STEROID-TRIGGERED, CELL-AUTONOMOUS PROGRAMMED CELL DEATH OF IDENTIFIED *DROSOPHILA* MOTONEURONS DURING METAMORPHOSIS

by

ARI WINBUSH

A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

December 2008

University of Oregon Graduate School

Confirmation of Approval and Acceptance of Dissertation prepared by:

Ari Winbush

Title:

"Steroid-triggered, cell-autonomous programmed cell death of identified Drosophila motoneurons during metamorphosis"

This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Eric Johnson, Chairperson, Biology
Janis Weeks, Advisor, Biology
Judith Eisen, Member, Biology
Christopher Doe, Member, Biology
Michael Wehr, Outside Member, Psychology

and Richard Linton, Vice President for Research and Graduate Studies/Dean of the Graduate School for the University of Oregon.

December 13, 2008

Original approval signatures are on file with the Graduate School and the University of Oregon Libraries.

An Abstract of the Dissertation of

Ari Winbush for the degree of Doctor of Philosophy

in the Department of Biology to be taken December 2008

Title: STEROID-TRIGGERED, CELL-AUTONOMOUS PROGRAMMED CELL DEATH OF IDENTIFIED *DROSOPHILA* MOTONEURONS DURING METAMORPHOSIS

Approved: _____
Dr. Janis C. Weeks

Programmed cell death (PCD) is a critical process during development and maturity of vertebrates and invertebrates. Aberrations in PCD are responsible for numerous developmental abnormalities and diseases in humans. Cell death pathways are surprisingly similar across species, so the study of PCD in simpler organisms such as insects provides important insight into the roles of cell death in higher animals including humans.

Metamorphosis of the fruit fly, *Drosophila melanogaster*, provides an excellent model system in which to study PCD. During metamorphosis, many obsolete larval structures undergo PCD, largely in response to changes in circulating levels of steroid hormones known as ecdysteroids. These effects of ecdysteroids are particularly striking

in the nervous system, where many larval neurons undergo PCD or functional remodeling during metamorphosis. One wave of neuronal PCD takes place during the first 24 hours of metamorphosis while a second follows adult emergence. Studies in another insect, *Manduca sexta,* suggested that the rise in ecdysteroids that initiates metamorphosis, the prepupal pulse, may trigger the first wave of neuronal PCD in *Drosophila.*

This dissertation investigated steroid-regulated neuronal PCD in *Drosophila* by studying an individually-identified larval motoneuron, RP2. Using molecular genetics, immunocytochemistry and primary cell culture, I showed that abdominal RP2s undergo PCD within the first 24 hours of *Drosophila* metamorphosis; identified a role for previously-identified PCD genes and ecdysteroid receptors in RP2's demise; and demonstrated that the prepupal pulse of ecdysteroids acts directly and cell-autonomously on RP2s to activate PCD. These experiments advance our understanding of hormonally-induced cell death and its regulation within the developing nervous system. This dissertation includes unpublished co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Ari Winbush

PLACE OF BIRTH: Los Angeles, CA

DATE OF BIRTH: May 8, 1979

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon
Macalester College

DEGREES AWARDED:

Doctor of Philosophy in Biology, 2008, University of Oregon
Bachelor of Arts in Biology, 2001, Macalester College

AREAS OF SPECIAL INTEREST:

Developmental Biology
Neurobiology

PROFESSIONAL EXPERIENCE:

Graduate Teaching Fellow, Department of Biology, University of Oregon, 2001-2002

Graduate Research Fellow, Department of Biology, University of Oregon, 2001-2008

GRANTS, AWARDS AND HONORS:

Developmental Biology Training Grant, National Institutes of Health, 2002-2004

Minority Predoctoral Fellowship, American Psychological Association, 2004-2007

ACKNOWLEDGMENTS

I wish to first express sincere thanks to my mentor and advisor, Dr. Janis C. Weeks for her guidance, instruction, and encouragement and help in the editing of my papers. I also thank her for sticking by me and allowing me to try new ideas even when things got rough and data seemed difficult to obtain. I also must acknowledge past lab members for their tremendous help and friendship. Included among these are: Dennis Hazelett, Johnathan Melville, Michele C. Zee, Christine Andrews, Julie Houck, Elizabeth Rodrigues, and Tregony Bucknell-Pogue. I also thank the members of my advisory committee: Dr Janis C. Weeks, Dr. Eric Johnson, Dr. Chris Doe, Dr. Judith Eisen, and Dr. Michael Wehr for their support and instruction. I also owe thanks to Dr. Richard Levine for pointing my research in the right direction. I am also indebted to Peg Morrow, Ellen McCumsey and Donna Overall who are true miracle workers and who kept me on track. I also acknowledge friends and family for their encouragement and comfort, especially during those rough and difficult times. This research was supported by an NIH Developmental Biology Training Grant (AW), an American Psychological Association Diversity Program in Neuroscience Predoctoral Fellowship (AW), The Oregon Medical Research Foundation (JCW) and Oregon Partnership for Alzheimer's Research (JCW).

Dedicated to my parents and family for their help and support these past years

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Significance

The elimination of cells by programmed cell death (PCD) is a critical process in the development and maintenance of both vertebrates and invertebrates (review in Baehrecke, 2002). During PCD, cells that are no longer required in the course of development or maintenance of homeostasis, are virally infected, or contain mutations that result in potentially dangerous dysfunction are eliminated in a form of self-destruction. In this process, cellular contents are degraded and prevented from spilling into the surrounding environment. PCD, which includes apoptosis and autophagy, is indispensable in embryonic and post-embryonic development and is required for proper development and function of anatomical structures, the nervous, immune and other systems (reviewed in Thompson, 1995; Vaux and Korsmeyer, 1999; Mattson, 2000; Yuan and Yanker, 2000).

Defects in PCD, whether due to its aberrant occurrence or failure to take place, have been implicated in developmental abnormalities, diseases and pathologies, making the study of this process critical. Examples of diseases associated with failure of PCD to take place include certain types of cancers (e.g., Soengas et al., 2001; reviewed in Green

and Evan, 2002), autoimmune disorders and viral infection (reviewed in Thompson, 1995). Undesired PCD is associated with Acquired Immune Deficiency Syndrome (AIDS), myocardial infarction and liver disease (reviewed in Thompson, 1995). Within the central nervous system of humans, diseases associated with aberrant PCD cause a significant burden. PCD of cortical neurons due to oxidative stress is a hallmark of Alzheimer's disease, while the degeneration of neurons within the substantia nigra and striatum underlie Parkinson's disease and Huntington's disease, respectively (reviewed in Mattson, 2000). Amyotrophic lateral sclerosis (ALS) is characterized by PCD of motoneurons within the spinal cord, leading to paralysis and death usually as a result of respiratory failure (reviewed in Mattson, 2000; Yuan and Yanker, 2000). Following stroke, areas surrounding the initial brain lesion show high levels of PCD due to oxidative stress following reperfusion (restoration of blood flow), which can result in additional brain damage (reviewed in Thompson, 1995; Won et al., 2002). For these reasons, an understanding of the mechanisms underlying PCD is of paramount importance. Indeed, much research into the treatment of these pathologies is centered on the development of drugs that interact with the PCD pathway (e.g., Amelio et al., 2008).

Although PCD occurs in many contexts, the internal or external cues and intracellular pathways are surprisingly similar among mammals, insects and nematode worms (*Caenorhabditis elegans*) whether during development, the maintenance of homeostasis, or in disease models (reviewed in Mattson, 2000; Baehrecke, 2002; Weeks, 2003; Danial and Korsmeyer, 2004; Hay and Guo, 2006; Kumar, 2007). Therefore research into PCD using simpler model systems can give insight into the mechanisms

behind disease pathologies. The metamorphosis of holometabolous insects (those that undergo complete metamorphosis) provides an excellent and relatively simple model. During metamorphosis, larvae whose behaviors are focused on feeding, locomotion, and defense are transformed into adults capable of flight and reproduction. During this process many larval structures are destroyed through PCD, providing a fruitful opportunity to study PCD in a natural context (reviewed in Baehrecke, 2000; Tissot and Stocker, 2000).

The greatest amount of study in this area has utilized the hawkmoth, *Manduca sexta*, and the fruit fly, *Drosophila melanogaster*. The nervous system of both insects is of particular interest. During metamorphosis the nervous system of the larva must undergo a dramatic reorganization to accommodate adult-specific behaviors. This reorganization includes the elimination of obsolete larval neurons through PCD. Metamorphosis of both insects is regulated by steroid hormones. Detailed studies have been undertaken investigating the role hormones play in the death of *Manduca* neurons (reviewed in Weeks, 2003) but little is known about PCD in the *Drosophia* nervous system during metamorphosis and its hormonal control. The available genetic tools in *Drosophila*, its experimental accessibility, and extensive data on its development from embryogenesis to adulthood (reviewed in Bangs and White, 2000), make the understanding of this process in the nervous system during metamorphosis a feasible area of study.

This dissertation focuses on PCD in the *Drosophila* nervous system during early metamorphosis. In particular it identifies a specific motoneuron that undergoes PCD and

the hormonal cues responsible for its demise. It also provides insight into the intracellular death pathway utilized by this motoneuron. Background information on PCD, steroid hormones, and *Drosophila* metamorphosis will first be provided.

Overview of PCD mechanisms

At the core of the death machinery is a family of cysteine proteases termed caspases that cleave target proteins at aspartate residues within the cell leading to its auto-degradation. This includes DNA fragmentation by caspase activated DNAases (CADs) (reviewed in Strasser et al., 2000; Danial and Korsmeyer, 2004; Kumar, 2007). The number of caspases varies between species and not all caspases function in PCD. Seven caspases have been identified in *Drosophila*, four in *C. elegans*, and at least eleven and ten in humans and mice, respectively (see Kumar, 2007 and references cited therein). Two types of caspases, the initiator and effector caspases, participate in PCD. Both normally remain inactive due to the presence of a stabilizing N-terminal prodomain that must be proteolytically cleaved. Initiator caspases have long prodomains that contain critical protein-protein interaction motifs that include caspase recruitment (CARD) domains or death effector (DED) domains, while effector caspases have short prodomains (reviewed in Strasser et al., 2000; Kumar, 2007). Effector caspases are activated through proteolytic cleavage by the upstream initiator caspase(s). Following cleavage, effector caspases become active heterodimers, containing two subunits which can dimerize with other heterodimers to form tetramers. These effector caspases then degrade the cell's

proteins through a series of intracellular cascades. The induction of PCD therefore lies in the activation of the initiator caspases, which is discussed below.

A detailed discussion of all the caspase-dependent PCD pathways in mammals, insects and worms and their regulation is beyond the scope of this dissertation. Therefore, a brief overview of the basic pathway will be described here for mammals and *C. elegans* before the focus shifts specifically to insects.

Initiator caspases in mammals include caspases-2, 8, 9, and 10 (reviewed in Danial and Korsmeyer, 2004; Kumar, 2007). In mammals, two pathways are prominent in PCD: the death receptor pathway and the mitochondrial pathway. In the death receptor pathway, the initiator caspase-8 (and/or possibly caspase-10) is activated following a complex signaling cascade mediated by extracellular cues acting on a death receptor. The death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily and include the TNFR1, Fas, and Trail receptor families. The cytoplasmic tail of the death receptor contains a protein-protein interacting death domain (DD). The activation of a death receptor via a TNFR ligand results ultimately in the recruitment of intracellular adaptor proteins that include the Fas-associating-death-domain-protein (FADD) and contain both a DD and DED. The cytoplasmic tails of the death receptor and adaptor proteins interact via their DDs to form a death-inducing-signalling-complex (DISC). The DED of the adaptor protein then interacts with the DED of caspase-8 resulting in the recruitment of caspase-8 proteins. The aggregation of caspase-8 proteins likely results in their auto-proteolytic activation possibly due to a proximity effect. Once activated, caspase-8 can then activate the effector caspases, of which caspase-3 and caspase-7 seem

to be most critical, leading to cell death (reviewed in Vaux and Korsmeyer, 1999; Strasser et al., 2000; Danial and Korsmeyer, 2004; Kumar, 2007).

The details of the mitochondrial PCD pathway remain controversial but strong evidence suggests that this pathway depends on the Bcl-2 family of proteins which includes both pro-and anti-apoptotic members (reviewed in Strasser et al., 2000; Danial and Korsmeyer, 2004; Kumar, 2007). In response to a death-activating stimulus, cytochrome c is released from mitochondria and interacts with the apoptotic-protease-activating-factor-1 (Apaf1) adaptor protein. Apaf1 contains a CARD and C-terminal domain containing WD40 repeats. Cytochrome c binds the WD40 domain of Apaf1, facilitating the subsequent binding of ATP/dATP to Apaf1. It is thought that this causes Apaf1 to undergo a conformational change that both exposes its CARD and causes the formation of a seven-member Apaf1-oligomer known as the apoptosome. The exposed CARDs recruit the initiator caspase-9, resulting in its aggregation and autoactivation and subsequent effector caspase activation (reviewed in Strasser et al., 2000; Danial and Korsmeyer, 2004; Kumar, 2007; Amelio et al., 2008). A detailed description of the Bcl-2 proteins and the roles they play in this process remain controversial and are beyond the scope of this dissertation. However, studies indicate that the release of cytochrome c requires permeabilization of the outer mitochondrial membrane. It is not known clearly how this occurs but in response to death activating stimuli, certain pro-apoptotic Bcl-2 proteins such as Bax and Bak may be able to insert into the outer mitochondria membrane and oligomerize to form pores. Indeed, certain anti-apoptotic Bcl-2 proteins

block PCD by preventing pore formation by the pro-apoptotic members (reviewed in Strasser et al., 2000; Green and Evan, 2002; Danial and Korsmeyer, 2004; Kumar, 2007).

In addition to regulation at the level of the Bcl-2 proteins and death receptor pathway proteins, caspases can also be inhibited by inhibitor of apoptosis proteins (IAPs) that bind caspases. The most prominent IAP in mammals, X-linked IAP (XIAP), can bind caspase-3, 7 and 9 and cause their degradation through ubiquitination. Other IAPs have also been characterized but their roles remain controversial. IAPs themselves are also subject to inhibition by other mitochondrial proteins that are released during permeabilization. Among these are Smac/DIABLO and Htra/Omi (reviewed in Green and Evan, 2002; Danial and Korsmeyer, 2004; Kumar, 2007).

PCD in *C. elegans* is regulated in a similar fashion (reviewed in Conradt and Xue, 2005). The proteins CED-3, CED-4 and CED-9 are at the core of the death machinery. CED-4, which is homologous to Apaf1, bears a CARD and is required for activation of the initiator caspase, CED-3. CED-9 has homology to Bcl-2 and inhibits the activity of CED-4 by binding it and keeping it localized to the mitochondria. The specification of a cell to die depends on the activity of a protein, EGL-1, that is expressed at high levels in doomed cells. The EGL-1 protein contains a BH3 domain that allows it to interact with CED-9, causing the liberation of CED-4 from the mitochondria. Liberated CED-4 may itself oligomerize at the perinuclear membrane and recruit CED-3 via its CARD domain. The aggregation of CED-3 likely results in its auto-activation, whereby it then activates downstream effector caspases (reviewed in Baehrecke, 2002; Danial and Korsmeyer, 2004; Conradt and Xue, 2005; Kumar, 2007).

Programmed cell death in *Drosophila*

The seven identified caspases in *Drosophila* are Dronc (Dorstyn et al., 1999a), Dredd (Chen et al., 1998), DCP-1 (Song et al., 1997), Drice (Fraser and Evan, 1997), Dream (Doumanis et al., 2001), Damm (Harvey et al., 2001), and Decay (Dorstyn et al., 1999b). Dronc, Dredd and Dream have long N-terminal pro-domains suggesting that they function as apical caspases. Only Dronc contains a CARD. Dredd contains two DEDs in its prodomain, while Dream contains no DEDs or CARDs, but has multiple Ser/Thr residues in its pro-domain (reviewed in Kumar, 2007). Of the three identified apical caspases, Dronc appears to be the most essential in developmental PCD (reviewed in Mills et al., 2005). Indeed, the majority of PCD that takes place during *Drosophila* development and metamorphosis appears to require Dronc (Chew et al., 2004; Daish et al., 2004; Waldhuber et al., 2005; Xu et al., 2005). The function of Dream remains obscure while Dredd appears to play a prominent role in the innate immune response (Leulier et al., 2000; reviewed in Cashio et al., 2005).

In contrast to mammals, the activity of caspases in *Drosophila* is strongly dependent on the balance between death-activating and death-inhibiting proteins. The critical death-activating proteins are encoded by genes that lie within the 75C1-2 genomic interval and include *reaper*, *hid* and *grim* (White et al., 1994; Grether et al., 1995; Chen et al., 1996). The *Df(3L)H99* (*H99*) deletion, which removes all three genes, results in complete failure of developmentally-dependent or stress-induced PCD during embryonic

development. *H99* homozygous embryos fail to hatch and the normal spatial and temporal patterns of PCD during embryogenesis are completely absent (White et al., 1994). Analysis of *H99* embryos reveals numerous extra cells especially in the central nervous system (White et al., 1994). Furthermore, transgenic mis-expression of Reaper, Grim or Hid results in ectopic PCD in the target tissue (reviewed in Rodriguez et al., 1998; Bergmann et al., 2003). Yet co-expression of the viral caspase inhibitor p35 is able to block PCD induced by Reaper, Grim or Hid, indicating that the protein products of these genes act upstream of caspases and are likely involved in their activation (White et al., 1994; Grether et al., 1995; Chen et al., 1996). These data together indicate a critical role for these genes and the proteins they encode in most modes of PCD in *Drosophila.*

PCD in *Drosophila* is negatively regulated by the *Drosophila* inhibitor of apoptosis protein 1 or 2 (Diap1/2) (Hay et al., 1995). In addition to Diap1 and 2, another protein, that bears IAP homology, dBruce, has been identified. Little is known about the roles of dBruce and Diap2 (reviewed in Hay and Guo, 2006). However, studies have revealed much about Diap1 and the critical role it plays in *Drosophila* PCD. In contrast to mammalian IAPs, Diap1 is a critical point of control in PCD in *Drosophila.* The N-terminal region of Diap1 contains two baculovirus inhibitor of apoptosis repeat (BIR) domains, and the C-terminus contains a critical RING domain (Hay et al., 1995).

The important role of Diap1 came from studies revealing that Reaper interacted with Diap1, and that mutations resulting in the loss of Diap1 resulted in embryonic lethality due to massive PCD of virtually all cells (Vucic et al., 1997; Wang et al., 1999). These studies revealed that the intracellular PCD machinery is constitutively expressed

and capable of becoming active, but is held in check by Diap1. An initial hypothesis was that Reaper, Hid, or Grim (RHG) somehow activates the apical and effector caspases and that Diap1 blocks PCD by binding RHG proteins thereby interfering with their activity (Vucic et al., 1997, 1998). Later studies, however, revealed that Diap1 is actually a caspase inhibitor and that RHG causes PCD by binding to Diap1 and antagonizing its inhibition (Goyal et al., 2000).

The BIR and RING domains of Diap1 are critical in its function. Reaper and Grim proteins specifically interact with BIR1 while evidence suggests that Hid interacts with BIR2 (Vucic et al., 1998; Lisi et al., 2000). Mutations in BIR1/2 that affect RHG binding result in inhibition of PCD induced by mis-expression of RHG protein (Goyal et al., 2000; Lisi et al., 2000). In contrast, loss of function mutations in *diap1* result in enhanced PCD (Wang et al., 1999).

The ability of Diap1 to bind and inhibit caspases lies in its BIR2 and RING domains. Studies have shown that the function of Diap1 lies in its ability to bind to the pro-domain of Dronc, and that this binding requires the BIR2 domain of Diap1 (Meier et al., 2000; Wilson et al., 2002). Mutations in BIR2 that affect the binding of Dronc to Diap1 result in enhanced PCD, indicating that this interaction prevents the activation of Dronc (Wilson et al., 2002). Furthermore, removal of the pro-domain from Dronc makes this protein resistant to inhibition by Diap1, demonstrating the critical role of this Diap1-Dronc interaction (Meier et al., 2000).

The RING domain of Diap1 allows it to act as an E3 ubiquitin ligase. This is confirmed by the short half-life of Diap1, indicating that this protein is subject to self-

ubiquitination leading to its degradation (Muro et al., 2002; Wilson et al., 2002). Mutations in the RING domain of Diap1 do not affect its ability to bind RHG or Dronc but still nevertheless result in enhanced PCD (Wilson et al., 2002). Mutations in the RING domain also increase the stability of Diap1 and prevent both the ubiquitination and inactivation of Dronc (Muro et al., 2002; Wilson et al., 2002). These data indicate that the binding of Dronc to Diap1 results in the ubiquitination and deactivation of Dronc or auto-processed Dronc, and its ability to undergo further catalytic autoprocessing (see below). This Diap-Dronc mechanism may also interfere with the required interaction between Dronc and Dark (see below). PCD occurs when RHG binds to the BIR of Diap1 and either prevents Diap1 from binding Dronc or causes the release of Dronc from Diap1. Diap1 along with RHG then undergoes self-ubiquitination and degradation, allowing for the accumulation of the more stable Dronc protein (reviewed in Martin, 2002). Diap1 levels can also be reduced through other mechanisms involving RHG (Yoo et al., 2002). Although a general mechanism of Diap1 function and inactivation by RHG has emerged, the details still are not concrete.

Diap1 also may block PCD by binding the effector caspases Drice and DCP1. In this model, Diap1 acts as a substrate for Drice/DCP1. The cleavage of Diap1 by either caspase exposes an unstable amino acid residue that promotes the ubiquitination of Diap1 and the bound effector caspase (reviewed in Bergmann et al., 2003). Thus Diap1 may also protect against PCD caused by spontaneous effector caspase activation.

The activation of Dronc remains poorly understood, but it appears that Dronc is able to undergo several auto-catalytic cleavage events (Yan et al., 2006). One cleavage

event occurs at a glutamate residue and is required for Dronc activation (Muro et al., 2002). The cleaved active sites then form a stable active dimer (reviewed in Hay and Guo, 2006). This activation requires the *Drosophila* Apaf1 homologue, Dark. Like Apaf1, Dark carries a CARD and WD-repeat domain (Rodriguez et al., 1999). Initial studies using hypomorphic mutations in *dark* revealed a requirement for Dark in several modes of PCD. *dark* hypomorphs were able to rescue PCD caused by loss of Diap1 or ectopic RHG expression (Rodriguez et al., 1999, 2002). Although the *dark* hypomorphs survived to adult stages, visible defects consistent with failed PCD were frequently observed (Rodriguez et al., 1999). Stronger null mutations in *dark* in the absence of any maternal contribution result in late embryonic lethality with loss of virtually all normally-occurring cell deaths (Akdemir et al., 2006). This phenotype strongly mimics that of *dronc* null mutants which, in the absence of any maternal contribution, also result in embryonic lethality and failure of almost all PCDs (Xu et al., 2005).

Studies have demonstrated formation of a double 8-member apoptosome by Dark (Yu et al., 2005). It is thought that apoptosome assembly with Dark recruits Dronc via a CARD interaction resulting in the auto-processing of Dronc to an active form. Auto-processed Dronc can then undergo further autocatalytic-activation leading to release from the apoptosome, Diap1 resistance, and activation of effector caspases (reviewed in Martin, 2002; Hay and Guo, 2006). The mechanisms behind apoptosome formation remain unresolved, particularly since apoptosome formation does not seem to require cytochrome c (Dorstyn et al., 2004; Yu et al., 2005). PCD in *Drosophila* depends therefore on the tight regulation of death-inducing and death-inhibiting factors. When the

balance is shifted in favor of death promoting factors (e.g., by loss of Diap1 activity) by some external or internal cue, caspases, normally held in-check, are activated.

PCD in *Drosophila* is prominent during embryogenesis, certain stages of larval development, metamorphosis, and the first 24 h following metamorphosis (reviewed in Bangs and White, 2000). One external cue that causes PCD in the developing nervous system is the withdrawal of trophic factors. For instance, developmental PCD of a subset of midline glial cells occurs when they are separated from neurons that secrete the epidermal growth factor (EGF) ligand SPITZ. In this case, SPITZ normally acts through EGF to mediate Ras-MAPK dependent signaling that results in the repression of hid expression. Loss of SPITZ results in hid expression and PCD (Bergmann et al., 2002).

Many of the dying cells during *Drosophila* development are neuroblasts that have completed the generation of their neural lineages. In this instance, PCD is used to regulate the number of neurons generated by embryonic and post-embryonic asymmetrical neuroblast divisions. Failure of such PCD results in the generation of extra neurons (Peterson et al., 2002; Ortmann et al., 2007; reviewed in Truman et al., 1993). PCD of a subset of postembryonic neuroblasts midway through larval development depends on the Hox protein Abdominal-A which acts to induce expression of RHG (Bello et al., 2003). There are also examples of PCD outside of the nervous system that depend on external cues. Following adult eclosion for example, wing epidermal cells undergo PCD in response to the peptide hormone bursicon (Kimura et al., 2004). However, the detailed cues for most cell deaths including neuroblasts and other cells

remain unclear. During metamorphosis, however, some cell deaths are regulated by steroid hormones as discussed below.

Steroid hormones and programmed cell death

Steroid hormones are small lipophilic molecules, resulting from fatty acid metabolism and synthesized from cholesterol derivatives, that have the ability to regulate gene expression. They do this by binding a special class of proteins belonging to the well-characterized nuclear receptor superfamily (reviewed in Aranda and Pascual, 2001). These nuclear receptors have the ability to act as transcriptional activators or repressors by binding special hormone response elements in DNA, in response to ligand. Numerous vertebrate nuclear receptors have been identified and classified into six subfamilies. These receptors include thyroid hormone, retinoic acid, vitamin D, hepatocyte proteins, glucocorticoid, androgen, estrogen, and retinoic X receptors. In addition, numerous orphan receptors whose ligands remain unknown have also been characterized (reviewed in Aranda and Pascual, 2001).

Receptors for steroid hormones belong to the third subfamily of nuclear receptors, and include receptors for estrogens, androgens, glucocorticoids, progestins and, in the case of insects, ecdysteroids (reviewed in Aranda and Pascual, 2001; Truman and Riddiford, 2002). Steroid hormone receptors normally bind palindromic sequences in the DNA response elements in response to ligand (reviewed in Aranda and Pascual, 2001). In vertebrate development steroid hormones play critical roles during pre-and postnatal

development and sexual maturation. Such critical roles include specification of systemic anatomical and local cellular sexual differences. Two prime examples of this are the sexual dimorphisms within the nervous and reproductive systems which ultimately affect sexual behavior (reviewed in Breedlove, 1992; Morris et al., 2004).

Since PCD is a critical component of organ system development and homeostasis, it is not surprising that organizational effects of steroid hormones include regulation of PCD. Circulating androgens for instance are indirectly responsible for protecting spinal motoneurons in spinal nucleus of the bulbocavernosus (SNB) (Freeman et al., 1996). These motoneurons normally innervate male-specific muscles in mammals. Hence, adult females have fewer SNB motoneurons. Studies in rats have demonstrated that, in females, SNB motoneurons and muscles undergo PCD around the time of birth. In this instance, it is the degeneration of the muscles that triggers death of the motoneurons. In male rats, testosterone prevents the degeneration the muscles, which in turn keeps the motoneurons intact (reviewed in Morris et al., 2004). Steroid hormones can also induce PCD in the immune system. Glucocorticoids are well known for their ability to cause PCD of lymphoid cells, making them useful in treating lymphoid cancers (reviewed in Schmidt et al., 2004). Other hormones are also known for their ability to cause PCD in both development and disease (reviewed in Kiess and Gallaher, 1998).

Metamorphosis provides an ideal opportunity to study developmentally-dependent PCD regulated by steroids and other hormones. During amphibian metamorphosis, for example, the destruction of tissues unique to the tadpole including the tail and cells in the intestine is mediated by thyroid hormone (reviewed in Su et al.,

1999). Studies have shown that thyroid hormone acts cell autonomously via nuclear receptors in target tissues to induce PCD (Su et al., 1997). The same hormone can also cause proliferation of adult-specific cells, adding to the complexity of the actions of hormones during metamorphosis (reviewed in Su et al., 1999). A similar situation is observed during insect metamorphosis in response to steroids, as discussed below.

Steroid hormones and PCD during insect metamorphosis

In holometabolous insects including *M. sexta* and *Drosophila*, circulating levels of steroid hormones regulate both growth and metamorphosis. *Drosophila* development consists of embryonic development and three larval stages (instars) and then metamorphosis. As in other insects, metamorphosis in *Drosophila* is driven by ecdyteroids, including the active metabolite 20-hydroxyecdysone (20E) (reviewed in Truman and Riddiford, 2002). Ecdysteroids are produced in *Drosophila* by the prothoracic ring gland in response to prothoracicotropic hormone, PTTH. PTTH is normally produced by a set of neurosecretory cells in the insect brain. In response to PTTH, the ring gland secretes ecdysteroids which are then converted into active metabolites including 20E by target organs or fat body (reviewed in Riddiford, 1993).

Levels of ecdysteroids are responsible for the growth and eventual transformation of the larva to an adult (see chapter II Fig. 1). Each of the three larval molts in *Drosophila* is preceded by a small pulse of ecdysteroids. However, metamorphic changes in response to these small pulses are held in check by juvenile hormones (JH)

(review in Truman and Riddiford, 2002). Small ecdysteroid fluctuations during the third (final) larval instar are responsible for the initiation of wandering behavior in which the larva leaves the food substrate and locates a suitable place for pupariation (Warren et al., 2006; reviewed in Riddiford, 1993). Pupariation is initiated by a larger, "late larval pulse" of ecdysteroids in the absence of JH during which the larva ceases locomotion and forms a white prepupa. This marks the onset of metamorphosis. A smaller "prepupal pulse" of ecdysteroids approximately 10 h after puparium formation (APF) causes pupation, marked by head eversion at 12 h APF (reviewed in Baehrecke, 1996). A much larger and long-lived "pupal pulse" is responsible for the bulk of metamorphosis. Following adult eclosion, ecdysteroid levels decline and remain low or absent during adult life (Handler, 1982; reviewed in Richards, 1981; Riddiford, 1993).

Ecdysteroids exert their response in *Drosophila* by binding to three isoforms of the ecdysone receptor (EcR): A, B1 and B2 which vary in their N-terminal domains. EcR-A is transcribed from a separate promoter while EcR-B1 and EcR-B2 are splice variants of the same transcript (Talbot et al., 1993). Like most nuclear receptors, the EcRs contain an N-terminal A/B domain, a DNA binding domain, ligand binding domain, and a C-terminal F domain (reviewed in Truman and Riddiford, 2002). Transcriptional activation lies within the N-terminal A/B region (AF1) and the ligand binding domain (AF2) (reviewed in Aranda and Pascual, 2001; Truman and Riddiford, 2002). All three isoforms have strong transcriptional activation in AF2 but, only EcR-B1 and B2 have strong activation in AF1. EcR-A may have transcriptional repressive function in this region (Hu et al., 2003). Despite being steroid hormone receptors, EcRs

form very poor homodimers and instead dimerize with the retinoic X receptor homologue, ultraspiracle (USP), similar to the heterodimerization of thyroid hormone receptor (Yao et al., 1992; Hall et al., 1998). Unliganded EcR/USP can act as a repressor, while liganded EcR/USP can strongly stimulate transcription (reviewed in Truman and Riddiford, 2002).

The metamorphic fates of different tissues, such as the larval midgut and salivary glands, and neurons within the nervous system, in response to changes in 20E, are determined in part by the spatial and temporal patterns of EcR expression, and include PCD (Talbot et al., 1993; Truman et al., 1994). For instance, the destruction of the larval midgut and salivary glands during *Drosophila* metamorphosis occurs in direct response to the respective late larval and prepupal pulses of 20E (Jiang et al., 1997). In the nervous system, many existing larval neurons are either remodeled to an adult phenotype, which may include dendritic regression and regrowth, or die by PCD. Both responses are hormone dependent as demonstrated in studies of *M. sexta* and *Drosophila* (reviewed in Tissot and Stocker, 2000). There is strong circumstantial evidence in *Drosophila* that dendritic regression is dependent on EcR-B isoforms while regrowth is EcR-A dependent (Truman et al., 1994).

In *M. sexta* two major waves of PCD take place in the nervous system during metamorphosis. At the beginning of metamorphosis, accessory planta retractor (APR) motoneurons, which innervate the larval prolegs in segments A5, A6 and A1, undergo PCD (Weeks and Truman, 1985; reviewed in Weeks, 2003). Studies have shown that the death of these motoneurons is a cell-autonomous response to the prepupal pulse of 20E

(Streichert et al., 1997). In this instance, a rise steroid hormone levels acts on the motoneurons to bring about their demise. At the end of metamorphosis, a second wave of neuronal PCD takes place that includes the remaining APR motoneurons in abdominal segments A2-A4 (Weeks and Ernst-Utzschneider, 1989). In this instance, it is the decline of 20E at the end of metamorphosis that activates PCD in APRs (Zee and Weeks, 2001). In both instances of PCD, the motoneurons, as demonstrated in cell culture, respond cell-autonomously to changes in 20E levels and not some other cue.

Dendritic regression is another response to 20E in *M. sexta.* The APR motoneurons in all abdominal segments, along with many other motoneurons, undergo dendritic regression during the prepupal pulse of 20E. Studies have shown this event to be a response to the prepupal rise in 20E, independent of cues from target muscles (Levine and Truman, 1985; Weeks and Truman, 1985; Weeks et al., 1992). Likewise the dendritic regrowth of motoneurons is also a 20E response (Weeks and Ernst-Utzschneider, 1989; Prugh et al., 1992).

During *Drosophila* metamorphosis, existing larval neurons are also remodeled in a hormone-dependent manner. At the onset of metamorphosis, motoneurons that innervate the dorsolongitudinal indirect flight muscles (DLM) undergo marked dendritic regression during the first 4 h APF (Consoulas et al., 2002). This is followed by the initiation of dendritic regrowth to an adult-specific pattern beginning at approximately 20 h APF (Consoulas et al., 2002, 2005). Dendritic regrowth of the DLM motoneurons requires an ecdysteroid-induced member of the *broad complex (BRC)* family of transcription factors. However, in this instance, the DLM motoneurons do not express

any *BRC* proteins, indicating a non-cell-autonomous role of ecdysteroids in dendritic regrowth (Consoulas et al., 2005). These experiments suggest that regression is induced by the late larval pulse of ecdysteroids while regrowth is mediated by the rise of ecdysteroids during the pupal pulse.

The dendritic pruning of thoracic ventral FMRFamide-expressing neurons also immediately follows the late larval pulse of ecdysteroids in *Drosophila*. Analysis of *EcR-B*-specific mutations reveals a requirement for EcR-B isoforms for this process to occur (Schubiger et al., 1998, 2003). Transcriptional de-repression of EcR-A by ecdysteroids may play a role in the dendritic regrowth of these FMRFamide neurons (Brown et al., 2006). The dendritic regression of certain mushroom body neurons during the same period requires EcR-B1 and can be rescued in *EcR-B1* mutants by cell-autonomous expression of either EcR-B isoform (Lee et al., 2000b). The dendritic re-growth of the neurons occurs during early pupal development during the period of the rise of the pupal pulse of ecdysteroids and may require the cell-autonomous action of ecdysteroids (Kraft et al., 1998).

PCD in the *Drosophila* nervous system during metamorphosis occurs in two waves. The second wave, involves roughly 300 "Type II" neurons that express high levels of EcR-A during the latter half of the pupal stage, and a subset of neurons that express the crustacean cardioactive peptide (CCAP). PCD of the Type II and CCAP neurons occurs during the first 24 h following adult eclosion and requires the decline in ecdysteroids that follows the pupal pulse. Injecting newly eclosed adults with 20E delays the death of both sets of neurons (Robinow et al., 1993; Draizen et al., 1999). Death of at

least some Type II neurons also requires an unknown cue that correlates with eclosion and subsequent wing inflation behaviors and can be perturbed by decapitation immediately following eclosion (Kimura and Truman, 1990; Robinow et al., 1997). Although it is not known for certain, evidence strongly suggests that ecdysteroids, acting through EcR-A, repress expression of Reaper and Grim in these neurons, until the hormones decline at the end of metamorphosis. Indeed, both Reaper and Grim play a role in the death of these neurons, and delaying the decline in ecdysteroids also delays the accumulation of *reaper* and *grim* transcripts in some doomed cells (Robinow et al., 1997; Draizen et al., 1999).

At the onset of metamorphosis, dying neurons are also observed shortly after the late larval pulse of ecdysteroids (reviewed in Truman et al., 1993). Dying neurons are observed through the first 24 h APF but their full identities and the hormonal cues responsible for their demise are unknown. Choi et al. (2006) identified a set of peptidergic neurons that express the neuropeptide corazonin (Crz), among these dying neurons. They provide evidence that the late larval pulse, acting through EcR-B isoforms, is responsible for the deaths of the Crz neurons. Interestingly, PCD of the larval midgut and salivary glands also occurs during this period, and is induced by ecdysteroids (Jiang et al., 1997). Since PCD in the nervous system during this period remains largely unexplored, it remains an intriguing area of study. For instance, it is not known for certain if all PCDs are hormonally dependent. If they are dependent on steroids, the exact hormonal cues remain unknown.

Bridge

The following chapter examines PCD within the *Drosophila* nervous system during early metamorphosis. It details findings surrounding the fate of the larval abdominal motoneuron, RP2, during this period. It also tests the hypothesis that PCD of the abdominal RP2 motoneuron is a cell-autonomous response to the prepupal pulse of ecdysteroids, acting via previously identified genes in the *Drosophila* PCD pathway. The chapter includes information that will be included in an upcoming publication with Janis C. Weeks as a co-author.

CHAPTER II

STEROID-TRIGGERED, CELL-AUTONOMOUS PROGRAMMED CELL DEATH OF IDENTIFIED *DROSOPHILA* MOTONEURONS DURING METAMORPHOSIS

Intended for publication with Janis C. Weeks as co-author. All experiments and writing were performed by me with J.C. Weeks providing editorial assistance.

Introduction

Metamorphosis of the fruitfly, *Drosophila melanogaster*, entails the transformation of a crawling, feeding larva into an adult capable of flight and reproduction. During this process the larval nervous system undergoes a systematic reorganization to accommodate these new adult-specific behaviors, including both neuronal remodeling and the elimination of obsolete larval neurons by programmed cell death (PCD) (reviewed in Truman et al., 1993; Tissot and Stocker 2000). Metamorphic events in larval tissues are regulated in large part by a class of steroid hormones collectively called ecdysteroids including the active metabolite, 20-hydroxyecdysone (20E). 20E exerts its effect by binding one of three isoforms of the ecdysteroid receptor (EcR): EcR-A, EcR-B1 or EcR-B2. Transcriptional activation or repression by liganded and unliganded EcRs depends on upon formation of a heterodimer with Ultraspiracle (USP) (Yao et al., 1992; Talbot et al., 1993).

Changes in circulating levels of ecdysteroids drive the transformation from larva to pupa to adult during metamorphosis (Fig. 1). A 'late larval pulse' of ecdysteroids is responsible for the transformation of the wandering larva into a white prepupa at puparium formation. A smaller 'prepupal pulse' of ecdysteroids at approximately 10 h APF initiates pupation whose hallmark, head eversion, occurs at about 12 h APF. A more prolonged 'pupal pulse' of ecdysteroids beginning at approximately 24 h APF drives remaining development of the adult fly. As adult eclosion (emergence) approaches, ecdysteroid levels decline and remain low or absent during early adult life (reviewed in Riddiford, 1993).

Programmed cell death is the process by which obsolete, excess or damaged cells are removed. In *Drosophila,* PCD in individual cells is controlled through a balance between death-activating and death-inhibiting proteins. The core cell death machinery includes a family of caspase proteases which, upon activation, target cellular proteins for degradation (reviewed in Hay and Guo, 2006). In most tissues, caspases are constitutively expressed but remain inactive until they are cleaved by an upstream initiator caspase. In *Drosophila*, the apical caspase Dronc appears to govern most PCD during embryonic development and metamorphosis (Daish et al., 2004; Xu et al., 2005). There is also strong evidence that Dronc activation requires the *Drosophila* Apaf-1-related killer (Dark) protein. The formation of an apoptosome by Dark and Dronc allows Dark to recruit Dronc via its caspase recruitment domain leading to Dronc activation.

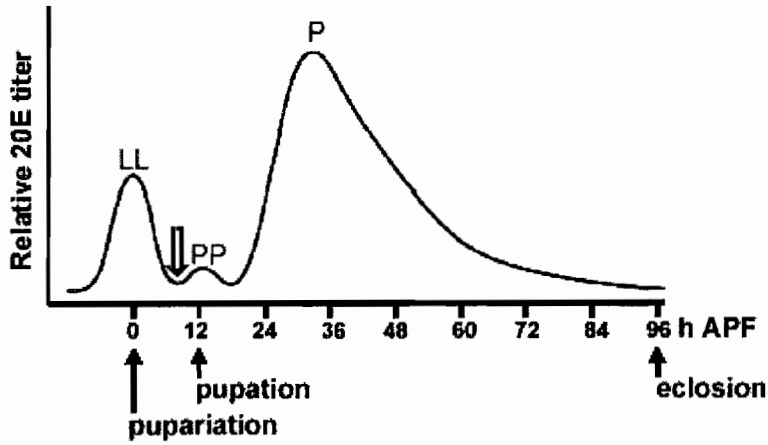


Figure 1 Endocrine events during *Drosophila* metamorphosis. Timeline illustrates changes in ecdysteroid levels during the larval-pupal-adult transformation, at 25°C. The late larval (LL) pulse of 20E triggers pupariation, with subsequent development timed by hours after puparium formation (APF). The prepupal (PP) pulse at approximately 12 h APF triggers pupation, and the prolonged pupal (P) pulse drives development of the adult fly. Emergence of the adult (eclosion) occurs at approximately 96 h APF. An open arrow indicates the time (8 h APF) at which neuronal cultures were prepared to test the effects of prepupal 20E on RP2s. Adapted from Kraft et al. (1998), based on hormone titers from Riddiford (1993) and Truman et al. (1994).

Animals carrying *dark* mutations show significant defects in PCD which are exacerbated in the presence of d*ronc* mutations (Rodriguez et al., 1999; Akdemir et al., 2006; Mills et al., 2006). Dronc is normally inhibited by the *Drosophila* inhibitor of apoptosis protein (Diap1), which may exert its effect through the ubiquitination Dronc (Meier et al., 2000; Wilson et al., 2002; reviewed in Bergmann et al., 2003). Death-activating stimuli cause the deactivation of Diap1 through the expression of a special class of proteins encoded by the genes *reaper, hid, and grim*. Reaper, Hid, or Grim cause PCD by antagonizing the activity of Diap1, resulting in the liberation of Dronc (Goyal et al., 2000; Yin and Thummel 2004; reviewed in Bergmann et al., 2003).

A role for 20E in both neuronal remodeling and PCD has been demonstrated in both *Drosophila* and the hawkmoth *Manduca sexta* (reviewed in Tissot and Stocker, 2000). The remodeling of motoneurons in *Manduca*, including those that innervate the abdominal body wall muscles is dependent on the prepupal and pupal pulses of ecdysteroids as opposed to some muscular cue (Levine and Truman, 1985; Weeks and Truman, 1985; Weeks and Ernst-Utzschneider, 1989; Prugh et al., 1992; Weeks et al., 1992; reviewed in Weeks, 2003). In *Drosophila,* the remodeling of larval motoneurons (Consoulas et al., 2002), mushroom body neurons in the larval brain (Lee et al., 2000b; Zheng et al., 2003), and the FMRFamide-expressing thoracic ventral neurons (Schubiger et al., 1998, 2003; Brown et al., 2006) is regulated by the late larval pulse of ecdysteroids. Re-growth and establishment of adult specific connections is dependent on the prepupal and pupal pulses of ecdysteroids (Kraft et al., 1998; Consoulas et al., 2005).

In *Manduca,* the PCD of accessory planta retractor (APR) motoneurons during metamorphosis is a direct, cell-autonomous response to 20E, as demonstrated by placing the motoneurons in cell culture and exposing them to appropriate changes in 20E levels (Streichert et al., 1997; Zee and Weeks, 2001). APRs that undergo PCD during the larval-pupal transformation die in response to the rise of the prepupal pulse of 20E (Streichert et al., 1997; Hoffman and Weeks, 1998, 2001). In like manner, APR motoneurons that undergo PCD at the end of metamorphosis do so cell-autonomously in response to the decline in 20E following the pupal pulse (Zee and Weeks, 2001).

In *Drosophila* PCD is highly prevalent in thoracic and abdominal neuromeres during embryogenesis, the first 24 h APF, and the first 24 h following adult eclosion (Kimura and Truman 1990; Abrams et al., 1993; White et al., 1994; reviewed in Truman et al., 1993). PCD after adult emergence includes approximately 300 "Type II" neurons identifiable by their high levels of EcR-A expression, and a subset of neurons that express the neuropeptide crustacean cardioactive peptide (CCAP) (Robinow et al., 1993; Draizen et al., 1999). In the case of the Type II and CCAP neurons, PCD appears to depend on both the decline in 20E at the end of metamorphosis and, for a subset of those neurons, an unknown cue that appears linked to eclosion behaviors and subsequent wing inflation (Kimura and Truman, 1990; Robinow *et al.*, 1993; Robinow *et al.,* 1997; Draizen et al., 1999). Less is known about the identities and potential hormonal cues responsible for neuronal PCD during the first 24 h of metamorphosis.

The findings in *Manduca* and *Drosophila,* that ecdysteroids drive PCD during certain periods throughout the insect's life cycle, suggests a possible role for ecdysteroids

in the death of larval neurons during early metamorphosis. Such a role has already been demonstrated in *Drosophila* for other larval tissues that degenerate during this period. For example, PCD of the larval midgut and salivary glands occurs in direct response to the late larval and prepupal pulses of ecdysteroids respectively (Jiang et al., 1997; Lee and Baehrecke, 2001; reviewed in Yin and Thummel, 2005). Furthermore, PCD of a subset of neurons in the larval nervous system (ventral ganglion) that express the neuropeptide Corazonin (Crz) and are eliminated within the first 6 h APF requires EcR-B isoforms (Choi et al., 2006). This study however did not determine whether 20E was acting cell-autonomously on the doomed Crz-positive neurons.

In the present experiments we sought to investigate the PCD of individually identified larval motoneurons in the *Drosophila* ventral ganglion during early metamorphosis. Our studies made use of the abdominal RP2 motoneurons which have been studied extensively in other contexts and whose location and pattern of peripheral muscle innervation is well characterized (e.g. Sink and Whitington, 1991; Choi et al., 2004; Hartwig et al., 2008). The goal of the present experiments was to investigate the hormonal cues, genes and proteins involved in their demise and test whether it's a cell-autonomous response to the prepupal pulse of ecdysteroids.

Methods

Drosophila Rearing and Staging

Drosophila were reared in 25x95mm polystyrene vials on standard cornmeal-yeast-agar medium as described in Lewis (1960), with the addition of propionic acid (4.71 μL/mL; Sigma-Aldritch, St. Louis MO) and tegosept (7 μg/mL; Genesee Scientific, San Diego, CA), on a 12 h light, 12 h dark photoperiod at 25°C. Animals were collected from vials at the onset of pupariation, held at 25°C on H_2O-moistened tissues in tissue culture dishes, and staged by hours after puparium formation (APF).

Stocks and Crosses

All stocks used to maintain reporter gene expression in RP2 and aCC neurons were obtained from the *Drosophila* stock center (Bloomington, IN). A Green Fluorescent Protein (GFP) reporter was driven in the RP2 and aCC neurons using flies homozygous for the *RN2-GAL4, UASmCD8GFP* transgene recombinants (2^{nd} or 3^{rd} chromosome). In these flies, GAL4, driven by upstream promoter fragments of the *even-skipped* gene, drives expression of membrane-bound GFP in RP2s and aCCs during embryonic development but not during subsequent larval and pupal stages (Lee and Lou, 1999; Fujioka et al., 2003). We therefore crossed males of this genotype to females doubly homozygous for a *UAS-FLP* (1^{st} chromosome) and *Act>y+>GAL4, UAS-GFP*

transgenes(s) (2^{nd} chromosome); in these flies, GAL4 falls under the control of the ubiquitous *Actin5C* promoter following the removal of an FRT cassette by *UAS*-driven FLP-recombinase (Ito et al., 1997; Duffy et al., 1998; Hartwig et al., 2008). F1 progeny from these crosses, which maintained GFP expression in the RP2s and aCCs, were used for all cell culture and TUNEL-staining experiments. For cell culture experiments, a subset of progeny in which GFP was expressed more strongly in RP2s than aCCs (see Results) was used.

For experiments using *reaper*, *hid*, *dark*, and *dronc* and *EcR* mutations, animals carrying both a *UAS-FLP, Act>y+>GAL4* and *RN2-GAL4, UASmCD8GFP* recombinant chromosome within the appropriate mutant background were generated to maintain GFP expression in the RP2s and aCCs. The following deficiencies and alleles were used to generate *reaper* and *hid* deletions: *Df(3L)H99* (*H99*) was obtained from the *Drosophila* stock center. The hid^{P05014} and *Df(3L)XR38,* (*XR38*) lines were generously supplied by Kristen White (Massachusetts General Hospital). The *XR38/H99* transheterozygous combination results in a deletion of *reaper* (Peterson et al., 2002) while $hid^{P05014}/H99$ results in a loss of *hid* function (White et al., 1994; Grether et al., 1995; Peterson et al., 2002). The following *dark* and *dronc* alleles were obtained from the *Drosophila* stock center: $dronc^{51}$, $dronc^{KG02994}$ and $dark^{CD4}$. Both $dark^{CD4}$ and $dronc^{KG02994}$ are hypomorphic alleles while $dronc^{51}$ is a null mutation (Rodriguez et al., 1999; Chew et al., 2004; Daish et al., 2004). $dark^{CD4}/dark^{CD4}$ homozygotes or $dronc^{51}/dronc^{KG02994}$ transheterozygotes were used in the assessment of *dark* and *dronc* function respectively. *EcR-A* mutants were generated by transheterozygous combination of the EcR^{139} and

EcR^{M554Fs} alleles (generously supplied by Michael Bender, University of Georgia) (Bender et al., 1997; Carney et al., 2004; Davis et al., 2005). For misexpression experiments, standard methods were used to generate progeny containing at least one *UAS-FLP, Act>y+>GAL4* and *RN2-GAL4, UASmCD8GFP* recombinant chromosome along with *UAS-p35* (Hay et al., 1994), *UAS-DIAP1* (Hay et al., 1995), or *UAS-EcR-B1*^{F645A} (Cherbas et al., 2003; Hu et al., 2003).

Immunohistochemistry and TUNEL processing

Using microsurgery forceps, ventral ganglia were dissected under ice-cold phosphate buffered saline (PBS; pH 7.2) and fixed overnight in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS at 4°C. Immunohistochemisty against GFP was performed using a rabbit anti-GFP polyclonal antibody at 1:500 dilution and an Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen, Eugene OR) at a 1:200 dilution. EcR-A and EcR-B1 staining was performed by double staining ventral ganglia for both GFP and either EcR-A using the monoclonal antibody 15G1a (1:10) or EcR-B1 using the monoclonal antibody AD4.4 (1:10) (Talbot et al., 1993). Both EcR antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa (Iowa City, IA). In some experiments ventral ganglia were subjected to Terminal Deoxynucleotidyl Transferase mediated dUTP nick end labeling (TUNEL) using the

Apop Tag ® Red In Situ Apoptosis Detection Kit (Chemicon Intl., Temecula, CA.) according to the manufacturer's instructions, using a rhodamine-conjugated antibody to detect TUNEL-labeled nuclei. Ganglia were imaged on a Bio-Rad Radiance 2100 confocal microscope; Alexa Fluor 488 was detected using a 488nm excitation maximum argon laser while rhodamine was detected using a 543nm excitation maximum helium-neon laser. Images were processed and converted into 8 bit Bitmap images using ImageJ 1.37 (NIH, Bethesda, MD).

Cell Culture

For all cell culture experiments, animals were allowed to develop to 8 h APF, a time that precedes the prepupal pulse (see Fig. 1). The dorsal surfaces of intact prepupae were examined under a Zeiss Axiovert 25 inverted microscope under epifluorescence and those in which GFP was expressed strongly in RP2s and weakly or not at all in aCCs (see Results) were selected for experiments. Neuronal cell cultures were generated using methods modified from those described previously (Kraft et al., 1993; Su and O'Dowd, 2003). Prepupae were sterilized in 95% ethanol (EtOH) and rinsed in sterile H_2O prior to dissections. All dissections were performed inside a laminar flow hood in glass wells (pre-sterilized with 95% EtOH) containing modified *Drosophila* defined medium (DDM2) which consisted of Ham's F-12 DMEM (high glucose) (Irvine Scientific, Santa Ana, CA) supplemented with 1.2 mg/mL sodium bicarbonate, 20 m*M* HEPES, 100 μM putrescine, 30 n*M* sodium selenite, 20 ng/mL progesterone, 50 μg/mL insulin, 100 μg/mL

transferrin (Sigma-Aldrich, St. Louis, MO) and 1% Pen-Strep (Invitrogen). Ventral ganglia were removed using microsurgery forceps. Following removal, the abdominal portion of each ventral ganglion was severed from the thoracic portion using a sterile 28 gauge needle (Monoject, St. Louis MO).

Either 6 or 8 abdominal ventral ganglia were then divided evenly into two groups and enzymatically treated in Rinaldini's saline (Wu et al., 1983) containing 50 U/mL of papain and 1.32 m*M* L-cysteine (Sigma) for 15 min, rinsed three times in DDM2 and mechanically dissociated using a fine-tipped, fire-polished Pasteur pipette. This produced two 20 μL suspensions each of which was dispensed into a well formed by punching an 8 mm hole into a 35 mm culture dish and attaching with Sylgard (Dow Corning Corp., Midland, MI) an alphanumeric gridded coverslip (Bellco Biotechnology, Vineland, NJ) coated with Concanavalin A (200 μg/mL) (Sigma) and laminin (3.55 μg/mL) (Invitrogen). Each well contained 80 μL DDM2 resulting in a final volume of 100 μL. Pairs of cultures prepared in this manner were termed "sister cultures." Cells were allowed to settle for 30 min before each dish was flooded with a 3:1 mixture of DDM2 and unconditioned neurobasal medium (Invitrogen) supplemented with B27 (20µl/mL; Invitrogen) to a final volume of 3 mL. One culture of each sister pair received medium containing 20-hydroxyecdysone (20E; Sigma) at a final concentration of 6 μg/mL while the control culture received 20E-free medium. Cultures were maintained in a humidified 23°C, 5% CO_2 ambient-O_2 incubator.

Cultured neurons were photographed immediately after flooding (Day 0) and again at 48 or 72 h *in vitro*. Fields containing one or more putative RP2 neurons,

identified by their strong GFP expression (see Results), were selected randomly and photographed under phase contrast and GFP epifluorescence optics using a Nikon Coolpix 4500 digital camera attached to a Zeiss Axiovert 25 inverted microscope. Typically, 5-13 fields were photographed per dish. Using the alphanumeric gridded coverslips, the same fields of cells were relocated and re-photographed 48 or 72 h later. For propidium iodide (PI; Invitrogen) staining, PI was added to cultures at a final concentration of 20 μg/mL, after 48 or 72 h *in vitro*. Live cells exclude PI from their somata while the nucleic acids of dead cells label strongly (e.g., Jordan et al., 1998). PI-treated cultures were photographed as described above and also under rhodamine epifluorescence optics.

Scoring Neurons as Alive or Dead

As in previous studies, cultured neurons were scored as alive or dead by morphological criteria (Streichert et al., 1997; Zee and Weeks, 2001). Live neurons had smooth somata, ovoid shapes and well-defined nuclei (see Results). Only putative RP2s that fulfilled these criteria were entered into the study on Day 0. In some experiments we also examined the effect of 20E on the survival of control "non-RP2" neurons. In this case, for each putative RP2 selected on Day 0, a GFP-negative neuron located in the same photographic field that was of similar size and appearance to the putative RP2, and that met the above morphological criteria, was entered into the study.

By definition, all putative RP2s and control neurons were alive on Day 0. The status of these neurons after 48 or 72 h was scored as follows, by an observer blind to the treatment conditions. The observer was provided digital images (phase-contrast and GFP fluorescence) of each putative RP2 or control neuron on Day 0, and of the same neurons at 48 or 72 h. Neurons were scored as dead if the soma was rounded and shrunken or showed significant degradation, and the nucleus was no longer discernable; many dead neurons also became uniformly phase bright (see Results). For cultures stained with PI, the observer was also provided a digital image taken under rhodamine fluorescence. These neurons were scored as dead or alive by morphological criteria and also, independently, by the presence of absence of PI staining.

Chi square with Yates Continuity Correction, or Fisher Exact statistical tests were used to compare the proportions of neurons surviving under different conditions.

Results

Loss of RP2 motoneurons during metamorphosis

To trace the fate of RP2 motoneurons during metamorphosis we used the GAL4/UAS system combined with FLP/FRT-mediated excision to drive and maintain the expression of membrane-bound GFP in RP2 and aCC motoneurons postembryonically (see Methods). The bilaterally-paired RP2s and aCCs were identified *in situ* by their characteristic locations and projections (Figure 2; Goodman et al., 1984; Sink and

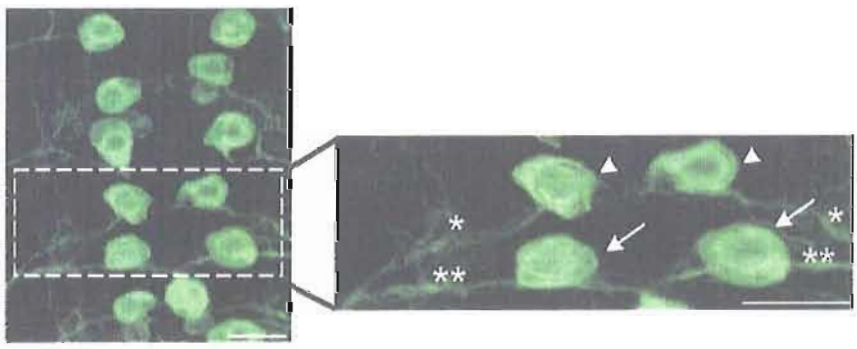


Figure 2 Identification of RP2 and aCC motoneurons in situ. Left, low-power confocal image shows repeating pattern of GFP-expressing neurons along the anterior-posterior axis of the abdominal region of the ventral ganglion, at 8 h APF. The dashed box demarcates one neuromere. Right, magnified view showing GFP expression in the somata and axons of a bilateral pair of RP2s (arrows) and aCCs (arrowheads) in one neuromere. The aCC somata lie anterior to those of RP2s and their axons (single asterisks) project posteriorly to join the peripherally-projecting intersegmental nerve (ISN). RP2 axons (two asterisks) project anteriorly from the somata to join the ISN. In some neuromeres GFP expression was observed in pCC interneurons, which lie posterior to the aCCs. Scale bars, 15μm.

Whitington, 1991; Fujioka et al., 2003). The somata of both neurons lie near the dorsal surface of the ventral ganglion, with RP2's soma located posterior to the aCC motoneuron of the next anterior neuromere. Both motoneurons produce ipsilateral axons; RP2's axon projects anteriorly while aCC's axon projects posteriorly, both exiting the CNS via the intersegmental nerve. RP2s and aCCs are present in both thoracic and abdominal neuromeres but the present study examined only abdominal neuromeres due to less reliable patterns of GFP expression in the thorax.

Two patterns of GFP expression were observed in larvae and young prepupae. In some animals, GFP expression was strong in both RP2s and aCCs [Fig. 2, 3(A1)] whereas, in others, GFP expression was strong in RP2s but weak or absent in aCCs [Fig. 3(A2)]. These two phenotypes occurred at approximately equal frequency (see below). In both phenotypes, unidentified GFP-labeled neurons were present in other regions of the ventral ganglia and appeared to increase in number in older prepupae and in pupae; these neurons were less salient in animals in which GFP expression in aCCs was weak or absent (data not shown). Ventral ganglia often had one or more neuromeres that lacked GFP-labeled RP2s and aCCs (Fig. 3A2), likely due to variable efficacy of the GAL4/UAS and FLP/FRT method (e.g., Duffy et al., 1998).

Previous reports documented an intense period of PCD in the ventral ganglion during the first 24 h after puparium formation (Truman et al., 1993; Choi et al, 2006). We examined the fate of RP2s in ventral ganglia removed from animals at various times between 0 and 30 h APF. GFP-labeled abdominal RP2s were present in 100% of ventral ganglia examined at 8 h APF [Fig. 3(A1, A2); n=45 animals]. In 51% of these ganglia

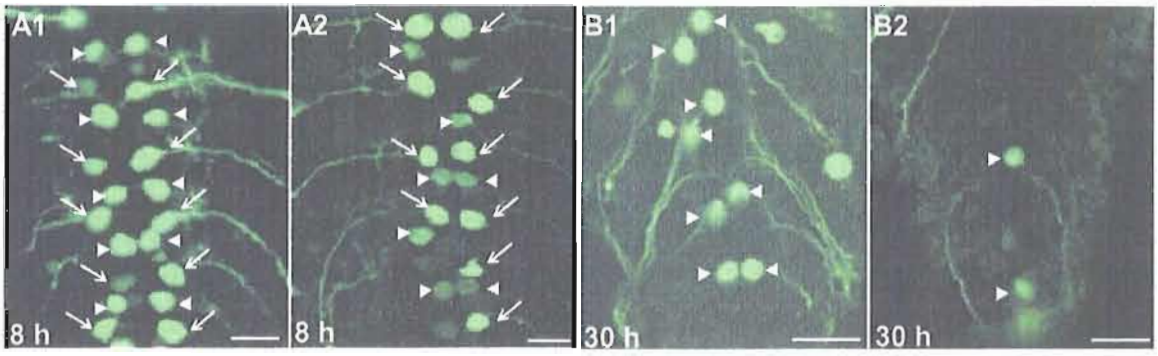


Figure 3. The fate of RP2s and aCCs during metamorphosis. A-D show confocal images of the abdominal region of ventral ganglia, with anterior up. RP2s (arrows) and aCCs (arrowheads) are marked. At 8 h APF (A), approximately 50% of animals exhibited strong GFP expression in both RP2s and aCCs while the remainder (B) had strong GFP expression in RP2s but weak or absent expression in aCCs (see Results). C, D. At 30 h APF, RP2s were invariably absent and the number of GFP-labeled aCCs varied. Scale bars, 20μm.

we also observed GFP-labeled aCCs [Fig. 3(A1); n=23 of 45 animals]. In contrast, in ventral ganglia examined at 30 h APF, GFP-labeled abdominal RP2s were never observed (n=39 animals). In 46% of these ganglia [Fig. 3(B1); n=18 of 39 animals], we observed GFP-labeled aCCs, suggesting that the developmental disappearance of abdominal RP2s was specific to these motoneurons.

RP2s die by programmed cell death

To determine whether the disappearance of the abdominal RP2s between 8 and 30 h APF resulted from PCD rather than, for example, the loss of GFP expression, we used the TUNEL method to label nuclei containing fragmented DNA, a hallmark of PCD (Gavrieli et al., 1992). Ventral ganglia examined at 6 to 10 h APF exhibited many TUNEL-positive nuclei, but no TUNEL-positive RP2s were observed during this period [Fig. 4(A); n=6 animals]. However, at 15 h APF, every ventral ganglion examined had one or more TUNEL-positive RP2 [Fig. 4(B); n=10 animals]. At no time did we observe TUNEL-positive aCC motoneurons (n=16 animals). These results indicate that abdominal RP2 motoneurons initiate PCD between 10 to 15 h APF, while the aCCs survive.

Embryonic and metamorphic PCD in *Drosophila* depends on protein products encoded by the death activating genes *reaper, hid,* and *grim* (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Animals homozygous for the *Df(3L)H99* deficiency, which removes all three genes, arrest during embryogenesis with numerous extra cells

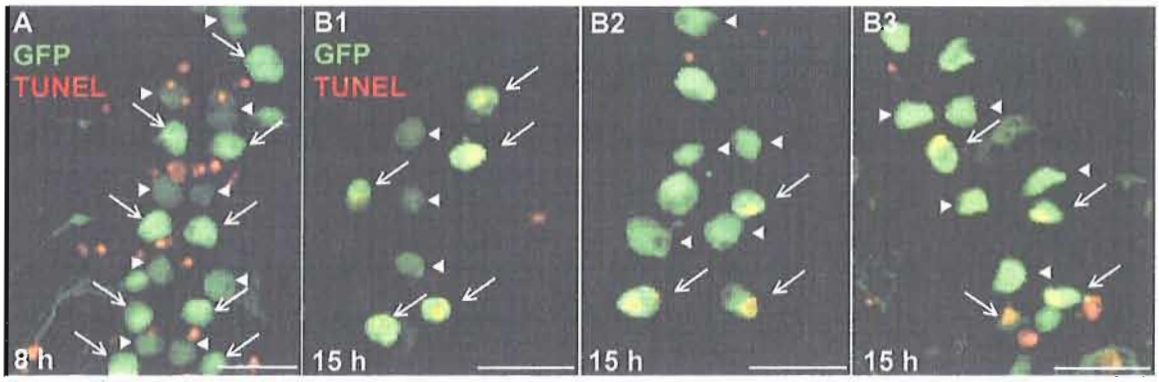


Figure 4 Developmental disappearance of RP2s is accompanied by TUNEL labeling. Confocal images of abdominal portions of ventral ganglia show GFP expression (green) and TUNEL labeling (red). Anterior is up. A. At 8 h APF, numerous TUNEL-positive nuclei were present but no RP2s or aCCs exhibited TUNEL labeling. B1-B3. Ventral ganglia from three different animals at 15 h APF are shown. TUNEL-positive RP2s (arrows) were present in all ventral ganglia examined at this time whereas TUNEL-positive aCC neurons were never observed. Scale bars, 20μm.

(White et al., 1994). The death of Type II and CCAP neurons at eclosion is impaired by mutations affecting *reaper* and *grim* (Draizen et al., 1999; Peterson et al., 2002). Likewise, PCD of the larval salivary glands is maximally impaired in a *reaper* mutant background following the targeted knockdown of *hid*, pointing to a role for both genes in their demise (Yin and Thummel, 2004). We therefore tested the requirements of *reaper* and *hid* in PCD of abdominal RP2s. In a $H99^{+/-}$ mutant background, in which *reaper*, *hid,* and *grim* have been reduced to one copy (White et al., 1994), we did not see any significant impairment in PCD of the abdominal RP2s. In all ventral ganglia examined at 8 h APF, the abdominal RP2 neurons were present at expected (Fig 5A; n=5 animals). In ventral ganglia removed from animals at 24 h APF, the abdominal RP2s were missing, leaving behind only the aCCs (Fig. 5B; n>17 ventral ganglia). Likewise in a $hid^{P05014}/H99$ background that removes *hid* function and reduces *reaper* and *grim* to one copy (White et al., 1994; Grether et al., 1995; Peterson et al., 2002), PCD of the abdominal RP2s occurred normally (Fig. 5C n=12 animals). In contrast, in ventral ganglia removed at 24 h APF from *Df(3L)XR38/H99* animals in which *reaper* was eliminated and *hid* and *grim* were reduced to one copy (Peterson et al., 2002), abdominal RP2s persisted (Fig. 5D; n=9 animals) along with aCCs, indicating that PCD of abdominal RP2s was impaired in this mutant. It must be noted that in all the *XR38/H99* ventral ganglia examined at 24 h APF, some neuromeres had missing RP2 neurons (data not shown). However, we could not determine whether this was due to PCD of those neurons or failure to transform them into *Act5C-GAL4* clones (see Methods). Therefore we could not quantify this result in greater detail. However, the presence of abdominal

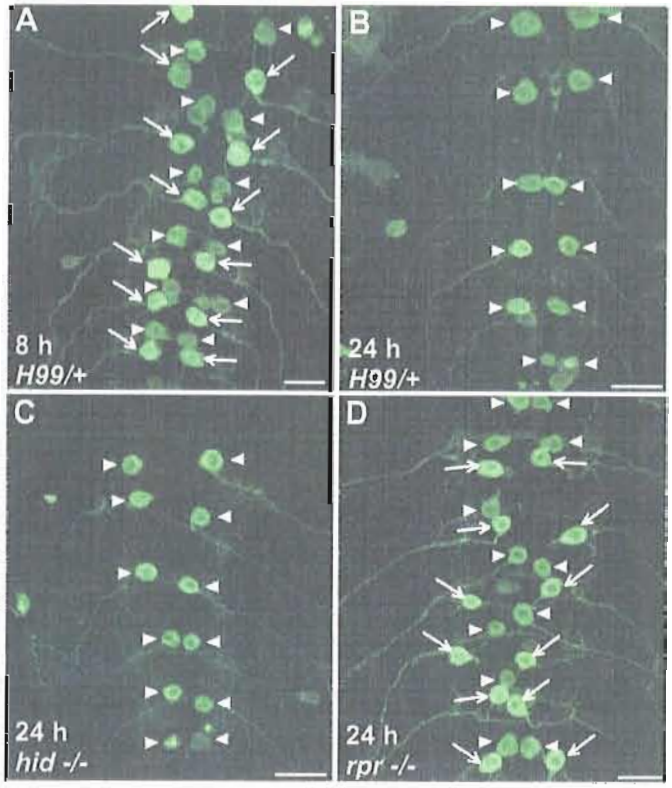


Figure 5 Reaper is necessary for PCD of abdominal RP2s. A. Representative abdominal ventral ganglion removed at 8 h APF from an *H99/+* animal that is heterozygous for a deletion that removes *reaper*, *hid*, and *grim* shows GFP-expressing RP2s (arrows) and aCCs (arrowheads). B. In a ventral ganglion removed from an *H99/+* animal at 24 h APF, the abdominal RP2 motoneurons have undergone normal PCD leaving only the aCCs (arrowheads). C. Ventral ganglion removed from a *hid* mutant ($hid^{P05014}/H99$), at 24 h. PCD of abdominal RP2s has taken place leaving behind the aCCs (arrowheads). D. In a deletion that removes *reaper* (*XR38/H99*), persisting abdominal RP2s (arrows) are present along with aCCs (arrowheads) in a ventral ganglion removed at 24 h APF indicating that PCD has failed to take place in this mutant. Scales bars, 20μm.

RP2s in *XR38/H99* animals at 24 h APF, strongly suggests that PCD of abdominal RP2s requires *reaper*.

The antagonization of Diap1 by Reaper, Grim, or Hid results in the liberation of the apical caspase, Dronc, which may in turn activates effector caspases such as Drice (Jiang et al., 1997; Meier et al., 2000; reviewed in Hay and Guo, 2006). Such is the case in the salivary glands whose PCD depends on the down-regulation and antagonization of Diap1 (Yin et al., 2007). We therefore first tested whether persistent Diap1 expression could block PCD of the abdominal RP2s by ectopically expressing Diap1 in the RP2s and aCCs. In ventral ganglia removed at 8 h APF from *UAS-DIAP1* animals (see methods) all abdominal RP2s were present as expected (Fig. 6A; n=5 animals). In ventral ganglia removed from at 24 h APF from these animals, abdominal RP2s underwent normal PCD, suggesting that Diap1 cannot antagonize their deaths (Fig. 6B; n=15 animals). However, this result could be the result of poor ectopic expression of Diap1 in the abdominal RP2s. As a positive control we repeated this experiment in the salivary glands using the GAL4 enhancer trap line, 4G, which we observed to express GFP in the salivary glands. In animals carrying a *4G-GAL4, UASmCD8GFP* and *UAS-FLP, Act>y+>GAL4* recombinant chromosome, the GFP-expressing salivary glands were present at 8 h APF (data not shown n=10) but showed considerable degeneration by 20 h APF with only faint levels of detectable GFP (Fig 6C1; n=3). Consistent with previous literature (Yin et al., 2007), ectopic expression of Diap1 in this same transgenic background using *UAS-DIAP1* resulted in persistent salivary glands at 20 h APF as indicated by persistent intact GFP expression (Fig 6C2; n=4). This result suggests that unlike the salivary glands, PCD

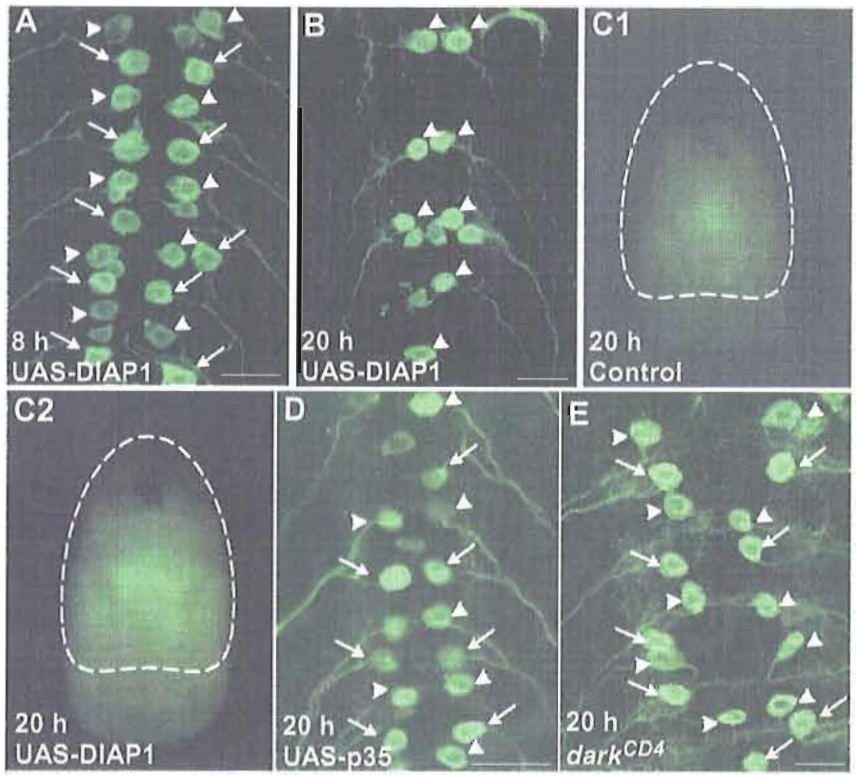


Figure 6 RP2 death is unaffected by Diap1 misexpression, but impaired by misexpression of p35 or in *dark* mutants. All panels are confocal images of abdominal portions of ventral ganglia showing GFP expression. A. Ventral ganglion at 8 h APF, removed from a *UAS-DIAP1* animal showing GFP-expressing RP2s (arrows) and aCCs (arrowheads). B. Ventral ganglion from a *UAS-DIAP1* animal at 24 h; abdominal RP2s are absent and only the aCCs (arrowheads) remain. C. Expression of Diap1 in salivary glands blocks their death. Both panels depict an intact pupa at 20 h APF viewed ventrally under fluorescent optics. Dashed outline identifies the upper thorax in which the salivary glands, visible due to GAL4-driven GFP (see results), reside. C1. At 20 h APF, the salivary glands have degenerated considerably. C2. Salivary glands expressing Diap1 *(UAS-DIAP1)* however, remain intact at 20 h APF. D. Ventral ganglion removed from animals in which p35 is expressed in RP2s and aCCs *(UAS-p35)* at 24 h APF shows persisting abdominal RP2s (arrows) in addition to the aCCs. E. In ventral ganglia removed from animals homozygous for a *dark* hypomorphic allele ($dark^{CD4}/dark^{CD4}$) at 24 h APF, abdominal RP2s (arrows) persist along with the aCCs (arrowheads), having failed to undergo PCD. Scale bars, 20μm.

of the abdominal RP2s may not depend on the on the antagonization or downregulation of Diap1.

To test whether PCD of abdominal RP2s was caspase dependent, we misexpressed the viral caspase inhibitor p35 in RP2s and aCCs using a *UAS-p35* responder (see Methods). P35 blocks PCD by acting as a cleavage substrate for the effector caspase, Drice (Hay et al., 1994; Bump et al., 1995). As expected, in the *UAS-p35* genetic background, abdominal RP2s and aCCs were present at 8 h APF (n=11 animals; data not shown). At 24 h APF, RP2s were still present in all *UAS-p35* animals examined (Fig. 6D; n=17 animals), consistent with a requirement for effector caspase activity for PCD of abdominal RP2s. Together these results demonstrate that PCD of abdominal RP2s is caspase dependent but cannot be blocked by ectopic expression of Diap1.

To test the role of Dronc in the demise of the abdominal RP2 neurons we assessed the survival of the RP2 neurons in $dronc^{51}/dronc^{KG02994}$ mutants. In ventral ganglia removed even as early as 18 h APF, abdominal RP2s were absent (n=6 ventral ganglia data not shown) indicating, PCD of the RP2s proceeded normally. Although $dronc^{51}$ is a null, the $dronc^{KG02994}$ allele represents a hypomorph from which low levels of dronc protein can still be produced (Chew et al., 2004) which may explain our results. The activation of Dronc requires apoptosome assembly, with the *Drosophila* apaf1 homologue Dark (reviewed in Mills et al., 2005), and mutations in *dark* appear to phenocopy null mutations in *dronc* (Akdemir et al., 2006). We tested for the requirement of *dark* in PCD of abdominal RP2s using animals homozygous for the $dark^{CD4}$ allele. In

ventral ganglia removed from *dark* mutants at 24 h APF we observed persistent abdominal RP2 neurons (Fig. 6E; n=8 ventral ganglia) suggesting that apoptosome assembly involving Dark and Dronc is required for PCD of abdominal RP2s.

Programmed cell death of RP2s is triggered by 20E

In *M. sexta*, the PCD of APR motoneurons during metamorphosis is triggered directly and cell-autonomously by 20E, as demonstrated by placing APRs in cell culture and exposing them to appropriate changes in 20E levels (Streichert et al., 1997; Zee and Weeks, 2001). In *Drosophila*, the PCD of RP2s occurs during the same time period as the degeneration of larval salivary glands, which has been shown in tissue culture to be triggered directly by the prepupal pulse of 20E (Fig. 1; Jiang et al., 1997). We therefore tested whether ecdysteroids triggered the PCD of abdominal RP2s.

Immunostaining of ventral ganglia between 8 and 10 hrs APF revealed both EcR-A and EcR-B1 expression in the abdominal RP2s and aCCs (Fig. 7A, B; n=6 and 5 animals respectively). However, levels of staining for both isoforms were low compared to the levels of EcR-A expression seen in the ventral ganglia during late metamorphosis, or EcR-B1 expression seen during puparium formation (unpublished observations). To test the possible requirement of EcR-A in PCD of abdominal RP2s we traced their fate in an *EcR-A* null (EcR^{139}/EcR^{M554Fs}) (Carney et al., 2004; Davis et al., 2005). Abdominal RP2s were present in ventral ganglia removed at 8 h APF (data not shown n=3 animals), and were missing at 24 h APF (Fig. 7C; n=10 animals) suggesting that their PCD did not

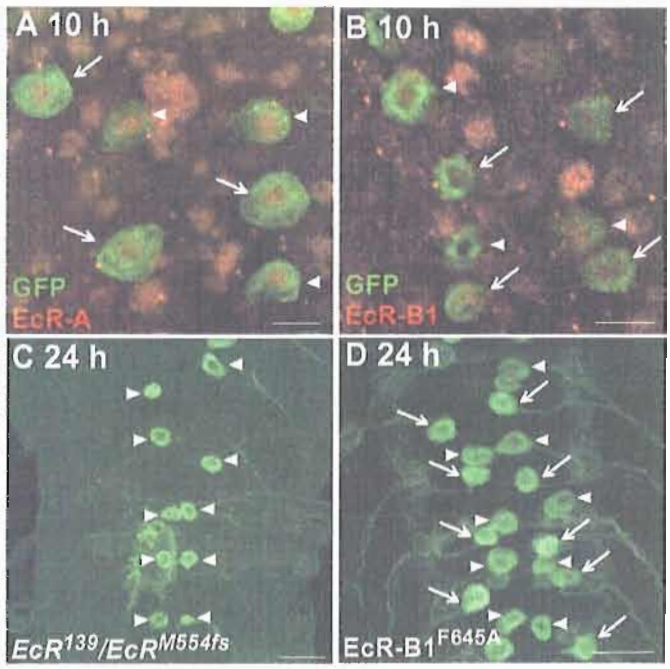


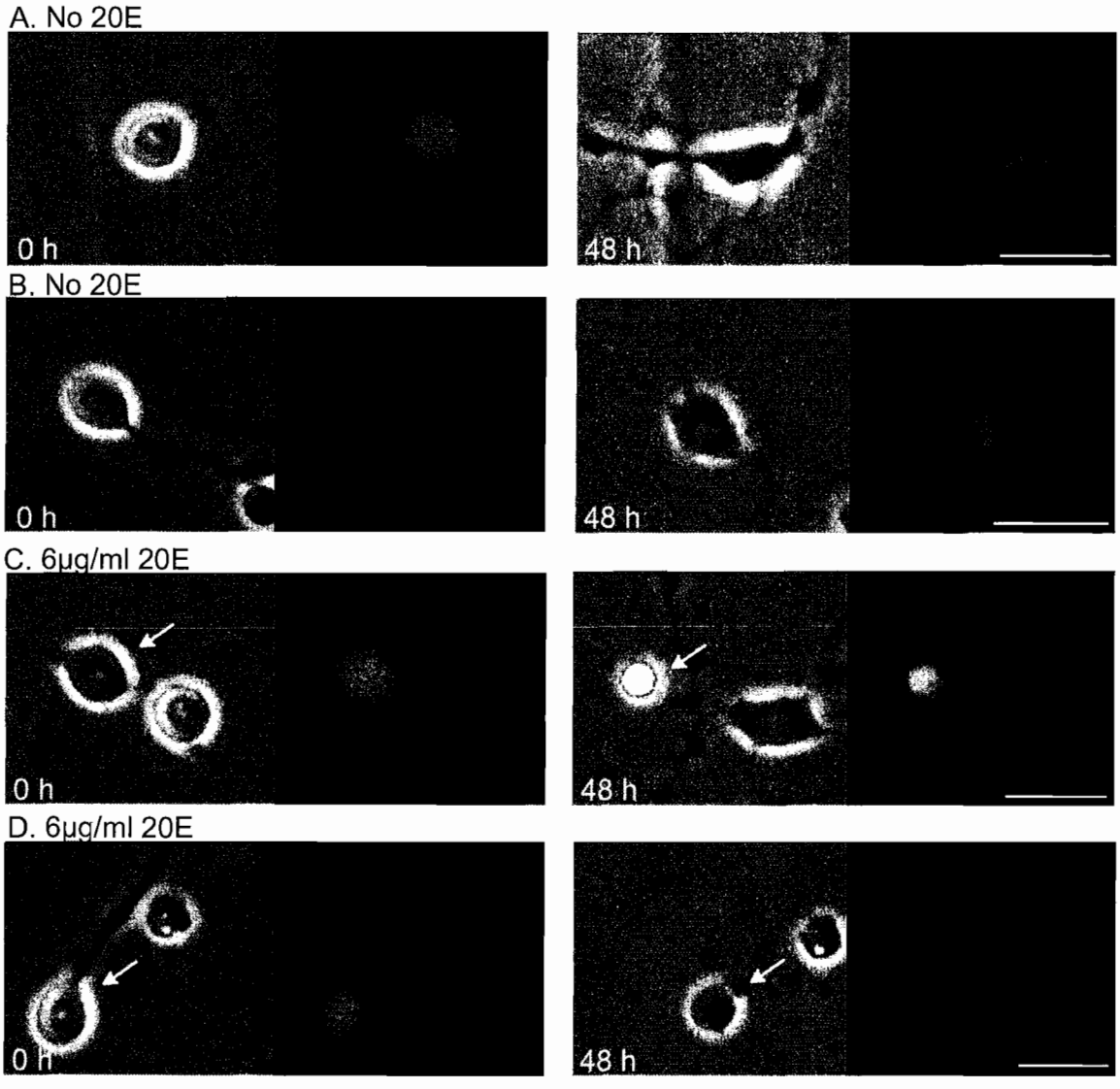
Figure 7 Programmed cell death of the abdominal RP2s is ecdysteroid-receptor dependent. A. Abdominal RP2 neurons express low levels of EcR-A at 10 h APF, just prior to head eversion. High-magnification image of two neuromeres within a abdominal ventral ganglion containing GFP-positive RP2s (arrows) and aCCs (arrowheads) stained for EcR-A (red) reveals low levels of EcR-A expression in both motoneurons. B. Similar to (A), ventral ganglia removed at 10 h APF and stained for EcR-B1 reveal low levels of EcR-B1 expression by both the abdominal RP2s (arrows) and aCCs (arrowheads). C, D Programmed cell death of abdominal RP2s proceeds through an EcR-B dependent pathway. C. In ventral ganglia removed from an EcR-A mutant (EcR^{139}/EcR^{M554fs}) at 24 h APF, normal PCD of the abdominal RP2s taken place and only the aCCs (arrowheads) remain. D. In ventral ganglia removed from a $UAS\text{-}EcR\text{-}B1^{F645A}$ background at 24 h APF in which a dominant-negative EcR-B1 mutation was expressed specifically in the RP2s and aCCs, PCD of the RP2s (arrows) has failed to take and they persist along with the aCCs (arrowheads). Scale bars in A, B, 10μm; C, D, 20μm.

depend on EcR-A function. Such was not the case in ventral ganglia in which the RP2s and aCCs were made to express a dominant-negative EcR-B1 mutant that binds ecdysteroids but lacks transcriptional activation (*UAS-EcR-B1*^{F645A}) (Hu et al., 2003). In ventral ganglia removed from this background at 24 h APF, abdominal RP2s persisted (Fig. 7D arrows; n=9 animals), due to failure of PCD. This indicates that ecdysteroids may act through one or both of the EcR-B isoforms to induce PCD of the abdominal RP2 neurons. However, the pattern of EcR isoform expression, does not appear to be a determinant as to whether a neurons dies or persists, as spatial and temporal patterns of EcR expression were similar between the RP2s and aCCs.

We next tested the hypothesis that PCD of the RP2s is a direct cell-autonomous response to the prepupal pulse of 20E. Low-density cell cultures were prepared from the abdominal portions of ventral ganglia at 8 h APF, a time immediately prior to the prepupal pulse of 20E (Fig 1). We used animals in which GFP expression was strong in RP2s and weak or absent in aCCs (Fig. 3B), to substantially reduce the number of GFP-labeled non-RP2 neurons in the cultures. Pools of dissociated neurons were divided into paired 'sister' cultures; one culture received medium containing 6 μg/mL 20E while the other culture of each pair received medium without 20E (see Methods).

Immediately after flooding the dishes (designated Day 0) we photographed fields of cells that contained one or more putative RP2, as identified by strong GFP expression. We use the term 'putative' because, although cultures were prepared from animals in which GFP expression was largely limited to RP2s, it is possible that a small proportion of neurons identified in this study as RP2s had other identities. Figure 8 A-D (left

Figure 8 Effects of 20E on putative RP2s in culture. Photomicrographs show putative RP2s at 0 h (left panels) and 48 h (right panels) in culture. All cultures were prepared at 8 h APF, immediately prior to the prepupal pulse. The pair of photomicrographs in each panel shows the same neuron(s) under phase contrast (left) and GFP epifluorescence (right). When more than one neuron is present, arrows mark putative RP2s (as identified by GFP expression) in the phase contrast image. A, B. Two examples of putative RP2s cultured without 20E. At 0 h, both had smooth, ovoid somata with well-defined nuclei. Neurite outgrowth is apparent in B. At 48 h, both neurons were still alive and exhibited extensive neurite outgrowth and GFP labeling. C, D. Two examples of putative RP2s cultured with 20E (6 μg/mL). At 0 h, both neurons resembled the RP2s in A and B. By 48 h (right), both were dead by morphological criteria (see Methods). The putative RP2 in C became shrunken and phase-bright, while retaining GFP fluorescence. The putative RP2 in D was shrunken, had an irregular, fragmented outline, and the nucleus was reduced to a small spot. Only faint, irregular GFP fluorescence remained. Scale bars, 10μm.


A. No 20E
0 h
48 h
B. No 20E
0 h
48 h
C. 6µg/ml 20E
0 h
48 h
D. 6µg/ml 20E
0 h
48 h

panels) shows representative phase contrast and fluorescence images of four putative RP2s on Day 0. For each putative RP2 included in the study, we also selected a non-GFP-labeled neuron in the same field of cells, of similar size and appearance as the putative RP2, to serve as a control (not shown). The same putative RP2s and control neurons were photographed at the end of the culture period (48 or 72 h). From photomicrographs, neurons were scored as alive or dead by morphological criteria (see Methods; Streichert et al. 1997; Zee and Weeks, 2001).

All neurons in the study were alive on Day 0, with smooth, ovoid somata and well-defined nuclei (Fig. 8). In many cases, neurite outgrowth was already underway. Neurons were scored as dead if the soma was rounded and shrunken or showed significant degradation, and the nucleus was no longer discernable (e.g., Fig. 8D); many shrunken neurons also became uniformly phase bright (e.g., Fig. 8C). The putative RP2s that underwent PCD in Figure 8 (C, D) were from cultures treated with 20E for 48 h whereas the putative RP2s that survived (Fig. 8A, B) were from hormone-free cultures; this effect is quantified below. Neurons scored as alive after 48 or 72 h typically maintained strong GFP labeling (Fig. 8A, B). Among neurons scored as dead, GFP fluorescence was sometimes strong (Fig. 8C) and sometimes weak or absent (Fig. 8D). Accordingly, GFP fluorescence was not a useful criterion for assessing neuronal viability: it was used only to identify putative RP2s on Day 0.

Figure 9A shows the percentage of control neurons alive after 48 h or 72 h in culture, in the presence or absence of 20E. The hormone had no effect on the survival of control neurons at either time point. Slightly fewer neurons were alive in the older

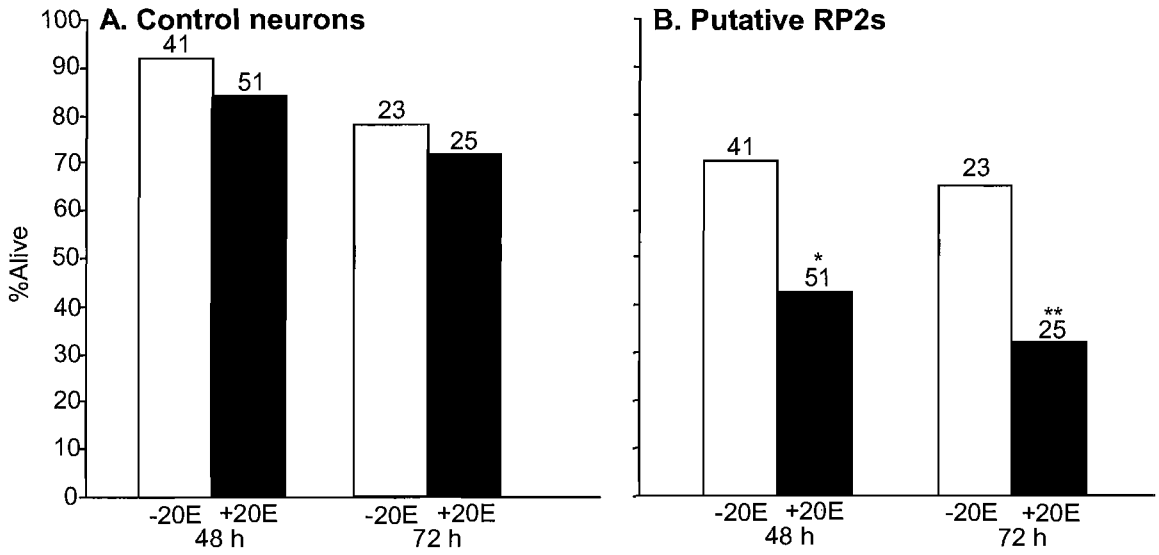


Figure 9 20E decreases survival of putative RP2s but not control neurons. Sister cultures were prepared at 8 h APF and treated with medium containing 6 μg/mL 20E (+20E) or no hormone (-20E). On Day 0, putative RP2 neurons were identified by GFP expression and a similar-appearing neuron in the same field of cells was selected to serve as a control for each putative RP2. At either 48 h or 72 h (4 pairs of sister cultures at 48 h and 2 pairs at 72 h) the putative RP2s and control neurons were scored as dead or alive by morphological criteria (see Methods). Histograms show percentage of neurons alive, with n given above each bar. A, The percentage of control neurons alive at 48 or 72 h did not differ significantly between +20 and -20E cultures. B, In contrast, at both 48 and 72 h, significantly fewer putative RP2s survived in cultures containing 20E (* $p<0.05$, ** $p<0.01$, two-tailed Chi square test with Yates Continuity Correction).

cultures but this was seen with or without 20E. In contrast, 20E significantly decreased the survival of putative RP2s at both 48 and 72 h; approximately twice as many putative RP2s died in cultures containing 20E (Fig. 9B). These results indicate that the PCD-inducing effect of 20E was specific to putative RP2s.

Propidium iodide confirmation of neuronal death

To independently assess the validity of the morphological criteria for PCD, we performed another set of experiments in which neurons were treated with propidium iodide (PI, a positive marker of dead cells; e.g. Jordán et al., 1998) at the end of the culture period (48 or 72 h; see Methods). Putative RP2s were photographed as described above, with the addition of PI imaging at the end of the culture period.

From photomicrographs, each putative RP2 was scored independently as dead or alive by morphological criteria and by PI staining. All putative RP2s were alive on Day 0 (data not shown). Figure 10 shows representative photomicrographs of putative RP2s after 48 h in culture without (Fig. 10A) or with (Fig. 10B) 20E. The putative RP2 in Figure 10A was alive by morphological criteria and lacked PI staining ("PI-negative") whereas the putative RP2 in Figure 10B was dead by morphological criteria and showed PI staining ("PI-positive"). Figure 11 compares the results obtained with the two scoring methods. As seen in the previous experiments (Fig. 11B), 20E significantly reduced the percentage of putative RP2s alive after 48 h (Fig. 11A) or 72 h (Fig. 11B) in culture. The

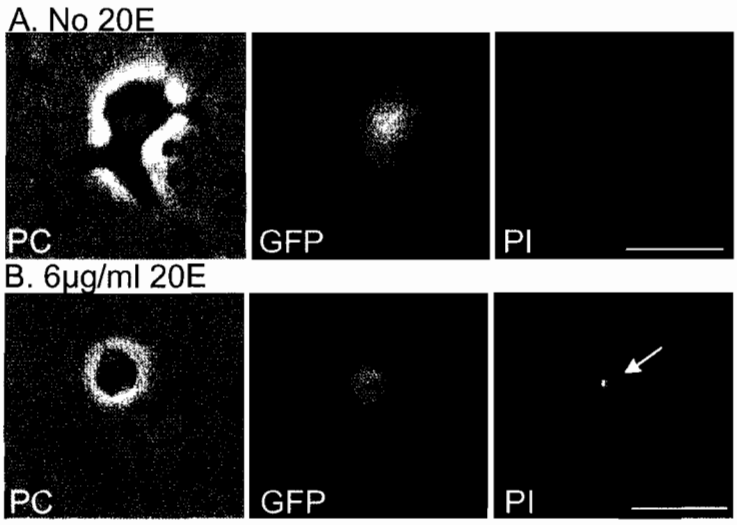


Figure 10 Comparison of morphology and PI staining of RP2s. Phase contrast (PC) and epifluorescent (GFP or PI) photomicrographs of an RP2 neuron grown in the absence (A) or presence of 20E (B) for 48 h and stained with PI. A, The neuron was scored as alive by morphological criteria, and shows uniform GFP fluorescence; however, no fluorescent PI staining is observed. B, By contrast, an RP2 neuron grown in the presence of 20E for 48 h was scored as dead and shows both GFP fluorescence and strong nuclear PI staining (arrow). Scale bars, 10µm.

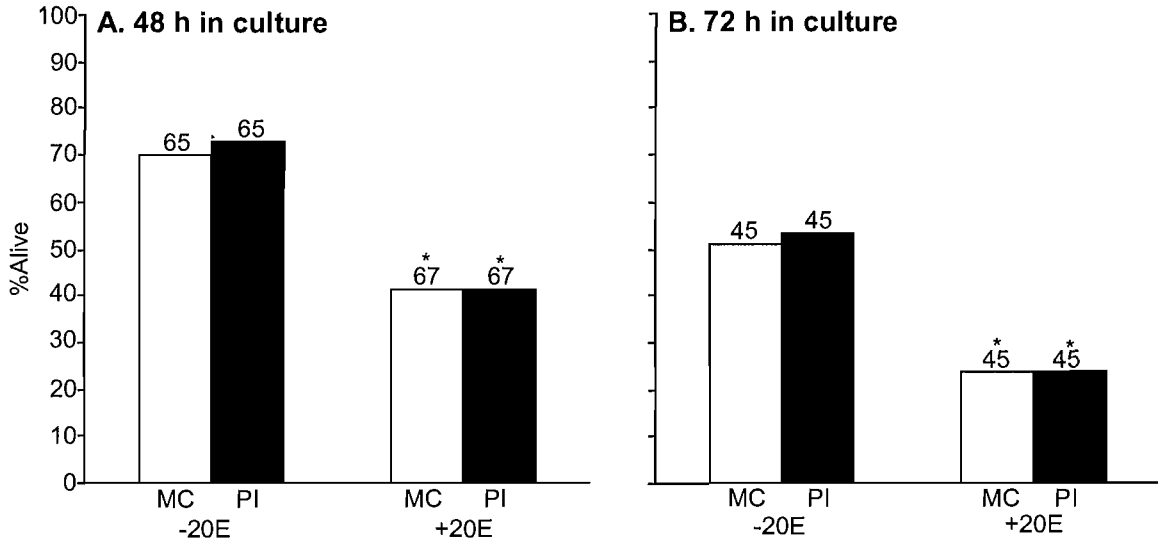


Figure 11 Comparison of morphological criteria and PI staining for scoring cell death. Putative RP2s were cultured for 48 or 72 h (two independent experiments) in the absence or presence of 20E (6 μg/mL). At the end of the culture period, percentages of surviving RP2 neurons were assessed independently, using either criterion. When scored using either morphological criteria or PI staining, the percentage of surviving RP2 neurons is significantly lower in the presence of 20E. *$p<0.01$ (Chi^2).

two scoring methods — morphological criteria and PI staining — yielded nearly identical results: neurons judged to be alive by morphological criteria were PI-negative whereas neurons judged to be dead by morphological criteria were PI-positive.

Role of neuronal contacts in programmed cell death

In *M. sexta*, contact with other neurons does not influence the ability of 20E to trigger appropriate PCD in cultured motoneurons (Streichert et al., 1997). The current experiments utilized relatively high-density cultures, so most putative RP2s had somatic and/or neuritic contact with other neurons during the culture period. To test the hypothesis that 20E acts directly on RP2s to trigger PCD, independently of neuronal contact, we analyzed separately a subset of putative RP2s that had no contact with other neurons throughout the culture period.

The number of putative RP2s without contacts was relatively small, so we pooled data from 48 and 72 hr experiments (same data as Figs. 9 and 11). Of 39 contact-free putative RP2s, 50% (5 of 10) cultured in hormone-free medium were alive at the end of the culture period whereas only 14% (4 of 29 neurons) cultured with 20E were alive at this time. These proportions differ significantly ($p = 0.028$; Fisher's Exact Test), consistent with the idea that neuronal contact was not required for RP2s to initiate PCD in response to 20E.

Taken together, the above results demonstrate that putative RP2s placed in cell culture immediately prior to the prepupal pulse of 20E undergo PCD when exposed to

20E, whether in contact with other neurons or completely isolated, supporting the hypothesis that this rise in 20E is the normal cue for PCD in vivo. Furthermore, our findings suggest that 20E acts directly and cell-autonomously on RP2s to trigger PCD.

Discussion

During *Drosophila* metamorphosis, most existing larval motoneurons are remodeled and some also undergo PCD. We have identified one such abdominal motoneuron, RP2, which undergoes PCD during the first 24 h of *Drosophila* metamorphosis. The abdominal RP2s survive pupariation but undergo PCD shortly after pupation. Their death is caspase-dependent, mediated through Reaper, and depends on the transcriptional activation of EcR-B1 or EcR-B2. Finally, we have also demonstrated that PCD of the abdominal RP2 neurons occurs cell-autonomously in response to the small prepupal pulse of ecdysteroids that induces pupation.

Time course of RP2 death

Our data indicate that PCD of the abdominal RP2s is initiated after 8 h APF. In all tissue examined at 8 h APF and earlier larval and prepupal stages, GFP-labeled abdominal RP2s are readily identifiable (Fig. 2A, B). However, in ventral ganglia removed from animals at 30 h APF, the RP2s were missing (Fig. 2C, D). That this elimination occurs through PCD is confirmed by TUNEL-labeling experiments. We

observed TUNEL-positive abdominal RP2 neurons in ventral ganglia removed at 15 h APF (Fig. 3B1-3), indicating that the RP2 neurons undergo PCD, and do not simply down-regulate their GFP expression. It must be noted that TUNEL staining did not positively label every abdominal RP2 neuron in a given ventral ganglion removed at this stage. This is likely due to minor temporal variations among RP2 neurons in their progress along the cell death pathway within and between animals. Similar asynchrony is reported for the PCD of *Manduca* motoneurons (Kinch et al., 2003). We conclude from these results that PCD of abdominal RP2s is initiated sometime after 8 h APF, most likely during pupation. A similar time course is observed in the larval salivary glands which become TUNEL-positive at 15 h APF and degenerate shortly thereafter (Jiang et al., 1997).

We were unable to accurately trace the fate of the thoracic RP2s in these experiments. It is not known therefore if the thoracic RP2s have a fate similar to their abdominal partners in terms of death vs. survival and time course. Limited observations of the thoracic RP2 neurons suggest that they may survive (unpublished observations). In *Manduca sexta*, PCD of the APR motoneurons during metamorphosis is determined in part by segmental identity (Choi and Fahrbach, 1992; Streichert et al., 1997; Zee and Weeks, 2001). A similar mechanism may regulate the fates of thoracic and abdominal RP2 motoneurons.

Cell death pathway in abdominal RP2s

PCD in *Drosophila* depends on the tight regulation of death-activating and death-inhibiting proteins. The three death activating genes *reaper, hid,* and *grim* are collectively or independently responsible for activating the cell death machinery in different cells and tissues (White et al., 1994; Grether et al., 1995; Chen et al., 1996). These three proteins exert their effect in part by liberating the apical caspase Dronc from inhibition by Diap1. PCD is initiated when levels of Reaper, Grim, or Hid become high enough to effectively antagonize Diap1 (Goyal et al., 2000). The destruction of the larval salivary glands at pupation depends on the induced expression of several early response genes by ecdysteroids including *E93, Broad complex (BR-C), and E74A,* which in turn induce maximal expression of Reaper and Hid (Jiang et al., 2000; Lee et al., 2000a; Yin and Thummel 2004). PCD of the Type II and CCAP neurons is mediated by *reaper* and *grim* but not *hid* (Robinow et al., 1997; Draizen et al., 1999).

In our experiments, death of the abdominal RP2s appears to depend exclusively on *reaper* function. The reduction of *reaper, hid,* and *grim* to one copy did not affect PCD of the abdominal RP2 neurons (Fig. 5A, B). This is in contrast to CCAP neurons, in which a reduction of all three genes caused a significantly delays the death of some neurons (Draizen et al., 1999). Likewise, the removal of *hid* function in the same background did not affect the death of the abdominal RP2 neurons (Fig. 5C). Only when *reaper* function was removed did we observe a rescue of these neurons (Fig. 5D).

Although we did not test the requirements of *grim,* it seems unlikely that *grim* is required. In the *reaper* mutants, there was still an intact copy of *grim,* yet death of the abdominal RP2s was still impaired.

Despite a role for *reaper* in the death of the abdominal RP2s, we were not able to block their degeneration by ectopic expression of Diap1. This is in contrast to the salivary glands whose death depends on the loss of Diap1 function; expression of a *UAS-DIAP1* transgene is sufficient to block salivary gland degeneration in both our experiments (Fig. 6C) and those reported previously (Yin et al., 2007). Based on this result alone however, it cannot be concluded that PCD of the abdominal RP2 neurons does not depend on Diap1 downregulation since expression of *UAS-DIAP1* in our genetic background may not have been effective. A more in-depth analysis of Diap1 expression in the RP2s, and analysis of *diap1* mutants (e.g. Lisi et al., 2000) are required.

Expression of the viral caspase inhibitor p35 in abdominal RP2s blocked their demise (Fig. 6D) demonstrating that their death depends in part on the activation of caspases. We also tested the requirement for the apical caspase dronc in the death of the abdominal RP2s. Although death of the RP2s still proceeded normally in the *dronc* mutants tested, there was significant impairment in the *dark* mutants (Fig. 6E). Apoptosome assembly requires both Dronc and Dark and our results suggest a possible role for both proteins in the death of abdominal RP2s. The rescue of PCD of abdominal RP2s in the $dronc^{51}/dronc^{KG02994}$ mutant was probably due to $dronc^{KG02994}$ not being a true null. Still, it would be wise to confirm that PCD of abdominal RP2s requires Dronc using a stronger *dronc* mutant.

20E as a hormonal cue for the death of RP2s

Pupation is initiated by the small prepupal pulse in 20E at ~10 h APF (Fig. 1). The hallmark of pupation is the eversion of the adult head which occurs by 12 h APF (Riddiford, 1993). The histolysis of the larval salivary glands is triggered cell-autonomously by this prepupal pulse (Jiang et al., 1997). Given the time course of PCD of the abdominal RP2 neurons, there was a high likelihood that their demise was initiated in response to the prepupal pulse as well.

This hypothesis was tested first by examining the requirements of EcRs for PCD of the abdominal RP2s. Both EcR-A and EcR-B1 are expressed in the abdominal RP2s at 8-10 h APF (Fig. 7A, B). However, in an *EcR-A* mutant, death of the abdominal RP2s was not affected (Fig. 7C). Although EcR-A is probably involved in PCD of the Type II neurons at the end of metamorphosis (Robinow et al., 1993), it would appear that this isoform plays a more substantial role in the growth of adult-specific neurons during early metamorphosis (Truman et al., 1994). Thus it is unlikely that EcR-A plays a role in the death of abdominal RP2s. The failure of the abdominal RP2s to die when they are made to express the dominant negative EcR-B1 (*UAS-EcR-B1*^{F645A}; Fig. 7D) however demonstrates a requirement for transcriptional activation from one or both EcR-B isoforms.

The strongest evidence for a cell-autonomous response to the prepupal pulse of ecdysteroids in the death of abdominal RP2s comes from our cell culture experiments.

Neuronal cultures generated from animals at 8 h APF and grown in the presence of 20E had a significantly lower percentage of surviving putative RP2 neurons after 48 or 72 h in culture (Fig. 9B). Our assessment of cultured putative abdominal RP2 neuron survival was verified by both morphological criteria and PI staining (Fig. 11). This effect of 20E on survival did not occur in randomly selected control neurons (Fig. 9A); no significant differences were observed between the survival of control neurons grown in the same cell cultures in the presence or absence of 20E for 48 or 72 h. This indicates that the 20E effect on neuronal survival was limited to the putative RP2 neurons in our cultures and was not a non-specific response. These data combined with our *in vivo* observations provide strong support for the hypothesis that the prepupal pulse in 20E causes the cell-autonomous death of the abdominal RP2 neurons during metamorphosis.

Some limitations in cell culture experiments should be noted. Even in cultures that were allowed to grow for 72 h, some putative abdominal RP2 neurons failed to degenerate (Fig. 9B; 11). Two factors may account for this finding: although we selected tissue in which strong GFP expression was limited to the RP2 neurons there was still low levels of GFP expression in the aCC neurons and a few unidentifiable neurons (Fig. 3). This raises the possibility that some of the neurons in our cell cultures that we identified as RP2s may have had a different identity (hence the use of the term "putative"). Furthermore, the time period during which cultures were maintained, may not have been sufficiently long for all the putative RP2s to complete PCD (see below). Similar results were obtained in *Manduca* cell culture experiments investigating PCD of the APRs. Not all APR(6) motoneurons, cultured during or prior to pupal ecdysis, underwent PCD in the

presence of hormone (Streichert et al., 1997; Hoffman and Weeks, 1998). Likewise, the removal of hormone from late pupal APR(4) motoneurons grown in culture also did not result in all of their deaths (Zee and Weeks, 2001). Since APR motoneurons were unambiguously identifiable in these cell cultures, there are likely other unknown limitations associated with cell culture

In our cell cultures, a significant number of putative abdominal RP2 neurons underwent PCD by 48 or 72 h *in vitro*. This is substantially slower than the observed time course *in vivo,* and is likely due to somewhat slower metabolic process in the cultured neurons due to temperature and other conditions associated with cell culture.

Mechanisms of RP2 fate

Based on the present experiments it can be concluded that PCD of abdominal RP2s is initiated by the prepupal pulse of ecdysteroids. The increased expression of Reaper and Dronc by ecdysteroids has been previously demonstrated in the salivary glands during the same period, and in other systems (Dorstyn et al., 1999; Jiang et al., 2000; Lee et al., 2000a; Cakouros et al., 2002, 2004) and a similar mechanism may take place in abdominal RP2s; however this has yet to be demonstrated. Several questions remain unresolved. Not all neurons, which die during early metamorphosis, do so at the same time as the abdominal RP2s pointing to other factors that control PCD during this period. For instance, pupariation is initiated in the wandering larvae by a "late larval pulse" in 20E (Fig. 1). This hormonal cue may be responsible for the death of the vCrz

neurons (Choi et al., 2006). PCD of the vCrz neurons is initiated very rapidly after pupariation and is complete by 6 h APF. Disruption of EcR B-specific expression in the ventral ganglion using EcR B specific mutants significantly impairs PCD of the vCrz neurons indicating that 20E likely plays a critical role in their demise (Choi *et al.,* 2006). Although it has not been shown conclusively that the late larval pulse of 20E is directly responsible for the PCD of these and other early dying neurons, the evidence strongly favors this possibility.

Taken together with our data, it would appear the same hormonal cues that initiate the morphological changes associated with pupariation and pupation are also responsible for the observed neuronal changes including at least two periods of PCD. The second period of PCD includes the abdominal RP2 neurons. In *Manduca* there is also a second period of PCD in the nervous system during pupation that includes sex-specific neurons (Weeks and Giebultowicz, unpublished observations). This raises the question of why the abdominal RP2 neurons survive the late larval pulse pulse of 20E but are eliminated by the prepupal pulse. A more in depth investigation into the function of Diap1 in the abdominal RP2s may provide answers to this question. Other factors however are likely involved in the persistence of the abdominal RP2 neurons until pupation as well. Cao et al. (2007) identified another transcription factor Fork head (Fkh) which appears to protect the salivary glands during pupariation through the inhibition of *reaper* and *hid* expression by ecdysteroids. Loss of Fkh expression during pupariation allows for the subsequent prepupal pulse of ecdysone to activate *reaper* and *hid.* Similar mechanisms may regulate the survival of the abdominal RP2 motoneurons as well.

The specification of neurons for death as opposed to remodeling during metamorphosis in response to the same hormonal cues remains unclear as well. The fate of a particular tissue or neuron during development and metamorphosis is determined in part by its EcR expression profile both spatially and temporally (Robinow et al., 1993; Talbot et al., 1993; Truman et al., 1994; Schubiger et al., 1998; Cherbas et al., 2003; Davis et al., 2005). However, the distinct role of EcR-B1 and EcR-B2 in either fate remains unclear since neurons that are remodeled and neurons destined for PCD both express EcR-B1. Differences in EcR-B2 expression remain unknown due to the lack of an available antibody, and may be a determining factor. A similar complexity occurs in *Manduca* as well: the prepupal pulse of 20E induces dendritic regression of APR motoneurons in all segments but selectively kills only a subset of those neurons (Streichert et al., 1997; Sandstrom and Weeks, 1998). Despite these unique responses to the same hormonal cue, no segmental differences in EcR expression were found (Ewer J, Kinch GA, and Weeks JC., unpublished data). This result is similar to the result obtained in our studies in which EcR expression did not differ between the doomed abdominal RP2s and persisting aCCs. It may be that differences were too minute to be detected in by our immunocytochemistry. Likewise, other factors downstream of EcRs probably also play a role.

This study has identified a unique motoneuron whose PCD occurs in a previously unexplored manner during metamorphosis. A more in depth investigation into the intracellular death pathway and its regulation by specific EcR isoforms, will provide

additional insight into how a systemic hormonal cue is uniquely interpreted individual neurons.

CHAPTER III

CONCLUDING REMARKS

Summary

The genetic tools available in *Drosophila* have made this insect an excellent model in which to study developmental processes at the cellular level. During metamorphosis, changes in circulating levels of ecdysteroids induce a variety of responses in the nervous system including PCD of some existing larval neurons. This has allowed for an opportunity to study steroid-induced PCD in its natural context. In contrast to *M. sexta* however, there has been little research into steroid-induced PCD in the *Drosophila* nervous system during the early stages of metamorphosis.

In this study, I have identified a motoneuron, RP2, which undergoes PCD during the first 24 h of metamorphosis. Its demise occurs in a caspase-dependent manner utilizing previously identified death-activating proteins. I have also provided strong evidence that their PCD is ecdysteroid-dependent by showing a requirement for EcR-B activity. Finally, using neuronal cell culture and *in vitro* hormonal manipulation, I have shown that PCD of the RP2s is a cell-autonomous response to ecdysteroids. These data lead to the conclusion that the prepupal pulse of ecdysteroids is responsible for the death of the RP2s during metamorphosis.

Future directions

This study has demonstrated a requirement of the death activating protein, Reaper, for the death of the RP2s. A reasonable hypothesis, that requires testing, is that ecdysteroids cause the upregulation of Reaper expression in the RP2s. Studies have shown this to be the case in the salivary glands which degenerate during the same period in response to the prepupal pulse (Jiang et al., 1997). In their case ecdysteroids cause ecdysteroid-specific transcription factors to be expressed. These transcription factors then upregulate the expression of Reaper and Hid (Jiang et al., 2000). In addition to Reaper, other proteins in the cell death pathway such as Dronc and Dark may also be upregulated by ecdysteroids in the RP2s.

Another unresolved issue concerns the survival of the RP2 neurons of the earlier late larval pulse of ecdysteroids. It is of importance to see if Diap1 is expressed in the RP2s at any time during this period and whether other transcriptional repressors such as Fkh are responsible for protecting the RP2s until the appropriate time.

Post-metamorphic PCD

As stated earlier, a second wave of neuronal PCD takes place at the end of *Drosophila* metamorphosis. Studies demonstrated that these deaths take place throughout the first 24 h following adult eclosion, and that the doomed neurons express

high levels of EcR-A (Kimura and Truman, 1990; Robinow et al., 1993). These Type II neurons begin expressing EcR-A at 24 h APF and, by 48 h APF, a maximum of roughly 300 Type II neurons are present (Robinow et al., 1993). Another set of neurons that express CCAP also die during the same period as the Type II neurons (Draizen et al., 1999). The post-metamorphic death of the Type II and CCAP neurons occurs following the decline in ecdysteroid levels that takes place during the final 24 h of metamorphosis. Intriguingly, injection of newly eclosed adults with 20E immediately following eclosion, is sufficient to delay to the death of both sets of neurons (Robinow et al., 1993; Draizen et al., 1999). Therefore, the decline of 20E at the end of metamorphosis is necessary for the deaths of these identified neurons. However, it is not known, if their deaths are triggered directly and cell-autonomously by this decline.

I attempted to test the hypothesis that the decline in 20E at the end of metamorphosis triggered the deaths of the Type II neurons. To drive GFP expression in a subset of Type II neurons, I used transgenic flies in which, GAL4 is driven by upstream regulatory elements of the *EcR-A* gene (GET-AG) (Sung and Robinow, 2000). These flies were crossed to a UAS-GFP reporter line resulting in flies in which GFP is expressed in a large number of Type II neurons. Using the same methods described in the previous chapter, sister neuronal cell cultures were generated from the dissociation of at least two ventral ganglia. However, in this case, sister cultures were generated at 72 h APF just prior to the completion of the decline in ecdysteroids at the end of metamorphosis.

One neuronal culture out of each sister pair, received 20E (1μg/ml), and cultures were monitored over a period of at least 48 h while tracking the survival of the GFP-positive neurons.

In these cell culture experiments, I did not observe any significant differences in the survival of GFP-positive neurons between cultures grown in the presence or absence of 20E. This result occurred even in cultures that were allowed to grow for 7 days. Two factors may have accounted for this result and warrant further investigation. When the post-eclosion behavior of wing inflation is disrupted in flies through decapitation or confinement immediately after adult eclosion, the death of many of these neurons is blocked (Kimura and Truman, 1990). This lends support to the likelihood that at least some of the Type II neurons require both the decline of ecdysteroids and some other "competence factor" associated with eclosion and wing inflation that I was unable to reproduce in my cultures. Furthermore, the GET-AG line drives GAL4 expression in numerous other neurons in addition to the Type IIs (Sung and Robinow, 2000). Many of these imposter neurons inevitably ended up in my cultures. These two compounding factors limited my ability to test my hypothesis using cell culture.

In order to effectively generate cultures in which only Type II neurons express GFP, it is necessary to specifically drive GAL4-expression exclusively in subsets of Type II neurons using the GET-AG line. This may be possible using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999). This method would allow one to limit GET-AG-driven GAL4 expression to a subset of Type II neurons. This would then allow for the generation of cultures in which the GFP-expressing neurons are

all Type II. In this setup, one can then determine if the decline in ecdysteroids can cell-autonomously cause PCD of at least some Type II neurons or whether an additional signal is required. The Type II neurons are an intriguing area of study, since they die in response to a hormonal cue that is distinct to that of the RP2s, and only express EcR-A. These studies will help to provide insight into the big question of how individual cells respond uniquely to universal hormonal cues.

BIBLIOGRAPHY

Abrams JM, White K, Fessler LI, Steller H. 1993. Programmed cell death during *Drosophila* embryogenesis. Development 117:29-43.

Akdemir F, Farkaš R, Chen P, Juhasz G, Medved'ová L, Sass M, Wang L, Wang X, Chittaranjan S, Gorski SM, Rodriguez A, Abrams JM. 2006. Autophagy occurs upstream or parallel to the apoptosome during histolytic cell death. Development 133:1457-1465.

Amelio MD, Tino E, Cecconi F. 2008. The apoptosome: emerging insights and new potential targets for drug design. Pharm Res 25(4):740-751.

Aranda A, Pascual A. 2001. Nuclear hormone receptors and gene expression. Physiol Rev 81:1269-1304.

Baehrecke EH. 1996. Ecdysone signaling cascade and regulation of *Drosophila* metamorphosis. Arch Insect Biochem Physiol 33:231-244.

Baehrecke EH. 2000. Steroid regulation of programmed cell death during *Drosophila* development. Cell Death Differ 7(11):1057-1062.

Baehrecke EH. 2002. How death shapes life during development. Mol Cell Biol 3:779-787.

Bangs P, White K. 2000. Regulation and execution of apoptosis during *Drosophila* development. Dev Dyn 218:68-79.

Bello BC, Hirth F, Gould AP. 2003. A pulse of the *Drosophila* hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. Neuron 37(2):209-219.

Bender M, Imam FB, Talbot WS, Ganetzky B, Hogness DS. 1997. *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. Cell 91:777-788.

Bergmann A, Tugentman M, Shilo B, Steller H. 2002. Regulation of cell number by MAPK-dependent control of apoptosis a mechanism for trophic survival signaling. Dev Cell 2(2):159-170.

Bergmann A, Yang AY-P, Srivastava M. 2003. Regulators of IAP function: coming to grips with the grim reaper. Curr Opin Cell Biol 15:717-724.

Breedlove SM. 1992. Sexual dimorphism in the vertebrate nervous system. J Neurosci 12(11):4133-4142.

Brown HLD, Cherbas L, Cherbas P, Truman JW. 2006. Use of time-lapse imaging and dominant negative receptors to dissect the steroid receptor control of neuronal remodeling in *Drosophila*. Development 133:275-285.

Bump NJ, Hackett M, Hugunin M, Seshagiri S, Brady K, Chen P, Ferenz C, Franklin S, Ghayur T, Li P, Licari P, Mankovich J, Shi L, Greenberg AH, Miller LK, Wong WW. 1995. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science 269(5232):1885-1888.

Cakouros D, Daish T, Kumar S. 2004. Ecdysone receptor directly binds the promoter of the *Drosophila* caspase *dronc,* regulating its expression in specific tissues. J Cell Biol 165(5):631-640.

Cakouros D, Daish T, Martin D, Baehrecke EH, Kumar S. 2002. Ecdysone-induced expression of the caspase DRONC during hormone-dependent programmed cell death in *Drosophila* is regulated by Broad-Complex. J Cell Biol 157(6):985-995.

Cao C, Liu Y, Lehmann M. 2007. Fork head controls the timing and tissue selectivity of steroid-induced developmental cell death. J Cell Biol 176(6):843-852.

Carney GE, Robertson A, Davis MB, Bender M. 2004. Creation of *EcR* isoform-specific mutations in *Drosophila melanogaster* via local P element transposition, imprecise P element excision, and male recombination. Mol Gen Genomics 271:282-290.

Cashio P, Lee TV, Bergmann A. 2005. Genetic control of programmed cell death in *Drosophila melanogaster*. Semin Cell Dev Biol 16(2):225-235.

Chen P, Nordstrom W, Gish B, Abrams JM. 1996. *grim*, a novel cell death gene in *Drosophila*. Genes Dev 10:1773-1782.

Chen P, Rodriguez A, Erskine R, Thach T, Abrams JM. 1998. *Dredd* a novel effector of the apoptosis activators *Reaper*, *Grim*, and *Hid* in *Drosophila*. Dev Biol 201:202-216.

Cherbas L, Hu X, Zhimulev I, Belyaeva E, Cherbas P. 2003. EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. Development 130:271-284.

Chew SK, Akdemir F, Chen P, Lu W-J, Mills K, Daish T, Kumar S, Rodriguez A, Abrams JM. 2004. The apical caspase *dronc* governs programmed and unprogrammed cell death in *Drosophila*. Dev Cell 7:897-907.

Choi Y-J, Lee G, Park JH. 2006. Programmed cell death mechanisms of identifiable peptidergic neurons in *Drosophila melanogaster*. Development 133:2223-2232.

Choi JC, Park D, Griffith LC. 2004. Electrophysiological and morphological characterization of identified motor neurons in the *Drosophila* third instar larva central nervous system. J Neurophysiol 91:2353-2365.

Conradt B, Xu D. 2005. Programmed cell death. In: Wormbook, editor. The *C. elegans* research community, wormbook, doi/10.1895/wormbook.1.32.1, http://www.wormbook.org.

Consoulas C, Levine RB, Restifo LL. 2005. The steroid hormone-regulated gene *Broad Complex* is required for dendritic growth of motoneurons during metamorphosis of *Drosophila*. J Comp Neurol 485:321-337.

Consoulas C, Restifo LL, Levine RB. 2002. Dendritic remodeling and growth of motoneurons during metamorphosis of *Drosophila melanogaster*. J Neurosci 22(12):4906-4917.

Daish TJ, Mills K, Kumar S. 2004. *Drosophila* caspase DRONC is required for specific developmental cell death pathways and stress-induced apoptosis. Dev Cell 7:909-915.

Danial NN, Korsmeyer SJ. 2004. Cell death: critical control points. Cell 116:205-219.

Davis MB, Carney GE, Robertson AE, Bender M. 2005. Phenotypic analysis of *EcR-A* mutants suggests that EcR isoforms have unique functions during *Drosophila* development. Dev Biol 282:385-396.

Dorstyn L, Colussi PA, Quinn LM, Richardson H, Kumar S. 1999a. DRONC, an ecdysone-inducible *Drosophila* caspase. Proc Natl Acad Sci 96:4307-4312.

Dorstyn L, Mills K, Lazebnik Y, Kumar S. 2004. The two cytochrome c species, DC3 and DC4, are not required for caspase activation and apoptosis in *Drosophila* cells. J Cell Biol 167(3):405-410.

Dorstyn L, Read SH, Quinn LM, Richardson H, Kumar S. 1999b. DECAY, a novel *Drosophila* caspase related to mammalian caspase-3 and caspase-7. J Biol Chem 274(43):30778-30783.

Doumanis J, Quinn L, Richardson H, Kumar S. 2001. STRICA, a novel *Drosophila melanogaster* caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2. Cell Death Diff 8(4):387-394.

Draizen TA, Ewer J, Robinow S. 1999. Genetic and hormonal regulation of the death of peptidergic neurons in the *Drosophila* central nervous system. J Neurobiol 38(4):455-465.

Duffy JB, Harrison DA, Perrimon N. 1998. Identifying loci required for follicular patterning using directed mosaics. Development 125(12):2263-2271.

Fraser AG, Evan GI. 1997. Identification of a *Drosophila melanogaster* ICE/CED-3-related protease, drICE. EMBO 16(10):2805-2813.

Freeman LM, Watson NV, Breedlove SM. 1996. Androgen spares androgen-insensitive motoneurons from apoptosis in the spinal nucleus of the bulbocavernosus in rats. Horm Behav 30(4):424-433.

Fujioka M, Lear BC, Landgraf M, Yusibova GL, Zhou J, Riley KM. 2003. Even-skipped, acting as a repressor, regulates axonal projections in *Drosophila*. Development 130:5385-5400.

Gavrieli Y, Sherman Y, Ben-Sasson SA. 1992. Identification of programmed cell death *in Situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 119(3):493-501.

Goodman CS, Bastiani MJ, Doe CQ, du Lac S, Helfand SL, Kuwada JY, Thomas JB. 1984. Cell recognition during neuronal development. Science 225(4668):1271-1279.

Goyal L, McCall K, Agapite J, Hartwieg E, Steller H. 2000. Induction of apoptosis by *Drosophila reaper, hid* and *grim* through inhibition of IAP function. EMBO 19(4): 589-597.

Green DR, Evan GI. 2002. A matter of life and death. Cancer Cell 1:19-30.

Grether ME., Abrams JM, Agapite J, White K, Steller H. 1995. The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. Genes Dev 9:1694-1708.

Hall BL, Thummel CS. 1998. The RXR homologue Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. Development 125:4709-4717.

Handler AM. 1982. Ecdysteroid titers during pupal and adult development in *Drosophila melanogaster*. Dev Biol 93:73-82.

Hartwig CL, Worrell J, Levine RB, Ramaswami M, Sanyal S. 2008. Normal dendrite growth in *Drosophila* motor neurons requires the AP-1 transcription factor. Dev Neurobiol 68(10):1225-42.

Harvey NL, Daish T, Mills K, Dorstyn L, Quinn LM, Read SH, Richardson H, Kumar S. 2001. Characterization of the *Drosophila* caspase, DAMM. J Biol Chem 276(27):25342-25350.

Hawkins CJ, Yoo SJ, Peterson EP, Wang SL, Vernooy SY, Hay BA. 2000. The *Drosophila* caspase Dronc cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. J Biol Chem 275(35) 27084-27093.

Hay BA, Guo M. 2006. Caspase-dependent cell death in *Drosophila*. Annu Rev Cell Dev Biol 22:623-650.

Hay BA, Wassarman DA, Rubin GM. 1995. *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell 83:1253-1262.

Hay BA, Wolff T, Rubin GM. 1994. Expression of baculovirus P35 prevents cell death in *Drosophila*. Development 120(8):2121-2129.

Hoffman KL, Weeks JC. 1998. Programmed cell death of an identified motoneuron *in vitro*: temporal requirements for steroid exposure and protein synthesis. J Neurobiol 35(3): 300-322.

Hoffman KL, Weeks JC. 2001. Role of caspases and mitochondria in the steroid-induced programmed cell death of a motoneuron during metamophosis. Dev Biol 229(2):517-536.

Hu X, Cherbas L, Cherbas P. 2003. Transcriptional activation by the ecdysone receptor (EcR/USP): identification of activation functions. Mol Endocrinol 17(4):716-731.

Ito K, Awano W, Suzuki K, Hiromi Y, Yamamoto D. 1997. The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurons and glial cells. Development 124(4):761-771.

Jiang C, Baehrecke EH, Thummel CS. 1997. Steroid regulated programmed cell death during *Drosophila* metamorphosis. Development 124:4673-4683.

Jiang C, Lambin A-FJ, Steller H, Thummel CS. 2000. A steroid-triggered transcriptional hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. Mol Cell 5:445-455.

Jordán J, Galindo MF, Miller RJ, Reardon CA, Getz GS, Ladu MJ. 1998. Isoform-specific effect of Apolipoprotein E on cell survival and β-amyloid-induced toxicity in rat hippocampal pyramidal neuronal cultures. J Neurosci 18(1):195-204.

Kiess W, Gallaher B. 1998. Hormonal control of programmed cell death/apoptosis. Eur J Endocrinol 138(5):482-491.

Kimura K, Kodama A, Hayasaka Y, Ohta T. 2004. Activation of the cAMP/PKA signaling pathway is required for post-ecdysial cell death in wing epidermal cells of *Drosophila melanogaster*. Development 131:1597-1606.

Kimura K, Truman JW. 1990. Postmetamorphic cell death in the nervous and muscular systems of *Drosophila melanogaster*. J Neurosci 10(2):403-411.

Kinch G, Hoffman KL, Rodrigues EM, Zee MC, Weeks JC. 2003. Steroid-triggered programmed cell death of a motoneuron is autophagic and involves structural changes in mitochondria. J Comp Neurol 457(4):384-403.

Kraft R, Restifo L. 1998. The steroid hormone 20-hydroxyecdysone enhances neurite growth of *Drosophila* mushroom body neurons isolated during metamorphosis. J Neurosci 18(21):8886-8899.

Kumar S. 2007. Caspase function in programmed cell death. Cell Death Diff 14:32-43.

Lee C-Y, Baehrecke EH. 2001. Steroid regulation of autophagic programmed cell death during development. Development 128(8):1443-1455.

Lee C-Y, Wendel DP, Reid P, Lam G, Thummel CS. 2000a. *E93* directs steroid-triggered programmed cell death in *Drosophila*. Mol Cell 6(2):433-443.

Lee T, Luo L. 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22(3):451-461.

Lee T, Marticke S, Sung C, Robinow S, Luo S. 2000b. Cell-autonomous requirement of the USB/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. Neuron 28(3):807-818

Leulier F, Rodriguez A, Khush RS, Abrams JM, Lemaitre B. 2000. The *Drosophila* caspase Dredd is required to resist gram-negative bacterial infection. EMBO Rep 1(4):353-358.

Levine RB, Truman JW. 1985. Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta*. J Neurosci 5(9):2424-2431

Lewis EB. 1960. A new standard food medium. Dros Info Serv 34:117-118.

Lisi S, Mazzon I, White K. 2000. Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila.* Genetics 154:669-678.

Martin SJ. 2002. Destabilizing influences in apoptosis: sowing the seeds of IAP destruction. Cell 109:793-796.

Mattson MP. 2000. Apoptosis in neurodegenerative disorders. Mol Cell Bio 1(2):120-129.

Meier P, Silke J, Leevers SJ, Evan GI. 2000. The *Drosophila* caspase DRONC is regulated by DIAP1. EMBO 19(4):598-611.

Mills K, Daish T, Harvey KF, Pfleger CM, Hariharan IK, Kumar S. 2006. The *Drosophila melanogaster* Apaf-1 homologue ARK is required for most, but not all, programmed cell death. J Cell Biol 172(6):809-815.

Mills K, Daish T, Kumar S. 2005. The function of the *Drosophila* caspase DRONC in cell death and development. Cell Cycle 4(6):744-746.

Morris JA, Jordan CL, Breedlove SM. 2004. Sexual differentiation of the vertebrate nervous system. Nat Neurosci 7(10)1034-1039.

Muro I, Hay BA, Clem RJ. 2002. The *Drosphila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. J Biol Chem 277(51):49644-49650.

Ortmann-Rogulja A, Lüer K, Siebert J, Rickert C, Technau GM. 2007. Programmed cell death in the embryonic central nervous system of *Drosophila melanogaster.* Development 134:105-115.

Peterson C, Carney GE, Taylor BJ, White K. 2002. *reaper* is required for neuroblast apoptosis during *Drosophila* development. Development 129:1467-1476.

Prugh J, Della Croce K, Levine RB. 1992. Effects of the steroid hormone 20-hydroxyecdysone, on the growth of neurites by identified insect motoneurons *in vitro*. Dev Biol 154(2):331-347.

Richards G. 1981. The radioimmune assay of ecdysteroid titres in *Drosophila melanogaster*. Mol Cell Endocrinol 21:181-197.

Riddiford LM. 1993. Hormones and *Drosophila* development. In: Bate M, Martinez Arias A, editors. The Development of *Drosophila melanogaster*. New York: Cold Spring Harbor Press, pp 899-939.

Robinow S, Draizen TA, Truman JW. 1997. Genes that induce apoptosis: transcriptional regulation in identified, doomed neurons of the *Drosophila* CNS. Dev Biol 190:206-213.

Robinow S, Talbot WS, Hogness DS, Truman JW. 1993. Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. Development 119:1251-1259.

Rodriguez A, Chen P, Abrams JM. 1998. Molecular prophets of death in the fly. Am J Hum Genet 62:514-519.

Rodriguez A, Chen P, Oliver H, Abrams JM. 2002. Unrestrained caspase-dependent cell death caused by loss of *Diap1* function requires the *Drosophila* Apaf-1 homologue, *Dark*. EMBO 21(9):2189-2197.

Rodriguez A, Oliver H, Zou H, Chen P, Wang X, Abram JM. 1999. Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. Nat Cell Biol 1:272-279.

Sandstrom DJ, Weeks JC. 1998. Segment-specific retention of a larval neuromuscular system and its role in a new, rhythmic, pupal motor pattern in *Manduca sexta*. J Comp Physiol 183(3):283-302.

Schmidt S, Rainer J, Ploner C, Presul E, Riml S, Kofler R. 2004. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Diff 11:S45-S55.

Schubiger M, Tomita S, Sung C, Robinow S, Truman JW. 2003. Isoform specific control of gene activity in vivo by the *Drosophila* ecdysone receptor. Mech Dev 120:909-918.

Schubiger M, Wade AA, Carney GE, Truman JW, Bender M. 1998. *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. Development 125:2053-2062.

Sink H, Whitington PM. 1991. Location and connectivity of abdominal motoneurons in the embryo and larva of *Drosophila melanogaster*. J Neurobiol. 22(3):298-311.

Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Optiz-Araya X, McCombie R, Herman JG, Gerald WL, Lazebnik YA, Cordón-Cardó C, Lowe SW. 2001. Inactivation of the apoptosis effector *Apaf-1* in malignant melanoma. Nature 409:207-211.

Song Z, McCall K, Steller H. 1997. DCP-1, a *Drosophila* cell death protease essential for development. Science 275(536):536-540.

Strasser A, O'Connor L, Dixit VM. 2000. Apoptosis signaling. Annu Rev Biochem 69:217-245.

Streichert LC, Pierce JT, Nelson JA, Weeks JC. 1997. Steroid hormones act directly to trigger segment-specific programmed cell death of identified motoneurons *in vitro*. Dev Biol 183(1):95-107.

Su H, O'Dowd DK. 2003. Fast synaptic currents in *Drosophila* mushroom body Kenyon cells are mediated by α-bungarotoxin-sensitive nicotinic acetylcholine receptors and picrotoxin-sensitive GABA receptors. J Neurosci 23(27):9246-9253.

Su Y, Damjanovski S, Shi Y, Shi YB. 1999. Molecular and cellular basis of tissue remodeling during amphibian metamorphosis. Histol Histopathol 14(1):175-183.

Su Y, Shi Y, Stolow MA, Shi Y-B. 1997. Thyroid hormone induces apoptosis in primary cell cultures of tadpole intestine: cell type specificity and effects of extracellular matrix. J Cell Biol 139(6):1533-1543.

Sung C, Robinow S. 2000. Characterization of the regulatory elements controlling neuronal expression of the A-isoform of the ecdysone receptor gene of *Drosophila melanogaster*. Mech Dev 91:237-248.

Talbot WS, Swyryd EA, Hogness DS. 1993. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73:1323-1337.

Thompson CB. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267(5203):1456-1462.

Tissot M, Stocker RF. 2000. Metamorphosis in *Drosophila* and other insects: the fate of neurons throughout the stages. Prog Neurobiol 62:89-111.

Truman JW, Riddiford LM. 2002. Insect developmental hormones and their mechanisms of action. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, editors. Hormones, Brain and Behavior, Vol. 2. San Diego: Academic Press, pp 841-873.

Truman JW, Talbot WS, Fahrbach SE, Hogness DS. 1994. Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. Development 120:219-234.

Truman JW, Taylor BJ. Awad TA. 1993. Formation of the adult nervous system. In: Bate M, Martinez Arias A, editors. The Development of *Drosophila melanogaster*. New York: Cold Spring Harbor Press, pp 1245-1275.

Vaux DL, Korsmeyer SJ. 1999. Cell death in development. Cell 96:245-254.

Vucic D, Kaiser WJ, Harvey AJ, Miller LK. 1997. Inhibition of Reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). Proc Natl Acad Sci 94:10183-10188.

Vucic D, Kaiser WJ, Miller LK. 1998. Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by *Drosophila* proteins HID and GRIM. Mol Cell Biol 18(6): 3300-3309.

Waldhuber M, Emoto K, Petritsch C. 2005. The *Drosophila* caspase DRONC is required for metamorphosis and cell death in response to irradiation and developmental signals. Mech Dev 122(7-8):914-27.

Wang SL, Hawkins CJ, Yoo SJ, Müller HA, Hay BA. 1999. The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. Cell 98(4):453-463.

Warren JT, Yerushalmi Y, Shimell MJ, O'Connor MB, Restifo LL, Gilbert LI. 2006. Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of *Drosophila melanogaster*: correlations with changes in gene activity. Dev Dyn 235(2):315-326.

Weeks JC. 2003. Thinking globally, acting locally: steroid hormone regulation of the dendritic architecture, synaptic connectivity and death of an individual neuron. Prog Neurobiol 70:421-442.

Weeks JC, Ernst-Utzschneider K. 1989. Respecification of larval proleg motoneurons during metamorphosis of the tobacco hornworm, *Manduca sexta*: segmental dependence and hormonal regulation. J Neurobiol 20(6):569-592.

Weeks JC, Roberts WM, Trimble DL. 1992. Hormonal regulation of segmental specificity of motoneuron phenotype during metamorphosis of the tobacco hornworm, *Manduca sexta.* Dev Biol 149:185-196.

Weeks JC, Truman JW. 1985. Independent steroid control of the fates of motoneurons and their muscles during insect metamorphosis. J Neurosci 5(8):2290-2300.

White K, Grether ME, Abrams JM, Young K, Farrell K, Steller H. 1994. Genetic control of programmed cell death in *Drosophila.* Science 264(5159):677-683.

Wilson R, Goyal L, Ditzel M, Zachariou A, Baker DA, Agapite J, Steller H, Meier P. 2002. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. Nat Cell Biol 4:445-450.

Won SJ, Kim DY, Gwag BJ. 2002. Cellular and molecular pathways of ischemic neuronal death. J Biochem Mol Biol 35(1):67-86.

Wu CF, Suzuki N, Poo MM. 1983. Dissociated neurons from normal and mutant *Drosophila* larval central nervous system in cell culture. J Neurosci 3(9):1888-1899.

Xu D, Li Y, Arcara M, Lackey M, Bergmann A. 2005. The CARD-carrying caspase Dronc is essential for most, but not all, developmental cell death in *Drosophila.* Development 132:2125-2134.

Yan N, Huh JR, Schirf V, Demeler B, Hay BA, Shi Y. 2006. Structure and activation mechanism of the *Drosophila* initiator caspase Dronc. J Biol Chem 281(13):8667-8674.

Yao TP, Segraves WA, Oro AE, McKeown M, Evans RM. 1992. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell 71(1):63-72.

Yin VP, Thummel CS. 2004. A balance between the *diap1* death inhibitor and *reaper* and *hid* death inducers controls steroid-triggered cell death in *Drosophila.* Proc Natl Acad Sci 101(21):8022-8027.

Yin VP, Thummel CS. 2005. Mechanisms of steroid-triggered programmed cell death in *Drosphila.* Semin Cell Dev Biol 16(2):237-243.

Yin VP, Thummel CS, Bashirullah A. 2007. Down-regulation of inhibitor of apoptosis levels provides competence for steroid-triggered cell death. J Cell Biol 178(1)85-92.

Yoo SJ, Huh JR, Muro I, Wang L, Wang SL, Feldman RM, Clem RJ, Müller HA, Hay BA. 2002. Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. Nat Cell Biol 4(6):416-424.

Yu X, Wang L, Acehan D, Wang X, Akey CW. 2005. Three-dimensional structure of a double apoptosome formed by the *Drosophila* Apaf-1 related killer. J Mol Biol 355(3): 577-589.

Yuan J, Yankner BA. 2000. Apoptosis in the nervous system. Nature 407:802-809.

Zee MC, Weeks JC. 2001. Developmental change in the steroid hormone signal for cell-autonomous, segment-specific programmed cell death of a motoneuron. Dev Biol 235:45-61.

Zheng X, Wang J, Haerry TE, Wu AY, Martin J. O'Connor MB, Lee CH, Lee T. 2003. TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. Cell 112(3):303-315.