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Programmed cell death mechanisms of identifiable peptidergic neurons in *Drosophila melanogaster*

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The molecular basis of programmed cell death (PCD) of neurons during early metamorphic development of the central nervous system (CNS) in *Drosophila melanogaster* are largely unknown, in part owing to the lack of appropriate model systems. Here, we provide evidence showing that a group of neurons (vCrz) that express neuropeptide *Corazonin* (*Crz*) gene in the ventral nerve cord of the larval CNS undergo programmed death within 6 hours of the onset of metamorphosis. The death was prevented by targeted expression of caspase inhibitor *p35*, suggesting that these larval neurons are eliminated via a caspase-dependent pathway. Genetic and transgenic disruptions of ecdysone signal transduction involving ecdysone receptor-B (EcR-B) isoforms suppressed vCrz death, whereas transgenic re-introduction of either EcR-B1 or EcR-B2 isoform into the *EcR-B*-null mutant resumed normal death. Expression of *reaper* in vCrz neurons and suppression of vCrz-cell death in a *reaper*-null mutant suggest that *reaper* functions are required for the death, while no apparent role was found for *hid* or *grim* as a death promoter. Our data further suggest that *diap1* does not play a role as a central regulator of the PCD of vCrz neurons. Significant delay of vCrz-cell death was observed in mutants that lack *dronc* or *dark* functions, indicating that formation of an apoptosome is necessary, but not sufficient, for timely execution of the death. These results suggest that activated ecdysone signaling determines precise developmental timing of the neuronal degeneration during early metamorphosis, and that subsequent *reaper*-mediated caspase activation occurs through a novel DIAP1-independent pathway.

KEY WORDS: Corazonin, Programmed cell death, Metamorphosis, Ecdysone receptor, Reaper, *Drosophila*

INTRODUCTION

In holometabolous insects that develop through complete metamorphosis, significant behavioral changes are observed from juveniles to adults. For example, *Drosophila* larvae display simple behaviors, including feeding, crawling and defensive thrashing, whereas adult flies lead more complicated lifestyles that involve foraging, flying, mating and aggression in order to survive and fulfill successful reproduction. This type of behavioral transition is accompanied by substantial reorganization of the nervous system, which establishes adult-specific neural circuitry, thereby accommodating new lifestyles (Truman et al., 1993; Levine et al., 1995; Consoulas et al., 2000; Tissot and Stocker, 2000). During metamorphosis, larval neurons face mainly two different fates: remodeling or programmed cell death (PCD). The former is a recycling process of persistent neurons and is characterized primarily by significant modifications in synaptic architectures, resulting from withdrawal of larval-specific connections, followed by reconnection with new targets (e.g. Kraft et al., 1998; Lee et al., 2000b). By comparison, other neurons are scheduled to die via developmentally regulated genetic programs, as their functions are no longer required for ensuing life stages.

Several studies have implicated ecdysone as a central endocrine regulator that initiates genetic programs orchestrating overall reorganization processes of the insect nervous system during metamorphosis (Truman et al., 1993). For example, genetic analyses have shown that ecdysone receptor activities are essential for cell-autonomous remodeling of mushroom body γ-neurons and SCP-immunoreactive (IR) neurosecretory cells (Schubiger et al., 1998; Schubiger et al., 2003; Lee et al., 2000b). In addition to the remodeling, ecdysone has been shown to cause apoptosis of obsolete larval neurons. In a moth, identified motoneurons innervating larval proleg muscles are degenerated in response to a prepupal ecdysone surge (Weeks, 2003). Treatment of isolated motoneurons with ecdysone in vitro also causes the death, suggesting that ecdysone directly induces a cell-autonomous death program (Streichert et al., 1997).

Although underlying molecular mechanisms for post-embryonic neuronal PCD are largely unknown, extensive genetic studies have identified key molecular players that either enhance or suppress PCD occurring in developing *Drosophila* embryos and compound eyes (reviewed by Cashio et al., 2005; Kornbluth and White, 2005). Apoptotic death is a direct consequence of massive destruction of cellular components mediated by specialized proteolytic enzymes: caspases (reviewed by Salvesen and Abrams, 2004). In living cells, the caspases are inactivated and/or degraded by the action of *Drosophila* inhibitor of apoptotic protein 1 (DIAP1) through forming a complex with caspases (Meier et al., 2000; Muro et al., 2002; Wilson et al., 2002). When cells are challenged with death stimuli, death activators (collectively referred to as RHG) encoded by *reaper* (*rpr*), *head involution defective* (*hid*; *W* – FlyBase) and *grim*, which are defined by deficiency *Df(3L)H99* (White et al., 1994; Grether et al., 1995; Chen et al., 1996), bind to DIAP1, liberating caspases from the DIAP1-caspase complexes, resulting in the activation of caspases (Wang et al., 1999; Goyal et al., 2000). Physical interactions between RHG proteins and DIAP1 are also known to downregulate DIAP1 levels via ubiquitin-mediated self-degradation of DIAP1, which further ensures an irreversible death pathway (Ryoo et al., 2002; Yoo et al., 2002). Products of two additional pro-apoptotic genes, *sickle* and *jafrac2*, are also implicated as DIAP1 antagonists (Christich et al., 2002; Srinivasula et al., 2002; Tenev et al., 2002; Wing et al., 2002).

Fundamental molecular cell death mechanisms just described appear to be conserved in the tissues that require ecdysone for their death at a precise developmental stage. Larval salivary glands and

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midgut are degenerated in response to an ecdysone stimulus during metamorphosis in which ecdysone activates transcription of *rpr* and *hid* directly, or indirectly via ecdysone-responsive transcription factors such as *Broad Complex* (*BR-C*), *E74* and *E93* (Jiang et al., 2000; Lee et al., 2002). Premature destruction of these tissues throughout larval growth is prevented by DIAP1, until ecdysone-induced RPR or HID proteins overcome the inhibitory action of DIAP1 (Yin and Thummel, 2004).

During prepupal stage, a number of unwanted larval neurons are removed from the CNS in *Drosophila*. Neuronal degeneration is particularly prominent in the abdominal ganglion, leading to significant shrinkage of this neuropil (Truman, 1990; Truman et al., 1993). Although ecdysone is an important developmental cue for the PCD of specific motoneurons in a moth (Weeks, 2003), ecdysone functions in the PCD of obsolete larval neurons in the CNS are largely unexplored. Here, we show that peptidergic neurons expressing *Corazonin* (*Crz*) in the ventral nerve cord (vCrz) are programmed to die during early metamorphosis in *Drosophila*. Our data further suggest that activated ecdysone signaling induces *rpr* expression, and subsequent activation of caspases does not involve *diap1* functions.

MATERIALS AND METHODS

Drosophila strains and genetic manipulations

Canton-S was used as a wild type. For visualization of Crz neurons, a *Crz-gal4* driver (see below) was crossed to a *UAS-lacZ* or *UAS-mCD8-GFP* reporter, which respectively produces GAL4-inducible β-galactosidase or membrane-targeted green fluorescence protein (GFP) (Phelps and Brand, 1998; Lee and Luo, 1999).

Double homozygous lines carrying both *Crz-gal4* and *UAS-lacZ* (or *UAS-mCD8-GFP*) transgenes were generated by genetic crosses. These flies were crossed to the following *UAS* responders to produce various types of transgenic manipulations: *UAS-EcR-A*, *UAS-EcR-B1* and *UAS-EcR-B2* to overexpress specific Ecdysone Receptor (EcR) isoforms (Lee et al., 2000b); *UAS-EcR-B1*^{F645A} and *UAS-EcR-B1*^{W650A} to express dominant-negative (DN) forms of the EcR-B1 (Cherbas et al., 2003); *UAS-diap1* (Hay et al., 1995) to produce inhibitors of apoptosis; and *symUAS-diap1*^{RNAi} to knockdown *diap1* mRNA (Huh et al., 2004). In some experiments, a double homozygous *Crz-gal4*, *UAS-p35* line was used for inhibition of caspases (Hay et al., 1995).

For *EcR-B*-null mutant, *EcR*^{31}/*CyO*, *y*$^{+}$ was crossed to *EcR*^{99}/*CyO*, *y*$^{+}$, from which F1 larvae lacking *y*$^{+}$ marker (*EcR*^{31}/*EcR*^{99}) were collected and dissected after completion of apolysis as described (Schubiger et al., 1998; Schubiger et al., 2003). To rescue the *EcR-B* mutation, *y w*; *EcR*^{31}/CyO, *y*$^{+}$; *UAS-EcR-B1* (or *B2*) were mated with *y w*; *EcR*^{99}/CyO, *y*$^{+}$; *Crz-gal4* to obtain *EcR*^{31}/*EcR*^{99}; *UAS-EcR-B1* (or *B2*)/*Crz-gal4*. The *EcR-A*-null (*EcR*^{112}/*EcR*^{M554fs}) and *EcR-B1*-null (*EcR*^{Q50st}/*EcR*^{31}) mutants were produced as described previously (Bender et al., 1997; Carney et al., 2004).

The following deficiencies were used to generate *rpr*- and *hid*-null mutations: *Df(3L)XR38*, *Df(3L)H99* and *Df(3L)X14* (for short, *XR38*, *H99*, and *X14*, respectively). Homozygous deletion of the *rpr* locus was obtained by combining *H99* with *XR38* in trans as described (cf. Peterson et al., 2002). Flies lacking *hid* functions were produced by transallelic combination of *H99* (or *X14*) with *hid*^{05014} allele (Grether et al., 1995). Although most of the *hid* mutants are embryonic lethal, a few escapers enabled us to assess vCrz neuronal death in these mutants (Peterson et al., 2002).

Null mutations of caspase-encoding *dronc* (*Nc* – FlyBase) gene were obtained by transallelic combinations of *dronc*^{I24} (or *dronc*^{I29}) with *dronc*^{51} (Chew et al., 2004; Xu et al., 2005). Two *thread* (*th*, *diap1*) alleles, *th*^{4} (loss-of-function) and *th*^{SL} (gain-of-function), were used in some experiments (Lisi et al., 2000). The mutant alleles used in this study are summarized in Table 1.

Transgenic lines

To construct *P*-element containing *Crz* promoter fused to the *gal4*-coding sequence (hereafter referred to as *Crz-gal4*), a genomic region upstream of the *Crz* gene (–1155 to +78 relative to the transcription start site +1) (Choi et al., 2005) was amplified by PCR, and subcloned into pBluescript at the *Sma*I site, from which an *Xba*I-*Eco*RI fragment was inserted into pPTGAL vector (Sharma et al., 2002). For *rpr-gal4* construct, previously defined 1.3 kb *rpr* upstream sequence (Jiang et al., 2000) was inserted into pPTGAL at *Bgl*II/*Eco*RI sites. Each resulting vector was mixed with pUChsπΔ2-3 helper plasmid (Laski et al., 1986) and injected into the *y w* or *w*^{1118} embryos for germ-line transformation (Rubin and Spradling, 1982). *Crz-gal4* (S2b, T2a) and *rpr-gal4* (III) lines were used in this study.

X-gal histochemistry and immunohistochemistry (IHC)

To detect β-galactosidase expression, CNSs were fixed in 0.2% glutaraldehyde, washed in PBS and then incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) solution at 37°C overnight. The tissues were rinsed in PBS, dehydrated in ethanol and mounted in glycerol (e.g. Park et al., 2000).

Whole-mount Crz immunohistochemistry was performed as described previously (Choi et al., 2005). The anti-Crz was previously referred to as anti-CAP, which was raised against Crz-Associated Peptide within the precursor (Choi et al., 2005). To co-localize EcR-immunoreactivity (IRy) in Crz neurons, rabbit anti-Crz and mouse monoclonal anti-EcR-A (15G1a) or anti-EcR-B1 (AD4.4) (Talbot et al., 1993) were simultaneously applied to the CNSs. The primary antibodies were detected by FITC- or TRITC-conjugated secondary antibodies (Jackson ImmunoResearch) at 1:200

Table 1. Mutant alleles used in this experiment

Locus	Allele	Type of mutation	Ref.
EcR	*EcR*^{112}	Amorphic (A isoform)	Carney et al. (2004)
	EcR^{M554fs}	Amorphic (A, B1, B2)	Bender et al. (1997)
	EcR^{Q50st}	Amorphic (B1)	Bender et al. (1997)
	EcR^{31}	Amorphic (B1, B2)	Schubiger et al. (1998)
	EcR^{99}	Amorphic (B1, B2)	Schubiger et al. (1998)
BR-C	*2Bc*^{1}	Amorphic	Belyaeva et al. (1980)
	br^{5}	Amorphic	Belyaeva et al. (1980)
	rbp^{5}	Hypomorphic	Belyaeva et al. (1980)
Cell death	*XR38*	Deletion (*rpr*, *sickle*)	Peterson et al. (2002)
	H99	Deletion (*hid*, *grim*, *rpr*)	White et al. (1994)
	X14	Deletion (*hid*)	White et al. (1994)
	hid^{05014}	Amorphic	Grether et al. (1995)
diap1	*Df(3L)brm11*	Deletion	Brizuela et al. (1994)
	th^{4}	Amorphic	Lisi et al. (2000)
	th^{SL}	Hypermorphic	Lisi et al. (2000)
dronc	*dronc*^{51}	Amorphic	Chew et al. (2004)
	dronc^{I24}	Amorphic	Xu et al. (2005)
	dronc^{I29}	Amorphic	Xu et al. (2005)
dark	*dark*^{CD4}	Amorphic	Rodriguez et al. (1999)

dilution. The specimens were then cleared in 60% glycerol and mounted in Vectashield medium (Vector Laboratory). Fluorescent signals were acquired by Leica TCS confocal microscopy or Olympus BX61 connected with CC-12 camera.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay

TUNEL was performed to detect DNA fragmentation in doomed vCrz neurons using a commercial kit (Deadend Fluorometric TUNEL system, Promega). CNSs were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature, washed in PBS containing 1% triton X-100, and then incubated in a reaction containing fluorescein-12-dUTP and terminal deoxynucleotidyl transferase at 37°C for 1 hour. Following termination of the reaction by washing tissues with 2× SSC, the tissues were subjected to Crz immunohistochemistry. Both TUNEL- and Crz-IR signals were visualized by epi-fluorescence.

Double labeling by in situ hybridization and immunohistochemistry

Chromogenic in situ hybridization of *Crz* mRNA was performed using digoxigenin (dig)-labeled cRNA probe as described (Lee et al., 2000a). Fluorescent in situ hybridization using TSA (Tyramide signal amplification) system was employed to detect *rpr* transcripts within Crz neurons (e.g. Zaidi et al., 2000). For this, CNS tissues were first hybridized with dig-labeled *rpr* cRNA probe, and then the tissues were incubated with anti-Crz (1:150,000) at 4°C overnight. After rinsing, FITC-conjugated secondary antibody was added to the tissues for 2 hours to obtain Crz-IRy. During the final 30 minutes of the incubation period, horseradish peroxidase-conjugated anti-dig was added to the incubation. Finally, Cy3-labeled tyramide substrate was added to produce in situ hybridization signals as recommended (Renaissance TSA Fluorescence Systems, Perkin Elmer).

RESULTS

Programmed death of vCrz neurons

Spatial *Crz* expression patterns change significantly during metamorphosis as previously reported (Choi et al., 2005). In third-instar larval CNSs, *Crz* gene products are detected in three pairs of DL neurons and in a pair of DM neurons in the brain, and in eight pairs of symmetrically positioned neurons (vCrz) in the ventral nerve cord (VNC) (Fig. 1A,C) (Choi et al., 2005). In the CNSs taken from pupae aged for ~36 hours after puparium formation (APF), *Crz* transcripts are found in 6-8 cells per hemi-brain (Fig. 1B), but none in the VNC. When immunohistochemistry was performed at an earlier stage (12-24 hours APF), protocerebral Crz-IR patterns remain unchanged from their larval patterns (arrowheads in Fig. 1D), while no vCrz neurons are visible (arrow in Fig. 1D). These temporal expression data suggest that adult-like protocerebral Crz neurons have been established between 24-36 hours APF, while vCrz expression disappeared within 12-24 hours APF.

The loss of vCrz expression could be due to programmed death of these neurons. We tested this possibility by transgenic rescue of cell death using *gal4/UAS* system (Phelps and Brand, 1998). As a preliminary step, we tested fidelity of the *Crz-gal4* driver. As shown in Fig. 1E, *Crz-gal4*-driven GFP signals are completely overlapped with Crz-IRy in third-instar larval CNS. *lacZ*-reporter expression also faithfully recapitulates endogenous *Crz* expression in all life stages (Fig. 2; data not shown), suggesting that the 1.2 kb upstream sequence contains *cis*-elements necessary for appropriate control of spatial and developmental *Crz* expression.

Using *Crz-gal4* driver, we induced expression of a caspase inhibitor, *p35* (Hay et al., 1995), to test whether *p35* can prevent cell death, thereby maintaining *Crz* expression in pupal VNC. Thus, a *Crz-gal4*, *UAS-p35* double homozygous strain was crossed to the *UAS-lacZ*, and then the progeny were processed for X-gal histochemistry. Remarkably, all vCrz neurons marked by *lacZ* expression are persistently present even at 24 hours APF (Fig. 2B, compare with 2A). These data strongly support that the loss of *vCrz* expression during pupal development is due to caspase-dependent cell death, not due to transcriptional silencing of the *Crz* gene.

Developmental timing of vCrz neuronal death

To determine developmental clock of the vCrz neuronal death, *lacZ*-reported *Crz* expression was examined in various prepupal stages. Elimination of vCrz neurons occurs progressively over a ~2- to 6-hour period after pupariation (Fig. 2C-F). At 2 hours APF, one or two cells are undetectable (Fig. 2D), suggesting that execution of the death has begun in some cells at around this stage. At 3 hours APF, ~40% of the neurons are lost in a random fashion, and staining in the projections are fainter and discontinuous, leaving broken line-like appearance (arrow in Fig. 2E). This perhaps reflects phagocytic elimination of non-functional cellular components possibly by glial cells (e.g. Sonnenfeld and Jacobs, 1995; Watts et al., 2004). At 6 hours APF, most of X-gal signals are undetectable, except for a few

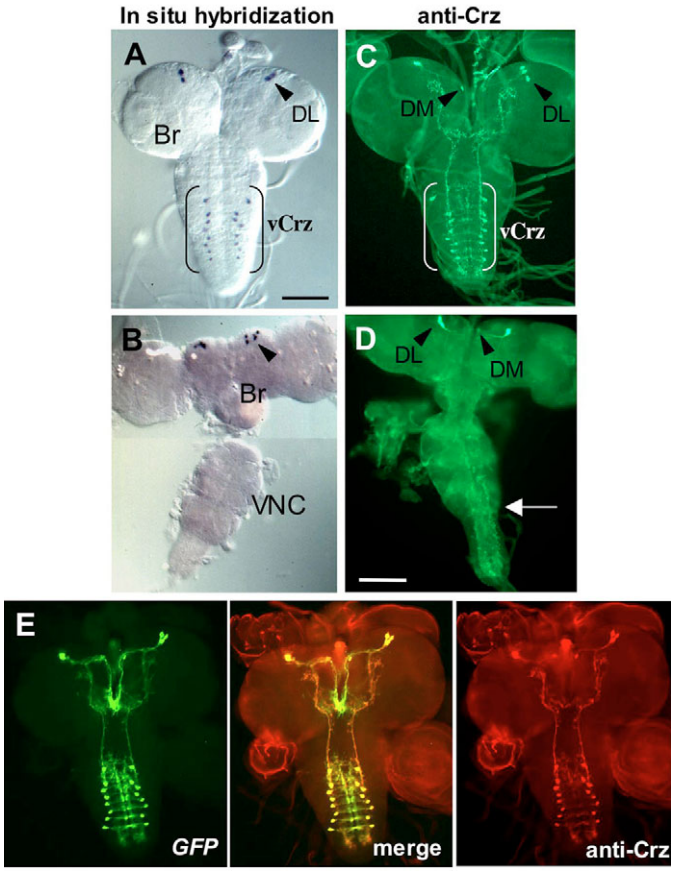

Fig. 1. *Crz* expression in the CNS during metamorphosis. (A,B) In situ hybridization of *Crz* mRNA. (**A**) Third-instar larval CNS, showing three pairs of DL neuron in the brain (Br) and eight pairs of vCrz neurons (brackets). (**B**) Pupal CNS at ~36 hours APF. The number of neurons in the brain (arrowhead) is found to be six to eight per lobe, whereas none of vCrz neurons are detectable. (**C**,**D**) Crz-IR neurons in third-instar larval CNS (C) and pupal CNS at 12-24 hours APF (D). Consistent with the in situ hybridization results, no Crz-IR neurons are detectable in the VNC of the pupal CNS (arrow). At least five specimens were processed for each panel. (**E**) Colocalization (center) of *Crz-gal4*-induced *GFP* expression (left) in Crz-IR neurons (right). Scale bars: 100 μm.

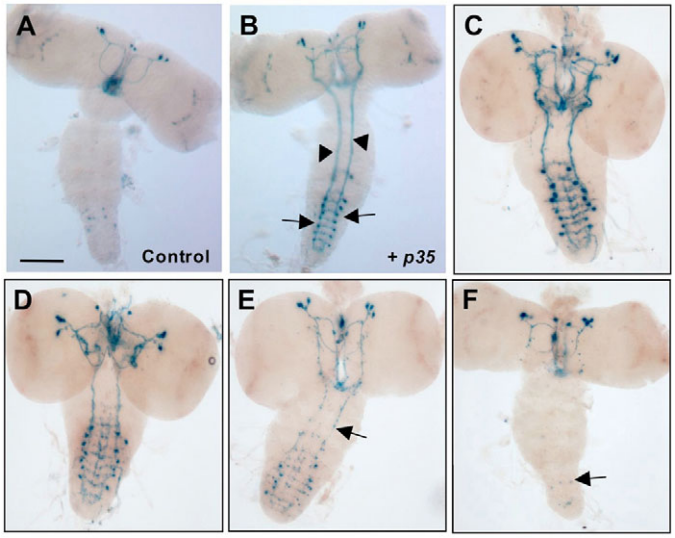

Fig. 2. Rescue of vCrz-cell death by transgenic expression of *p35*. (**A**) Control 1-day old pupa (*UAS-lacZ*/+; *Crz-gal4*/+). (**B**) One-day-old pupa expressing *p35* in Crz neurons (*UAS-lacZ*/+; *Crz-gal4*, *UAS-p35*/+). Persistent vCrz neuronal somata and ascending projections are designated by arrows and arrowheads, respectively. (**C-F**) Progressive removal of *lacZ*-marked vCrz neurons (*UAS-lacZ*/+; *Crz-gal4*/+) during metamorphosis. White prepupae were aged at 25°C for durations designated in each panel, and then processed for X-gal staining. (C) White prepupa (*n*=5). (D) 2 hours APF (*n*=6). (E) 3 hours APF (*n*=4). Arrow points to discontinued projection. (F) 6 hours APF (*n*=9). Faint dots (arrow) most probably come from residual β-galactosidase activity rather than from live cells. Scale bar: 100 μm.

faint dots (arrow in Fig. 2F). This type of signals, which most probably reflects residual β-galactosidase activity, was seen in some tissues taken even after 12 hours APF (arrowhead in Fig. 4A).

Suppression of cell death by *p35* suggests that vCrz neurons die through the activation of caspases. Another hallmark of the apoptosis is degradation of genomic DNA into nucleosomal units, which is catalyzed by a caspase-activated DNase (Wyllie, 1980; Enari et al., 1998). To determine whether such a biochemical event occurs in doomed vCrz neurons, we performed TUNEL to detect fragmented DNAs (Prochazkova et al., 2003). As our foregoing data imply that pro-apoptotic machinery comes into play almost immediately after pupariation (Fig. 2), 0- to 3-hour-old prepupal CNSs were processed for TUNEL, followed by Crz immunohistochemistry. As a result, TUNEL signals are detected in ~4% of total vCrz neurons examined (Fig. 3). Lack of the signals in other vCrz neurons may be due to unsynchronized onset of the death or inefficient labeling reaction for whole-mounted tissues. Nevertheless, these findings, together with the caspase-dependent cell death suggest that vCrz neuronal death occurs in an apoptotic fashion; however, precise determination of the type of cell death (apoptotic versus autophagic) requires electron microscopic identification of specific cytological markers (Baehrecke, 2003).

Ecdysone signaling initiates the death program in vCrz neurons

As the beginning of vCrz death is approximately coincident with a surge of ecdysone at pupariation (Riddiford, 1993), and the ecdysone is a key endocrine signal orchestrating overall metamorphic reformation of the CNS in insects including *Drosophila* (Weeks, 2003), ecdysone could be a developmental cue, activating genetic death program in doomed vCrz neurons. It is well documented that ecdysone signal is transduced by a heterodimeric receptor complex consisting of EcR and ultraspiracle (reviewed by King-Jones and Thummel, 2005). The EcR-encoding gene produces three isoforms (A, B1 and B2) via usage of different promoters and alternative splicing, which share common C-terminal ligand and DNA-binding domains but variable N termini (Talbot et al., 1993).

Recently, Cherbas et al. (Cherbas et al., 2003) developed two dominant-negative (DN) forms of EcR-B1 ($B1^{F645A}$ and $B1^{W650A}$) that lack transcriptional activator functions, but still retain DNA-binding ability. Ectopic expression of these mutant variants effectively blocked ecdysone-led physiological processes, perhaps via competitively inhibiting wild-type receptor functions (Cherbas et al., 2003). This prompted us to test whether ectopic EcR-$B1^{DN}$ expression interferes with normal vCrz-cell death. For this, progeny from *UAS-lacZ*; *Crz-gal4* × *UAS-EcR-*$B1^{F645A}$ (or *UAS-EcR-*$B1^{W650A}$) were subjected to X-gal histochemistry. Intriguingly, all of vCrz neurons are detectable at 12 hours APF (compare Fig. 4B,C with 4A), showing that both EcR-$B1^{DN}$s are (equally) capable of blocking vCrz PCD. The results strongly support our hypothesis that EcR-mediated signaling plays a decisive role in the initiation of death pathway within vCrz neurons.

EcR-B isoforms are major players for vCrz PCD

Distinct temporal expression profiles of each EcR-isoform suggest that each isoform exhibits developmental stage-specific functions for adult CNS formation during metamorphosis (Truman et al., 1994). For example, EcR-B-isoforms are involved in modifications of some larval neurons during early metamorphosis (Lee et al., 2000b; Schubiger et al., 2003). To determine types of EcR involved in vCrz-cell death, we examined isoform-specific immunoreactivity in vCrz neurons, except for the EcR-B2 because of lack of available antibody. Consistent with a previous report (Truman et al., 1994), widespread EcR-B1-IRy was observed in white prepupal CNS (Fig. 4D), whereas EcR-A-IRy was almost undetectable (data not shown). Furthermore, EcR-B1-IRy was evident in the nuclei of vCrz neurons (Fig. 4D), implicating a role of the EcR-B1 as a signal transducer for the PCD of vCrz.

To gain more insight into isoform-specific functions, we examined Crz-IRy in various *EcR* loss-of-function mutants. In animals heterozygous for the *EcR*-null mutant allele (EcR^{M554fs}) that lacks one copy of all EcR isoforms (Bender et al., 1997), Crz immunohistochemistry results indicated normal cell death, suggesting that a half dose of the EcR is sufficient for mediating vCrz-cell death (data not shown, $n>5$). Consistent with the absence of EcR-A-IRy, an *EcR-A*-null mutation (EcR^{112}/EcR^{M554fs}) did not affect normal vCrz PCD (compare Fig. 4F with 4E). Therefore, we conclude that EcR-A isoform is not a signal transducer for this type of cellular event.

As documented previously, animals carrying a null mutation for both EcR-B1 and EcR-B2 were generated by a trans-heterozygous combination of EcR^{31} and EcR^{99} alleles (Schubiger et al., 1998). Although most of mutant animals are developmentally arrested during the course of larval growth, a few escapers develop into prepupae (Schubiger et al., 2003), thereby permitting us to assess vCrz-cell death in this genetic background. Remarkably, Crz immunohistochemistry revealed ~12 vCrz neurons at stages when vCrz neurons were normally absent (compare Fig. 4G and Fig. 5A with Fig. 4E), suggesting that the death pathway in the majority of vCrz neurons is unable to proceed in the absence of EcR-B

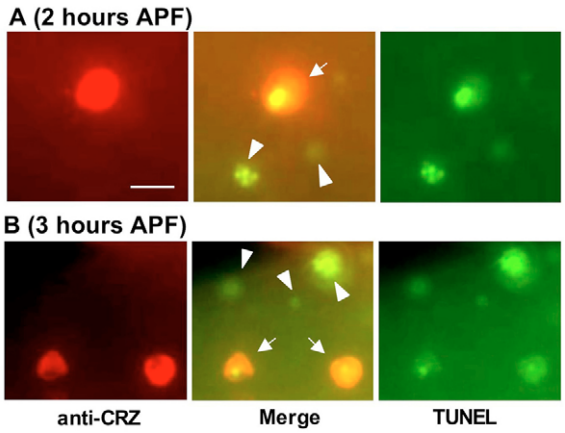

Fig. 3. DNA fragmentation in vCrz neurons. Wild-type prepupal CNSs at 2 hours APF (**A**) or 3 hours APF (**B**) were subjected to TUNEL assay (right), followed by Crz immunohistochemistry (left). These images were merged to show superimposition of the two signals (center). TUNEL-positive vCrz neurons are indicated by arrows and TUNEL signals in non-vCrz cells by arrowheads. Scale bar: 10 μm.

receptors. Therefore, our histological and genetic evidence together demonstrates that EcR-B-isoforms play significant roles for vCrz-cell death.

Both EcR-B1 and EcR-B2 are involved in vCrz PCD

We further determined whether both EcR-B1 and EcR-B2 are required or whether either of them is sufficient for vCrz PCD. To test this, an EcR-B1-specific mutant (EcR^{Q50st}/EcR^{31}) was examined for Crz-IRy (Bender et al., 1997). Surprisingly, complete lack of EcR-B1 showed no deficit in vCrz PCD (Fig. 4H). This result may suggest that only EcR-B2 is necessary for the death. Alternatively, EcR-B1 and EcR-B2 may have redundant functions, so that either isoform alone is sufficient for normal PCD.

To address this issue, it would be necessary to analyze *EcR-B2* specific mutants. As such mutants are unavailable, we employed transgenic rescue of the *EcR-B* mutant. For this attempt, we generated *y w*; EcR^{31}/*CyO*, y^+; *UAS-EcR-B1* (or *EcR-B2*) and *y w*; EcR^{99}/*CyO*, y^+; *Crz-gal4* strains. These lines were crossed with each other and F1-larvae carrying *y* marker [i.e. EcR^{31}/EcR^{99}; *Crz-gal4/UAS-EcR-B1* (or *B2*)] were collected and dissected after complete apolysis (separation of the old cuticle from the underlying epidermis) for Crz immunohistochemistry. As individual CNS morphology was variable depending on their developmental progress, the CNSs were classified as 'early', 'intermediate' or 'late' phenotypes (cf. Schubiger et al., 2003). In the 'early' group, CNSs retain white prepupa-like morphology in which the optic lobes are not yet extended and subesophageal ganglia are broadly attached to the VNC. In the 'late' group, optic lobes are highly developed, and areas between the subesophageal ganglia and VNC begin to constrict; this is nearly equivalent to wild-type at 12 hours APF (Truman et al., 1993). In the 'intermediate' group, CNSs show various levels of development between 'early' and 'late' groups.

Crz immunohistochemistry revealed significant differences between *EcR-B* mutant and transgene-rescued lines. Complete lack of PCD was seen in 18% of the *EcR-B* mutant CNSs, and partial cell death in the remaining 82% of specimens. Overall, 75% of vCrz neurons did not undergo PCD in the *EcR-B* mutant (Fig. 5A). By contrast, expression of *EcR-B1* or *EcR-B2* in the mutant restored complete cell death in 73% or 87% of tissues, respectively, while four to 11 neurons were detectable in the remaining specimen (Fig. 5B,C). These data suggest that either EcR-B1 or EcR-B2 alone is capable of mediating PCD of vCrz neurons, and support the idea that EcR-B1 and EcR-B2 have redundant functions for this purpose.

Reaper (*rpr*) is a proapoptotic executor of vCrz PCD

The pro-apoptotic genes *rpr*, *hid* and *grim*, defined by *H99* deletion, are well-known death activators in *Drosophila* (White et al., 1994). As *rpr* and *hid* have been shown to promote ecdysone-mediated PCD of the salivary glands and midgut (Yin and Thummel, 2004), we hypothesize that one or both of these genes might induce vCrz PCD.

The PCD of vCrz neurons was unaffected by heterozygous *H99* deletion, suggesting that one copy of the wild-type *rpr*, *hid* and *grim* alleles is sufficient for inducing vCrz-cell death (Fig. 6A). Heterozygosity for *XR38* deletion, which removes *rpr* and *sickle* (Peterson et al., 2002; Wing et al., 2002), also did not affect normal PCD (Fig. 6B). Intriguingly however, approximately seven pairs of vCrz neurons survived in a *rpr*-null mutant (*XR38/H99*) (Fig. 6C,

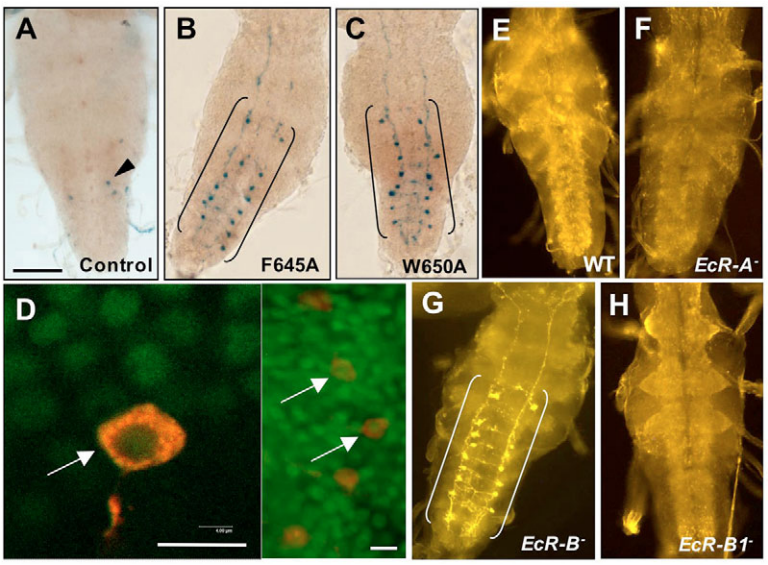

Fig. 4. Inhibition of vCrz-cell death by targeted expression of EcR-B1 dominant-negative forms. (**A-C**) CNSs were dissected at 12 hours APF and processed for X-gal staining. (A) Control (*UAS-lacZ*/+; *Crz-gal4*/+). An arrowhead indicates the staining from residual β-galactosidase activity. (B) *UAS-lacZ*/+; *Crz-gal4*/*UAS-EcR-*$B1^{F645A}$ (*n*=4). (C) *UAS-lacZ*/+; *Crz-gal4*/*UAS-EcR-*$B1^{W650A}$ (*n*=4). Live vCrz neurons are shown within the brackets. (**D**) (Left) A confocal image showing nuclear EcR-B1-IRy (green) in a vCrz *neuron* (orange, arrow) at white prepupal stage of wild-type. (Right) An independent specimen showing two vCrz neurons containing EcR-B1-IRy (arrows). (**E-H**) Crz-IRy in various *EcR* mutants at 12-17 hours APF. (E) Wild type. (F) *EcR-A* mutant (EcR^{M554fs}/EcR^{112}). (G) *EcR-B* mutant (EcR^{31}/EcR^{99}) after completion of apolysis. Surviving vCrz neurons are shown in the brackets (see also Fig. 5A). (H) *EcR-B1* mutant (EcR^{31}/EcR^{Q50st}). Scale bars: in A, 50 μm for A-C,E-H; in D, 10 μm for D.

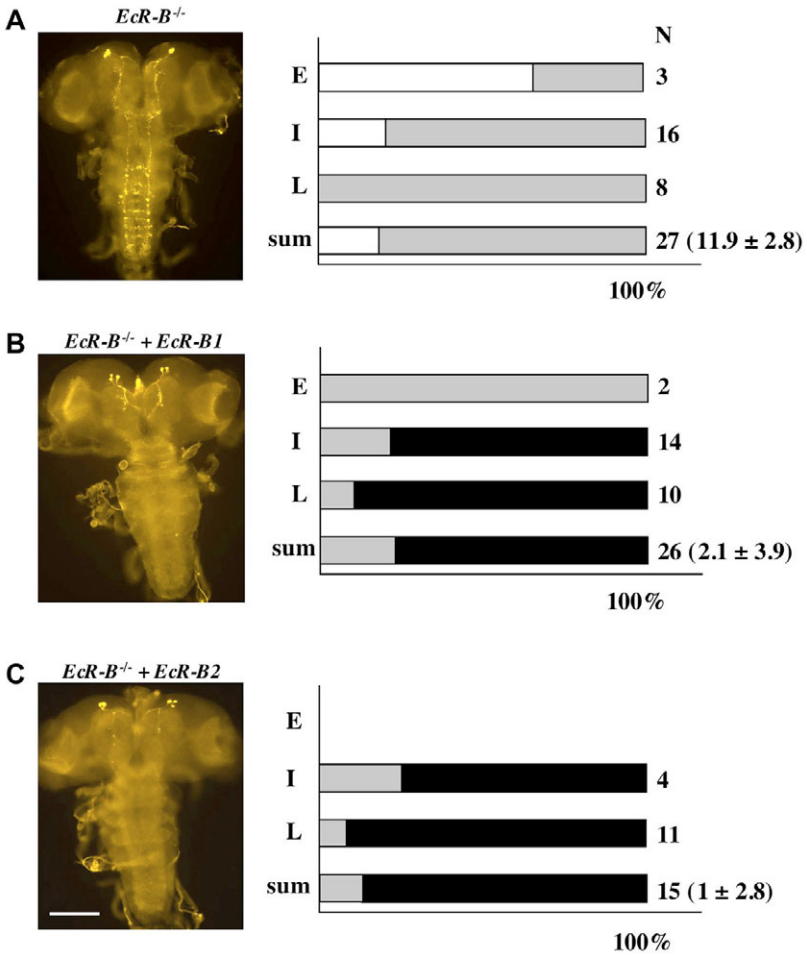

Fig. 5. Rescue of *EcR-B* mutant phenotype by transgenic expression of *EcR-B1* or *EcR-B2*-isoform. (**A**) *EcR-B* mutant control (EcR^{31}/EcR^{99}). (**B**) EcR^{31}/EcR^{99}; *UAS-EcR-B1/Crz-gal4*. (**C**) EcR^{31}/EcR^{99}; *UAS-EcR-B2/Crz-gal4*. The histograms indicate percentages of the CNSs showing no cell death (open bar), partial (gray bar), or complete death (black bar). As described in the text, the CNSs were grouped into 'E' (Early), 'I' (Intermediate), or 'L' (Late), depending on their developmental status. Numbers of tissues examined in each category are noted on the right of each bar (N), and mean numbers of vCrz neurons (± s.d.) are in parentheses. The CNSs belonging to the 'E' group were not found in the EcR-B2 rescue group. Representative images taken from the 'I' group are shown in the left. Scale bar: 100 μm.

n>5), and a similar result was obtained by *Crz-gal4*-mediated *GFP* expression in the same mutant background (*n*=4, data not shown). These data strongly support a crucial role that *rpr* plays in the destruction of vCrz neurons.

By contrast, vCrz neurons in mutants that lack *hid* functions ($X14/hid^{05014}$ and $H99/hid^{05014}$) (Grether et al., 1995; Peterson et al., 2002) showed normal PCD (Fig. 6D, and data not shown), suggesting that *hid* is not a death promoter for vCrz neurons. Owing to a lack of specific mutants, possible roles for *grim* were not tested. However, as *grim* in situ hybridization signals were hardly detectable in larval as well as early prepupal vCrz neurons in wild type (data not shown), we suppose that *grim* might not be involved in vCrz PCD.

To determine cell-autonomous roles of *rpr*, we investigated whether *rpr* is expressed in vCrz neurons by in situ hybridization. To validate our *rpr* cRNA probe, in situ hybridization was performed on the CNSs taken from the progeny of [*Crz-gal4*, *UAS-p35* × *UAS-rpr*] cross. In this genetic context, *rpr* transcripts are overproduced in Crz neurons but *rpr*-induced death is suppressed by co-expression of the *p35*. As a result, we detected faint but distinct *rpr* in situ hybridization signals seemingly in vCrz neurons in the third-instar larva (arrowheads in Fig. 6E). Except for these cells, in situ hybridization signals were nearly blank in the entire larval CNS, suggesting that *rpr* is transcriptionally silent in most cells of this tissue. Having validated the *rpr* cRNA probe, we extended in situ hybridization to wild-type prepupal CNS. At 1.5 hours APF, numerous *rpr* in situ hybridization signals were revealed particularly in abdominal ganglionic region (circle in Fig. 6F), while the sense probe did not produce any signals (data not shown). Together these results suggest that *rpr* becomes transcriptionally active soon after pupariation, thereby inducing death of numerous obsolete larval neurons in this CNS region.

We applied *rpr* in situ hybridization and Crz immunohistochemistry simultaneously to see if endogenous *rpr* mRNAs are also expressed in vCrz neurons prior to death. At 1.5 hours APF, *rpr* in situ hybridization signals were detectable in ~10% of vCrz neurons (17 out of 177) examined (Fig. 6G). Unsynchronized or transient expression of *rpr* may explain such a minor proportion of *rpr*-positive vCrz neurons.

To detect *rpr* expression in a more sensitive manner, we generated *rpr-gal4* transgenic flies. Previously, the 1.3 kb *rpr* upstream sequence was shown to contain an ecdysone response element (EcRE) and to be sufficient for ecdysone-dependent *rpr* expression in salivary glands (Jiang et al., 2000). When F1 progeny from *rpr-gal4* × UAS-mCD8-*GFP* cross were processed for Crz immunohistochemistry, 17 out of 80 vCrz neurons (21%) produced GFP signals at 1 hour APF (Fig. 6H). To further corroborate this result, *p35* expression was induced by *rpr-gal4*. We reasoned that if *rpr* is expressed in vCrz neurons, then *rpr-gal4*-induced *p35* would prevent vCrz PCD (compare with Fig. 2B). Remarkably, Crz immunohistochemistry revealed ~10 neurons even at 7-9 hours APF (Fig. 6I). Incomplete rescue of the death by *p35* is somewhat similar to the results from *rpr*-null mutation (cf. Fig. 6C). These data overall support the model in which EcR-B-induced *rpr* expression is a primary cause for vCrz PCD.

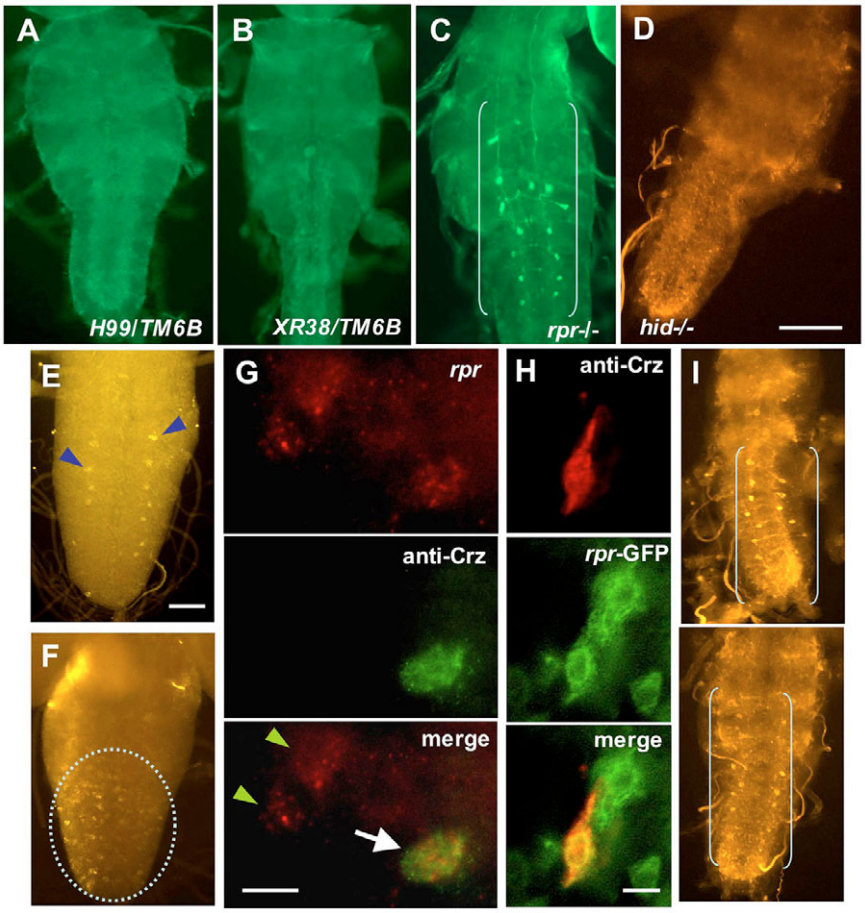

Fig. 6. The role of *rpr* in vCr2 PCD. (**A-D**) Crz-IRy in (A) *H99/TM6B*, (B) *XR38/TM6B*, (C) *rpr*-null mutant (*XR38/H99*) at 12~16 hours APF, and (D) *hid* loss-of-function mutant (*X14/hid*^{05014}) at 8 hours APF. Note that ~14 vCrz neurons are still detectable in the *rpr* mutant (brackets in C). (**E**,**F**) *Rpr* in situ hybridization of the CNSs dissected from (E) third-instar larva (*Crz-gal4*, *UAS-p35/UAS-rpr*) and (F) wild-type prepupa at 1.5 hours APF. The hybridization signals are indicated by arrowheads or by a circle. (**G**) Double labeling of wild-type CNS with *rpr* in situ hybridization probe (top) and anti-Crz (middle) at 1.5 hours APF. Merge of the two images (bottom) shows co-localization of *rpr* mRNA in a vCrz neuron (arrow). Expression of *rpr* in non-vCrz neurons is indicated by arrowheads. (**H**) *rpr* promoter activity in vCrz neurons at 1 hour APF. Crz-IRy (top), *rpr-gal4*-driven GFP expression (middle) and merge of the two (bottom). A vCrz neuron positive for both GFP and Crz-IRy appears in yellow. (**I**) *Rpr-gal4*-mediated *p35* expression (*rpr-gal4/UAS-p35*) suppresses the death of ~10 vCrz neurons (brackets) at 7-9 hours APF. Two different specimens are shown here. Scale bars: in D, 50 μm for A-D,I; in E, 50 μm for E,F; 10 μm in G,H.

diap1 is not required for survival of vCrz neurons

Although vCrz-cell death is most likely to be a cellular response to an ecdysone surge occurring at pupariation, this event does not occur prematurely during ecdysone-led larval-to-larval moltings (Riddiford, 1993). This could be due to anti-apoptotic factors that counteract the pro-apoptotic machinery in larval vCrz neurons. The best candidate for this role is *diap1*, the anti-apoptotic activity of which comes from inhibition of caspase activity (reviewed by Bergmann et al., 2003). If *diap1* is important for the survival of vCrz neurons during larval growth, then these larval neurons would contain high levels of DIAP1. Our immunohistochemistry, however, did not detect any DIAP1-IRy within larval vCrz neurons of wild type (data not shown, n=12). Despite this, as undetectable levels of DIAP1 still could be sufficient for this role, we attempted to knockdown *diap1* transcript levels via RNA interference (RNAi). For this, progeny from *symUAS-diap1*^{RNAi} × *UAS-mCD8-GFP*; *Crz-gal4* cross was examined for GFP signals. Such a transgenic manipulation was intended to produce double-stranded RNAs from complementary RNAs symmetrically transcribed from the *diap1* cDNA, which then mediate degradation of *diap1* mRNA specifically in Crz neurons (Giordano et al., 2002; Huh et al., 2004). Our data showed that GFP expression at 1 hour APF in *diap1*^{RNAi} was essentially the same as that in wild type (compare Fig. 7B with 7A), indicating that *diap1*-knockdown did not cause premature cell death. Similar results were obtained when two copies of the *Crz-gal4* transgene were employed to increase *diap1*^{RNAi} dose or by heat-shock-induced *diap1*^{RNAi} (data not shown) (cf. Yin and Thummel, 2004).

As the negative results could be due to the lack of effective *diap1*^{RNAi}, we checked the validity of *symUAS-diap1*^{RNAi} in salivary gland PCD. In doing so, the *34B-gal4*; *UAS-GFP* (salivary gland-specific driver) (Yin and Thummel, 2004) was crossed to the *symUAS-diap1*^{RNAi}, and then GFP signals were examined in white prepupa. In contrast to intense signals seen in control, such signals were significantly reduced by *diap1*^{RNAi} expression, reflecting premature death of this tissue (Fig. 7C). This is consistent with a previous report employing *heat shock-diap1*^{RNAi} (Yin and Thummel, 2004), thus validating *symUAS-diap1*^{RNAi}.

Genetic reduction of *diap1* levels in half also did not accelerate death of vCrz neurons, as mutants heterozygous for *th*^{4} or *Df(3L)brm11* alleles showed normally scheduled death of vCrz neurons (data not shown), although these alleles dominantly exacerbate *rpr*-induced death of eyes (e.g. Lisi et al., 2000). These overall results support that RPR-mediated caspase activation does not involve the role of RPR as a DIAP1 antagonist.

As the pro-apoptotic action of RPR can be nullified by DIAP1 (e.g. Lisi et al., 2000), we tested whether gain-of-*diap1* functions can suppress vCrz PCD. This was accomplished in two ways: one is *th*^{SL} allele that was shown to suppress *rpr*-induced photoreceptor cell death (Lisi et al., 2000); the other is from transgenic *diap1* expression in Crz neurons (*Crz-gal4/UAS-diap1*). Surprisingly, neither *th*^{SL} nor ectopic *diap1* expression suppressed vCrz PCD (Fig. 7D,E). Similar results were obtained from ectopic *diap2* expression (data not shown). The negative results from ectopic *diap1* expression is unlikely to be due to the lack of active DIAP1, as the same transgenic construct rescued the PCD of salivary glands (Fig. 7F).

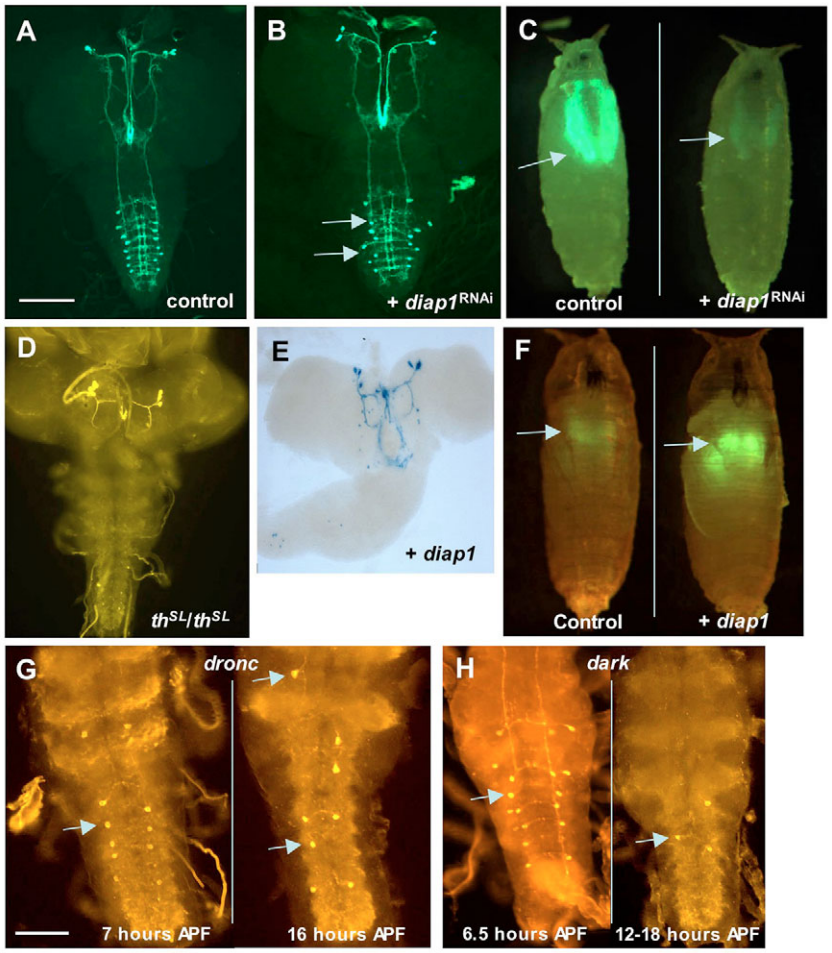

Fig. 7. Lack of role for *diap1* in the vCrz PCD. (**A**) Control (*UAS-mCD8-GFP/+;;Crz-gal4/+*). (**B**) Targeted expression of the *diap1*^{RNAi} in Crz neurons (*UAS-mCD8-GFP/+*; *symUAS-diap1*^{RNAi}*/+*; *Crz-gal4/+*) (cf. Huh et al., 2004). Intact GFP signals (vCrz) at 1 hour APF suggest that knockdown of endogenous *diap1* does not cause premature cell death. (**C**) Effect of *diap1*^{RNAi} on premature death of the salivary glands. The *symUAS-diap1*^{RNAi} or *y w* (control) was crossed to the *34B-gal4*; *UAS-GFP*, and then white prepupal progeny were examined for GFP. In the control, intense GFP signals are seen in a pair of the salivary glands (left, arrow), whereas the signals are significantly weakened by *diap1*^{RNAi} (right, arrow). (**D**) Crz-IRy in homozygous *th*^{SL} at 6.5 hours APF. (**E**) X-gal histochemistry of UAS-*lacZ*/+; *Crz-gal4/UAS-diap1* at 6 hours APF. Both genetic and transgenic *diap1* gain of function do not prevent vCrz death. (**F**) Rescue of the salivary gland degeneration by *diap1*. In a control pupa (*34B-gal4/+*; *UAS-GFP/+*, *n*=5) at 18 hours APF, faint GFP signals (left, arrow) reflect the PCD in this tissue. By contrast, strong GFP signals (right, arrow) are maintained by *diap1* expression (*34B-gal4/+*; *UAS-GFP/UAS-diap1*, *n*=8). (**G**,**H**) Delayed PCD of vCrz neurons (G) in a *dronc*-null mutant (*dronc*^{51}*/dronc*^{I24}) or (H) in a homozygous *dark*-null mutant (*dark*^{CD4}). Arrows indicate Crz-IR neurons that still survive at stages indicated in each panel. Scale bars: in A, 100 μm for A,B,D,E; in G, 50 μm for G,H.

As the apical caspase DRONC is a primary target of DIAP1 (Meier et al., 2000; Muro et al., 2002; Wilson et al., 2002), the lack of DIAP1 function may suggest that DRONC is not responsible for vCrz death. Contradictory to this prediction, vCrz PCD was significantly delayed in *dronc*-null mutants (*dronc*^{51}*/dronc*^{I24}) (Chew et al., 2004; Xu et al., 2005). Approximately 12 neurons still survived at 7 hours APF, while the number was reduced to ~8 at 16 hours APF (Fig. 7G), and none at 48 hours APF (data not shown). Comparable delay of the death was observed in another *dronc*-null mutant (*dronc*^{51}*/dronc*^{I29}) (data not shown), suggesting that DRONC is a caspase that executes vCrz-cell death. However, the delay of death in the absence of DRONC indicates that activities from other caspases are also required for timely execution of vCrz PCD.

Activation of DRONC may require the adaptor protein DARK, a fly homolog of vertebrate Apaf1 (Rodriguez et al., 1999). If so, then lack of DARK may phenocopy *dronc*-null mutants. Remarkably, Crz-IR patterns in a homozygous *dark*-null mutant (*dark*^{CD4}) were comparable with those of *dronc* mutants (compare Fig. 7H with 7G). These data suggest that an apoptosome consisting of DRONC and DARK is an essential component for vCrz PCD.

DISCUSSION

During post-embryonic CNS development in *Drosophila*, two prominent waves of neuronal cell death have been observed mostly in the VNC: the first during prepupal stage; and the second within 24 hours after adult emergence (reviewed by Truman et al., 1993). As for the latter, a group of ~300 neurons (termed type II), which had been characterized by high levels of EcR-A expression throughout the latter half of pupal development and a subset of neurons expressing CCAP neuropeptide in the VNC, undergo PCD in response to the fall in ecdysone levels (Robinow et al., 1993; Robinow et al., 1997; Draizen et al., 1999). Such hormonal change triggers accumulation of *rpr* and *grim* transcripts (Robinow et al., 1997; Draizen et al., 1999); at least for CCAP neurons, *rpr* was verified to be a death activator (Peterson et al., 2002). It is, however, not known how the fall in ecdysone levels is signaled to activate *rpr* expression and whether *rpr* promotes the death by antagonizing DIAP1 functions.

In contrast to the post-eclosion neurons just described, vCrz neurons are removed via PCD soon after the onset of metamorphosis (this study). Although we have no direct evidence for ecdysone as a death signal for this event, our data suggest that activation of EcR-B in response to a surge of ecdysone at pupariation might be a key upstream molecular event that, in turn, stimulates an irreversible death pathway in which *rpr* plays a crucial role. Therefore, these two comparative model systems show how ecdysone regulates the PCD of distinct neuronal groups at different developmental stages.

Interestingly, EcR-B receptors are also major signal transducers for remodeling of persistent larval neurons during early metamorphosis. For example, SCP-IR neurons and mushroom body γ-neurons initially lose their neurites at this stage (Schubiger et al., 1998; Schubiger et al., 2003; Lee et al., 2000b). Genetic ablations of *EcR-B* prevent such processes, and the mutant phenotype is rescued by transgenic expression of EcR-B isoforms. Therefore, EcR-B-

mediated signal transduction probably controls remodeling of persistent neurons, as well as PCD of obsolete neurons during this crucial developmental period. These observations then raise the important issue of how distinct neuronal fates (remodeling versus death) are determined in response to the same hormonal stimulus and receptor types involved. Perhaps activated EcR-B in persistent neurons might silence the death pathway or turn on anti-apoptotic activities.

Apoptotic pathways downstream of activated EcR

In the case of ecdysone-triggered salivary gland and midgut degeneration, northern blotting has shown that *rpr* and *hid* are transcriptionally induced just prior to death (Jiang et al., 1997; Jiang et al., 2000). Such induction requires BR-C functions, as *rpr* and *hid* expression in these tissues is impaired by rbp^5 and $2Bc^2$ mutant alleles of the *BR-C* locus (Jiang et al., 2000; Lee et al., 2002). Ecdysone also directly activates *rpr* transcription in the salivary glands via an interaction between activated EcR and its consensus binding sequence (EcRE) within 1.3 kb upstream of the *rpr* (Jiang et al., 2000). Our various findings support *rpr* as an intracellular death promoter for vCrz PCD. Although we do not have definitive evidence for direct activation of *rpr* by ecdysone, as the 1.3 kb *rpr* promoter apparently drives *p35* expression in vCrz neurons (Fig. 6I), we favor the hypothesis that activated EcR-B directly induces *rpr* transcription in the doomed vCrz neurons. Upregulation of *rpr*, however, does not seem to require BR-C, as we found normal vCrz PCD in the *BR-C* mutants (data not shown).

Another downstream target of the ecdysone signaling is DRONC. In vitro treatment of salivary glands and midgut with ecdysone induces *dronc* expression (Dorstyn et al., 1999), perhaps through a direct interaction between activated EcR-B1 and a consensus EcRE found in the *dronc* promoter (Cakouros et al., 2002; Cakouros et al., 2004). Upregulation of *dronc* transcription may be important for supplying doomed cells with a sufficient amount of DRONC in order to conduct massive cellular destruction in response to death signals. Further analysis will be necessary to determine if this also occurs in doomed vCrz neurons.

Involvement of RPR and caspases in the vCrz PCD raises another fundamental issue of how RPR leads to caspase activation. According to a current model, interactions between RPR and DIAP1 antagonize the inhibitory action of DIAP1, resulting in the accumulation of free active caspases (reviewed by Bergmann et al., 2003). By contrast, we did not find any evidence for DIAP1 as a survival factor for vCrz neurons. Although other DIAP1-like proteins in *Drosophila* (DIAP2, BRUCE and DETERIN) are possibly functional in this system, we speculate that this might not be the case. This is because failure of ectopic *diap1* expression to block vCrz PCD suggests that other DIAP family members would not be effective in inhibiting vCrz-cell death, as anti-apoptotic functions of these proteins are mediated by consensus BIR domains (Bergmann et al., 2003). Thus, we propose that RPR-mediated caspase activation occurs independently of DIAP1 in vCrz neurons.

RPR perhaps mediates an assembly of the apoptosome, as both DRONC and DARK – two essential components of the apoptosome – are necessary for vCrz PCD (Fig. 7G,H). In vertebrate cells, the formation of the apoptosome triggered by the release of cytochrome C (cytC) from mitochondria is an essential step towards caspase activation (Liu et al., 1996; Cain et al., 1999). Although such molecular events are not evident in flies yet, a recent report showed that cytC-d is able to activate caspases during spermatogenesis (Arama et al., 2006). In this regard, it will be interesting to determine roles of cytC as an upstream regulator for caspase activation for vCrz PCD.

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