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Nuclear receptors and *Drosophila* neuronal remodelling

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ABSTRACT

During the development of both vertebrates and invertebrates, neurons undergo a crucial remodelling process that is necessary for their new function. Neuronal remodelling is composed of two stages: first, axons and dendrites are pruned without the loss of the cell body; later, this process is most commonly followed by a regrowth step. Holometabolous insects like the fruitfly *Drosophila* exhibit striking differences between their larval and adult stages. These neuronal remodelling processes occur during metamorphosis, the period of transformation from a larva to an adult. All axon and dendrite pruning events ultimately depend on the *EcR* nuclear receptor. Its ligand, the steroid moulting hormone ecdysone, binds to heteromeric receptors comprising the nuclear receptor ECR and USP, and this complex regulates target genes involved in neuronal remodelling. Here we review the nuclear receptor-mediated genetic control of the main neuronal remodelling events described so far in *Drosophila*. These events consist of neurite degeneration in the mushroom bodies (MBs: the brain memory center) and in the dendritic arborizing sensory neurons, neurite retraction or small scale elimination in the thoracic ventral neurosecretory cells, olfactory circuits and in the neuromuscular junction as well as MB axon regrowth after pruning and the role of MB neuron remodelling in memory formation.

Highlights:

EcR is required in the glia and neurons during MB axon pruning

EcRB1 is required in both the muscle and motor neurons during NMJ dismantling

EcRB1 is required for both axon retraction and degeneration

The *unf* nuclear receptor is required for MB axon regrowth possibly by repressing *EcRB1*

MB neuronal remodelling during metamorphosis is necessary for adult memory

Keywords:

Ecdysone

Neurite degeneration

Neurite retraction

Axon regrowth

Memory

Mushroom bodies

da sensory neurons

T_v neurons

Olfactory circuit

Motor neuron

1. Introduction

Drosophila melanogaster nuclear receptors are regulated transcription factors that play important roles in key metabolic and developmental pathways. The activity of some nuclear receptors is ligand regulated, and known ligands can be lipophilic molecules or steroids hormones. Other receptors, known as orphan receptors, may function in a ligand-independent fashion. The *Drosophila* genome contains only 18 canonical nuclear receptor genes that belong to six different nuclear receptor families: *E75* (NR1D3), *E78* (NR1E1), *Hr3* (NR1F4), the ecdysone receptor (*EcR*; NR1H1), *Hr96* (NR1J1), *Hnf4* (NR2A4), *ultraspiracle* (*usp*), *Hr78* (NR2D1), *tailless* (*tll*; NR2E2), *dissatisfaction* (*dsf*, NR2E4), *Hr83* (NR2E5), *unfulfilled* (*unf*, also known as *Hr51* and *Nr2e3*), *seven up* (*svp*), *ERR* (NR3B4), *Hr38* (NR4A4), *ftz-f1* (NR5A1), *Hr39* (NR5B1), and *Hr4* (NR6A2) [1].

Most effects of the steroid hormone 20-hydroxyecdysone (ecdysone) are mediated through the ECR-USP receptor. Many direct targets of the ecdysone-ECR-USP complex receptor are themselves nuclear receptors. Therefore, most of these nuclear receptors are indirectly regulated by ecdysone. In flies, a peak of ecdysone induced at the end of the third larval stage initiates diverse metamorphic responses in neuroblasts (neuronal precursor cells) and postmitotic neurons. Many postmitotic neurons are remodelled in response to this trigger, to generate adult circuits. The remodeling occurring during this developmental period is expected to be necessary

for the normal functioning of these new circuits. In holometabolous insects, the difference in lifestyle is particularly apparent between the larval and the adult stages. A larva is chiefly constantly eating in a virtually constant environment. An adult has to eat but also to fly, to behave in an ever-changing environment, to find mates, to copulate and, for the females, to lay eggs in an appropriate area.

Seven *Drosophila* nuclear receptors have been shown to regulate various aspects of neural development: ECR, USP, TLL, UNF, SVP, FTZ-F1 and HR39. ECR binds to ecdysone and heterodimerizes with USP (an RXR-ortholog) to mediate pruning and outgrowth in the *Drosophila* nervous system. The ECR/USP heterodimer binds to specific ecdysone response elements (EcRE) in DNA to regulate transcription of target genes. As such, the ECR/USP complex is able to both activate and repress target genes, depending on the presence or absence of ecdysteroids [2,3]. The three *EcR* isoforms, *EcR-A*, *EcR-B1* and *EcR-B2*, differ at their N-terminal ends but share the same ecdysone and DNA binding domains. Studies in *Manduca sexta* and *Drosophila* have shown that *EcR-B1* is expressed in neurons that undergo reorganization, and is required for neuron remodelling during metamorphosis, whereas *EcR-A* is frequently expressed in differentiating neurons [4,5,6,7]. It was shown, in the bee *Apis mellifera*, that a high dose of ecdysone injected in Kenyon cells induced a nuclear receptor expression cascade similar to the canonical cascade induced in late third instar fly larvae in response to ecdysone at the onset of metamorphosis (*AmEcR*, *Amusp* and *Amftz-f1* are also expressed in *Apis mellifera*) [8].

During nervous system development in flies, neuroblasts acquire different fates through spatial patterning [9] and generate different cell types at different time points. This mechanism is controlled by a sequentially acting cascade of transcription factors, including SVP, a temporal factor during neuroblast development that acts early in this cascade [10]. In addition, *svp* leads to a switch in transcription factors that specify temporal identities of neuroblasts and regulates the time at which neuroblasts undergo either cell-cycle exit to produce a specific neuron type or apoptosis [11,12]. More recently, it has been shown that *unf* have a role in neuronal identity [13]. Furthermore, Kusuru *et al* demonstrated that *tll* was required for the maintenance of neuronal progenitors [14].

Postmitotic neurons ultimately undergo morphogenesis, and many of them are remodelled during metamorphosis to generate adult circuits. A recent study is considering that neuronal remodelling involves four major forms [15]: programmed cell death (PCD), transdifferentiation, temporally-tuned differentiation and remodelling-pruning-regrowth. Furthermore, it has been shown that *Drosophila* nuclear receptors are implicated in all four forms. This review will examine the mechanisms of remodelling – pruning – regrowth, to which nuclear receptors are essential.

PCD is triggered by ecdysone during metamorphosis to kill specific neuronal subsets that are not utilized in adult insects. PCD has been reported in the ventral neurons, where neuronal death is correlated with high levels of ECR-A expression [4]. By contrast, *EcR-B1* and *EcR-B2* have been shown to mediate PCD in peptidergic neurons that express the neuropeptide corazonin [16]. Furthermore, both *EcR-B1* and *ftz-f1* induce PCD in the optic lobes [17]. However, contrarily to what happens in the MBs (see below), in the optic lobe, the expression of *EcR-B1* did not appeared to be controlled by the prepupal expression of FTZ-F1.

Transdifferentiation involves the differentiation from one stable cell identity into another. Only one case of transdifferentiation has been observed in *Drosophila*, in which photoreceptor Rh5 (expressing the blue-sensitive Rhodopsin Rh5) transdifferentiates into Rh6 (expressing the green-sensitive Rhodopsin Rh6) under the effect of *EcR* [18].

Temporally tuned differentiation includes terminal differentiation of temporally frozen neurons at early embryonic or larval stages. This process is observed for instance in CCAP-expressing efferent neurons that differentiate under the effect of ECR-A, ECR-B1 and FTZ-F1 [19].

Remodelling – pruning – regrowth at metamorphosis occurs during the development of both vertebrates and invertebrates. By this mechanism neurons undergo a crucial remodelling process that is necessary for their new function. Axon and dendrite elimination over a certain distance (pruning), without the cell death of the neuron itself, is a recurrent theme in many biological processes [20]. Importantly, it was shown that developmental axon pruning is linked (at the molecular and cellular levels) to axonal response to injury or disease in the adult. During an individual's development, some early-born neurons are still present much later in adult life, although the nervous system of the individual (and particularly its brain) has been

subjected to profound anatomical and physiological changes. For instance, holometabolous insects such as *Drosophila* exhibit striking life history differences between their larval and adult stages. Finally, the culmination of neuron remodelling consists of dendritic and axonal regrowth, a necessary step for the neuron to connect with its new synaptic partners. The neuronal remodelling that occurs during metamorphosis is therefore expected to be vital to the normal functioning of the new circuits.

Neurite elimination can be divided into two main classes, relative to the scale of the events [20]. These include small-scale events such as the local pruning of axon and dendrite arbors, and large-scale events such as the removal of a significant length of the axon. Usually, small-scale events are associated with neurite retraction mechanisms and large-scale events are associated with axon degeneration. In the MB, the disruption of the microtubule cytoskeleton is a clear molecular marker that precedes this axon pruning. Also, axon degeneration is associated with membrane fragmentation along the whole length of the process to be pruned. On the contrary, motor neuron retraction during dismantling of the neuromuscular junction (NMJ) is neither associated with substantial membrane fragmentation nor with an early disappearance of Tubulin. Furthermore, a mix of these two cellular mechanisms can be present in the same particular neuron during its remodelling (see *ddaC* sensory neurons below).

The mechanisms of remodelling – pruning – regrowth at metamorphosis will be described below for five different types of neurons: MBs, the class IV dendritic arborization sensory neurons (named *ddaC*), the thoracic ventral (Tv) neurons, the projection neurons (PNs) of the olfactory circuit and the motor neurons.

In this review we will discuss gene regulatory and cellular pathways involving nuclear receptors that operate in neurons to direct axonal and/or dendritic pruning. In particular, we will focus our attention on the regulation of *EcR-B1*, as this nuclear receptor is required for different processes and mechanisms that regulate neuronal remodelling.

2. Neurite degeneration

In this section, we review neuron remodelling mechanisms that have been described as large scale degeneration events, *e.g.* in the MB γ axons [21], or in which

degeneration events have been well-described, even if a combination of both degeneration and retraction has been observed [22].

2.1. Mushroom bodies

MBs are prominent structures in the *Drosophila* brain involved in olfactory learning and memory. MBs are composed of three different types of neurons: γ , $\alpha'\beta'$, and $\alpha\beta$, all of which are sequentially generated from the same neuroblast [23]. MB γ neurons, born in early larva, initially establish a projection consisting of a dendritic tree and an axon peduncle, which bifurcates to give rise to a dorsal and medial branch. Only the γ axons [23] and dendrites [24] are remodelled by degeneration at metamorphosis. During this step the γ neurons retain only the part of their axon in the peduncle and the cell body. The first signs of pruning begin at 4 hours after puparium formation (APF) with the appearance of dendrite and axon blebbing. Blebs in dendrites appear disconnected while axon blebs remain continuous. Thus, dendritic pruning precedes axon pruning. At 8 hours APF most dendrite fragments have disappeared, whereas the axons have begun to fragment. Finally, many axon fragments have disappeared by 12 to 18 hours APF. The γ axon regrows later in pupal development (24 hours APF), and projects only into the medial γ lobe of the adult.

The ecdysone receptor *EcR-B1* is necessary for γ axon pruning [25]. ECR-B1 and its co-receptor USP bind ecdysone, and the resulting complex regulates the expression of genes involved in pruning during the onset of metamorphosis. ECR-B1 is specifically expressed in γ neurons before remodelling but not in neurons where axonal remodelling is absent, as in $\alpha'\beta'$ or $\alpha\beta$ neurons. Thus, its transcriptional regulation must be precisely regulated, both temporally and by cell type.

2.1.1. *EcR-B1* is required in γ axons for pruning to occur

Three independent pathways orchestrate the expression of *EcR-B1* in γ axons: the TGF β pathway, the cohesin complex and the nuclear receptor pathway [26]. The TGF β pathway was the first positive regulator of *EcR-B1* to be discovered [27]. Subsequently, it was shown that components of the TGF β pathway such as the *baboon* (*babo*), *wishful thinking* (*wit*) and *punt* (*put*) receptors, as well as *Smad*

proteins, were essential for the activation of *EcR-B1* expression. A crucial question at this point was to determine the nature and cell origin of the ligand that activates the TGF β pathway. Even though preliminary studies pointed to a role for the activin ligand in *EcR-B1* brain expression, further research has clearly shown that myoglianin, secreted essentially by central glia [28], is the main activator of the TGF β pathway in γ neurons. Correspondingly, blocking myoglianin production in glial cells with a *myoglianin-RNAi* transgene driven by the pan-glia-specific *repo-GAL4* decreased *EcR-B1* expression in γ neurons, consequently blocking γ axon pruning. Moreover, it was found that myoglianin was correctly positioned in the BABO-WIT/PUT complex *via* the transmembrane immunoglobulin family protein PLUM [29].

The cohesin complex has a known role in the cohesion of sister chromatids during meiosis. Unexpectedly, Schuldiner *et al.* [30] demonstrated that several proteins of the cohesin complex (including *SMC1* and *SA*) are involved in γ axon pruning and positively regulate *EcR-B1* expression. Although these authors did not demonstrate a direct binding of the cohesin complex to the *EcR-B1* locus, additional studies by Misulovin *et al.* [31] have shown that the cohesin complex binds to the *EcR* locus. These results strongly suggest that the cohesin complex could act as a direct transcriptional activator of *EcR-B1* in γ neurons during remodelling.

Boulanger *et al.* revealed yet a third pathway, the nuclear receptor module including *ftz-f1* and its paralogue *Hr39*, responsible for the regulation of *EcR-B1* [32]. This work establishes that the nuclear receptor FTZ-F1 directly binds to the *EcR* locus and activates the expression of *EcR-B1*. To demonstrate that FTZ-F1 activates *EcR-B1* expression, Boulanger *et al.* used the MARCM technique to generate γ neuron *ftz-f1^{-/-}* clones and analysed the expression of ECR-B1 in these clones by immunostaining. It was shown that ECR-B1 expression was lost in these clones, suggesting that FTZ-F1 was a transcriptional activator of *EcR*. This activation of *EcR-B1* expression by FTZ-F1 was unexpected since ECR-B1 is considered to regulate the overall expression of *ftz-f1* and other early-late nuclear receptors during metamorphosis [1].

It remains to be explained why *EcR-B1* transcriptional regulation works in an opposite way in γ neurons. Further immunostaining experiments demonstrated that artificial expression of *Hr39* in γ neurons leads to a decrease in ECR-B1 expression, which in turn blocks γ axon pruning. These results signify that *EcR-B1* expression is activated by FTZ-F1 and inhibited by HR39. Indeed, Boulanger *et al.* also evidenced

that artificial expression of both *Hr39* and *ftz-f1* rescued the unpruned γ axon phenotype obtained by *Hr39* overexpression alone and this rescue was correlated to an increased *EcR-B1* expression. Finally, FTZ-F1 was shown to be essential in blocking *Hr39* expression, in order to avoid *EcR-B1* down-regulation. The nuclear receptor module and the TGF β signalling, were shown to be independent of each other by additional genetic interaction experiments and expression data.

FTZ-F1, the TGF β pathway components and the cohesin pathways are mostly present throughout the central nervous system. The down-regulation of *Hr39* via FTZ-F1 does not appear to be sufficient to explain the MB specific expression of *EcR-B1* in γ neurons at metamorphosis. A putative MB γ axon-specific ligand or cofactor for FTZ-F1 may ensure such specificity. Consequently, the role for *EcR-B1* cell-specific regulation in γ axon remodelling remains unknown. Nevertheless, although the γ neurons are the only class in the MB that express *EcR-B1*, virtually every neuron in the larval brain is *EcR-B1*⁺ at the start of metamorphosis and they all are either pruned or die in response to ecdysone. Only the arrested immature neurons lack *EcR-B1* expression. Therefore, it may be more appropriate to find how *EcRB1* is specifically repressed in $\alpha\beta$ ' neurons rather than how *EcRB1* is expressed in the γ neurons.

At the protein level, it is interesting to cite that in the fat body of the mosquito *Aedes aegypti*, a protein-protein interaction between the coactivator FISC and ECR is facilitated by FTZ-F1 in an ecdysone dependent manner and this interaction is essential for the binding of ECR to the corresponding promoters [33].

2.1.2. *EcR* expression in both γ neurons and glial cells influences glial behaviour during γ axon pruning

In addition to the cell autonomous role of *EcR-B1* in γ axon pruning, extrinsic signals mediated by glial cells also have an important role in this mechanism [34,35,36]. Remarkably, glial extensions approach and penetrate into both γ lobes in the early pupa, engulfing neuronal varicosities by a phagocytosis mechanism. This suggests that a diffusible factor is produced by γ axons in order to recruit glia around the γ lobes. Even if the existence of such a diffusible factor has not been established in γ axons, general models explaining overall glia-mediated synaptic pruning converge on the hypothesis that glial cells may be attracted to sites of activity-dependent pruning by a soluble recruitment signal that binds receptors at the glial

cell surface [37]. Glial infiltration in the γ lobes occurs before any sign of γ axon degeneration. Interestingly, blockade of glial cell endocytosis by the thermosensitive mutant *shibire^{ts}* (which acts as a dominant negative of dynamin at restrictive temperatures) inhibits glial γ lobe penetration, resulting in a delayed pruning. Furthermore, Awasaki and Ito [34] revealed that the engulfment and phagocytosis of γ axons by glial cells depends on *EcR-B1* expression in γ axons. Thus, inhibition of *EcR-B1* function by an *EcR*-dominant negative (*EcR-DN*) form, driven in γ axons blocks the penetration of glial processes into the γ lobes. This suggests that *EcR-B1* expression in γ axons induces a signal that promotes glial infiltration leading to γ axon pruning. Regarding the function of *EcR* in glia, recent results published by two independent groups [38,39] have demonstrated that *EcR* is essential in astrocyte-like glia for γ axon pruning. To accomplish this, ~~the authors used~~ a GAL4 line specific for astrocyte-like glia, *almr-GAL4* was used to drive the expression of *EcR-DN* in this particular glial cell type. Under these conditions, the authors successfully showed that astrocyte-like glia do not infiltrate into the γ lobes during the pupal stages, and that unpruned γ axons remain in adult flies. Consequently, *EcR-B1* is required in both γ axons and astrocyte-like glia for γ axon pruning to occur.

2.1.3. Molecular targets of *ECR* in γ neurons and glial cells leading to γ axon pruning

Very little is known about the *ECR-B1* target genes in γ axons or glia. Curiously, the general primary *EcR* response genes *E74* and *E75* were not required in MB γ axon pruning [25]. To date, only the RNA-binding protein BOULE (BOL) has been molecularly characterized as an *ECR-B1* target in γ axons, although several other putative targets of *EcR-B1* have been suggested. *bol* was isolated, as a negative target of *ECR-B1*, by microarray analysis from mushroom body neurons in which *EcR* activity was genetically blocked by an *EcR-DN*. The expression of *bol* must be down-regulated in order to allow γ axon pruning to occur [40]. The ubiquitin proteasome system (UPS) and the α -Tubulin (α -*Tub*) are *ECR-B1* candidate targets. Indeed, UPS proteins are required for γ axon pruning, and mutations in some members (e.g. ubiquitin activating enzyme (*Uba1*), *Mov34* and *Rpn6*) have been shown to block γ axon remodelling [21]. Even though it was clearly demonstrated, in this study, that UPS does not act upstream of *EcR-B1*, it was not establish if UPS acts in parallel to, or downstream of, the *EcR-B1* pathway. Additional studies by Hoopfer *et al.* [40] have shown the down-regulation of the proteasome subunits when

EcR-B1 function was blocked, which pinpoints a positive regulation of UPS by *EcR-B1* rather than a role in an *EcR-B1* parallel pathway. Expression of an α -*Tub-Myc* construct in γ axons demonstrated that the microtubule cytoskeleton completely disappears during normal development in γ axons by 8 hours APF, before the first signs of γ axon degeneration are observed [21]. α -*Tub-Myc* did not disappear when *EcR-B1* function was blocked in γ axons with an *EcR-DN* construct [41]. This strongly suggests that ECR-B1 controls the transcription of α -*Tub* in γ axons.

The only identified potential ECR target in glia is the engulfment receptor *draper* (*drpr*) [41]. Showing increased expression in glia by 6h APF, *drpr* is needed in glia for normal γ axon pruning. Expressing *EcR-DN* in glia with *repo-GAL4* lowered expression of DRPR, suggesting that *drpr* is an ECR target in glia.

Although no other ECR targets are known in glial cells, a recent study by Hakim *et al.* [39] alludes to the presence of additional ECR glial targets acting in a pathway independent of *drpr*. First, the authors showed that the function of *drpr* is restricted to axon clearance in adult flies, since its mutation only blocks γ axonal debris elimination but not γ axon fragmentation. Second, they clearly demonstrate that *EcR* loss-of-function in astrocytes inhibits both γ axon debris cleaning and γ axon fragmentation. Based on these data, it can be hypothesized that ECR acts in astrocyte-like glia as a master regulator of two pathways: the *drpr* pathway for debris clearance, and an additional unknown pathway for active axon fragmentation.

Surprisingly, blocking *drpr* function in glia does not affect the microtubule cytoskeleton in γ axons, even though pruning is delayed. These results suggest that α -*Tub* loss in γ axons is independent of the *EcR*-mediated regulation of *drpr* in glia. One pursuit for future research would be to examine if α -TUB also does not disappear from γ axons when *EcR* function is blocked in glia. Nevertheless, it seems likely that the loss of α -TUB is an essential step in γ axon pruning. This step may mediate the putative γ axon expression of a ligand that allows glia infiltration, as well as the γ axon autonomous expression of an unknown receptor that would be required for the induction of an active axon fragmentation program by glial cells (Fig. 1) [34]. Different examples of using MARCM to induce single γ axon clones that do not show remodelling, in which *EcR-B1* expression is lost, have been described in the literature [25,27,40,30,32]. This indicates that the glia must specifically recognize and fragment only those γ axons expressing such a receptor. Consequently, a single γ neuron that

does not express this putative *EcR-B1*-targeted receptor will not be pruned, even if all surrounding γ axons are remodelled.

2.2. *ddaC*

Most larval sensory neurons die during metamorphosis, although some persist and become functional adult neurons. Among those that survive are the dendritic arborizing sensory neurons (*da*), which include the class IV dendritic arborization sensory neurons (*ddaC*). The first step in dendritic pruning of *ddaC* consists of severing (*i.e.* thinning and breaking) the dendrites from 5 to 12 hours APF. Over the next 6 hours, severed dendrites undergo rapid fragmentation and clearance, such that dendritic arbors are completely removed by 16 to 18 hours APF (whereas the soma and axons remain intact). In a final step, the dendritic arbors regrow by 24 hours APF. Dendritic removal involves both local degeneration and branch retraction mechanisms induced by ecdysone [42,22]. Remarkably, inhibition of steroid action by *EcR-DN* blocks local degeneration, although retraction events still occur. Two possibilities have been proposed to explain why only local degeneration is affected when *EcR* action was blocked: this could be due to an unblocked ecdysone-induced cellular program; or it is a secondary response of neurons to the metamorphic changes occurring in the abdominal epidermis, where the *ddaC* arborize. It is important to note that the initiation of pruning by *EcR* results in the destabilization of the microtubule cytoskeleton within the dendritic arbor of *ddaC*, similar to what occurs in MB γ axons. This has led to the hypothesis that α -TUB destabilization weakens axons and dendrites in a general way, preparing them to be “attacked” by extrinsic cells (macrophage and epithelial cells) that allow pruning to continue. As previously observed for the MBs, it was deduced by MARCM clones of *Uba1* and *Mov34* that *ddaC* remodelling requires intrinsic activation of UPS [42,43]. Here, UPS was presumed to sever dendrites and initiate dendrite breakage.

Several ECR targets have been revealed in *ddaC*, including the transcription factor *Sox14* and the cytosolic protein *Mical* [44,45]. *Sox14* was isolated in an RNAi screen that comprised genes from previous microarray analyses with the potential to respond to ecdysone signalling. Attenuation of *Sox14* by RNAi was shown to result in a severe blockade of pruning in all *ddaC* neurons, and no SOX14 expression was observed when *EcR-DN* was driven in *ddaC*, suggesting that *Sox14* is an ECR target

in *ddaC*. In addition, overexpression of *Sox14* in *ddaC* was sufficient to initiate premature dendritic pruning before the ecdysone pulse, indicating that *Sox14* induction is necessary and sufficient to induce dendritic pruning. Moreover, it was also demonstrated that MB γ axon pruning was blocked by *Sox14 RNAi*, suggesting that *Sox14* might also be an ECR target in γ neurons. How *Sox14* functions in these different cells to execute pruning is still unclear. The authors subsequently performed a similar screen to identify targets of *Sox14*, and isolated MICAL. *Mical* loss-of-function nearly blocked *ddaC* pruning but did not appear to have a role in γ axon pruning. Thus, it was proposed that ecdysone/ECR/USP activates transcription of *Sox14* in MBs and *ddaC*, and the resulting SOX14 protein activates the expression of *Mical* by binding to its promoter only in *ddaC*.

Following this work, another target of ECR, *headcase (hdc)*, was identified in a genome-wide interaction screen in *ddaC* neurons [46]. *hdc* is a gene responsible for dendrite severing isolated by designing genetic interactions between an *EcR* mutant and various deficiency sets, and subsequent screening for flies in which pruning was affected. Although its real function in the cell is unknown, *hdc* is cell-autonomously required to control dendrite severing, and the cell autonomous knockdown of *EcR* results in a lack of *hdc* expression. Since knockdown of *Sox14* in *ddaC* neurons did not affect HDC expression and no SOX14 expression was observed in *hdc* mutant flies, the authors could conclude that *hdc* acts downstream of *EcR* and that it belongs to a new *EcR*-regulated pathway independent of *Sox14*. Finally, it was demonstrated that *hdc* was not required for MB γ axon pruning.

The function of intrinsic chromatin remodelers in *ddaC* pruning was recently uncovered in a study [47] that examined the potential requirements of 81 chromatin remodelers for *ddaC* dendrite pruning, using dominant-negative and RNAi approaches. In this study, the chromatin remodeler *brahma (brm)* and a CREB binding protein (CREB-BP) with histone acetyltransferase (HAT) activity were isolated. Surprisingly, two factors that activate ecdysone response gene expression during metamorphosis, the ISWI chromatin remodeler and the dGc5 HAT, were not involved in dendrite pruning. Since ECR-B1 formed a protein complex with CREB-BP in the presence of ecdysone, it was suggested that CREB-BP is a *bona fide* ECR-B1 coactivator. Furthermore, they showed that the formation of this complex was facilitated by BRM, and that the resulting complex specifically activated *Sox14*. To explain the mechanism by which this complex activates *Sox14*, the authors

performed very elegant ChIP assay and co-IP experiments and demonstrated that ECR-B1 and BRM co-ordinately promote CREB-BP-mediated histone acetylation at *Sox14*-specific regions in response to ecdysone. Based on these results they proposed a global model in which BRM and CREB-BP are acting together with ECR-B1 to establish a transcriptionally active chromatin state, allowing the expression of their common target *Sox14*. Interestingly, overexpressing dominant negatives of *brm* and the gene expressing CREB-BP in MBs also blocked γ axon pruning. Thus, epigenetic factors chromatin remodelers are critical for the initiation of pruning in both dendrites and axons. More recent experiments have added to this body of evidence, showing that a *Cullin1*-based SCF E3 ubiquitin ligase targets and inactivates the InR/PI3K/TOR pathway to regulate neuronal pruning. This pathway also acts downstream of *EcR-B1* and *Sox14* and is independent of *Mical* (Fig. 2) [48].

Additional studies have shown that phagocytes (macrophage-like haemocytes) are required for ddaC pruning [22]. Phagocytes play an active role in severing intact dendritic branches, attacking the branches either at their distal tips or at proximal sites near the cell body. In contrast, when phagocytes encounter da neurons expressing *EcR-DN* they fail to attack the dendritic arbor. This behaviour is analogous to the one observed when astrocyte-like glia encounter MB γ neurons expressing *EcR-DN*. Even though circulating phagocytes play a clear role in ddaC pruning, a recent study from Han *et al* [49] has shown that epidermal cells, acting via a DRPR mediated recognition, are the primary phagocytes in the fragmentation and clearance of these dendrites.

3. Neurite retraction and small scale elimination

In this section, we describe neuron remodelling events that are *bona fide* retraction events: thoracic ventral neurosecretory cells [50], motor neuron during metamorphosis [51,52] or that are small scale events but were neither assigned as retraction nor degeneration [53].

3.1. Thoracic ventral neurosecretory cells (Tv neurons)

The Tv neurons are paired neurosecretory cells located in each of the larval thoracic neuromeres. The axons from each Tv neuron pair form a neurohemal organ by wrapping around support cells. The remodelling of these Tv neurons takes place in three different steps. First, the dendrites of these neurons are severely pruned back by 5 to 6 hours APF. Then, axonal remodelling takes place around 10 hours APF and ends by 18 hours APF. Finally, outgrowth of adult branches starts between 18 to 20 hours APF; by 48 hours APF, a larger and more extensive mesh-like adult arbor is formed. Subsequently the authors [50] followed the regression of individual axon branches by confocal live imaging in explanted nervous systems and observed retraction but no sign of local degeneration.

Control of Tv neuron remodelling by the ecdysone receptor has been described using *in vitro* imaging techniques and immunocytochemistry, as well as the expression of *EcR-DN* and *EcR-RNAi* constructs. A two-step model of Tv neuron remodelling is proposed [50]. The first step is a pruning phase where ecdysone activates the ECR-USP heterodimer, which in turn activates the transcription of target genes of the core retraction program. A sprouting phase follows, in which ECR-USP is predominantly a repressor of the target genes of the core outgrowth program, and the role of ecdysone is to relieve this repression. Nevertheless, during this phase ecdysone also activates, *via* ECR-USP, the transcription of target genes involved in an enhancement program of axon outgrowth.

3.2. Olfactory circuit

In the adult fly, odours are detected by olfactory receptors on the dendrites of olfactory receptor neurons (ORNs) located in the antennae and maxillary palps. ORN

axons converge in the glomeruli of the antennal lobes (ALs), where olfactory information is relayed by projection neurons (PNs) to higher brain centres, *i.e.* the calyx of the MB and the lateral horn (LH). One class of PNs is embryonic-born and participates in the adult olfactory system [53]. These embryonic-born PNs also participate in the larval olfactory system and undergo local pruning of their dendrites and axons during early metamorphosis. The dendrites are pruned, here also, (from 2 to 4 hours APF) before the axons (from 4 to 10 hours APF). Therefore, the dendrites of PNs connect the ORN axons in the AL glomeruli, while their axons connect the MB and LH dendrites.

The best-studied case of genetic control of neuronal remodelling in *Drosophila* is the MB γ neuron, in which TGF β signalling is required for *EcR-B1* up-regulation. This *EcR-B1* up-regulation is required cell-autonomously for γ neuron pruning (see above). These observations make it particularly compelling to follow what happens to MB neuron synaptic partners while they reorganize their dendrites and axons. Notably, these two pruning events (embryonic-born projection neurons and MB γ neurons) are coincident and therefore raise the possibility that they might be coordinated. MARCM clone analysis has been used to produce embryonic-born projection neurons homozygous for the TGF β Type I receptor *babo* in a heterozygous (*i.e.* functionally wild-type) background; these mutant projection neurons failed to show high-level expression of ECR-B1 and a normal reorganization of their processes [53]. Therefore, the presence of ECR-B1 is required autonomously in these two pruning programs, implying that the pruning of one synaptic partner cannot be entirely dependent on the pruning of the other partner. Although the two pruning programs use the same mechanism involving TGF β -induced expression of *EcR-B1*, they are developmentally independent.

3.3. Motor neuron retraction at metamorphosis

The motor neurons innervating the larval muscles at the NMJ are also remodelled during *Drosophila* metamorphosis. To date, only two studies have treated this NMJ dismantling during metamorphosis [51,52]. This has revealed that the NMJ motor neuron is pruned predominantly through a retraction mechanism from 4 to 8 hours APF. This retraction can be directly observed during live imaging [51], and exhibits molecular markers of retraction as opposed to degeneration [52]. Indeed, α -TUB is

present in the motor neuron at the NMJ throughout all stages of axonal pruning. This is in clear contrast to the cessation of α -*Tub* expression observed before the first signs of MB γ axon degeneration (see above). Surprisingly, the same gene cascade underlies axon pruning of both the MB and the NMJ, despite the fact that it leads to two different cellular outcomes (degeneration versus retraction).

A proposed model of NMJ dismantling during metamorphosis [52] is summarized as follows (Fig. 3). The first sign of the NMJ dismantling process comes from the postsynaptic partner (muscle). Interestingly, in *Manduca*, it was earlier shown that muscle degeneration precedes motor neuron retraction and synapse loss [54,55]. The nuclear receptor pathway involving FTZ-F1 and HR39 regulates *EcR-B1* expression in muscle and initiates muscle dismantling. *EcR-B1* expression at the postsynapse is positively and negatively controlled by FTZ-F1 and HR39, respectively. FTZ-F1-mediated *EcR-B1* expression leads to the transcriptional regulation of target genes involved in postsynaptic degradation. Then, a TGF β -like ligand is supposed to be secreted by the postsynapse. The authors propose that this signal produced by the dismantling muscle is received by the motor neuron. There TGF β signalling and the *ftz-f1/Hr39* nuclear receptor pathways induce *EcR-B1* expression that triggers axon retraction. This is similar to what happens in the MB γ neurons. According to the NMJ dismantling model (Fig. 3), *EcR-B1* must therefore be expressed both pre- and postsynaptically. An important feature to this model is that *EcR-B1* in the postsynapse induces the expression of *EcR-B1* in the presynapse via a TGF β -like ligand that is secreted from the postsynapse and received by the presynapse. Thus, this mechanism insures the temporality of the two processes by the same ecdysone wave at metamorphosis, and prevents motor neuron pruning before post-synaptic degradation. Finally, macrophages (macrophage-like haemocytes), and possibly glia, are required for NMJ dismantling, although the role of *EcR-B1* in these cells remains unknown.

4. MB γ axon regrowth

In most cases of neuronal remodelling, a final step of dendrite and axon regrowth takes place following neurite pruning. This insures new connections (and their likely attendant functionality) to the remodelled neuron. In the particular case of the MBs,

where most larval γ axons are completely pruned by 18 hours APF, γ axon regrowth is clearly seen from 24 hours APF [23].

However, until recently, nothing was known about the genetic control of this step in γ axon regrowth. The first identified genetic component involved in this axon regrowth process is a nuclear receptor. The *Drosophila* gene *unf* was recently shown to be required cell-autonomously for γ axon regrowth once γ axon pruning has taken place [56]. This orphan nuclear receptor appears to be involved, in an autonomous way, specifically in regrowth, as it is not autonomously necessary for initial axon outgrowth or guidance of any MB neuronal type (see also the *unf* review from Bates et al. in this BBA-Special Issue). It is proposed that UNF promotes developmental regrowth by activating the TOR pathway, which is an attractive hypothesis since the TOR pathway is known to be involved in axon regeneration after injury. Therefore, axon regrowth may share common molecular features with regeneration following injury. Finally, UNF is believed to promote another unknown pathway that regulates axon guidance during axon regrowth. Since overexpressed UNF partially represses *EcR-B1*, this might be a way to prevent pruning of the re-growing axon.

5. MB γ axon remodelling and memory

Hr39 overexpression in the MBs leads to a dramatic down-regulation of *EcR-B1* expression in the late third instar larval brain [32]. The lack of ECR-B1 expression in larval MBs almost completely blocks γ neuron remodelling and results in adult flies that are viable and healthy enough to perform behavioural tests. This is therefore a unique condition in which adult flies with an otherwise wild-type adult brain retain nearly all of their MB γ neurons in an unpruned larval state.

These adult flies with unpruned larval γ neurons were tested for both short-term (STM) and long-term memory (LTM), within the context of courtship memory [57]. Quite unexpectedly, the LTM performance of these adult flies was similar to normal wild-type flies, yet they were totally impaired for STM. This result shows that, at least for certain forms of *Drosophila* memory, functional γ neurons are necessary for STM, although LTM can be produced without functional γ neurons. This indicates a probable specialization for short- versus long-term memory pathways within MB neuron classes. Indeed, using the appetitive olfactory paradigm, another fly memory assay, it was shown that short- and long-term memories are formed and processed

in parallel, with γ neurons required for STM and $\alpha\beta$ neurons required for LTM [58]. On a related note, there is some indirect evidence that pruning defects in humans might cause neurological disorders such as chronic epilepsy, schizophrenia and autism [59]. An excess of immature synapses could explain the disorganized and hyper-excitable brains of these patients, as well as their behavioural and cognitive dysfunctions.

Notably, ecdysone signalling regulates the formation of long-term courtship memory in adult flies [60]. The levels of ecdysone were found to be increased after courtship conditioning and, depending on the timing of exogenous administration, ecdysone either enhanced or suppressed LTM. Clearly, LTM requires functional *EcR-A* in adult neurons, but the specific STM defect described above is strictly developmental. Therefore, it can be proposed that ecdysone signalling is required during development to insure normal MB γ neuron remodelling (necessary for STM formation), whereas later (once development is over), ecdysone signalling is required in the adult for normal LTM formation. Interestingly, a recent study shows that a G-protein Coupled Receptor, *DopEcR*, that binds either ecdysone or dopamine is also involved in courtship memory [61].

6. Conclusion and perspectives

The ecdysone/ECR/USP complex is central to all neuronal remodelling processes during *Drosophila* metamorphosis that have been described to date. This body of work has uncovered an unexpected regulatory relationship between *EcR-B1* and two other nuclear receptors, *ftz-f1* and *Hr39*. Indeed, it was expected that *ftz-f1* and *Hr39* would be ECR target genes, based on numerous studies that examined the role of the nuclear receptor gene cascade during the main developmental transitions in *C. elegans* and *Drosophila* [62,63]. In fact, it has been clearly shown during remodelling of MB γ neurons and the NMJ that *EcR-B1* expression is activated by FTZ-F1, and repressed by HR39 (if present). In the case of the MB γ neuron (and likely as well for the NMJ), *Hr39* is not required although it must be repressed by FTZ-F1. The *ftz-f1* and *Hr39* nuclear receptor pathway is independent of TGF β signalling (another *EcR-B1* activating pathway), which may insure the *EcR-B1* cell-specific regulation necessary for axon remodelling.

EcR-B1 expression has been observed to be required in every cell type involved in the different remodelling events. It is required in the MBs (likely to activate the UPS degradation of α -*Tub*), and also in the astrocyte-like glia (to activate the *drpr* membrane receptor); *EcR-B1* expression is thus necessary in these two cell types for γ axon fragmentation. During the NMJ dismantling process, the presynaptic motor neuron and the postsynaptic muscle also require *EcR-B1* expression; it is likely that this ensures the temporality of the two dismantling events. Notably, even though the genetic cascade of remodelling is conserved between the MBs and the NMJ, their cellular outcomes are distinct (*i.e.* degeneration of the MB γ neuron vs. retraction of the NMJ motor neuron).

The persistent projection neuron axons from the ALs connect the dendrites of the MB γ neurons. The pruning of these dendrites and axons appears to be coincident, raising the possibility that the remodelling of two synaptic partners might be dependent on each other. Nevertheless, these two remodelling events are independent, despite the fact that they both use TGF β -induced expression of *EcR-B1*.

Future research into this challenging developmental regulation must complete the identification of the different target genes (either direct or indirect) of the nuclear receptors involved in neuronal remodelling. For example, it has been proposed that during MB γ neuron remodelling an unknown secreted ligand (necessary to attract glia cells) and an unknown intrinsic receptor (necessary for cell-autonomous axon fragmentation by the glia cells) are both targets of ECR. Likewise, the UNF nuclear receptor is required for the regrowth of the γ axon to activate the TOR pathway, although another downstream UNF unknown pathway is hypothesized to be required for γ axon guidance during regrowth. Finally, this is also a salient field of research as developmental axon pruning has been linked (at the molecular and cellular levels) to axonal response to injury or disease in the adult; complementary to this, axon regrowth may also share common molecular features with regeneration following injury. Therefore, studying these mechanisms in a genetically malleable organism like *Drosophila* should provide well-needed information for proposing therapeutic targets.

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Figures

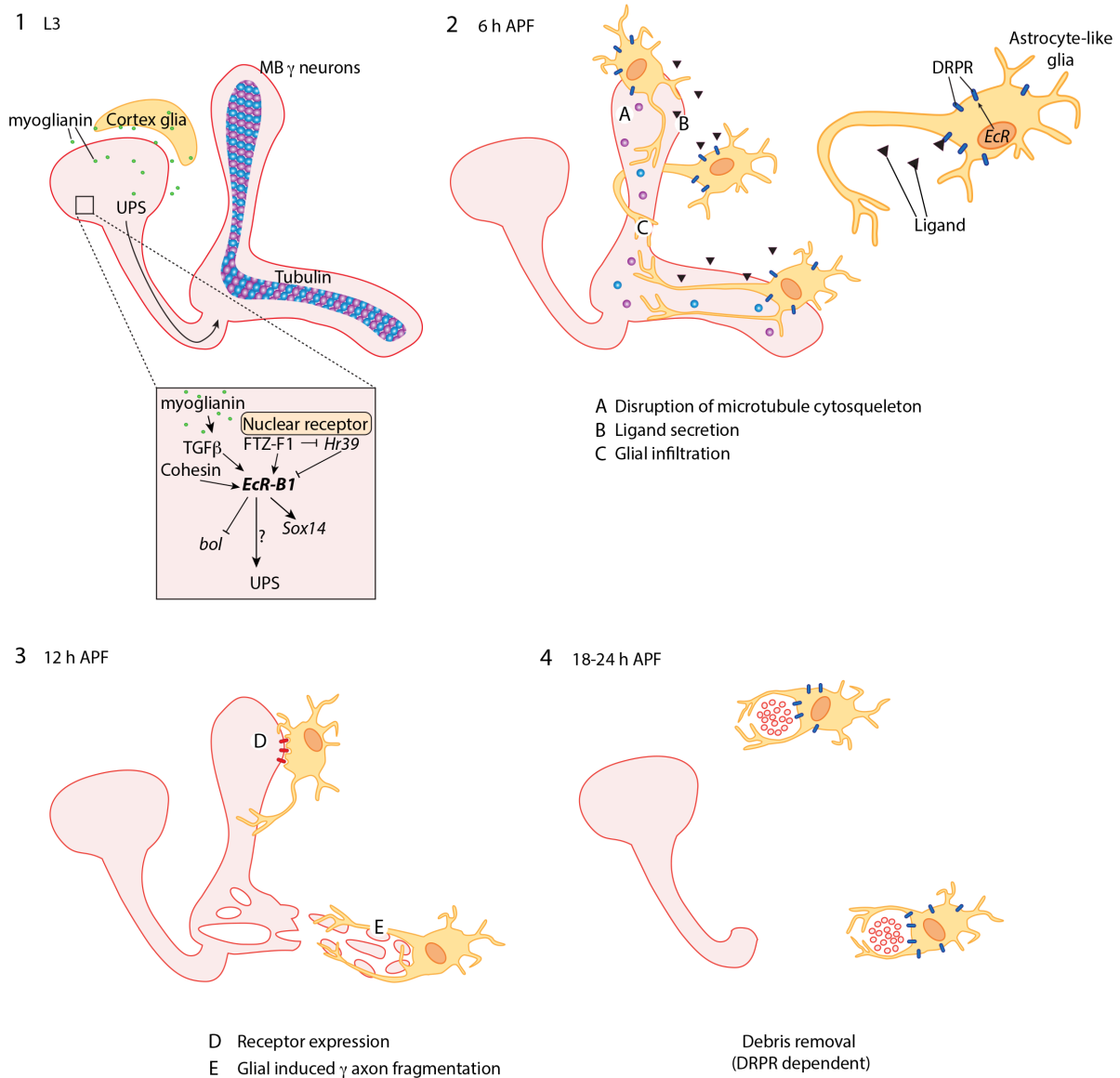


Figure 1. The MB γ axon pruning model. 1. During the late larval stage (L3), the cortex glia secretes the TGF β ligand myoglianin to direct γ axon remodelling, through the activation of TGF β signalling. Three independent pathways (the cohesin complex, TGF β signalling, and the nuclear receptor pathway) are essential for the up-regulation of *EcR-B1* before the prepupal ecdysone peak. *EcR-B1* expression in γ axons is positively and negatively controlled by FTZ-F1 and HR39, respectively. *ECR-B1* activates *Sox14* and represses *bol* for γ axon pruning to occur. Then, it is presumed that a probable *ECR-B1*-mediated activation of UPS (*via* an unknown pathway) targets the tubulin cytoskeleton for degradation. 2. By 6 h APF, disruption

of the microtubule cytoskeleton in γ axons (A) induces the γ axon production of a ligand (B) that in turn allows the infiltration of astrocyte-like glia processes into the γ axon lobes. In parallel, ECR activates the expression of *drpr* in astrocyte-like glia. DRPR might recognize the γ axon-derived ligand and trigger infiltration of glial processes (C). 3. After glial infiltration, another type of neuron-glia interaction might exist. At 12 h APF a putative γ axon-specific membrane receptor is expressed (D). This receptor is required for cell autonomous γ axon fragmentation by glial cells, and leads to a DRPR-independent γ axon phagocytosis and fragmentation step (E). 4. Between 18 and 24 h APF, the glia clear out the remnants of axons in a second DRPR-dependent mechanism [21,36,41,28,39].

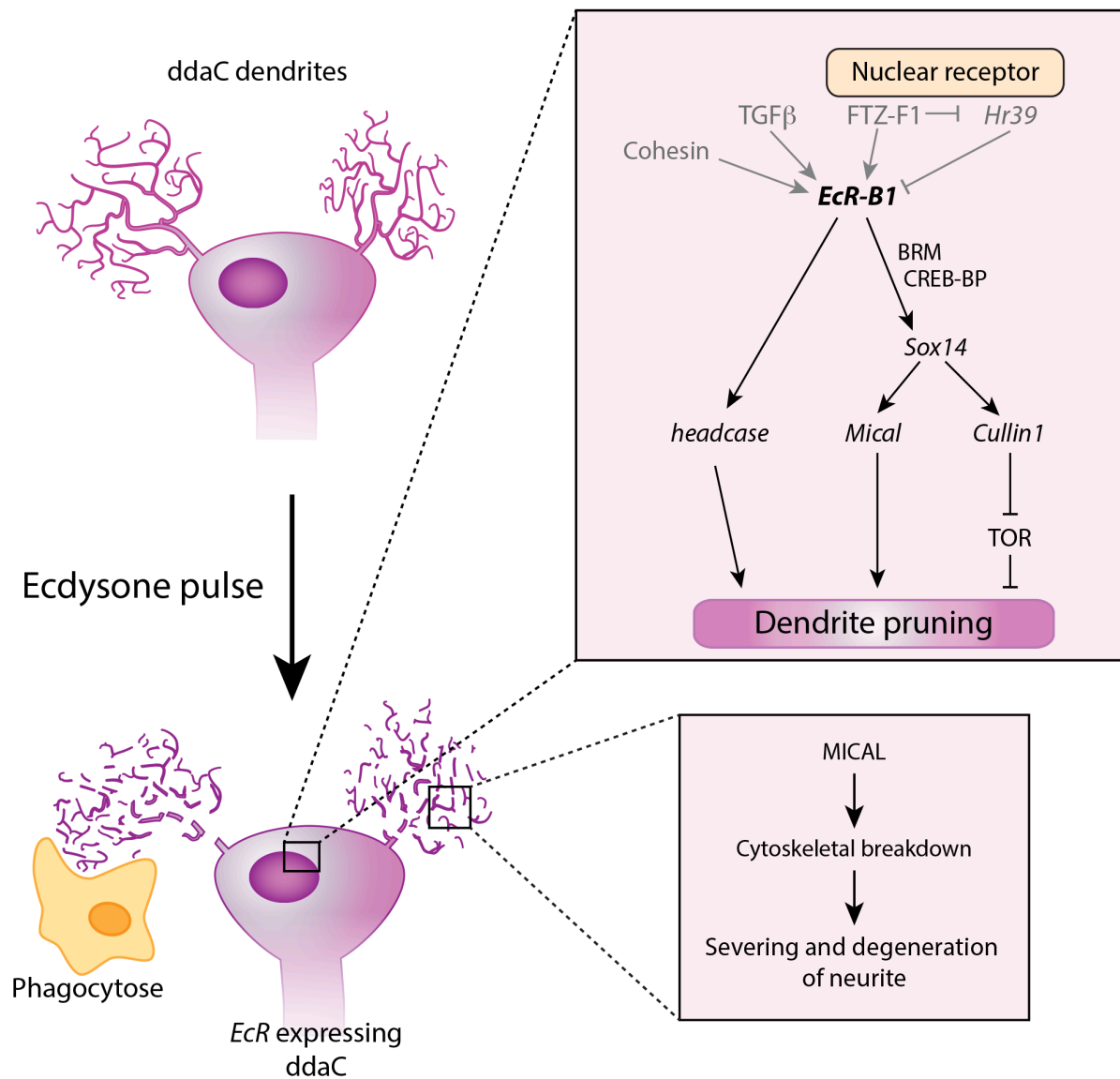


Figure 2. The ddaC pruning model. *EcR-B1* expression in ddaC dendrites may be regulated by cohesin, TGF β , and nuclear receptors (as in the MB), although this has not been directly shown. ECR-B1 expression results in the activation of target genes including *headcase* (*hdc*) and *Sox14*. A BRM-containing chromatin remodeller facilitates the formation of a complex between ECR-B1 and a histone acetyltransferase CREB-binding protein (CREB-BP), which specifically activates *Sox14*. Moreover, SOX14 activates two independent pathways: *Mical* and a *Cullin 1*-based SCF ubiquitin ligase (*Cullin 1*) pathway. HDC and MICAL activate dendrite pruning. The *Cullin 1* pathway facilitates ddaC dendrite pruning primarily through inactivation of the InR/PI3K/TOR (TOR) pathway. Thus, the TOR pathway negatively regulates ddaC dendrite pruning. In addition, phagocytes (macrophage-like

haemocytes and epidermal cells) actively attack dendritic arbors in an EcR-expressing ddaC-dependent manner [22,45,47,48].

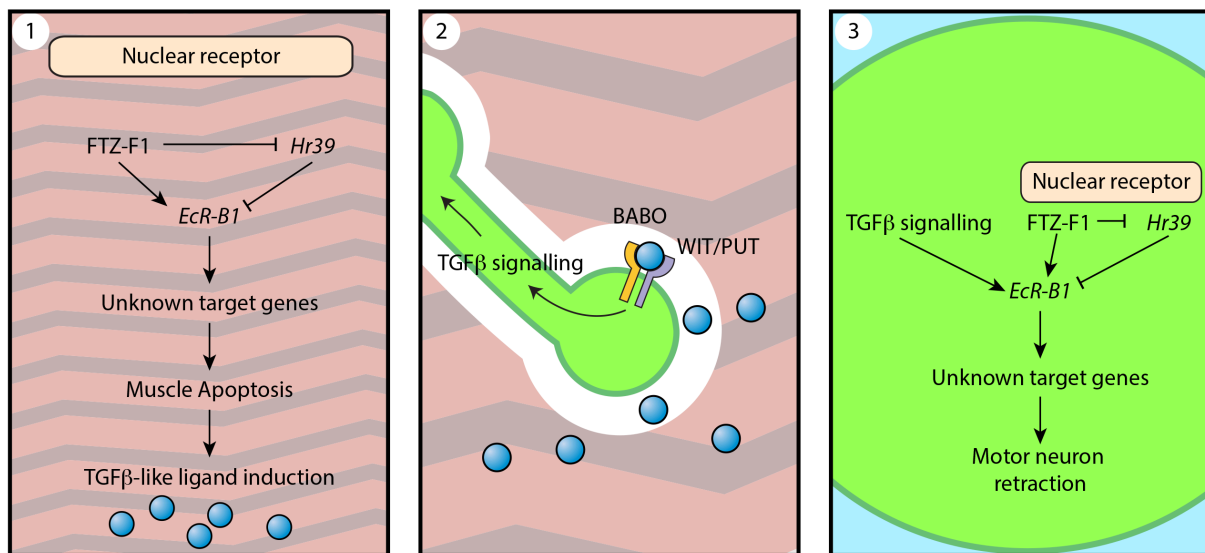
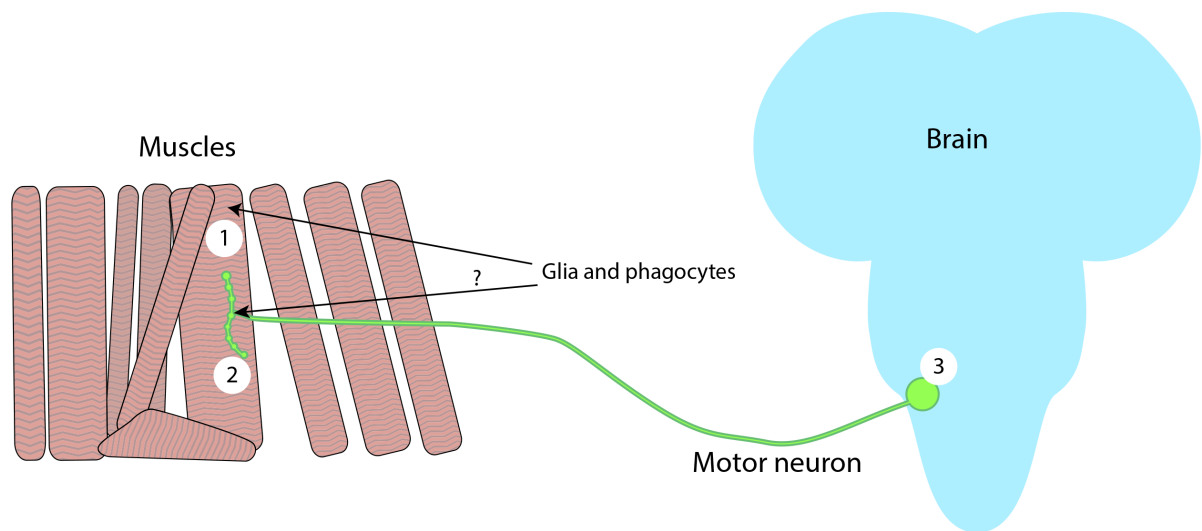


Figure 3. The neuromuscular junction (NMJ) dismantling model. 1. The nuclear receptor pathway involving FTZ-F1 and HR39 regulates EcR-B1 expression in muscle during the late larval stage and initiates dismantling in pupae. EcR-B1 expression in muscle is positively and negatively controlled by FTZ-F1 and HR39, respectively. FTZ-F1-mediated EcRB1 expression regulates unknown target genes involved in postsynaptic degradation and finally leads to muscle apoptosis that triggers the release of TGF β -like ligands. 2. It has been proposed that the TGF β -like ligand is next received by the presynaptic motor neuron (via BABO and WIT/PUT receptors). 3. In the motor neuron, activation of EcR-B1 expression (by TGF β signalling and nuclear receptors) triggers axon retraction. In addition, glial cells and phagocytes participate in the NMJ dismantling process [51,52].