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Two-factor specification of apoptosis: TGF-β signaling acts cooperatively with ecdysone signaling to induce cell- and stage-specific apoptosis of larval neurons during metamorphosis in *Drosophila melanogaster*

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Abstract

Developmentally regulated programmed cell death (PCD) is one of the key cellular events for precise controlling of neuronal population during postembryonic development of the central nervous system. Previously we have shown that a group of corazonin-producing peptidergic neurons (vCrz) undergo apoptosis in response to ecdysone signaling via ecdysone receptor (EcR)-B isoforms and Ultraspiracle during early phase of metamorphosis. Further utilizing genetic, transgenic, and mosaic analyses, we have found that TGF-β signaling mediated by a glia-produced ligand, Myoglianin, type-I receptor Baboon (particularly Babo-A isoform) and dSmad2, is also required autonomously for PCD of the vCrz neurons. Our studies show that TGF-β signaling is not acting epistatically to EcR or vice versa. We also show that ectopic expression of a constitutively active phosphomimetic form of dSmad2 ($dSmad2^{PM}$) is capable of inducing premature death of vCrz neurons in larva but not other larval neurons. Intriguingly, the $dSmad2^{PM}$-mediated killing is completely suppressed by coexpression of a dominant-negative form of EcR (EcR^{DN}), suggesting that EcR function is required for the proapoptotic $dSmad2^{PM}$ function. Based on these data, we suggest that TGF-β and ecdysone signaling pathways act cooperatively to induce vCrz neuronal PCD. We propose that this type of two-factor authentication is a key developmental strategy to ensure the timely PCD of specific larval neurons during metamorphosis.

Keywords Glia · Apoptosis · Metamorphoptosis · Myoglianin · TGF-beta · Babo · Corazonin

Introduction

Adolescence in mammals is a critical developmental period that is characterized by diverse physiological and morphological changes in response to gonadal steroid hormones to acquire capabilities as a reproductive adult [1]. This period is also associated with significant changes in brain anatomy and functions. For example, magnetic resonance imaging studies (MRI) have shown significant volume decrease in the prefrontal cortex (PFC), a region primarily involved in executive function and emotional regulation [2–5]. In the rat mPFC model, it was found that such reduced volume is due to a loss of significant number of neurons [6–8]. Although the steroid hormones are shown to cause the neuronal loss, the molecular mechanisms underlying this type of regional event by the globally acting hormones are largely unknown.

Insect metamorphosis, primarily orchestrated by steroid molting hormone, 20-hydroxyecdysone (hereafter,

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ecdysone), is a developmental period that is analogous to the mammalian puberty and adolescence with respect to the development and maturation of adult-specific organs that are required for reproduction and adulthood activities. In conjunction with the reproductive maturation, the central nervous system (CNS) is also significantly remodeled to develop much more complicated neural circuits that support the dynamic adult life style. One of the key events occurring in the metamorphosing CNS is programmed cell death (PCD) of 'obsolete' larval neurons and glia in response to ecdysone signaling [9–13]. Hence, the insect CNS metamorphosis offers an excellent system to elucidate the molecular mechanisms underlying PCD of specific doomed neurons in response to globally affecting steroidal hormones.

We previously found that a group of peptidergic neurons producing a neuropeptide corazonin (Crz) in the larval ventral nerve cord (VNC), namely vCrz neuronal group, along with many other unidentified larval neurons, undergo apoptosis shortly after puparium formation (AFP) in response to a surge of ecdysone that is also responsible for entering metamorphic development [10, 14]. The proapoptotic ecdysone signal in the doomed vCrz neurons is transduced by a canonical heterodimer of ecdysone receptors (EcR), in particular EcR-B1 and EcR-B2 isoforms, and Ultraspiracle (Usp) [10, 15]. The EcR-Usp is likely to elevate the expression of *grim*, one of the key death-promoting genes in *Drosophila*, and then Grim antagonizes *Drosophila* inhibitor of apoptosis 1 (DIAP1), thereby activating three major caspases, Dronc, Ice, and Dcp-1 [14, 16]. Besides vCrz neurons, there are other identified groups of doomed larval neurons but their death occurs at later metamorphic phases. For example, PCD of RP2 motoneurons residing in dorso-medial region of the VNC is triggered by a relatively small ecdysone peak that drives pupal formation [13]. In another example, apoptosis is not executed until adult emergence. Approximately 300 neurons (type-II) and CCAP neuropeptide-producing neurons in the VNC are eliminated shortly after adult eclosion that marks the end of metamorphosis, in response to significantly reduced ecdysone level below threshold [11, 15, 17–20]. While the ecdysone signaling promotes PCD of vCrz and RP2 neurons, the same serves to protect the type-II and CCAP neurons from their premature death prior to the adult emergence.

Ecdysone also induces non-apoptotic changes of persisting larval neurons during metamorphosis. For example, larval mushroom body (MB) γ neurons are remodeled during early metamorphosis by pruning neurites at 6–18 h APF, followed by forming adult-type projections. The pruning process requires ecdysone signaling involving EcR-B1 and Usp [21, 22]. Currently it is not clearly understood as to how globally acting ecdysone signal is capable of eliciting diverse cell type-specific responses in the metamorphosing CNS and how specific groups of larval neurons respond selectively to a certain ecdysone pulse and ignore others during post-embryonic development.

In addition to ecdysone signaling, TGF-β signaling plays important roles in the metamorphosis-associated CNS development. It was found that the axon pruning of MB γ neurons requires TGF-β signaling to upregulate EcR-B1 expression [22, 23]. TGF-β signaling is also involved in the morphogenesis of immature larval neurons [24] and pruning of motoneurons at the neuromuscular junction during metamorphosis [25]. The involvement of TGF-β signaling in metamorphosis-associated remodeling of juvenile neurons is noteworthy, since the members of TGF-β superfamily are evolutionarily conserved signaling molecules and play diverse regulatory roles in cellular growth, differentiation, proliferation, and morphogenesis during development in a context- and cell type-dependent manner [reviewed in Ref. 26]. TGF-β signaling is also implicated in the death of various cell types and such TGF-β-induced cell death is important for homeostatic maintenance of immune cells and tissue formation/remodeling [reviewed in Ref. 27, 28]. Despite extensive studies of the TGF-β signaling on diverse biological functions, little is known about its roles in the apoptotic neuronal cell death during post-embryonic development of the nervous system, except for the ontogenetic death of neurons in the developing vertebrate retina [29, 30]. Hence, the molecular mechanisms underlying TGF-β-regulated neuronal cell death during post-embryonic CNS development remain largely elusive. Moreover, whether TGF-β signaling plays a role in the metamorphosis-regulated apoptosis [metamorphoptosis; Ref. 19] of juvenile neurons in *Drosophila* has not been investigated.

In this study, we used vCrz neuronal apoptosis as a model system to elucidate the proapoptotic roles of TGF-β signaling. We found that this signaling, mediated by glia-derived Myoglianin (Myo) (ligand), Babo-A isoform type-I receptor, and dSmad2 (effector), is essential for the metamorphoptosis of vCrz neurons. Furthermore, our data suggest that both TGF-β and ecdysone signaling pathways cooperate to initiate the cell death program in the vCrz neurons for their timely death during metamorphosis.

Materials and methods

Fly strains and genetic crossing

The *crz-gal4* lines were from our previous studies [10, 31]. Following fly lines were used for *babo* MARCM analysis: (1) *hs-FLP, UAS-mCD8GFP; FRT*^{G13}, *tubulin-gal80/CyO, y*$^{+}$, (2) *FRT*^{G13}, *babo*^{52}*/CyO, y*$^{+}$; *crz-gal4*, (3) *FRT*^{G13}, *babo*^{Fd4}*/CyO, y*$^{+}$; *crz-gal4*, and (4) *FRT*^{G13}, *babo*^{Fd4}; *crz-gal4, UAS-babo. Df(2R)NP4* (*Df*) is a deficiency line that uncovers the *babo* locus. For *babo* transgenic manipulation

we used *UAS-babo-isoforms* for isoform-specific overexpression and *UAS-mi-babo*-isoforms for cell-specific silencing [23]. To analyze *dSmad2* function, $dSmad2^{1}$ [22], $UAS\text{-}dSmad2^{DN}$ (dominant negative), *UAS-dSmad2-RNAi*, and $UAS\text{-}dSmad2^{PM}$ (*a.k.a.* $UAS\text{-}dSmad2^{SDVD}$) were used [32, 33]. For the type-II receptors, wit^{G15} [34], $punt^{62}$ and a temperature-sensitive $punt^{135}$ [35], *UAS-punt-RNAi*, and *UAS-wit-RNAi* (gifted from M. O'Connor) were used individually or in combination. For studying *myo* function, $myo^{\Delta 1}$, *UAS-mi-myo*, and *myo-gal4* lines were used [23]. For glia-specific *gal4* drivers [23, 36] we used *repo-gal4* (pan-glia), *moody-gal4* (subperineurial glia, Ref. 37), *alrm-gal4* (astrocyte-like glia), *Mz97-gal4* (ensheathing glia), *NP2222-gal4* (cortex glia) and *NP6293-gal4* (perineurial glia). For *plum*, $plum^{\Delta 1}$ [38] and *UAS-plum-RNAi* (BDSC #60062) were used. For EcR and Usp signaling pathway, we used $UAS\text{-}EcR^{DN}$, *UAS-EcR* isoforms, and *UAS-mi-usp* [15]. For blocking PCD, *UAS-p35*, *UAS-mi-Ice* + *mi-dcp-1*, and *UAS-mi-grim* were used [14, 16, 17].

Generation of MARCM clones

Embryos (0–8 h after egg laying) were collected and placed on standard fly food. A single 60-min heat shock at 37 °C was applied to induce the expression of the *hs-FLP* transgene. After the heat pulse, the animals were kept at 25 °C and then CNSs were dissected and processed for immunohistochemical analyses at desired stages.

Histochemistry

Whole-mount Crz immunohistochemistry was performed, as described previously [14]. Anti-Crz polyclonal rabbit antisera [10] and anti-EcR-B1 monoclonal mouse antisera (DSHB) were used at 1:3000 and 1:50 dilutions, respectively. The primary antibodies were detected with TRITC-conjugated secondary antibodies (Jackson ImmunoResearch) at 1:200 dilution. The specimens were then mounted in 80% glycerol containing 2% *n*-propyl gallate. Fluorescent signals were acquired either by Olympus BX61 microscope or Keyence BZ-X710.

Results

Essential role of a type-I TGF-β receptor Babo-A isoform for vCrz PCD

Crz neuropeptide is produced by two groups of protocerebral neurons as well as eight bilateral pairs of neurons located from the 2nd thoracic to 6th abdominal neuromeres in the larval VNC (Fig. 1a). The ventral Crz neuronal group (vCrz) is removed by developmentally regulated activation of caspases during early phase of metamorphosis, whereas the protocerebral neurons persist into adulthood (arrowheads in Fig. 1b). Recently, by using a newly developed neuron-specific caspase sensor (Casor) we detected caspase activation in the vCrz neurons as early

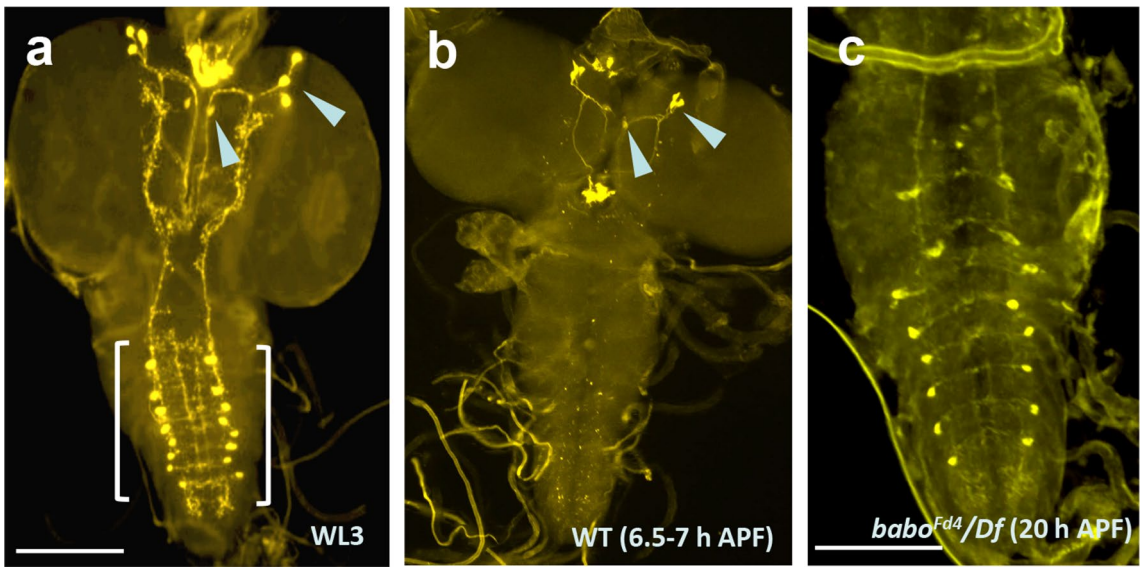
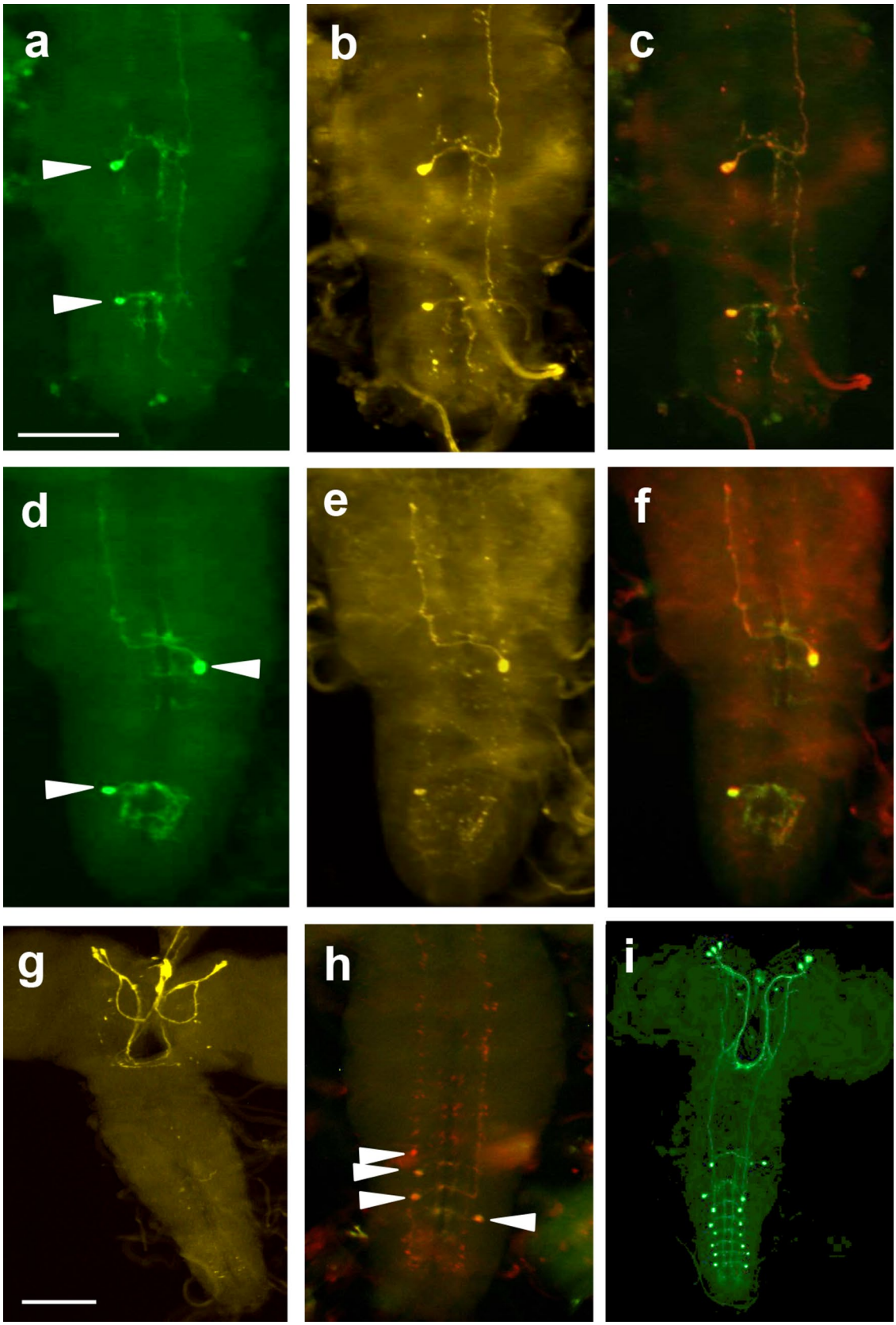


Fig. 1 Essential role of Babo for vCrz neuronal PCD. The Crz neurons were visualized by Crz-immunohistochemistry (Crz-IR). **a** Two different groups of protocerebral Crz neurons (arrowheads) and eight pairs of vCrz neurons (brackets) in the ventral nerve cord (VNC) are normally seen in the CNS of wild-type (WT) at wandering 3rd instar larva (WL3) (n > 16). **b** In WT, all vCrz neurons and their neuronal projections were removed via apoptosis by 6–7 h APF (n > 14). Note that the two groups of protocerebral neurons remained (arrowheads). **c** All 16 vCrz neurons remained at 20 h APF in a *babo*-null mutant, $babo^{Fd4}/Df$ (n = 5). Scale bars, 100 µm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◂**Fig. 2** Autonomous role of Babo-A for PCD of vCrz neurons. Mitotic recombination of $babo^{Fd4}$ or $babo^{52}$ allele was induced by heat-shock treatment and then vCrz neurons bearing homozygous mutation were doubly labeled by mCD8GFP and Crz-IR at 6–7 h APF. **a–c** $babo^{Fd4}$ mutant vCrz neurons are labeled by mCD8GFP (arrowheads in **a**) as well we Crz-IR (**b**). **c** Merged image of (**a**) and (**b**). (genotype: *hs-FLP*, *UAS-mCD8GFP/+*; FRT^{G13}, $babo^{Fd4}/FRT^{G13}$, *tubP-gal80; crz-gal4/+*). The results show that vCrz PCD requires Babo function autonomously. **d–f** Same as (**a–c**), except for $babo^{52}$ allele used (genotype: *hs-FLP, UAS-mCD8GFP/+*; FRT^{G13}, $babo^{52}/FRT^{G13}$, *tubP-gal80; crz-gal4/+)*. Arrowheads indicate $babo^{52}$ mutant neurons. **g** Expression of *UAS-babo-A* rescued PCD-defect in vCrz neurons carrying $babo^{Fd4}$ mutation, as no mCD8GFP-labeled mutant vCrz neurons were detectable (genotype: *hs-FLP*, *UAS-mCD8GFP/+*; FRT^{G13}, $babo^{Fd4}/FRT^{G13}$, *tubP-gal80*; *crz-gal4, UAS-babo-A/+*). **h** PCD defect of $babo^{Fd4}$ mutant vCrz neurons was not rescued by overexpression of either Babo-B or Babo-C isoform (genotype: *hs-FLP, UAS-mCD8GFP/+;* FRT^{G13}, $babo^{Fd4}/FRT^{G13}$, *tubP-gal80; crz-gal4, UAS-babo-isoform*). Arrowheads indicate surviving vCrz neurons. **i** Expression of *babo-a miRNA* (*mi-babo-A*) by *crz-gal4*. All 16 vCrz neurons remained at 6–8 h APF. Scale bars, 100 µm

as 30 min APF [19]. From this time point on, vCrz neurons gradually degenerate and their cellular debris are cleared by 6–8 h APF at 25 °C (Fig. 1b, see also Ref. 10, 14). We further showed that vCrz PCD requires ecdysone signaling mediated by redundant function of EcR-B1 and EcR-B2 isoforms as well as Usp [10, 15].

Our further screening to find additional molecular components involved in vCrz PCD discovered *baboon* (*babo*), a type-I receptor in the TGF-β signaling pathway, as a proapoptotic factor. Trans-heterozygous *babo* mutants ($babo^{Fd4}/Df$) die mostly during early metamorphosis, but a small number of escapers survive slightly longer [39], allowing us to examine its mutational effect on the vCrz PCD. It should be noted, however, that the developmental stages of the mutants cannot be compared directly to those of wild-type, because developmental progression of the *babo* mutants is systemically compromised. Hence we selected the latest stage of the mutants that we could obtain. In $babo^{Fd4}/Df$ animals, vCrz neurons failed to proceed into apoptotic death and the surviving neurons showed relatively normal neuronal architecture around 20 h APF (Fig. 1c), suggesting that Babo is required for the vCrz PCD. To determine if Babo acts autonomously, we employed MARCM to produce a few vCrz neurons bearing homozygous *babo* mutant alleles [40]. The homozygous *babo* mutant vCrz neurons were labeled by mCD8GFP signals (Fig. 2a, d). About 45% of specimens processed revealed a few normal-looking vCrz neurons bearing the $babo^{Fd4}$ allele without apoptotic signs at 6–7 h APF (n = 50, Fig. 2a–c), while 35% of $babo^{52}$ specimens did so (n = 50, Fig. 2d–f). Other vCrz neurons that did not carry homozygous *babo* mutations were undetectable as they were eliminated via normal apoptotic process. Together, the data strongly support that autonomous Babo function is indeed essential for the developmental PCD of vCrz neurons.

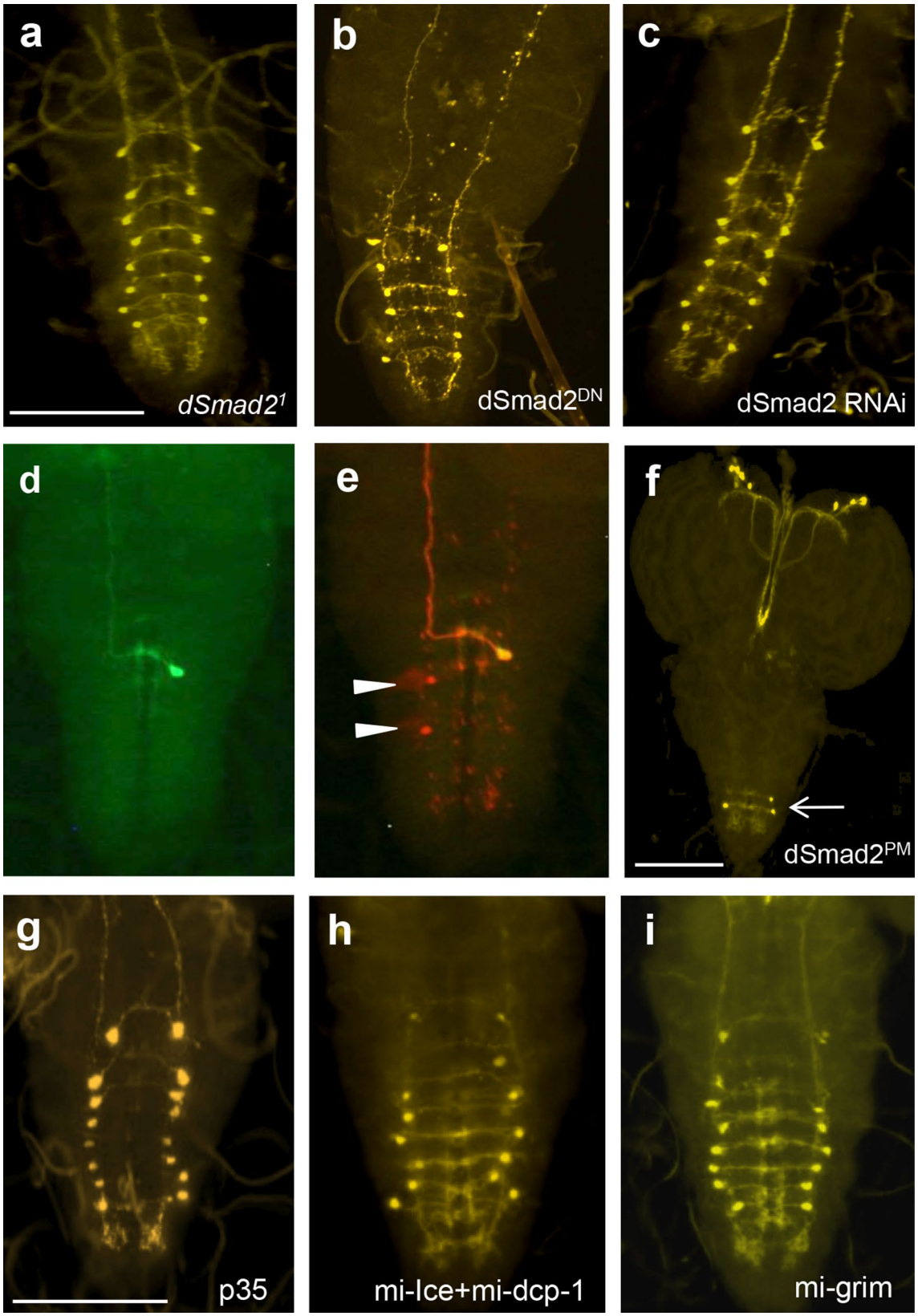
The *babo* locus encodes three isoforms, Babo-A, Babo-B, and Babo-C, varying the fourth exon through differential splicing so that the isoforms differ only in the extracellular domain that dictates ligand binding specificity [41]. To determine particular Babo isoform acting for vCrz neuronal PCD, each isoform was expressed in $babo^{Fd4}$ mutant vCrz neurons. Expression of *babo-A* complemented PCD defect by $babo^{Fd4}$ mutation, leaving no detectable vCrz neurons in all specimen examined at 6–7 h APF (n = 50, Fig. 2g). In contrast, expression of *babo-B* or *babo-C* isoform did not rescue the PCD defect, as ~40% of samples of *babo-B* expression (n = 32) and 30% of *babo-C* expression (n = 50) showed defective PCD phenotype (Fig. 2h). To further corroborate the autonomous role of *babo-A*, we performed isoform-specific *microRNA*-based *babo* knockdown driven by *crz-gal4* and found that PCD of vCrz neurons was blocked only by *babo-A* knockdown (n = 14, Fig. 2i). These data together strongly support that Babo-A receptor is a proapoptotic factor essential for the PCD of vCrz neurons. The results are also consistent with the fact that *babo-A* is the predominant isoform expressed in the CNS [41].

dSmad2 is a proapoptotic effector of Babo-A receptor

In mammals, ligand-bound TGF-β receptors activate Smad2 and Smad3 (R-Smads) via phosphorylation. The phosphorylated Smad2/3 forms a complex with Smad4 (co-Smad), which subsequently enters the nucleus and regulates expression of a variety of target genes. However, there are some TGF-β regulated events that are independent of R-Smad [42].

A fly ortholog of R-Smad is dSmad2 encoded by *Smox* [33]. To determine if dSmad2 is a proapoptotic factor working in the downstream of Babo-A, we investigated vCrz PCD in various *dSmad2* mutants. As shown in Fig. 3a, all 16 vCrz neurons remained alive in $dSmad2^{1}$ mutants without overt signs of apoptotic degeneration at 6–7 h APF. Furthermore, the expression of a dominant-negative dSmad2 ($dSmad2^{DN}$) by *crz-gal4* driver showed that an average of 12.5 neurons remained intact at 6–7 h APF; however, the cell bodies and axonal projections showed signs of progressing apoptosis, implying mild PCD defect (Fig. 3b). In response to RNAi-mediated knockdown (*dSmad2-RNAi*), an average of 13.5 vCrz neurons were detectable at 6–7 h APF (Fig. 3c), suggesting that PCD was not completely blocked by *dSmad2-RNAi* expression either. Nonetheless, these results support that dSmad2 is an essential TGF-β signaling component autonomously functioning for vCrz neuronal PCD.

Next we examined if *dSmad2* restores PCD defect of $babo^{Fd4}$-mutant vCrz neurons that were generated by MARCM. Ectopic expression of *dSmad2* in this background still showed intact vCrz neurons in about 22% of samples,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dSmad2¹
b
dSmad2DN
c
dSmad2 RNAi
d
e
f
dSmad2PM
g
p35
h
mi-Ice+mi-dcp-1
i
mi-grim

◂**Fig. 3** Apoptotic roles of dSmad2 for vCrz PCD. The vCrz neurons were visualized by either Crz-IR or *Crz>mCD8GFP*. **a** PCD of all vCrz neurons were prevented in $dSmad2^{1}$ homozygous mutants at 6–8 h APF (n = 5). **b** Expression of a dominant negative $dSmad2^{DN}$ showed 12.5 ± 1.0 (n = 6) vCrz neurons remained at 6–7 h APF. **c** d*Smad2-RNAi* expression by *crz-gal4* showed 13.5 ± 1.0 (n = 5) vCrz neurons remained at 6–7 h APF. **d, e** Expression of d*Smad2* did not rescue PCD defect in $babo^{Fd}$ mutant clone, as indicated by mCD8GFP-labeled neuron in (**d**) (genotype: *hs-FLP, UAS-mCD8GFP/+; FRT^{G13}, tubP-gal80/FRT^{G13} $babo^{Fd4}$; crz-gal4, UAS-dSmad2/+*). **e** Double-labeling of (**d**) with crz-IR. Arrowheads indicate residual cell bodies of the $babo^{Fd4}$ heterozygous vCrz neurons labeled only by Crz-IR. It is not uncommon to see a few dying neurons that are not completely cleared at this time point. **f** Expression of a phosphomimetic form of dSmad2 ($dSmad2^{PM}$) led to premature loss of most vCrz neurons at WL3 stage, except a few indicated by an arrow (n = 16, genotype: *UAS-$dSmad2^{PM}$/+; crz-gal4/+*). **g–i** $dSmad2^{PM}$-mediated loss of larval vCrz neurons was rescued by *crz-gal4*-driven ectopic expression of indicated transgenes (genotype: *crz-gal4/+; UAS-$dSmad2^{PM}$/UAS-gene*). Mean numbers ± sd of vCrz neurons detected are 16 ± 0 (n = 10, **g**), 12.7 ± 1.2 (n = 3, **h**), and 14.1 ± 1.5 (n = 7, **i**). Scale bars, 100 µm

indicating that the overexpression of *dSmad2* did not rescue defective PCD phenotype of $babo^{Fd4}$ mutant (n = 50, Fig. 3d, e). This is perhaps because dSmad2 is not phosphorylated in the absence of functional Babo receptor. To find evidence supporting the role of phosphorylated dSmad2, we tested a phosphomimetic form of dSmad2 ($dSmad2^{PM}$; a.k.a. $dSmad2^{SDVD}$) that was shown to be constitutively active [32]. Ectopic expression of the $dSmad2^{PM}$ eliminated most vCrz neurons in wandering third instar larvae (WL3) (Fig. 3f). Furthermore, co-expression of a caspase inhibitor *p35* or miRNA-induced silencing of two major effector caspases, *dcp-1* and *ice*, blocked similarly the $dSmad2^{PM}$-mediated premature killing of vCrz neurons (Fig. 3g, h). These data suggest that $dSmad2^{PM}$-mediated killing effect is accomplished by active caspases. Since the caspase activation in vCrz neurons is mediated by *grim* cell death gene [16], we tested if *grim* is involved in the $dSmad2^{PM}$-mediated killing. Consistent with this prediction, silencing of *grim* blocked $dSmad2^{PM}$-mediated vCrz cell death (Fig. 3i). These data together suggest that Babo-dSmad2 pathway promotes vCrz PCD by activating *grim* expression.

To further see if $dSmad2^{PM}$ causes the death of other larval neurons non-specifically, we expressed it in three different groups of peptidergic larval neurons that do not undergo PCD (dilp2 and mip) or die after adult eclosion (ccap). Of interest, we did not observe the premature death of these neurons at the WL3 stage (suppl. Fig. 1), suggesting that $dSmad2^{PM}$-induced killing is specific to the neurons that are programmed to die during early metamorphosis.

Obscure function of type-II receptors, Punt and Wit

According to the canonical signaling model, binding of TGF-β ligand to type-II receptors recruits type-I to form a functionally active complex that phosphorylates R-Smads. There are two type-II receptors in *Drosophila*, Punt and Wishful thinking (Wit). To gain insights into the roles for these type-II receptors in the vCrz PCD, simultaneous RNAi-mediated knockdown of *punt* and *wit* was driven by two copies of *crz-gal4*. However, no significant effect by the double knockdown was observed on the vCrz neuronal death at 6–7 h APF (Fig. 4a). We also examined *punt* and *wit* mutants. Homozygous $punt^{62}$ or $punt^{135}$ mutants are embryonic lethal but temperature-shift manipulation (16 °C to 25 °C) of *trans*-heterozygous $punt^{62/135}$ mutants in early larval stages permitted us to test this gene's role at prepupal stage [35]. Crz-immunohistochemical assays (Crz-ir) showed normal progression of vCrz PCD in $punt^{62/135}$ prepupae at 6–7 h APF with slight delay of the clearance of apoptosed debris (Fig. 4b). Similar results were found in homozygous wit^{G15} mutants (Fig. 4c) and even in *punt-wit* double mutants ($punt^{62/135}$; $wit^{G15/G15}$) (Fig. 4d). In contrast, the $punt^{62/135}$; $wit^{G15/G15}$ mutant clearly reduced EcR-B1 expression in the larval MB γ neurons, thus proving its effectiveness [22]. We speculate the possibility that leaky (or residual) expression of the type-II receptors due to hypomorphic nature of the mutants is still sufficient for the signaling to support normal PCD of vCrz neurons, but not for the MB γ neuron pruning.

Essential role of Plum

A member of immunoglobulin superfamily protein (IgSF), Plum, was previously shown to have an essential role in TGF-β signal transduction for axon pruning of MB γ neurons [38]. Evidence in this study proposed that Plum assists Myo binding to the type-I/II receptors, thereby facilitating Myo signaling. To test the roles of Plum in the PCD of vCrz neurons, we examined a *plum*-null mutation, $plum^{\Delta 1}$ [38]. As a result, we found that most vCrz neurons remained alive in the CNS of $plum^{\Delta 1}$ homozygous mutant at 6–8 h APF (Fig. 4e). We also knocked down *plum* by using *RNAi*-based silencing to see its autonomous function. We observed an average of seven vCrz neurons with *plumRNAi* expression by a single copy of *crz-gal4* at 6–8 h APF (Fig. 4f). The knockdown by two copies of *crz-gal4* produced stronger PCD defect (Fig. 4g), the results of which were comparable to those observed in $plum^{\Delta 1}$ homozygotes. These data indicate that Plum is an important factor autonomously working for vCrz PCD, presumably via a similar mechanism proposed for the axonal pruning of MB γ neurons [38].

Glia-derived Myo is a ligand of Babo-A for vCrz PCD

Three Activin type ligands, Actvinβ (Actβ), Dawdle (Daw) and Myoglianin (Myo), are known to signal through type-I receptor Babo [22, 23, 43–45]. To find the candidate ligand

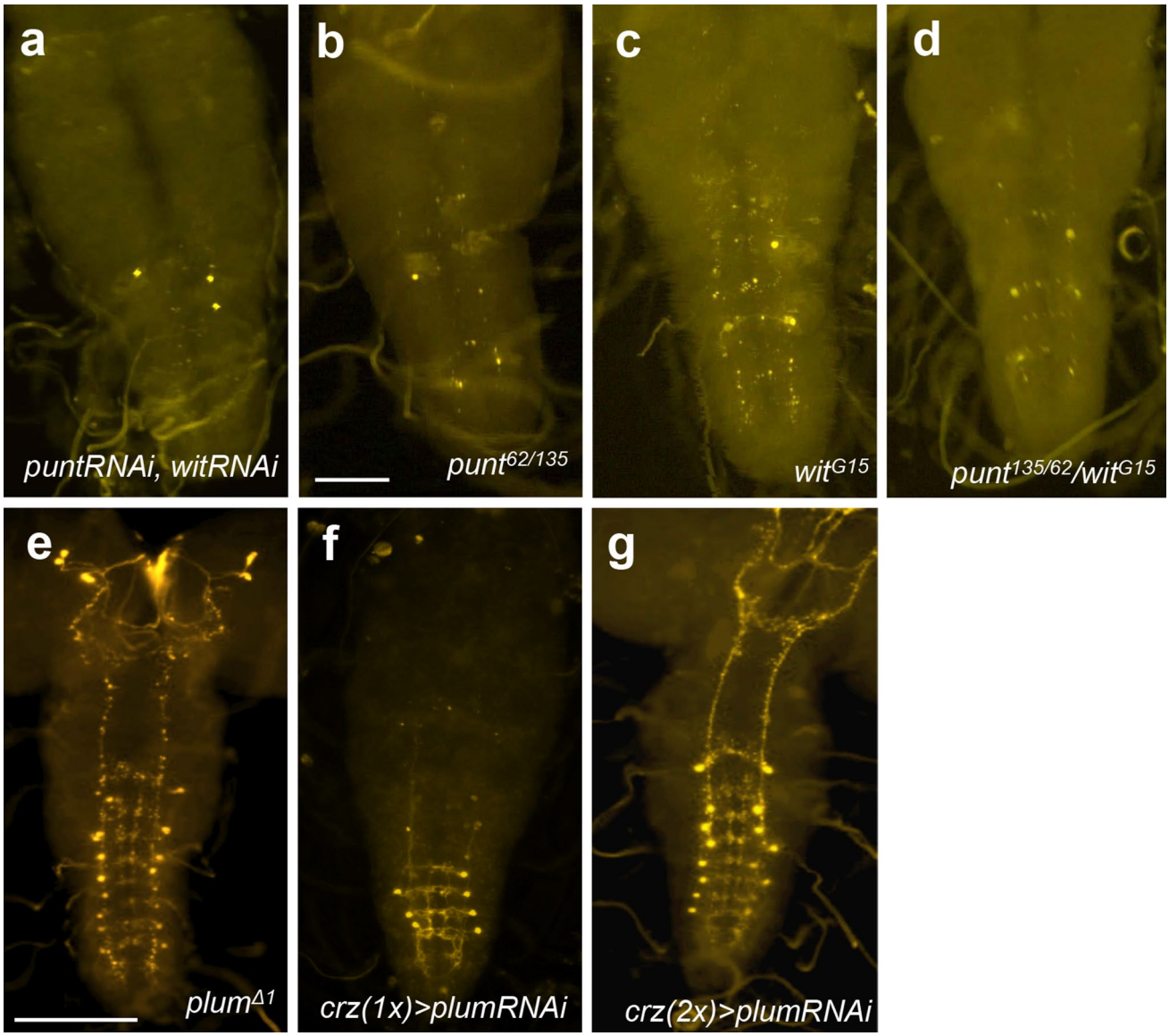

Fig. 4 Role of type-II receptors and Plum for PCD of vCrz neurons. The Crz neurons were detected by Crz-IR at 6–8 h APF. **a** Double RNAi-mediated knockdown of *punt* and *wit* by two copies of *crz-gal4* at 6–7 h APF (n = 5, genotype: *crz-gal4/UAS-puntRNAi*; *crz-gal4/UAS-witRNAi*). **b** $punt^{62/135}$ (n = 3). **c** $wit^{G15/G15}$ (n = 12). **d** $punt^{62/135}$; $wit^{G15/G15}$ (n = 3). vCrz PCD phenotype is mostly normal in these mutants. Only residual uncleared cellular debris are detected. **e** PCD defect in homozygous $plum^{\Delta 1}$ mutant at 7–8 h APF (n = 5). **f, g** RNAi-mediated knockdown of *plum* using one copy and two copies of *crz-gal4* driver at 7–8 h APF (genotypes: *UAS-plumRNAi/+; crz-gal4/+* and *UAS-plumRNAi/ crz-gal4; crz-gal4/+*). Mean numbers ± sd of surviving vCrz neurons are 10.6 ± 3.0 (n = 7, **f**) and 15.7 ± 0.6 (n = 14, **g**). Scale bars, 50 µm (**b**), 100 µm (**e**)

that triggers TGF-β signal transduction in the vCrz neurons, we examined knockdown or null-mutants available for each ligand for their vCrz PCD phenotype. For Actβ, we have initially tested systemic knockdown (HLdAct) and expression of a dominant-negative form (CMdAct) driven by *heat shock (hs)-gal4* [22] and observed 7.7 and 12.4 vCrz neurons remaining at 6–7 h APF, respectively (Fig. 5a, b). Contradictory to these results, however, vCrz PCD occurred normally in an *act*β-null mutant, $act\beta^{ed80}$ [45], suggesting that Actβ is not the ligand acting for vCrz PCD (Fig. 5c). Similar phenomena were also described for the remodeling of MB γ neurons [23]. vCrz PCD phenotype was also normal in a *daw*-null (daw^{ex32}) mutants [44], disproving Daw's role for the vCrz PCD (Fig. 5d). Remarkably, unlike in *actβ* and *daw* mutants, PCD of vCrz neurons was completely abolished in *myo*-null ($myo^{\Delta 1}$) mutants at 6 h APF (Fig. 5e). Even at 20 h APF, all vCrz neurons survived and retained normal-looking appearance without overt sign of apoptosis (Fig. 5f), indicating that Myo is the acting ligand for Babo-A receptor.

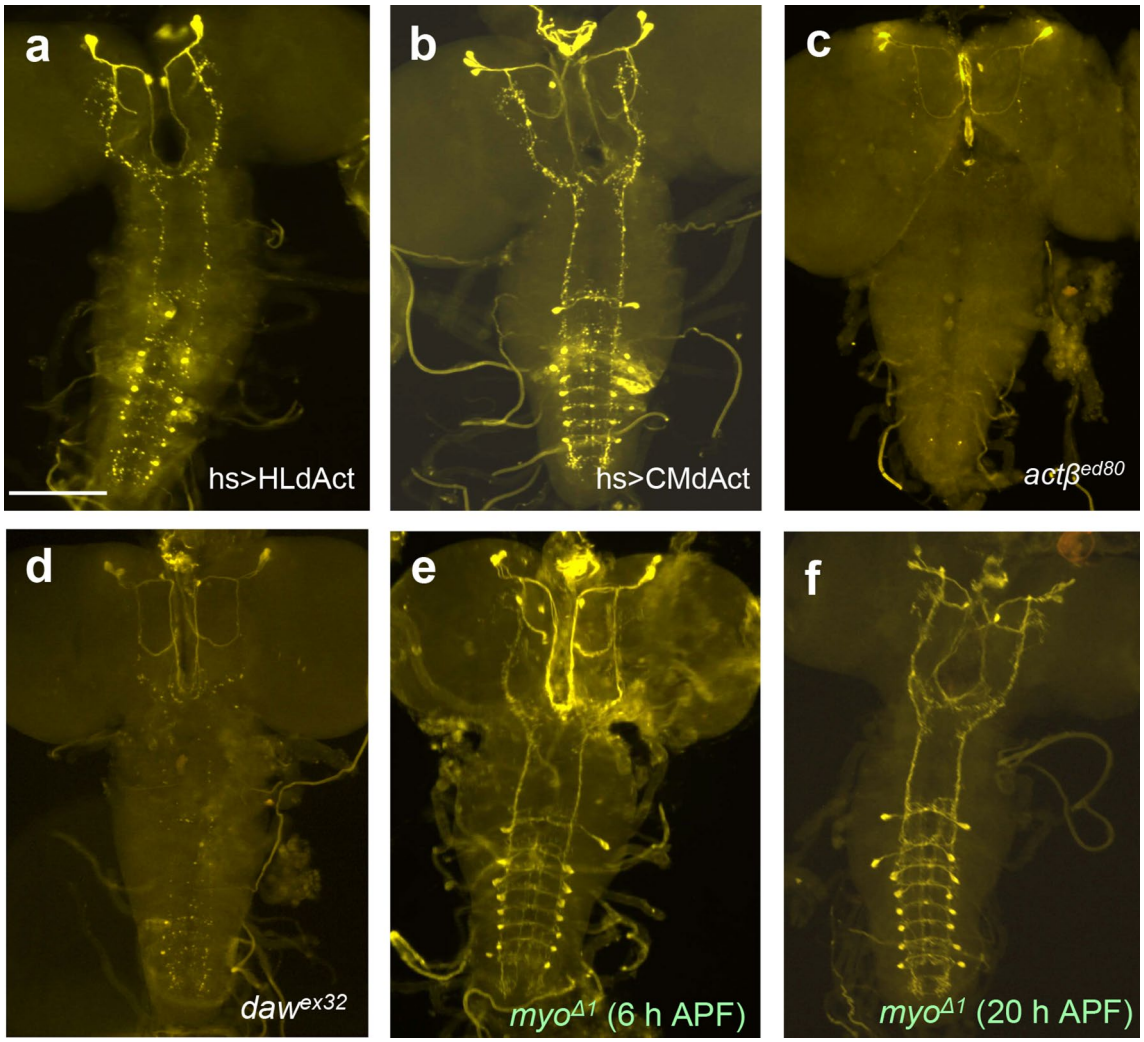

Fig. 5 Essential role of *myo* for PCD of vCrz neurons. The Crz neurons were detected by Crz-IR. **a, b** Systemic knockdown or inactivation of *activin* with *heatshock* (*hs*)-*gal4* suppressed PCD of vCrz neurons. *hs-gal4* was crossed to a *UAS-HLdActivin* (knockdown) or a *UAS-CMdActivin* (dominant negative). Mean numbers ± sd of vCrz neurons remained at 6–7 h APF are 7.7 ± 1.0 (n = 7, **a**) and 12.4 ± 1.4 (n = 5, **b**). **c** Normal vCrz PCD in *activin* mutants ($act\beta^{ed80}$) at 6–8 h APF (n = 5). **d** Normal vCrz PCD in *dawdle* (daw^{ex32}) mutants at 6–8 h APF (n = 7). **e**, **f** PCD of vCrz neurons was completely blocked in homozygous *myo*-null mutants at 6 h (n = 5, **e**) and 20 h APF (n = 3, **f**). Scale bar, 100 μm

Primary expression sites of *myo* are muscles and glia [46]. In the larval CNS, *myo* is expressed in two subtypes of glial cells: cortex glia and astrocyte-like glia [23]. To see if vCrz PCD-inducing Myo is derived from the glial cells, we first knocked down *myo* expression using *myo miRNA* (*mi-myo*) in all glial cells by a *repo-gal4* driver. Such panglial *myo* knockdown (*repo* > *mi-myo*) delayed pupal development slightly. Although pharate adults formed normally, they failed to eclose and died within the pupal case (Fig. 6a). We dissected CNSs of *repo* > *mi-myo* at 10–12 h APF which is morphologically comparable to 6–8 h APF of wild-type. Remarkably, *repo* > *mi-myo* blocked vCrz PCD completely as all normal-looking vCrz neurons were detectable (Fig. 6b). The results demonstrate that glia-derived Myo acts as 'the ligand' for the Babo-A receptor to induce vCrz neuronal PCD.

We furthered to determine specific glia subtype that produces apoptogenic Myo. Knockdown of *myo* in either astrocyte-like (*alrm-gal4*) or cortex (*NP2222-gal4*) glia did not disrupt normal course of PCD, since all vCrz neuronal structures were eliminated by 6–8 h APF (Fig. 6c, d). Similar results were also obtained with *myo* knockdown by *myo-gal4* (Fig. 6e). This is not surprising, since the *myo-gal4* incompletely reflects endogenous expression patterns of the gene [23]. Taken together, we suggest that Myo production from either glial source (astrocyte-like or cortex glia) is sufficient to promote PCD of vCrz neurons.

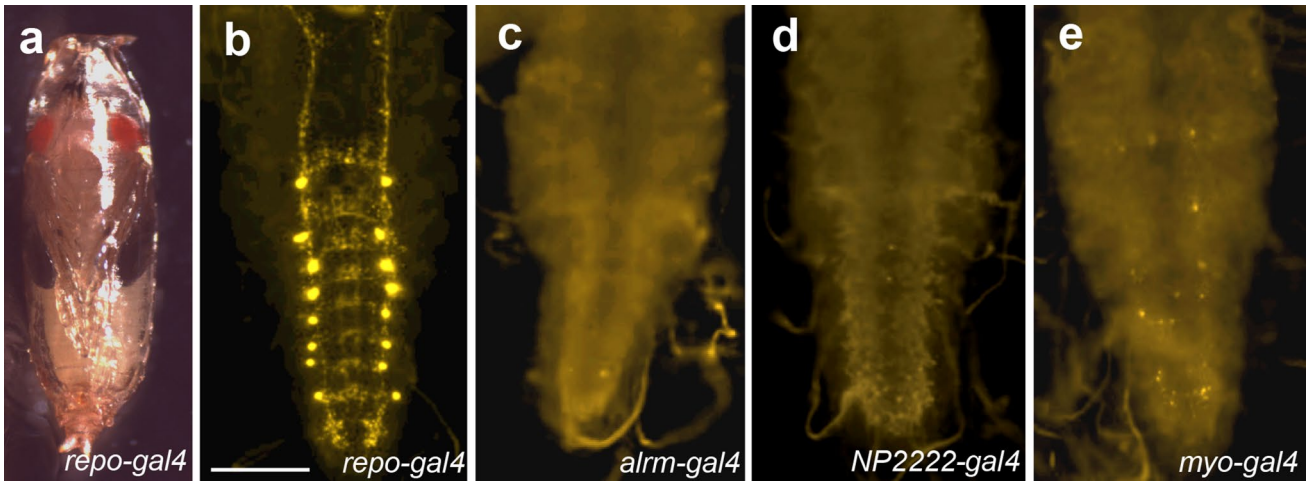

Fig. 6 Glia-specific knockdown of *myo* inhibited PCD of vCrz neurons. The vCrz neurons were detected by Crz-IR. *myo* knockdown was done with an indicated *gal4* driver. **a** Panglial knockdown (*repo* > *mi-myo*) delayed pupal development slightly but did not affect the formation of pharate adults (n > 100). **b** Panglial knockdown blocked PCD of vCrz neurons at 10–12 h APF (n = 5). **c–e** Normal vCrz PCD in *myo* knockdown driven by glia-subtype specific *gal4*: Astrocyte-like glia, *alrm-gal4* (n = 14, **c**); cortex glia, *NP2222-gal4* (n = 16, **d**); *myo-gal4* (n = 16, **e**). Scale bar, 100 µm

Glial EcR signaling for *myo* expression and clearing activity

Since *myo* expression in the larval CNS is approximately coincident with the increase in ecdysone levels at 3rd instar larval stage [23], we wondered if ecdysone signaling plays a role in the regulation of temporal *myo* expression. To test this, glial EcR function was disrupted by expressing a dominant-negative form of EcR (EcR^{DN}) with *repo-gal4* (*repo* > EcR^{DN}). Panglial EcR^{DN} expression did not show noticeable interference with larval growth and development but led to a developmental arrest between late prepupal and early pupal stages (Fig. 7a). Intriguingly, we found that all vCrz neurons remained alive in the pupae aged from 7 to 16 h APF (Fig. 7e). To gain further insight into the role of EcR as an upstream of *myo* expression, we attempted to rescue *repo* > EcR^{DN} by co-expression of *myo* (*repo* > EcR^{DN} + *myo*). As a result, about 70% of *repo* > EcR^{DN} + *myo* animals advanced to pharate adult stage, although they showed varying degrees of morphological defects (Fig. 7b–d). Consistent with the developmental rescue, we observed similar variability in rescuing vCrz PCD defect at 7–8 h APF (Fig. 7f–h). In about 30% of samples, all vCrz cell bodies were completely removed, although some residual neuronal projections remained uncleared (Fig. 7h). In the remaining samples, the rescue was incomplete as variable numbers of vCrz somata along with neuronal projections were detectable (Fig. 7f, g). Nevertheless, the results strongly support that EcR function in glia is necessary for the *myo* expression.

Glial cells are also involved in the engulfment of apoptotic bodies. Normally both vCrz cell bodies and their projections are completely removed by around 7 h APF in wild-type [14]. However, incomplete clearance of the projections observed in *repo* > EcR^{DN} + *myo* (arrowheads in Fig. 7h) suggests that phagocytic activities of the glia cells also require EcR function.

To determine a particular EcR isoform required for the regulation of *myo* expression, each EcR isoform was co-expressed with EcR^{DN}. Glial EcR^{DN}-mediated PCD defect of vCrz neurons was completely rescued by all EcR isoforms at 6–8 h APF (Fig. 8a–d). Since all EcR isoforms are present in the CNS at this stage [47], we speculate that *myo* expression is regulated by redundant function of EcR isoforms. We also questioned if Usp, as a partner of EcR, plays a role for *myo* expression in glial cells. Panglial knockdown of *usp* (*repo* > *mi-usp*) did not affect overall pupal development but normal-looking pharate adults failed to eclose (n > 100). To our surprise, in contrast to *repo* > EcR^{DN}, *repo* > *mi-usp* did not block vCrz PCD (Fig. 8a vs e), suggesting that EcR-dependent *myo* expression for vCrz PCD does not involve Usp function. Interestingly however, despite the lack of detectable vCrz cell bodies, neuronal projections remained (Fig. 8e), as observed with *repo* > EcR^{DN} + *myo* (Fig. 7h). Furthermore, similar clearance defect was found in prepupae carrying either EcR^{DN} or *mi-usp* expression by *alrm-gal4* in astrocyte-like glial subtype (Fig. 8f, g). In other words, the phagocytic clearance of apoptosed axons by the astrocyte-like glial cells requires both EcR and Usp function. This is consistent with a previous report showing that astrocyte-like glial cells require EcR function to remove vCrz

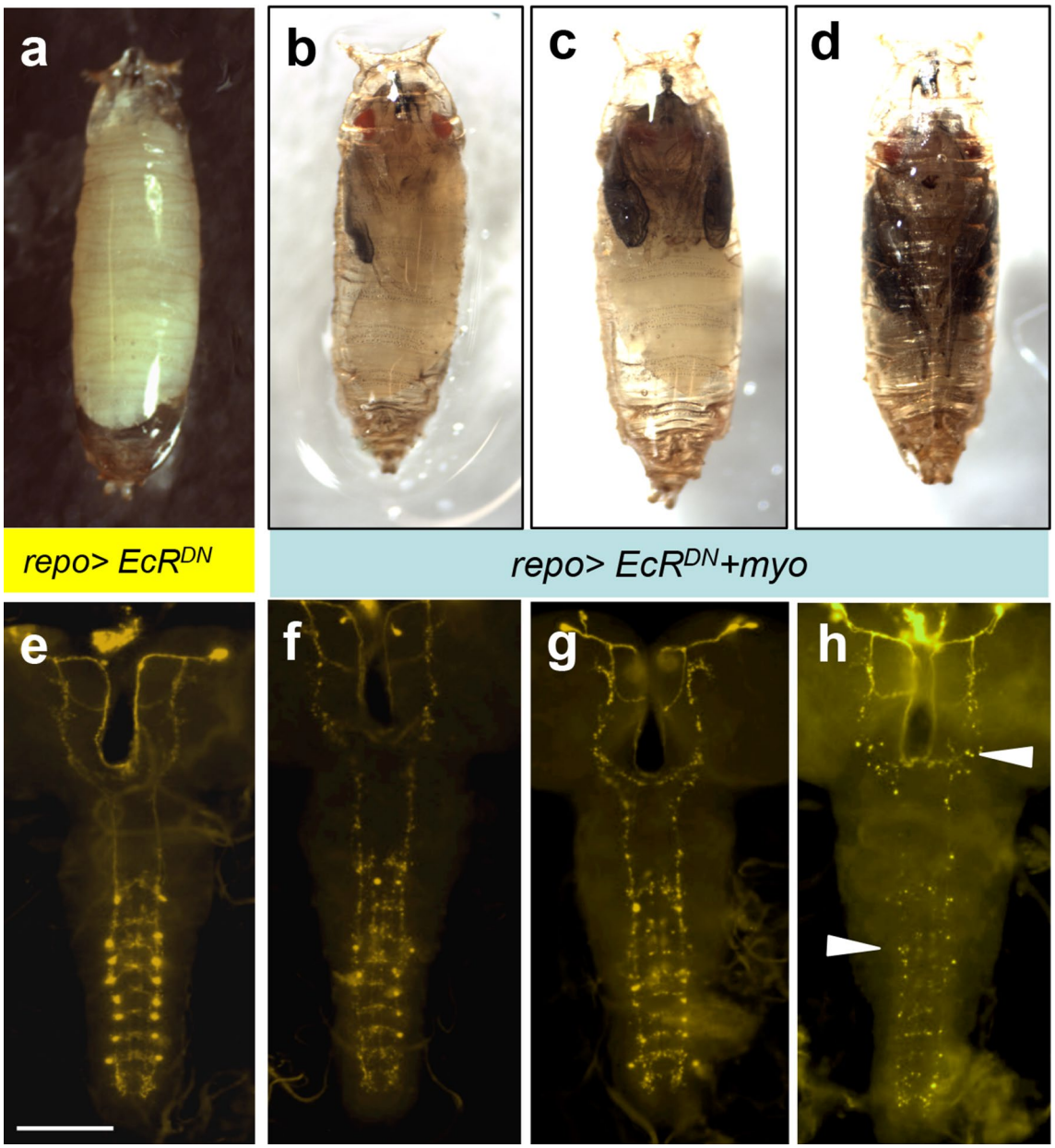

Fig. 7 Glial EcR signaling for the regulation of *myo* expression. The Crz neurons were detected by Crz-IR. **a** Panglial expression of EcR^{DN} (*repo* > EcR^{DN}) caused severe developmental defect between late prepupal and early pupal stages (n > 50). **b–d** Co-expression of *myo* (*repo* > EcR^{DN} + *myo*) rescued EcR^{DN}-caused developmental defect in varying degrees: pharate adults with short and deformed appendages (**b**, **c**) and relatively normal appearance (**d**). **e** PCD defect of vCrz neurons in *repo* > EcR^{DN} at 7–16 h APF (n = 16). **f–h** Co-expression of *myo* rescued EcR^{DN}-caused PCD defect of vCrz neurons to varying degrees (n = 10). All specimen showed neuronal projections that are not completely cleared. Some samples showed several cell bodies remained (**f, g**) while others had no cell bodies detectable (**h**). Arrowheads indicate uncleared neuronal projections. Scale bar, 100 μm

neuronal projections [48]. From these data, we propose dual roles of EcR in the glial cells: One is to induce *myo* expression to promote vCrz PCD, and Usp is dispensable for this role. The other is to stimulate phagocytic activity of the astrocyte-like glia for clearing of the apoptosed neuronal projections, and this function requires Usp.

TGF-β signaling is not epistatic to EcR signaling

Our data thus far showed that PCD of vCrz neurons requires activation of both TGF-β signaling by glia-derived Myo and ecdysone signaling via EcR-B:Usp [10, 15]. Since it has been demonstrated that the Myo-Babo-dSmad2 signaling pathway positively regulates the

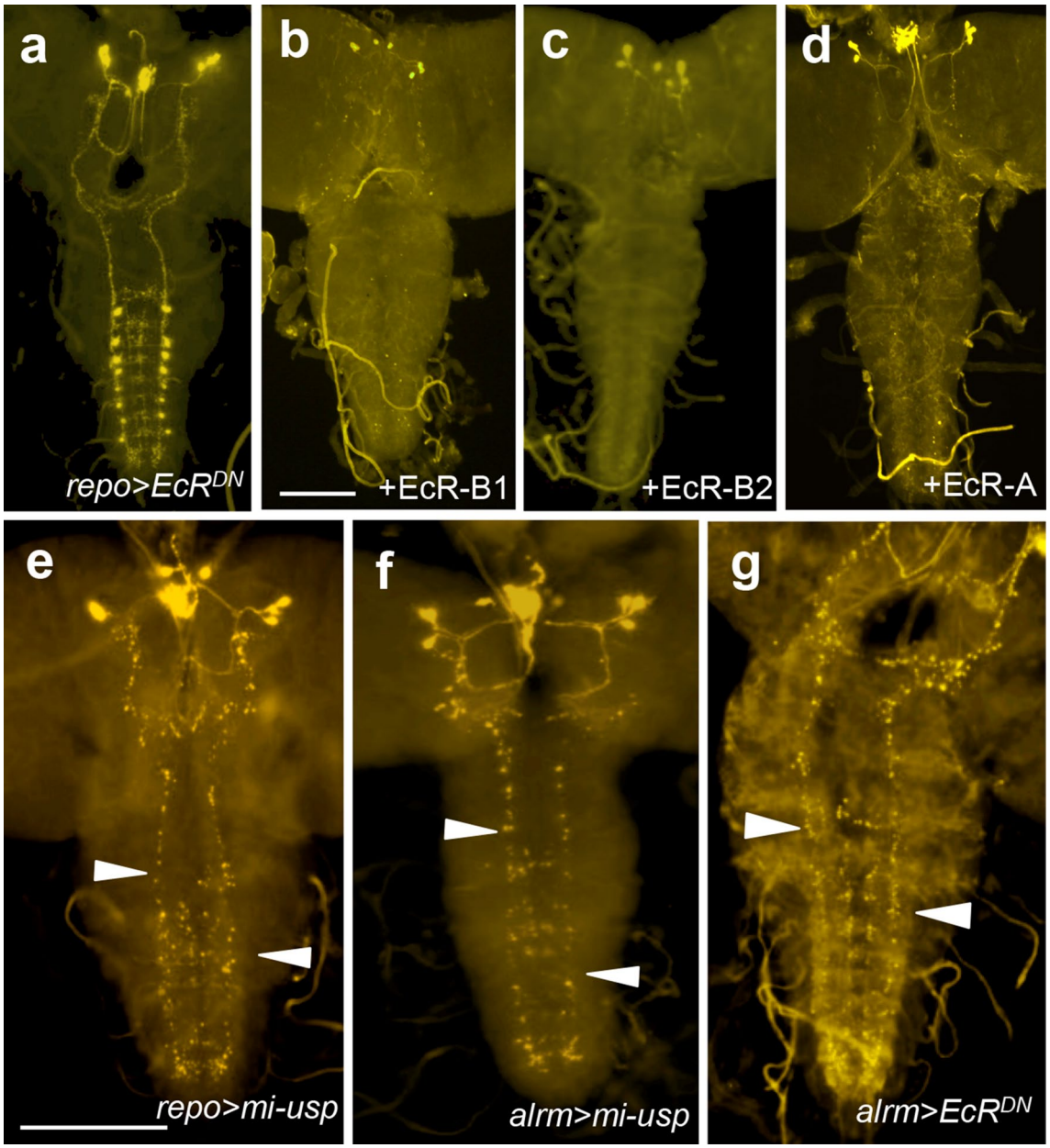

Fig. 8 Differential functions of EcR and Usp. The vCrz neurons were visualized by Crz-IR. **a** Inhibition of vCrz PCD by panglial expression of EcR^{DN}. **b–d** PCD defect of vCrz neurons by *repo* > EcR^{DN} was rescued by co-expression of EcR-B1 (**b**), EcR-B2 (**c**), or EcR-A (**d**) at 6–8 h APF (n > 8 for each isoform). **e** Panglial knockdown of *usp* (*repo* > *mi-usp*) showed normal vCrz PCD but significantly defective clearance of the neuronal projections, as indicated by arrowheads (n = 14). **f, g** Defective clearance of vCrz neuronal projections (arrowheads) as a result from the expression of either *mi-usp* (**f**) or EcR^{DN} (**g**) in the astrocyte-like glial cells by *alrm-gal4* (n = 9 for both genotype). Scale bars, 100 µm

expression of EcR-B1 in the MB γ neurons, thus acting epistatically to the EcR signaling [22, 23], we questioned whether this is the case for PCD of vCrz neurons. To test this, we first examined EcR-B1 levels in the MARCM clones of *babo*-null vCrz neurons at WL3 stage, since high levels of EcR-B1 are observed in the CNS at this stage. In contrast to the case of MB γ neurons, EcR-B1 immunoreactivities (EcR-B1-IR) in the *babo*-null vCrz neurons were comparable to those of neighboring cells (Fig. 9a, ai). Secondly, we examined EcR-B1-IR in the vCrz neurons carrying *mi-babo*-A expression which efficiently blocked vCrz PCD. Unexpectedly we noticed that EcR-B1-IR were not uniform among control vCrz neurons (n = 16, Fig. 9b, bi) as well as those carrying *babo-A* knockdown (n = 16, Fig. 9c, ci). The cause of cell-to-cell variability of EcR-B1-IR is not known. Nonetheless, overall EcR-B1-IR

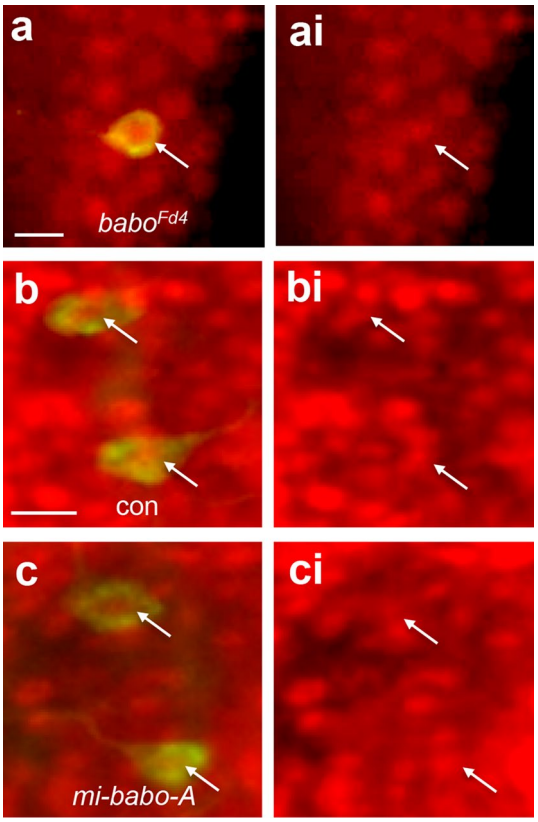

Fig. 9 EcR-B immunoreactivity (IR) is not downregulated in vCrz neurons carrying a *babo*-null mutation or *babo-A* knockdown. CNSs of WL3 were double-labeled by anti-EcR-B1 (red) and mCD8GFP (green) driven by *crz-gal4*. Arrows indicate EcR-B1-IR in the nuclei of vCrz neurons. **a**, **ai** EcR-B1-IR in a $babo^{Fd4}$-mutant vCrz neuron and neighboring cells (n > 12, genotype: *hs-FLP*, *UAS-mCD8GFP/+*; FRT^{G13}, $babo^{Fd4}/FRT^{G13}$, *tubP-Gal80/+*; *crz-gal4/+*). **b**, **bi** EcR-B1-IR in control (con) vCrz neurons and neighboring cells (n = 14, genotype: *UAS-mCD8GFP/+*; *crz-gal4/+*). **c**, **ci** EcR-B1-IR in vCrz neurons with *babo-A* knockdown (*crz > mi-babo-A*) and neighboring cells (n = 14, genotype: *UAS-mCD8GFP/+; UAS-mi-babo-A/+; crz-gal4/+*). Scale bar, 10 μm

seemed comparable between wild-type and *mi-babo-A* expressing vCrz neurons.

We also found that the PCD defect of $babo^{Fd4}$ mutant vCrz neurons was not rescued by EcR-B1 expression (n > 35, Fig. 10a-c). Furthermore, co-expression of EcR-B1 with *mibabo-A* did not rescue PCD defect either (n > 21, Fig. 10d, e), although overexpressed EcR-B1-IR was clearly detectable in the vCrz neurons (Fig. 10h–j). Similar results were obtained with co-expression of EcR-B2 (Fig. 10f), implying that TGF-β signaling does not act through the upregulation of EcR-B isoforms. Since canonical EcR-B:Usp heterodimer is required for PCD of vCrz neurons, the disruption of Usp function alone abrogates PCD of vCrz neurons [15]. Thus, we questioned if TGF-β signaling is involved in the regulation of *usp* expression instead of EcR-B. However, co-expression of *usp* with *mibabo-A* did not rescue the PCD defect either (n = 16, Fig. 10g). With all data combined, we conclude that TGF-β signaling does not upregulate the expression of *EcR-B* or *usp* in order to induce vCrz neuronal death. Conversely, EcR signaling might act in the upstream of the TGF-β signaling pathway by regulating expression of key factors in the TGF-β pathway. However, EcR^{DN}-mediated PCD defect was not rescued by overexpressing *babo-A* or *dSmad2* (Fig. 11a–c), suggesting that Babo-A-mediated signaling is not a downstream of EcR function.

The lack of epistatic relationship between EcR and TGF-β signaling might suggest a paralleled mechanism. Intriguingly, we observed that $dSmad2^{PM}$-induced premature killing of vCrz neurons was completely suppressed by co-expression of EcR^{DN} at WL3 and even at 6–8 h APF (Fig. 11d–g). These data suggest that pro-apoptotic function of $dSmad2^{PM}$ requires an input from EcR signaling. Hence, vCrz PCD is developmentally specified by cooperative and timely action of EcR and TGF-β signaling.

Discussion

TGF-β signaling is required for vCrz neuronal PCD

In *Drosophila*, TGF-β signaling within the prothoracic gland cells (PGC) promotes production of the most important developmental hormone, ecdysone, to drive metamorphosis, thus rendering a systemic effect on the metamorphosing CNS [49]. Utilizing genetic and transgenic manipulations of key factors, we revealed that the TGF-β signaling also plays an essential and autonomous role in promoting the PCD of vCrz neurons. For instance, vCrz PCD was blocked in neurons carrying homozygous *babo*-null mutation or silencing of *babo* or *dSmad2*. Moreover, expression of $dSmad2^{PM}$ killed vCrz neurons prematurely and that was suppressed by RNAi of Ice/Dcp-1 caspases or *grim* (a major death gene acting in the nervous system), all of which are the endogenous factors essentially required for the PCD of vCrz neurons [14, 16]. Hence, timely activation of TGF-β signaling is important to set off the PCD events in vCrz neurons at a defined phase of metamorphosis. Importantly, the $dSmad2^{PM}$-mediated killing is specific to vCrz neurons, as it did not induce the death of other peptidergic larval neurons that are not destined for PCD or die at later stage, suggesting that proapoptotic TGF-β signaling acts in a cell-specific fashion.

Roles of Type II receptors and Plum

According to the canonical signaling model, binding of TGF-β ligand recruits type-II and type-I receptors to form a functional complex that phosphorylates R-Smad. There

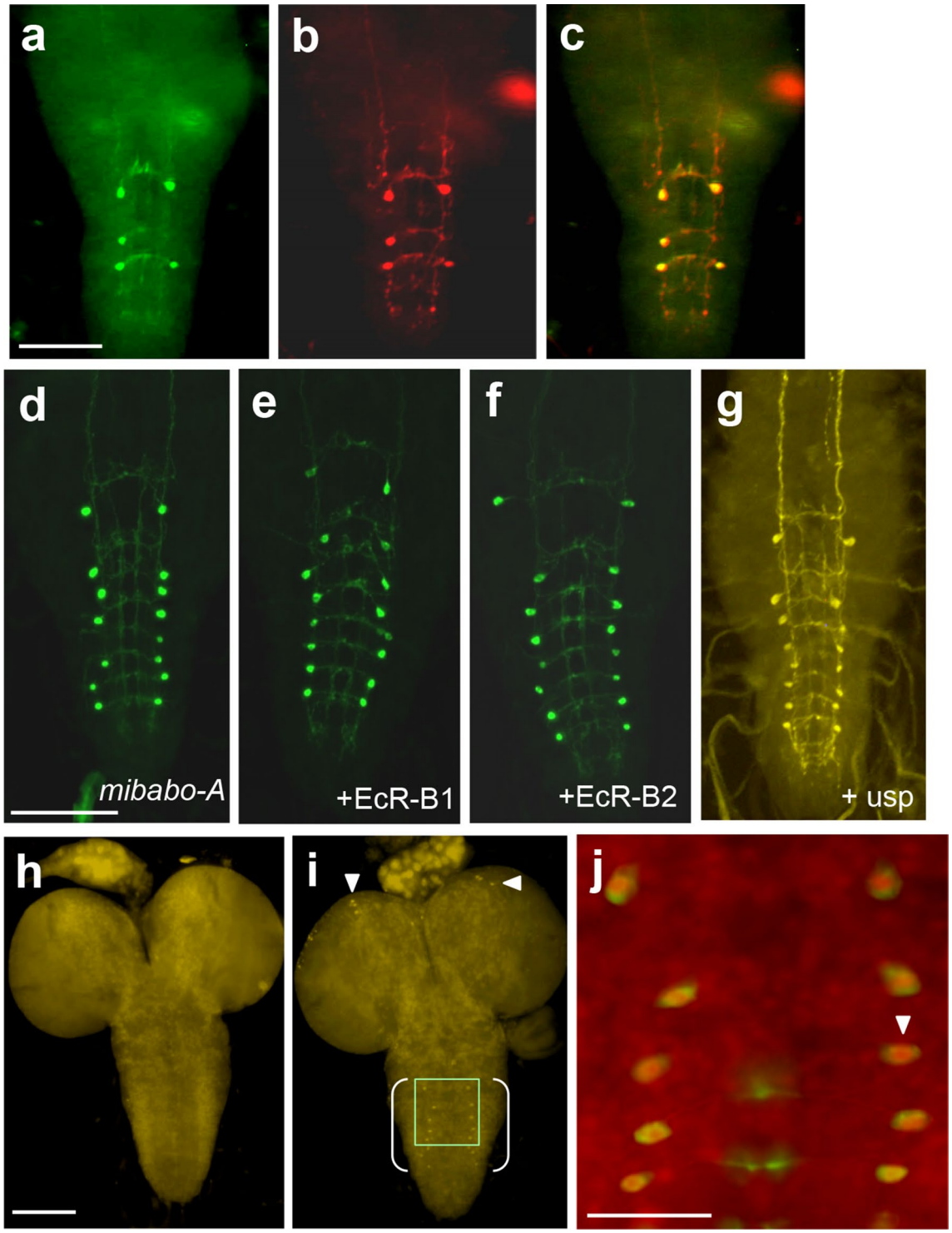

Fig. 10 EcR-B isoforms and Usp are not downstream of TGF-β signaling. The Crz neurons were visualized by mCD8GFP expression driven by *crz-gal4* or crz-IR. **a–c** $babo^{Fd4}$ mutant vCrz neurons were positive for mCD8GFP expression (**a**), crz-IR (**b**). A merge of (**a**) and (**b**) images is shown in (**c**). Expression of EcR-B1 did not rescue PCD defect of $babo^{Fd4}$ mutant vCrz neurons at 6–8 h APF (Genotype: *hs-FLP*, *UAS-mCD8GFP*/+; FRT^{G13}, $babo^{Fd4}/FRT^{G13}$, *tubP-gal80*; *crz-gal4*/*UAS-EcR-B1*). **d–g** PCD defect caused by *mibabo-A* expression at 6–8 h APF (**d**) was not rescued by co-expression of *EcR-B1* (**e**), *EcR-B2* (**f**), or *usp* (**g**). A triple homozygous line (*UAS-mCD8GFP, crz-gal4; UAS-mi-babo-A*) was crossed to indicated UAS lines. **h** EcR-B1-IR in the wild-type CNS of WL3 (n = 14). **i** Detection of overexpressed EcR-B1 in the vCrz neurons was indicated in a bracket. EcR-B1-IR in a cluster of protocerebral Crz neurons was also indicated by arrowheads (n = 16, Genotype: *UAS-mCD8GFP*, *crz-gal4*/+; *UAS-mi-babo-A*/+; *UAS-EcR-B1*/+). **j** The vCrz neurons located in a box (**i**) were double-labeled for mCD8GFP (green) and EcR-B1-IR (red). Higher levels of EcR-B1 were detected in the nuclei of vCrz neurons than in neighboring cells. Scale bars, 100 μm (**a**, **d**, **h**), 50 μm (**j**) (Color figure online)

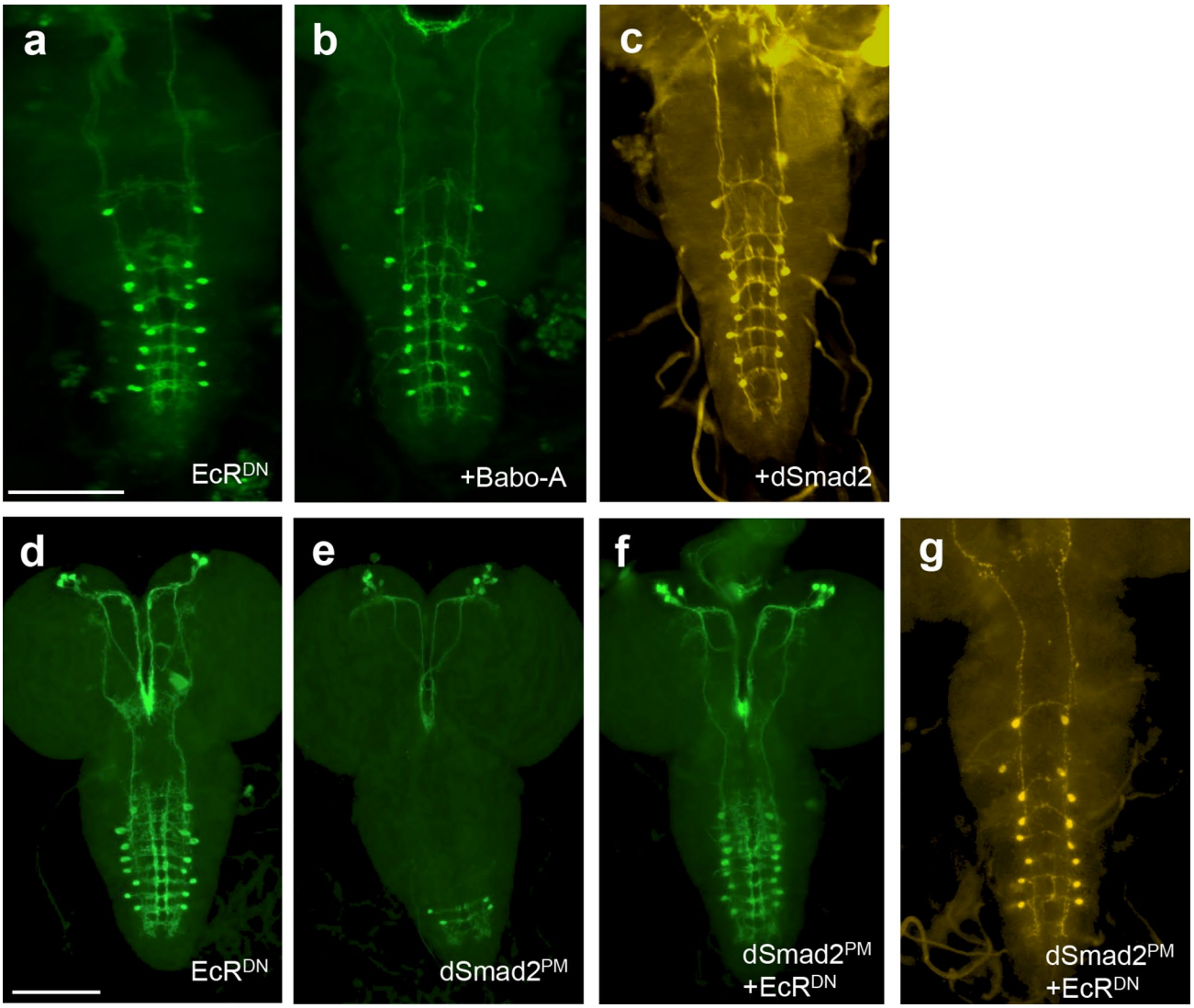

Fig. 11 Rescue of EcR^{DN}-mediated PCD defect. Triple homozygous line (*crz-gal4*, *UAS-mCD8GFP*; *UAS-EcR*^{DN}) was crossed to indicated UAS lines. Resulting progeny were dissected either at WL3 stage or 6–8 h APF to examine vCrz neurons labeled with mCD8GFP or Crz-IR. **a–c** EcR^{DN}-mediated PCD defect (n = 14, **a**) was not rescued by co-expression of *babo-A* (n = 8, **b**) or *dSmad2* (n = 10, **c**) at 6–8 h APF. **d** vCrz neurons carrying EcR^{DN} expression at WL3 stage (n = 8). **e** Premature killing of larval vCrz neurons by *dSmad2*^{PM} at WL3 stage (n = 16). **f**, **g** EcR^{DN} expression blocked $dSmad2^{PM}$-mediated killing at WL3 stage (n > 16, **f**) and at 6–8 h APF (n = 12, **g**). Scale bar, 100 μm

are only two type-II receptors, Punt and Wit, in *Drosophila*. Cell-based assays showed that both Myo and its mammalian relative Myostatin bind to the Babo-Wit (type I-type II) receptor complex [50]. However, our attempts using mutations or knockdown of *wit* and *punt* individually or together did not produce any significant effect on vCrz PCD, although similar genetic assays showed redundant function of *wit* and *punt* for axon pruning of MB γ neurons [22]. One possible explanation is that different types of neurons might have different sensitivities to the threshold levels of type-II receptors. Thus, residual or leaky expression of the receptors in double mutants or knockdown is sufficient to mediate Myo signaling in vCrz neurons. In line with this, it is notable that TGF-β ligands bind sequentially to the receptors; first to high affinity type-II receptors, and then to the recruited low affinity type-I receptors [51]. Because of the high affinity, a threshold level of the type-II receptors is expected to be much lower than that of the type-I receptors.

We also showed that Plum, a member of IgSF, is required for PCD of vCrz neurons. It was first identified as an important TGF-β signaling factor for the axon pruning of MB γ neurons [38]. Although Plum's biochemical function is little known, genetic studies done by Yu et al. [38] proposed that Plum facilitates Myo binding and stabilizes type-I/II

receptor complex, which functions are similar to the type-III receptors (a.k.a. co-receptors) as described in mammals [52].

Relationship between TGF-β and EcR signaling

For the pruning event of MB γ neurons, TGF-β signaling acts epistatically to EcR by upregulating EcR-B1 expression [22]. In contrast, we did not find evidence supporting the hierarchical relationship between EcR and TGF-β signaling pathways for the vCrz neuronal apoptosis. An alternative possibility is that the two signaling pathways act 'cooperatively' to induce cell death pathway in the vCrz neurons. In this model, both signaling components must be present and act together otherwise PCD cannot be induced. A very important clue supporting this model comes from the results showing that dSmad2^{PM}-mediated premature death is completely abolished by coexpression of EcR^{DN} (Fig. 11). In other words, in the absence of EcR function, activated TGF-β signaling alone cannot promote the cell death program, and *vice versa*. The molecular mechanism of how these two transcriptional regulators (EcR and dSmad2) cooperate is a subject of future research. Because transcriptional induction of *grim* is likely a rate-limiting step toward the activation of caspases in the PCD of vCrz [14, 16], we speculate that the cooperation of EcR and dSmad2, either directly or indirectly, leads to eventual activation of *grim* expression.

Dual roles of glia for PCD of vCrz neurons

Completion of the apoptotic cell death requires three consecutive steps: initiation of the death program, execution of cellular degeneration by caspases, and clearance of the apoptotic bodies. In the CNS, it has been well documented that glial cells play critical roles in the clearance of neuronal projections derived from either pruning or apoptosis [48, 53]. However, the roles of glia as a 'direct inducer of apoptosis' has not been explored until our studies. For the vCrz PCD, Myo derived from either cortex or astrocyte-like glia subtype acts redundantly to induce the cell death, as *myo* knockdown in either glia subtype alone did not block PCD of vCrz neurons but pan-glial *myo* knockdown did so. This is also the case for the MB γ axon pruning [22]. In addition to the production of Myo, astrocyte-like glia was known to play a key role in clearing of apoptosed vCrz neuronal projections [48]. Interestingly, autonomous EcR signaling is required for glia to produce Myo as well as to remove apoptosed projections. However, the molecular mechanisms underlying this signaling appear quite different between these two glial activities (Fig. 12). While panglial EcR^{DN} expression completely blocked vCrz PCD, likely due to the lack of Myo production, panglial knockdown of *usp* did not

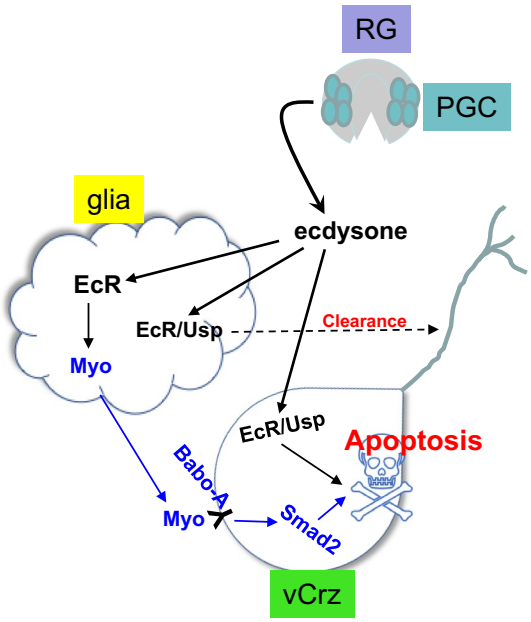

Fig. 12 A diagram illustrating coordinated action of the three different cell types for timely vCrz PCD. (PGC, prothoracic gland cells; RG, ring gland). Global metamorphic signal, ecdysone hormone, produced from the PGCs activates local EcR signaling cascades in a cell-specific manner. In glia (astrocyte-like and cortex glia subtypes) EcR, independently of Usp, leads to Myo production. Secreted Myo binding to its cognate receptor Babo-A in the vCrz neurons transduces TGF-β signaling pathway activating dSmad2. In the vCrz neurons, activated EcR signaling (mediated through EcR:Usp complex) acts cooperatively with dSmad2 to initiate PCD. Astrocyte-like glial cells also remove neuronal projections of apoptosed vCrz neurons (broken arrow), and this activity requires EcR:Usp complex

do so, implying that Usp function is dispensable for promoting Myo expression. In contrast, phagocytic activities of the astrocyte-like glial cells autonomously require both EcR and Usp functions, since the lack of either one resulted in defective clearance of the neural projections. In both cases, cell bodies of the vCrz neurons are normally removed, indicating that the clearance by astrocyte-like glial cells is specific to neural projections, which is consistent with previous observation [48]. These findings provide an interesting case in which the same receptor molecule elicits multiple responses in the same cell via differential modes of signal transduction.

Regulating PCD timing of vCrz neurons

Distinct groups of larval neurons undergo PCD at different metamorphic phases in response to ecdysone signaling. Since we expect that all CNS neurons are simultaneously exposed to changes in the ecdysone titer during development, how the doomed larval neurons select their time of death has been a mystery. Our studies on the vCrz PCD provide a hint. Initiation of PCD in vCrz neurons requires two distinct signaling molecules produced from different

tissues; ecdysone from PGC and Myo from glia. The most critical matter for determining the timing of vCrz neuronal PCD is sequential arrival of these two ligands in order. The globally acting ecdysone surge at the end of larval stage serves as an initial cue by activating local (or cell-specific) EcR signalings that carry out different outcomes depending on the cell type or stage. In cortex and astrocyte-like glial cells, the EcR signaling stimulates Myo production, which subsequently transacts to trigger TGF-β signaling pathway in vCrz neurons. The activated TGF-β signaling, cooperatively with activated EcR-Usp, initiates cell death cascade in the vCrz neurons (summarized in Fig. 12). In this sense, Myo becomes the second time cue that works regionally as a secondary ripple generated by global ecdysone surge. Therefore, it is likely that the EcR signaling sets a booby trap but it is the TGF-β signal that pulls a trigger to ignite the apoptotic program. Our studies demonstrate that a seemingly simple PCD event of vCrz neurons is an outcome of intricate coordination of multiple signaling tissues. Such coordinated efforts seem to be a key in order to remove vCrz neurons timely as well as rapidly, otherwise defective (premature, delayed, or deficient) PCD of neuronal cells could be detrimental to the accurate construction of adult-specific neural circuits in a rapidly metamorphosing CNS and thus compromises neural functions for adult life. Considering a wide diversity of doomed neuronal cell types in the metamorphosing CNS, we predict that other types of extracellular signals work cooperatively with EcR to carry out the apoptosis of these neurons at different metamorphic phases. It will also be interesting to see if similar two-factor specification of apoptosis works for the PCD of vertebrate neurons during pubertal development.

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