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Ultraspiracle-independent anti-apoptotic function of ecdysone receptors is required for the survival of larval peptidergic neurons via suppression of *grim* expression in *Drosophila melanogaster*

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Abstract

In *Drosophila melanogaster* a significant number of heterogenous larval neurons in the central nervous system (CNS) undergo metamorphosis-associated programmed cell death, termed metamorphoptosis. Interestingly distinct groups of doomed larval neurons are eliminated at different metamorphic phases. Although ecdysone hormonal signaling via nuclear ecdysone receptors (EcRs) is known to orchestrate the neuronal metamorphoptosis, little is known about how this signaling controls such diverse neuronal responses. Crustacean cardioactive peptide (CCAP)-producing neurons in the ventral nerve cord (VNC) are developmentally programmed to die shortly after adult emergence. In this study, we show that disruption of endogenous EcR function by ectopic expression of dominant negative forms of EcRs (EcR^{DN}) causes premature death of larval CCAP neurons in a caspase-dependent manner. This event is rescued by co-expression of individual EcR isoforms. Furthermore, larval CCAP neurons are largely normal in *ecr* mutants lacking either EcR-A or EcR-B isoforms, suggesting that EcR isoforms redundantly function to protect larval CCAP neurons. Of surprise, a role of Ultraspiracle (Usp), a canonical partner of EcR, is dispensable in the protection of CCAP neurons, whereas both EcR and Usp are required for inducing metamorphoptosis of vCrz neurons shortly after prepupal formation. As a downstream, *grim* is an essential cell death gene for the EcR^{DN}-mediated CCAP neuronal death, while either *hid* or *rpr* function is dispensable. Together, our results suggest that Usp-independent EcR actions protect CCAP neurons from their premature death by repressing *grim* expression until their normally scheduled apoptosis at post-emergence. Our studies highlight two opposite roles played by EcR function for metamorphoptosis of two different peptidergic neuronal groups, proapoptotic (vCrz) vs. antiapoptotic (CCAP), and propose that distinct death timings of doomed larval neurons are determined by differential signaling mechanisms involving EcR.

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Keywords

metamorphoptosis; ultraspiracle; ecdysone receptor; central nervous system; peptidergic neurons; grim; apoptosis

Introduction

Toward the end of growth period, juvenile animals undergo significant changes in the body as well as in the CNS in order to accommodate adult life style that is different from juvenile one. Most dramatic metamorphic changes are seen in holometabolous insects and amphibians in which obsolete juvenile tissues and organs are degenerated in conjunction with *de novo* formation of adult ones. It is known that endocrine hormones drive such developmental reprogramming and underlying mechanisms of hormonal actions have been a subject of extensive researches [1, 2].

In holometabolous insects including the fruit fly *Drosophila melanogaster*, metamorphosis is primarily orchestrated by a steroid hormone, ecdysone. In *Drosophila*, consecutive pulses of ecdysone at the end of third instar larval and prepupal stages induce massive cell death of larval organs [2]. This metamorphosis-regulated programmed cell death (PCD) has been referred to as *metamorphoptosis* in order to distinguish it from PCD events occurring during embryonic development that do not involve ecdysone signaling [3]. In contrast to the annihilation of larval organs, the larval CNS continuously functions throughout metamorphosis; however, it is sculptured extensively to form adult CNS. At the cellular level, the larval neurons are subjected to two different fates. Persisting neurons undergo remodeling process that sheds larval-specific neural projections and acquires adult-specific ones, while unnecessary neurons are programmed to die mostly via apoptosis. However, PCD of doomed larval neurons initiates at different phases of metamorphosis depending on the neuronal types [4–8], suggesting that heterogeneous molecular death mechanisms operate in distinct neuronal groups. Little is known how ecdysone signaling induces such diverse responses of doomed larval neurons.

In order to assist future investigations on the upstream molecular events that trigger apoptosis in a metamorphic phase-specific manner, we propose to distinguish the doomed larval neurons largely into three classes based on the time of their death. Death class-I includes the neurons dying shortly after puparium formation (APF) that is driven by late larval ecdysone pulse. This class is represented by eight pairs of ventral corazonin neurons (vCrz) [3, 9–11], eleven pairs of CEv motoneurons located in the ventral median area of thoracic and abdominal segments, and two pairs of CE_d neurons in the second and third thoracic segments [3]. Death class-II encompasses the doomed neurons in which their death is initiated by a rather small ecdysone peak that leads to pupal formation approximately at 12 h APF. A group of RP2 motoneurons located in the dorsal median area of the VNC represents this class [12]. Death class-III includes the larval neurons that survive throughout metamorphosis and die shortly after adult eclosion. A group of ~300 neurons, previously dubbed as type-II neurons, belongs to this class [13]. These neurons are characterized by a high level of immunoreactivities specific to ecdysone receptor A isoform (EcR-A)

throughout pupal development. Majority of these neurons undergo PCD within 24 h after adult emergence [13]. Another group of neurons producing CCAP neuropeptide is also subject to postemergence PCD in a manner similar to the type-II neurons; however, these neurons show weak or moderate EcR-A immunoreactivities, and thus they are branded as type-I neurons [13–15].

It has been shown that PCD of vCrz neurons and RP2 motoneurons, each representing death class-I and -II neurons, respectively, is triggered by ecdysone signaling and EcR-B isoforms are essential to activate downstream apoptotic pathway [9, 12]. By contrast, PCD of the death class-III neurons is triggered by the reduction of ecdysone titers below the threshold, which results from the degeneration of the prothoracic glands, a source of ecdysone production [13, 16, 17]. When ecdysone titers are maintained high by an injection of ecdysone within 30 min after eclosion, PCD of both type-II and CCAP neurons is aborted along with transcriptional suppression of two major death promoting genes, *reaper* (*rpr*) and *grim* [14, 17]. These data suggest that ecdysone signaling protects the death class-III neurons via suppression of these death genes. However later studies showed that *grim* plays a major role for the PCD of CCAP neurons [15].

CCAP neurons provide an excellent model system to comprehend the protective role of ecdysone signaling for the death class-III type, as they are readily identifiable using various histological tools as well as malleable to transgenic manipulations [18, 19]. Intriguingly, we found that precocious death of CCAP neurons is brought about by ectopic expression of dominant-negative form of EcR (EcR^{DN}) that disrupts endogenous EcR function. We further delved into the molecular mechanisms underlying EcR-mediated protection of larval CCAP neurons and differences between antiapoptotic and proapoptotic roles played by EcR signaling.

Materials and methods

Fly Stocks

Flies were raised at 25°C on cornmeal-yeast-agar medium. Canton-S was used as a wild type strain and either *yellow white* (*y w*) or *w¹¹¹⁸* as genetic controls. Three *gal4* drivers, *ccap-gal4* [18], *bursicon-gal4* [15], and *crz-gal4* [9, 20], were used to express various *UAS-responders* in CCAP and Crz neurons. The following homozygous or *trans*-heterozygous mutant alleles were used; *ecr³¹/ecr⁹⁹* (*ecr^{31/99}*) for *ecrB* [21], *ecr¹¹²/ecr^{M554fs}* (*ecr^{112/M554fs}*) and *ecr¹³⁹/ecr^{M554fs}* (*ecr^{139/M554fs}*) for *ecrA* [22, 23], *X14/hid^{P05014}* (*X14/hid^P*) for *hid* [24] and *rpr⁸⁷* for *reaper* (*rpr*) [25]. To simultaneously knockdown *rpr*, *hid*, and *grim*, a *UAS-miRGH* line was used. This line carries micro RNA-based hairpin constructs targeting the three cell death genes simultaneously [11, 26]. Similarly, *UAS-migram* [11] and *UAS-miusp* [27] were used for the silencing of *grim* and *ultraspiracle* (*usp*), respectively. Two *UAS-EcR^{DN}* lines were described in Cherbas et al. [28]. To generate vCrz neurons carrying homozygous *usp³* mutation by MARCM technique [29], two recombinant fly lines were crossed: (1) *FRT^{19A}, tubP-gal80, hs-FLP; crz-gal4, UAS-mCD8GFP*, (2) *FRT^{19A}, usp³/FM7*. Embryos collected from 0–8 h after egg laying were heat-shocked for 1 h at 37°C to induce mitotic recombination and then incubated at 25°C.

Construction of *UAS-Usp³*

To generate *UAS-Usp³* construct, genomic DNA was extracted from *usp³* heterozygous mutant. The *usp³* allele carries a point mutation, substituting a conserved Arg160 for His (R160H) at the base of the second zinc finger domain, which disables *Usp³* to bind DNA [30]. PCR was employed to obtain 1657-bp-long *usp* genomic region (−43 to +1614 relative to translation start site +1) by using Fwd (ACCggtaccTCCAATATACCCA, KpnI site in lower-case) and Rev (AGGGAtctagaGGAGAAATGC, XbaI site in lower-case) primers. The PCR products were subcloned into pGEMTeasy vector, and then a recombinant plasmid clone carrying *usp³* allele was confirmed by sequencing analysis (pGEMT-*Usp³*). Because of the methylation of XbaI site, the pGEMT-*Usp³* was amplified in *dam*-negative JM110 *E. coli* cells. The purified plasmid was digested with KpnI and XbaI, and then the *Usp³* fragment was inserted into the pUAST vector. The resulting *UAS-Usp³* was used for germ-line transformation. Two independent lines each inserted in II and III chromosome were used in this study.

Histochemistry of whole-mount CNS

To detect *LacZ* expression in specific neurons, CNSs were dissected in PBS containing 0.2% Triton X-100 and 10% ethanol, and then fixed in 0.5-mL of 0.2% glutaraldehyde for 5–10 min. Following washing in PBS, the tissues were incubated in 300 µL of X-gal solution (final 2 mg/mL) for overnight at 37°C. The tissues were rinsed in PBS, dehydrated in ethanol and mounted in 90% glycerol. Whole-mount bursicon and Corazonin immunohistochemistry (antibursicon was kindly provided by Dr. Ben White, NIMH) was performed, as described previously [15]. To assess co-localization of EcR isoforms in CCAP neurons, *mCD8GFP*-reported CCAP neurons were co-labeled with mouse monoclonal anti-EcR-A, anti-EcR-B1, or anti-EcR-COM [31]. Fluorescent images were obtained with Olympus BX61 or Keyence BZ-X710 microscopes.

Results

Premature death of CCAP neurons induced by EcR^{DN}

Three subgroups of CCAP neurons located in the subesophageal (sCCAP), dorsal abdominal (dCCAP), and ventral thoracic (vCCAP) regions of the CNS are programmed to undergo apoptosis within 24 h after adult emergence [14, 15]. Because the apoptosis of these neurons is coincident with lowest ecdysone titers and it is prevented by administration of ecdysone immediately after adult eclosion, it has been postulated that ecdysone signaling through EcR-A isoform provides these neurons with a protective measure against premature PCD during pupal development when EcR-A expression is prevalent.

To delve into the molecular mechanisms underlying EcR-A isoform-mediated protection, we attempted to disrupt EcR function in the CCAP neurons via expression of dominant-negative forms of EcRs (*EcR^{DN}*), *EcR^{W650A}* and *EcR^{F645A}*, by using a *ccap-gal4* driver. Expression of *EcR^{DN}* caused severely defective pupal development, producing a failure in head eversion and shortened adult appendages such as wings and legs (Fig. 1a-c). Severity of the pupal defects was greater with *EcR^{W650A}* than with *EcR^{F645A}* (n=50 for both, Fig. 1b, c).

Because the defective pupal phenotypes by *EcR^{DN}* expression are comparable to those of flies having CCAP neurons either functionally inactivated [19, 32] or prematurely ablated during larval growth [18, 33], we carried out histological analysis of larval CCAP neurons in the wandering third instar larval stage. In control (*ccap>lacZ*), CCAP neuronal somata were shown in the protocerebral and subesophageal regions of the brain and in both thoracic and abdominal ganglia of the ventral nerve cord (VNC) (Fig. 1d). However, in *EcR^{W650A}*-expressing larvae nearly all of CCAP neurons in both subesophageal and VNC regions disappeared except for a pair of CCAP neurons in each brain hemisphere that seemed largely normal (n=16, Fig. 1e). *EcR^{F645A}* expression produced similar pattern, except for a few and weakly stained somata remained in the VNC (n=16, Fig. 1f). Nonetheless, these results indicate that the disappearance of the CCAP neurons by *EcR^{DN}* expression was specific to those that normally undergo apoptosis after adult eclosion.

Undetectable CCAP neurons in response to *EcR^{DN}* expression could be a result from transcriptional repression of *ccap* gene or ablation of CCAP neurons. To test if the latter is the case, we employed p35, a virally originated pan-caspase inhibitor. As a result, co-expression of *p35* completely prevented *EcR^{DN}*-mediated defective pupal development (n=150, Fig. 1g, h). Consistent with the developmental rescue, normal patterns of larval CCAP neurons were restored (n=20, Fig. 1i, j). These data support that the loss of EcR function causes the precocious death of CCAP neurons in a caspase-dependent manner. Therefore, we suggest that EcR function is required to suppress a premature onset of death program in larval CCAP neurons which function is required for normal pupal development.

Developmental timing of *EcR^{DN}*-mediated apoptosis

To determine precise developmental timing of *EcR^{DN}*-induced apoptosis of CCAP neurons, we performed time-course detection of CCAP neurons labeled with GFP-reporter. To do this, a double transgenic line bearing both *ccap-gal4* and *UAS-mCD8GFP* was generated and crossed it to the *UAS-EcR^{W650A}*. Notably, CCAP neurons looked normal in the first instar (L1) and early 2nd instar (L2) larvae (n>6 for L1 and L2, Fig. 2a, b, ai), but we found a few neurons missing in more mature L2 larvae (n=5, Fig. 2c, ci). In early feeding 3rd instar (FL3), more neurons became undetectable and nearly all disappeared in later wandering larvae (WL3) (n>7 for FL3 and WL3, Fig. 2d-g). Since GFP expression appeared clearly visible in L1 stage (Fig. 2a, ai), the survival of CCAP neurons in such early larvae is unlikely due to the lack of *ccap-gal4* activity. A likely possibility is that *EcR^{DN}* has not been accumulated sufficiently to counteract wild-type EcR function at this stage.

Redundant roles of EcR isoforms for the protection of CCAP neuron death

The *ecr* locus produces three EcR isoforms, EcR-A, B1 and B2, varying in N-termini but bearing identical DNA and ligand binding domains. Previous studies with isoform-specific mutations and expression suggested differential roles played by each isoform in a stage- and tissue-specific manner during post-embryonic development [21–23, 31, 34]. In general, EcR-B1 isoform is predominant in late larval organs, whereas EcR-A is prevalent in adult progenitor cells [31].

To gain insight into the roles of each isoform for the protection of CCAP neurons, EcR isoforms were detected in the larval CCAP neurons by isoform-specific immunohistochemistry. Consistent with previous report [34], we detected weak expression of EcR-A, but conspicuous EcR-B1 expression broadly in the larval VNC (Fig. 3a, b). Immunoreactivity with anti-EcR-com, which detects all isoforms, was stronger than that of either EcR-B1 or EcR-A, implying a presence of EcR-B2 (Fig. 3c). Co-labeling experiments indicate that both EcR isoforms are present in the CCAP neurons (Fig. 3d-f). These results raise the possibility of protective roles played by all three isoforms in the larval CCAP neurons.

Next, we tested if co-expression of each EcR isoform is able to rescue *EcR^{W650A}*-induced CCAP neuron death. Expression of either *EcR-B1* or *EcR-B2* fully rescued the death (Fig. 4a-c), whereas *EcR-A* did so partially (Fig. 4d). Consistent with the neuronal rescue phenotypes, normal pupal phenotype was fully restored by either *EcR-B1* or *EcR-B2* isoform but to a lesser extent by *EcR-A* (Fig. 4e-g).

To further verify the roles of EcR isoforms for the protection of CCAP neurons, we employed genetic mutations lacking either *EcR-A* or *EcR-B* isoforms. Since a majority of CCAP neurons co-expresses another neuropeptide bursicon (burs), we visualized them by using anti-burs [15, 19]. In two of heteroallelic mutations lacking *EcR-A* isoform expression, *ecr^{112/M554fs}* and *ecr^{139/M554fs}*, burs-immunoreactivities were comparable to wild-type, implying that CCAP neurons were protected by EcR-B isoforms (n>6, Fig. 5a-c). In *ecr^{31/99}* mutants lacking both *EcR-B1* and *EcR-B2* isoforms, we observed one or two burs-immunoreactive neurons missing particularly in the thoracic segments of some specimen (n=7, bracket in Fig. 5d), suggesting that CCAP neurons were also mostly protected by EcR-A. Taking these results together supports that all three EcR isoforms redundantly function to maintain larval CCAP neurons alive, but EcR-B isoforms play slightly more prominent roles than does EcR-A particularly during larval stages.

EcR-B levels are higher than EcR-A in the CNS during larval development. However, during metamorphosis, EcR-B decreases precipitously while EcR-A gradually increases, thus becoming a dominant isoform in pupal CNS [34]. Since CCAP neurons are immunoreactive only with anti-EcR-A in later pupal stage [14], EcR-A is likely the main protector of CCAP neurons at this stage. If so, it is expected that CCAP neurons might be prematurely eliminated in *ecrA* mutants during pupal development. To test this, we examined pupal CCAP neurons in *ecrA* mutants. However, this is a challenge, as both *ecrA* mutants (*112/M554fs* and *139/M554fs*) died mostly during larval stages and rare escapers were developmentally arrested and died in prepupal and early pupal stages, respectively. We managed to obtain a small number of pupal escapers from both mutant alleles for the immunohistochemical analysis.

During early pupal development, numbers of burs-immunoreactive CCAP neurons are reduced but sizes of somata increase in wild-type [35], because of transcriptional silence of *burs* in about a half of larval neurons in the VNC. Consistent with this report, we observed about seven pairs of strong burs-immunoreactive CCAP neurons in the abdominal ganglia of control (+/*M554fs*) at 24–48 h APF (n=14, Fig. 5e). Interestingly both *112/M554fs* (n=5)

and *139/M554fs* (n=3) mutants showed more neurons compared to the control and some of these neurons retained larval features as judging from the small soma size and larva-like projections (Fig. 5f-i). These suggest that transcriptional regulation of *burs* expression is somehow altered in the *ecrA* mutants. Because of this factor, it was difficult to recognize dead burs neurons. Nonetheless, we observed one or two missing pupal neurons in *139/M554fs* mutant VNC, as indicated by arrows (Fig. 5h-i). We speculate that residual EcR-B is still capable of blocking premature PCD of CCAP neurons to some extent at this stage. The results, however, need to be interpreted cautiously, because it is possible that the missing pupal neurons in the *ecrA* mutant could be a result from the systemic effect of developmental arrest.

Ultraspiracle (Usp) is required for the normal apoptosis of vCrz, but not for the protection of CCAP neurons

Since EcR is known to form a functional heterodimeric complex with another nuclear receptor Usp, a fly ortholog of vertebrate retinoid X receptor (RXR) [36, 37], we wanted to determine if the EcR-Usp complex provides CCAP neurons with antiapoptotic function. Because EcR is known to be essential for PCD of vCrz peptidergic neurons [9], we first investigated if Usp is required for this event.

As we reported previously, all vCrz neurons died apoptotically by 6–7 h APF and their death was completely suppressed by *EcR^{DN}* expression (Fig. 6a-c). Because of embryonic and L1 lethality of the *usp*-null mutants [38], we employed microRNA-based knockdown of *usp* using a pre-existing *UAS-miusp* line [27]. Remarkably, targeted expression of *miusp* in Crz neurons completely blocked vCrz apoptosis as effectively as *EcR^{DN}* did (Fig. 6c, d).

To further corroborate a Usp's autonomous role, we performed mosaic analysis (MARCM), as described in Lee et al. [29]. vCrz neurons bearing homozygous *usp³* mutation, as detected by GFP expression, showed normal-looking appearance in both cell body and neural projections at 6 h APF, indicating that PCD of these vCrz neurons was blocked in the absence of Usp function (arrows in Fig. 6e, n=5). A few vCrz neurons that are weakly positive for corazonin immunoreactivity but GFP-negative in some specimen at this stage (Fig. 6f, g) are normally dying neurons having smaller cell bodies and degenerating axons, as we reported previously [10]. These data together strongly support that metamorphoptosis of vCrz neurons requires ecdysone signaling conveyed by a conventional EcR-Usp complex.

Next, we investigated if Usp is also essential for the survival of larval CCAP neurons. To our surprise, *miusp* expression did not induce premature killing of the CCAP neurons (Fig. 7a-c). This result can be due to insufficient reduction of endogenous *usp* expression in the CCAP neurons, although it is unlikely based on the *miusp* results shown for the vCrz neurons. Anyway, we designed a putatively dominant negative form of Usp as an alternative way to interfere with endogenous Usp function like EcR^{DN}. A candidate is Usp³ mutant protein that carries R160H substitution in the second zinc finger motif [30]. This study has shown that Usp³ can compete effectively with wild-type one for the dimerization with EcR, but the EcR-Usp³ complex fails to bind its target DNA binding sites.

To test *Usp³*'s dominant negative action *in vivo*, we generated *UAS-Usp³* transgenic lines, and then the results from ectopic *Usp³* expression driven by various tissue-specific *gal4* drivers were compared to those from *miusp* and *EcR^{DN}* expression. Ubiquitous expression of *miusp* or *Usp³* using a *tub-gal4* caused embryonic lethality (Table 1) and similar results were found for *EcR^{W650A}* as reported previously [28]. Expression of *miusp* or *Usp³* in the fat body using *r4-gal4* driver [39] similarly caused pupal lethality, whereas *EcR^{W650A}* severely retarded larval growth leading to larval death at L1-L2 stage (Table 1). Expression by *A9-gal4* [40] also showed different effects between *EcR^{DN}* and *Usp³* expression on larval and pupal development (supplemental Fig. 1). *EcR^{W650A}* caused lethality broadly ranging from early larvae to pupae. Dying larvae frequently showed melanotic spots in the digestive system; pupal development was severely compromised and all pupae failed to reach adulthood (supplemental Fig. 1a-e). By comparison, both *Usp³* and *miusp* expression similarly led to the developmental arrest at late pupal stage and majority of pupae died inside the pupal case (Table 1, supplemental Fig. 1f). Comparable developmental arrest phenotypes produced by ectopic *Usp³* and *miusp* expression in various *gal4* drivers suggest that *Usp³* mutant form effectively competes with wild-type *Usp* protein to disrupt normal *Usp* functions. Because wild-type *EcR* and *Usp* expression patterns do not completely overlap in some tissues (modENCODE tissue expression data; <http://flybase.org/reports/FB1c0000206.html>), different effects by *EcR^{DN}* and *Usp³* (and *miusp*) on the development suggest that some *EcR* functions are independent of *Usp*, or *vice versa*.

In addition to the gross developmental effects, we tested if *Usp³* expression suppresses the metamorphosis of vCrz neurons. As expected, targeted expression of *Usp³* in the Crz neurons completely inhibited vCrz cell death (n=12, Fig. 6h), which is consistent with the results observed from *miusp*-mediated knockdown. These results verify that *Usp³* is capable of disrupting *EcR-Usp* function for PCD of vCrz neurons by acting as a dominant negative form. In contrast, *Usp³* did not cause premature death of larval CCAP neurons (Fig. 7d, e), which was consistent with *miusp* expression. Based on these data, we conclude that the *EcR* signaling plays a role, independently of *Usp*, for the prevention of premature onset of CCAP apoptosis.

***grim* is the cell death gene mediating *EcR^{DN}*-induced CCAP-neuron death**

In *Drosophila* cells, a death-inducing signal promotes transcriptional activation of a single or a combination of cell death genes *rpr*, *grim*, and *head convolution defective (hid)*, commonly referred to as *RGH*. These death promoters antagonize endogenous caspase inhibitor Diap1, thereby leading to the activation of caspases [41]. Since *EcR^{DN}*-mediated CCAP-neuronal death also involves caspase activation (Fig. 1), it is conceivable that disruption of *EcR* signaling by *EcR^{DN}* triggers expression of the cell death genes. The *RGH* genes have been shown to work in a cell-type specific manner [e.g., ref. 2], prompting us to identify specific death genes responsible for the *EcR^{DN}*-induced CCAP death.

We first employed a *UAS-miRGH* that knocks down *rpr*, *grim*, and *hid* simultaneously [11, 26]. As a result, we found that *EcR^{W650A}*-induced death was rescued effectively by *miRGH* (n=8, Fig. 8a, b). Furthermore, knockdown of *grim* alone by *migram* [11] rescued CCAP death as comparably as *miRGH* did (n=12, Fig. 8c). In contrast, no roles were found for *hid*

and *rpr*, as the death was not rescued in either *hid*-null mutants (*hid^P/X14*) or *rpr*-null homozygous mutants (*rpr⁸⁷*) (n>7 for both, Fig. 8d, e). These findings suggest that *grim* is the major cell death gene whose expression is suppressed by EcR-mediated function in larval CCAP neurons.

Since Grim is known to antagonize Diap1 to activate caspases, we further tested if Diap1 also plays an important role for the protection of larval CCAP neurons. To do this, we used a *burs-gal4* to co-express *Diap1* and *EcR^{W650A}* because of the convenience of transgenic manipulations. *burs-gal4* activity is limited to one pair of subesophageal and abdominal CCAP neurons in the larval CNS (n=14, Fig. 8f). Expression of *EcR^{W650A}* alone prematurely eliminated most *burs*-expressing CCAP neurons (n=12, Fig. 8g), whereas *Diap1* overexpression blocked it in many neurons (n=7, Fig. 8h). These results suggest that *grim* acts as an upstream death inducer required for the activation of caspases by antagonizing Diap1.

Discussion

The final steps in insect metamorphosis include adult emergence, expansion of the adult wings, and cuticular hardening and darkening. CCAP neurons play important roles for these events by producing multiple neuropeptides and a neuropeptide receptor [42–44]. Then as soon as their roles are complete, these neurons undergo apoptosis. Although CCAP neurons in the subesophageal brain area are also programmed to die, two particular CCAP subgroups, ventral CCAP and dorsal CCAP (a.k.a. *bursCCAP*), are relatively well studied with respect to their apoptotic events [3, 14, 15]. Falling ecdysone titers toward the end of pupal development [13, 14, 17, 45] are considered to be a key developmental cue inducing the PCD of both CCAP and type-II neurons. It was initially thought that changes of ecdysone titers *per se* trigger the PCD event, but further studies suggest that it is initiated when insufficient ecdysone signaling fails to repress transcription of the death promoting genes, thus implying ecdysone signaling acting as a protective agent [14, 17]. Since EcR-A is the only detectable isoform in type-II and CCAP neurons in pharate adults, this isoform has been suggested to repress cell death genes [13, 17].

In this study, we further pursued anti-apoptotic aspects (protection theory) of ecdysone signaling by disrupting EcR function in CCAP neurons. From these experiments, we anticipated premature induction of CCAP neuronal death during pupal development when EcR-A levels are moderate. The expression of *EcR^{DN}* indeed led to the premature death; surprisingly however, it took place much earlier during larval development. In addition, the results from rescue experiments and isoform-specific mutants suggested that not only EcR-A but also both EcR-B isoforms protect CCAP neurons in a redundant manner. Since CCAP neuronal functions are required for larval molting, pupal development, and pupal ecdysis in addition to adult eclosion and maturation [18, 44], the functional redundancy of EcR isoforms seems important in order to provide continuous protection for CCAP neurons from larval development and onward.

Previous studies found expression of *grim* and *rpr* in dying CCAP neurons and n4 type-II neurons [14, 17], implying important roles of both genes for the PCD of these neurons.

However, data from gene-specific mutations or knockdown of individual death genes have clearly demonstrated that it is not *rpr* but *grim* that is primarily responsible for post-emergence PCD of CCAP neurons [15]. As shown here, *grim* is also a key player for inducing premature death of larval CCAP neurons by *EcR^{DN}*. Therefore, we suggest that *EcR^{DN}*-mediated premature death of CCAP neurons mirrors postemergence PCD of them and premature onset of CCAP apoptosis is prevented by continuous suppression of *grim* expression by redundant function of EcR isoforms either directly or indirectly. Interestingly, *grim* is also the major death gene responsible for the metamorphoptosis of another doomed larval peptidergic neuronal group, vCrz neurons [11]. For this event, however, *grim* expression is induced by EcR signaling.

How does the same ecdysone signaling induce *grim* expression in one type of larval neurons but represses it in other type? Our studies provide an insight into the differential molecular mechanisms of EcR action. One major difference is a role played by Usp. Metamorphoptosis of vCrz neurons is blocked by either *Usp³* or *miusp* expression as well as *EcR^{DN}*, indicating that Usp is an essential component for the vCrz death as a canonical EcR-Usp complex. In contrast, Usp function appears to be dispensable for the protection of larval CCAP neurons, as disruption by a dominant-negative *Usp³* did not cause CCAP death. One might argue that EcR-Usp³ complex is still capable of repressing *grim* expression because DNA binding domain in Usp is not needed for such negative action. However, similar results obtained from a *miusp*-mediated knockdown support that Usp is not an essential component for the EcR's repressive function. Hence, we propose that EcR acts independently of Usp to render protection for larval CCAP neurons and perhaps type-II larval neurons that similarly die after adult eclosion. One possible mechanism of Usp-independent function of EcR is that EcR functions with an unknown partner. Having said this, Usp was reported to form a dimer with DHR38 other than EcR [46, 47]. Additional difference between pro- and anti-apoptotic roles of EcR signaling comes from a differential use of EcR isoforms. While only EcR-B isoforms are necessary for vCrz PCD [9], all three EcR isoforms are redundantly required for anti-apoptotic function in larval CCAP neurons. EcR-A might provide an additional layer of protection to maintain the CCAP neurons alive in larvae. And then, because EcR-A is only detectable isoform during later pupal development [13], its role is likely to protect CCAP neurons in the pupal CNS until their destined demise at post-eclosion. Therefore the antiapoptotic role of ecdysone/EcR signaling plays an important role in determination of developmental PCD timing of CCAP neurons.

Lastly, our studies raise the possibility that accidental loss of steroid-mediated endocrine signaling can cause abnormal CNS development by altering fates of doomed postembryonic neurons, as exemplified by continued survival of vCrz neurons and premature death of CCAP neurons in *Drosophila*. In this respect, it is worthy to note that many artificial chemicals, collectively termed endocrine-disrupting chemicals (EDCs), mimic steroid hormones. In vertebrates, these chemicals are well known to disrupt development and reproduction by interfering with endogenous steroid hormones. Neurons in the brain are not immune to EDCs either. A growing body of evidence shows that steroid hormones play important roles in neuroprotection following brain injuries via various mechanisms including suppression of cell death [48]. Hence, such neuroprotective roles can be compromised by EDCs, as demonstrated by *in vitro* and *in vivo* studies [49–51].

Alternatively, EDCs can interfere with normal course of developmentally-regulated neuronal cell death. In the primary visual cortex, male rats have 19% more neurons than do females due to a greater degree of steroid-mediated cell death in the females [52, 53]. Sex-biased apoptosis also contributes to sexually dimorphic development of other brain areas [54]. Either premature degeneration or interference with developmentally regulated normal apoptosis can result in the formation of abnormal neural architectures and/or activities, which potentially cause various impairments of the neurological conditions such as defective cognition, abnormal behaviors or mental retardation. Further studies will shed more lights on the effects of EDCs on the steroid-associated neuronal apoptosis and neuroprotection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AE	After eclosion
APF	After puparium formation
CNS	Central nervous system
PCD	Programmed cell death
VNC	Ventral nerve cord
CCAP	Crustacean cardioactive peptide
Crz	Corazonin
EcR	Ecdysone Receptor
USP	Ultraspiracle

References

1. Ishizuya-Oka A, Hasebe T, Shi Y-B (2010) Apoptosis in amphibian organs during metamorphosis. *Apoptosis* 15:350–364 [PubMed: 20238476]
2. Yin VP, Thummel CS (2005) Mechanisms of steroid-triggered programmed cell death in *Drosophila*. *Sem Cell Dev Biol* 16:237–243
3. Lee G, Kim J, Kim Y, Yoo S, Park JH (2018) Identifying and monitoring neurons that undergo metamorphosis-regulated cell death (metamorphoptosis) by a neuron-specific caspase sensor (Casor) in *Drosophila melanogaster*. *Apoptosis* 23:41–53 [PubMed: 29224041]

4. Truman JW, Taylor BJ, Awad TA (1993) Formation of the adult nervous system. In *Development of Drosophila melanogaster* (ed. Bate M, Arias AM). pp. 1245–1275. Cold Spring Harbor Press, NY
5. Truman JW (1996) Metamorphosis of the insect nervous system. In *Metamorphosis: Postembryonic reprogramming of gene expression in Amphibian and insect cells* (ed. by Gilbert LI, Tata JR, Atkinson BG). pp.283–320. Academic Press.
6. Weeks JC (2003) Thinking globally, acting locally: steroid hormone regulation of the dendritic architecture, synaptic connectivity and death of an individual neuron. *Prog Neurobiol* 70:421–442 [PubMed: 14511700]
7. Yaniv SP, Schuldiner O (2016) A fly's view of neuronal remodeling. *WIREs Dev Biol* 5:618–635
8. Pinto-Teixeira F, Konstantinides N, Desplan C (2016) Programmed cell death acts at different stages of *Drosophila* neurodevelopment to shape the central nervous system. *FEBS Lett* 590:2435–2453. [PubMed: 27404003]
9. Choi Y-J, Lee G, Park JH (2006) Programmed cell death mechanisms of identifiable peptidergic neurons in *Drosophila melanogaster*. *Development* 133:2223–2232 [PubMed: 16672345]
10. Lee G, Wang Z, Sehgal R, Kikuno K, Chen C-H, Hay B, Park JH (2011) *Drosophila* caspases involved in developmentally regulated programmed cell death of peptidergic neurons during early metamorphosis. *J Comp Neurol* 519:34–48 [PubMed: 21120926]
11. Lee G, Kikuno K, Sehgal R, Wang Z, Nair S, Chen C-H, Hay B, Park JH (2013) Essential role of grim-led programmed cell death for the establishment of Corazonin-producing peptidergic nervous system during embryogenesis and metamorphosis in *Drosophila melanogaster*. *Biol Open* 2:283–294 [PubMed: 23519152]
12. Winbush A, Weeks JC (2011) Steroid-triggered, cell-autonomous death of a *Drosophila* motoneurons during metamorphosis. *Neural Dev* 6:15 [PubMed: 21521537]
13. Robinow S, Talbot WS, Hogness DS, Truman JW (1993) Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. *Development* 119:1251–1259 [PubMed: 8306887]
14. Draizen TA, Ewer J, Robinow S (1999) Genetic and hormonal regulation of the death of peptidergic neurons in the *Drosophila* central nervous system. *J Neurobiol* 38:455–465 [PubMed: 10084681]
15. Lee G, Kikuno K, Nair S, Park JH (2013) Mechanisms of postecdysis-associated programmed cell death of peptidergic neurons in *Drosophila melanogaster*. *J Comp Neurol* 521:3972–3991 [PubMed: 23784845]
16. Dai JD, Gilbert LI (1991) Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval–pupal–adult transformation of *Drosophila melanogaster*: A cytophysiological analysis of the ring gland. *Dev Biol* 144:309–326 [PubMed: 1901285]
17. Robinow S, Draizen TA, Truman JW (1997) Genes that induce apoptosis: Transcriptional regulation in identified, doomed neurons of the *Drosophila* CNS. *Dev Biol* 190:206–213 [PubMed: 9344539]
18. Park JH, Shroeder AJ, Helfrich-Förster C, Jackson FR, Ewer J (2003) Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of ecdysis behavior. *Development* 130:2645–2656 [PubMed: 12736209]
19. Luan H, Lemon WC, Peabody NC, Pohl JB, Zelensky PK, Wang D, Nitabach MN, Holmes TC, White BH (2006) Functional dissection of a neuronal network required for cuticle tanning and wing expansion in *Drosophila*. *J Neurosci* 26:573–584 [PubMed: 16407556]
20. Choi S-H, Lee G, Monahan P, Park JH (2008) Spatial regulation of Corazonin neuropeptide expression requires multiple cis-acting elements in *Drosophila melanogaster*. *J Comp Neurol* 507:1184–1195 [PubMed: 18181151]
21. Schubiger M, Wade A, Carney GE, Truman JW, Bender M (1998) *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development* 125:2053–2062 [PubMed: 9570770]
22. Bender M, Imam FB, Talbot WS, Ganetzky B, Hogness DS (1997) *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* 91:777–788 [PubMed: 9413987]

23. Carney GE, Robertson A, Davis MB, Bender M. (2004) Creation of EcR isoform-specific mutations in *Drosophila melanogaster* via local P element transposition, imprecise P element excision, and male recombination. *Mol Genet Genomics* 271:282–290 [PubMed: 14747942]
24. Grether ME, Abrams JM, Agapite J, White K, Steller K (1995) The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev* 9:1694–1708. [PubMed: 7622034]
25. Moon N-S, Stefano LD, Morris EJ, Patel R, White K, Dyson NJ (2008) E2F and p53 induce apoptosis independently during *Drosophila* development but intersect in the context of DNA damage. *PLoS Genet* 4(8), e1000153 [PubMed: 18688282]
26. Chen C-H, Huang H, Ward CM, Su JT, Schaffer LV, Guo M, Hay BA (2007) A synthetic maternal-effect selfish genetic element drives population replacement in *Drosophila*. *Science* 316, 597–600 [PubMed: 17395794]
27. Lin S, Huang Y, Lee T (2009) Nuclear receptor unfulfilled regulates axonal guidance and cell identity of *Drosophila* mushroom body neurons. *PLoS One* 4(12): e8392 [PubMed: 20027309]
28. Cherbas L, Hu X, Zhimulev I, Belyaeva E, Cherbas P (2003) EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development* 130:271–284. [PubMed: 12466195]
29. Lee T, Marticke S, Sung C, Robinow S, Luo L (2000) Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. *Neuron* 28:807–818 [PubMed: 11163268]
30. Henrich VC, Szekely AA, Kim SJ, Brown NE, Antoniewski C, Hayden MA, Lepesant J-A, Gilbert LI (1994) Expression and function of the ultraspiracle (*usp*) gene during development of *Drosophila melanogaster*. *Dev Biol* 165:38–52 [PubMed: 8088449]
31. Talbot WS, Swyryd EA, Hogness DS (1993) *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* 73:1323–1337 [PubMed: 8324824]
32. Diao F, Mena W, Shi J, Park D, Diao F, Taghert P, Ewer J, White BH. (2016) The splice isoforms of the *Drosophila* ecdysis triggering hormone receptor have developmentally distinct roles. *Genetics* 202:175–189 [PubMed: 26534952]
33. Yoo S, Lam H, Lee C, Lee G, Park JH (2017) Cloning and functional characterizations of an apoptogenic *Hid* gene in the Scuttle Fly, *Megaselia scalaris* (Diptera; Phoridae). *Gene* 604:9–21 [PubMed: 27940109]
34. Truman JW, Talbot WS, Fahrbach SE, Hogness DS (1994) Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development* 120:219–234 [PubMed: 8119129]
35. Zhao T, Gu T, Rice HC, McAdams KL, Roark KM, Lawson K, Gauthier SA, Reagan KL, Hewes RS. (2008) A *Drosophila* gain-of-function screen for candidate genes involved in steroid-dependent neuroendocrine cell remodeling. *Genetics* 178:883–901 [PubMed: 18245346]
36. Yao T, Segraves WA, Oro AE, McKeown M, Evans RM (1992) *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* 71:63–72 [PubMed: 1327536]
37. Thomas HE, Stunnenberg HG, Stewart AF (1993) Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature* 362:471–475 [PubMed: 8385270]
38. Oro AE, McKeown M, Evans RM (1992) The *Drosophila* retinoid X receptor homolog ultraspiracle functions in both female reproduction and eye morphogenesis. *Development* 115:449–462 [PubMed: 1330482]
39. Lee G, Park JH (2004) Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. *Genetics* 167:311–323 [PubMed: 15166157]
40. Haerry TE, Khalsa O, O'connor MB, Wharton KA (1998) Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in *Drosophila*. *Development* 125:3977–3987 [PubMed: 9735359]
41. Goyal L, McCall K, Agapite J, Hartwig E, Steller H (2000) Induction of apoptosis by *Drosophila* reaper, *hid*, and *grim* through inhibition of IAP function. *EMBO J* 19:589–597 [PubMed: 10675328]

42. Dewey EM, McNabb SL, Ewer J, Kuo GR, Takanishi CL, Truman JW, Honegger H-W (2004) Identification of the gene encoding bursicon, an insect neuropeptide responsible for cuticle sclerotization and wing spreading. *Curr Biol* 14:1208–1213 [PubMed: 15242619]
43. Luo C-W, Dewey EM, Sudo S, Ewer J, Hsu SY, Honegger H-W, Hsueh AJW (2005) Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2. *Proc Natl Acad Sci USA* 102:2820–2825 [PubMed: 15703293]
44. Kim YJ, Zitnan D, Galizia CG, Cho KH, Adams ME (2006) A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. *Curr Biol* 16:1395–1407 [PubMed: 16860738]
45. Handler AM (1982) Ecdysteroid titers during pupal and adult development in *Drosophila melanogaster*. *Dev Biol* 93:73–82 [PubMed: 6813165]
46. Sutherland J, Kozlova T, Tzertzinis G, Kafatos F (1995) *Drosophila* hormone receptor 38: A second partner for *Drosophila* USP suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. *Proc Natl Acad Sci USA* 92:7966–7970 [PubMed: 7644522]
47. Buszczak M, Segraves WA (1998) *Drosophila* metamorphosis: The only way is USP? *Curr Biol* 8:879–882
48. Siddiqui AN, Siddiqui N, Khan RA, Kalam A, Jabir NR, Kamal MA, Firoz CK, Tabrez S (2016) Neuroprotective role of steroidal sex hormones: an overview. *CNS Neurosci Ther* 22:342–350 [PubMed: 27012165]
49. Sasaya H, Yasuzumi K, Maruoka H, Fujita A, Kato Y, Waki T, Shimoke K, Ikeuchi T (2012) Apoptosis-inducing activity of endocrine-disrupting chemicals in cultured PC12 cells. *Adv Biol Chem* 2:92–105
50. Oka T, Adati N, Shinkai T, Sakuma K, Nishimura T, Kurose K (2003) Bisphenol A induces apoptosis in central neural cells during early development of *Xenopus laevis*. *Biochem Biophys Res Commun* 312:877–882 [PubMed: 14651953]
51. Masuo Y, Ishido M (2011) Neurotoxicity of endocrine disruptors: possible involvement in brain development and neurodegeneration. *J Toxicol Environ Health part B* 14:346–369
52. Nunez JL, Lauschke DM, Juraska JM (2001) Cell death in the development of the posterior cortex in male and female rats. *J Comp Neurol* 436:32–41 [PubMed: 11413544]
53. Nunez JL, Sodhi J, Juraska JM (2002) Ovarian hormones after postnatal day 20 reduce neuron number in the rat primary visual cortex. *J Neurobiol* 52:312–321 [PubMed: 12210098]
54. Tsukahara S (2009) Sex differences and the roles of sex steroids in apoptosis of sexually dimorphic nuclei of the preoptic area in postnatal rats. *J Neuroendocrinol* 21:370–376 [PubMed: 19226350]

Fig. 1.

Effect of *EcR^{DN}* expression by *ccap-gal4* on pupal development and CCAP neurons. **a** Control (*ccap>lacZ*) shows normally developing appendages, wings (W) and legs (L) as indicated by arrows. **b, c** *EcR^{DN}* expression in CCAP neurons. **(b)** *ccap>lacZ, EcR^{W650A}* and **(c)** *ccap>lacZ, EcR^{F645A}* compromised pupal development, resulting in the defective formation of wings and legs (arrows). *EcR^{W650A}* produced more severe phenotypes than *EcR^{F645A}* did. **d** *LacZ*-reported control CCAP neurons (*ccap>lacZ*) in the wandering third instar larval CNS (Br, brain; VNC, ventral nerve cord; SEG, subesophageal ganglion). **e, f** Double transgenic *ccap-gal4; UAS-lacZ* line was crossed to each *UAS-EcR^{DN}* line. Nearly complete elimination of CCAP neurons by *EcR^{W650A}* expression (**e**) and partial removal by *EcR^{F645A}* expression (**f**). **g, h** Rescue of *EcR^{DN}*-mediated defective pupal development by *p35* expression. **(g)** *EcR^{W650A} + p35* and **(h)** *EcR^{F645A} + p35*. **i, j** Rescue of *EcR^{DN}*-induced loss of CCAP neurons by *p35* expression (*ccap>lacZ, EcR^{DN}, p35*). **(i)** *EcR^{W650A} + p35* and **(j)** *EcR^{F645A} + p35*. Scale bar in (d), 100 μ m

Fig. 2.

Gradual loss of CCAP neurons by *EcR^{DN}* expression during larval development. Double transgenic *UAS-mCD8GFP*, *ccap-gal4* line was crossed to *UAS-EcR^{W645A}*. **a-g** GFP-reported CCAP neurons are shown from L1 to WL3 stages, as indicated above each panel. Arrows over L2 and L3 indicate aging within the same instar. L3 stages are termed as feeding L3 (FL3) and wandering (WL3). **ai, ci** Magnified images of **a** and **c**, respectively. Loss of CCAP neurons in the VNC began to be detectable in late L2 (**c, ci**) and early L3 (**d**). Scale bars, 100 μ m

Fig. 3.

Expression of EcR isoforms in larval CNS and CCAP neurons. **a-c** EcR isoform-specific immunoreactivities in the WL3 VNC. **(a)** EcR-A, **(b)** EcR-B1, **(c)** all EcR isoforms by anti-EcR-com. A doublet of CCAP neurons located from 1st to 3rd abdominal neuromeres (indicated by brackets) was selected to show double-labeling for the EcR and *ccap>GFP* as shown below panels. **d-f** Detection of EcR isoforms (red signals) in the GFP-reported CCAP neurons (green) by anti-EcR-A **(d)**, anti-EcR-B1 **(e)**, and anti-EcR-com **(f)**. Scale bars for (a) and (d) are 100 μ m and 10 μ m, respectively.

Fig. 4.

Rescue of EcR^{DN}-induced PCD by EcR isoforms. **a** Control *LacZ*-reported CCAP neurons (*ccap>lacZ*). **b** EcR^{W650A}-induced precocious CCAP cell death (*ccap>lacZ*, EcR^{W650A}). **c**, **d** Complete rescue of EcR^{W650A}-mediated CCAP neuronal death by co-expression of either EcR-B1 or EcR-B2 (**c**) and partial rescue by EcR-A (**d**). *ccap-gal4; UAS-lacZ* line was crossed to *UAS-EcR^{W650}*, *UAS-EcR*. **e-g** EcR^{W650A}-led defective pupal phenotypes are rescued by co-expression of *EcR* isoforms. Complete rescue by EcR-B1 (**e**) and EcR-B2 (**f**)

but partial rescue by EcR-A (g). An arrow in (g) points to short legs. Scale bar for (a), 100 μm

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Fig. 5. CCAP neurons in *ecr* mutants devoid of either EcR-A or EcR-B isoforms. CCAP neurons were detected by anti-bursicon. Since bursicon expression is limited to subsets of CCAP neurons, bursicon-negative CCAP neurons in the protocerebrum were not detectable. **a-d** Larval CNS. **(a)** Wild-type. **(b, c)** Heteroallelic *ecrA* mutants, *112/M554fs* **(b)** and *139/M554fs* **(c)**, showed wild-type pattern of bursicon-positive neurons. **(d)** Heteroallelic *ecrB* mutant, *31/99*. A few bursicon-positive neurons in thoracic neuromeres (bracket) were frequently lost. **e-i** Bursicon-immunoreactive neurons in the pupal VNCs at 24–48h APF. **(e)** Control *M554fs/+*. **(f, g)** *112/M554fs* and **(h, i)** *139/M554fs* mutant. Arrows in **(h, i)** indicate missing bursicon neurons. Scale bars, 100 μm

Fig. 6.

Essential role of Usp for vCrz apoptosis. **a, b** Eight pairs of corazonin-immunoreactive neurons in the wild-type VNC of WL3 indicated by a box (**a**) are completely removed by 7 h APF (**b**). **c, d** Complete blocking of vCrz PCD by expression of *EcR^{W650A}* (**c**) and *miusp* (**d**). **e-g** Mosaic analysis (MARCM) of *usp³* in vCrz neurons (*FRT^{19A}, usp³/FRT^{19A}, hs-FLP, tubp-gal80, UAS-mCD8GFP, crz-gal4/+*). Arrows indicate that two vCrz neurons bearing homozygous *usp³* mutation did not undergo PCD at 6 h APF. These neurons were labeled by both GFP (**e**) and Crz-immunosignals (**f**). A merge of (**e**) and (**f**) (**g**). **h** Ectopic expression of *Usp³* completely blocked vCrz PCD at 7 h APF (*crz-gal4/UAS-Usp³*). Scale bar, 100 μ m

Fig. 7.

Dispensable role of Usp for the protection of larval CCAP neurons. *UAS-mCD8GFP;ccap-gal4* line was crossed to indicated UAS lines. **a** GFP-reported larval CCAP neurons in control (*ccap>mCD8GFP*). **b** Premature CCAP neuron death by *EcR^{W650A}*. **c-e** CCAP cell death was not caused by either *mlusp* (**c**) or *Usp³* expression (**d, e**). Two independent *UAS-Usp³* lines inserted in 2nd (II) and 3rd (III) chromosomes showed the same results. Scale bar, 100 μ m

Fig. 8.

Essential role of *grim* for EcR-mediated protection of larval CCAP neurons. **a** Control (*UAS-mCD8GFP; ccap-gal4* x *y w*). **b, c** *EcR^{W650A}*-caused premature cell death was blocked by knockdowns of *RGH* (*UAS-mCD8GFP; ccap-gal4* x *UAS-EcR^{W650A}, UAS-miRGH*) (**b**) and by *grim* knockdown (*UAS-mCD8GFP; ccap-gal4* x *UAS-EcR^{W650A}, UAS-migrim*) (**c**). **d, e** *EcR^{W650A}*-caused cell death was not blocked in either a heteroallelic *hid*-null mutant (*hid^P/X14*) (*UAS-mCD8GFP; ccap-gal4; X14/TM6B* x *UAS-EcR^{W650A}; hid^P/TM6B*) (**d**), or *rpr⁸⁷* homozygosity (*UAS-mCD8GFP; ccap-gal4; rpr⁸⁷/TM6B* x *UAS-EcR^{W650A}; rpr⁸⁷/TM6B*) (**e**). **f-h** Rescue of *EcR^{W650A}*-induced CCAP cell death by *Diap1* expression. Control (*UAS-mCD8GFP, burs-gal4* x *y w*) (**f**). *EcR^{W650A}* expression by *burs-gal4* (*UAS-mCD8GFP, burs-gal4* x *UAS-EcR^{W650A}*) (**g**). *Diap1* expression significantly rescued *EcR^{W650A}*-mediated premature cell death (*UAS-mCD8GFP, burs-gal4; UAS-diap1* x *UAS-EcR^{W650A}*) (**h**). Scale bar, 100 μm

Table 1.

Comparison of developmental defects by ectopic expression of *EcRW650A*, *usp³* and *miusp*.

gal4	expression	<i>EcRW650A</i>	<i>Usp³</i>	<i>miusp</i>
tubulin	ubiquitous	emb. lethal	emb. lethal	emb. lethal ***
r4	fat body	L1-L2 lethal	pupal lethal **	pupal lethal ****
A9	imaginal discs	larva-pupal lethal *	pupal lethal *	pupal lethal ****

* see also supplemental Fig. 1

** During early pupal development, fat bodies do not seem to be distributed to the head region, making it transparent. Also, normally granule-shaped fat bodies are not clearly developed. Survival rate into adults was ~12%.

*** Very few larval escapers were found, but none survived beyond L2 stage.

**** Most of them were died in pharate adult stage. Survival rate into adults was~10%.