STEROID HORMONE REGULATION OF NEURONAL REMODELING IN *DROSOPHILA*

by

Katherine R. Small

(Under the direction of Michael Bender)

ABSTRACT

This thesis describes the role that the steroid hormone ecdysone plays in the regulation of neuronal remodeling. In *Drosophila*, ecdysone binds to the ecdysone receptor (EcR), which has three protein isoforms, EcR-A, B1 and B2 which previous studies have indicated are involved in the process of neuronal remodeling. The dendrites of neurons are originally extensively branched and as the development of the organism progresses, the dendrites are pruned back and then re-grow to establish new adult specific connections. I examined neuronal remodeling in the thoracic ventral (Tv) neurons of *EcR-A* mutants by using confocal microscopy. I found that *EcR-A* is not required for initial dendritic growth nor for dendritic pruning but that *EcR-A* is required for dendritic re-growth. *EcR-A* mutants are also not developmentally delayed. Overall, this dissertation has clarified the role of EcR-A in neuronal re-growth, further showing the importance of hormonal signaling in the regulation of developmental processes.

INDEX WORDS: *Drosophila melanogaster*, Steroid hormone, *EcR-A*, Neuronal remodeling

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DEDICATION

To all my family and friends for all their love, encouragement and never ending support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Every aspect of a multicellular organism requires regulation (Snyder, 1985). An organism needs to know everything from what developmental stage they are in, to how much lipid is in circulation in their body (Gronke et al., 2003; Gronke et al., 2007). Since a complex multicellular organism can be composed of billions of cells, these cells need to be able to communicate with one another. The way in which cells communicate is through cell signaling (Scharrer, 1990).

There are two main systems of cell communication, the nervous system and the endocrine system (Snyder, 1985; Scharrer, 1990). The nervous system conducts high-speed electrical signals along cells known as neurons. These rapidly sent messages control body movement in response to environmental stimuli, such as when you jerk your foot away after stepping on a tack. The electric signals are sent from one neuron to another through direct cell-to-cell contact. Activated neurons release neurotransmitters into the synaptic cleft and the binding of the neurotransmitters to the receptors on the neighboring cell cause a change in ion permeability or activation of intracellular second messenger cascades, thereby potentiating the electrical signal (Silverthorn, 2001).

The endocrine system regulates developmental processes and coordinates slower and longer lasting responses to stimuli, such as decreasing amounts of stored lipid, through the use of chemical messengers known as hormones. Hormones are not limited to communicating with only neighboring cells, like the electrical signals sent through the nervous system, but can travel long distances. Cell signaling in the endocrine system involves three main events: reception,

signal transduction and response. Reception occurs when the hormone binds to a specific receptor in the target cell. This binding triggers events in the cell called signal transduction that result in a response, or a change in the cell's behavior (Silverthorn, 2001).

There are three major classes of hormones: amines, peptide and steroids (Silverthorn, 2001). Amines are derived from amino acids and include catecholamines and the thyroid hormones. Peptide hormones are synthesized from amino acids and some examples are insulin and glucagon (Berne and Levy, 1999; von Eggelkraut-Gottanka and Beck-Sickinger, 2004). Steroid hormones are derived from cholesterol and some examples include progestins, estrogens and glucocorticoids (Berne and Levy, 1999; Sanderson, 2006).

Mammalian Steroid Hormone Biosynthesis

Steroid hormone biosynthesis is an important aspect of cell communication. Interference with steroid biosynthesis can result in impaired reproduction, alterations in sexual differentiation, growth and development, and the development of certain cancers (Miller, 2005). The cytochrome P450 enzymes and several steroid dehydrogenases and reductases control the synthesis of these hormones (Silverthorn, 2001; Sanderson, 2006).

In mammals, cholesterol is either obtained from the diet or synthesized from acetate. An average human being consumes 300mg of cholesterol from their diet each day with about 600mg of cholesterol being synthesized from acetate. Mammalian cholesterol is nonpolar and insoluble in aqueous solutions causing it to be transported in a lipoprotein complex from its main synthesis site, the liver, to either the adrenal cortex, testis or ovary (Nussey and Whitehead, 2001; Sanderson, 2006).

In the adrenal cortex, eighty percent of the cholesterol needed for steroid hormone synthesis binds to low-density lipoprotein receptors. The remaining twenty percent is made from acetate within the adrenal cells. Cholesterol can either be stored as esters in lipid droplets or used immediately (Nussey and Whitehead, 2001).

Adrenal steroids are first hydrolyzed and then transferred to the outer mitochondrial membrane. Cholesterol is chaperoned into the inner mitochondrial membrane by a steroidogenic acute regulatory protein (StAR) and is then converted into pregnenolone by a side chain cleavage enzyme, P450. Pregnenolone is then transferred from the mitochondria to the smooth endoplasmic reticulum where it is converted into progesterone. Progesterone can be converted into corticosterone and aldosterone through subsequent hydroxylations. Pregnenolone and progesterone are the precursors for all other steroid hormones (Nussey and Whitehead, 2001; Payne and Hales, 2004).

There is rarely storage of steroids within the adrenal gland, so their secretion requires activation of a biosynthetic pathway. The major secretions of the adrenal cortex are cortisol and aldosterone (Payne and Hales, 2004). Cortisol is an important metabolic hormone and aldosterone is a hormone involved in salt and water homeostasis (Nussey and Whitehead, 2001).

Invertebrate Steroid Hormone Biosynthesis and Ecdysone

Remarkably, steroid hormone synthesis in insects and higher vertebrates is quite similar. The most notable differences are due to how cholesterol is obtained and the chemical structure and properties of the active hormones. As mentioned previously, vertebrates synthesize cholesterol from small carbon units such as acetate and their steroid hormones are nonpolar and are restricted in movement within the organism. Invertebrates must obtain cholesterol from their

diet and the active steroid hormones, the ecdysteroids, are soluble, polar steroids moving easily throughout the open circulatory system (Gilbert et al., 2002).

In insects, dietary cholesterol is dehydrogenated to 7-dehydrocholesterol and is then translocated to the mitochondria of the prothoracic gland. 7-dehydroecdysone is oxidized to 3-dehydroecdysone by a complex, multistep catalysis involving insect-specific P450 monooxygenases. The prohormone 3-dehydroecdysone is then synthesized from the prothoracic gland, under the control of prothoracicotropic hormone (PTTH). Once released from the prothoracic gland into the hemolymph, 3-dehydroecdysone is rapidly converted into ecdysone by a ketoreductase. In the target tissues, ecdysone is converted into the active steroid hormone 20-hydroxyecdysone (20E) (heretofore referred to as ecdysone) by a monooxygenase system (Henrich et al., 1999).

Ecdysone and Development

Ecdysone triggers developmental events in invertebrates and belongs to a class of related ecdysteroids that control more of the world's biomass and living species than any other class of steroid hormones. Many insects have been used to detail the effect of ecdysone on its target tissues with *Drosophila* being used extensively for molecular and genetic characterization (Henrich et al., 1999).

During the development of *Drosophila melanogaster*, a peak of ecdysone occurs during embryogenesis (Figure 1.1) and is required for germ band retraction and head involution, two morphogenetic events that prepare the organism to become a first instar larvae (Kozlova and Thummel, 2003). Ecdysone pulses during each of the first two larval instars trigger molts to the next larval instar as the organism increases in size. Larval molting involves the separation of the

larval cuticle from the underlying epidermis, the formation of the new cuticle and the shedding of the old larval cuticle (Henrich et al., 1999).

A series of ecdysone pulses drives the metamorphic transitions between larval, prepupal, pupal and adult stages. In the late third instar, a small ecdysteroid peak is believed to trigger larval wandering, a behavioral change characterized by the departure of the larvae from the food source in search of a suitable site for puparium formation. This pulse is followed several hours later by a large larval peak that triggers formation of the puparium, the hardened structure in which metamorphosis will take place. The formation of the puparium involves the shortening of the larvae, eversion of the anterior spiracles, and formation of the operculum, a structure through which the adult fly will eventually emerge. Eight to twelve hours later, a small peak of ecdysone drives head eversion. Head eversion marks the end of the prepupal period (Henrich et al., 1999).

The largest pulse of ecdysone occurs about twenty four hours after pupariation and initiates pupal-adult development. The function of this pulse has not been definitively determined, but is thought to play a role in morphogenesis and cuticle deposition (Henrich et al., 1999).

During metamorphosis, the larva is completely reorganized from an organism specialized in feeding and growth, to an adult specialized for reproduction. Consequently, many larval tissues degenerate and are replaced by adult specific tissues. Death of the larval cells varies, depending on the tissue. The larval midgut cells, for example, initiate cell death in response to the ecdysone pulse that triggers puparium formation. The salivary glands initiate cell death when triggered by the prepupal ecdysone peak (Henrich et al., 1999).

Adult structures such as the head, thorax and genitalia are formed from the imaginal disc cells that proliferate throughout larval development. During puparium formation, the imaginal

disc cells evaginate and elongate to form the rudiments of the adult fly appendages, the legs and the wings. The prepupal ecdysone pulse which triggers head eversion also causes the further elongation of these appendages (Henrich et al., 1999).

The adult abdomen and internal tissues, including the adult foregut, midgut, hindgut and salivary glands, arise from the imaginal histoblast cells. The histoblast cells respond to ecdysone by rapidly proliferating and migrating to form the appropriate adult structure (Henrich et al., 1999).

Nuclear Receptor Classes and Structure

In *Drosophila*, ecdysone causes the aforementioned diverse cell and tissue specific responses by binding to the *Drosophila ecdysone receptor* (*EcR*). The ecdysone receptor is functional when bound with Ultraspiracle (USP) (Yao et al., 1993). Both EcR and USP belong to the nuclear receptor superfamily and in vertebrates this family is divided into four divisions which are based upon DNA binding properties (Henrich et al., 1999).

Class I includes the vertebrate steroid receptors that form homodimers after binding their ligand. Class II receptors form heterodimers with an RXR protein. Class II receptors include the vertebrate thyroid hormone receptors, receptors for vitamin D and receptors for the eicosinoids. Classes III and IV are receptors in which no ligand has been determined and are known as the orphan receptors. Class III receptors bind DNA as dimers whereas Class IV receptors bind DNA as monomers (Henrich et al., 1999).

Nuclear receptors have a similar structure regardless of class (Figure 1.2). All nuclear receptors are composed of a highly conserved DNA-binding domain connected to a ligand-binding domain by a flexible hinge region. The DNA-binding domain contains two zinc fingers

which provide DNA-binding specificity and weak dimerization ability. The ligand-binding domain provides strong dimerization. Also found within the structure of nuclear receptors are two activating functions. Activating function 1 (AF-1) is found in the amino terminal domain and can act in a ligand-independent manner while activating function 2 (AF-2), found in the C-terminal domain, is often ligand-dependent and recruits transcriptional co-activators (King-Jones and Thummel, 2005).

EcR

EcR is a member of the Class II nuclear receptors and is most closely related to the vertebrate farnesol X receptor which functions in bile acid homeostasis in vertebrates. As mentioned previously, the functional ecdysone receptor is composed of two subunits, ECR and USP, with the latter being an RXR homolog. EcR is not a typical member of Class II though. All vertebrate steroid hormones bind to the homodimer receptors of Class I, whereas ecdysone binds to the EcR/USP heterodimer belonging in Class II. The EcR receptor also differs from the Class II receptors in that the EcR/USP complex has been shown to interact with molecular chaperones which in vertebrates interact with Class I receptors.

EcR was cloned by homology to another *Drosophila* ecdysone receptor, E75, in the early 1990s. Supporting the hypothesis that EcR was a component of the *Drosophila* ecdysone receptor, EcR was found to bind active ecdysteroids, bind with high specificity at ecdysone response elements, and rescue ecdysone responsiveness when transfected into a mutant cell line lacking EcR (Koelle et al., 1991). EcR by itself was incapable of high affinity DNA binding and transcriptional activation though, these activities were dependent on heterodimer formation with Ultraspiracle (USP) (Yao et al., 1993).

In order for the EcR/USP complex to initiate transcription, ecdysone must bind and co-repressors such as SMRTER must dissociate (Tsai et al., 1999) and be replaced with proteins such as heat shock proteins 90 and 70 (Hsp90 and Hsp70) (Arbeitman and Hogness, 2000) . Once bound to its receptor, ecdysone directly induces the expression of a small set of early regulatory genes (Figure 1.3). The protein products of these genes repress their own expression and induce a much larger set of late target genes. These late target genes can be divided into two classes, the early-late genes and the late-late genes. The early-late genes are rapidly induced after the presence of ecdysone and require its continued presence. The late-late genes are induced later upon ecdysone withdrawal (Ashburner and Richards, 1976).

The EcR encoding gene is over 70 kilobases (kb) in size and its levels of transcripts fluctuate with each peak of ecdysone during larval and pupal development. The EcR gene contains two alternative promoters giving way to three EcR isoforms (EcR-A, B1 and B2) with unique amino-terminal domains and conserved C-terminal domains (Figure 1.4) (Talbot et al., 1993). The three EcR isoforms are hypothesized to have unique functions based upon temporal and spatial expression studies. EcR-A is found in cells that undergo metamorphosis into adult structures such as the imaginal discs whereas EcR-B1 is associated with larval tissues that do not contribute to adult structures (Talbot et al., 1993). Within the central nervous system (CNS), EcR-A is predominantly localized to neurons that survive metamorphosis only to die shortly after the adult emerges (Robinow et al., 1993) whereas EcR-B1 localizes to neurons during outgrowth and regression (Truman et al., 1994). Expression of EcR-B2 has not yet been determined due to an antibody not being available.

The unique functions of the three EcR isoforms are not only due to their individual temporal and spatial expression patterns, but also due to their amino-terminal domains. The

amino terminal domains of both EcR-B1 and EcR-B2, when fused to the GAL4 DNA binding protein, are sufficient to activate gene transcription whereas the amino terminal domain of EcR-A represses gene transcription (Mouillet et al., 2001). It is therefore thought that EcR-B1 and EcR-B2 contain a ligand-indepent activation (AF1) in their amino termini (Mouillet et al., 2001; Hu et al., 2003) and that EcR-A contains an inhibitory function (IF) in its amino terminal (Mouillet et al., 2001).

Mutational Analyses of the EcR Isoforms

Mutational analyses of the *EcR* genes has supported the hypothesis that the three EcR isoforms are functionally different. *EcR-B1* mutants in which the B1 isoforms were truncated by stop codons near their amino termini, arrested at the onset of metamorphosis with problems in their developmental progression whereas EcR-A predominant tissues initiated their standard ecdysone response. Experiments studying the larval midgut and midgut imaginal islands, which predominantly express EcR-B1, found that neither of these cell types followed its typical developmental pathway. In contrast, the leg imaginal discs, which predominantly express EcR-A, initiated the normal process of disc elongation (Bender et al., 1997).

Mutations lacking EcR-B1 and B2 were made by deletion of the *EcR-B* transcription start site. The majority of these mutants died at the first to second instar larval molts with a few escaping into the third instar only to have problems with the metamorphosis of the CNS (Schubiger et al., 1998).

EcR-A mutants made by imprecise P element excision (Carney et al., 2004) and shown to lack EcR-A expression while retaining EcR-B1 expression died during pupal development (Davis et al., 2005). The mutants completed pupariation and progressed through head eversion,

eye development and leg and wing extension, but frequently started to degenerate in their pupal cases during this stage. *EcR-A* mutants were often defective in puparium formation, failing to shorten normally and failing to properly position the anterior spiracles. Failure to darken the cuticle was a normal occurrence as was failure to exhibit hardening of the cuticle. Investigations of internal tissues showed that *EcR-A* mutants had salivary glands persisting past the stage in which they should have been present. *EcR-A* mutants surviving to be adults often exhibited malformed legs with kinks in the femur and tarsal segments that were shortened and rounded (Davis et al., 2005).

Mutants lacking *EcR-A* also exhibited behavioral defects during pupariation. At the onset of pupariation, wild type larvae become motionless, shorten in length to form the puparium, attach to a solid surface and harden the cuticle. In contrast, *EcR-A* mutants sometimes continued to feed and seemed to physically resist the changes of pupariation until the larval cuticle hardened and formed the puparium. This caused the puparium to be misshapen. Many *EcR-A* mutants also failed to exit the food, suggesting that they either did not receive or did not respond to the signal that stimulates the wandering behavior normally preceding pupariation (Davis et al., 2005).

EcR and Neuronal Remodeling

The varied cellular responses to the three EcR isoforms are evident in the remodeling insect nervous system. Nervous system remodeling is an important part of growth and development not just in invertebrates though, but in vertebrates as well. During early vertebrate development, the retinal neurons from the eye project to the occipital lobe. Initially, dendrites from the left and right eye overlap, so that the brain is not able to distinguish between what the

left eye is seeing versus the right. As the eyes are used, the dendrites from the left and right retinal neurons are pruned so that eventually there are stacked columns within the cortex of the brain with dendrites from only the left eye in one column and dendrites from only the right eye in another column. This neuronal remodeling of the retinal neurons is what allows vertebrates to know what the left eye is viewing versus what the right eye is viewing (Bear et al., 2001).

Neuronal remodeling is also involved in the process of learning and memory in both vertebrates and invertebrates. New memories are achieved through the pruning back of both dendrites and axons and their subsequent re-growth (Lee et al., 2000; Bear et al., 2001).

Besides its implication in growth and development, neuronal remodeling has also been hypothesized to play a role in the development of certain diseases such as epilepsy, autism, and Fragile X mental retardation (Dong and Greenough, 2004). In epilepsy and autism, lack of neuronal pruning is thought to lead to too much synaptic input, thereby causing patients to have seizures and to seem disassociated with the world (Dong and Greenough, 2004), whereas the mental retardation associated with Fragile X is hypothesized to be caused by aberrant neuronal remodeling due to a mutation affecting actin reorganization (Schenck et al., 2003).

The study of remodeling neurons in insects was initiated in the hornworm *Manduca sexta.* The large size of *Manduca* allowed individual neurons to be identified, marked, and followed throughout the course of metamorphosis. The abdominal motorneuron 3 (MN-3) has been extensively studied. MN-3 innervates the body wall muscles and during metamorphosis, undergoes remodeling. During the final larval instar, MN-3 innervates the larval abdominal body wall and has full dendritic branching (Figure 1.5). Early in pupal development though, MN-3 undergoes severe dendritic pruning. Regrowth of adult specific processes takes place during pupal development and the adult MN-3 cells that innervate the newly created adult

abdominal muscles has extensive branching. These morphological changes are correlated with changes in the ecdysteroid titer. Dendritic pruning is initiated after the late larval ecdysone pulse and regrowth occurs during the broad pupal ecdysone pulse (Truman, 1996).

As in Manduca, many Drosophila neurons also undergo neuronal remodeling during the metamorphosis from the larval stage to the adult stage. During the larval-pupal transition, most larval neurons lose their specializations by responding to surges in ecdysone with synapse elimination and the loss of dendritic and axonal arbors through a process known as pruning. Surges of ecdysone during the pupal-adult transition then induce these cells to have dendritic and axonal re-growth followed by synaptogenesis (Truman et al., 1994).

High levels of EcR-B1 have been associated with the pruning of larval dendritic branches. As EcR-B1 expression decreases in the remodeling neurons, expression of EcR-A increases as the dendritic branches begin to re-grow (Truman et al., 1994). Experiments studying pruning in both the Tv neuron dendrites (Schubiger et al., 1998) and mushroom body axons (Lee et al., 2000) of *Drosophila* have shown that EcR-B mutants fail to adequately prune but can be rescued by expression of either EcR-B1 or EcR-B2 but not EcR-A (Schubiger et al., 1998; Lee et al., 2000; Schubiger et al., 2003). EcR-B1 mutants exhibit normal pruning indicating that either EcR-B1 and B2 are functionally redundant or that B2 is the primary isoform driving pruning (Schubiger et al., 1998).

Expression of an EcR dominant negative construct for any of the three EcR isoforms or an EcR inverted repeat construct results in incomplete pruning of the larval axonal arbors of the Tv neurons. Unlike pruning, outgrowth of the Tv neuron axons depends on the EcR construct being expressed. Cells expressing EcR-B1 that can bind ligand but not mediate activation or cells expressing EcR-B2 that cannot bind ecdysone have a normal pattern of adult-like branching

during outgrowth although branching is slightly reduced. Cells expressing EcR-B1 or EcR-A that cannot bind ecdysone form a poorly branched, larval-like axonal arbor (Brown et al., 2006). These studies emphasize the role of the EcR-A and B1 receptors in the regulation of dendritic re-growth in the Tv neurons.

The Role of EcR-A During Neuronal Remodeling of the Tv-neurons

Chapter 2 focuses on extending the analysis of EcR function during neuronal remodeling by using *EcR-A* mutants made by imprecise P element excision (Carney et al., 2004) and shown to lack EcR-A expression while retaining EcR-B1 expression (Davis et al., 2005). Through the use of confocal microscopy, I show that EcR-A is required during neuronal remodeling and that delayed development and early cell death are not responsible for the mutant phenotype. This study contributes to our evolving understanding of steroid hormone signaling and its effect on neuronal remodeling and is significant on many levels. My results emphasize the importance of neuronal remodeling by supporting the hypothesis that it is a highly regulated process controlled by the three isoforms of the ecdysone receptor and strengthen our knowledge of neuronal remodeling and its regulation by a steroid hormone.

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Figure 1.1. Ecdysone and the *Drosophila* Life Cycle

Surges in the ecdysteroid titer regulate developmental events throughout the *Drosophila* life cycle. A peak of ecdysone is found during embryogenesis and during the first two larval instars with the latter two triggering molting. Three subsequent peaks are found during the prepupal and pupal stages. The prepupal pulse triggers puparium formation, followed eight to twelve hours later by a smaller pulse triggering head eversion. A subsequent large peak of ecdysone triggers metamorphosis. Figure from (Thummel, 2001).

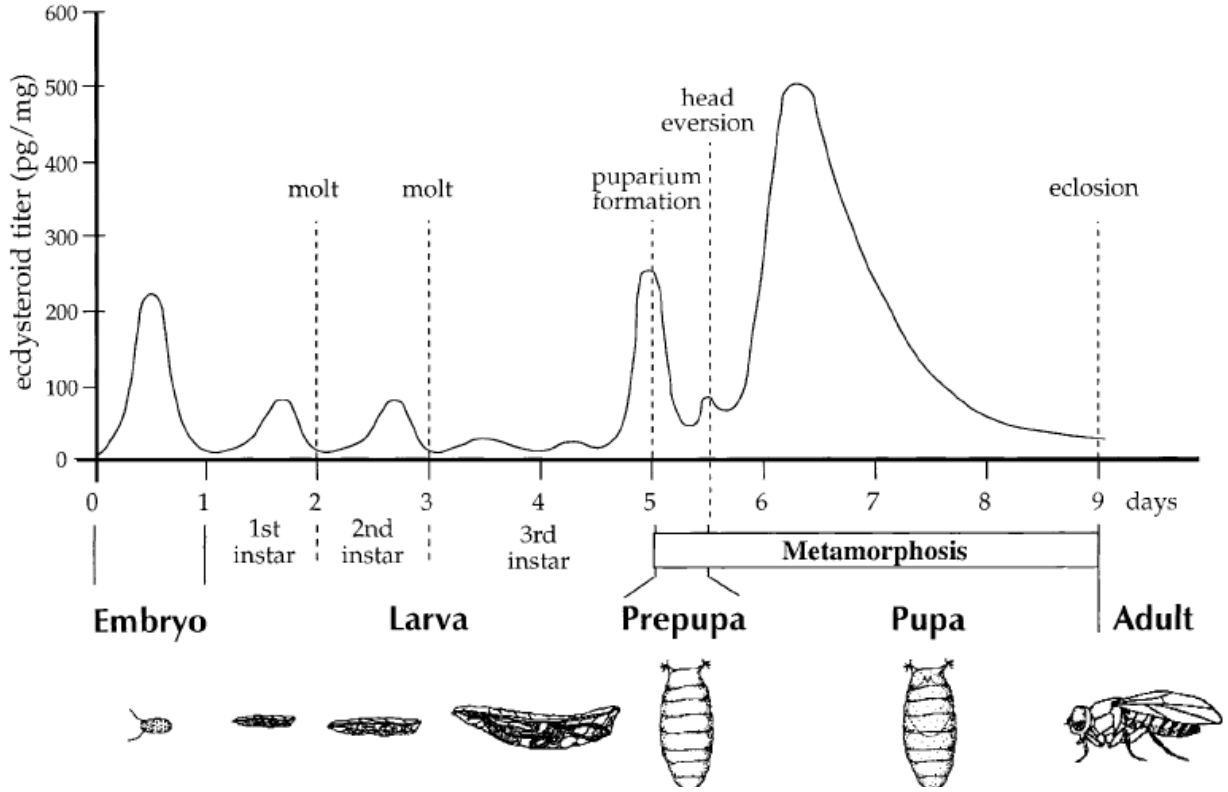

ecdysteroid titer (pg/mg)
600
500
400
300
200
100
0
molt
molt
puparium formation
head eversion
eclosion
0
1
2
3
4
5
6
7
8
9
days
1st instar
2nd instar
3rd instar
Metamorphosis
Embryo
Larva
Prepupa
Pupa
Adult

Figure 1.2. Nuclear Receptor Structure

Nuclear receptors are composed of a similar structure containing a highly conserved DNA-binding domain connected to a ligand-binding domain by a flexible hinge region. The DNA-binding domain contains two zinc fingers providing DNA-binding specificity and weak dimerization ability. The lingand-binding domain confers the principal dimerization ability. The activating function 1 (AF-1) domain can act in a ligand-independent fashion and is located at the N-terminus whereas the activating function 2 (AF-2) domain is located in the ligand-binding domain and is often ligand-dependent. Figure from (King-Jones and Thummel, 2005).

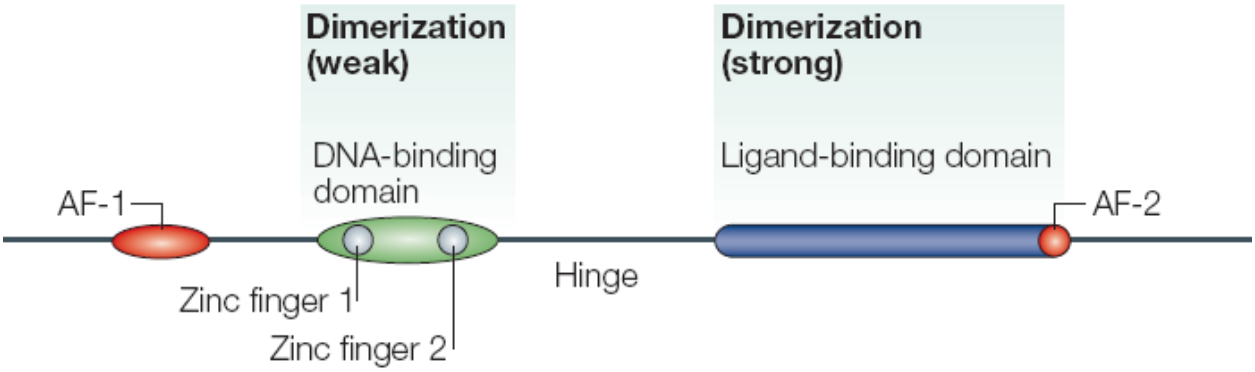

Dimerization
(weak)
DNA-binding
domain
Dimerization
(strong)
Ligand-binding domain
AF-1
AF-2
Zinc finger 1
Zinc finger 2
Hinge

Figure 1.3. Ecdysone Gene Activation

Ecdysone binds to its receptor forming the ecdysone/receptor complex and initiating the early puff gene transcription. The activated ecdysone/receptor complex also initiates early-late puff gene transcription as well as inhibiting late-late puff gene transcription. The protein products of the early puff genes repress their own transcription and activate the transcription of both the early-late puff genes and the late-late puff genes. Figure from (Thummel, 2002).

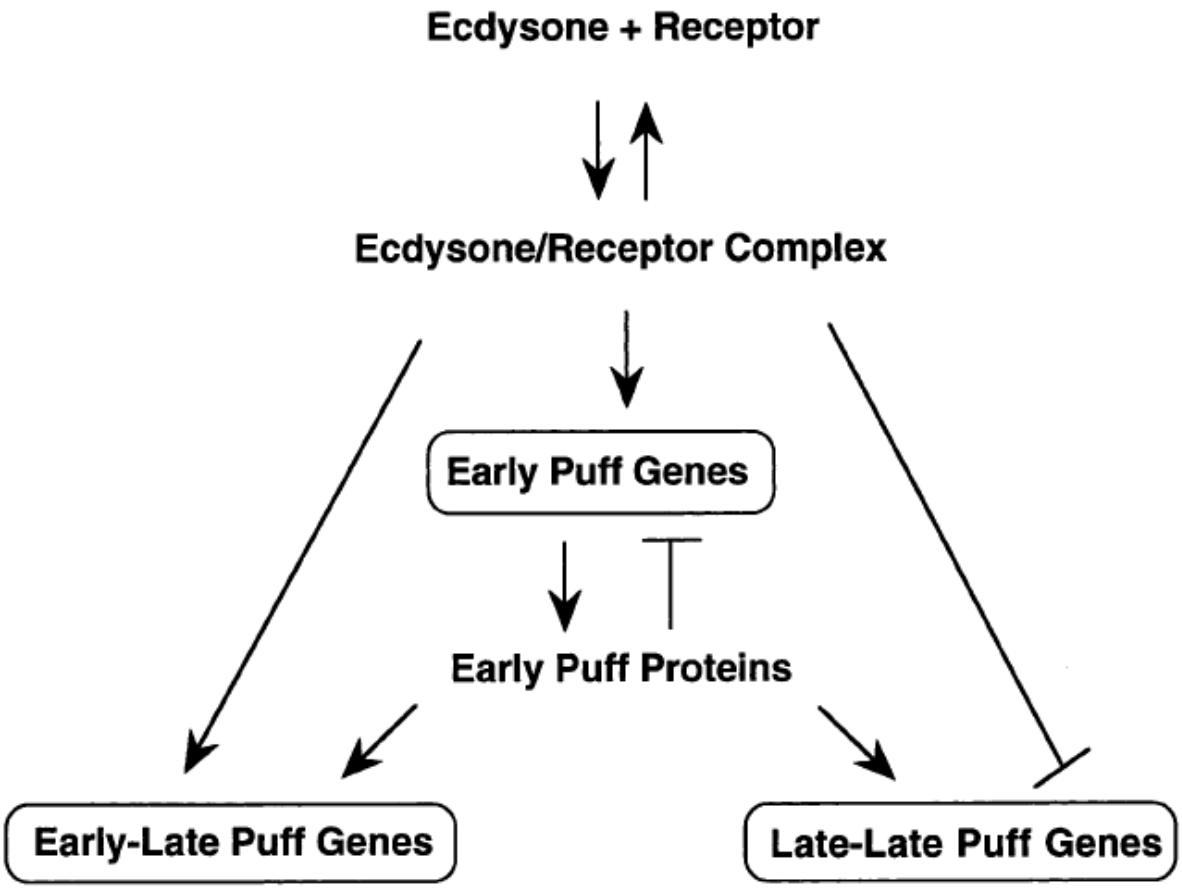

Ecdysone + Receptor
Ecdysone/Receptor Complex
Early Puff Genes
Early Puff Proteins
Early-Late Puff Genes
Late-Late Puff Genes

Figure 1.4. EcR Genomic Structure

The genomic structure of EcR contains two transcription start sites resulting in three protein isoforms due to alternative splicing of the transcript produced from the second transcription start site. The first transcription start site produces an mRNA that encodes the EcR-A protein while the second transcription start site produces mRNAs that encode the EcR-B1 and EcR-B2 proteins. All three isoforms have unique amino termini but share the same carboxy terminals. Modified from (Talbot et al., 1993).

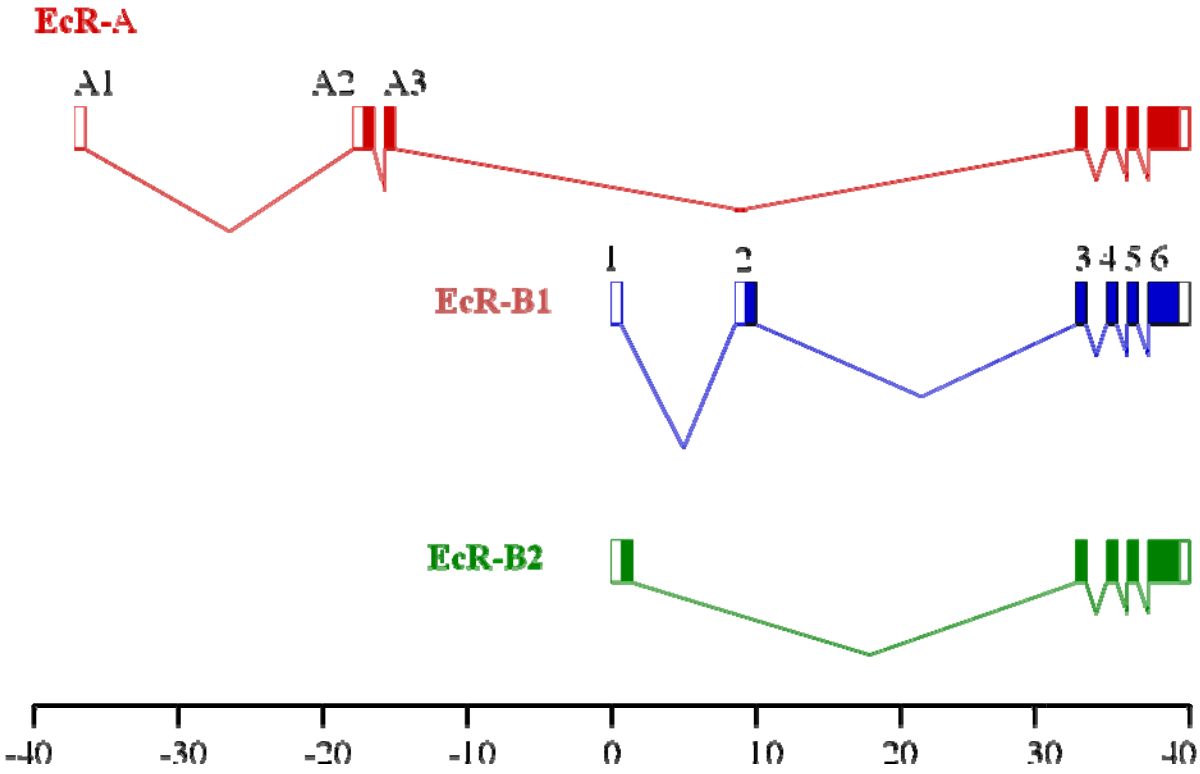
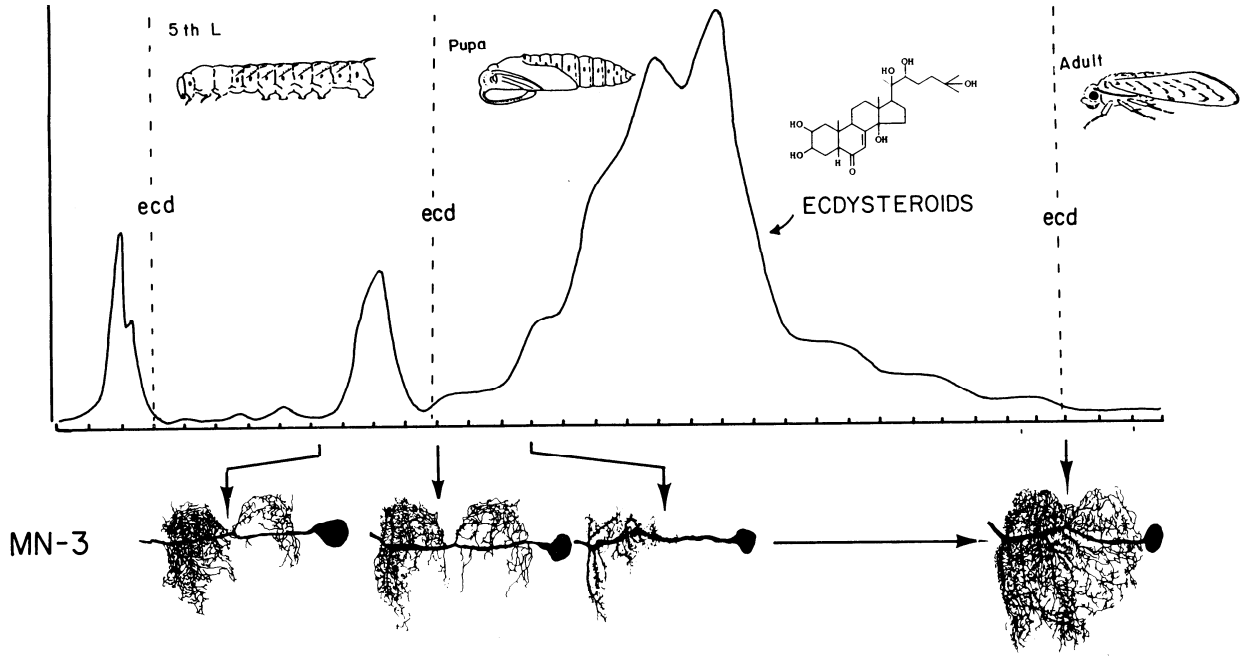

EcR-A
A1
A2
A3
EcR-B1
1
2
3 4 5 6
EcR-B2
-40
-30
-20
-10
0
10
20
30
40

Figure 1.5. Neuronal Remodeling in *Manduca sexta*

Surges in the ecdysteroid titer control development in the tobacco hornworm, *Manduca sexta*. A peak in the ecdysteroid titer triggers molting to the fifth larval stage whereas peaks in ecdysone also trigger the start of pupal formation and metamorphosis. Changes in the shape of the abdominal motorneuron 3 (MN-3) has also been associated with changes in the ecdysteroid titer. During the fifth larval stage, the dendrites of MN-3 are thickly branched. As the ecdysteroid titer peaks and the hornworm pupariates, MN-3 dendrites are pruned. During metamorphosis, another surge in the ecdysteroid titer is thought to trigger dendritic re-growth. Figure from (Truman, 1996).


5th L
Pupa
Adult
ecd
ecd
ecd
ECDYSTEROIDS
MN-3

CHAPTER 2

EcR-A IS REQUIRED FOR DENDRITIC RE-GROWTH OF THE TV-NEURONS DURING STEROID HORMONE INDUCED NEURONAL REMODELING IN *DROSOPHILA*[1]

[1] Small, K. R. and M. Bender. To be submitted to *Developmental Dynamics*.

ABSTRACT

The steroid hormone 20-hydroxyecdysone acts through a heterodimeric receptor composed of two nuclear receptors, EcR and USP, to control neuronal remodeling during metamorphosis in *Drosophila*. The *ecdysone receptor (EcR)* gene encodes three isoforms (EcR-A, EcR-B1, and EcR-B2) with stage and tissue specific expression during metamorphosis. Studies performed in the nervous system utilizing *EcR-B* mutants and dominant negative constructs for the three EcR isoforms suggest EcR-B is required for neuronal pruning while EcR-A may be required for neuronal re-growth. We have tested the hypothesis that EcR-*A* is required for dendritic re-growth but not for initial dendritic out-growth or pruning in the Tv neurons of *Drosophila* by examining neuronal growth in *EcR-A* mutants. Of the two *EcR-A* mutant alleles examined, both alleles had initial dendritic out-growth and underwent dendritic pruning with the $EcR\text{-}A^{112}$ allele exhibiting an overpruning phenotype suggesting that EcR-A is not required for the initiation of dendritic outgrowth or pruning. The $EcR\text{-}A^{112}$ allele exhibited no dendritic re-growth whereas the $EcR\text{-}A^{139}$ allele exhibited a variable phenotype of no re-growth observed to full re-growth, suggesting that functioning EcR-A is needed to regulate dendritic re-growth. Whole animal developmental comparisons, dissections of the central nervous system (CNS) and lethal analysis studies of the *EcR-A* mutants supported the hypotheses that the lack of dendritic re-growth seen in the *EcR-A* mutants is neither due to developmental delays nor early stage lethality. Our whole animal analysis of *EcR-A* mutants establishes that EcR-A is not required for initiation of dendritic pruning but is required for the regulation of dendritic re-growth of the Tv neurons in *Drosophila* and complements previous experiments performed using dominant negative constructs of *EcR-A.*

INTRODUCTION

The steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) regulates the *Drosophila* life cycle. Pulses of ecdysone punctuate each stage of the life cycle and trigger the transition into the subsequent stage. Ecdysone acts through the ecdysone receptor complex that is a heterodimer of two nuclear hormone receptors, the ecdysone receptor (EcR) and the RXR-ortholog ultraspiracle (USP) (Koelle et al., 1991; Yao et al., 1992; Thomas et al., 1993). The *EcR* gene encodes three different isoforms, EcR-A, EcR-B1 and EcR-B2, that share the same carboxy-terminal domain containing the DNA-binding and ligand-binding domains but have unique amino termini. The three isoforms are thought to have unique functions based on experiments defining their distinct temporal and spatial expression patterns (Robinow et al., 1993; Talbot et al., 1993; Truman et al., 1994; Kim et al., 1999; Sung and Robinow, 2000) and the distinct biochemical properties of their unique amino terminal domains (Dela Cruz et al., 2000; Mouillet et al., 2001; Hu et al., 2003). From these experiments it has been proposed that EcR-A is principally responsible for adult differentiation while EcR-B1 directs metamorphosis of larval tissues (Talbot et al., 1993; Truman et al., 1994).

Many larval neurons undergo neuronal remodeling and persist into the adult. Studies in both *Manduca sexta* and *Drosophila melanogaster* have shown that the neuronal remodeling process is characterized by the pruning back of larval dendritic processes and axonal arbors and subsequently the re-growth and formation of adult specific synapses (Truman et al., 1994; Levine and Weeks, 1996). The pruning and re-growth taking place has been shown to be under the control of ecdysone in *Drosophila melanogaster* (Robinow et al., 1993; Truman et al., 1994; Schubiger et al., 1998; Schubiger et al., 2003; Brown et al., 2006).

Studies of EcR expression in the CNS from larval hatching through adult eclosion have revealed a temporal and spatial pattern of EcR-A and EcR-B1 expression through metamorphosis. EcR-B1 is expressed predominantly in cells undergoing regressive responses while EcR-A is mainly expressed in cells showing maturation (Truman et al., 1994). Experiments studying neuronal pruning in *EcR-B* mutants have shown that early pruning back of larval-specific processes fail to take place in *EcR-B* mutants (Schubiger et al., 1998). Dendritic pruning can be cell autonomously rescued by expressing either EcR-B1 or EcR-B2. Expression of EcR-A results in limited pruning (Robinow et al., 1993; Schubiger et al., 2003).

Recently, Brown et al. (2006) found that the expression of an EcR dominant negative construct for any of the three EcR isoforms or an EcR inverted repeat construct resulted in incomplete pruning of the larval axonal arbors of the Tv neurons. Unlike pruning, outgrowth of the Tv neuron axons depended on the EcR construct being expressed. Cells expressing EcR-B1 that could bind ligand but not mediate activation or cells expressing EcR-B2 that could not bind ecdysone had a normal pattern of adult-like branching during outgrowth although branching was slightly reduced. Cells expressing EcR-B1 or EcR-A that could not bind ecdysone formed a poorly branched, larval-like axonal arbor (Brown et al., 2006). These studies emphasize the role of activation of the EcR-A and EcR-B1 receptor in the regulation of dendritic re-growth in the Tv neurons.

We have extended the analysis of EcR function during neuronal remodeling by using *EcR-A* mutants made by imprecise P element excision (Carney et al., 2004) and shown to lack EcR-A expression while retaining EcR-B1 expression (Davis et al., 2005). Confocal microscopy images show that *EcR-A* mutants dissected at 0 and 15 hours after puparium formation (APF) initiate dendritic outgrowth and pruning suggesting that EcR-A is not required for the initiation

of these two stages. *EcR-A* mutants dissected at 30 hours APF fail to show complete dendritic re-growth suggesting that EcR-A is required for the regulation of dendritic re-growth. Development comparisons and lethal analysis studies indicate that delayed development and early cell death are not responsible for the lack of dendritic re-growth. Our results establish that EcR-A is not required for the initiation of dendritic outgrowth or pruning but is required for the regulation dendritic re-growth of the Tv neurons in *Drosophila*.

MATERIALS AND METHODS

Fly strains

EcR-A mutants were obtained by crossing *yw UAS-CD8-GFP; FG5-GAL4 EcR M554fs/CyO, y+* virgin females to *yw; EcR-A^{139} or EcR-A^{112} /CyO, y+* males. *EcR-A* mutant progeny (*yw UAS-CD8-GFP; FG5-GAL4 EcR M554fs/EcR-A^{139}* or *EcR-A^{112}*) were identified by their yellow phenotype. The GAL4-UAS system was used to drive expression of GFP in the Tv neurons through the use of the FG5-GAL4 driver (Suster et al., 2003) and UAS-CD8-GFP reporter (Brand and Perrimon, 1993). *EcR-A^{139}*and *EcR-A^{112}* each lack detectable expression of the EcR-A protein (Davis et al., 2005). The *EcR* M554fs mutation is a predicted null for EcR-A, EcR-B1, and EcR-B2 functions (Bender et al., 1997). The control genotype was a *Canton S* wild-type strain.

Dissections and Microscopy

EcR-A mutants and controls were selected as white prepupa (Bainbridge and Bownes, 1981) and dissected immediately or held on grape juice agar plates at 25° C and then dissected at 15 or 30 hours APF. After dissection in 1 X PBS the CNS were washed 2 times in 1 X PBS and

then mounted on a slide in 1:1 glycerol/TBS. Confocal microscopy with a step size of 1.4μm was used to view the Tv neurons.

Scoring

For blind scoring of Tv neuron pruning, three rows (corresponding to control, *EcR-A*^{112}, and *EcR-A*^{139} genotypes) of ten printed images per genotype of the Tv neurons at 0 hour were presented to ten observers (Genetics graduate students). Each observer then ranked the rows relative to one another for extent of dendritic branching. A similar protocol was used for blind scoring of 15 hour and 30 hour images except that observers ranked extent of pruning or extent of regrowth for these time points.

Developmental Progression and Lethal Phase Analysis

EcR-A mutant progeny (*yw UAS-CD8-GFP; FG5-GAL4 EcR M554fs/EcR-*^{A139}*)* were obtained as described previously. Control genotypes were a *Canton S* wild-type strain and a third fly strain containing the *EcR-A*^{139} mutation but not the GAL4-UAS system. This third fly strain was of the genotype *yw; EcR M554fs/EcR-A*^{139} and was obtained by crossing *yw; EcR M554fs/CyO, y+* virgin females to *yw; EcR-A*^{139}*/CyO, y+* males. Timing and stages of *Drosophila* development were as described previously (Bainbridge and Bownes, 1981). A four hour egg collection was done three days after the flies were mated. 36 hours after egg lay (AEL) mutants were identified by their *yellow* phenotype. At 96 hours AEL, stage L3, mid third instar larvae were scored for survival. At 116 hours AEL, stage P1, animals were checked for white prepupa formation. At 24hrs APF, stage P5, animals were checked for head eversion. At 48 hours APF, stage P6, animals were examined for green Malpighian tubules. At 72 hours APF, stage P11, animals were examined for eye color and for bristle formation. At 84hrs APF, stage

P13, animals were checked for black wings. At 108hrs APF, stage P15, animals were examined for eclosion.

To dissect pupae out of the pupal case, pupae were placed in 4% paraformaldehyde. The pupal case was gently ripped open and the pupa removed. A paintbrush was used to place the pupa onto a slide containing 1:1 glycerol/TBS. CNS were dissected as described previously. Photographs of pupae and dissected CNS were taken using a Wild Heerbrugg dissecting scope with a Hamamatsu color chilled 3 CCD camera. Percent survival equals the number of animals alive at a given developmental stage divided by the total number of mutants collected at hatching.

RESULTS

EcR-A is not required for initial dendritic growth nor for the initiation of dendritic pruning of the Tv neurons.

Dendritic pruning and regrowth of the Tv neurons can be followed by expression of a CD8-GFP fusion protein in these cells using the FG5-GAL4 driver (Schubiger et al., 1998). To ask whether *EcR-A* is required for neuronal remodeling of the Tv cells, *EcR-A* mutant heterozygotes carying these constructs (*yw UAS-CD8-GFP; EcR-A*^{139} *or EcR*-A^{112}*/FG5-GAL4 EcR null*) were compared to sibling control strains (*yw UAS-CD8-GFP; FG5-GAL4 EcR null/CyO, y+* or *yw UAS-CD8-GFP; EcR-A*^{139} *or EcR*-A^{112}*/CyO, y+).* Mutant and control progeny were selected at the white prepupa stage of the *Drosophila* life cycle (Bainbridge and Bownes, 1981), which we will refer to here as 0 hour. At this stage, dendrites of wild type pupae are densely branched and still have their larval connections (Truman, 1990; Truman et al., 1994). The dendrites of both the *EcR-A*^{139} and *EcR*-A^{112} 0 hour pupae were branched (Figure 2.1), but

not as densely as control dendrites (Figure 2.2). The results from this experiment suggests that EcR-A expression is not required for the initial dendritic branching of the Tv neurons.

To study the effect of EcR-A on dendritic pruning, *EcR-A* mutant heterozygotes were collected at the white prepupa stage and aged for 15 hours on grape juice agar plates. In wild type pupae larval dendrites are pruned back so that the new dendritic connections that are needed for the adult stage of life can form (Truman, 1990; Truman et al., 1994). A panel of 10 graduate students blindly scored 10 images of the Tv neurons for each genotype and found that at 15 hours the dendrites of the $EcR\text{-}A^{139}$ pupae were pruned back relatively similar to the control (Figure 2.1 and 2.2). Out of the 10 images scored, 3 $EcR\text{-}A^{139}$ pupae looked more pruned than the control, 4 pupae looked the same as the control, and 3 $EcR\text{-}A^{139}$ pupae looked slightly less pruned than the control (Figure 2.2). The dendrites of the and $EcR\text{-}A^{112}$ allele looked slightly more pruned than the control. Out of the 10 images scored, 8 and $EcR\text{-}A^{112}$ pupae looked more pruned than the control and 2 pupae looked the same as the control. The results from this experiment suggest that EcR-A expression is not required for the initiation of dendritic pruning of the Tv neurons.

***EcR-A* is required for dendritic re-growth of the Tv neurons.**

To study the effect of EcR-A on dendritic re-growth, *EcR-A* mutant heterozygotes were collected at the white prepupa stage and aged for 30 hours on grape juice agar plates. At this time, the new outgrowth of the dendrites needed to establish connections necessary for the activities of an adult fly can be seen in wild type pupae (Truman, 1990; Truman et al., 1994). A panel of 10 graduate students blindly scored 10 images of the Tv neurons for each genotype. The $EcR\text{-}A^{112}$ mutants did not have any dendritic re-growth (Figure 2.1 and 2.2). The $EcR\text{-}A^{139}$ mutants had a variable phenotype. Overall, the dendrites of the $EcR\text{-}A^{139}$ mutants were not as re-

grown as the control pupae (Figure 2.1 and 2.2) but had more re-growth than the *EcR*-A^{112} mutants. Due to this variable phenotype 14 *EcR-A*^{139} mutants were dissected at one time and then classified as having dendrites with the same amount of re-growth as the control, less re-growth than the control, or more re-growth than the control. Out of the 14 *EcR-A*^{139} mutants analyzed, 4 pupae had full dendritic re-growth comparable to that of the control, 3 showed a little re-growth that was not as densely branched as control dendrites, and 7 showed no dendritic re-growth at all (Figure 2.3). The results of this experiment suggest that *EcR-A* expression is required for dendritic re-growth necessary to establish the dendritic connections needed for adult life.

EcR-A^{139} animals are not developmentally delayed in growth and have a high frequency of survival until 50hrs APF.

Development comparison experiments were performed for two reasons. First, we wanted to ensure that the lack of dendritic re-growth seen in mutant pupae was not due to the *EcR-A* mutants being developmentally delayed. Secondly, we wanted to further characterize the developmental progression of *EcR-A* mutants (Davis et al., 2005) and ensure that the FG5-GAL4 driver and UAS-CD8-GFP construct did not alter the development of *EcR-A* mutants. At 36 hours AEL *EcR-A*^{139} heterozygous mutants were selected by lack of the *yellow* marker. As previously described by Davis et al., 2005, at 110 hours AEL the mutants were compared to controls. As seen in Figure 2.4, the mutant pupae are in the white prepupa stage as are the *CS* control pupae at 110 hours AEL. However, *EcR-A*^{139} mutant pupae are longer in length than controls. 14 hours APF the pupae were checked for head eversion (Figure 2.4). The mutants and control pupae both had undergone head eversion with the only differences being the mutant pupal cases had not tanned as much as the control pupal cases and the operculum of the mutants

had not properly formed. At 57 hours APF the Malpighian tubules were green in both the mutant and control pupae (Figure 2.4). At 78 hours APF the mutant and control pupae both had color pigmentation in the wings (Figure 2.4). The mutant pupae had brown colored wings due to having the *yellow* marker.

Percent survival of the *EcR-A*^{139} mutants was scored during the development experiment (Figure 2.5). *EcR-A*^{139} mutants were found to have a high frequency of survival until 50 hours APF. The survival of the *EcR-A*^{139} animals expressing the GAL4-UAS system did not drastically differ from the survival of *EcR-A*^{139} animals not expressing the GAL4-UAS system. Our results suggest that the lack of dendritic re-growth seen in the *EcR-A*^{139} mutants is neither due to the mutants being developmentally delayed nor to early stage lethality.

The CNS of *EcR-A*^{139} animals develops normally.

To further ensure that *EcR-A*^{139} mutants are not developmentally delayed and to study in more detail their developmental progression, we took a closer look at the central nervous system (CNS) of the *EcR-A*^{139} mutants. The development of the *Drosophila* CNS has been extensively described by Truman (Truman, 1990; Truman et al., 1994). The CNS of wild type animals initially is compact and as time progresses the optic lobes separate, the ventral nerve cord elongates, and eye discs develop on the ends of the optic lobes. 0 hours APF both the mutant and control CNSs were compact (Figure 2.6). The optic lobes had not separated and the ventral nerve cord had not lengthened. 15 hours APF the ventral nerve cord in both the mutant and control pupae had started to elongate and the optic lobes had started to separate (Figure 2.6). 30 hours APF the mutant and control pupae optic lobes had started to develop eye discs (Figure 2.6). By 48 hours APF the eye discs had completely developed on both the mutant and control optic lobes (Figure 2.6). The results from this experiment suggest that the lack of dendritic re-

growth seen in the *EcR-A*^{139} mutants is not due to the CNS of the mutants being developmentally delayed.

DISCUSSION

Through the use of *EcR-A* mutants, we have found that EcR-A is not required for the initiation of dendritic out-growth and pruning but is required for the regulation of dendritic re-growth of the Tv-neurons in *D. melanogaster*. *EcR-A* mutants dissected at 0 and 15 hours APF initiated dendritic outgrowth and pruning suggesting that EcR-A is not required for these stages. *EcR-A* mutants dissected at 30 hours APF failed to show complete dendritic re-growth suggesting that EcR-A is required for the regulation of dendritic re-growth.

Understanding the mechanisms through which neurons reorganize their existing processes and subsequently make new projections is important for understanding how neuronal networks are regulated. In insects, the steroid hormone ecdysone initiates and coordinates diverse tissue-specific developmental programs at different developmental stages (Thummel, 1996). Previous studies on *Manduca* and *Drosophila* have shown the involvement of ecdysone in neuronal remodeling (Schubiger et al., 1998; Schubiger et al., 2003). Our finding that EcR-A is required for dendritic re-growth of the Tv neurons provides further genetic evidence to support the importance of ecdysone in orchestrating neuronal remodeling.

There are three different isoforms of the ecdysone receptor, EcR-A, EcR-B1 and EcR-B2. The three isoforms share common DNA binding and hormone binding domains but differ in their N-terminal domains. These isoforms are generated through alternative use of promoters (EcR-A versus EcR-B) and alternative splicing (EcR-B1 versus EcR-B2) and exhibit distinct patterns of tissue-specific expression (Robinow et al., 1993; Talbot et al., 1993; Kim et al., 1999; Sung and

Robinow, 2000). EcR-B1 is expressed in neurons that are undergoing regressive responses while EcR-A is expressed in differentiating neurons (Robinow et al., 1993; Truman et al., 1994). We found that EcR-A is required in the Tv neurons during the differentiating stage of dendritic re-growth but was not required during the regressive response stage of dendritic pruning.

Our results correlate with previous experiments which utilized *EcR-B* mutants. *EcR-B* mutants failed to undergo dendritic pruning of the Tv neurons (Schubiger et al., 1998). Dendritic pruning could be cell autonomously rescued by expression of either EcR-B1 or EcR-B2. Expression of EcR-A only resulted in limited pruning (Schubiger et al., 2003). The fact that EcR-A could not rescue dendritic pruning but was found by us to be required for dendritic re-growth further supports the idea that each of the three EcR isoforms has a unique function with some isoform redundancy occurring (Robinow et al., 1993; Talbot et al., 1993; Truman et al., 1994; Kim et al., 1999; Sung and Robinow, 2000).

Our results also correlate and support a previous study employing EcR dominant negative constructs and an EcR inverted repeat construct. The expression of an EcR dominant negative construct for any of the three EcR isoforms or an EcR inverted repeat construct resulted in incomplete pruning of the larval axonal arbors of the Tv-neurons. Unlike pruning, outgrowth of the Tv neuron axons depended on the EcR construct being expressed. Cells expressing EcR-B1 that could bind ligand but not mediate activation or cells expressing EcR-B2 that could not bind ecdysone had a normal, but a bit reduced, adult-like branching during outgrowth. Cells expressing EcR-B1 or EcR-A that could not bind ecdysone formed a poorly branched, larval-like axonal arbor (Brown et al., 2006).

We believe the lack of re-growth that is seen in the *EcR-A* mutants is not due to the animals being developmentally delayed based on several of our findings. First, while $EcR\text{-}A^{139}$

mutants did have some developmental defects, overall the development of the *EcR-A*^{139} mutants was not delayed. Second, dissections of the CNS of the mutant pupae found that the CNS follows the same developmental progression as wild type CNS development. Third, lethal analysis studies showed that the *EcR-A*^{139} mutants have a high frequency of survival until 50 hours APF. All these findings support our conclusion that *EcR-A* mutant animals lack dendritic re-growth due to a loss of the *EcR-A* gene function and not due to a developmental delay.

We are not sure exactly why the two *EcR-A* alleles cause somewhat different phenotypes. The *EcR*-A^{112} allele exhibits an over-pruning phenotype whereas the *EcR*-A^{139} allele does not, and as mentioned above, the *EcR*-A^{139} allele has a variable re-growth phenotype that is not seen in the *EcR*-A^{112} allele. We believe the different re-growth phenotypes might be explained by the deletion of an *EcR-B* repressor regulatory element in the *EcR*-A^{139} allele. If an *EcR-B* repressor is located around *EcR-A* exons 2 and 3, then the deletion of this chromosomal region in the *EcR*-A^{139} allele, but not the *EcR*-A^{112} allele, could explain the variable re-growth phenotype seen in the *EcR*-A^{139} allele. In essence, the lack of the *EcR-B* repressor could be causing leaky *EcR-B* expression. The variable phenotype could therefore be due to *EcR-B* expression substituting in for the lack of *EcR-A* expression.

The scoring of the Tv neurons was another way in which we ensured the validity of our results. Ten graduate students from labs not involved in the study of neuronal remodeling were employed to blindly score the pictures of the Tv neurons to guarantee there was no prejudice in our results.

Neuronal remodeling is seen not only in invertebrates but in vertebrates as well. In mammals, most layer 5 pyramidal neurons projecting to subcortical areas undergo neuronal remodeling (Stanfield et al., 1982). Similar to ecdysone, vertebrate steroid hormones can

significantly influence the development and function of diverse tissues including the nervous system. It has been previously reported that gonadal steroids have effects on neuronal survival and dendritic arborization in certain regions of the brain and spinal cord (Arnold and Gorski, 1984). In rats, thyroid hormone has been linked with the regulation of spinal cord pruning in certain cortical neurons (Li et al., 1995). The study of steroid hormones and neuronal remodeling is therefore not just important to understanding invertebrate development, but to the development of both vertebrates and invertebrates.

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Figure Legends

Figure 2.1. *EcR-A* mutants exhibit dendritic pruning but no dendritic re-growth.

A) Schematic drawing of the central nervous system (CNS). Green box outlines the area shown in panels B-J. (Brain = Br, Optic lobes = OL, Thoracic Segments 1, 2, 3 = T1-T3, Abdominal neurons = A). (B-J) Tv-neurons at times 0hr, 15 or 30hrs APF. (C = control, 139 = $EcR\text{-}A^{139}$, 112 = $EcR\text{-}A^{112}$). (B, E, and H) Control Tv-neurons at times 0hr, 15hrs and 30hrs APF, respectively. Panels represent initial dendritic state (B), dendritic pruning (E) and dendritic re-growth (H) of the Tv-neurons. (C, F, and I) and (D, G, and J) $EcR\text{-}A^{139}$and $EcR\text{-}A^{112}$ Tv-neurons at times 0hr, 15hrs and 30hrs APF, respectively.

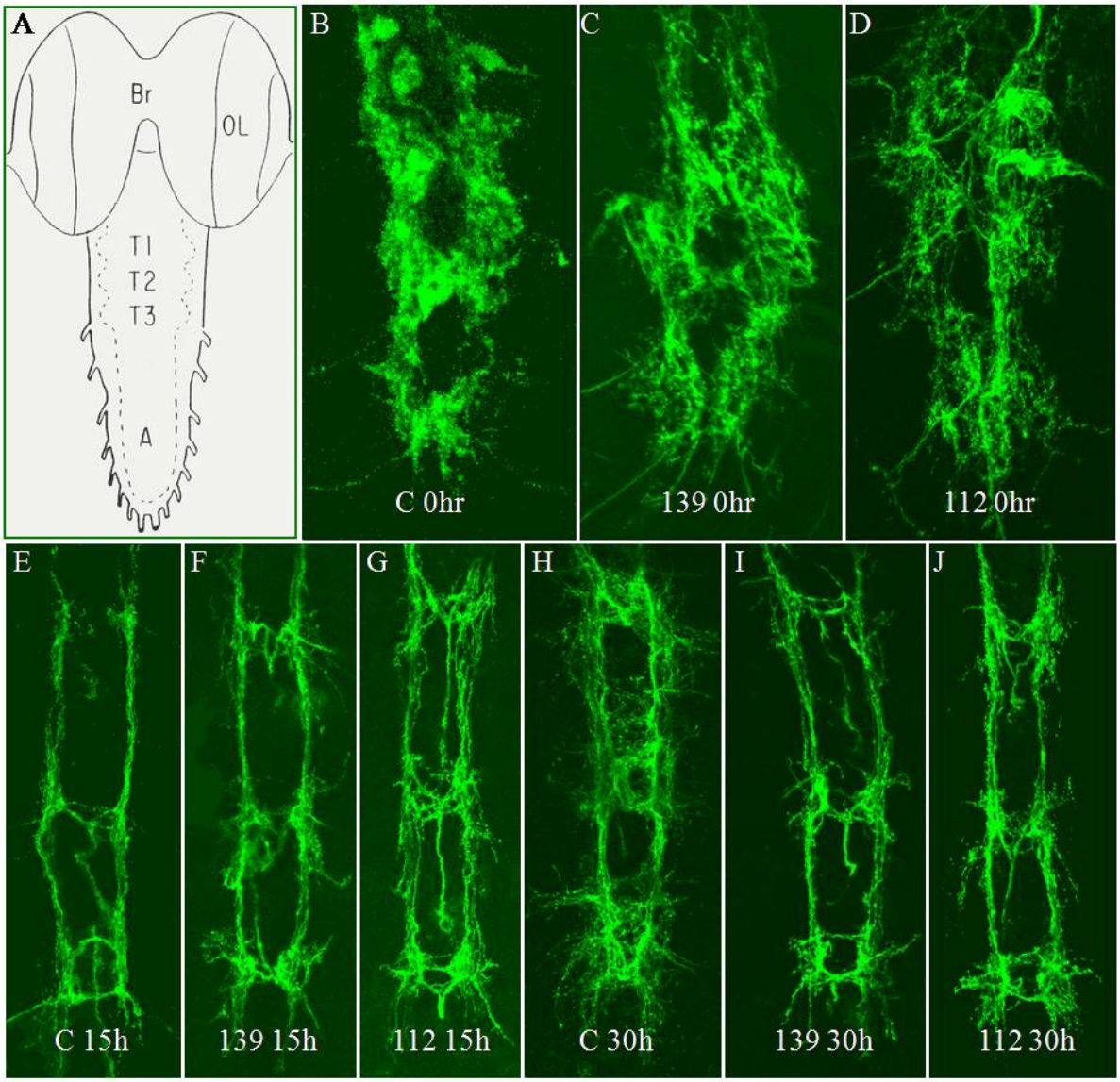
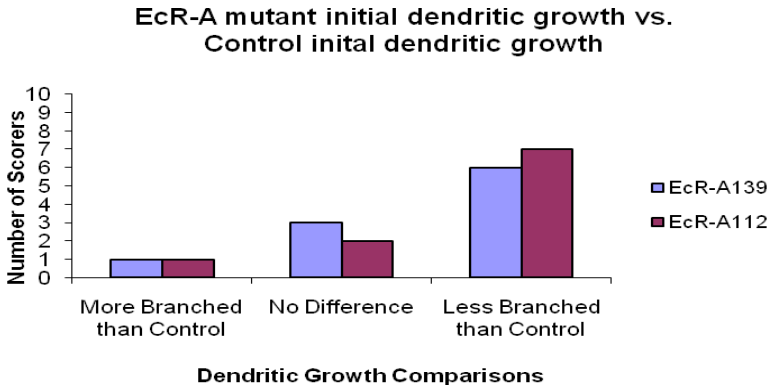

A
Br
OL
T1
T2
T3
A
B
C 0hr
C
139 0hr
D
112 0hr
E
C 15h
F
139 15h
G
112 15h
H
C 30h
I
139 30h
J
112 30h

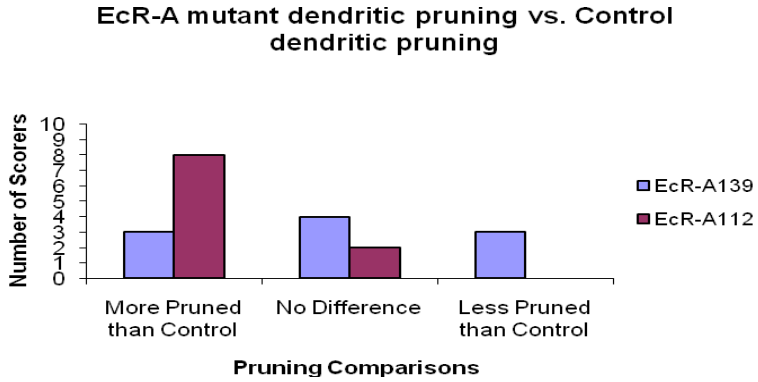
Figure 2.2. *EcR-A* mutant scoring.

EcR-A mutant animals were scored blindly by ten observers. (A) Six out of ten students indicated that the $EcR\text{-}A^{139}$ mutants were less branched initially than control animals while seven out of ten believed $EcR\text{-}A^{112}$ mutants were less branched initially than control animals. (B) Three out of ten students believed the $EcR\text{-}A^{139}$ mutants were more pruned than control animals while eight out of ten believed $EcR\text{-}A^{112}$ mutants were more pruned than control animals. (C) Eight out of ten students believed the $EcR\text{-}A^{139}$ mutants were less re-grown than control animals while ten out of ten believed the $EcR\text{-}A^{112}$ mutants were less re-grown than control animals.

A.


EcR-A mutant initial dendritic growth vs. Control inital dendritic growth
Number of Scorers
0
1
2
3
4
5
6
7
8
9
10
More Branched than Control
No Difference
Less Branched than Control
EcR-A139
EcR-A112
Dendritic Growth Comparisons

B.


EcR-A mutant dendritic pruning vs. Control dendritic pruning
Number of Scorers
0
1
2
3
4
5
6
7
8
9
10
More Pruned than Control
No Difference
Less Pruned than Control
EcR-A139
EcR-A112
Pruning Comparisons

C

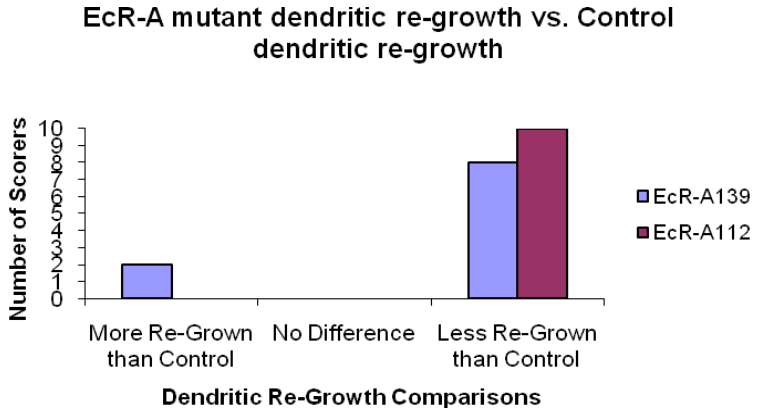

EcR-A mutant dendritic re-growth vs. Control dendritic re-growth
Number of Scorers
0
1
2
3
4
5
6
7
8
9
10
More Re-Grown than Control
No Difference
Less Re-Grown than Control
EcR-A139
EcR-A112
Dendritic Re-Growth Comparisons

Figure 2.3. *EcR-A*^{139} mutants have a variable 30hr phenotype.

(A, B, and C) controls, (D, E, and F) *EcR-A*^{139} mutants from the most branched found to the least branched found at the 30hr time point. Fourteen images were obtained and analyzed for the *EcR-A*^{139} mutants. Four *EcR-A*^{139} mutants had full re-growth (D) as compared to (A). Three *EcR-A*^{139} mutants had a little re-growth (E) as compared to (B). Seven *EcR-A*^{139} mutants had no re-growth at all (F) as compared to (C).

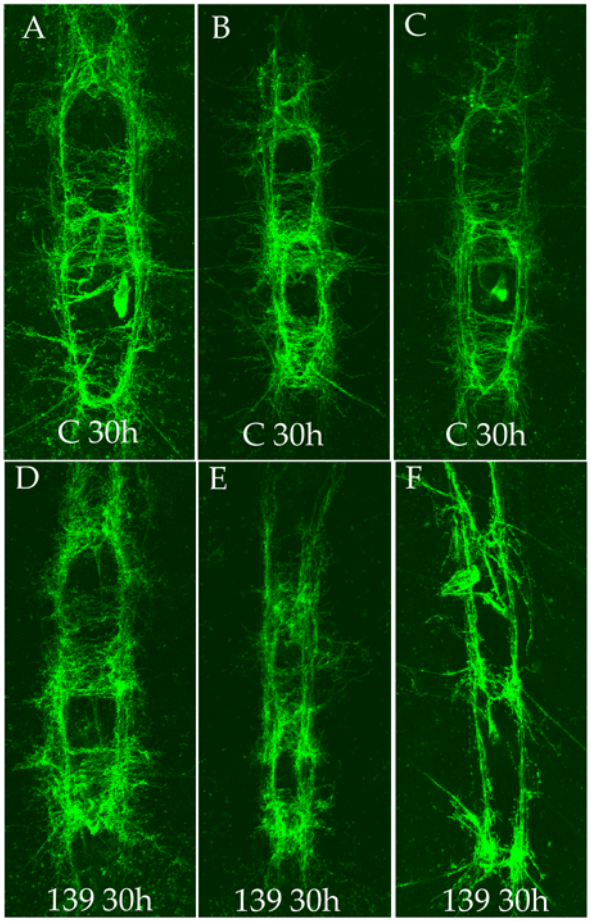

A
B
C
C 30h
C 30h
C 30h
D
E
F
139 30h
139 30h
139 30h

Figure 2.4. Surviving *EcR-A*^{139} mutants are not developmentally delayed.

(A, C, E, G) *CS* controls, (B, D, F, H) *EcR-A*^{139} mutants at time points 0hr, 14hrs, 57hrs and 78hrs APF, respectively. (B) *EcR-A*^{139} mutants failed to evert spiracles properly (indicated by arrow) and failed to shorten as compared to (A). (D) *EcR-A* mutants underwent head eversion, but did not completely tan or form operculum properly (operculum indicated by arrow), as compared to (C). (E and F) control and *EcR-A*^{139} mutants had green/white malpigian tubules. (G and H) control had gray wings due to their wild type phenotype while *EcR-A*^{139} mutants had developed brown wings due to their *yellow* phenotype.

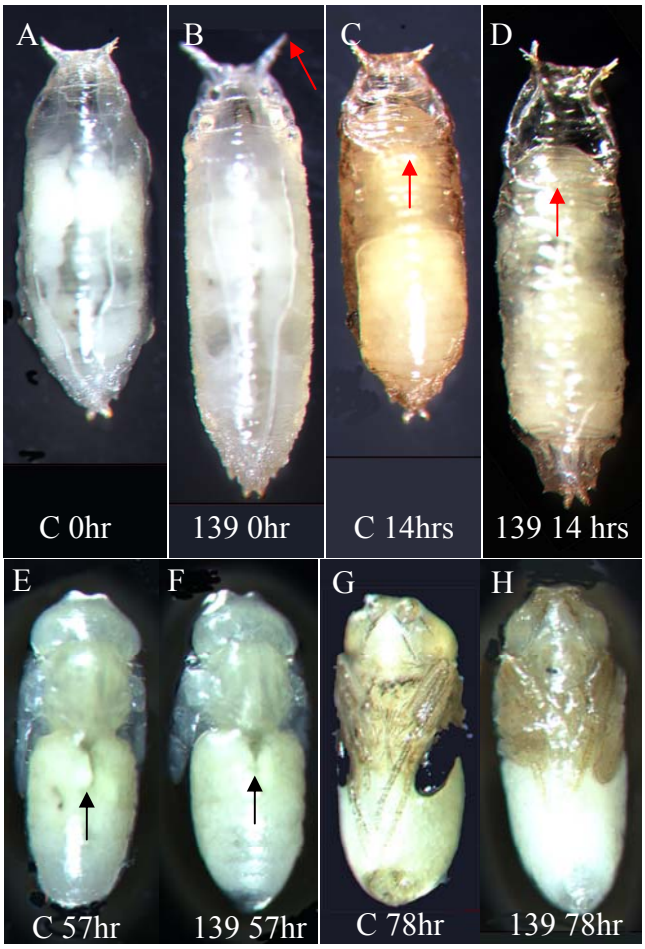

A
B
C
D
C 0hr
139 0hr
C 14hrs
139 14 hrs
E
F
G
H
C 57hr
139 57hr
C 78hr
139 78hr

Figure 2.5. *EcR-A*^{139} mutants start to decrease in numbers of survival between 57 and 78hrs APF. Staging during prepual and pupal development (P1 through P15) is according to Bainbridge and Bownes (1981). L3 = late third instar stage.

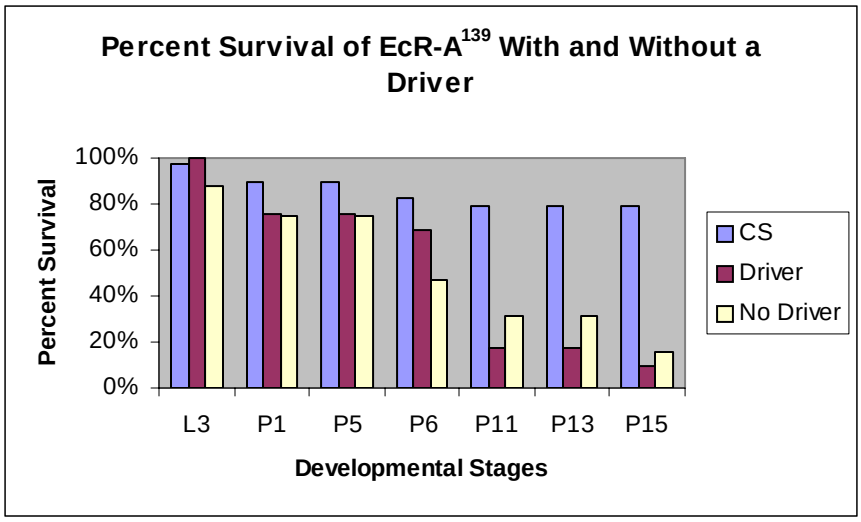
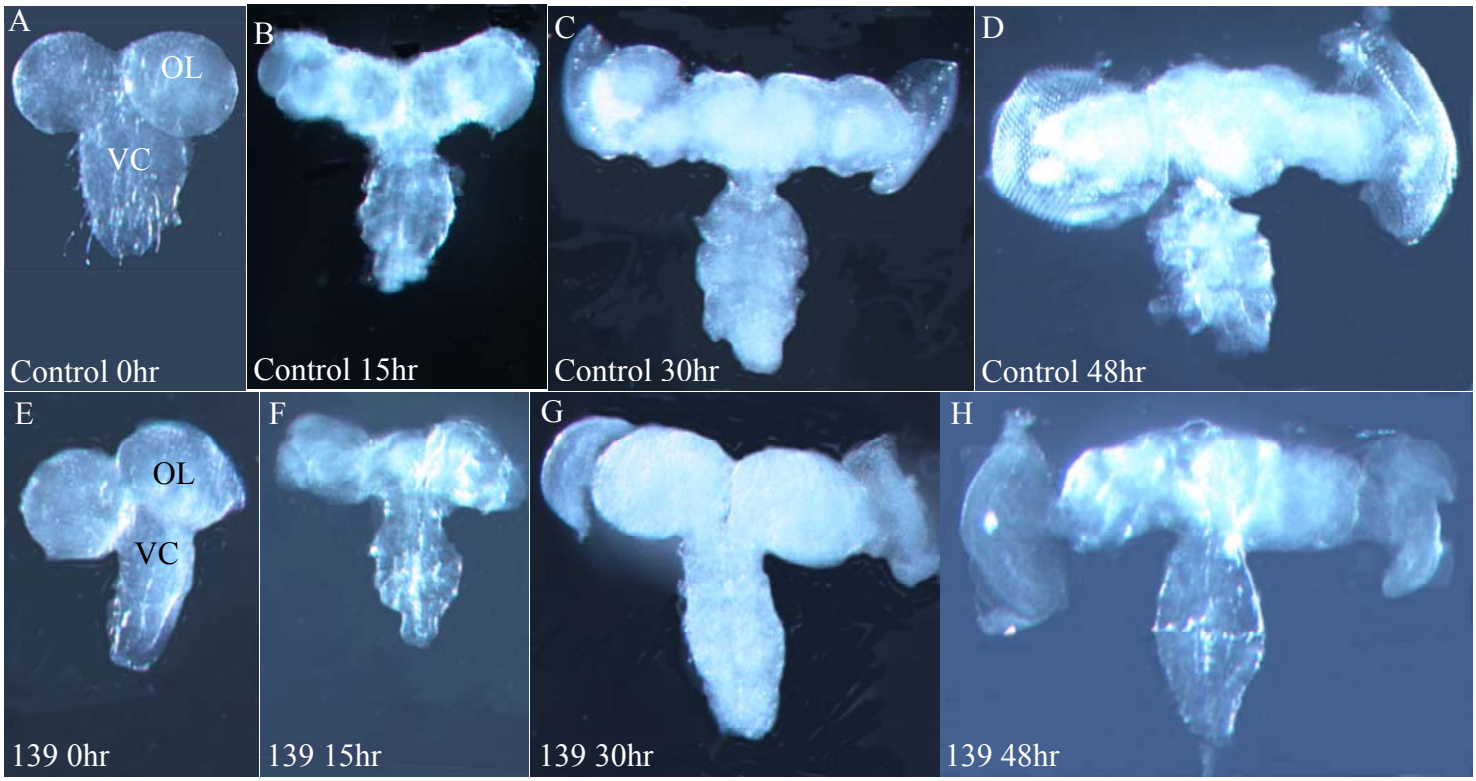

Percent Survival of EcR-A139 With and Without a Driver
100%
80%
60%
40%
20%
0%
Percent Survival
L3
P1
P5
P6
P11
P13
P15
Developmental Stages
CS
Driver
No Driver

Figure 2.6. *EcR-A*^{139} mutant CNS development is not delayed or altered.

(A-D) *CS* control, (E-H*) EcR-A*^{139} mutant. (A and E) optic lobes (OL) are close together and ventral chord (VC) is not elongated in 0hr mutant and control. (B and D) OLs start to separate and VCs start to elongate. (C and G) eye discs start to appear. (D and H) eye discs fully formed.


A
OL
VC
Control 0hr
B
Control 15hr
C
Control 30hr
D
Control 48hr
E
OL
VC
139 0hr
F
139 15hr
G
139 30hr
H
139 48hr

CHAPTER 3

CONCLUSION

In this thesis, I have examined a type of signaling involving steroid hormones. In Chapter 2, I investigated the role of the steroid hormone ecdysone and its regulation of neuronal remodeling. I have shown the importance of an isoform of the ecdysone receptor (EcR), EcR-A, during the process of neuronal re-growth of the Tv-neurons but the lack of a role for this receptor during initial dendritic outgrowth and the subsequent pruning of the dendrites. These results support the hypothesis that each of the three EcR isoforms have independent functions.

From organisms as diverse as invertebrates to vertebrates, the need to understand the mechanisms through which neurons prune back existing processes to accommodate new projections is important for understanding how neuronal networks are regulated. In insects, the steroid hormone ecdysone is responsible for regulating many developmental processes occurring in a wide variety of tissues at multiple times (Thummel, 1996). One ecdysone-regulated process is neuronal remodeling (Schubiger et al., 1998; Schubiger et al., 2003) and our finding that EcR-A is required for the dendritic re-growth of the Tv neurons provides further genetic evidence to support the importance of ecdysone in orchestrating this process.

The ecdysone receptor has three protein isoforms, EcR-A, EcR-B1 and EcR-B2, which are generated through alternative use of the promoters EcR-A and EcR-B and through the alternative splicing of EcR-B1 and EcR-B2. Each of the isoforms share common DNA binding and hormone binding domains but differ in their N-terminal domains (Talbot et al., 1993). Not surprisingly, the three isoforms have distinct expression patterns (Robinow et al., 1993; Talbot et al., 1993; Kim et al., 1999; Sung and Robinow, 2000) with EcR-A being predominantly

expressed in tissues undergoing metamorphosis to develop into structures needed in the adult and EcR-B1 being the principal isoform expressed in larval-like structures (Talbot et al., 1993). Similarly, during neuronal remodeling, EcR-B1 is the primary isoform expressed during pruning while EcR-A is predominantly expressed in neurons undergoing re-growth to establish adult connections (Robinow et al., 1993; Truman et al., 1994). Supporting this, we found that EcR-A is required in the Tv neurons during dendritic re-growth but is not required during initial dendritic outgrowth nor dendritic pruning.

The finding that EcR-A is not required for dendritic outgrowth or pruning but is required for dendritic re-growth is consistent with previous experiments involving *EcR-B* mutants and further supports the idea that each of the three EcR isoforms has a unique function with some isoform redundancy occurring (Robinow et al., 1993; Talbot et al., 1993; Truman et al., 1994; Kim et al., 1999; Sung and Robinow, 2000). *EcR-B* mutants failed to undergo dendritic pruning of the Tv neurons (Schubiger et al., 1998) but could be cell autonomously rescued by expression of either EcR-B1 or EcR-B2 but not EcR-A. Expression of EcR-A only resulted in limited pruning (Schubiger et al., 2003). My work on EcR-A and the work of others on EcR-B1 and EcR-B2, supports a model in which EcR-B1 and EcR-B2 are necessary and sufficient for dendritic pruning, with EcR-A being able to substitute to some extent when there's no active EcR-B. For dendritic outgrowth, EcR-A is the only protein isoform necessary. This model indicates that the effect of ecdysone on neuronal remodeling is regulated by the expression of the individual protein isoforms.

To strengthen this model and to ensure that the lack of dendritic re-growth seen in the *EcR-A* mutants is not due to the animals being developmentally delayed, I performed a series of developmental assays. I first studied the overall development of the $EcR\text{-}A^{139}$ mutants and

found there were no significant developmental defects and that development was not delayed. For a more in depth approach, I next dissected the CNS of the mutant pupae and found that the CNS followed the same developmental progression as wild type CNS development, again supporting my hypothesis that the lack of re-growth seen in the *EcR-A* mutants is due to there being no active EcR-A. Lastly, a lethal analysis study indicated that the $EcR\text{-}A^{139}$ mutants had a high frequency of survival until 50 hours APF, well beyond the time-frame in which dendritic re-growth occurs. All these findings support our conclusion that the *EcR-A* mutants lack dendritic re-growth due to a loss of the *EcR-A* gene function and not due to a developmental delay, thus supporting the previously stated model.

My work studying the regulation of ecdysone during neuronal remodeling is significant on many levels. First, my results emphasize the importance of neuronal remodeling by supporting the hypothesis that it is a highly regulated process controlled by the three isoforms of the ecdysone receptor. Without the tight control of the individual isoforms, dendritic synapses might be formed at the incorrect times or needed synapses might be pruned prematurely. Second, my results strengthen our knowledge of neuronal remodeling and its regulation by a steroid hormone. The role of steroid hormones in neuronal remodeling is not just limited to invertebrates, again indicating the significance of steroid hormones and their impact on the function and development of multiple organisms.

Steroid hormones have been implicated in the control of many developmental processes requiring neuronal remodeling in both vertebrates and invertebrates. In vertebrates, neuronal remodeling is involved in the ability to distinguish between what the right and left eye are viewing. Most layer 5 pyramidal neurons projecting to subcortical areas undergo neuronal remodeling so that instead of the dendrites from the right and left eye intermingling, they become

stacked with a layer of right eye dendrites followed by a layer of left eye dendrites (Stanfield et al., 1982). Many aspects of vertebrate neuronal remodeling are controlled by steroid hormones similar to ecdysone and its role in Tv-neuron remodeling. For example, both gonadal steroids and thyroid hormone are involved in the neuronal remodeling of the spinal cord neurons. Gondal steroids are important for normal dendritic outgrowth (Arnold and Gorski, 1984) whereas thyroid hormone is necessary for pruning (Li et al., 1995). Understanding how the steroid hormone ecdysone coordinates neuronal remodeling in insects therefore also contributes to not just invertebrate studies but to vertebrate ones as well.

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