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Axon and Dendrite Pruning in *Drosophila*

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Abstract

Pruning, a process by which neurons selectively remove exuberant or unnecessary processes without causing cell death, is crucial for the establishment of mature neural circuits during animal development. Yet relatively little is known about molecular and cellular mechanisms that govern neuronal pruning. Holometabolous insects, such as *Drosophila*, undergo complete metamorphosis and their larval nervous systems are replaced with adult-specific ones, thus providing attractive models for studying neuronal pruning. *Drosophila* mushroom body and dendritic arborization neurons have been utilized as two appealing systems to elucidate the underlying mechanisms of axon and dendrite pruning, respectively. In this review we highlight recent developments and discuss some similarities and differences in the mechanisms that regulate these two distinct modes of neuronal pruning in *Drosophila*.

Introduction

During an early phase of neurogenesis, neurons often generate superfluous connections. Selective elimination of unnecessary neurites without death of the parental neurons, referred to as pruning, is critical for proper wiring of the nervous systems [1]. Pruning occurs extensively in many neuronal systems including the hippocampus, cortex, spinal cord and neuromuscular junction in mammals [1]. In *Drosophila*, many larval neurons undergo pruning during metamorphosis [2, 3], a developmental stage induced by the steroid molting hormone 20-hydroxyecdysone (ecdysone) [4]. In the central nervous system (CNS), mushroom body (MB) γ neurons, embryonic olfactory projection neurons and serotonergic neurons prune their larval axons and dendrites, and subsequently re-extend their adult-specific processes [5–7]. In the peripheral nervous system (PNS), a subset of dendritic arborization (da) sensory neurons engage in either apoptosis [8] or dendrite-specific pruning [8, 9]. The stereotyped occurrence of axon and dendrite pruning, coupled with the plethora of genetic tools available in *Drosophila*, allows an unprecedented dissection of the underlying molecular and cellular mechanisms. Here we will highlight the recent findings involving axon and dendrite pruning in *Drosophila* and discuss some similarities and differences in the underlying mechanisms.

Axon Pruning of MB γ neurons

Mushroom body development

The remodeling of MB γ neurons during metamorphosis has become an attractive model system to study developmental axon pruning. The MB is composed of about 2000 neurons that belong to three types of neurons (γ , α'/β' and α/β) born sequentially from four identical neuroblasts per hemisphere [10, 11]. The γ neurons are the only type that undergoes remodeling [11, 12]. MB γ neurons initially send out small dendritic arbors and bifurcated axons that project to the medial (m) and dorsal (d) lobes (Figure 1). During metamorphosis, the dendrites are completely eliminated and the axons are pruned up to a specific and stereotypic point. At 18h after puparium formation (APF), γ neurons complete pruning and subsequently undergo developmental axon regrowth in a process distinct from initial axon growth [13], resulting in mature axons projecting only to the medial lobe [11].

Cellular mechanisms of MB γ neuron pruning

By following the pruning of γ neurons at a single cell resolution, Watts and colleagues [14] found that neurites are eliminated via local degeneration beginning with dendrite fragmentation at ~4h APF, followed by axon fragmentation at ~8h APF. The spatial and temporal regulations of axon fragmentation, where and how it is initiated (distal vs. proximal) and whether it depends on extrinsic signals, remain to be determined. The first observable event during axon pruning is the elimination of microtubules (MTs) [14]. However, the mechanisms of MT elimination and its requirement for axon pruning of MB neurons remain unknown.

The fact that pruning involves axon fragmentation posits the existence of phagocytic cells to clear the remaining debris. Indeed, glial cells have been shown to engulf axonal debris [15, 16], dependent on the engulfment receptor Draper (Drpr, CED-1 homolog) [17, 18] and CED-6 [17]. Following engulfment, axonal debris is degraded within glia by the lysosomal pathway [15, 16]. Recently, two studies have highlighted astrocytes as the major phagocytic glia [19, 20] in a process that depends on Drpr [19, 20] functioning in parallel to CED-12 [19]. While during normal development astrocytes are the primary cells that engulf degenerated axons, inhibiting *drpr* or both *drpr* and *ced-12* in astrocytes results in a delay or modest defect of fragment clearance [19, 20], suggesting that other unknown cells might take over when astrocytes are defective.

A second role of glia is to instruct the initiation of axon pruning [21]. In an elegant study, Awasaki and colleagues show that cortex glia and to a lesser extent also astrocytes, secrete the TGF- β ligand Myoglianin (Myo) which likely functions through the TGF- β receptor complex on γ neurons and initiates a transcription program that is essential for pruning initiation (Figure 3; see more below). While cortex glia surround the cell bodies, astrocytes send extensions that occupy the degenerating lobes [20]. The relative contribution of different glial cell types to pruning initiation still remains to be resolved. Glia may also actively regulate the axon fragmentation process, which seems to be regulated by glial expression of the ecdysone receptor (EcR) [19, 20]. Finally, during metamorphosis, 6-8 glial cells are adjacent to the degenerating lobes [16, 20] out of which only 1-2 are astrocytes

[20]. The identity or the role of the 4-6 additional glial cells is not known. Therefore, glia-neuron interaction may play complex roles during axon pruning and many aspects of these interactions remain to be discovered.

Molecular mechanisms of MB γ neuron pruning

Although neuron-glia interactions are required for axon pruning, in essence, it is a cell-autonomous process. Most of the molecules required for axon pruning in MB γ neurons were identified in clonal assays using the Mosaic Analysis with a Repressible Cell Marker (MARCM) technique [22]. The first player identified is the ecdysone receptor heterodimer composed of Ultraspiracle (Usp) and EcR-B1 (Figure 3) [5]. MARCM clones homozygous for *usp* as well as γ neurons mutant for *EcR-B1* fail to prune. However, ectopic expression of EcR-B1 in α'/β' neurons is not sufficient to induce pruning (Schuldiner, unpublished), suggesting additional pathways/factors required in parallel to EcR-B1.

EcR-B1 is specifically expressed in γ neurons [5], which requires the TGF- β pathway [23], the cohesin complex [24] and the nuclear receptors Ftz-f1 and Hr39 [25]. TGF- β signaling is initiated by glial secretion of Myo [21] functioning through the type I receptor Baboon (Babo) and either one of the type II receptors Punt or Wishful thinking (Wit) (Figure 3) [23]. Recently, another transmembrane receptor, Plum, belonging to the immunoglobulin superfamily, has been shown to facilitate TGF- β signaling, most likely functioning as a TGF- β accessory receptor (Figure 3) [26]. How does TGF- β regulate the expression of EcR-B1? While it was shown that dSmad2 (also known as SmoX) [23] and Smad4 (also known as Medea; Schuldiner, unpublished) are required for axon pruning, the precise mechanisms of EcR-B1 activation by the TGF- β pathway remains to be determined.

Surprisingly, not much is known about the molecular mechanisms that regulate axon pruning downstream of EcR-B1. Recently, studies originating from forward genetic screens in da neurons (see below) have uncovered that the transcription factor Sox14 appears to be essential for axon pruning of MB neurons (Figure 3) [27]. Knockdown of *sox14* via RNA interference resulted in an axon pruning defect in MB γ neurons [27]. Furthermore, a Cullin1-based SCF E3 ubiquitin ligase complex, regulated by *sox14*, is also essential for MB γ neuron axon pruning (Figure 3) [28] but the downstream targets and precise mechanisms remain to be uncovered. MB γ neurons require an intact ubiquitin-proteasome system (UPS) for axon pruning [14]. MARCM clones for *uba1*, the only *Drosophila* E1 gene, or the proteasome subunits *rpn6* and *mov34* exhibited axon pruning defects (Figure 3) [14]. The transcript levels of *cullin1*, *uba1* and *rpn6* are regulated by *EcR-B1* [28, 29] and *sox14* [28]. Regardless, the substrate(s) that is degraded by the UPS to promote MB axon pruning is not yet known.

Dendrite pruning of ddaC neurons

Dendritic arborization sensory neurons

The remodeling of da sensory neurons during metamorphosis has emerged as another appealing model system to study developmental dendrite pruning. da neurons are classified into classes I-IV based on their arbor size/complexity [30, 31]. While da neurons extend

highly-branched dendrites underneath the epidermis, their axons project ventrally to the ventral nerve cord. Peripheral glia wrap the soma, axons, and proximal dendrites of da neurons [32, 33]. During metamorphosis, da neurons undergo extensive remodeling. While some of da neurons are eliminated via apoptosis [8], the class IV ddaC neurons survive but prune the larval dendrites with the axons remaining intact [8, 9]. Dendrite-specific pruning of sensory neurons contrasts with pruning of both axons and dendrites in MB γ neurons and other central neurons [5–7]. ddaC neurons subsequently regrow new dendrites to be integrated into the adult nervous system [9, 34, 35]. The molecular mechanisms regulating this regrowth and whether they are similar to MB axon regrowth are not yet known.

Cellular mechanisms of dendrite pruning in class IV ddaC neurons

da neurons undergo a stereotyped pruning process mainly involving local degeneration [8]. Shortly after puparium formation, ddaC neurons form small blebs along the proximal dendritic branches, leading to dendritic thinning, microtubule disassembly and subsequently severing of the dendrites from the soma (Figure 2) [8, 27, 36]. Dendrite severing, a hallmark step of the pruning process, initiates at approximately 5h APF, which involves the microtubule severing protein Katanin p60-like 1 [36]. Peripheral glial cells that wrap the proximal dendrites of da neurons regulate the location of initial dendrite severing [33]. Upon severing, detached dendrites undergo rapid fragmentation and clearance of cellular debris which is completed by 16h APF (Figure 2). Phagocytic hemocytes were shown to be involved engulfment and degradation of the dendritic fragments and debris [8], which involves activation of extracellular matrix metalloproteases [9]. However, a recent study reported that epidermal cells are the primary phagocytes in debris clearance [37].

Ecdysone receptor, epigenetic regulators and their target genes during dendrite pruning

EcR-B1 and its co-receptor Usp are cell-autonomously required to regulate dendrite pruning of da neurons (Figure 3) [8, 9]. In ddaC neurons, overexpression of the dominant negative form of EcR (EcR^{DN}) or loss of *usp* function prevents the initiation of dendrite severing [8, 9]. Similar to MB γ neurons, EcR-B1 expression is induced by TGF- β signaling in ddaC neurons at the late third in-star larval stage (Yu, unpublished). However, the identity and source of the TGF- β ligand remain unknown during ddaC dendrite pruning. The first EcR-B1 target gene that regulates ddaC dendrite pruning is *sox14* (Figure 3) [27], an ecdysone early-response gene [38]. Sox14, a HMG domain transcription factor, binds to the *mical* promoter and induces the expression of Mical, a cytoskeletal regulator (Figure 3) [27]. Both Sox14 and Mical play important roles in dendrite severing in ddaC neurons [27]. Mical was previously shown to mediate Sema/Plexin-dependent axonal repulsion by disassembling F-actin cytoskeleton [39, 40]. However, the possible role of Mical in the rearrangement of actin cytoskeleton during dendrite severing remains to be determined. Another target gene of EcR-B1 during ddaC dendrite pruning is *headcase* (*hdc*) which is expressed independently of Sox14 [41]. Hdc is important for dendrite severing in ddaC neurons and thus represents the Sox14-independent pathway [41]. However, its exact role during dendrite pruning remains unknown.

How is the expression of Sox14 induced by EcR-B1? Two epigenetic factors, the chromatin remodeler Brahma (Brm) and the histone acetyltransferase CREB-binding protein (CBP),

were identified for their roles to facilitate Sox14 expression and dendrite pruning [42]. A model has been proposed that the interplay among EcR-B1, Brm and CBP results in local enrichment of histone acetylation at the *sox14* regulatory locus and thereby induces Sox14 expression and dendrite pruning [42]. Future studies towards identification of the repressive complex that suppresses Sox14 expression prior to ecdysone release might allow us to elucidate the developmental timing control of dendrite pruning.

Ubiquitin-proteasome system and its substrates during dendrite pruning

The UPS cell-autonomously regulates dendrite pruning in ddaC neurons [9], similar to MB axon pruning [14]. When the UPS was inhibited, ddaC neurons exhibited severe dendrite severing defects [9]. The specificity of substrates that are degraded by the UPS is determined by a variety of E3 ubiquitin ligases. One E3 ligase is *Drosophila* inhibitor of apoptosis protein 1 (DIAP1), which has been shown to suppress the Dronc caspase activity and negatively regulate ddaC dendrite pruning (Figure 3) [43]. Although inhibition of caspases activity does not lead to a notable axon pruning defect in MB γ neurons [14], the studies in dendrite pruning have highlighted an essential role of caspases [43–45]. Local caspase activity is detectable in the dendrites of ddaC neurons during pruning [43, 44]. Moreover, DIAP1 and caspases are regulated by Valosin-containing protein (VCP) [46]. Inhibition of VCP causes the defects in ddaC dendrite pruning, occurring concomitantly with reduced caspase activity and high DIAP1 levels.

Another E3 ligase that has recently been identified for its roles in both ddaC dendrite pruning and MB axon pruning is the Cullin1-based SCF E3 ubiquitin ligase complex (Figure 3) [28]. This E3 ligase acts downstream of Sox14 but in parallel to Mical and facilitates ddaC dendrite pruning through inactivation of the InR/PI3K/TOR pathway (Figure 3) [28]. To achieve this, the F-box protein Slimb, a component of the SCF E3 ligase, associates with Akt, an activator of the InR/PI3K/TOR pathway, resulting in its ubiquitination and proteasomal degradation [28]. The UPS and the Cullin-1 SCF E3 ligase govern both axon and dendrite pruning, implying a role in the initiation step, rather than the execution step.

Calcium signaling during dendrite pruning

In an elegant study, Kanamori and colleagues employed the genetically encoded calcium indicator GCaMP3 and detected compartmentalized calcium transients in specific dendrite branches prior to branch elimination [47]. Suppression of calcium transients in mutant neurons devoid of the voltage gated calcium channels (VGCCs) caused impaired dendrite pruning [47]. Calcium transients activate the calpain proteases to promote dendrite pruning [47]. Thus, compartmentalized calcium transients provide temporal and spatial cues to induce dendrite pruning. However, the precise mechanisms of the calpain proteases and their activation by the calcium pathway during dendrite pruning remain to be determined.

Conclusions

Axon and dendrite pruning is still an emerging field. In this review, we have discussed considerable progress made recently in our understanding of the underlying cellular and molecular mechanisms in *Drosophila*. Both types of pruning are initiated by ecdysone-

dependent activation of EcR-B1/Usp pathway. TGF- β signaling as a means to regulate expression of EcR-B1 is conserved in axon and dendrite pruning. The cohesin complex and the Ftz-F1/Hr39 nuclear receptors are conserved in regulating both axon and dendrite pruning (Yu, unpublished). Epigenetic factors and their common target *sox14* are required for both ddaC dendrite pruning and MB axon pruning. UPS and the Cullin-1 SCF E3 ligase govern both types of pruning although the downstream targets destined for degradation are likely distinct. On the other hand, although its expression is upregulated by EcR-B1 and Sox14 in MB γ neurons and ddaC neurons, Mical is required for ddaC dendrite pruning but not for MB axon pruning. Likewise, Hdc and Ik2 specifically regulate ddaC dendrite pruning. Another fundamental difference between these two systems is the involvement of caspases, which are activated during ddaC dendrite pruning but not MB axon pruning. These specific requirements may suggest the differences in the execution phase for these two types of pruning. Therefore, we raise a few non-mutually exclusive hypotheses as for these differences. First, the execution of axon and dendrite pruning are likely to utilize different mechanisms. At least in the case of caspases, though, this is not a sufficient explanation as they are not required for axon or dendrite pruning of MB neurons[43] (Schuldiner, unpublished). Second, these differential mechanisms might result from the differences in the CNS vs PNS, resulting in structural and environmental differences between MB γ neurons and ddaC neurons. Finally, potential parallel pathways in MB neurons might circumvent their identification experimentally.

In summary, we are still far from having a complete understanding of the underlying mechanisms of axon and dendrite pruning. Future studies will undoubtedly provide further evidence of conserved and differential mechanisms for axon and dendrite pruning in MB and ddaC neurons in *Drosophila*. These mechanisms might be evolutionarily conserved and also exploited to facilitate the neurite pruning processes across species.

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** of outstanding interest

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[47]. Kanamori T, Kanai MI, Dairy Y, et al. Compartmentalized calcium transients trigger dendrite pruning in *Drosophila* sensory neurons. *Science*. 2013; 340:1475–8. [PubMed: 23722427] ** [19, 20] In these two studies, the authors show that astrocytes are the major phagocytic cell during development of the nervous system. While the paper [21] shows that astrocytes engulf synaptic and axonal fragments throughout the nervous system, the paper [22] focuses on their role during MB remodeling. Both studies show that astrocytes engulf pruned MB neurons in a process that depends on Drpr [21, 22] and Ced-12 [21]. Astrocytic EcR-B1 is important for their morphological change as well as their active induction of pruning via an unknown mechanism. ** [21] In this elegant study the authors have focused on identification of the TGF-beta ligand that is required for pruning induction. They found that myoglianin, secreted from cortex glia and to a lesser extent also astrocytes, instructs axon pruning of MB γ neurons. Thus, glia regulate developmental axon pruning not only by engulfing unwanted axons but not by activating EcR-B1 expression and enabling the initiation of the pruning process. ** [26] In this study, the authors performed a large forward genetic screen and identified Plum, an uncharacterized transmembrane immunoglobulin superfamily (Ig-SF) protein, which is required for MB axon pruning. Plum facilitates TGF-beta signaling, most likely by functioning as an accessory receptor. Remarkably, Plum, the TGF-beta signaling pathway and EcR-B1 are also important for axonal refinement at the larval neuromuscular junction (NMJ). Thus, MB pruning and NMJ refinement, unlike previously thought, share common mechanisms. ** [28] This paper identifies a Cullin1-based SCF E3 ligase that governs both dendrite pruning of ddaC neurons and axon pruning of MB γ neurons. The authors show that the SCF E3 ligase attenuates the InR/PI3K/TOR pathway by ubiquitination of Akt, an activator of the InR/PI3K/TOR pathway, and thereby promotes ddaC dendrite pruning. The F-box protein Slimb, a component of the SCF E3 ligase, forms a complex with Akt. Thus, Akt is a novel substrate of the SCF E3 ligase complex during dendrite pruning. * [41] This paper describes a genetic interaction screen which identified *headcase*, a novel target gene of EcR-B1. Headcase expression is upregulated at the wandering 3rd instar stage, independently of Sox14. Headcase is important for dendrite severing in ddaC neurons, representing a Sox14-independent pathway. This study raises interesting questions about the role of Headcase in dendrite pruning. ** [42] This paper shows that two epigenetic factors Bramha and CBP govern both dendrite pruning in ddaC neurons and axon pruning in MB γ neurons. CBP interacts with EcR-B1 in an ecdysone dependent manner, which is facilitated by Brm, resulting in Sox14 expression by the enhancement of histone acetylation at the *sox14* locus in cultured cells. Thus, epigenetic regulators are important for the developmental timing control of dendrite pruning in ddaC neurons. * [46] This paper illustrates a role of Valosin-containing protein (VCP) in dendrite pruning and apoptosis of *Drosophila* da sensory neurons. Reduced VCP function cause impaired caspase activation and high DIAP1 levels. VCP is able to associate with DIAP1 in a ubiquitin- and BIR domain-dependent manner and promotes its degradation in cultured cells. ** [47] This paper provides the appealing evidence for compartmentalized calcium transients in specific dendritic branches of class IV da (ddaC) neurons before branch elimination. In dendritic branches, calcium signaling is activated via the voltage-gated calcium channels (VGCCs). Moreover, the authors show the calcium-activated protease calpain function downstream of the calcium transients to regulate dendrite pruning. Thus, compartmentalized calcium transients provide temporal and spatial cues to induce dendrite pruning.

Figure 1.

A schematic representation of axon pruning in MB γ neurons. At larval stage, MB γ neurons project a single axon that branches to form dendrites (den), a tightly fasciculated axon peduncle (p) that bifurcates to the dorsal (d) and medial (m) lobe. Cortex glia (yellow) instruct MB axon pruning by secreting the TGF- β ligand Myoglianin. At 6 h after puparium formation (APF), MB γ dendrites are mostly eliminated, axons begin to undergo disassembly and astrocytes (magenta) infiltrate the dorsal and medial lobes. The role of the remaining unidentified glia or cortex glia during these time points is not known (gray). At 18 h APF fragmentation is complete and axonal fragments are being engulfed by astrocytes. Subsequently, γ neurons project new, adult specific axons, that project only to the medial lobe.

Figure 2.

A schematic representation of dendrite pruning in ddaC neurons during metamorphosis. At the white prepupal stage (0 h APF), ddaC neurons extend highly-branched dendrites (green) as well as a single axon projecting ventrally to the ventral nerve cord. Peripheral glia (red) wrap the soma, axon and also the proximal dendritic area in ddaC neurons. Dendrites undergo blebbing, thinning, subsequently detachment starting from approximately 5 h APF, followed by fragmentation and debris clearance via phagocytes. By 16-18 h APF, all dendritic debris is eliminated. Open arrowheads point to proximal severing of the dorsal dendrite branches of ddaC neurons. Red arrowheads point to ddaC somas. An asterisk indicates a phagocyte.

	MB	da
TGF- β Myo, Babo, Punt/Wit, Plum, Smox, Med	●	● *
EcR-B1; Usp	●	●
Sox14	●	●
Hdc	●	●
MICAL	●	●
UBA1	●	●
Cullin 1	●	●
AKT, InR/PI3K/TOR	● *	●
UbcD1, DIAP, Caspases	● *	●

Figure 3. Ecdysone signaling and its regulators govern axon and dendrite pruning in *Drosophila*. A table summarizes the conserved and differential mechanisms between MB axon pruning and ddaC dendrite pruning. Green circles indicate ‘required’, red circles indicate ‘not required’, and grey circle indicates ‘unknown’. Asterisks highlight the unpublished observations.