

**Nuclear Receptors in Ecdysone-mediated Programmed Cell
Death in *Drosophila melanogaster***

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

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August 2011

This thesis is dedicated to my parents,
Brij Mohan Sehgal and Neelam Sehgal,
without whose endless hard work and values,
this would not have been possible.

Acknowledgments

I would like to thank my advisor, Dr. Jae Park for providing me scientific support, academic guidance, and his time. I am thankful to my committee members, Dr. Bruce Mckee, and Dr. Mariano Labrador, for their help and critical evaluation of my research.

I am grateful to all members in Dr. Park's laboratory including Dr. Gyunghye Lee, Zixing Wang, Kai Sha and Sudershana Nair, for the stimulating discussions and making lab a fun place to work. I would also like to thank Faith Creekmore for helping me with some cloning work.

A special thanks goes to my friends, Sangeetha Rajagopalan and Ritin Sharma for their invaluable help and for being there whenever I needed them. Lastly, I would like to thank my brother, Siddharth Sehgal and my sister-in-law, Tripti Bhaskar, without whose love and encouragement I could not have succeeded in my study.

Abstract

The steroid hormone ecdysone plays vital roles during *Drosophila* development. Pulses of 20E during *Drosophila* life cycle function as temporal cues, signaling the onset of metamorphic processes, including the stage specific programmed cell death of larval tissues. Ecdysone is the critical developmental cue orchestrating the metamorphic reformation of CNS, resulting in the formation of adult-specific neural circuitry. Ecdysone signaling is transduced by a heterodimeric receptor complex formed between two nuclear receptors: EcR and Ultraspiracle (USP). There are 18 nuclear receptors known in *Drosophila* and EcR is the only receptor whose functions in neuronal PCD have been well recognized. Therefore, the current study is aimed to define the role of nuclear receptors in neuronal cell death mechanisms in *Drosophila*. Here, I examine the function of nuclear receptors in PCD of two groups of peptidergic neurons: vCrz and CCAP.

EcR and USP receptor complex on activation results in the coordinated transcriptional regulation of a host of transcription factors regulating genes essential for PCD. USP plays a dual role in ecdysone response, as its function is necessary for both activation and repression of ecdysone primary response genes. I have developed a possible dominant-negative mutant USP (*usp³*), and expressed it in flies using the GAL4-UAS system to illustrate the role of USP in ecdysone mediated PCD of vCrz neurons. Targeted expression of *usp³* in corazonin neurons results in a complete blockage of PCD pathway. Another interacting partner of USP, *Drosophila* Hormone Receptor 38, however shows no involvement in PCD of vCrz neurons. I have also designed an ecdysone sensor to monitor the developmental timing of EcR activation in vCrz neurons.

Further, I investigate the survival factors required for preventing the untimely PCD of these two groups of neurons. The study reveals that DIAP1 is required for the survival of larval vCrz and CCAP neurons. Also, the nuclear receptor E75 is shown to be critical for preventing premature PCD of CCAP neurons.

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List of abbreviations

20E: 20-HydroxyEcdysone

aCCAP: abdominal ganglia CCAP

APF: After puparium Formation

BR-C: Broad Complex

CCAP: Crustacean CardioActive Peptide

CNS: Central Nervous System

Crz: Corazonin

DBD: DNA binding domain

dCCAP: dorsal CCAP

Dcp-1: Drosophila caspase-1

DHR38: Drosophila Hormone Receptor 38

DIAP: Drosophila Inhibitor of Apoptosis

DL: Dorsal Lateral

DM: Dorsal Medial

drICE: Drosophila Interleukin-1 β -Converting Enzyme

EcR: Ecdysone Receptor

EcRE: Ecdysone response element

ELAV: Embryo Lethal Abnormal Vision

GFP: Green Fluorescent Protein

GMR: Glass Multiple Repeat

Hid: Head Involution Defective

IAP: Inhibitor of Apoptosis

IHC: Immunohistochemistry

LBD: Ligand binding domain

mCCAP: Medial protocerebrum CCAP

PCD: Programmed Cell Death

Pdf: Pigment Dispersing Factor

RHG: Rpr, Hid, Grim

Rpr: Reaper

sCCAP: subesophageal ganglia CCAP

SCP: Small Cardioactive Peptide B

Skl: Sickie

USP: Ultraspiracle

vCCAP: ventral CCAP

vCrz: Crz in the ventral nerve cord

VNC: Ventral Nerve Cord

Chapter I

Background and Significance

I.1 Nuclear Receptors

I.1.1 Overview

Nuclear receptors are a class of proteins that act as ligand-regulated transcription factors. Nuclear receptors are ancient proteins, identified throughout metazoa, but are absent in plants and yeast (Thornton 2003). They comprise of a large superfamily of evolutionarily related DNA-binding transcription factors that regulate a wide variety of metabolic and developmental processes. Members of the nuclear receptor superfamily are characterized by a common protein structure, comprising of a highly conserved globular domain for DNA-binding and a less conserved α -helical globular domain at the C-terminus responsible for ligand binding and dimerization (LBD). The DNA-binding domain (DBD) is followed by a structurally variable N-terminal domain involved in transcriptional regulation.

Nuclear receptor genes have been classified into six subfamilies based on the sequence similarity between their respective DBDs and LBDs. Small lipophilic molecules like steroids, thyroid hormone, retinoic acid and vitamin D bind to and activate nuclear receptors. Nuclear receptors having no known endogenous ligand, act in a ligand-independent manner and are referred to as orphan receptors (Mangelsdorf & Evans 1995).

Nuclear receptors play key regulatory roles in various biological processes,

including lipid and glucose homeostasis, detoxification, cellular differentiation and embryonic development. Mutations in nuclear receptor genes are associated with many human diseases, including cancer, diabetes, and heart disorders (Francis et al 2003).

Nuclear receptors comprise of six domains; A/B domain at the N-terminal, a central DBD, a LBD located in the C-terminal, a hinge region joining the DBD and LBD, and a region F at the C-terminal (Kumar & Thompson 1999) (Fig. 1-1). The N-terminal A/B domain (NTD) is highly variable in sequence and structure between various nuclear receptors. The NTD is a regulatory domain, containing the transactivation domain, activation function 1 (AF-1). AF-1 functions in a ligand-independent fashion and is also implicated in protein-protein interaction.

The DBD comprises of two C4 type zinc finger domains, containing four cysteine residues each. The first zinc finger is mainly involved in site-specific recognition, via a stretch of five amino acids, termed P-box making specific amino acid-nucleotide contacts (Umesono & Evans 1989). The second zinc finger provides the DBD homodimerization interface, allowing the DBDs to dimerize when a target DNA molecule is present (Renaud & Moras 2000). Nuclear receptors regulate gene expression by binding to DNA response elements (REs) of the target genes. The response elements comprise half-sites that are derived from, or identical to the archetypical AGGTCA sequence. The receptor specificity is provided by the sequence and arrangement of half-sites, which can be arranged as direct, inverted, or everted repeats (Acevedo & Kraus 2004).

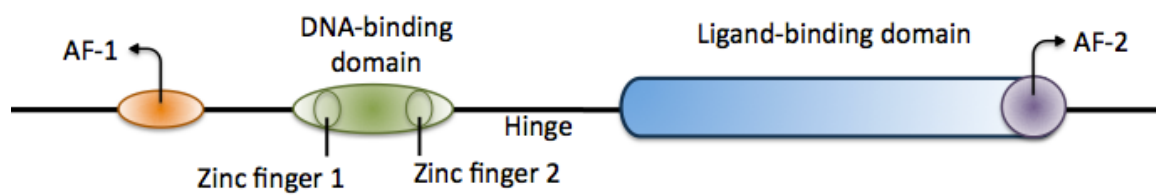
The ligand-binding domain consists of 12 α -helices (H1-H12), folded such that a hydrophobic pocket is formed for ligand binding (Egea et al 2000). The C-terminal part of LBD has a transactivator domain termed AF 2, which functions in a ligand-dependent

manner. Structural studies have shown that, on ligand binding, helix 12 of LBD rotates and acquires a configuration where it can recruit transcriptional co-activators through its AF-2 domain, thereby switching LBD in an active state (King-Jones & Thummel 2005). In addition to binding ligands and co-regulatory proteins, LBD also functions as the principal dimerization interface of the receptor, allowing the dimerization of different receptors (Bourguet et al 2000; Nolte et al 1998).

I.1.2. Nuclear Receptor Mechanisms of Action

Extensive studies have provided insights into the molecular mechanism of nuclear receptor regulation as well as understanding on how the nuclear receptors transduce hormonal signals into a transcriptional response (King-Jones & Thummel 2005). In the absence of ligands, free nuclear receptors occur either in the cytoplasm or in the nucleus. Nuclear receptor ligands are small lipophilic molecules that diffuse through plasma and nuclear membranes to bind nuclear receptors. Ligand binding initiates structural changes within the receptor and facilitates its binding to response elements of target genes.

Based on the mechanism of action, nuclear receptors are classified into type I, type II, and type III. Type I receptors, such as, progesterone (PR), estrogen (ER), androgen (AR), glucocorticoid (GR), and mineralocorticoid (MR) receptors, are inactive in the absence of ligand, and usually reside in the cytoplasm (Chawla et al 2001). Upon hormone binding, type I receptors form homodimers and translocate to the nucleus, where they bind to the specific response elements (inverted repeats) and regulate transcription of the target gene (Fig. 1-2A).



(Adapted from (Nikolenko Iu & Krasnov 2007))

Figure 1-1. **Domain organization of Nuclear Receptors.**

Type II receptors, such as, 9-cis retinoic acid (RXR), trans retinoic acid (RAR), and vitamin D3 (VDR) receptors, are localized in the nucleus regardless of their ligand binding status. They are constitutively bound to REs as heterodimers. In the absence of ligand, some type II receptors are associated with co-repressor complexes to silence the target gene activity. Ligand binding triggers the release of co-repressor and simultaneous recruitment of co-activator to induce the transcriptions of target gene (McKenna et al 1999) (Fig. 1-2B).

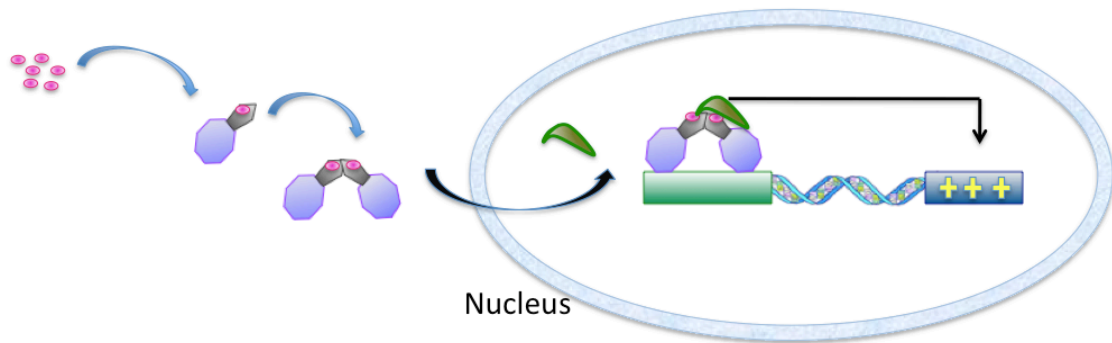
Type III includes the orphan nuclear receptors, such as, farnesoid X receptor (FXR), liver X receptor (LXR), steroidogenic factor 1 (SF-1) and peroxisome proliferator-activated receptor (PPAR). Type III SF-1 receptor functions as a constitutive transcription activator, binding to its REs as a monomer. The transcriptional activity of SF-1 is regulated via a specific kinase, which phosphorylates and stabilizes the active conformation of SF-1 to enhance its activating properties (Hammer et al 1999) (Fig. 1-2C).

1.1.3 Nuclear Receptors in *Drosophila*

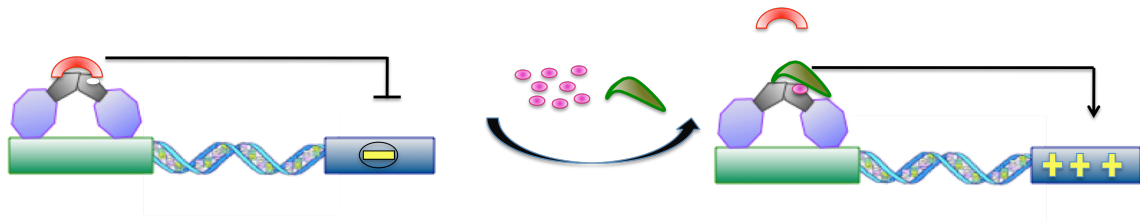
In contrast to 48 nuclear receptors found in humans, *Drosophila melanogaster* only has 18 nuclear receptors, which represent all six subfamilies of vertebrate receptors (Laudet 1997). Also, only two known physiologically active lipophilic hormones exist in *Drosophila*, the steroid hormone 20-hydroxyecdysone (20E) and the sesquiterpinoid juvenile hormone (JH).

Steroid hormone 20E is an active metabolite of ecdysone and plays vital roles during *Drosophila* development, including molting and metamorphosis

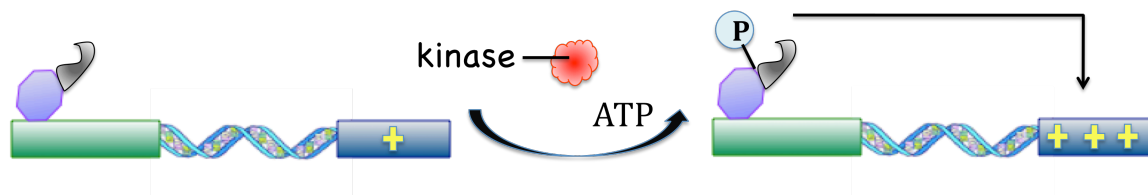
A



B



C



(Adapted from King-Jones et al., 2005)

Figure 1-2 **Molecular mechanism of nuclear receptor action.** (A) Type I receptors. (B) Type II receptors. (C) Type III receptors.

(Gilbert et al 2002; Riddiford 1993). Pulses of 20E during *Drosophila* life cycle function as temporal cues, signaling the onset of metamorphic processes, including the stage specific programmed cell death of larval tissues.

Ecdysone signaling is transduced via a heterodimer of two nuclear receptors, the ecdysone receptor (EcR) and ultraspiracle (USP), which together make up a functional ecdysone receptor complex (Yao et al 1993). EcR and USP share the typical domain structure common to all nuclear receptors and are orthologues of the vertebrate farnesoid X receptor (FXR) or liver X receptor (LXR), and RXR receptors, respectively. Ecdysone receptor is constitutively localized in the nucleus and regulates the transcription of wide variety of genes. EcR/USP belong to type II class of nuclear receptors, with liganded EcR/USP functioning as a transcriptional activator and the unliganded receptor acting as a repressor of transcription (Hu et al 2003; Schubiger & Truman 2000).

EcR exists in three protein isoforms, A, B1, and B2, while *usp* produces only one isoform. The three isoforms of EcR differ in their amino terminal domains, particularly the isoform specific A/B domain. EcR-B isoforms function as ligand-dependent transcriptional activator, whereas EcR-A is a weak transcriptional activator (Mouillet et al 2001). USP does not impart any activation function, but acts in an allosteric manner, facilitating the DNA and ligand-binding activities of EcR. Structural studies of USP LBD suggest that, helix 12 is locked in an inactive conformation and is thus unable to bind 20E (Billas et al 2001; Clayton et al 2001). EcR, on the other hand is incapable of ligand binding on its own. The ligand-binding domain of EcR mediates binding to 20E as well as dimerization with USP, the two processes being interdependent. EcR must dimerize with USP, such that the highly flexible ligand-binding pocket of EcR is stabilized in a

conformation capable of binding hormone (Billas et al 2003).

The DNA binding domains of EcR and USP bind to specific DNA sequences called ecdysone response elements (EcREs) in the promoters of ecdysone responsive genes. Interestingly, genes encoding six nuclear receptors - *Drosophila* hormone receptor *DHR3*, *DHR4*, *DHR39*, *E75*, *E78*, and *FTZ-f1* are transcriptionally regulated by EcR/USP complex.

E75 is an orphan nuclear receptor, similar in sequence to the human REV-ERBA receptor. The *E75* gene encodes three protein isoforms, each distinguished by a unique N-terminal sequence (Segraves & Hogness 1990). *E75A*-specific null mutants show reduced ecdysteroid titres in their body, defining a feed-forward pathway between *E75A* and 20E (Bialecki et al 2002). The ligand-binding domain of *E75* contains a heme group, through which, it can bind small gas molecules such as nitric oxide and carbon monoxide that function in stress-response pathways (Reinking et al 2005).

DHR3 is the *Drosophila* orthologue of mammalian RAR-related orphan receptor (ROR), which plays essential roles during embryogenesis. It functions to activate the transcription of orphan nuclear receptor *βFTZ-f1*, via direct binding to its promoter (Kageyama et al 1997). The heterodimerization of *DHR3* with *E75B* results in suppression of *βFTZ-f1* transcription. Thereby, interplay between *DHR3* accumulation and *E75B* decay, determines the precise timing of *βFTZ-f1* activation (White et al 1997). *DHR4*, encoding the closest homologue of the vertebrate orphan receptor germ-cell nuclear factor (GCNF), functions in redundant manner to *DHR3* (Zhang & Dufau 2004).

The *FTZ-f1* receptor exists in two isoforms, α *FTZ-f1* and β *FTZ-f1*, expressed during early embryogenesis and mid-prepupa, respectively (Yamada et al 2000). *FTZ-f1*

shares high sequence and functional similarity to nuclear receptor DHR39, with both binding to identical DNA sequences (Ayer et al 1993; Ohno et al 1994). FTZ-F1 and DHR39 are very similar to vertebrate liver receptor homologue 1 (LRH-1) and steroidogenic factor 1 (SF-1). β FTZ-f1 acts as a competence factor, directing appropriate stage specific responses to the second 20E pulse.

Nuclear receptor Tailless (TLL) and Dissatisfaction (DSF), function as a transcriptional repressors, and are similar to their mammalian orthologue (Pitman et al 2002), TLX. TLL is involved in embryonic patterning as well as required for the development of all protocerebral neuroblasts and the embryonic visual system (Miyawaki et al 2004; Shi et al 2004). The DSF receptor on the other hand, plays critical role in maintaining adult sexual behaviour (Finley et al 1998).

Similar to TLL, nuclear receptor Sevenup (SVP) has critical roles in neuronal development. SVP is required for the development of photoreceptor cells and for the proper establishment of embryonic CNS. Chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1), represents the vertebrate orthologue of SVP receptor. Interestingly, SVP heterodimerizes with USP and competes with EcR-USP for DNA binding.

DHR38, the *Drosophila* orthologue of mammalian NGFI-B subfamily of orphan receptors, is responsible for mediating a second ecdysteroid signaling pathway in *Drosophila*. DHR38 receptor is able to dimerize with USP and be activated by a distinct class of ecdysteroids in an EcR independent manner. The crystal structure of DHR38 reveals that it lacks both a co-activator binding site and a ligand-binding pocket. The activation of DHR38-USP heterodimer by ecdysteroids, requires a transactivated USP

and does not involve direct binding of the agonist to the receptor complex. Like USP, DHR38 is widely expressed during development, and is critical for cuticle formation.

DHR78 receptor, the orthologue of vertebrate TR2 (NR2C1) and TR4 (NR2C2), plays essential function during tracheal development. Also, DHR78 is able to bind a subset of EcR-USP binding site and inhibit 20E-induced transcription of certain target genes. The functions of DHR51, DHR83, DHR96, HNF4, and ERR, remains elusive due to the lack of availability of mutants or valid phenotype.

I.2 Programmed Cell Death

I.2.1 Significance of Programmed Cell Death

Programmed cell death, or apoptosis, is a widespread and conserved cellular process, occurring in all metazoans including nematodes, flies and mammals. The process of apoptosis is generally characterized by cell shrinkage, nuclear fragmentation, chromatin condensation and DNA fragmentation. Apoptosis plays a vital role during development and for maintaining the homeostasis of metazoan organisms.

During development, apoptosis helps in removing superfluous cells and eliminating tissues that have outlived their function. Therefore, proper execution of PCD is critical for normal developmental processes such as sculpturing bodies and metamorphosis. For example, the differentiation of digits occurs by the timely elimination of interdigital mesenchymal tissue via apoptosis (Zuzarte-Luis & Hurlle 2002). In *Xenopus*, the resorption of tail and dorsal muscles of tadpole during metamorphosis occurs by apoptosis (Dodd MHI 1976). The formation of correct synaptic connections in the vertebrate nervous system requires the elimination of surplus neurons

by means of apoptosis (Raff et al 1993). More than half of the neurons die prior to the completion of neural architecture during development (Ohsawa & Miura 2008). PCD helps in sexual dimorphism by deleting sex-specific cells or organs that are required in one sex but not in the other (Jacobson et al 1997). PCD also performs a proofreading function, by removing gametes and embryonic cells containing damaged DNA or abnormal chromosomal content during development (Vaux & Korsmeyer 1999).

PCD contributes to the proper development and functioning of the immune system. During lymphocyte development, the cells that fail to generate functional and nonautoreactive antigen receptors, lose developmental signals and undergo PCD (Melchers et al 2000). PCD also provides defense against the cells that are damaged by pathogens or the cells that proliferate inappropriately (Benedict et al 2002).

Deregulation of PCD is associated with many developmental defects and pathological conditions. Defects in apoptotic machinery may cause autoimmune diseases, such as lupus erythematosus and rheumatoid arthritis (Vaux & Flavell 2000). Inappropriate cell death is linked to neurodegenerative disorders, stroke, and cardiac ischaemia (Thompson 1995). Conversely, suppression of naturally occurring cell death or its failure in removing damaged cells, contributes to cancer (Green & Steinmetz 2002).

1.2.2 Programmed Cell Death Mechanisms in *Drosophila*

The components of apoptotic pathway and its mechanisms of regulation are highly conserved from flies to humans. Members of cysteine protease family, caspases, constitute the central components of the cell death machinery. Caspases are synthesized as inactive zymogens, which become activated in response to different death signals.

Physiological signals such as steroid hormones and cytokines or cellular stresses resulting from radiation, viral infection or nutrient deprivation, trigger programmed cell death in organisms. Since most cells constitutively express caspase zymogens, the key to cell death and survival signaling lies with the regulation of active caspase levels in the cells. The molecular mechanism underlying PCD constitutes a complex interaction among pro-death factors, including proapoptotic proteins and caspases, and anti-apoptotic factors such as inhibitor of apoptosis proteins.

The identification of genes involved in *Drosophila* PCD resulted from extensive genetic screening studies. The screening of homozygous deficiencies for loss of embryonic PCD resulted in identification of the *H99* deficiency, which removes approximately 300 kb of genomic DNA in the 75C1 region on chromosome arm 3L (White et al 1994). Analysis of the *H99* region revealed the presence of three proapoptotic genes: *reaper* (White et al 1994), *hid* (Grether et al 1995), and *grim* (Chen et al 1996), collectively known as the RHG genes. Subsequently, three additional proapoptotic genes were identified in *Drosophila*: *sickle* (*skl*) (Christich et al 2002; Srinivasula et al 2002), *jafrac2* (Tenev et al 2002), and *dOmi/HtrA2* (Khan et al 2008).

The ectopic expression of any of the proapoptotic genes is sufficient to induce cell death. RHG genes encode novel proteins, varying considerably in size and lacking substantial homology among each other. However, they share a conserved N-terminal region, which is referred to as RHG domain or IBM (IAP binding motif). This domain is critical for the death activating functions of the RHG proteins (Bergmann et al 2003; Srinivasula et al 2002).

Another genetic screening study in *Drosophila*, resulted in the identification of inhibitor of apoptosis proteins (IAPs) (Hay et al 1995). IAPs inhibit PCD by binding to caspases (Salvesen & Duckett 2002). The binding to caspases as well as RHG proteins is mediated by the baculoviral IAP repeat (BIR) motif. BIR motif is essential for the anti-apoptotic function of IAPs. Some IAPs also contain a C-terminal RING domain, responsible for E3-ubiquitin ligase activity (Yang et al 2000). RHG proteins, caspases, and IAPs themselves are the targets for RING-mediated ubiquitylation (Bergmann et al 2003).

Four IAPs have been identified in *Drosophila*: DIAP1, DIAP2, Deterin, and dBruce. DIAP1 is the most important anti-apoptotic protein in *Drosophila*. It contains two BIR and one RING domain. The loss of function mutations in *diap1* occurs in either of the BIR domains, affecting DIAP1's ability to bind and inhibit caspases, and consequently resulting in uncontrolled cell death. Conversely, mutations in BIR domains that affect its ability to interact with RHG proteins, result in suppression of RHG-mediated apoptosis (Goyal et al 2000). In living cells, DIAP1 binds to caspases through its BIR2 domain, and this interaction allows the RING domain to target caspases for degradation via ubiquitin-mediated proteolysis (Wilson et al 2002). However, the binding of RHG proteins to DIAP1 in the dying cells alters the substrate specificity of RING domain, resulting in autoubiquitylation and self-degradation of DIAP1 (Yoo et al 2002).

Studies on DIAP2 suggest that it is not a critical component for *Drosophila* PCD. Though DIAP2 overexpression is able to suppress RPR and HID mediated cell death (Wing et al 1998), null mutations in *diap2* have no effect on developmental apoptosis (Huh et al 2007; Leulier et al 2006). The anti-apoptotic protein, dBruce, has been shown

to inhibit *rpr* and *grim*, but not *hid*-induced cell death (Vernooy et al 2002). The role of Deterin as an anti apoptotic factor is elusive due to lack of mutants (Jones et al 2000). Caspases are the main executioners for PCD, consisting of a prodomain and two catalytic subunits. Based on their mode of activation and the length of prodomain, caspases are classified into two types: initiators and effectors. The initiator caspases are characterized by a long N-terminal prodomain that harbors CARD (caspase activation and recruitment domain) or DED (death effector domain) domains. The initiators cleave and activate the effector caspases, which on the other hand can cleave a number of cellular components, resulting in apoptotic cell death.

The *Drosophila* genome encodes seven caspases: DRONC, DREDD, STRICA being the initiator caspases and DCP-1, DRICE, DECAY, and DAMM, the effectors (Kumar 2007). DRONC is the most important initiator caspase, essential for PCD during embryogenesis and metamorphosis (Dorstyn et al 1999). STRICA plays a minor role in cell death, its function being redundant with DRONC (Baum et al 2007). DREDD, on the other hand, is known to be more involved in non-apoptotic processes like the innate immune responses (Hultmark 2003). Among effector caspases, DCP-1 and DRICE are known to play major role in PCD, with the two functioning in a tissue-specific manner.

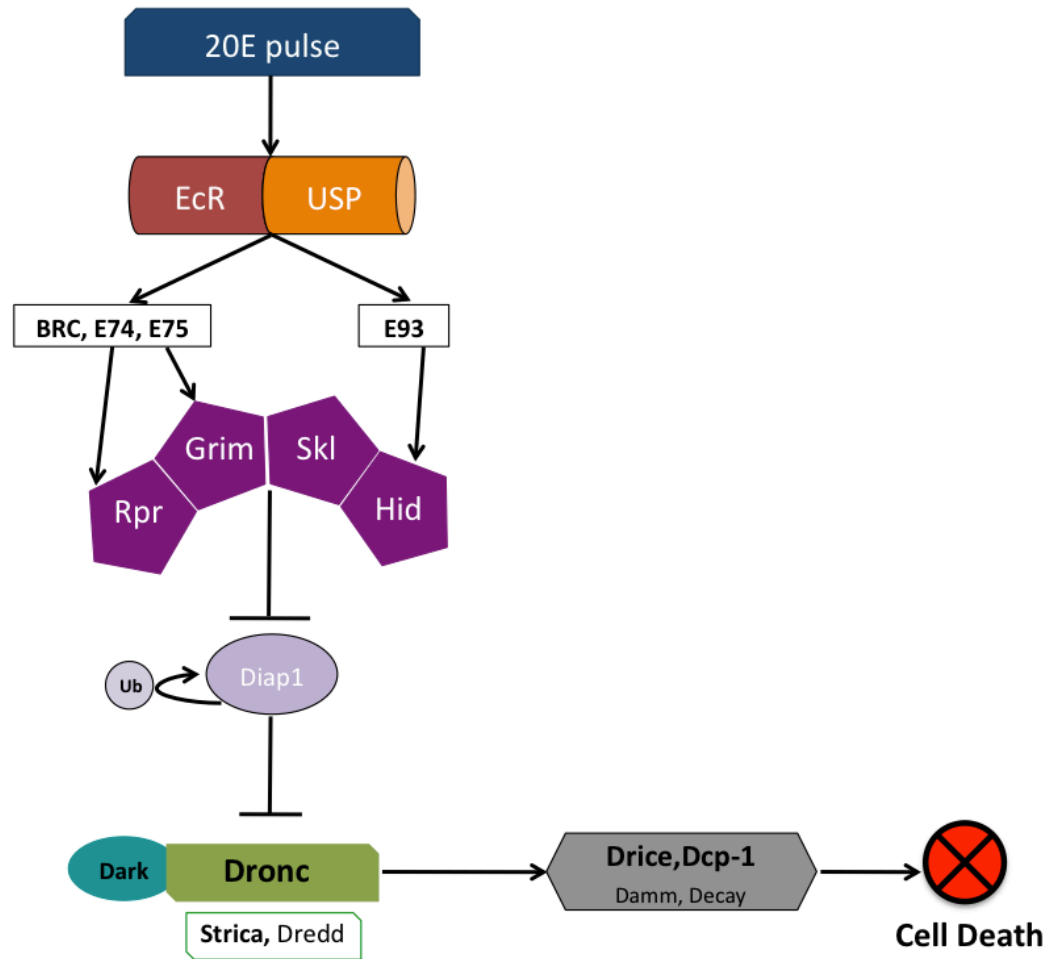
I.2.3 Ecdysone-mediated Developmental Cell Death in *Drosophila*

Drosophila life cycle, includes embryonic, three larval instars, prepupal, pupal, and adult developmental stages. The steroid hormone ecdysone, acts as a critical temporal cue, regulating the developmental transitions during each stage. Two consecutive pulses of ecdysone during larval-pupal transition, direct the transform of a crawling larva into a

highly mobile adult fly. The late larval pulse of ecdysone triggers larval-to-prepupal transition while the prepupal pulse ~10 hours after puparium formation, signals prepupa-to-pupa transition.

During metamorphosis, ecdysone is responsible for the destruction of obsolete larval tissues via PCD, as well as for the differentiation of stem cells to form adult structures. Ecdysone mediated PCD removes larval tissues such as salivary glands, midgut, larval muscles and neurons in a stage-specific manner, with the anterior muscles and larval midgut degenerating in response to late larval ecdysone pulse, and the larval salivary glands and abdominal muscles dying in response to pre-pupal peak.

Ecdysone exerts its effects by binding to a heterodimeric ecdysone receptor complex composed of ecdysone receptor (EcR) and ultraspiracle (USP). The ecdysone receptor complex activates the transcription of primary-response genes, *Broad Complex* (BRC), which encodes a zinc finger transcription factor, *E74*, encoding an ETS-domain transcription factor, and *E93*, which encodes a site-specific DNA binding protein. The early genes regulate the expression of a number of late genes, which are involved in specific biological responses, including PCD. BR-C is required for the transcription of cell death genes *rpr*, *hid* and *dronc*. *E74* is essential for inducing *hid* expression and *E93* induces the expression of *rpr*, *hid*, *dronc* and *croquemort* (*crq*) (Lee et al 2002). Therefore, ecdysone-mediated PCD involves a two-step regulatory hierarchy in which ecdysone-induced transcription factors regulate the expression of key death activators (Fig. 1-3). Apart from the indirect activation, ecdysone also directly induces the expression of *rpr* and *dronc* by binding to an EcR/USP response element in their promoters (Jiang et al 2000).



(Modified from (Cashio et al 2005))

Figure 1-3. **Programmed cell death mechanism in *Drosophila*.**

I.3.1 Neuronal PCD in *Drosophila*

During metamorphosis, *Drosophila* central nervous system undergoes extensive reformation in order to establish adult-specific neural circuitry (Levine et al 1995; Truman 1993). This process involves remodeling and programmed death of larval neurons. Remodeling of larval neurons is essential for acquiring adult-specific projection patterns, and is characterized by the loss of larval-specific connections followed by outgrowth of adult-specific projections (Lee et al 2000). Conversely, obsolete larval neurons, whose functions are no longer required in the later stages of development, undergo programmed cell death.

Like the metamorphosis of other larval tissues, steroid hormone ecdysone acts as the critical developmental cue orchestrating the metamorphic reformation of CNS. For example, ecdysone initiates the remodeling of mushroom body γ -neurons (Lee et al 2000).

The mechanism of neuronal PCD is best studied in a group of peptidergic neurons expressing the neuropeptide Corazonin (Crz) in the ventral nerve cord. These neurons undergo ecdysone mediated PCD shortly after the onset of metamorphosis (Choi et al 2006; Lee et al 2011). Corazonin expression is detectable in three groups of neurons in the larval CNS: three pairs of dorsolateral (DL) neurons, one pair of dorso-medial (DM) neurons and eight bilateral pairs of neurons in the ventral nerve cord (referred to as vCrz neurons). During metamorphosis, there occurs a significant change in the Crz expression patterns, the vCrz and DM neurons are eliminated via PCD while the DL neurons survive till adulthood (Choi et al 2006). It has been shown that Crz-immunoreactive signals in vCrz neurons progressively disappear in the course of 3–7 hours APF, but the cytological

signs of PCD start appearing as early as 1h APF (Lee et al 2011). The overexpression of caspase inhibitor p35 in the corazonin neurons suppresses cell death, maintaining the Crz expression in pupal VNC. Thereby elucidating that the loss of Crz expression is a result of caspase-mediated cell death, and not due to transcriptional silencing of *Crz* gene. The functional disruption of ecdysone receptor (EcR) via the ectopic expression of EcR dominant-negative and null mutant forms inhibits vCrz PCD, indicating the role of Ecdysone signaling in initiating the death pathway within doomed Crz neurons. Recent genetic studies have also shown that PCD of vCrz neurons requires *dronc*, *strica*, *dcp-1*, and *ice* as the major caspases (Lee et al 2011).

The current study is aimed to define the role of nuclear receptors in neuronal cell death mechanisms in *Drosophila*. Out of the 18 nuclear receptors known in *Drosophila*, EcR is the only receptor whose functions in neuronal PCD have been well recognized. Therefore, I intended to examine the functions of remaining nuclear receptors in PCD of two groups of peptidergic neurons (vCrz and CCAP) (Chapters II, III, IV). Further, I focus this study to determine the survival factors required for preventing the untimely PCD of these neurons (Chapter IV and V).

Chapter II

Role of Ultraspiracle (USP) in vCrz PCD

II.1 INTRODUCTION

Ultraspiracle protein (USP) is an orphan nuclear receptor and *Drosophila* homolog of vertebrate retinoid X receptor (RXR) (Oro et al 1990). USP plays essential roles during multiple stages of development including embryogenesis, larval development and pupation (Hall & Thummel 1998; Henrich et al 2000). USP heterodimerizes with EcR to form a functional ecdysone receptor, which signals appropriate transcriptional and developmental responses mediated by steroid hormone ecdysone (Hall & Thummel 1998; Yao et al 1992). The expression of USP, unlike EcR, varies only modestly throughout development (Henrich et al 1994) and the temporal profiles of the two do not show a complete overlap (Sullivan & Thummel 2003). This and the ability of USP to form heterodimers with other nuclear receptor partners such as DHR38 and Sevenup (SVP), raises the possibility of USP having functions independent of EcR (Baker et al 2003; Sutherland et al 1995).

In addition to its role in the activation of ecdysone-response genes in the presence of hormone, USP is also required for the repression of some genes in the absence of 20E. USP thus plays a dual role in ecdysone response, with its function being necessary for both activation and repression of ecdysone primary response genes (Schubiger & Truman 2000). USP is required for the upregulation of hormone responsive genes like *EcR*, *E75B* and *DHR3*. On the other hand, USP is able to suppress the premature induction of ecdysone-mediated responses during larval growth (Schubiger & Truman 2000).

Removal of USP from wing imaginal disc cells causes premature activation of key 20E-regulated target genes like β -*FTZ-fl* and *BRC-Z1* (Ghbeish et al 2001; Schubiger & Truman 2000). Similar results have also been observed in eye discs as well as in cultured cells, where USP represses some target promoters. These studies also strongly suggest that USP represses gene activity when ecdysteroid titres are low. It is, however unknown if USP exerts its repressive function in combination with EcR.

USP along with EcR has been demonstrated to play cell-autonomous roles in neuronal remodeling (Lee et al 2000), suggesting towards the heterogeneous functions of USP in different tissues that respond to ecdysone during metamorphosis. Since, previous studies have revealed the requirement of EcR for the PCD of vCrz neurons (Choi et al 2006), we aimed to define the roles of USP in ecdysone-mediated vCrz PCD. The early lethality associated with all the mutant alleles of *usp* has restricted the genetic studies elucidating the functions of USP. By over-expressing one of the mutant *usp* alleles specifically in corazonin neurons, we show that USP is critical for developmentally regulated vCrz cell death. Our data further suggests that the *usp* allele functions in a putative dominant-negative manner.

Additionally, the interaction of USP with nuclear receptor DHR38 can interfere with the formation of EcR:USP heterodimer via competition for USP (Sutherland et al 1995). The activation of DHR38 in CNS at late L3 and prepupal stages, suggests towards its function in developing CNS (Palanker et al 2006). It is possible that DHR38 by dimerizing with USP acts as a repressor of premature induction of metamorphic processes including vCrz PCD during early larval stages when the ecdysone levels are low. The high level of EcR in later stages of larval development (Truman et al 1994),

may possibly displace DHR38 to form the functionally active EcR:USP receptor complex. To this end, we analyzed the role of DHR38 in PCD of vCrz neurons. We found that both the overexpression and knockout of DHR38 have no effect on developmentally regulated cell death of vCrz neurons.

II.2 MATERIALS AND METHODS

II.2.1 *Drosophila* Stocks and Transgenic Fly Lines

Flies were maintained at 25°C under 12-h:12-h light:dark cycles on food containing yeast, cornmeal, dextrose, agar and methyl paraben (Tegosept) as a preservative. *UAS-usp³* and *UAS-usp^{WT}* were inserted into yw flies via P element transformation. Three *UAS-usp³* transformants were tested to reveal quantitatively similar results. For most of the study, we have used a transformant line of *UAS-usp³* on chromosome II. *UAS-EcR-B1^{W650A}* (Cherbas et al 2003) was used to express dominant-negative form of EcR-B1 in various tissues. For targeted expression in corazonin neurons, *UAS-usp³*, *UAS-usp^{WT}*, and *UAS-EcR-W650A* were crossed to a *Crz-gal4* driver (Choi et al 2008). The *Crz-gal4* driver used in this study is a double transgenic *Crz-gal4* line (2X) comprising of two independent transgenes in the 2nd and 3rd chromosomes. The UAS responders were crossed to the following gal4 drivers to produce various transgenic manipulations: *actin-gal4*, *tubulin-gal4*, *elav-gal4*, *GMR-gal4*, *201Y-gal4*, *UAS-mCD8-GFP*; *34B-gal4*, *UAS-mCD8-GFP*; *FG5-gal4*. *EcRE-lacZ* flies (stock# 4517) carry a reporter gene that consists of 7 copies of 30bp sequence from Hsp27, including the 23-bp EcRE region followed by the *lacZ* gene.

II.2.2 Plasmid Constructs

To generate *UAS-usp³* construct, genomic DNA was extracted from *usp³* heterozygous mutant (*usp³¹⁰*, *FRT^{19A}/FM7*, provided by Dr. Liquan Luo) having one copy of wild type and mutant *usp³* allele. A 1657-bp-long *usp* genomic region (-43 to +1614 relative to translation start site +1) was amplified by PCR using *usp*-Fw (ACCGGTACCTCCAATATACCCA) & *usp*-Rv (AGGGATCTAGAGGAGAAATGC) primers and GoTaq PCR premix (at PCR conditions: 94°C for 2 min, 94°C for 45 sec, 50°C for 45 sec, 72°C for 2 min, 33 cycles, final extension at 72°C for 5 min). The PCR products were subcloned into pGEMTeasy vector. Mutant and wild type pGEMT-*usp* clones were selected via sequence analysis (hereafter referred to as pGEMT-*usp³* and pGEMT-*usp^{WT}*, respectively). Subsequently, a *KpnI/XbaI* fragment from pGEMT-*usp³* and pGEMT-*usp^{WT}* was inserted into pUAST vector to get *UAS-usp³* and *UAS-usp^{WT}* construct, respectively.

II.2.3 Whole Mount CNS Immunohistochemistry (IHC)

Rabbit anti-Crz (Choi et al 2005; Choi et al 2006) was generated against a synthetic peptide corresponding to a region of Crz-Associated Peptide (VDPDPENSAHPRLSN) within the Crz precursor. For whole mount immunohistochemistry, anti-Crz was used at a dilution of 1: 1,200. Mouse monoclonal anti-EcR-B1 (AD4.4) (Talbot et al 1993) was used at a dilution of 1: 100 to label EcR in Crz neurons.

CNSs were dissected in phosphate-buffered saline (PBS) and fixed in fixative containing 4% paraformaldehyde and 7.5% saturated picric acid in 0.1 M sodium

phosphate buffer (SPB, pH7.4) for 2-3h at room temperature (Helfrich-Forster et al 2000). After fixation, samples were rinsed thrice in SPB and TNT buffer (0.1 M Tris, pH7.4, 0.3M NaCl, 0.5% Triton X-100) for 15 minutes each. Tissues were then incubated in blocking buffer containing 4% normal donkey serum and 0.02% NaN₃ in TNT at room temperature for 0.5–2 hours. Primary antibody was applied and incubated overnight at 4°C followed by rinsing in TNT 6 times for 10 minutes each at room temperature. To detect primary antibody, CNSs were incubated in a solution containing tetramethyl rhodamine (TRITC)-conjugated secondary antibody (Jackson ImmunoResearch) at 1:200 dilution in TNT containing 2% normal donkey serum for 1–2 hours at room temperature in dark. After rinsing in TNT (3X 15 minutes) and subsequently in SPB (3X 15 minutes), the samples were mounted in medium containing 80% glycerol, SPB, and 2% n-propyl gallate. Images were acquired by Olympus BX-61 epifluorescence microscope equipped with a CC12 camera.

II.2.4 X-gal Histochemistry

For detection of β -galactosidase expression, CNSs were dissected in PBS and fixed for 5 minutes in 0.2% glutaraldehyde in PBS at room temperature. The fixed samples were washed thrice in PBS for 5 minutes and then incubated at 37°C overnight in the buffer (10 mM Sodiumphosphate (pH 7.4), 0.15 M NaCl, 1 mM MgCl₂, 5 mM Pottassium ferrocyanide, 5mM Pottassium ferricyanide, 0.1% Triton X-100) containing X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside in dimethylformamide) with the final concentration of 1 mg/ml. After briefly rinsing in PBS, samples were dehydrated in ethanol to dissolve X-gal crystals and finally mounted in glycerol.

II.2.5 Green fluorescent protein (GFP) Staining

CNS samples were dissected in PTE (PBS/0.1% Triton X-100/25% ethanol), followed by fixation in 4% paraformaldehyde in PBS for 30 minutes at room temperature. Subsequently the samples were rinsed thrice in PBS for 5 minutes each, cleared in 30% and 60% glycerol in PBS and mounted in vectashield (Vector Laboratory).

II.3 RESULTS

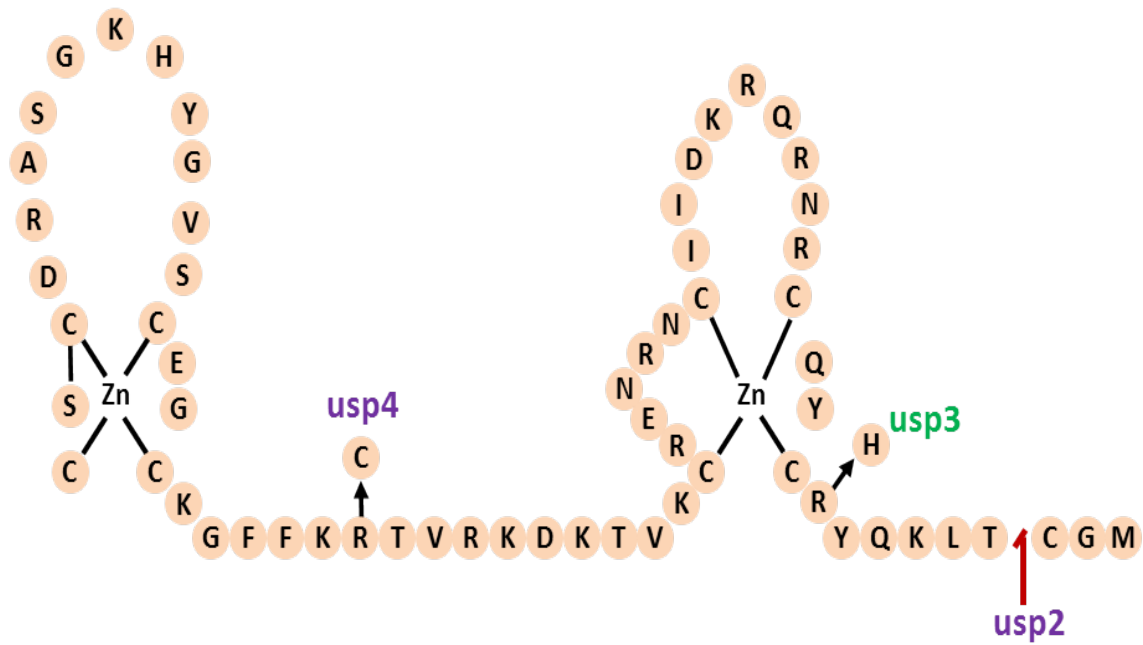
II.3.1 Dominant negative USP Mutant

The previously described *usp* mutations, *usp*³ and *usp*⁴, are point mutations near the zinc finger DNA binding domain of *usp* gene, substituting the highly conserved Arg160 for His (R160H) and Arg130 for Cys (R130C) respectively (Fig. 2-1) (Henrich et al 1994). It has been showed that these mutations affect the DNA binding ability of USP, since the mutant proteins are unable to bind ecdysone response elements (EcRE) but retain their ability to bind EcR. These properties are comparable to dominant negative EcR mutant (*EcR*^{W650A}), which has a point mutation in the highly conserved helix 12 of the LBD. *EcR*^{W650A} is able to dimerize with USP and bind DNA normally but it is unable to activate transcription and thereby acts as competitive inhibitor of wild-type EcR (Cherbas et al 2003). Based on these results, it is plausible that overexpression of USP mutant proteins might function in a dominant-negative manner and block EcR signaling. To this end, we cloned the *usp*³ mutant cDNA into *pUAST* vector to generate *UAS-usp*³ transgenic lines. To test if USP³ could act as competitive inhibitor of endogenous USP, we expressed *usp*³ ectopically in different tissues using the specific *gal4* drivers. The

effects of targeted *usp*³ expression will be discussed in following sections.

II.3.2 Targeted expression of *usp*³ in corazonin neurons results in a complete blockage of the PCD pathway

Preliminary results using MARCM revealed that *usp* mutant clones in Crz neurons could block cell death, which is most likely due to the lack of functional EcR-USP receptor complex. To overexpress the mutant *usp*³ allele in corazonin neurons, *UAS-usp*³ transgenic lines were crossed to *Crz-gal4* driver. We expected that if USP functions as an activator as a functional partner of EcR, the overexpression of mutant allele will block normal vCrz PCD, and if it is acting as a repressor, vCrz neurons would undergo premature cell death. For this, we performed immunostaining of CNS samples from larval and 7 hours prepupal stages with corazonin specific antibody to analyze the effect on vCrz PCD. Remarkably, at 7 hours APF, ectopic expression of *usp*³ in Crz neurons completely blocked its PCD (Fig. 2-2) with all 8 pairs of vCrz surviving till adulthood. This result suggests that USP as a partner of EcR, functions as an activator for vCrz PCD. It also suggests that USP³ most likely acts as competitive inhibitor of endogenous USP, resulting in functional disruption of receptor.



(Modified from Ghbeish et al 2001)

Figure 2-1. Mapped mutations in *usp* gene. *usp*²: null mutation, *usp*³: R160H in DBD, *usp*⁴: R140C in DBD.

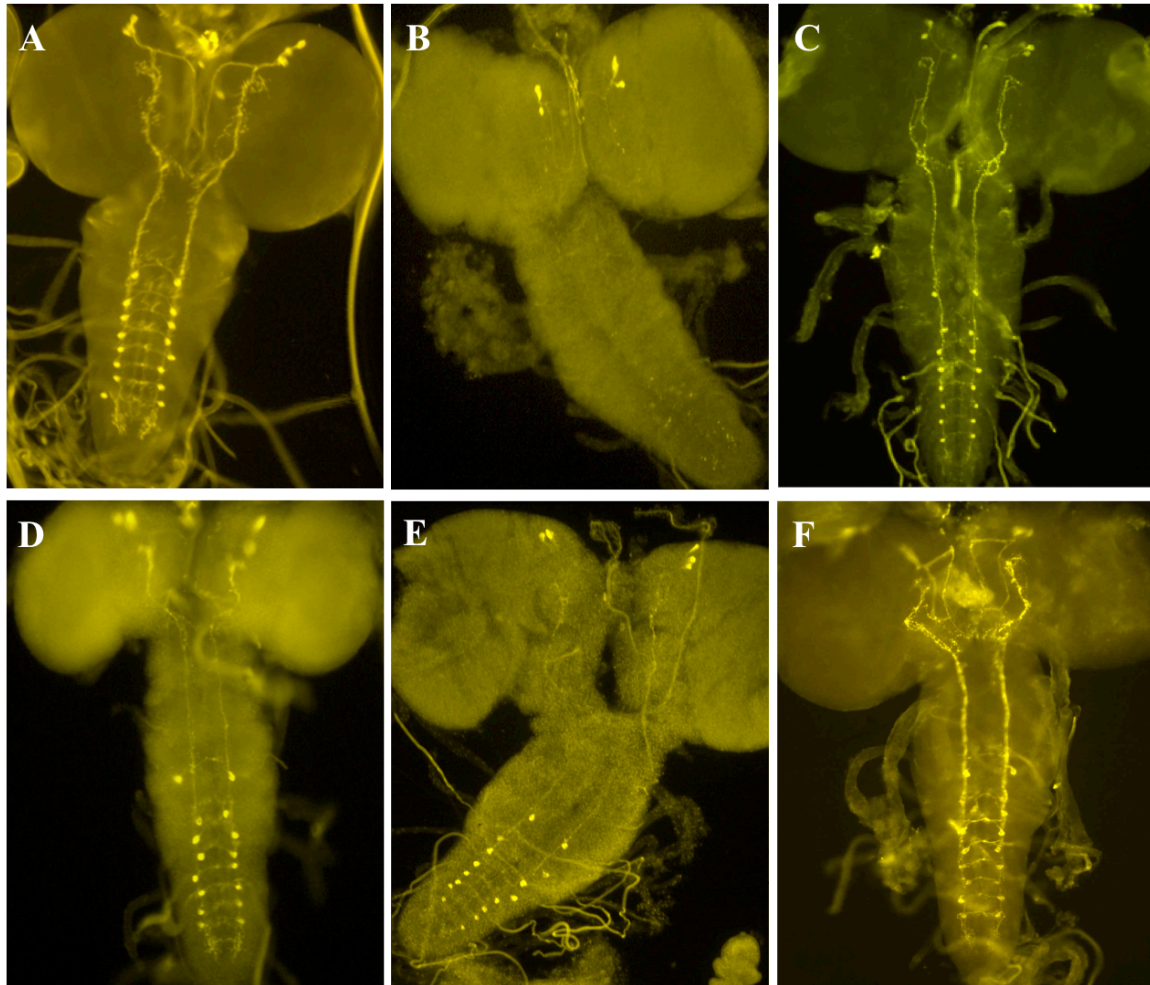


Figure 2-2. **Inhibition of vCrz cell death by targeted expression of *usp*³.** CNSs were dissected at WL3 or 7 hours APF and processed for Crz-immunoreactivity. (A) Control (*Crz-gal4/+; Crz-gal4/+*) at WL3 and (B) 7 hours APF. Surviving vCrz neurons at 7 hours APF on ectopic expression of EcR^{DN} and USP³. (C) *Crz-gal4; UAS-EcR^{W650A}*. (D) *UAS-usp³/Crz-gal4; Crz-gal4/+*. (E) *Crz-gal4/+; UAS-usp³(2t)/Crz-gal4* (F) *Crz-gal4/+; UAS-usp³(8t)/Crz-gal4*

II.3.3 Effects of *usp*³ expression in targeted tissues:

To further characterize the potential dominant negative role of the *usp*³ mutant, we used *gal4* driver system to arrest USP receptor function in different developmental domains (Table 2-1).

Ubiquitous expression: Since USP plays an important role during metamorphosis; we expected that ubiquitous expression of *usp*³ would result in developmental delay or defects. To test this, we expressed *usp3* using universal *gal4* drivers, *actin-gal4* and *tubulin-gal4*, and found that ubiquitous expression of *usp*³ was indeed detrimental as it caused embryonic lethality (100%) similar to what was observed for homozygous *usp*³ mutants and with ectopic expression of EcR^{W650A}.

Salivary Glands: Using a *34B-gal4* driver, *usp*³ was ectopically expressed in the salivary glands. Ecdysone mediated PCD of the salivary glands starts at 12 hours APF and the tissue is completely degenerated and undetectable by 18 hours APF (Jiang et al 1997). Targeted expression of *usp*³ blocked PCD in these tissues similar to vCrz neurons as intact glands could still be detected at 18 hours APF (Fig. 2-3).

Photoreceptors: We also looked for the effect of *usp*³ expression in photoreceptors since *sgmr-gal4* driven expression of EcR^{W650A} in *Drosophila* eye, caused early pupal lethality (50%). Like EcR^{W650A}, *usp*³ expression resulted in ~80% embryonic lethality. Most escapers had defective eyes, showing reduced eye phenotype and a few had normal eyes.

SCP neurons: Next we tested the effect of *usp*³ expression in neuronal pruning. A set of neurosecretory cells producing the small cardioactive peptide (SCP) undergo remodeling at metamorphosis. In the VNC of 0 h prepupa, the SCP-immunoreactive

signals are detectable in a pair of ventral neurons in each thoracic neuromere as well as several neurons in subesophageal ganglion. A characteristic projection pattern is formed by the neurites from these cells that is particularly dense in the region of the subesophageal ganglion, forming a posteriorly directed branch. The dendritic arbors of these neurons begin to regress shortly after pupariation and are highly reduced by the time of head eversion (around 12 h APF) (Schubiger et al 1998). This process has been shown to be blocked in EcR-B mutants (Schubiger et al 1998). To examine if the heterodimeric partner of EcR, USP is also required for the remodeling of these neurons, mutant USP³ protein was overexpressed in SCP neurons. We observed that the expression of *usp³* disrupts ecdysone-mediated pruning in SCP neurons, since compared to control animals, extensive arborization could still be detected when *usp³* was ectopically expressed in these neurons using the *FG5-gal4* driver (Fig. 2-4).

II.3.4 Effect of *usp³* on EcR expression in CNS

Since USP is known to be required for the up-regulation of hormone responsive genes such as EcR, we compared the expression levels of EcR-B1 in the CNS of animals expressing *usp³* using pan-neuronal driver *elav-gal4*. Surprisingly, no significant difference could be detected between control and *usp³* expressing CNS samples. However, *elav-gal4* driven expression of *usp³* resulted in 50% pupal lethality.

Similarly, no difference in EcR-B1 could be detected on ectopic expression of *usp³* in mushroom body γ neurons, using *201Y-gal4* driver (Fig. 2-5).

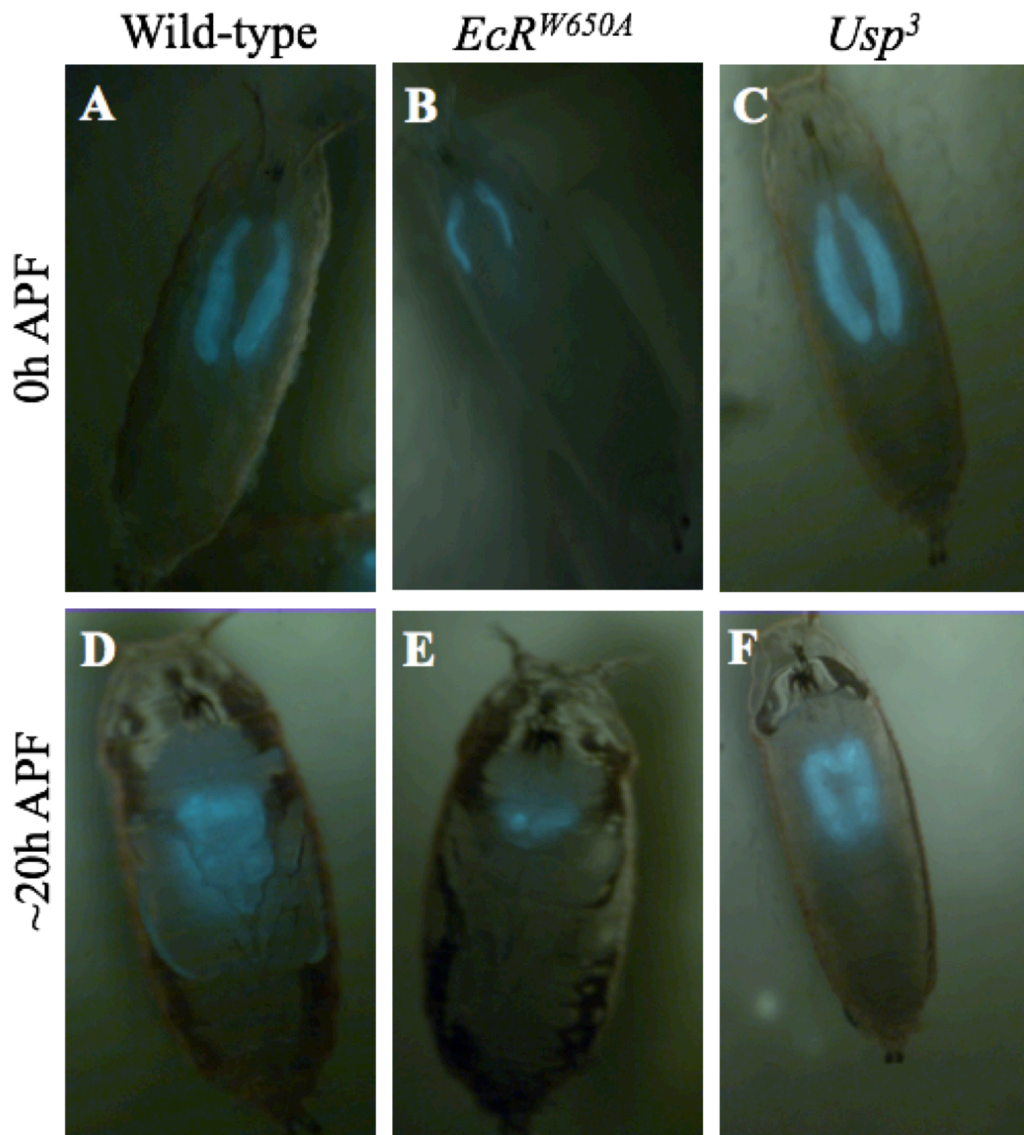


Figure 2-3. Expression of *usp*³ results in persistent larval salivary glands. Larval salivary glands were visualized at 0h APF (A-C) and 20h APF (D-F), using the salivary gland-specific *34B-gal4* driver combined with *UAS-gfp*. (A & D) Control *34B-gal4*/+; *UAS-gfp*/+. (B & E) *34B-gal4/UAS-EcR^{W650A}*; *UAS-gfp*/+. Consistent with the normal completion of salivary gland cell death, only residual GFP is detected in control pupa and pupa expressing *EcR^{W650A}* at 20h APF. (C & F) *34B-gal4/UAS-usp*³; *UAS-gfp*/+. Pupa expressing *usp*³ show intact larval salivary gland at 20h APF.

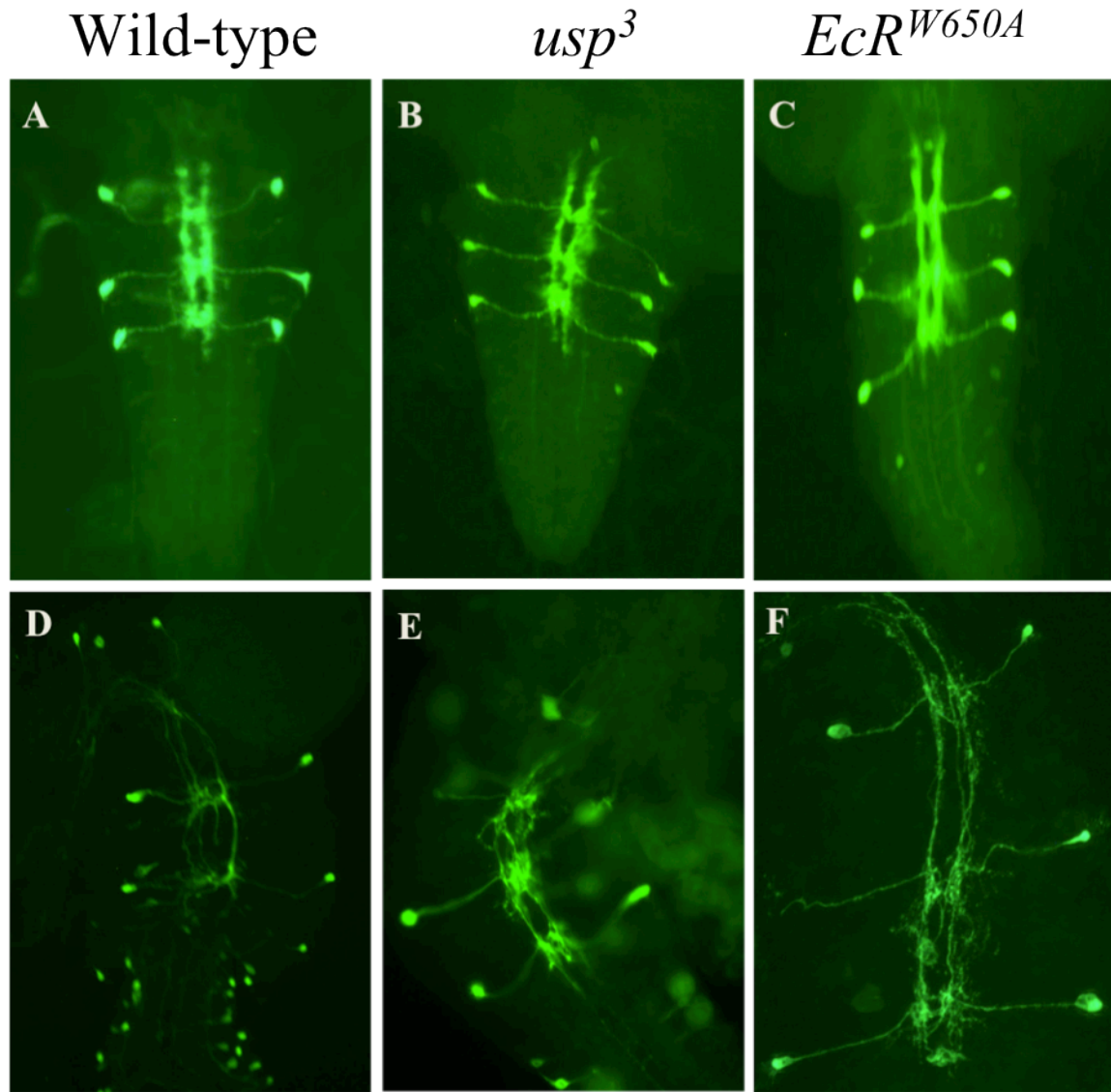


Figure 2-4. Disruption of SCP neuronal remodeling by targeted expression of *usp³*.
 SCP expressing neurons are labeled with GFP at white pre pupa (A-C) and 13h APF (D-F). (A, D) *UAS-gfp/+; FG5-gal4/+* (B, E) *UAS-gfp/+; FG5-gal4/UAS-usp³* (C, F) *UAS-gfp; FG5-gal4/UAS-EcR^{W650A}*

Table 2-1. Effects of *usp*³ and *EcR*^{W650A} expression in targeted tissues.

gal4 driver	Domain of expression	Phenotype	
		<i>usp</i> ³	<i>EcR</i> ^{W650A}
<i>actin</i>	Ubiquitous	embryonic lethal	embryonic lethal
<i>tubulin</i>	Ubiquitous	embryonic lethal	embryonic lethal
<i>elav</i>	Pan-neuronal	pupal lethal (50%)	pupal lethal
<i>GMR</i>	Photoreceptors	embryonic lethal	pupal lethal
<i>Crz</i>	Corazonin neurons	viable, blocks vCrz PCD	viable, blocks vCrz PCD
<i>CCAP</i>	CCAP neurons	viable, no premature PCD	pupal lethal, causes premature PCD
<i>34B</i>	Salivary glands	viable, blocks larval of salivary gland PCD	viable, normal PCD larval salivary glands
<i>FG-5</i>	SCP expressing neurons	viable, disrupts pruning of SCP neurons	viable, disrupts pruning of SCP neurons

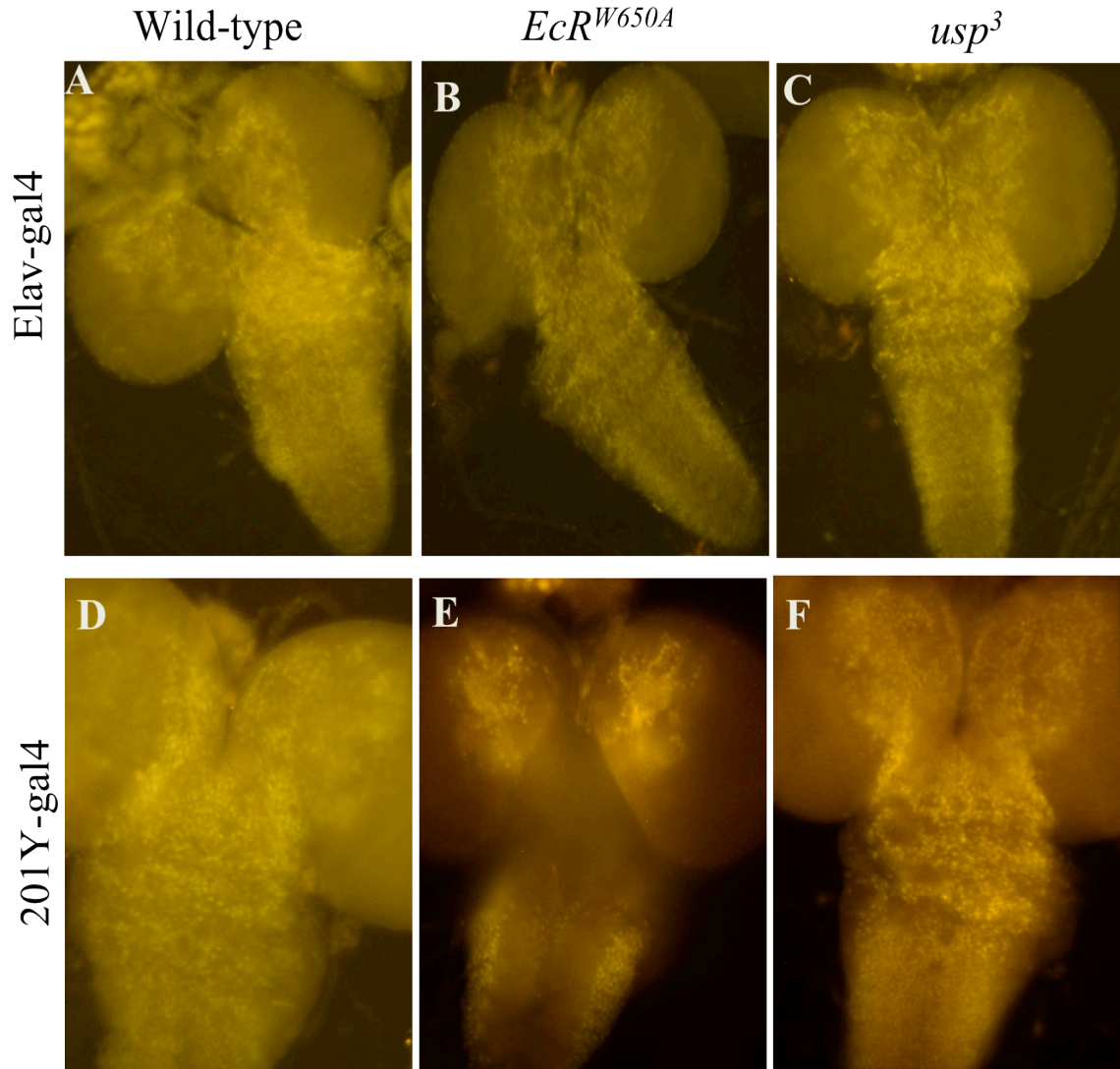


Figure 2-5. **Effect of *usp³* on EcR expression.** CNSs were dissected from third-instar wandering larvae and processed for EcR-IRy. **(A-C)** EcR expression in neurons expressing *usp³*. **(A)** Control *Elav-gal4/+* **(B)** *Elav-gal4/+; UAS-EcR^{W650A}/+* **(C)** *Elav-gal4/+; UAS-usp³/+* **(D-F)** EcR expression in mushroom body neurons. **(D)** *201Y-gal4/+* **(E)** *201Y-gal4/ UAS-EcR^{W650A}* **(F)** *201Y-gal4/ UAS-usp³*

II.3.5 DNA-binding ability of USP³

To gain further insight into the dominant-negative character of *usp³*, we proposed that USP³ is able to form heterodimer with EcR but unable to bind promoter DNA of the target genes, resulting in the disruption of ecdysone signaling. To examine this, we performed a DNA-binding assay using EcRE-lacZ transgenic flies (described previously). We expected that the inability of USP³ to bind EcRE would result in reduced lacZ expression compared to the control. However, our data did not reveal any conclusive result (Fig. 2-6).

II.3.6 DHR38 is not required for cell death of vCrz neurons

The function of ecdysone receptor complex (EcR-USP) in *Drosophila*, can be modulated by other members of steroid receptor superfamily, either by competition for binding to certain DNA EcREs or by interaction with one of the receptor subunits (Thummel 1995; 1997). Nuclear receptor DHR38 is one such modulator, which is known to interact with USP (Sutherland et al 1995). As in vivo activation of DHR38 has been revealed in CNS at late larval and prepupal stages, we hypothesize that DHR38 has a function associated with ecdysone-mediated PCD of vCrz neurons.

As mentioned previously, USP-DHR38 complex can function as a repressor of premature induction of PCD during L3 stage. If this holds true, the loss of DHR38 would accelerate the interaction between EcR and USP and trigger early PCD of vCrz neurons and the overexpression of DHR38 would disrupt the formation of EcR-USP complex resulting in delay or blocking of vCrz PCD. Apparently, DHR38 loss-of-function mutants (*DHR38⁵⁶/Df(2)Ketel^{RX32}* and *DHR38^{Y214}/Df(2)Ketel^{RX32}*) examined for vCrz PCD at

white prepupa stage, revealed no signs of early cell death (Fig. 2-7A, B). All the eight pairs of vCrz neurons that undergo developmental cell death during metamorphosis are intact in the L3 CNS of animals lacking DHR38. Correspondingly, the ectopic expression of DHR38 in corazonin neurons analyzed for Crz immunohistochemistry at 7 hours APF did not reveal any surviving Crz neuron in the VNC (Fig. 2-7C). Together, these results suggest that DHR38 is not involved PCD of vCrz neurons.

II.4 DISCUSSION

USP can function both as an activator and a repressor in response to ecdysone; therefore the present study was intended to establish the functions of USP in neuronal cell death. We show here, that the functional disruption of USP is able to inhibit the developmental cell death of vCrz neurons, thereby establishing the activator functions of USP in the PCD pathway of vCrz neurons.

Since homozygous mutant alleles for *usp* are lethal during the early larval stages, a putative-dominant negative form of *usp*, referred to as *usp*³, was used for the current study. We demonstrate that the targeted expression of *usp*³ in different tissues, like *EcR*^{W650A}, is associated with developmental defects and lethal phenotypes. We suspect that the heterodimerization of USP³ with EcR but the inability to bind EcREs contributes to the dominant negative phenotype and disrupts the ecdysone signaling. However, our DNA-binding study does not support this hypothesis. Hence, further analysis is necessary to determine how USP³ functions as the competitive inhibitor of endogenous USP.

Furthermore, our studies with DHR38 suggest that USP-DHR38 complex does not function as a repressor for PCD of vCrz neurons and as a result we propose that DHR38 does not play a role in this process.

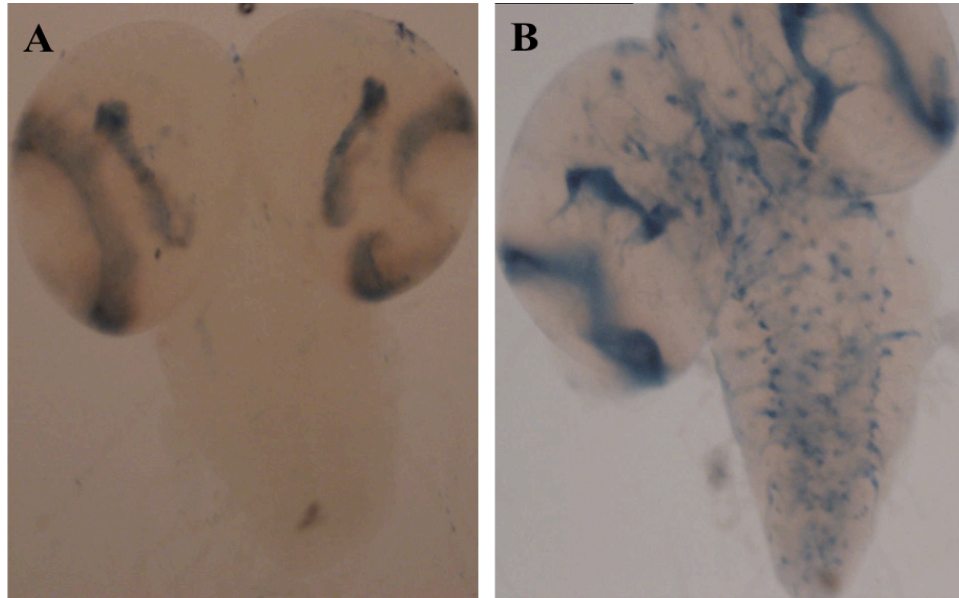


Figure 2-6. **DNA-binding ability of USP³**. CNSs were dissected from white pre pupae (0h APF) and processed for X-gal histochemistry. **(A)** Control *Crzgal4/+; EcRE-lacZ/+* **(B)** *Crzgal4/+; UAS-usp³/EcRE-lacZ*

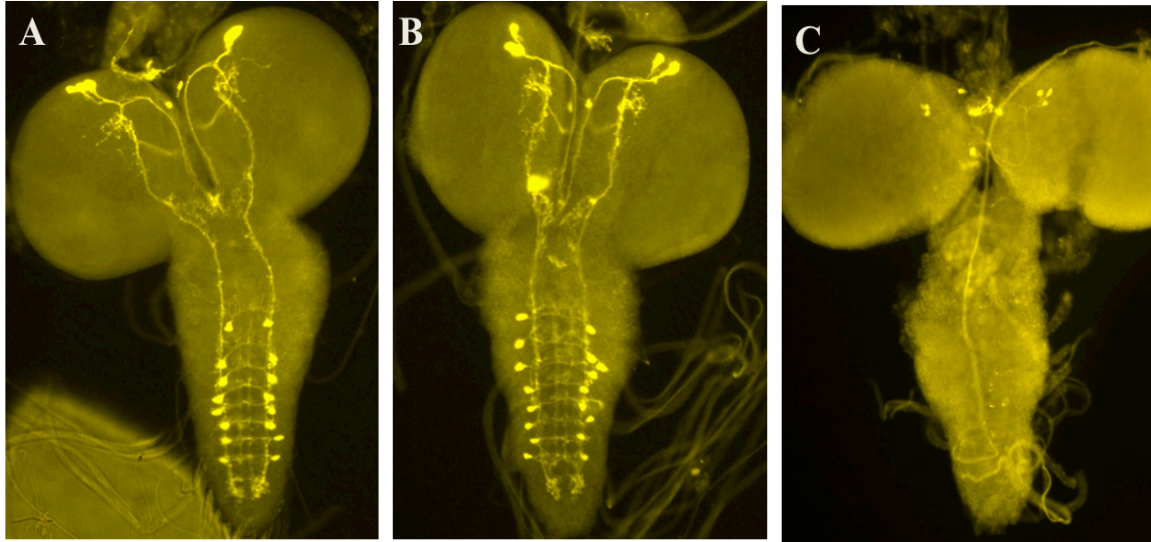


Figure 2-7 **Lack of role DHR38 in PCD of vCrz neurons.** (A, B) Loss of DHR38 does not trigger premature vCrz PCD. Crz-IHC of CNS samples from white prepupa of DHR38 mutants: **(A)** *DHR38⁵⁶//Df(2)Ketel^{RX32}* **(B)** *DHR38^{Y214}/Df(2)Ketel^{RX32}*. **(C)** Ectopic expression of DHR38 does not suppress vCrz PCD at 7h APF (*UAS-DHR38/Crz-gal4; Crz-gal4/+*)

Chapter III

Temporal activation patterns of EcR within corazonin neurons during metamorphosis

III.1 INTRODUCTION

Ecdysone signaling is transduced by EcR-USP heterodimer that regulates the transcription of downstream target genes. USP is expressed widely during development, present in most of the tissues at the onset of metamorphosis (Henrich et al 1994). Since the expression of EcR in CNS begins at third instar larval stage, it is believed that functional ecdysone receptor complexes are formed during final stages of larval development (Truman et al 1994). The activation of EcR-USP complex requires binding of ecdysone, which is synthesized in the larval endocrine organ, the prothoracic gland and released into the body as inactive precursor (Riddiford 1993). The biologically active form of ecdysone (20-hydroxyecdysone) is then formed in the peripheral tissues, such as fat body and gut, mediated by the action of *shade*-encoded cytochrome P450 enzyme (Petryk et al 2003). Previous studies revealed that only 20E and not the unhydroxylated alpha-ecdysone is able to activate the EcR-USP heterodimer (Baker et al 2003; Kozlova & Thummel 2002). Since, *shade* is not expressed in the CNS, it is assumed that CNS receives 20E via diffusion (Petryk et al 2003). Consequently, the temporal activation of ecdysone signaling in the neurons might not necessarily overlap with the high-titre pulses of ecdysone in the whole body. Addressing this problem would require a sensing system that can report the developmental timing of EcR activation in specific neurons.

The beginning of vCrz PCD coincides with ecdysone pulse at pupariation, taking place progressively between 1-7 hours after puparium formation (Choi et al 2006; Lee et al 2011). It is, however, expected that activation of ecdysone signaling pathway leading to vCrz PCD precede the actual death of vCrz neurons, indicating a time-lapse between the two events. To monitor the developmental timing of EcR activation within vCrz neurons prior to their death, we have designed an ecdysone sensor specific for Crz neurons.

Previously, a ligand-sensor (also known as Gal4-LBD) system was developed to monitor the tissue- and stage-specific activation patterns of nuclear receptors in *Drosophila* (Kozlova & Thummel 2002; Palanker et al 2006). Transgenic lines expressing the ligand-binding domain (LBD) of each nuclear receptor fused to the DNA-binding domain (DBD) of yeast Gal4, in presence of a Gal4-responsive reporter gene, revealed spatial and temporal activation patterns. Since ligand binding induces a conformational change that promotes the binding of transcription coactivators, the ability of Gal4-LBD to activate the transcription of reporter gene is directly related to the availability of specific ligand.

Likewise, ecdysone sensor using a heat-inducible promoter (hsp70) was described (Kozlova & Thummel 2002). Two potential problems with this system for our purpose are: first, heat treatment can affect normal developmental progress. Second, broad expression by the hsp70 promoter may not allow us to examine the expression specifically in Crz neurons. To overcome these problems, we employed endogenous *Crz* promoter to drive expression of the ecdysone sensor.

III.2 MATERIALS AND METHODS

III.2.1 *Drosophila* Stocks and Transgenic Fly Lines

The following reporter lines were used: *UAS-mCD8-GFP* (a membrane targeted green fluorescent protein, Lee and Luo, 1999) and *UAS-nlacZ*^{J312} (nucleus-specific expression β -galactosidase).

III.2.2 Generation of *Crz-Gal4*^{DBD}-*EcR*^{LBD} Transgenic Lines

DNA encoding the EcR ligand binding domain (EcR^{LBD}) and hinge region (330 to 655 amino acids) was amplified by PCR from *pCMXGAL4-EcR* (provided by K. Baker) using forward and reverse primers flanked by *Sac II* and *Xba I* sites respectively (5'-atccgcgccggaatgcgtcgtc and 5'-gctctagaggtgcgactagaccagatg). DNA encoding *Crz* promoter and Gal4 DNA binding domain (Gal4^{DBD}; 1 to 148 amino acids) was amplified by PCR from *504-Crz-gal4* plasmid (described in Choi et al., 2008) using Gal4 forward and *Sac II* containing reverse primer (and 5'-ggccgcgccgatacagtcactg). To generate Gal4^{DBD}-EcR^{LBD} fusion proteins, we used overlapping primers for the two PCRs. The PCR products encoding the EcR^{LBD} along with hinge region and *Crz* promoter fused to Gal4^{DBD} were digested with *Sac II/Xba I* and *Not I/Sac II* respectively and inserted between the *Not I* and *Xba I* sites of the *pBluescript* vector. The fusion between Gal4^{DBD} and EcR^{LBD} was verified by DNA sequence analysis. The resulting 2.1-kb *Not I/Xba I* fragment corresponding to *Crz-Gal*^{DBD}-*EcR*^{LBD} was cloned into P[-UAS] *P*-element transformation vector (described below). This recombinant *P*-element construct was mixed with *pUChs π 42-3* helper plasmid (Laski et al 1986) and injected into the *y w* embryos for germ-line transformation (Rubin & Spradling 1982). Two independent

homozygous viable transgenic lines (inserted in second and third chromosome) were used.

III.2.3 Generation of *pP[-UAS]* plasmid

pP[-UAS] plasmid was generated by deleting the *UAS* sequence from *pP[UAST]* plasmid, which is flanked by two *Pst I* sites. To remove the *UAS* sequence, *pP[UAST]* was digested with *Pst I*, which produced a 230 bp containing the *UAS* sequence and 8.8 kb vector. The latter fragment was gel-purified and self ligated. This new vector works as another *P*-element transformation vector.

III.2.4 Histochemistry

X-gal, GFP histochemistry and immunohistochemistry (IHC) were performed as described in Chapter 2. Mouse anti- β -galactosidase antibody was used at a dilution of 1:750 at 4°C overnight. The primary antibody was detected with TRITC-labeled secondary antibody at 1:200 dilution (Jackson Immunoresearch).

III.2.5 Ecdysone Feeding Assay

Larvae fed on regular food were collected at wandering third-instar stage and divided into two groups. One group was fed on yeast paste containing 3.3% ethanol (50 mg dry yeast, 90 μ l water, 10 μ l 100% ethanol), while the other group was fed yeast paste with a final concentration of 0.33 mg/ml 20E in 3.3% ethanol (50 mg dry yeast, 90 μ l water, and 10 μ l of 5 mg/ml 20-hydroxyecdysone [Sigma, H5142] in 100% ethanol) for 12 hours.

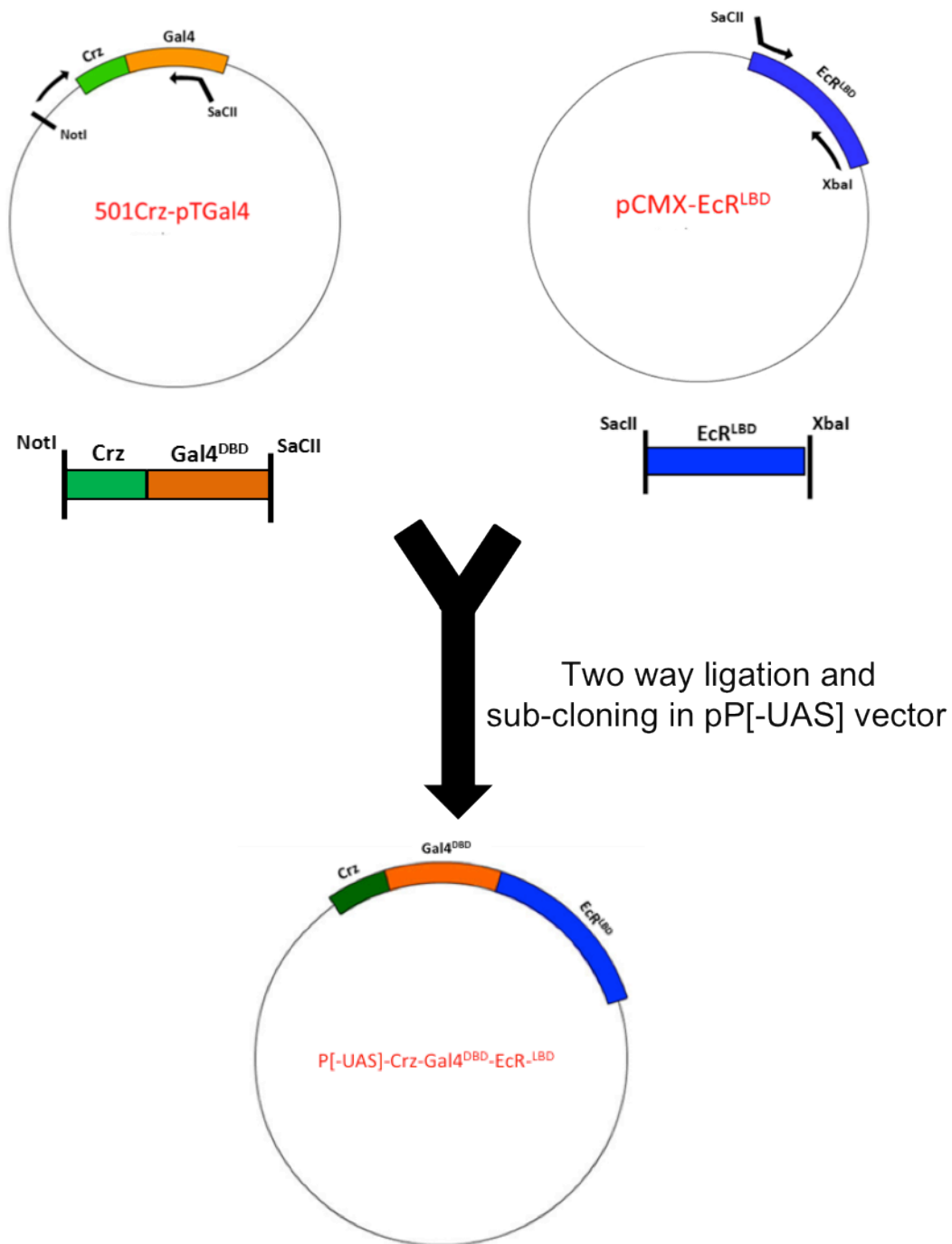


Figure 3-1. Cloning scheme for the Generation of Crz-GAL4^{DBD}-EcR^{LBD} construct.

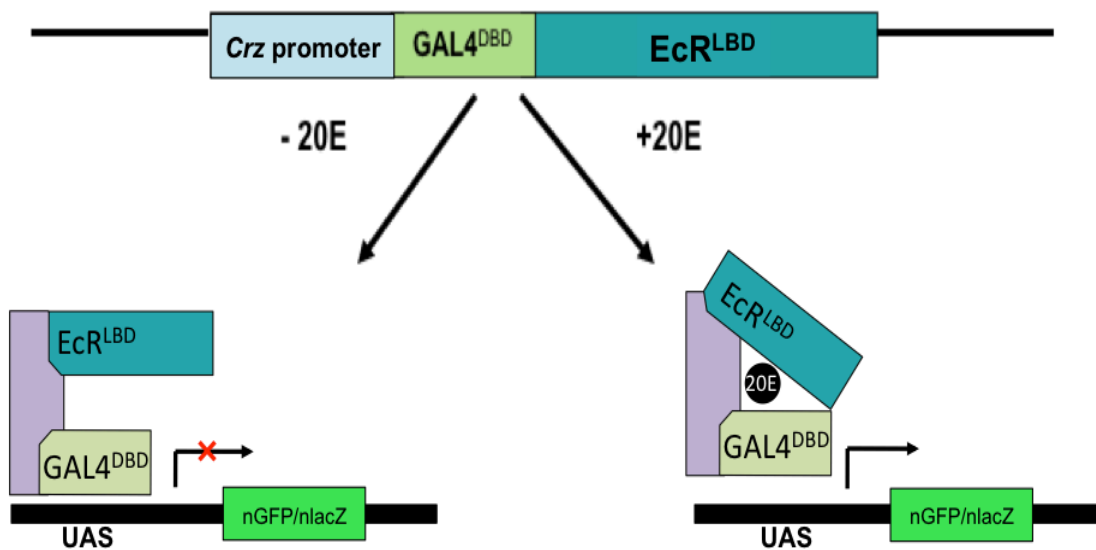
III.3 RESULTS

III.3.1 Ecdysone Sensor for Crz Neurons

To monitor the temporal activation of EcR-mediated signaling specifically in Crz neurons, we have generated an ecdysone sensor by fusing the *Crz* regulatory region to the *Gal4-EcR* coding sequence (Fig. 3-1). This Crz-specific ecdysone sensor involves the use of transgenic flies that have two *P*-element insertions, *Crz-Gal4^{DBD}-EcR^{LBD}* and *UAS-reporter* gene. *Crz* promoter directs the expression of Gal4^{DBD}-EcR^{LBD} fusion protein exclusively in Crz neurons. In the absence of 20E, Gal4^{DBD}-EcR^{LBD} fusion protein does not activate reporter gene transcription; however, in the presence of 20E, it can achieve an active conformation and induce reporter gene expression in Crz neurons (Fig. 3-2).

III.3.2 Developmental Timing of EcR Activation

Because PCD of vCrz neurons begins as early as 1 h after puparium formation (Lee et al 2011), we expected that activation of EcR would precede this event. Supporting this, the levels of ecdysone are also high in later stages of larval development as a result of high-titre ecdysone pulse during this stage. Therefore to determine the developmental timing of EcR activation in Crz neurons, we crossed *Crz-Gal4^{DBD}-EcR^{LBD}* transgenics to *UAS-reporter* (*UAS-nlacZ* and *UAS-Stinger*). CNS samples from the progeny were dissected at third-instar larval and white-prepupal (0 h APF) stage to examine reporter expression. Strangely however, we did not detect any expression of either β -galactosidase or GFP in the Crz neurons (Fig. 3-3).



(Adapted from Kozlova & Thummel 2002)

Figure 3-2. **Ecdysone sensor.** A schematic representation depicting the functioning of GAL4-EcR as ecdysone sensor in corazonin neurons.

Since, 20E likely reaches the neurons via diffusion, it is possible that the levels of 20E are not high enough to activate the transgenic sensor. To address this issue, we fed the third-instar larvae expressing the ecdysone sensor (*Crz-Gal4^{DBD}-EcR^{LBD} /+; UAS-nlacZ/+*) on yeast paste containing 0.33 mg/ml 20E for 12 hours. CNS samples were dissected and processed for β -galactosidase-IHC to examine activation of EcR. Similar to our previous results, no β -galactosidase signals could be detected in Crz neurons (Fig. 3-4).

III.4 DISCUSSION

In contrast to the widespread activation observed with the previously described ecdysone sensor in response to late larval ecdysteroid pulse, we observe no activation in the Crz neurons during this stage. It is known that CNS is unable to convert ecdysone into 20E, because of the lack *shade*-encoded cytochrome P450 enzyme (Petryk et al 2003). Therefore, the absence of Gal4^{DBD}-EcR^{LBD} activation in Crz neurons may be a result of low endogenous levels of active ecdysteroids in these neurons. The inactivity of ecdysone sensor in Crz neurons of animals fed on 20E is likely due to the levels of active ecdysone being lower than threshold.

The study by Kozlova et al, demonstrated that GAL4-EcR is able to exert a dominant negative effect on ecdysteroid signaling, resulting in reduced receptor activation and lethal phenotypes. This dominant-negative activity of GAL4-EcR is stage-specific, resulting in higher degree lethality when GAL4-EcR is expressed during late third instar larvae. It was also proposed that GAL4-EcR could dimerize with endogenous USP in the cell, forming an inactive complex that fails to mediate 20E-inducible target

gene transcription. Therefore, it is possible that within Crz neurons, GAL4-EcR functions in a dominant negative manner leading to inactivation of EcR. To confirm this, we can examine the DNA-binding ability of GAL4-EcR using EcRE-lacZ reporter. Also, it would be interesting to see if expression of GAL4-EcR interferes with the PCD of vCrz neurons.

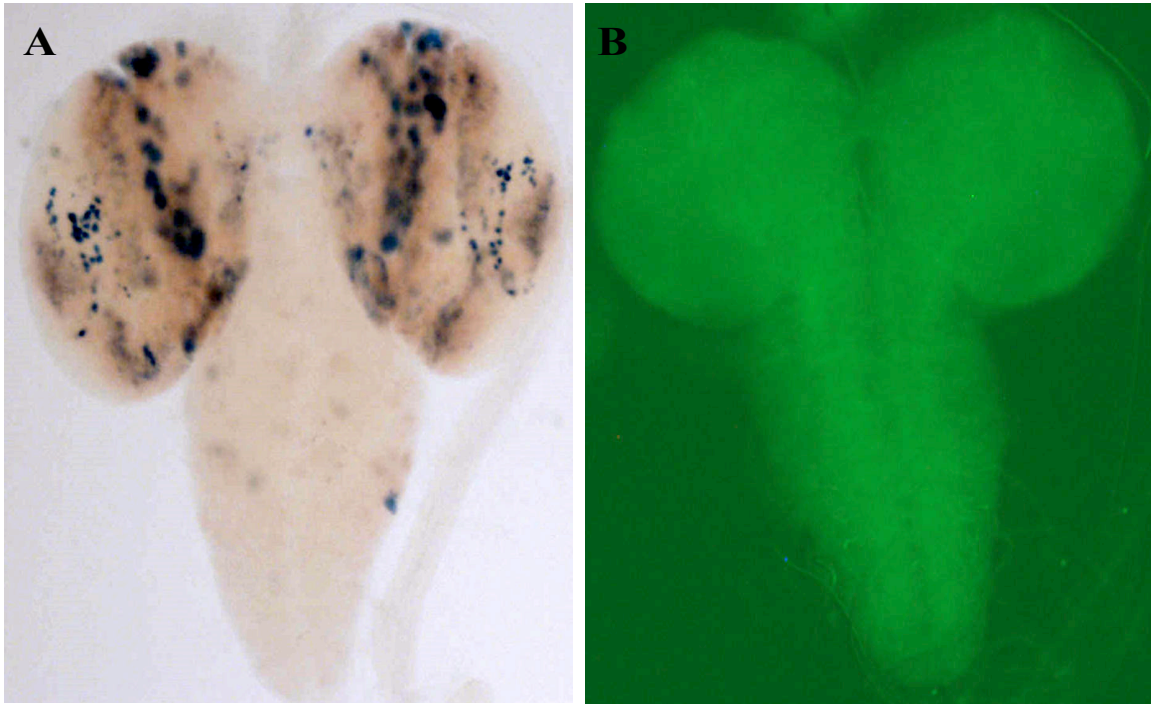


Figure 3-3. **GAL4-EcR activation in corazonin neurons.** CNSs were dissected at third-instar larval stage (L3) and processed for X-gal (A) and GFP histochemistry (B). **(A)** *Crz-Gal4^{DBD}-EcR^{LBD} /+; UAS-nlacZ/+*. **(B)** *Crz-Gal4^{DBD}-EcR^{LBD} /+; UAS-Stinger/+*.

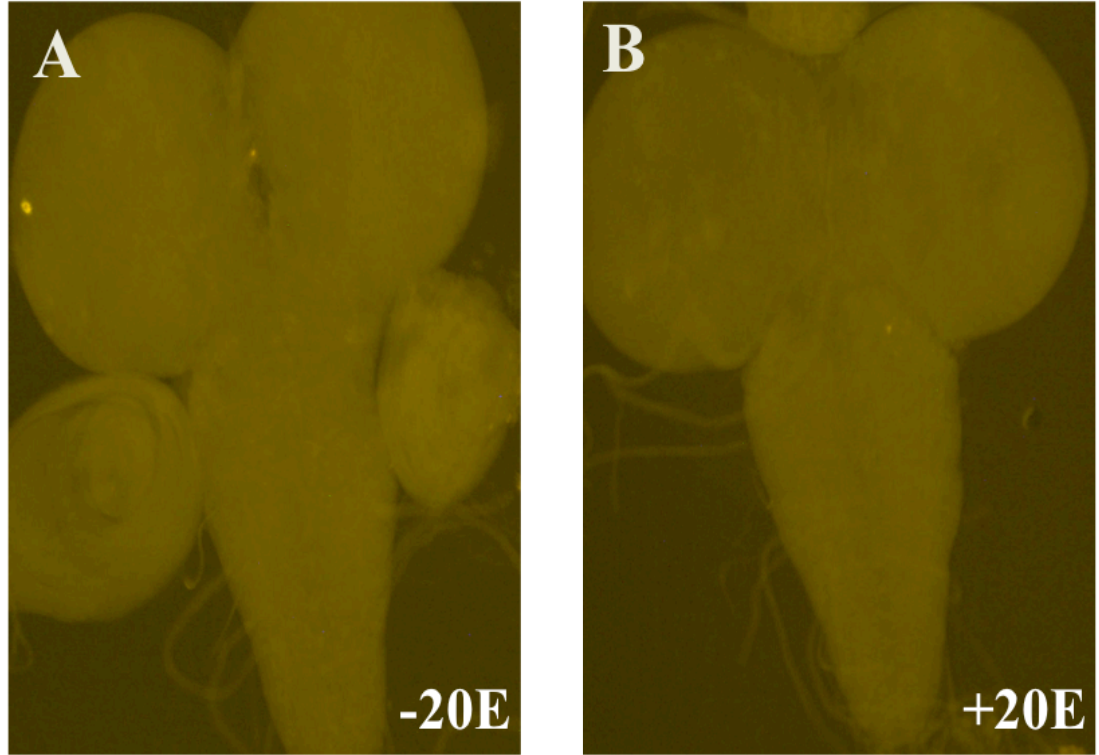


Figure 3-4. **GAL4-EcR activation in 20E-fed animals.** β -galactosidase-IHC for third-instar larval CNS from *Crz-Gal4^{DBD}-EcR^{LBD}/+; UAS-nlacZ/+* animals fed on yeast paste lacking 20E (**A**) and yeast paste containing 0.33 mg/ml 20E (**B**) for ~ 12 hours.

Chapter IV

Premature activation of PCD in CCAP-expressing peptidergic neurons

IV.1 INTRODUCTION

A group of peptidergic neurons expressing the Crustacean cardioactive peptide (CCAP) are observed in the CNS of pharate fly. CCAP neuropeptide is responsible for controlling the ecdysis behavior and is essential for turning on ecdysis in *Drosophila* (Ewer J 2002; Park et al 2003). CCAP neurons in the VNC of pharate adults are classified into three groups; 24 neurons in the dorsal side of the metathoracic and abdominal ganglia (dCCAP), 6-8 neurons in the ventral surface of the thoracic neuromeres (vCCAP), and 4-6 neurons in the abdominal ganglia (aCCAP) (Draizen et al 1999). A subset of about 14 dCCAP neurons has been reported to co-express an additional neuropeptide, bursicon, and is thus referred to as bursCCAP neurons (Luan et al 2006). In brain, CCAP neurons comprise of two pairs in the median region of the protocerebrum (mCCAP) and about 4 pairs in the subesophageal area of the brain (sCCAP) (Luan et al 2006).

Previously, it has been shown that both vCCAP and dCCAP neurons are eliminated via PCD within 24 hours after adult eclosion while aCCAP neurons remain intact in the VNC (Draizen et al 1999). Additionally, the sCCAP neurons in the brain are also observed to undergo cell death within 3 days after adult eclosion (Choi et al., unpublished data). By comparison with the PCD of vCrz neurons, the developmental timing of the death of CCAP neurons coincides with the second wave of neuronal death

that occurs after adult emergence, removing the neurons, which might have roles in developing pupae or newly eclosed adults (Truman et al 1994).

CCAP neurons have been shown to accumulate critical levels of *grim* and *reaper* transcripts prior to the onset of apoptosis (Draizen et al 1999). Unlike vCrz neurons, the PCD of CCAP neurons occurs in response to a decline in ecdysone levels during later stages of pupal development. Studies by Draizen et al. (1999) have also demonstrated that 20E treatment suppresses the accumulation of *reaper* and *grim* transcripts and prevents the death of vCCAP neurons. Presumably, the high concentration of ecdysone during earlier developmental stages plays a protective role, ensuring the survival of these neurons. Here we show that ecdysone signaling is essential for preventing the premature death of CCAP neurons. Our data also suggests that USP is not involved in the ecdysone-mediated protection of CCAP neuronal cell death.

IV.2 MATERIALS AND METHODS

IV.2.1 *Drosophila* Stocks Transgenic Fly Lines

A *CCAP-gal4* line was used to drive the gene expression in CCAP neurons (Park et al 2003). Double homozygous lines carrying both *CCAP-gal4* and *UAS-lacZ* (or *UAS-mCD8-GFP*) were used for some experiments. *UAS-miRNA* transgenic fly lines against *dhr3*, *dhr4*, *dhr96*, *dhr38*, *dhr83*, *dhr39*, *dsf*, *tll*, *svp*, *usp*, *E78*, *E75*, *ftz-fl* were obtained from Tzumin Lee. According to their study, *UAS-miRNA* constructs were generated by cloning the miRNA sequence targeting specific nuclear receptor into the pCa4B vector (a pCaSpeR4 based vector which has a 375bp fragment containing attB sequence amplified from pUASTB vector) (Lin et al 2009; (Markstein et al 2008). To generate transgenic

flies, *UAS-miRNA* constructs were inserted into the attp-16 site on the second chromosome (Lin et al 2009). In some experiments, double homozygous *CCAP-gal4*, *UAS-p35* and *CCAP-gal4*; *UAS-EcR-BI* lines were used to rescue cell death. The E75 isoform specific mutants, *E75^{A81}/TM6b Tb Ubi-GFP*, *E75^{Δ1}/TM6B, Tb, Ubi-GFP*, and *E75^{X37}/TM6B Tb Ubi-GFP* as well as E75 null mutant (*E75^{Δ51}/TM6B Tb Ubi-GFP*) were obtained from C S Thummel. *UAS-EcR^{W650A}* and *UAS-usp³* were used to express dominant-negative forms of *EcR* and *usp* respectively.

IV.2.2 Histochemistry

X-gal histochemistry and immunohistochemistry (IHC) were performed as described in Chapter 2. Rabbit Anti-Bursicon was used at 1:600 dilution (Luan et al 2006) at 4°C overnight. The primary antibodies were detected with TRITC-labeled secondary antibodies at a 1:200 dilution (Jackson Immunoresearch).

IV.3 RESULTS

IV.3.1 Ecdysone Signaling Protects Premature Cell Death of CCAP Neurons.

To investigate whether ecdysone signaling is responsible for the protection of CCAP neurons from premature cell death, we induced ectopic expression of dominant-negative form of the ecdysone receptor, *EcR^{W650A}*, in CCAP neurons. We postulated that a loss of ecdysone signaling as a result of functional disruption of ecdysone receptor would cause CCAP neurons to undergo premature cell death. CCAP expressing neurons were detected by *CCAP-gal4* driven expression of lacZ reporter gene. In addition, for some experiments, anti-bursicon was used to label bursCCAP neurons.

Targeted expression of EcR^{W650A} removed most of *lacZ*-reported CCAP neurons except for the protocerebral neurons at wandering third instar larval stage (Fig. 4-1B). The lack of CCAP neurons in these animals resulted in defective pupal development and lethality during late pupal stages, similar to CCAP Knock-out animals (Park et al 2003). To further elucidate that the lack of *lacZ*-reported CCAP neurons is indeed a result of caspase-mediated cell death and not due to transcriptional suppression of CCAP-promoter activity, we coexpressed the caspase inhibitor *p35* along with *EcR^{W650A}*. Ectopic expression of *p35*, efficiently rescued the EcR^{DN}-mediated death of CCAP neurons (Fig. 4-1C). Similarly, the restoration of ecdysone signaling by expressing EcR-B1 in CCAP neurons is able to rescue the premature cell death. These results establish the protective role of ecdysone signaling for the survival of CCAP neurons until adult emergence.

IV.3.2 USP is not involved in the premature cell death of CCAP neurons

Since, USP is the heterodimeric partner for EcR, forming the functional ecdysone receptor, we examined if the functional disruption of USP has the same effect as the EcR dominant negative mutant. To test this, we induced ectopic expression of putative dominant-negative form of ultraspiracle receptor, *usp³*, in CCAP neurons. Interestingly, however the targeted expression of *usp³* did not result in premature elimination of CCAP neurons. All the *lacZ*-reported CCAP neurons as well as the bursicon labeled dCCAP neurons survived during the larval stage (Fig. 4-2). Also, the developmental defects associated with the expression of *EcR^{W650A}*, were not observed in *usp³* expressing animals, which were in fact healthy and viable. This result suggests that USP might not be playing a role in protecting CCAP neurons from premature cell death. The lack of

involvement of USP is surprising because previous studies as well as our results have sufficiently established the significance of ecdysone and its signaling in the survival of CCAP neurons until adult eclosion.

IV.3.3 Nuclear receptor E75 is required for the survival of larval CCAP neurons

Since the protective function of ecdysone is in contrast to its usual role as a PCD inducing signal, it is likely that the two responses are mediated through different downstream pathways, possibly involving distinct genetic components. Also the lack of role of USP in ecdysone-mediated protection of larval CCAP neurons indicates towards the possible involvement of other nuclear receptors in the process. To examine whether nuclear receptors apart from EcR are required for the survival of larval CCAP neurons, we screened 12 out of the total 18 nuclear receptors found in *Drosophila*, for their role in the protection process. For this, we used *UAS-miRNA* transgenic lines against each of these receptors (Lin et al 2009).

CCAP-gal4 driven expression of miRNA, knocks-down individual NRs in CCAP neurons (Fig. 4-3). Evidently, the silencing of NR genes essential for the survival of larval CCAP neurons would result in premature cell death of these neurons along with the associated developmental defects. Our screening result shows that silencing of *dhr96*, *dsf*, *dhr39*, *svp*, *tll*, *E78*, *dhr83*, *dhr38*, and *βftz-f1* does not result in untimely PCD of CCAP neurons. Also the developmental defects associated with the lack of CCAP neurons are not observed on knocking down any of these receptors. The silencing of *dhr3* and *dhr4* shows most of the larval neurons surviving except for a few missing dCCAP neurons, with no apparent developmental defects.

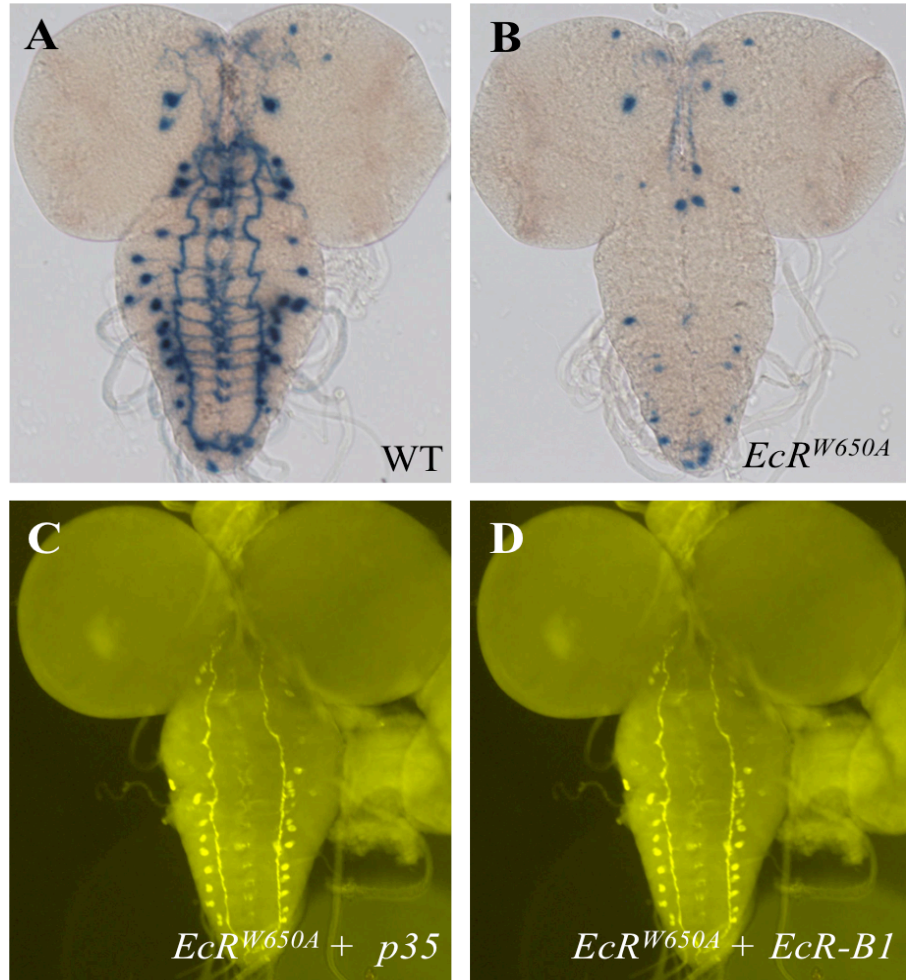


Figure 4-1. **Functional disruption of EcR results in premature cell death of CCAP neurons.** CNSs were dissected from third-instar wandering larvae and processed for X-gal histochemistry (A, B) and Burs-IHC (C, D). **(A)** Wild-type pattern of CCAP expressing neurons. (*UAS-lacZ/+; CCAP-gal4/+*). **(B)** CCAP neurons undergo premature cell death on ectopic expression of EcR^{DN} (*UAS-lacZ/+; CCAP-gal4/EcR^{W650A}*). Rescue of EcR^{DN}-mediated death of bursCCAP neurons by **(C)** Ectopic expression of p35 (*CCAP-gal4, UAS-p35/ UAS-EcR^{W650A}*) **(D)** Ectopic expression of EcR-B1 (*CCAP-gal4/ UAS- EcR^{W650A}; UAS-EcR-B1/+*)

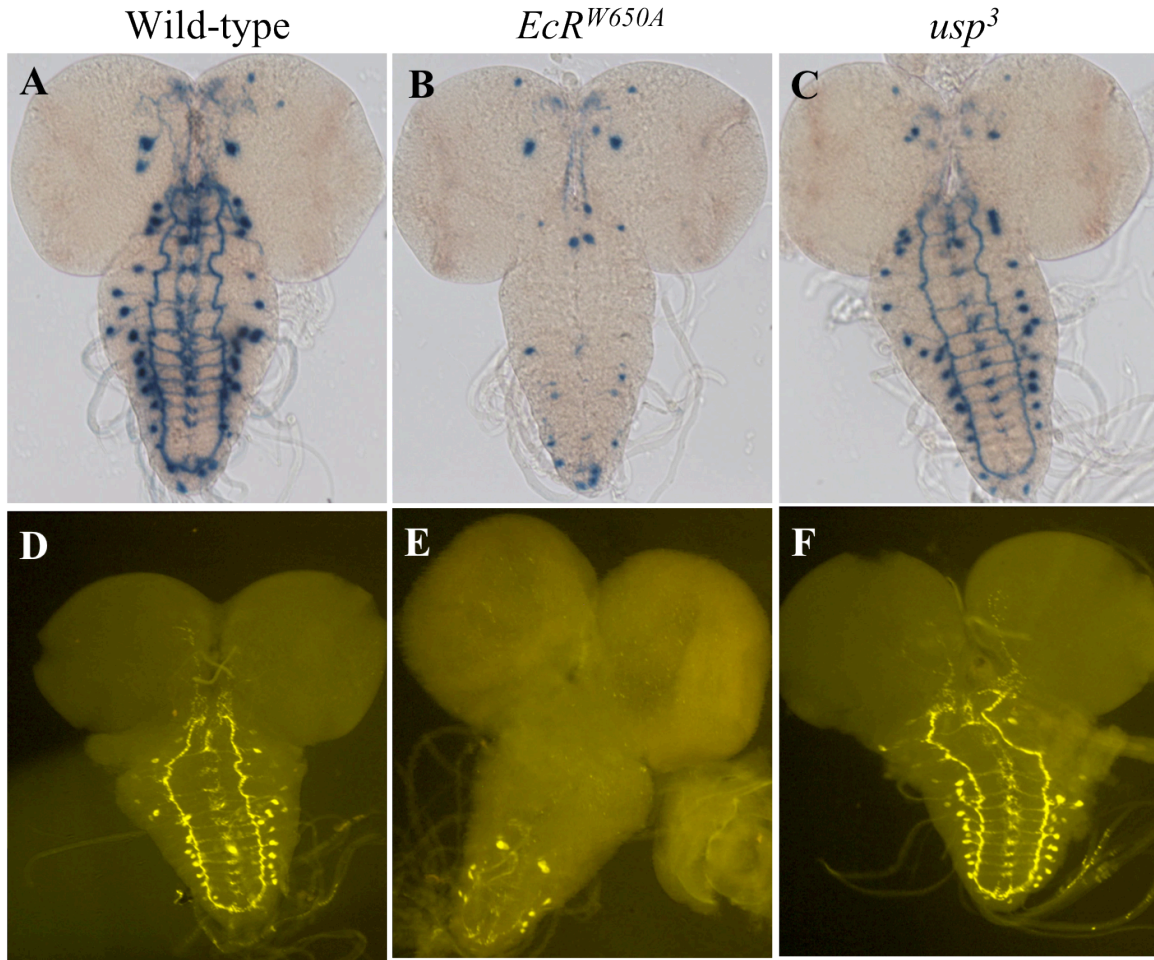


Figure 4-2. *usp* is not involved in the early cell death of CCAP neurons. CNSs were dissected from third-instar wandering larvae and processed for X-gal histochemistry (A-C) and Burs-IHC (D-F). Wild-type pattern of (A) CCAP expressing neurons (*UAS-lacZ/+; CCAP-gal4/+*) and (D) bursCCAP neurons (*UAS-lacZ/+; CCAP-gal4/+*). Removal of *lac-Z*-reported CCAP neurons (B) and burs-IR neurons (E) on ectopic expression of *EcR^{DN}* (*UAS-lacZ/+; CCAP-gal4/EcR^{W650A}*). (C, F) Targeted expression of *usp³* in CCAP neurons (*UAS-lacZ/+; CCAP-gal4/usp³*). *lac-Z*-reported CCAP neurons (C) and burs-IR neurons (F) survive during larval stage.

Remarkably, the targeted knockdown of *E75* in CCAP neurons eliminated most of the larval CCAP neurons (Fig. 4-4 A, B). Like the expression of *EcR^{W650A}*, the disruption of *E75* in CCAP neurons also resulted in defective pupal development and lethality during late pupal stages. This result suggests that nuclear receptor *E75* is required for the survival of CCAP neurons during larval and pupal stages.

E75 exists in three protein isoforms, *E75A*, *E75B* and *E75C*, each containing canonical DBD and LBD, as well as a unique N-terminal domain encoded by a distinct 5' exon (Segraves & Hogness 1990). The *E75* miRNA used here targets two sites in the LBD coding sequence, knocking down all three isoforms (Lin et al 2009). To determine types of *E75* involved in the survival of larval CCAP neurons, we examined Bursicon immunoreactivity in isoform specific mutants of *E75*. Bursicon immunohistochemistry of specific mutants for *E75A* (*E75^{A81}/E75^{Δ51}*) *E75B* (*E75^{Δ1}/E75^{Δ1}*) and *E75C* (*E75^{X37}/E75^{Δ51}*), revealed all dCCAP neurons surviving at the larval stage (Fig. 4-4D, E, F). This could be a result of the redundant functions of these isoforms or the requirement of more than one isoform in the protection process. The *E75* null mutants that are missing all the three isoforms are lethal during the early first-instar larva stage, hindering the examination of CCAP neurons. Nevertheless, our miRNA data strongly indicates the role of *E75* in protecting the premature cell death of CCAP neurons.

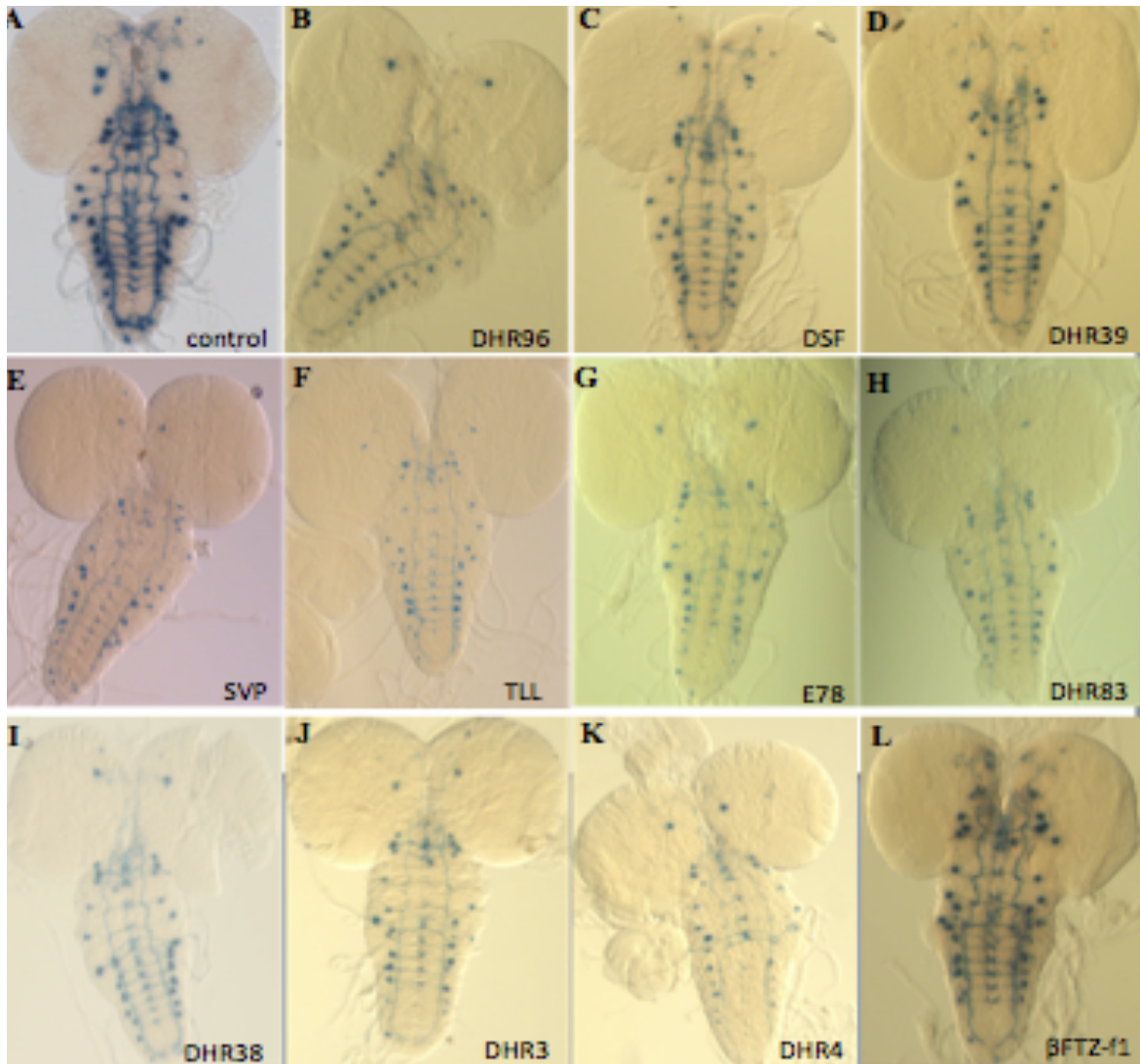


Figure 4-3. **Roles of nuclear receptors in premature cell death of CCAP neurons.** (A) Control (*UAS-lacZ/+; CCAP-gal4/+*). To knockdown nuclear receptors in CCAP neurons, *UAS-lacZ; CCAP-gal4* was crossed to (B) *UAS-mi-DHR96* (C) *UAS-mi-DSF* (D) *UAS-mi-DHR39* (E) *UAS-mi-svp* (F) *UAS-mi-tll* (G) *UAS-mi-E78* (H) *UAS-mi-DHR83* (I) *UAS-mi-DHR38* (J) *UAS-mi-DHR4* (K) *UAS-mi-DHR3* (L) *UAS-mi-βFTZ-f1*. CNS samples were dissected from third-instar wandering larvae and processed for X-gal histochemistry.

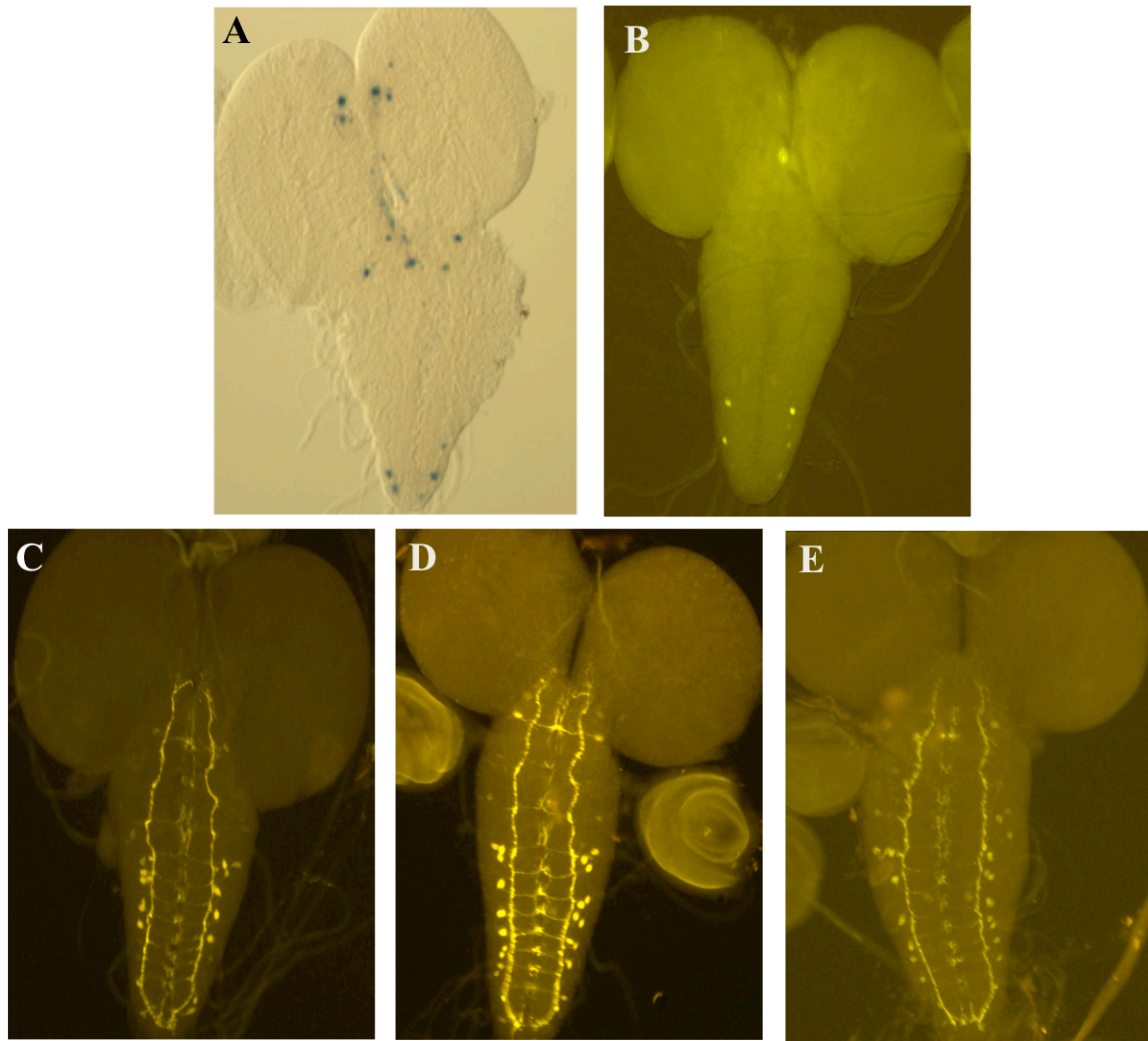


Figure 4-4. **Role of E75 in premature cell death of CCAP neurons.** (A, B) E75 knockdown in CCAP neurons leads to cell death at third-instar wandering larval stage. (A) X-gal histochemistry of *UAS-lacZ/+; CCAP-gal4/UAS-mi-E75*. Bursicon-IHC of CNS samples from third-instar wandering larvae of (B) *CCAP-gal4/UAS-mi-E75* (C) *E75A*-specific mutant (*E75^{A81}/E75^{Δ51}*) (D) *E75B* specific mutant (*E75^{Δ1}/E75^{Δ1}*) (E) *E75C*-specific mutant (*E75^{X37}/E75^{Δ51}*).

IV.4 DISCUSSION

The post-embyonic CNS development in *Drosophila* is marked by two waves of neuronal cell death. The first wave occurs during the prepupal stage and the second one takes place within 24 hours of adult emergence (Truman 1993). vCrz neurons undergo PCD in response to larva-to-pupa ecdysone peak during the first wave, at the onset of metamorphosis (Choi et al 2006). CCAP neurons, on the other hand undergo PCD during the second wave of neuronal death in response to the fall in ecdysone levels (Draizen et al 1999). Ecdysone is responsible for inducing both activation and repression of proapoptotic genes (Jiang et al 1997(Robinow et al 1993), underscoring the involvement of distinct genetic components in the two events.

In contrast to its function as a stimulus for apoptosis, our current study has shown that ecdysone signaling is also essential for the protection of CCAP neurons during larval and pupal stages. This raises the important issue of how two contrasting events are regulated by the same hormonal signal. It is presumable that distinct genetic pathways govern the two events. Our finding, that the functional disruption of EcR but not USP causes the untimely cell death of CCAP neurons supports this hypothesis. Since, EcR is unable to bind ecdysone on its own, it is possible that it forms heterodimer with a different nuclear receptor. A potential candidate could be E75, which was found to be essential for the survival of CCAP neurons. To date, EcR has not been shown to heterodimerize with any nuclear receptor except USP; therefore further biochemical studies have to be done to examine actual physical interaction between EcR and E75.

Furthermore, *E75* is known to be one of the early ecdysone response genes, essential for regulation of apoptosis (Buszczak & Segraves 2000). *E75A* and *B* have been

shown to induce apoptosis of larval salivary glands (Jiang et al 2000). However, according to our study, *E75* functions as a suppressor of apoptosis in larval CCAP neurons. Interestingly, a recent study showed that *E75B* is able to suppress apoptosis in *Drosophila* egg chamber (Terashima & Bownes 2006). Therefore, it is likely that suppression of premature apoptotic death in CCAP neurons is mediated by *E75B*. On the contrary our results show that the isoform-specific *E75B* mutant does not cause premature cell death of CCAP neurons. The possibility of functional redundancy of the *E75* isoforms and the lethality associated with the null mutants has hampered our current study. Consequently, further studies would be needed to dissect the genetic machinery involved in ecdysone-mediated protection of CCAP neurons and the roles played by nuclear receptor *E75*.

Chapter V

Role of DIAP1 in vCrz neuronal cell death

V.1 INTRODUCTION

The survival of vCrz neurons during ecdysone-mediated larval-to-larval moltings, suggests the involvement of anti-apoptotic factors that counteract the pro-apoptotic machinery in larval vCrz-neurons. DIAP1 is the most important anti-apoptotic factor known in *Drosophila*, and the lack of DIAP1 is associated with extensive cell death leading to embryonic lethality (Hay et al 1995).

Previously, studies were carried out to define its role in PCD of vCrz neurons. According to these studies, the over-expression of wild-type *diap1* did not completely rescue vCrz death and the knockdown of *diap1* transcript using RNAi did not cause premature cell death (Choi et al 2006). On the other hand, the same genetic manipulations rescued and accelerated PCD of larval salivary glands, respectively. Therefore, it was concluded that DIAP1 is not involved in the developmental cell death of vCrz neurons (Choi et al 2006).

Although none of the evidence supported DIAP1 as the survival factor for vCrz neurons, this study did reveal that the intensity of DIAP1-immunoreactive signals in vCrz neurons show a gradual decline from early third-instar larvae to wandering third-instar larvae, indicating that DIAP1 undergoes gradual degradation within these neurons. It is already known that during apoptosis, DIAP1 is proteolytically removed via RPR-mediated auto-ubiquitination process, leading to the activation of caspases (Ryoo et al 2002). Since the amount of DIAP1 in a cell is a critical factor determining the threshold

to cell death, it is possible that DIAP1 is indeed the protective factor for vCrz in early larval stages and its declining levels in the later stages is associated with the susceptibility of these neurons to undergo apoptosis.

This study is aimed to further define the role of DIAP1 in PCD of vCrz neurons. Here, we report that the amount of DIAP1 in vCrz neurons is a determining factor for the survival of these neurons and the efficient knockdown of *diap1* does indeed cause premature PCD.

V.2 MATERIALS AND METHODS

V.2.1 *Drosophila* Stocks and Transgenic Fly Lines

For transgenic manipulations, a *Crz-gal4* driver (Choi et al., 2008) was crossed to a *UAS-diap1* (Hay et al 1995) to overexpress wild-type *diap1* and *UAS-mi-diap1* (Huh et al 2007) to knockdown *diap1*. A double homozygous line carrying both *Crz-gal4^{S2b}* and *UAS-diap1* transgenes was generated by genetic crosses and used for super ectopic expression of DIAP1. The gain-of-function *thread* allele (*th, diap1*), *th^{6-3S}*, was used for some experiments (Goyal et al 2000). The recombined lines, *Crz-gal4, dr^{J24}/TM6B* and *UAS-mi-diap1, dr^{J24}/TM6C* were crossed to silence *diap1* in *dronc*-null mutant background. We obtained *UAS-diap1^{WT}* (III) from Bloomington stock center and *UAS-midiap1* from B. Hay at Caltech.

V.2.2 Generation of UAS- *diap1^(6-3S)* and UAS- *diap1^{ARF}* Transgenic Lines

To generate *UAS- diap1^(6-3S)* *P*-element construct, genomic DNA was extracted from *th^{6-3S}* mutant flies (Goyal et al 2000). A 1314-bp-long *th^{6-3S}* open reading frame was

amplified by PCR using *diap1*-forward (CCAGAGAAGAATTCAAGAGTCAGGCA) and *diap1*-reverse (TCTAGATTTGAGGATTGGGTGCGCA) primers. For *UAS-diap1^{ΔRF}* construct, the preexisting *UAS-diap1* plasmid was amplified by PCR using *UAS*-forward (GAATACAAGAAGAGAACTCTG) and *diap1^{ΔRF}*-reverse (CCGATGATCTAGATTACTACTGCTCTTCC) primers. The *diap1^{ΔRF}*-reverse primer introduced premature stop codons in the *diap1* ORF, in order to generate a truncated *diap1^{ΔRF}* (Q388STOP), lacking the 300 bp fragment encoding ring finger domain. The PCR products were cloned into pGEM-Teasy vector, from which a *EcoRI/XbaI* fragment was subcloned into pUAST vector to obtain *UAS-diap1^(6-3s)* and *UAS-diap1^{ΔRF}*, respectively. Germ-line transformation was done as described previously. For *UAS-diap1^(6-3s)*, we established five transgenic lines (one on the X, one on the second, and three on the third chromosome, one of which is homozygous lethal) and for *UAS-diap1^{ΔRF}*, we generated three homozygous lines (one on the second and two on the third chromosome). *UAS-diap1^(6-3s)* (II) and *UAS-diap1^{ΔRF}* 17 (III) lines were used in this study.

V.3 RESULTS and DISCUSSION

V.3.1 *diap1* is Required for Survival of vCrz Neurons

Previously, knockdown of the *diap1* using RNAi employed symplectic UAS vector system (Giordano et al 2002; Huh et al 2007) in which *diap1* coding sequence was flanked by a UAS promoter at both ends. As a result, sense and antisense RNAs are transcribed at the same time and form dsRNA, thereby inducing RNAi-mediated

knockdown of *diap1*. Although this construct works in some tissues, we did not have positive results, when expressed in neuronal cells.

As an alternative approach, we attempted to knockdown *diap1* transcript levels by using microRNA (*midiaap1*). To knockdown *diap1* mRNA specifically in Crz neurons, Crz-gal4 was crossed to a *UAS-midiaap1*, and then the progeny was examined for Crz-immunoreactive signals.

Crz-IHC of the CNSs from wandering third-instar larvae did not show any Crz neurons in the VNC as well as in the protocerebrum (Fig. 5-1B), indicating that *diap1*-knockdown results in premature Crz cell death, regardless of their positions. Similarly, CCAP and PDF expressing neurons also undergo premature cell death on targeted expression of *midiaap1* (Fig. 5-2). Constitutive activation of caspases in the absence of DIAP1 is the most likely cause of this untimely death of vCrz neurons. Since the initiator caspase, DRONC plays a major role in vCrz PCD (Lee et al 2011), we examined if *midiaap1* in the absence of DRONC would not be able to cause the premature cell death phenotype. Indeed, we found that *diap1* knockdown in *dronc*-null mutants (*dronc*^{I24}) did not cause killing of larval vCrz neurons (Fig. 5-1C).

Two things are noteworthy from these results. First, the death is default for neurons that have distinct developmental fates; vCrz neurons that normally die of apoptosis and protocerebral DL neurons that normally survive and persist into adulthood. In other words, cells are equipped with suicidal weapons thus ready to die whenever someone (=death signal) pulls the trigger.

Second, our results verify that DIAP1 is indeed an antiapoptotic factor that prevents vCrz neurons from their premature death during larval development. Then why

over-expression of wild-type *diap1* does not rescue vCrz PCD? Perhaps, a degradation machinery prevents DIAP1 from its accumulation of even overproduced DIAP1 toward the end of larval stage.

To address the second question, we turned our attention to the gain-of-function of *diap1*. This was accomplished in two ways; first, we used *th*^{6-3S} allele that was shown to cause the gain-of-function phenotype. The mutant protein, DIAP1^{6-3S}, carries the glycine to serine mutation at the 88th position (G88S) in the BIR1 domain, resulting in its greatly diminished binding to proapoptotic proteins REAPER and HID (Goyal et al 2000). According to our results, *th*^{6-3S} resulted in significant delay of vCrz PCD. At 7 h APF, approximately 10 neurons (n=5) still survived in *th*⁶⁻³ mutants (Fig. 5-1E), suggesting that the mutant protein is less vulnerable to degradation, compared to wild-type DIAP1, thus rescuing a few vCrz neurons from their apoptotic death.

V.3.2 High Level of DIAP1 in vCrz Neurons Results in Enhanced Protection of vCrz Neurons

The incomplete rescue of vCrz PCD on ectopic expression of *diap1* could be a result of DIAP1 degradation via ubiquitination. During apoptosis, DIAP1 levels in the cell are regulated by auto-ubiquitination mediated by proapoptotic proteins. DIAP1 contains a conserved RING finger domain at the C terminus that functions as an E3 ubiquitin ligase, responsible for recruiting E2 ubiquitin-conjugating enzymes, and promoting the degradation of DIAP1 in the presence of death signals (Silke et al 2005). Also, our previous study revealed that DIAP1 undergoes gradual degradation within vCrz neurons (Choi et al 2006; Lee et al., unpublished data). Therefore it is predictable that

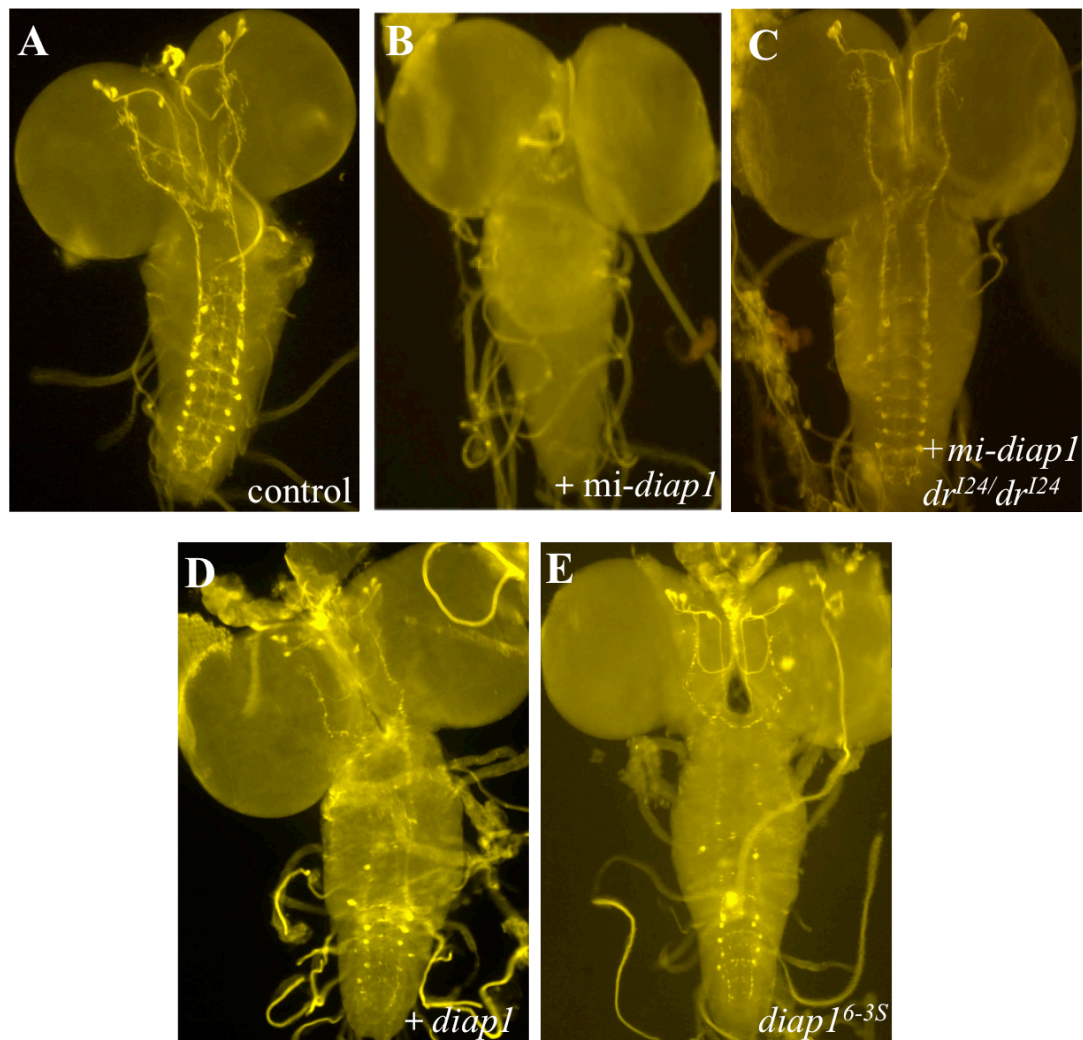


Figure 5-1. ***diap1* is required for survival of vCrz neurons.** (A) Control (B) Targeted expression of mi-*diap1* in Crz neurons (*Crz-gal4/+;; UAS-mi-diap1/+*). Absence of Crz neurons at L3 stage, suggests that knockdown of endogenous *diap1* causes premature cell death. (C) Rescue of premature cell death in *dronc*-null-mutants (*Crz-gal4, dr^{l24}/UAS-mi-diap1, dr^{l24}*). Delayed PCD of vCrz neurons (D) on ectopic *diap1* expression (*Crz-gal4/+; UAS-diap1/+*) or (E) in *diap1* gain-of-function mutant (*diap1^{6-3S}*). CNS samples were dissected from either third-instar larvae (A, B, C) or 7 hours APF (D, E) and processed for Crz-IHC.

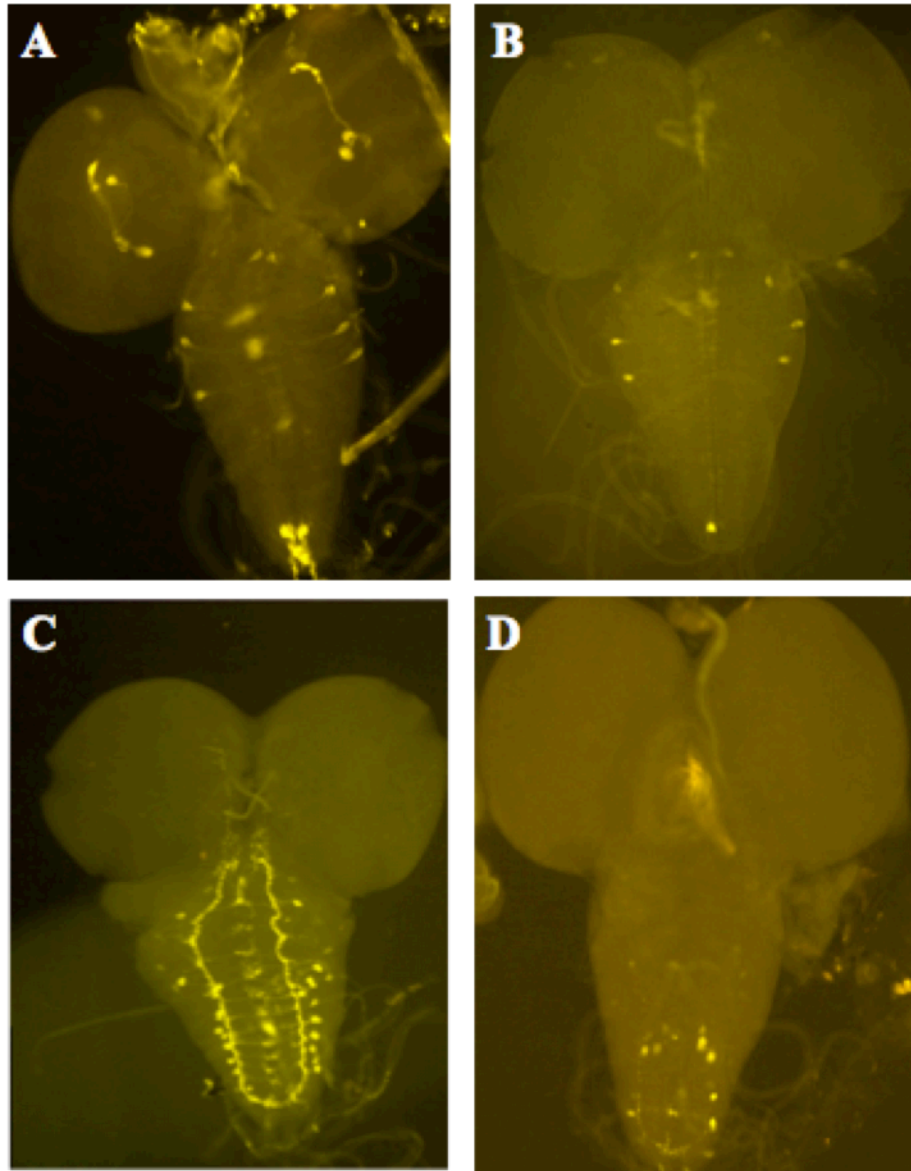


Figure 5-2. ***diap1* knockdown results in premature killing of CCAP and PDF expressing neurons.** Wild-type pattern of (A) Pdf neurons and (C) dCCAP neurons during wandering larval stage. Targeted expression of mi-*diap1* in (B) Pdf neurons (*BmRj-gal4/+; UAS-midiap1/+*) and (D) CCAP neurons (*CCAP-gal4/+; UAS-midiap1/+*)

high levels of DIAP1 must be maintained in vCrz neurons to be able to completely suppress their PCD.

To examine this, we designed experiments to induce super-ectopic and potentially hyperactive DIAP1 expression in Crz neurons. Super-ectopic expression of DIAP1 was achieved by using transgenic manipulations. Two copies each of *UAS-diap1* and *Crz-gal4^{S2b}* transgenes were able to significantly delay vCrz PCD. Crz-IHC of the CNSs obtained from *Crz-gal4^{S2b}; UAS-diap1* revealed that all 16 vCrz neurons survived at 7 h APF (n=5) and 9.5 ± 0.7 (n=2) at 18 h APF (Fig. 5-3C, H). Crz-IHC from animals containing one copy of *Crz-gal4^{S2b}* and two copies of *UAS-diap1* showed similar result at 7 h APF (i.e. 16 neurons surviving) but fewer neurons were detected at 18 hours APF (7.5 ± 0.7 , n=2) (Fig. 5-2 B, F). One copy of each transgene could only weakly delay vCrz PCD, with 12.4 ± 1.67 (n=5) neurons surviving at 7 h APF and 1.3 ± 0.6 (n=3) neurons 18 h APF (Fig. 5-3A, D). Therefore, we observed that the extent of delayed vCrz PCD increased with the number of transgenes.

For hyperactive DIAP1 expression, we designed two transgenic constructs, *UAS-diap1^{6-3S}* and *UAS-diap1^{ΔRF}*, which would produce potentially hyperactive mutant DIAP1 proteins. *UAS-diap1^{6-3S}* and *UAS-diap1^{ΔRF}* transgenic flies crossed to *Crz-gal4* driver would result in overproduction of DIAP1^{6-3S} gain-of-function and DIAP1^{ΔRF} loss-of-function mutant specifically in Crz neurons. DIAP1^{6-3S} protein has reduced binding ability for proapoptotic proteins and therefore it can strongly inhibit caspase activity even in the presence of apoptotic signals. DIAP1^{ΔRF} on the other hand, lacks the C-terminal ring finger domain and is expected to be resistant to REAPER/HID/GRIM-mediated auto-ubiquitination. The expression of *diap1^{6-3S}* resulted in partial protection of vCrz

PCD, with 13.2 ± 2.6 (n=4) neurons surviving at 7 h APF (Fig. 5-4C). In comparison, the expression of *diap1^{ARF}* resulted in enhanced protection against cell death, with 15.1 ± 0.69 (n=7) neurons surviving at 7 h APF (Fig. 5-4D). These overall results conclude that DIAP1 does play a role in vCrz PCD, acting as a survival factor during larval stages and high levels of DIAP1 must be maintained in these neurons to suppress cell death.

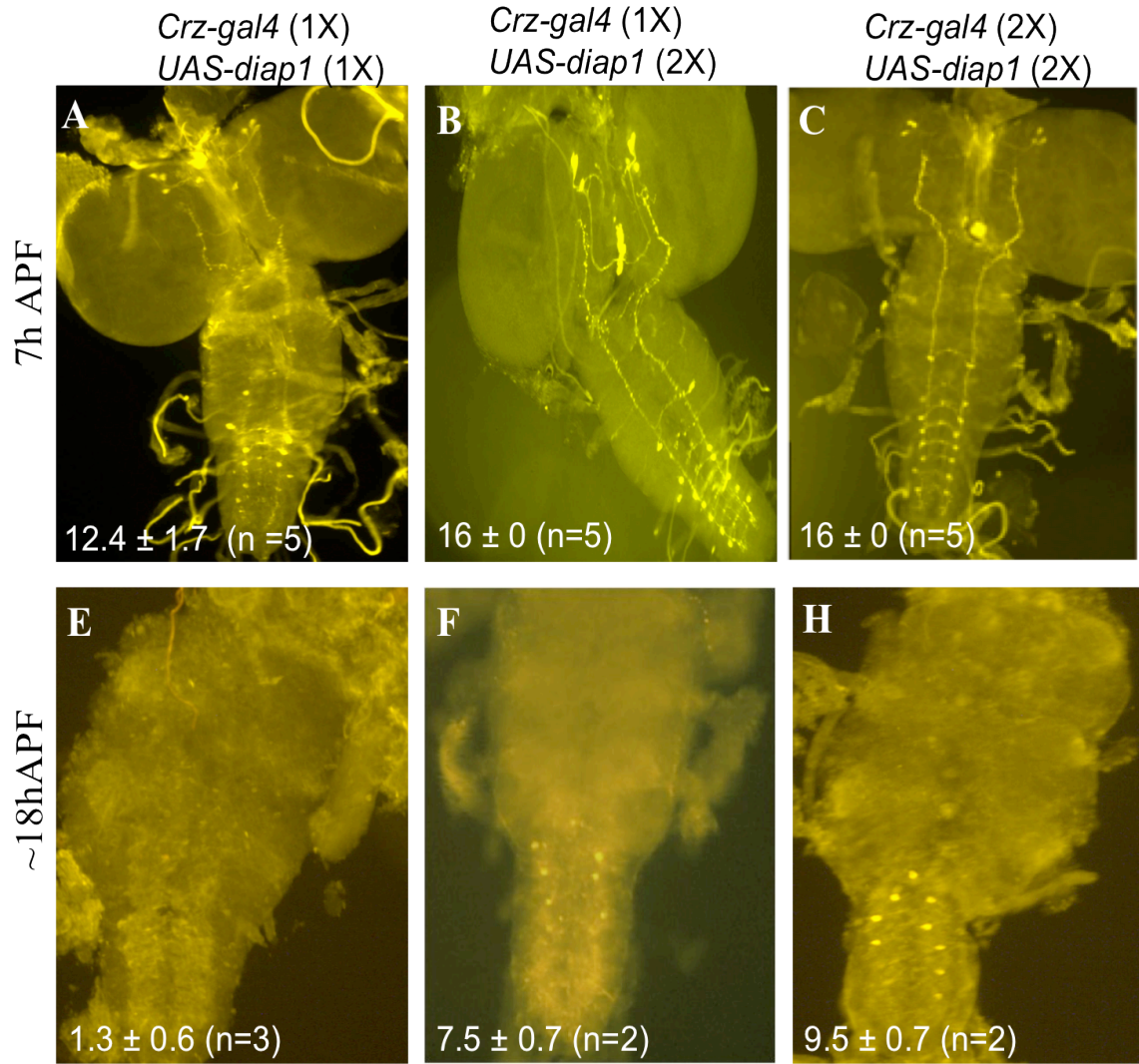


Figure 5-3. **Super ectopic expression of DIAP1 in vCrz neurons.** CNS samples were dissected from either 7 h (A, B, C) or 18 h APF (D, E, F) and processed for Crz-IHC. (A, E) *Crz-gal4*^{S2b}/+; *UAS-diap1*/+ (B, F) *Crz-gal4*^{S2b}/+; *UAS-diap1/UAS-diap1* (C, H) *Crz-gal4*^{S2b}; *UAS-diap1*

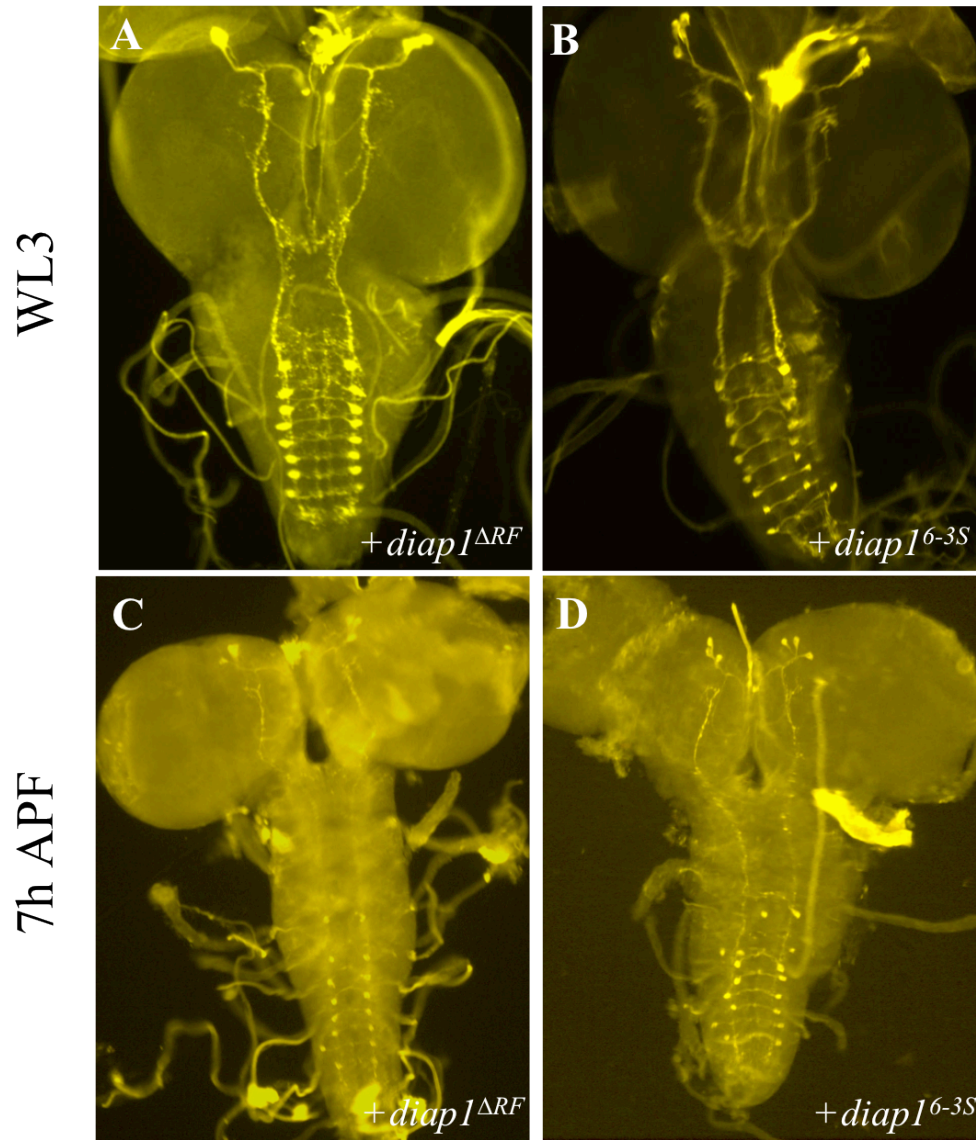


Figure 5-4 **Hyperactive DIAP1 expression in vCrz neurons.** (A, C) *Crz-gal4/UAS-diap1^{ΔRF}* (17) (B, D) *UAS-diap1^{6-3S}/+; Crz-gal4/+*. CNS samples were dissected from either wandering third-instar larvae (A, B) or 7 h APF (C, D) and processed for Crz-IHC.

Chapter VI

Summary And Conclusions

Because neuronal PCD mechanisms can be very different from non-neuronal cell death mechanisms, largely this study is aimed to identify the genetic components regulating neuronal PCD. The primary focus is to gain insight into the nuclear receptors involved in programmed neuronal cell deaths.

Since, EcR is the only nuclear receptor in *Drosophila*, recognized to play important role in neuronal PCD, the present study is expected to provide significant information on the roles played by other nuclear receptors and the associated signaling pathway.

vCrz and CCAP represent two distinct sets of peptidergic neurons that undergo PCD at different developmental stages. Ecdysone is the common hormonal signal that on one hand triggers the PCD of vCrz neurons and on the other, protects the premature killing of CCAP neurons. This is indicative of distinct genetic components and signaling pathway regulating the two events. Our study demonstrates that USP as a partner of EcR activates vCrz PCD, but has no role to play in survival of CCAP neurons. Secondly, it shows that nuclear receptor E75 is critical for preventing premature PCD of CCAP neurons, while the similar functions are not associated with the PCD of either vCrz neurons or larval salivary glands. Lastly, the possibility of DHR38 functioning as a ecdysteroid receptor is not supported in our studies, which show that DHR38 is not required for either ecdysone-mediated vCrz PCD or the survival of larval CCAP. Together, our study sheds light on the distinctive functions performed by the nuclear

receptors with respect to PCD mechanism in two sets of neurons. In future, it will be interesting to investigate whether hormonal signals other than ecdysone are involved in regulating the neuronal PCD. Juvenile Hormone is a potential candidate sine it has been demonstrated to suppress PCD in the prothoracic glands of *Manduca sexta* (Dai & Gilbert 1999) and also suggested to be a likely ligand for USP.

Lastly, it would be interesting to understand why some neurons like vCrz and DM neurons but not DL neurons, are selectively destined to die.

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