocytic volumes were reconstructed using the Imaris "Surface" function, and synapses were identified using the "Spots" function (Figure 2a'-c', see Methods for details). Astrocyte volume increased markedly across development, increasing in size by a 20-fold factor within the first 24h of development (y=2851.5, $R^2 = 0.8857$) (Figure 2d). Likewise, astrocyte-associated synapses increased across development following a linearly fitted regression line (y=10033, $R^2 = 0.998$) (Figure 2h). By 72h, a single astrocyte supported an average of 5500 neuropil synapses at once. Taken together, I observed that instead of increasing the number of astrocytes as is seen in vertebrate models and adult *Drosophila* models[123,124], *Drosophila* larval neural development favors stereotyped astrocyte numbers that dramatically increase their neuropil territory as a function of time.

Astrocytes regulate global synapse number in the mushroom body

I showed above that astrocytes are capable of expanding and supporting a large number of synapses over time. Neuropathies such as Alzheimer's disease are marked by insults to synapse homeostasis indiscriminate of synapse type[125]. Synapse loss occurs in part due to atrophies in astroglia, some which likely arise during development[126]. Recent studies have shown that astrocytes influence glutamatergic synapse development by secreting pro- and anti-synaptogenic proteins[127]. The degree to which astrocytes regulate other synapse types is poorly understood, but is of profound clinical

importance. For this reason, I assessed the role of astrocytes in cholinergic synapse development. First, I used the Kenyon cells of the fly Mushroom Body (MB), a known center of learning and memory in *Drosophila*[128], to determine if astrocytes are required to instruct and maintain cholinergic synapse numbers at 72h ALH. I used a MB-specific Flippase tool (FLP) to label both neuronal membranes (*myr::TdTomato*, magenta) and their pre-synaptic sites (*Brp+*, green), using Synaptic Tagging with Recombination (STaR) to assess non-cell autonomous changes in synapse number following astrocyte ablation (Figure 3a-b'). Subsequently, I reconstructed the MB neuronal membranes alongside pre-synaptic puncta to assess the number of MB synapses (Figure 3b''-b'''). I found astrocyte ablation, achieved using *alrm-gal4* paired with *UAS-hid* (death factor), significantly reduced the number of cholinergic synapses within the mushroom body ($p<.05$, Figure 3c-e). These data suggest that astrocytes perform essential roles in modifying synaptic connectivity, and resonate with the devastating symptoms experienced by patients that develop astroglia atrophies.

Astrocytes instruct normal synapse number in dorsal dendritic bipolar neurons

Having established the importance of astrocytes in regulating cholinergic synapse number in center of learning and memory in *Drosophila*, I next asked whether they performed a similar role in sensorimotor circuit development. To address this question, I took a similar approach to label the cholinergic synapses of dorsal bipolar dendritic (Dbd) sensory neurons in control and astrocyte ablation conditions (Fig. 4a-a'''). I found that astrocyte ablation drives a significant reduction in Dbd presynapses, demonstrating that astrocytes broadly regulate cholinergic synapse development/stability ($p<.01$, Figure 4b-d).

A screen to identify astrocyte-secreted proteins that regulate cholinergic synapse number

To further characterize the mechanisms astrocytes employ to instruct synaptogenesis in cholinergic synapses, I took part in a large-scale, reverse genetic RNAi screen to knockdown CSM and secreted proteins expressed in astrocytes and conserved in humans. We used *alrm-gal4* to drive expression of commercially available UAS-RNAi lines[149], and assayed in larvae for both the MB and Dbd neuron presynapses via STaR at 72h ALH. Excitingly, we were able to identify 23 genes, some known and some novel, that regulate synapse formation in cholinergic neurons (MB and DBD), and have potential to regulate synaptogenesis in vertebrates (Preliminary data shown in Figure 5). Continuing work in the Doe lab has recently refined these results and extended into defining the consequence of target gene knockdown to astrocyte health (Dawson 2020 Senior Thesis). Future work will entail characterization of the neuronal receptors of each of these astrocyte-derived synaptogenesis genes, and will define whether they're required specifically for cholinergic synapse development, or universally for generation of all synapse types.

Discussion

Complex neuronal networks are assembled through the birth of immature synapses between axons and dendrites, followed by synapse maturation and refinement. This three-step process relies heavily on molecular mechanisms from glia to establish functional networks. Astrocytes, an abundant subtype of glia, can monitor and alter synaptic function directly, thus actively controlling synaptic regulation in the brain. Quantification of astrocytic domains in mice revealed that a single astrocyte is capable of enwrapping close to 600 dendrites and, through fine processes, ~100,000 synapses[129]. Accumulating evidence is redefining the importance of neuron–glial interactions at synapses in the CNS. For example, *in vitro* studies have shown that most synapses are concurrently generated alongside glial association, and astrocyte ablation alone reduces the global number of functional synapses[130]. More recently it was also discovered that purified glutamatergic spinal motor neurons form few synapses unless cultured with astrocytes[131]. Similarly, synapses at the hippocampal cortex, the center of learning and memory in vertebrates, are greatly reduced following astrocyte elimination *in vitro*[132]. This study recapitulates our current understanding of the critical role of astrocyte-synapse dynamics from an *in vivo* approach. Further, our findings expand on our knowledge of glia-derived synaptogenetic molecules involved in locomotion and learning & memory circuit formation.

Astrocyte processes invade the neuropil during late *Drosophila* embryogenesis, concurrent with synaptogenesis. [46] We observed a 20-fold marked increase in astrocyte volume just within the first 24h of larval life. Moreover, astrocyte-associated synapses increased linearly with the expansion of glial volume. In rodents, astrocytes and

the majority of excitatory synaptic structures appear during the second and third postnatal weeks[109]. This developmental window of extensive synapse formation coincides with the expansion of astrocytes that we observed in fly larvae from 0h to 22h ALH, considering that *Drosophila* embryos hatch at twenty-one hours after egg-laying (25°C). Together, these observations indicate that unlike mammalian models[135], the number of astrocytes in *Drosophila* larvae remains constant across development by expanding astrocytic territories and engulfing a larger number of synapses[136].

Neuronal culturing systems have shed light into the critical role of astrocytes in the formation of many types of synapses, including glutamatergic and GABAergic[137]. These findings have been reported in numerous models ranging from *C. elegans*[138] to humans[139]. Until recently, *in vivo* characterization of astrocyte-neuron dynamics using behaviorally relevant circuits has been largely restricted to glutamatergic circuits[46]. The extensive *Drosophila* genetic toolkit allowed us to manipulate astrocytes while monitoring synapse development for two sets of cholinergic neurons, Kenyon cells involved in learning & memory (MB) and Dbd neurons involved in stretch perception. Astrocyte ablation caused a significant reduction of synapses in both systems, which is in accordance to several neurodevelopmental pathologies that affect circuit development. For example, although autism spectrum disorders have diverse behavioral manifestations with varying degrees of severity, studies have identified global CNS synapse number disruption as central to the pathogenesis of Autism[140]. Similarly, Amyotrophic Lateral Sclerosis studies in cultured mouse spinal cord neurons have shown that disruptions to motoneuron-glia communication via mutations in TNFα/TNFR1 signaling reduced synapse survival[141].

Excitingly, we have determined that the major astrocyte-secreted molecules that induce synapse formation (e.g. TGF-β) or inhibit synapse development (e.g. SPARC) in vertebrates are conserved in fly. Some variants of the TGF-β family (e.g fly *dpp*) had opposing effects in knock down animals, were MB synapses were significantly increased and Dbd synapses were pronouncedly decreased, suggesting that TGB-β may be pro- or anti-synaptogenic depending on the synaptic context[142]. Indeed, glial derived TGB-β ligands promote synaptogenesis at the larval neuromuscular junction[143], but promote pruning of synapses in the MB during morphogenesis[144]. Finally, the advance of sequencing techniques has uncovered the identity of previously unknown astrocyte-enriched genes[145], some even differentially expressed among astrocytes in neurodegenerative disease models[146]. Our screen has identified a subset of CSMs with previously unknown role in astrocytes that yielded strong synaptogenetic phenotypes, including teneurins (Ten-A) and connexins (Innexin 2), stressing the importance of screening to further identify and characterize astrocyte-derived genes that regulate synapse formation. Together, our findings support a large regulatory role for astrocytes in synapse establishment, and sheds light into largely unknown astrocyte-mediated mechanisms of synaptogenesis.

Methods

Fly husbandry

Flies were reared at 25°C on cornmeal fly food.

Genetics

1. *alrm*-gal4 [98] (Chromosome 3)
2. *hs-FLPG5;; 10xUAS(FRT.stop)myr::smGdP-HA, 10xUAS(FRT.stop)myr::smGdP-V5, 10xUAS-(FRT.stop)myr::smGdP-FLAG* (hs-MCFO, BDSC# 64085)
3. *MB-FLP* (this study)
4. *Dbd-FLP* (this study)
5. STaR (BDSC# 56142)
6. TRiP Chromsome 2 Control (BDSC# 36304)
7. TRiP Chromsome 3 Controls (BDSC# 36303, 31603)
8. *UAS-hid*[148]

RNAi lines from the Transgenic RNAi Project at Harvard Medical School[149] (BDSC#)

34661, 29566, 32964, 55276, 34974, 34898, 29597, 34650, 25782, 58128, 38264, 58119, 33952, 34091, 51723, 40885, 55929, 37514, 29627, 28744, 33409, 27991, 28782, 29626, 41656, 29604, 28293, 38965, 38310, 28654, 38229, 38227, 38231, 38936, 30483, 28911, 28588, 33690, 27989, 25873, 27543, 27735, 29439, 34895, 34027, 28071, 42616, 55388, 42783, 62902, 35640, 34700, 32424, 27502, 51741, 29457, 31966, 36732, 27249, 53342, 51788, 44579, 53318, 62918, 32904, 29566, 40888, 32964, 26022, 35248, 28654, 34000, 38536, 38231, 34039, 33416, 64573, 55870, 35024, 25790, 38936, 34084, 40826, 64541, 28724, 25840, 35290, 53253, 64917, 60894, 35628, 50692, 31871, 28519, 30488, 30498, 29565, 57399, 28020, 32490, 51438, 27566, 43988, 34945, 63575, 31874, 57000, 61212, 28770, 35432, 34320, 41949, 56012, 38965, 52110, 34965, 42785, 51460, 28043, 52905, 55242, 42811, 53257, 34880, 34370, 44080, 55657, 55906, 57429, 51480, 52883, 61257, 41913, 25837, 34321, 27568, 64990, 41704, 55386, 41817, 62910, 41817, 53990,

25947, 51787, 44462, 34551, 50678, 34035, 44110, 56866, 27524, 43134, 50693, 54461, 61897, 29452, 35008, 41686, 28581, 36638, 57436, 61309, 62490, 43169, 43236, 63035, 53243, 30483, 28911, 28588, 33690, 36760, 64867, 62368, 34354, 64018, 40946, 40829, 51706, 41681, 39046, 28515, 40901, 51403, 25933, 36131, 35653, 25949, 63578, 35251, 37469, 25781, 29617, 29441, 32429, 43281, 58158, 44116, 34970, 28008, 50911, 42795, 36809, 33618, 28331, 38265, 28747, 50540, 62364, 57813, 32910, 44553, 33642, 62002, 29440, 35213, 28713, 28715, 32408, 36691, 50737, 38207, 34970, 28008, 35050, 57038, 28940, 60013, 58289, 55869, 61985, 28990, 37496, 57299, 28511, 29557, 50901, 32901, 28528, 28924, 29519, 29554, 40938, 33722, 39013, 58256, 38985, 40831, 40947, 42481, 36714, 27536, 34821, 53348, 38894, 57380, 38237, 57813, 32910, 39047, 40866, 55865, 35166, 41906, 51518, 36773, 36722, 32430, 53879, 57235, 29422, 36732, 42645, 30501, 28042, 44663, 26297, 33637, 51503, 28332, 43287, 29306, 60112, 31889, 35600. From VDRC: 101977, 103521, 106791, 106788, 102628, 103185, 107402, 104056.

Larval Collection (MCFO and Ablation)

Fly crosses were kept at 25 º C in collection bottles capped with 3.0% agar apple juice carrying plain yeast paste. Embryonic collections were done for 1.5 hours in new caps to time collections. Larvae were collected at 0h ALH and brains were dissected immediately after reaching their target developmental time.

Larval Collection (Screen)

Fly crosses were maintained at 25 º C in standard food bottles (cornmeal fly food). After 72h, flies were transferred to fresh food bottles for embryo collections (1.5h intervals). At 24h after egg laying, bottles were supplied with wet yeast paste to promote larval growth. Larvae were collected at dissected at 93h after egg laying (72h ALH).

MultiColor FlpOut (MCFO) Generation

The MCFO construct is temperature sensitive and requires short term exposure to 37 º C to increase the likelihood of inducing stochastic FlpOut events[150]. To obtain

sparse labeling of astrocytes, we transferred embryos at 15h after egg laying on thinly sliced, ~2mm thick, apple caps (3.0% agar apple juice) to petri dishes. The caps were sealed with parafilm and floated in a water bath reading 37 º C for 15 minutes. The heat-shock treatment was followed by a 15-minute incubation in an 18 º C chamber to prevent further FlpOut events. Animals were returned to 25 º C until dissections.

Immunochemistry

Brains were extracted in ice-cold, sterile-filtered 1X PBS and mounted on 12mm #1 thickness poly-D-lysine coated round coverslips (Corning® BioCoat™, 354085). Tissues were fixed for 12 minutes (4h ALH samples), 15 minutes (24h ALH samples), or 23 minutes (72h ALH) using fresh 4% paraformaldehyde (Electron Microscopy Sciences, 15710) in .3% PBSTriton, and subsequently washed in .3X PBSTriton to eliminate the fix solution. Blocking was done overnight at 4°C in a .3% PBSTriton mixture with 1% BSA (Fisher, BP1600-100), 1% normal donkey serum and 1% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., 017-000-121 and 005-000-121). Primary antibody was added to the wells and allowed to incubate for one-two days at 4°C. Primary was washed away overnight at 4°C with 0.3% PBST. Secondary antibodies were added and incubated overnight at 4°C following wash. The secondary antibodies were removed from the sample, and round coverslips containing the brains were transferred to .3% PBSTriton overnight for DPX mounting the next day. An ethanol series preluded the DPX mounting: 30%, 50%, 70%, 90%, each for 5 minutes, then twice in 100% ethanol for 10 minutes (Decon Labs, Inc., 2716GEA). Samples were rested in xylenes twice (Fisher Chemical, X5-1) for 10 minutes, and sequentially mounted onto slides containing DPX (Millipore Sigma, 06552). DPX was allowed to

solidify for 1-2 days at room temperature before imaging. Slides were stored at 4°C following DPX hardening. Note: for astrocyte ablation experiments, successful ablation was assessed via anti-Gat staining[109].

Antibodies used

1º antibody (concentration)	Source	Figures
Rabbit anti-V5 (1:1000)	Cell signaling 13202S	1
Rat anti-HA (1:100)	Roche Cat. 11867423001	1, 2
Chicken anti-V5 (1:1000)	Cell signaling 13202S	2
Mouse anti-Brp (1:100)	DSHB Nc82	2
Chicken anti-GFP (1:1000)	Aves Cat. GFP-1010	3, 4, 5
Ms anti-cherry (1:500)	Takara Bio #632543	3, 4, 5
Rabbit anti-Gat (1:4000)	M. Freeman lab	3, 4, 5

Light Microscopy

Fixed brain preparations for MB/Dbd neurons and astrocyte morphology analyses were imaged with a Zeiss LSM 800 laser scanning confocal fitted with a 63x/1.40 NA Oil Plan-Apochromat DIC m27 objective lens and GaAsP photomultiplier tubes.

Data processing and analysis

Quantification of astrocyte-associated synapses & volumetric analyses: Data were acquired with a voxel size of .076 x .076 x .27 μm^3 and de-convoluted in Imaris. A surface ROI was made encompassing only one astrocytic membrane. To quantify synaptic puncta, the same ROI coordinates were used to annotate neuropil synapses (Brp+) using Imaris "Spots". Spots were binned as "direct" if they fell within 90 nm of the Astrocyte "Surface", which accounts for Chromatic aberration between the two channels[151]imaged and only takes in account synapses in direct contact with astrocytic membranes.

MB/Dbd volumetric analyses and synapse counting: Both the MB and Dbd neuronal membranes were reconstructed in Imaris "Surface" module (no smoothing, thresholds varied with fluorescence intensity). For the MB, a standard ROI spanned 300 x 300 pixels in XY, and 50 slices (.34 μm each) in Z. Dbd analyses included only one neuron within the ROI. A distance transformation was then performed on the "Surface". STaR-labeled presynapses within the ROI were annotated using the Imaris "Spots" functions and considered a putative synapse if they fell within 90 nm of the "Surface".

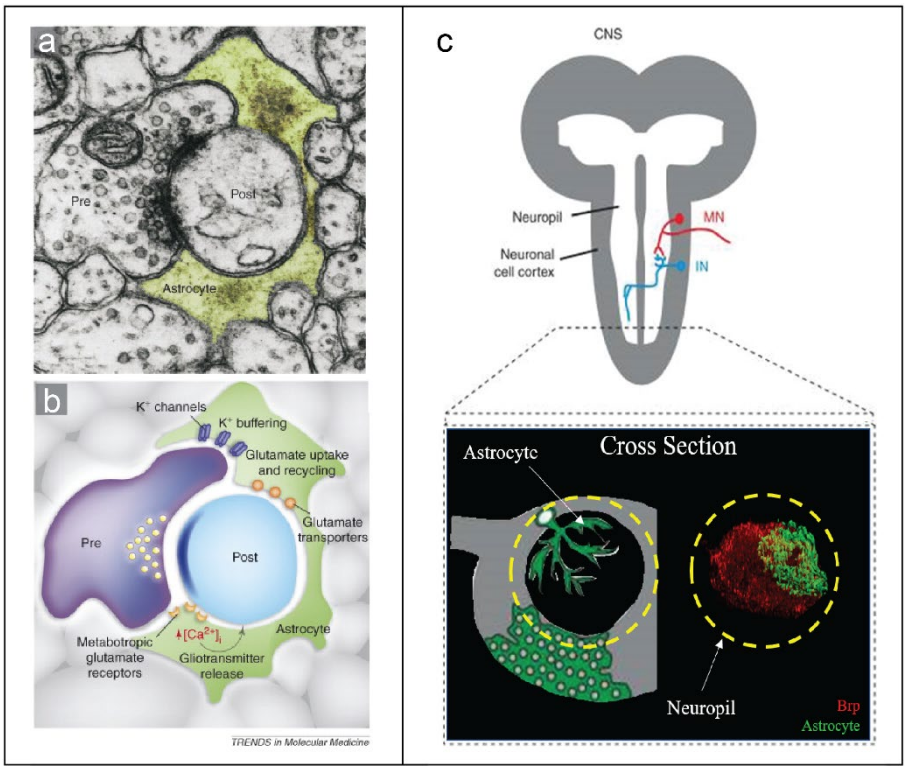
Figure Preparation

The images on the figures were prepared by taking a snapshot of 3D projections in Imaris 9.2.0 (Bitplane AG) or 3D projections in FIJI (ImageJ 1.52h) and assembled using Adobe Illustrator. Schematics were drawn in Adobe Illustrator.

Statistical analyses

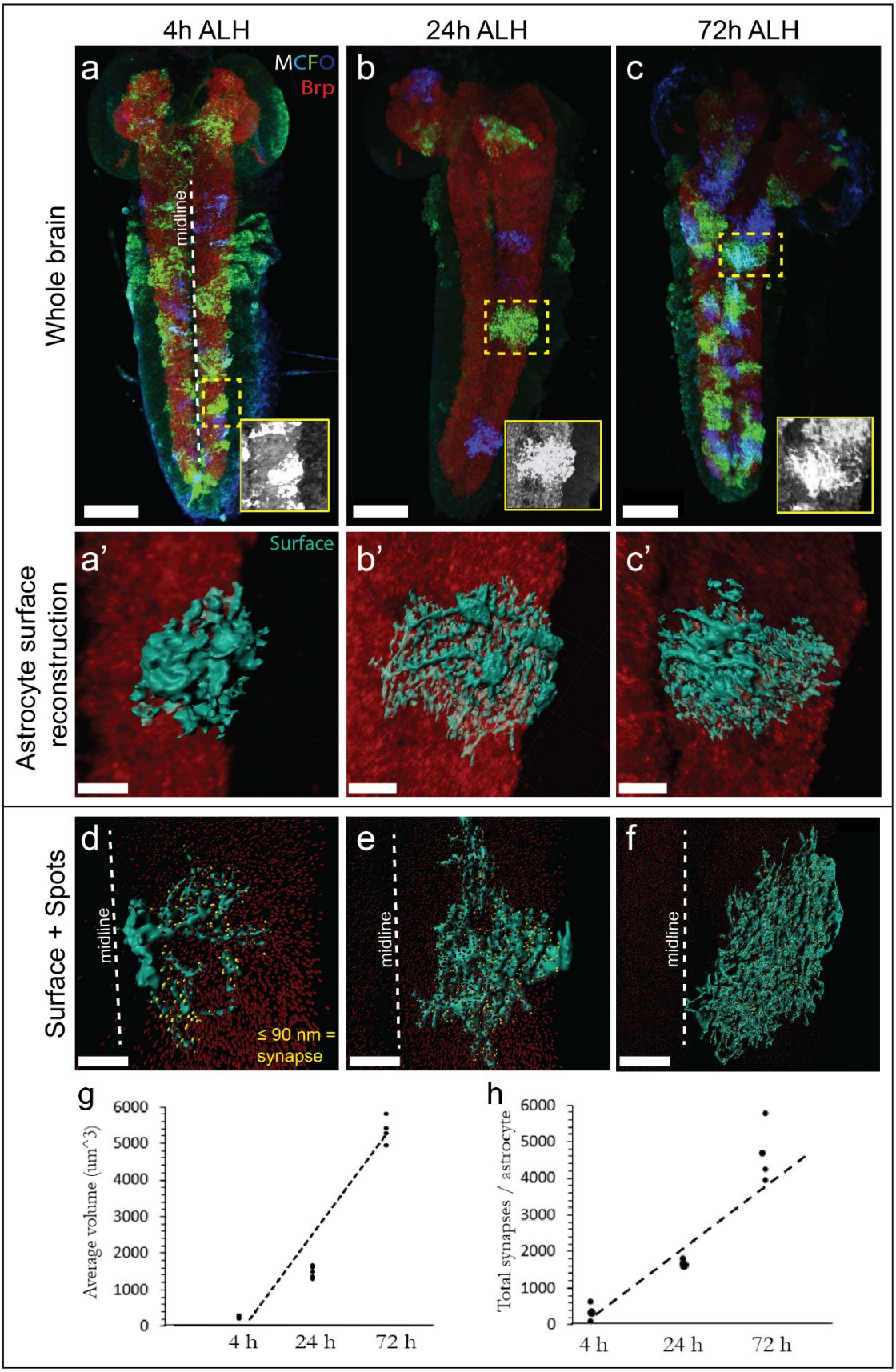
Significance analyses were performed using Microsoft Excel, and Prism (GraphPad). One-way ANOVA was used unless noted otherwise. Alpha values were set to 0.05 to define the level of significance. Significance: *, $p<.05$; **, $p<.01$; ***, $p<.001$; ****, $p<.0001$, NS= not significant. Figure legends and figure labels contain sample size, statistical test employed, and variance results.

Chapter 3 Figures and Legends



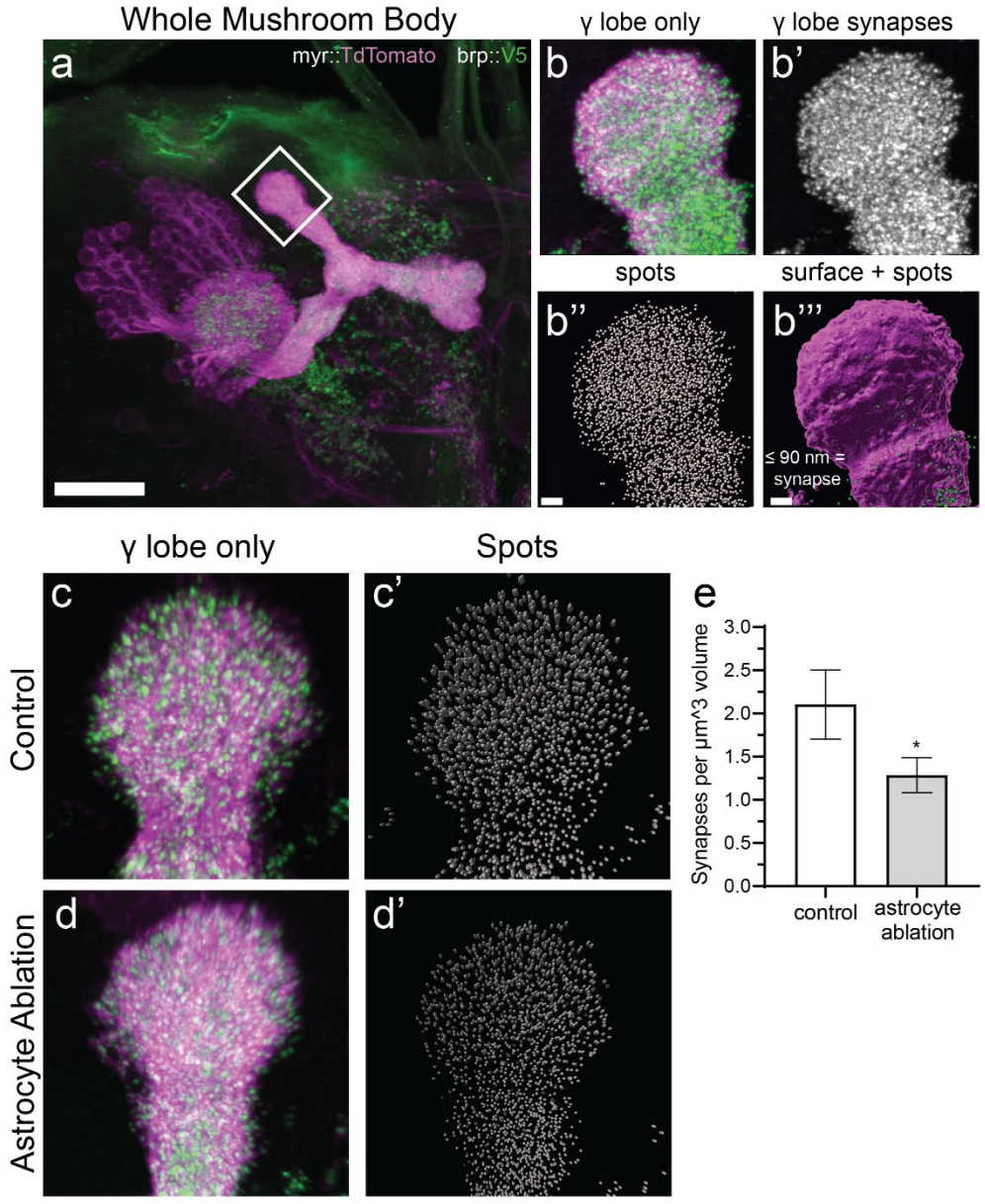
Ch3 Figure 1. Astrocytes are a member of the tri-partite synapse.

(**a**) Astrocytes are a type of glial cell that closely associates with synapses. This EM reconstruction shows that they are part of the "tripartite synapse" composed of two synapsing neurons (pre- and post-synaptic zones labeled accordingly) and an astrocyte (yellow). (**b**) A schematic of astrocyte-dependent processes at the synapse. (**c**) A schematic representing a fly brain, the two lobes and VNC. A simple circuit is depicted. Motor neuron in red. Interneuron in blue. Bottom left panel: A schematic of a VNC cross section, an astrocyte domain is depicted in green. Bottom right panel: Reconstruction of a VNC cross section corresponding to the highlighted region in the schematic. Brp labels synapses within the neuropil. Green is a surface reconstruction of an astrocyte membrane extending its processes into the neuropil. Animal corresponds to 4h ALH. (**a-b**) Reproduced from Allen and Eroglu, 2017[127]. (**c**) Modified from M. Freeman, 2015[113].


4h ALH
24h ALH
72h ALH
Whole brain
Astrocyte surface reconstruction
Surface + Spots
a
b
c
MCFO
Brp
midline
a'
b'
c'
Surface
d
e
f
≤ 90 nm = synapse
g
Average volume (um^3)
6000
5000
4000
3000
2000
1000
0
4 h
24 h
72 h
h
Total synapses / astrocyte
6000
5000
4000
3000
2000
1000
0
4 h
24 h
72 h

Ch3 Figure 2. Astrocytes expand to support increased synapse numbers during development.

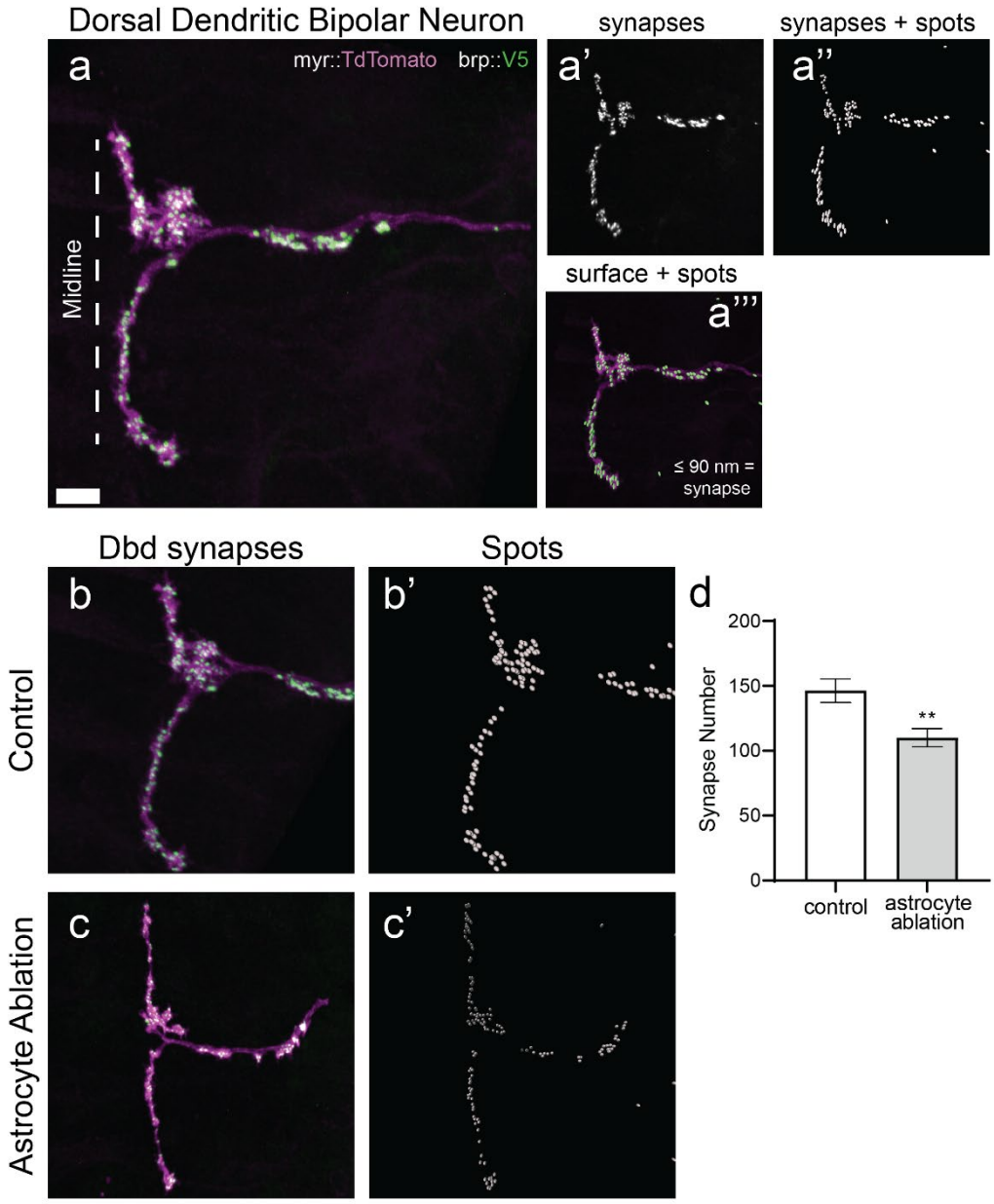
(**a-c**) Multi Color Flip-Out (MCFO) experiments reveal the morphology of astrocytes in the fly neuropil across development (4 h, 24 h, 72 h ALH). (**a'-c'**) Imaris software "Surface" reconstruction of astrocyte membranes shows cell volume growth as a function of developmental time. (**d-f**) Imaris "Surface" reconstruction of wildtype astrocyte membranes across development, along with Brp punta via Imaris "Spots" (neuropil synapses). Yellow correspond to puncta within 90 nm of the astrocyte membrane, and therefore associated with the astrocyte. (**g**) Astrocytic volume increase across development (N= 5 brains per stage, N=3 astrocyte reconstructions per brain). (**h**) Quantification of synapses within <90nm of astrocyte membrane across development. (N=5 cells per timepoint).



Ch3 Figure 3. Ablation of astrocytes decreases MB synapse density.

(**a**) Representative 3D projection of a larval brain lobe (70h ALH) expressing STaR under a FLP line that is active in the MB. Neuronal membranes marked by myr::TdTomato (magenta), pre-synapses by Brp-V5 (green). (**b-b'''**) Imaris pipeline for reconstructing presynaptic spots and neuronal surfaces. (**b**) 3D projection of a γ-lobe

(MB) marked by myr::TdTomato (magenta), pre-synapses by Brp-V5 (green) (white box in a). (**b'**) 3D projection of a γ-lobe displaying pre-synapses only. (**b''**) synaptic puncta reconstructions (spots). (**b'''**) Reconstruction of neuronal surfaces in the MB marked by myr::TdTomato (magenta) + presynaptic spots localized within 90 nm of the surface. (**c-c'**) 3D projection of a control Mb γ-lobe labeled with the STaR method (see above). (**d-d'**) 3D projection of a Mb γ-lobe labeled with the STaR method. Astrocyte ablation was caused by *alrm-gal4* paired with *UAS-hid* (death factor). (**e**) Bar graph displaying ratio of synapses per μm^3 (obtained from synapses/total volume of the membrane). Both conditions were averaged and compared using an unpaired Student's T-test (p=0.01, N=6 control lobes, N=4 lobes for ablation).



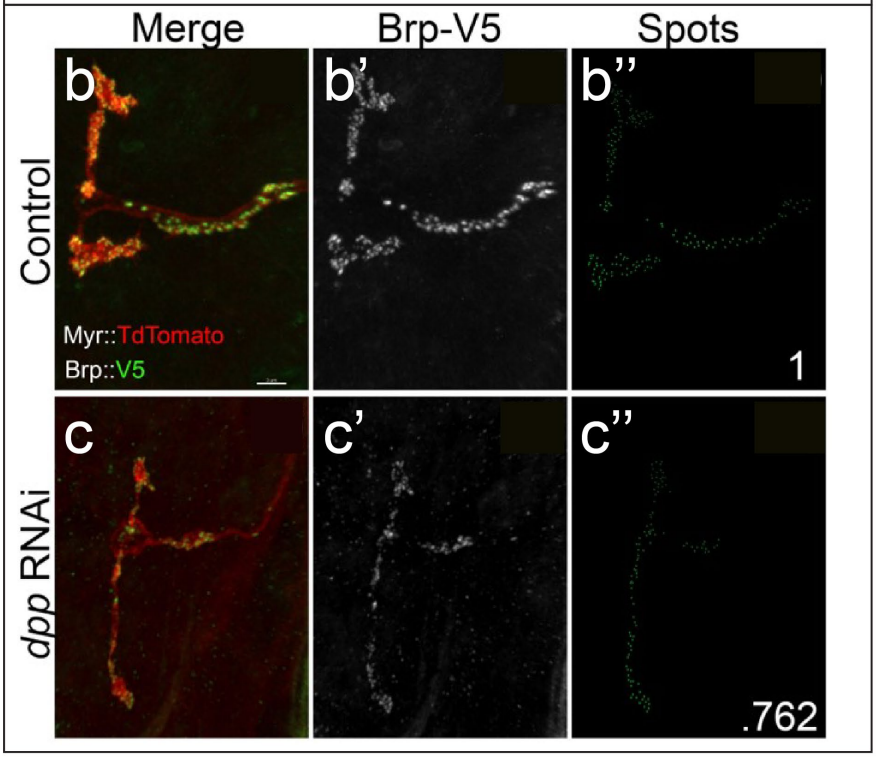
Ch3 Figure 4. Ablation of astrocytes decreases Dbd synapse number.

(a) Representative 3D projection of a Dbd sensory neuron, Dbd extends axons to innervate the CNS and form synapses close to the midline. Neurons labeled with STaR (Brp::V5 in green, myr::TdTomato in magenta). (**a'**) 3D projection of a Dbd neuron displaying pre-synapses only. (**a''**) Reconstruction of pre-synaptic puncta utilizing Imaris "Spots" function. (**a'''**) Neuronal membranes reconstructed (Magenta) via Imaris "Surface" function along with pre-synaptic "Spots" within the same ROI.

(**b-b'**) Control Dbd labeled via STaR. (**c-c'**) Dbd following astrocyte death by *alrm-Gal4, UAS-hid* (death factor). Prime panels show Imaris spots reconstruction of synapses. (**d**) Quantification of synapse numbers at 72h ALH. Unpaired T-test P=0.002, N=5 brains with a total of N=10 neurons per condition.

a

	Protein (Family)	Astrocyte Function	Fly Homologs	MB synapses	Dbd synapses
previously defined in mammals	TGF-β	+ synaptogenesis &	*mav*	Increased**	NS
		+ synapse pruning	*dpp*	Increased**	Decreased**
			daw	Decreased*	TDB
			gbb	Decreased**	NS
	TSP	+ synaptogenesis	*tsp*	NS	Decreased****
	γ-Protocadherins	+ synaptogenesis	*fat*	Increased*	NS
			dachsous	Increased*	NS
	TNF-α	+ synaptogenesis	*egr*	Decreased*	Decreased*
	Glypican	+ synaptogenesis	*dally*	Increased**	NS
	Neuroligins	+ excitatory, - inhibitory synapses	*neuroligin 2*	Increased*	Decreased****
			neuroligin 4	TBD	Decreased****
	SPARC	- synaptogenesis	*sparc*	Increased**	NS
astro+	FGFR	neuropil infiltration	*heartless*	Increased**	Decreased*
	STAT92E	+ engulfment	*stat92e*	Increased**	NS
	GAT3	GABA turnover	*gat*	Increased*	Decreased*
unknown	TENM-3	unknown	*ten-a*	Decreased**	Increased*
	Connexins	unknown	*Innexin 2*	Increased***	Decreased*
	7B2 (neuropep)	unknown	*7B2*	Increased*	TBD
	Notch	various	*N*	Increased**	TBD
DIP/Dpr	IG superfamily	unknown	*dpr5*	Increased*	TBD
	IG superfamily	unknown	*dip-beta*	Increased*	TBD
	IG superfamily	unknown	*dpr-17*	TBD	Decreased*
	IG superfamily	unknown	*dip-beta*	NS	Decreased*



Ch3 Figure 5. Astrocyte KD of CSM and secreted proteins disrupts cholinergic synapse number.

(**a**) Table summary of screen preliminary data. Knockdown of genes previously identified in mammalian astrocytes as regulators of synapse number produced phenotypes in one or both circuits (grey). Genes known to affect astrocyte function and/or morphology (Astro+) shown in blue. Novel genes shown in orange. Genes belonging to the IG superfamily of DIP/Dprs, putative synapse specificity molecules, shown in green. (**b-c**) Representative images of control (**b-b''**) and *dpp* RNAi (**c-c''**) neurons. Knockdown neurons have ~.762 times less synapses than control (N>5 brains, > 16 neurons per genotype, p<.01).

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