Steroid hormone receptor regulation of neuronal pruning and outgrowth in the *Drosophila* central nervous system

Heather L. D. Brown

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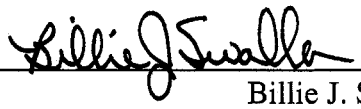

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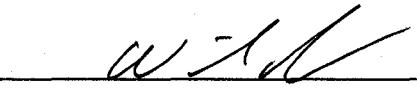

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Abstract

Steroid hormone receptor regulation of neuronal pruning and outgrowth in the *Drosophila* central nervous system

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During metamorphosis, the reorganization of the CNS of *Drosophila melanogaster* occurs via death of most larval neurons, remodeling of surviving larval neurons, and production of de novo adult neurons. This restructuring is driven by the steroid hormone ecdysone, and its receptor, EcR. In this study, I examined the role of EcR in regulating the development of both remodeling and de novo neurons within the CNS. I expressed various EcR constructs in the remodeling Thoracic-ventral (Tv) neurons and examined both pruning and outgrowth. Axons of these neurons prune their larval arbors early in metamorphosis and a larger, more extensive adult arbor is established via branch outgrowth. Both knockdown of total EcR and expression of EcR dominant negative constructs resulted in pruning defects of larval axons, but variable effects on outgrowth were seen depending on the construct expressed. When the blend of wild type EcR isoforms was altered, the timing of remodeling phases was changed. Pruning was accelerated by overexpression of the EcR-B isoforms. Overexpression of EcR-B2 resulted in precocious outgrowth of the Tv axons, possibly indicating a derepressive role for this isoform. I also examined the function of EcR in development of de novo neurons. When EcR-DN was expressed in clones of the adult-specific lineages,

neuroblasts were retained longer in clones expressing EcR-DN. There was no alteration in the lineages' primary, initial projection. We did see defects in secondary arbors of both central and peripheral projections, which varied across the lineages and included clumping and cohesion of fine branches, misrouting, smaller arbors and some defasciculation.

The results from this study are consistent with the role of EcR activation versus derepression for successive phases of neuronal remodeling, and suggest that functional ecdysone receptor is necessary for some, but not all, remodeling events. Additionally, they point to previously unknown roles for the EcR isoforms in driving outgrowth in remodeling neurons. Finally, the data from the adult-specific neuron lineages indicate that EcR plays a lesser role in regulating their outgrowth than in neurons that remodel.

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Chapter 1

Introduction

An organism's nervous system must change to meet the demands of progressive life stages throughout development. These changes often continue after birth, as the nervous system matures in response to environmental pressures and the continuing development of the organism. The plasticity of the postnatal human brain is now well documented as certain neural pathways are not concrete until early adulthood. Modifications take place on the level of individual neurons, as synaptic connections are pruned and refined. These alterations in neuronal morphology can be driven by a variety of both intrinsic and extrinsic factors. The role of hormones in regulation of the nervous system development is common to most animals. For example, sex steroids play a crucial role in regulating genderization of the nervous system. Exposure of the human fetal brain to estrogen causes permanent differentiation of brain structures towards a male phenotype (Arnold and Breedlove, 1985; Arnold and Gorski, 1984; Pilgrim and Hutchison, 1994). In rodents, the number and size of neurons in the spinal nucleus of the bulbocavernosus is regulated by androgens (Breedlove et al., 1999). Hormones are a critical factor in promoting normal brain development in addition to the genderizing effects of sex steroids. In humans, the dendritic growth, spinogenesis and synaptogenesis of Purkinje cells are promoted by estrogen and progesterone (Tsutsui et al., 2004). Thyroid hormone, in particular, has also long been known to affect development of the nervous system. In amphibians, thyroid hormone influences axon guidance and growth, cell division and apoptosis (Tata, 2000).

Thyroid deficiencies in early human development, either from decrease of hormone or defects in the thyroid hormone receptor, lead to global defects in cerebral, cortical, cerebellar and ear development, and more specific effects on neuronal development, maturation and synaptic connectivity (reviewed in Smith et al., 2002).

Classically, steroid hormone function is modulated through the action of nuclear receptors, one of the most prevalent transcriptional regulators in metazoans. Nuclear receptors share a common structure and make up the nuclear receptor superfamily (Lavery and McEwan, 2005; Mangelsdorf et al., 1995; Robinson-Rechavi et al., 2003). The N-terminus is composed of a highly variable A/B domain, and may contain a ligand-independent activation region (AF1). The C domain binds DNA through two conserved zinc fingers and may also have some dimerizing capability, while the D domain functions as a hinge and a linker to the ligand-binding domain, E. The E domain contains another activation region (AF2) contingent on ligand binding, as well as the main dimerization domain. At the C-terminal end, nuclear receptors may have an additional, non-conserved F domain with unknown function. Nuclear receptors work through mechanisms of transcriptional activation and/or transcriptional repression. Switching from a repressive mode to a derepressive or activational role depends on the spatial and temporal expression of ligand, cofactors, and receptor isoforms.

Drosophila melanogaster has been an extremely useful model to study the role of hormones and nuclear receptors in directing nervous system development. *Drosophila* is a holometabolous insect, undergoing complete metamorphosis, and its

postmetamorphic development is orchestrated by the steroid hormone ecdysone. As the larva develops through three instars, the larval tissues that will degrade during metamorphosis grow via cell enlargement, while the nervous system and other imaginal precursor cells remain diploid (Nothiger, 1972). At the start of metamorphosis, the larval tissues undergo degeneration, while the diploid imaginal discs and parts of the nervous system persist and mature. Many larval neurons do undergo programmed cell death, but not all die at metamorphosis. Some, particularly neuromodulatory neurons and motorneurons, survive and are remodeled (Truman, 1990). These remodeled neurons prune back their larval arbors both centrally and peripherally and grow new arbors to make functional connections with the developing adult tissue.

Remodeled larval neurons make up only a small portion of the adult nervous system in *D. melanogaster*. Most neurons are born during the larval instars and only mature during metamorphosis. These adult-specific neurons are born from persisting embryonic neuroblasts that resume divisions in the first larval instar. After each neuron is born, it extends a primary neurite, then arrests until metamorphosis. During metamorphosis, these neurons sprout to their final targets, establishing connections that are necessary to support the large range of behaviors and increased sensory processing in the adult fly (Truman et al., 2004). The neurons of the mushroom bodies differ in two respects from the above pattern. First, the mushroom body neuroblasts do not have a period of transient dormancy but divide continuously from their initial appearance during embryogenesis until late in metamorphosis. Second, the neurons of

the mushroom body that are produced during larval life become functional neurons after they are born, rather than arresting until metamorphosis (Truman and Bate, 1988).

The large-scale metamorphic reorganization of *D. melanogaster* is driven by the steroid hormone ecdysone and the ecdysone receptor, a heterodimer of EcR and ultraspiracle (USP), a homolog of the vertebrate retinoid X receptor (Koelle et al., 1991). EcR and USP form an obligate heterodimer, and must be complexed in order to bind DNA and ligand (Yao et al., 1992). EcR is a member of the nuclear receptor superfamily, and is similar in sequence and structure to other RXR heterodimeric partners such as thyroid hormone receptor and retinoic acid receptor (Mangelsdorf et al., 1995). Activation through the ligand-binding domain (AF2) of EcR only occurs when ligand is bound to the ecdysone receptor. In the absence of ligand, corepressors present in the nucleus bind to EcR and repress transcription by reducing the accessibility of chromatin. SMRTER and Alien, two corepressors that bind to EcR, recruit Sin3 and histone deacetylase to the transcriptional complex (Dressel et al., 1999; Tsai et al., 1999). Ligand binding facilitates both the release of corepressors and the recruitment of coactivators such as Taiman, a histone acetyltransferase, or TTR, a methyltransferase (Bai et al., 2000; Sedkov et al., 2003). The association of these cofactors with EcR therefore determines the transcriptional state of target genes: repression, derepression or activation. Derepression is a biologically relevant state of EcR; in the sensory neurons of the wing margin, loss of function of either EcR or USP causes premature differentiation, indicating that ecdysone binding to EcR may function

as a regulatory timer for developmental processes in these cells, rather than acting purely in an activation capacity (Schubiger et al., 2005; Schubiger and Truman, 2000).

Translation of the ubiquitous ecdysone signal into tissue-specific responses is driven not only by cofactors, but by the distribution of the three different EcR isoforms in *Drosophila*. EcR-B1 and EcR-B2 are splice variants, while EcR-A is transcribed from a separate promoter (Talbot et al., 1993). The isoforms have common C, D, E and F regions, but vary in their N-terminal A/B regions. EcR-B1 and EcR-B2 have an additional AF1 activational domain in their A/B region, while the A/B region of EcR-A is repressive (Mouillet et al., 2001). The expression patterns of the three different isoforms have been correlated with specific nervous system responses during metamorphosis (Truman et al., 1994). The mushroom body neurons and the larval neurons that are remodeled for adult function show high levels of EcR-B1 at pupariation, just prior to pruning. This high level of EcR-B1 expression during early metamorphosis is necessary for the pruning of larval arbors. EcR-B mutant animals show a loss of dendritic pruning in the Tv neurosecretory neurons and the mushroom body γ neurons. This pruning defect can only be rescued by expression of either EcR-B1 or EcR-B2, but not EcR-A (Lee et al., 2000; Schubiger et al., 2003; Schubiger et al., 1998). Larval neurons that remodel have low EcR-A early in metamorphosis, but increase A levels later, as the adult arbors are forming, correlating EcR-A with sprouting and maturation. Adult-specific neurons that are born in the larva express only EcR-A during most of metamorphosis, as they mature their functional adult arbors (Truman et al., 1994). An interesting exception to this pattern is the neurons of the

optic lobe (OL). High levels of EcR-B1 are seen in OL neurons starting about 10 hours after the start of metamorphosis. These are imaginal neurons born during larval life, and do not prune. However, the asynchronous development of the optic lobe demands a period of extended neuronal plasticity in order to establish proper connections. Since EcR-B1 is high during this phase, it has been suggested that EcR-B1 may facilitate keeping the OL neurons in an immature, plastic state through this period, rather than maturing in concert with the other imaginal neurons (Truman et al., 1994).

I investigated the relationship between EcR and the formation of the adult nervous system of *Drosophila melanogaster* for my dissertation research. Previous studies on EcR function have explored some of the aspects of EcR action and isoform specificity on pruning of remodeling neurons. However, a comprehensive study of the role of EcR activation and derepression in remodeling and how the various EcR isoforms impact both pruning and outgrowth of neurons during metamorphosis was needed. I expressed EcR-dominant negative constructs, EcR-RNAi constructs and EcR isoform constructs in both remodeling larval neurons and the lineages of adult-specific neurons. In remodeling Tv cell axons, I found that EcR activation was necessary for pruning of larval arbors. Pruning of the Tv axons required the presence of both the EcR-B1 and EcR-B2 isoforms. The requirements for formation of a complete adult arbor were different for remodeling neurons compared to adult-specific neurons. In the Tv cell axons, derepression of EcR was sufficient for moderate sprouting and outgrowth. The outgrowth phase of these neurons was also prematurely initiated by shifting the blend of isoform in the cell towards EcR-B2, indicating a novel function for this isoform.

However, EcR appears to have little influence on the final arbor of the adult-specific neuron lineages. Blocking ecdysone signaling in these neurons resulted in only minor defects in fasciculation and branching. This implies that other factors besides the ecdysone signal may be involved in driving sprouting and outgrowth in this class of neurons.

This study emphasizes the complexity of EcR function in *D. melanogaster.* Both the derepression and activation functions of this nuclear receptor are functionally significant during development, indicating that differential states of target gene transcription are involved regulating CNS development. Specificity is added to the system as temporal and spatial expression of isoforms and cofactors translates the ubiquitous developmental signal of hormone titer fluctuation into precise cellular responses. Despite the many layers of the *Drosophila* ecdysone signaling pathway, investigations such as this study are resulting in a progressive understanding of the function of nuclear receptors, the role of hormones in the development of the nervous system, and the basic processes of neuronal pruning and growth.

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Chapter 2

Use of time-lapse imaging and dominant negative receptors to dissect the steroid receptor control of neuronal remodeling in *Drosophila*

Introduction

Steroid hormones control the body plan changes of holometabolous insects during metamorphosis. Ecdysone and its metabolite 20-hydroxyecdysone (20E) drive the remodeling of the larval body during this period, including the rewiring of the nervous system for adult function (Weeks and Truman, 1985). This rewiring proceeds through programmed cell death of larval neurons, maturation of adult-specific neurons, and remodeling of persistent larval neurons for adult use (Tissot and Stocker, 2000; Truman, 2005; Weeks, 1999). These persistent neurons prune back their axonal and dendritic branches early in metamorphosis and then grow new, adult-specific arbors, establishing the neuronal circuitry necessary for the adult. The ecdysteroids, particularly 20E, act through their receptor to mediate pruning and outgrowth in the nervous system of *Drosophila melanogaster.* The ecdysone receptor is an obligate heterodimer formed from two nuclear receptors: EcR and the RXR-ortholog ultraspiracle (USP) (Koelle et al., 1991; Yao et al., 1992). The EcR-USP heterodimer binds to specific ecdysone response elements (EcRE) in DNA to regulate transcription of target genes (Cherbas et al., 1991).

The EcR/USP complex, referred to in this paper as the ecdysone receptor, is able to both activate and repress target genes, depending on the presence or absence of ecdysteroids (reviewed in (Kozlova and Thummel, 2000). When ecdysteroid is absent

and sufficient amounts of a corepressor such as SMRTER are present, the ecdysone receptor is able to repress transcription (Dressel et al., 1999; Tsai et al., 1999). When ecdysteroid is present, it binds to the ecdysone receptor complex, causing a conformational change that promotes the release of corepressors and the binding of coactivators, initiating transcription of early response genes (Bai et al., 2000; Sedkov et al., 2003). Both the activational and repressive actions of the receptor are functionally significant in vivo. For example, USP null clones in the wing disc show a failure to express some ecdysone target genes while others are expressed prematurely, and the clones show precocious differentiation of sensory neurons (Schubiger and Truman, 2000). This mixed response suggests that although the steroid acts via the EcR/USP complex, in some cases it is through activation while in others it is through derepression.

EcR is a member of the nuclear receptor superfamily. Its N-terminus is composed of a variable A/B domain, and may contain a ligand-independent activational region (AF1). The E domain contains the main dimerization domain and another activational region (AF2) contingent on ligand-binding (Robinson-Rechavi et al., 2003). There are three EcR isoforms in *Drosophila melanogaster:* EcR-A, EcR-B1 and EcR-B2. Studies have shown that EcR-B1 and EcR-B2 both have strong activation functions in their A/B regions, while EcR-A may have an inhibitory function (Hu et al., 2003; Mouillet et al., 2001). All three EcR isoforms bind equally well to the DNA EcRE (Cherbas et al., 1991; Mouillet et al., 2001) and to ecdysteriod (Dela Cruz et al., 2000),

but have different spatial and temporal expressions and induce different cellular responses (Cherbas et al., 2003; Talbot et al., 1993; Truman et al., 1994).

Variable cellular responses to the three EcR isoforms are particularly evident in the remodeling nervous system. High levels of EcR-B1 early in metamorphosis have been associated with pruning of larval branches (Truman et al., 1994). In many remodeling neurons, EcR-B1 expression decreases and EcR-A expression becomes prominent as the neuron begins its adult outgrowth. In contrast to remodeling larval neurons, arrested imaginal neurons born during larval life express only EcR-A at the start of metamorphosis as they begin their adult outgrowth (Truman et al., 1994). Experiments examining pruning in both thoracic ventral (Tv) neuron dendrites (Schubiger et al., 1998) and mushroom body axons (Lee et al., 2000) showed that EcR-B mutants lose their ability to prune, and can be rescued by expression of either EcR-B1 or EcR-B2 but not EcR-A (Lee et al., 2000; Schubiger et al., 2003; Schubiger et al., 1998). EcR-B1 specific mutants prune normally, indicating that either B1 and B2 are functionally redundant or B2 is the primary isoform driving pruning (Schubiger et al., 1998). These experiments suggest that the EcR B isoforms are associated with pruning and reorganizational responses while EcR-A is responsible for outgrowth of arbors. However, the specific role each EcR isoform has in directing precise cellular responses during neuronal remodeling is still not understood.

In this study, I investigated the role of EcR in Tv cell axonal remodeling. Our results highlight the specific axonal events during Tv cell remodeling, allowing us to analyze in detail both pruning and outgrowth. Results from cell autonomous

expression of EcR dominant negative constructs and EcR RNAi show while activation is necessary for early pruning events, it may not be required for later remodeling events.

Materials and Methods

Fly stocks

CMA-EcR-B1-d655-F645A, CMA-EcR-d655-F645A-W650A, and their P-element vector derivatives UAS-EcR-B1-d655-F645A (referred to in this paper as UAS-EcR-B1^{F645A}) and UAS-EcR-B1-d655-W650A (referred to in this paper as UAS-EcR-B1^{W650A}) were described in Hu et al., 2003 and Cherbas et al., 2003. Similar constructs based on the A and B2 isoforms of EcR, UAS-EcR-A^{F645A}, UAS-EcR-A^{W650A}, and UAS-EcR-B2^{W650A} were made as follows: The 5' portion of the EcR-B1 coding sequence was excised from a mutant CMA-EcR-B1 plasmid with BamH I (which cuts upstream of the start of translation) and Asc I (which cuts within the coding sequence for the common-region DNA-binding domain), and replaced with the corresponding BamH I-Asc I fragment from either CMA-EcR-A or CMA-EcR-B2 (Hu et al., 2003) to generate a coding sequence containing the A or B2 isoform-specific amino terminus and the dominant-negative mutation (F645A or W650A). The entire coding region for the mutant EcR was then excised with BamH I and Nhe I and inserted between the Bgl II and Xba I sites of the vector pUAST, to generate the responder transposon. UAS-EcR-B1^{F645A}, UAS-EcR-B1^{W650A}, UAS-EcR-B2^{W650A} and UAS-EcR-A^{W650A} were inserted into flies via P element transformation to create homozygous fly stocks

(Cherbas et al., 2003). EcR RNAi was performed using the CA104 stock containing an inserted EcR core inverted repeat construct (UAS-IR-EcR) from C. Antoniewski (Schubiger et al., 2005). To facilitate cell autonomous expression of these constructs in the Tv cells, the above stocks were crossed with a fly stock containing both the GAL4 driver (Brand and Perrimon, 1993) under control of the FMRF-amide promoter and a UAS-GFP insert (Lee and Luo, 1999) that targets CD8::GFP to the membrane. The ywUASmCD8::GFP; FG10 stock made use of a FMRF-amide promoter construct created by S.Robinow, U. of Hawaii (Schubiger et al., 2003; Suster et al., 2003). Progeny expressed both membrane-bound GFP and either EcR dominant negative construct or IR-EcR (core) cell autonomously in the Tv neurosecretory cells.

Immunocytochemistry

Central nervous systems were dissected and fixed for 20-30 minutes in 4% paraformaldehyde, followed by multiple rinses with phosphate buffered saline containing 1% Triton X-100 (PBS-Tx). Tissue was blocked with 5% normal donkey serum for 15 minutes followed by transfer to a solution of primary antibodies and incubation overnight. Primary antibodies used were monoclonal mouse anti-SCP (Masinovsky et al., 1988), rat anti-mCD8 (1:200, CALTAG), and mouse anti-EcR (IID9.6, common) (Talbot et al., 1993). After several rinses with PBS-Tx, the tissues were incubated overnight in secondary antibody (1:200 in PBS-Tx of Texas red conjugated donkey anti-mouse IgG, Texas red conjugated donkey anti-rabbit IgG, or FITC conjugated donkey anti-rat IgG; Jackson Immunoresearch). Nervous systems were rinsed several times in PBS and attached to polylysine-coated coverslips, then

dehydrated, cleared in xylene and mounted in DPX (Fluka). For quantification of EcR or mCD8 immunoreactivity, all genotypes to be compared were dissected, antibody stained and imaged as one batch.

Staging and Imaging

Animals were collected at white puparia and maintained at either 25°C or 29°C. Late wandering larvae were distinguished by their enlarged salivary glands. For live imaging, whole intact nervous systems were dissected from staged animals, leaving the ring gland intact. Tissue was attached to a polylysine-coated coverslip and inverted onto a metal imaging chamber (Kiehart et al., 1994). This chamber was bounded on the bottom by oxygen-permeable membrane (model 5793, YSI, Yellow Springs, OH) and filled with Shields and Sang M3 insect culture media (Sigma, St Louis, MO) with 7.5% fetal bovine serum (Sigma). For live imaging, a Bio-Rad Radiance 2000 confocal microscope equipped with a 488nm Kr/Ar laser was used. Individual Z-stacks with a step size of 1.05μ were taken every 20 minutes over a 3-18 hour period. The development of explanted nervous systems slowed in culture and 2 hours of time in vitro was roughly equivalent to 1 hour in vivo (Gibbs and Truman, 1998). The age of Z-stack projections from time-lapse video was calculated by referring to the time of explantation. Time-lapse movies were created from the Z-stacks using NIH ImageJ (http://rsb.info.nih.gov/ij/).

Analysis

Arbor area, arbor footprint, filopodial quantification and quantification of EcR and mCD8 immunoreactivity were calculated using NIH ImageJ. For arbor area, all images

were adjusted to a similar threshold, converted to black and white, and quantified by counting the number of pixels. To obtain the arbor footprint, a polygon was created by connecting the outer edges of the axon arbor with straight lines, then the total number of pixels in the area inside the polygon were counted. Filopodia were quantified by manual counting at each hour for each neuromere. For more developed arbors that were already fasciculated, all filopodia were counted and divided by three to get a filopodia count for each neuromere. To quantify relative EcR and mCD8 immunoreactivity, fixed cell bodies were imaged from each genotype. Each nucleus was selected and the mean intensity determined using ImageJ. The mean intensity for the background area was subtracted from the selected area to get the net mean intensity for each nucleus. Since all animals used had one copy of mCD8::GFP, the immunoreactivity of mCD8 should be similar among all genotypes. The ratio of EcR to mCD8 immunoreactivity was calculated by dividing the net mean EcR intensity by the net mean mCD8 intensity. To calculate statistical significance, a non-paired t-test (α=0.05) was run using SigmaStat and SPSS software.

Results

Remodeling of the Tv neuron axon arbors

The Thoracic-ventral (Tv) neurons are paired neurosecretory cells located in each of the thoracic neuromeres. During larval life, the axons of each segmental pair of Tv cells wrap around 2 support cells in a compact, globular structure, forming a neurohemal organ (Nassel et al., 1988). The neurohemal organs project above the

dorsal surface of the CNS, while the somata and dendrites of the Tv cells reside within the neuromere (Fig. 2.1A). Although the central dendrites of these cells had severely pruned back by 5-6 hours after puparium formation (APF) (Schubiger et al., 1998), the start of axonal remodeling did not begin until approximately 10 hours APF, with the larval arbor being completely pruned by 18h APF. Outgrowth of adult branches began between 18-20 hours APF, and was essentially finished by 48 hours APF, forming a complex mesh-like arbor spreading out over the dorsal surface of the CNS (Fig. 2.1C). The branching of the arbor changed little between 48 hours and adult eclosion (approximately 96 hours APF).

I used confocal live imaging to visualize axon pruning and growth in short-term explanted *Drosophila* nervous systems. The Tv cells were vitally labeled using the FG10-GAL4 driver to express the membrane marker CD8::GFP in these cells. (Fig. 2.1B). The superficial location of the Tv cell axons on the dorsal surface of the explanted CNS aided live imaging. At pupariation, the neurohemal sites appeared much the same as in the larva and I observed no morphological changes from late 3rd instar through 3 to 5 hours APF. Beginning around 5 hours APF, however, fine filopodia started sprouting from the axon arbor (Fig. 2.2A) and approximately 5-7 hours later, the neurons began pruning back their axonal arbor. During pruning, I saw active filopodia both on the regressing larval portion of the arbor and in an adult growth zone that was forming beneath the neurohemal organ (Fig. 2.2B). Most filopodia present on the neurohemal organ and the growth zone changed considerably in length during each 20 minute interval, either extending, shortening, or branching. In

instances in which I could follow the regression of individual axon branches, I saw only retraction and no sign of branch fragmentation (local degeneration) as described for mushroom body axons (Watts et al., 2003) and dendrites of the dendritic arborizing neurons (Williams and Truman, 2005). Addition of hemolymph to the culture, supplying a source of hemocytes, did not affect pruning, and no severing of branches occurred (data not shown). Filopodia in the growth zone area continued to be active as the larval axonal arbor disappeared, and eventually stabilized to form branches at the base of the pruned neurohemal organ (Fig. 2.2B-C). I observed filopodia on both the shaft and tip of primary branches as they extended to form a mesh-like network of axonal branches. Filopodia persisted as late as 56 hours APF, as the arbor continued to expand through activity on the outer-most branches of the network (not shown).

Effects of Dominant Negative EcR isoforms on axonal pruning

Since the ecdysone receptor is key in directing neuronal remodeling, cell-autonomous expression of dominant negative EcR (EcR-DN) should poison the cell response to ecdysteroid and disrupt pruning of Tv cell axons. Two types of EcR-DN constructs were used, both containing a point mutation in helix 12 of the ligand-binding domain (Cherbas et al., 2003). The F645A mutation is in the co-activator binding groove in the AF2 domain and results in a receptor that binds ligand, but cannot mediate activation. The W650A mutation prevents steroid binding and, consequently, also results in a lack of ligand-dependent activation. In vitro, these EcR-DNs bind DNA and USP normally, and act as competitive inhibitors of endogenous wild-type EcR (Cherbas et al., 2003). Since both types of EcR-DNs are equally

effective at suppressing ligand-dependent activation, differences between the biological effects of the two are likely due to the fact that one can bind steroid and the other cannot. The latter ability is significant because the unliganded EcR/USP complex can act as a repressor. Both the F645A and W650A-based EcR-DNs are capable of mediating this repression (Hu et al., 2003), but their differences in ligand binding may mean that one is a conditional repressor whereas the other is a constitutive repressor (see Discussion).

I compared the effects of the F645A or W650A substitution in EcR-B1 and the W650A substitution in all three isoforms (EcR-B1, -B2, and -A). Since endogenous EcR shows no isoform specificity in binding to DNA (Mouillet et al., 2001), I assumed that expressing EcR dominant negative at high levels in the cell displaced the endogenous EcR in a non-isoform-specific manner. The different A/B regions of the EcR-DNs should have little effect on the activation capacities of the modified receptor because the AF2-mediated activation functions are blocked in all of them. However, since the EcR-DNs retain their repressor function, the use of isoform-specific W650A-based EcR-DNs allowed us to probe the role of the A/B region of the isoforms in processes that might be mediated via derepression. Comparison of the F645A mutation to the W650A mutation in EcR-B1 let us analyze the need for activation over derepression of the AF2 domain.

Cells expressing the individual EcR-DN constructs showed similar pruning responses, regardless of which construct was present. Figure 2.3 shows the progression of pruning for neurons expressing each of the EcR-DN constructs. All cells expressing

the EcR-DN were of normal size and larval morphology at the start of metamorphosis. By 18 hours APF, though, the cells expressing EcR-DN showed only modest pruning compared to control cells (expressing GFP only) and by 24 hours APF, they still had a reduced larval neurohemal organ with no sign of branch outgrowth. Neurons expressing CD8::GFP only (control cells), by contrast, had completely pruned their neurohemal organs and were beginning branch outgrowth by this time (Fig. 2.3). Live imaging through this pruning period also gave similar results for all EcR-DN constructs. Notably, neurons expressing any EcR-DN construct lacked filopodia before and during the pruning time period (Fig. 2.4A). As with the immunocytochemistry, live imaging revealed slow and incomplete pruning of the larval neurohemal organ. These larval arbors were retained well past the time of pruning into the period when the cell should switch to outgrowth (Fig. 2.4B-C)

Effects of Dominant Negative EcR isoforms on axon outgrowth

Although expression of the various EcR-DN constructs resulted in similar effects during the pruning phase, they were quite different in how they affected outgrowth. Neurons expressing EcR-B1^{F645A} or EcR-B2^{W650A} showed relatively mild effects on adult outgrowth characteristics, whereas neurons expressing EcR-A^{W650A} or EcR-B1^{W650A} were much more severely affected. At both 48 hours APF and in the adult, the neurons expressing EcR-B1^{F645A} or EcR-B2^{W650A} had a moderately elaborated, branched arbor that formed a loose reticulum at the junction of the T2 and T3 neuromeres and had 2 to 4 branches that extended anteriorly, often into the neck connectives. Unlike in control animals, cells expressing these EcR-DN's often had a

clumped tangle of branches in the center of the adult arbor (Fig. 2.5A). Neurons that expressed EcR-A^{W650A} had a clumped arbor at 48 hours APF, reminiscent of the larval arbor, but also showed a few adult-like fasciculating branches. Arbors of cells expressing EcR-B1^{W650A} were the most severely affected, with prominent larval-like neurohemal organs and occasional branches that extended over the surface of the CNS. These branches only rarely showed higher order branching. Measurements of the area covered by the new arbor under the various conditions show that none of the EcR-DN's made an arbor as extensive as seen in controls, but the most reduced arbors were seen in cells expressing EcR-A^{W650A} or EcR-B1^{W650A} (Fig. 2.5B).

Live imaging data emphasized the differences seen between the outgrowth patterns of cells expressing EcR-B1^{F645A} or EcR-B2^{W650A} versus the other EcR-DN constructs. Although cells expressing the EcR-DNs showed no filopodia during the pruning phase, those expressing EcR-B1^{F645A} or EcR-B2^{W650A} started extending filopodia after 24h APF (Fig. 2.6). Filopodia formed on both the remnant of the neurohemal organ and below it, in the growth zone. Filopodia in both locations stabilized into branches that fasciculated to form an arbor with both adult and larval characteristics. As filopodia located on the neurohemal organ extended and stabilized into branches, the remnant neurohemal organ partially fragmented, resulting in an adult-like arbor with clumped varicosities and a dense center. Figure 2.4B shows filopodia emanating from the remaining larval arbor of cells expressing EcR-B1^{F645A}. Although cells expressing EcR-B1^{F645A} and EcR-B2^{W650A} formed branches during this outgrowth phase, these axon arbors differed in appearance from branches on cells expressing CD8::GFP-only.

Axons from cells expressing these two dominant negatives were blebbier, with swollen regions along the branches. Interestingly, the cells expressing EcR-B1^{F645A} and EcR-B2^{W650A} had fewer filopodia in the meso and metathoracic neuromeres as compared to their prothoracic counterparts (Fig. 2.6).

In contrast to the above EcR-DN's, cells expressing EcR-B1^{W650A} or EcR-A^{W650A} failed to extend filopodia during the outgrowth phase. The rare filopodial-like structures that I saw were very short, stubby, and less active than those seen on control cells or cells expressing EcR-B1^{F645A} or EcR-B2^{W650A} (Fig. 2.4C). They did not change extensively in length or branching during each 20 minute interval and once established, they persisted without a significant length change for 60 minutes or more. The arbors of these cells remained very larval-like through the end of imaging and did not show the extended branches seen on the arbors of cells expressing EcR-B1^{F645A} or EcR-B2^{W650A}.

Effects of Dominant Negative EcR expression are qualitative

The differences seen during outgrowth of neurons expressing the various EcR-DN's could represent a qualitative difference reflecting the molecular action of EcR or a simple quantitative effect due to varying expression levels of the EcR-DN inserts. I addressed this issue in three different ways: expression of the same EcR-DN constructs from different insertion sites, comparison of neurons from animals raised at 25°C vs. 29°C, and quantitative immunocytochemistry to determine the relative amounts of protein expressed in Tv cell bodies for all EcR-DN constructs.

I examined the arbors of cells expressing two different inserts of EcR-B2^{W650A} and of EcR-A^{W650A}. There was no significant difference seen between the arbor areas for the individual inserts of the same construct. This was also true for the arbor footprints; the cells expressing either of the EcR-B2^{W650A} inserts were similar and cells expressing either of the EcR-A^{W650A} inserts were similar (Table 2.1).

Since increasing temperature boosts the efficiency of GAL4-UAS expression, raising animals at 29°C leads to an increase in target protein production (Brand and Perrimon, 1993). Therefore I compared the arbor areas and footprints of the adult axonal arbors for cells expressing the various EcR-DN's at 25°C versus 29°C. The only significant difference seen was between the arbor footprints of cells expressing the EcR-B1^{W650A} at the different temperatures. At 25°C, the footprint pixel count was 38578 (n=8) while at 29°C it was reduced to 12422 (n=10, P=0.01). The difference in the arbor area of cells expressing EcR-B1^{W650A} at 25°C versus 29°C was not significant, with pixel counts of 3306 and 3766 respectively (P=0.41). All of the other EcR-DN's were not significantly different for either arbor area or arbor footprint at the different temperatures (Table 2.1).

I also used quantitative immunocytochemistry to determine the relative protein levels in nuclei of cells expressing each individual EcR-DN's. EcR levels were significantly higher in cells expressing EcR-DN than in control cells ($P<0.05$, Fig. 2.7A). Although EcR levels varied between the cells expressing different EcR-DN's, the differences in protein level did not correspond with the severity of axonal phenotype. Neurons expressing EcR-B1^{F645A} had a relatively higher EcR level than

those expressing EcR-B1^{W650A}, but the cells expressing EcR-B1^{W650A} had a more severe phenotype. CD8 levels in all cells examined did not differ significantly; the ratio of EcR to CD8 immunofluorescence confirmed that the higher levels of EcR in cells expressing EcR-DN was not due to differences in imaging (Fig. 2.7A, inset). All of the above data support our conclusion that the qualitative differences I see for the different EcR-DN's are not based on quantitative differences in the level of EcR expression.

Effects of IR-EcR(core) on remodeling of the Tv neurons

To further investigate the role of EcR in directing remodeling of the Tv cell axons, I knocked down the level of all EcR isoforms in these cells via an RNAi approach (Roignant et al., 2003). I selectively expressed UAS-IR-EcR (core) and UAS-CD8::GFP in the Tv cells, using the FG10-GAL4 driver. I quantified total EcR protein levels as described above. In the late 3rd instar, Tv cell nuclei from IR-EcR (core) expressing animals showed total EcR levels below the level of detection compared to EcR levels in control animals (Fig. 2.7B). The ratio of EcR to CD8 protein was much lower for cells containing IR-EcR (core) than those without (Fig. 2.7C, inset), indicating that the decrease in EcR immunoreactivity was due to a true suppression of EcR protein and not due to immunocytochemistry or imaging artifact. To further insure adequate levels of IR-EcR (core) to knock down EcR level, I raised animals at 29°C until the time of dissection. I compensated for difference in developmental time when comparing IR-EcR (core) data to that from animals maintained at 25°C (Powsner, 1935).

Axonal pruning in the cells expressing IR-EcR (core) resembled the reduced pruning seen in cells expressing the dominant negative EcR constructs. Immunostained nervous systems showed normal larval neurohemal organs at pupariation, but by 24 hours APF, the cells still had larval-like arbors, although they were clearly reduced in size as compared to larval stages. I did not see adult-like branches at 24 hours APF (Fig. 2.8A), but by 48 hours APF and in the adult a new arbor was present that appeared similar to EcR-B1^{F645A} and EcR-B2^{W650A} expressing cells. The neurons expressing IR-EcR (core) also had a dense, clumped axon arbor that was surrounded by the reduced adult-like arbor. Comparison of the arbor footprints of cells expressing IR-EcR (core) to control cell arbor footprints emphasizes the decreased branching in these cells (Fig. 2.8B).

Data from live imaging of cells expressing IR-EcR (core) showed no filopodia during the normal pruning phase (12-18 hours APF) along with a slowed retraction of the larval branches. I first observed filopodia around 24 to 28 hours APF (Fig. 2.8C). These filopodia formed stable, fasciculated branches, resulting in an adult arbor with both larval and adult characteristics, similar in appearance to arbors of cells expressing EcR-B1^{F645A} and EcR-B2^{W650A}. Figure 2.8D shows quantification of filopodial activity of cells expressing IR-EcR (core).

Discussion

Remodeling of Tv Neurons

Previous studies on neurite pruning in *Drosophila* at the start of metamorphosis showed the removal of processes by fragmentation (Watts et al., 2003) or a combination of fragmentation and retraction (Williams and Truman, 2005). In both of these cases, surrounding cells participate in the fragmentation process. For the axons of the mushroom body gamma neurons, glial invasion of the axon lobes is necessary for proper pruning (Awasaki and Ito, 2004), whereas for the peripheral dendritic arborizing neurons, phagocytic blood cells are seen to sever proximal and distal regions of the dendritic arbor (Williams and Truman, 2005). In both immunocytochemistry of fixed preparations and time-lapse movies of pruning in the Tv neurons, I saw no sign of fragmentation, even with the addition of hemolymph. In instances where I could visualize individual branches, they were gradually retracted. The fact that Tv axon pruning occurs via retraction only may be partially due to their peripheral location underneath the basal lamina (Lundquist and Nassel, 1990). This location may isolate them from invading glia within the CNS and phagocytic blood cells circulating in the hemolymph.

Confocal imaging of live Tv cell axons during metamorphosis showed that filopodial activity is a widespread feature of the remodeling neuron. Abundant filopodia were observed during pre-pruning, pruning and outgrowth phases of remodeling. The appearance of filopodia during the pre-pruning period coincides with the time that the Tv cells prune back their dendrites (Schubiger et al., 1998). The axonal structure, however, appears normal at this time so it is unlikely that these filopodia result from a disruption of the axonal cytoskeleton. The difference in timing

between dendrite and axon retraction is also seen in motoneurons MN1-4 that innervate the larval mesothoracic muscles of the larva and remodel to innervate the dorsolongitudinal indirect flight muscles of the adult fly. The dendrites of these neurons undergo retraction between 2 and 4 hours APF, while the axons begin to prune at 6 hours APF and finish at 10 hours APF (Consoulas et al., 2002). In the Tv neurons, filopodia continue to be present as the axonal arbor is retracted. The presence of filopodia during pruning was also observed in the dendritic arborizing neurons (Williams and Truman, 2005). In the mammalian hippocampus, filopodia are associated with both formation and reduction of dendritic spines. Synaptic activation results in growth of dendritic filopodia (Maletic-Savatic et al., 1999) while deafferentation causes filopodial activity followed by reduction of spine density (Benshalom, 1989). It is probable that the presence of filopodia is either a reflection of cytoskeletal instability or plays an active role in disrupting or transporting the cytoskeleton of axons being pruned by retraction.

After pruning is completed, filopodial activity continues as the cell switches to its outgrowth phase. The filopodia present during outgrowth behave differently than earlier filopodia in that many are precursors to the stable branches that form the adult arbor. Branch stabilization most likely occurs via selective invasion of filopodia by microtubules, as seen in other systems (Sabry et al., 1991; Schaefer et al., 2002). It is unknown if these later filopodia represent a different pharmacological or molecular class from the structures seen during pruning.

Role of Ecdysone Signaling in Pruning

The expression of the EcR dominant negative constructs and the IR-EcR (core) construct in the Tv neurons resulted in incomplete pruning of the larval axonal arbors. Cells expressing any one of these constructs showed a reduction in neurohemal organ size during the pruning period, but substantial larval material was retained into the outgrowth phase. In a study by Williams and Truman (2005) examining pruning in the dendritic arborizing neurons, proximal destabilization of microtubules and thinning of the dendrites was seen before fragmentation. When dominant negative EcR was expressed, the dendrites did not thin proximally. Shortening dendrites with retraction bulbs at their tips were observed, however, indicating that some aspects of pruning still persisted in cells whose steroid-response system was blocked. This indicates that proximal cytoskeletal destabilization may depend on ecdysone signaling in the da neurons, while distal tip retraction may not (Williams and Truman, 2005). One possibility is that distal retraction results from 20E-evoked changes in the epidermis on which the neuron resides. Although fragmentation of Tv cell axons does not occur, the pruning process in these cells may nevertheless be similar to that seen in the da neurons. Destabilization of the cytoskeleton in proximal regions of the axon arbor may facilitate rapid axon retraction. Without activation via ecdysteroid (cells expressing dominant negative EcR or EcR RNAi), cytoskeletal destabilization along the axon shaft may not occur and any pruning must take place via tip retraction with slow retrograde progression. Additionally, cells expressing EcR-DN or IR-EcR (core) lacked filopodia during the pre-pruning and pruning stages. Since filopodia are

associated with cytoskeletal instability (Benshalom, 1989; Maletic-Savatic et al., 1999), this lack of filopodia may indicate that the cytoskeleton is not destabilizing properly, thereby slowing retraction. Because pruning and early filopodial activity were deficient in cells expressing either the EcR-DNs or IR-EcR (core), I conclude that these events must be mediated by the ecdysteroid activation of transcription (Fig. 2.9). The fact that the Tv cells prune at all suggest that either the EcR-DN's and the EcR-RNAi are not inactivating the endogenous EcR sufficiently, or that there may also be a non-cell autonomous component to pruning. Since the EcR-DN's are not expressed in the supporting glial cells of the neurohemal organ or in surrounding tissue, these support cells are still responsive to ecdysone. The modified pruning response I see could be due to ecdysone-mediated death of the supporting glial cells or signaling from the surrounding tissue, causing the Tv axons to retract, albeit slowly as discussed above.

Earlier studies on dendritic pruning of the Tv cells and axonal pruning in the mushroom body showed that the EcR-B isoforms are necessary to support pruning, indicating the need for AF1 activation in this process. That all of the dominant negative EcR isoforms as well as the IR-EcR (core) construct also fail to prune effectively argues that activation through the AF2 domain of EcR is also essential for this process. The EcR-B AF1 and AF2 activation domains likely work cooperatively to bring about the rapid deconstruction of the axonal arbor.

Role of Ecdysone Signaling in Outgrowth

Unlike pruning, outgrowth of Tv cell axons depends on the EcR construct being expressed. Cells expressing EcR-B1^{F645A}, EcR-B2^{W650A} or IR-EcR (core) started to extend filopodia during the outgrowth phase, which began after 24 hours APF. This outgrowth occurred both from filopodia that formed on the remaining larval arbor and from the growth zone underneath. However, new branches that extended from both the larval arbor and the neural outgrowth growth zone were unusual in their morphology. Compared to controls, branches from both sites had small varicosities along their length, resulting in a "blebby" appearance. In a study by Jacobs and Stevens on cultured PC12 cells, neurites with microtubules depolymerized by Nocodazole showed formation of varicose expansions filled with randomly oriented membranous organelles, in contrast to untreated neurites where organelles are uniformly distributed and longitudinally oriented (Jacobs and Stevens, 1986). The Tv neurons in this treatment group may have suffered similar microtubule abnormalities, resulting in formation of similar irregular varicosities.

Cells expressing EcR-B1^{W650A} or EcR-A^{W650A} showed a qualitatively different type of outgrowth, forming very few filopodia and finishing with adult arbors that appeared larval-like and did not form a net-like structure over the surface of the nervous system. This draws attention to the importance of filopodia in guiding proper outgrowth to form the adult arbor. Although I observed the extension of rare branches, these seldom sub-branched and did not fasciculate with those from other Tv cells to form the adult-like meshwork. Filopodia actin bundles guide microtubule polymerization in *Aplysia* (Schaefer et al., 2002), while inhibition of F-actin in cultured hamster neurons leads to

inhibition of directed growth (Dent and Kalil, 2001; Schaefer et al., 2002). Eradication of filopodia through cytochalasin treatment of grasshopper pioneer neuron growth cones results in disoriented and improper branch formation, but did not eliminate axon extension (Bentley and Toroian-Raymond, 1986). The latter result is similar to our results with the Tv cells, in that growth is not eliminated by lack of filopodia, but instead results in a more larval-like arbor in the adult.

EcR Dominant Negatives, IR-EcR (core) and Remodeling

All of the constructs produced similar results for pruning, but they had varied effects on outgrowth. These differences in the cellular response to the various EcR constructs indicate that the molecular action of EcR on these neurons may change during the course of their remodeling. The ability of cells expressing IR-EcR (core) to produce filopodia and adult-like branches during the outgrowth period is surprising in the context of 20E acting via activation through the ecdysone receptor. It is possible that the hypomorphic levels of EcR are nevertheless sufficient to support this activation and induce outgrowth. However, similar types of outgrowth were seen in cells expressing EcR-B1^{F645A} and activation is strongly suppressed by this dominant negative receptor (Cherbas et al., 2003). A more likely hypothesis is that during the outgrowth phase, the ecdysone receptor acts predominantly as a repressor and the role of 20E is to relieve this repression (Fig. 2.9). The function of the ecdysone receptor as a developmental repressor has been best studied in the developing wing imaginal disc. USP-null clones, which cannot respond to 20E, show precocious sensory neuron differentiation that is no longer dependent on ecdysteroid (Schubiger and Truman,

2000). Expression of IR-EcR (core) in the wing disc similarly leads to precocious neuron development (Schubiger et al., 2005). It is notable that expression of EcR-B1^{F645A} produces a phenotype in the Tv cells that is similar to receptor removal. Since this dominant negative can bind 20E (Cherbas et al., 2003) our results suggest that ligand binding by this EcR-DN may relieve the transcriptional repression that it enforces on its target genes, thereby enabling a core program of axonal outgrowth that does not depend on activation. The ability of the EcR-B1^{F645A} dominant negative to support derepression, however, may be highly context dependent since there were no differences seen in activation or inhibition in Kc167 cells expressing either EcR-B1^{F645A} or EcR-B1^{W650A} (Hu et al., 2003).

This hypothesis that the ecdysone receptor plays a permissive role as a repressor during the phase of neuronal outgrowth during remodeling is reinforced by the results from cells expressing EcR-A^{W650A} and EcR-B1^{W650A}, since these cells show a more severe phenotype than that seen in cells with knocked-down EcR levels (Figs. 2.5 and 2.8). However, the phenotype of cells expressing EcR-B2^{W650A} was less severe in both immunocytochemistry and live imaging than those expressing EcR-A^{W650A} and EcR-B1^{W650A}. Experiments testing the activation potential of each of the wild type EcR isoforms indicated the presence of a repression domain in the A/B region of EcR-A (Mouillet et al., 2001). Only an activation domain (AF1) has been ascribed to the A/B region of EcR-B1 (Hu et al., 2003; Mouillet et al., 2001), but this region is quite long and incompletely characterized. I suggest that the A/B region of EcR-B1 may also possess a repression domain, although this needs to be confirmed biochemically. If

this were the case, then either of these isoforms would then be able to tether a strong corepressor complex that could not be removed without ecdysteroid binding. Under this hypothesis, the weak phenotype seen with expression of the EcR-B2^{W650A} construct may be a function of its short A/B region. This isoform has a strong AF1 activational function in its 17 amino acid A/B region but it is unlikely that this short region also contains a repressive domain. Because of this, the EcR-B2 isoform may not be able to assemble the strong corepressor complexes that are seen with the other isoforms. Hence, the EcR-B2 isoform may be able to mediate strong activation but be a very poor repressor. This model would account for the fact that in axon outgrowth, a situation where the critical step is the relief of transcriptional repression by ecdysteroid binding to its receptor, cells expressing EcR-A^{W650A} or EcR-B1^{W650A} have a much more severe phenotype than cells expressing EcR-B2^{W650A}, EcR-B1^{F645A} or IR-EcR (core).

These studies indicate that EcR function is complex, with expression of isoforms and cofactors, hormone titer, and derepression versus activation of target genes varying through space and time. However, studies of the effects of EcR on neuronal remodeling in combination with new technology are yielding invaluable information on the role of nuclear receptors in development of the nervous system.

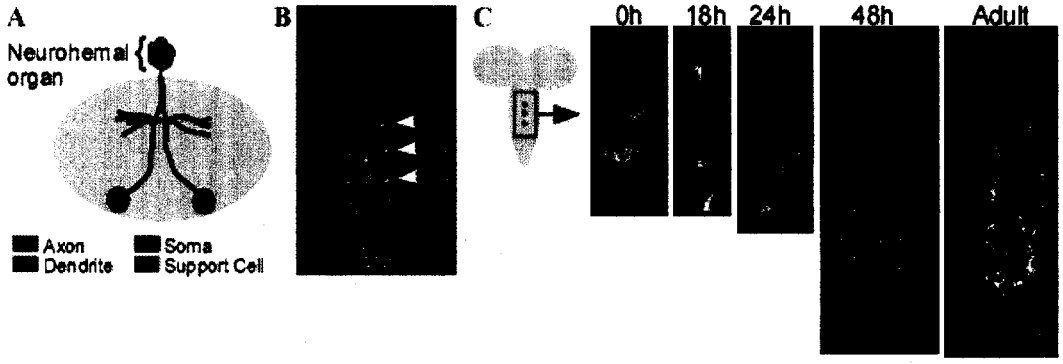


Figure 2.1. *Metamorphosis of the Thoracic ventral (Tv) neurosecretory cells.* A) Schematic transverse section of the ventral nervous system showing the axons, dendrites and cell bodies of a pair of Tv cells, before metamorphosis. The axons are wrapped around a pair of support cells (dorsal neurohemal organ), situated outside the neural sheath. B) Projected confocal Z-stack of CD8::GFP expression in the thoracic neuromeres of an early metamorphic individual (18 hours APF) of the genotype UAS-CD8::GFP;FG10-GAL4. Expression in the thorax is limited to the three pairs of Tv neurons (arrows point to somata). C) Dorsal view of the Tv cell axons, immunostained with α-SCP, at various time points during metamorphosis.

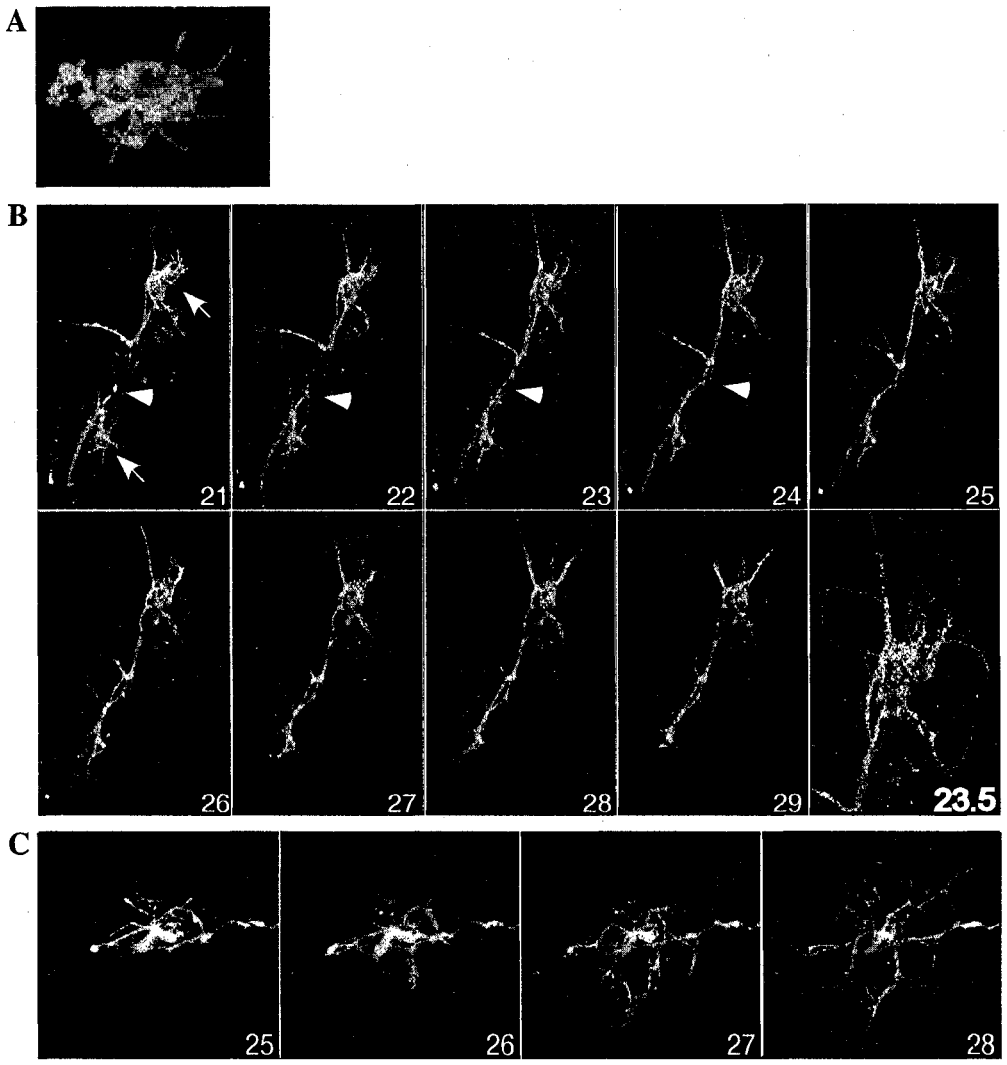


Figure 2.2. *Z-stack projections from live imaging time-lapse videos showing changes in the axonal arbors of Tv neurons.*
A) Enlarged still of the most anterior axon arbor (T1) at 6.5 hours APF showing filopodia extending from the neurohemal organ. B) Z-stack time-lapse projection of the T2 (bottom) and T3 (top) neurohemal organs (arrows) from 21 to 29 hours APF, every 60 minutes. Pruning can clearly be seen as T2 decreases in size from 21 to 29 hours APF. Filopodia from the growth zone under each neurohemal organ fasciculate (arrowheads). Last panel is an enlarged still of the T3 neurohemal organ at 23.5 hours APF, showing the large number of filopodia. C) Z-stack time-lapse projection of a pruned T3 axon arbor from 25 to 28 hours APF, showing the dramatic outgrowth that occurs between 25 and 28 hours APF.

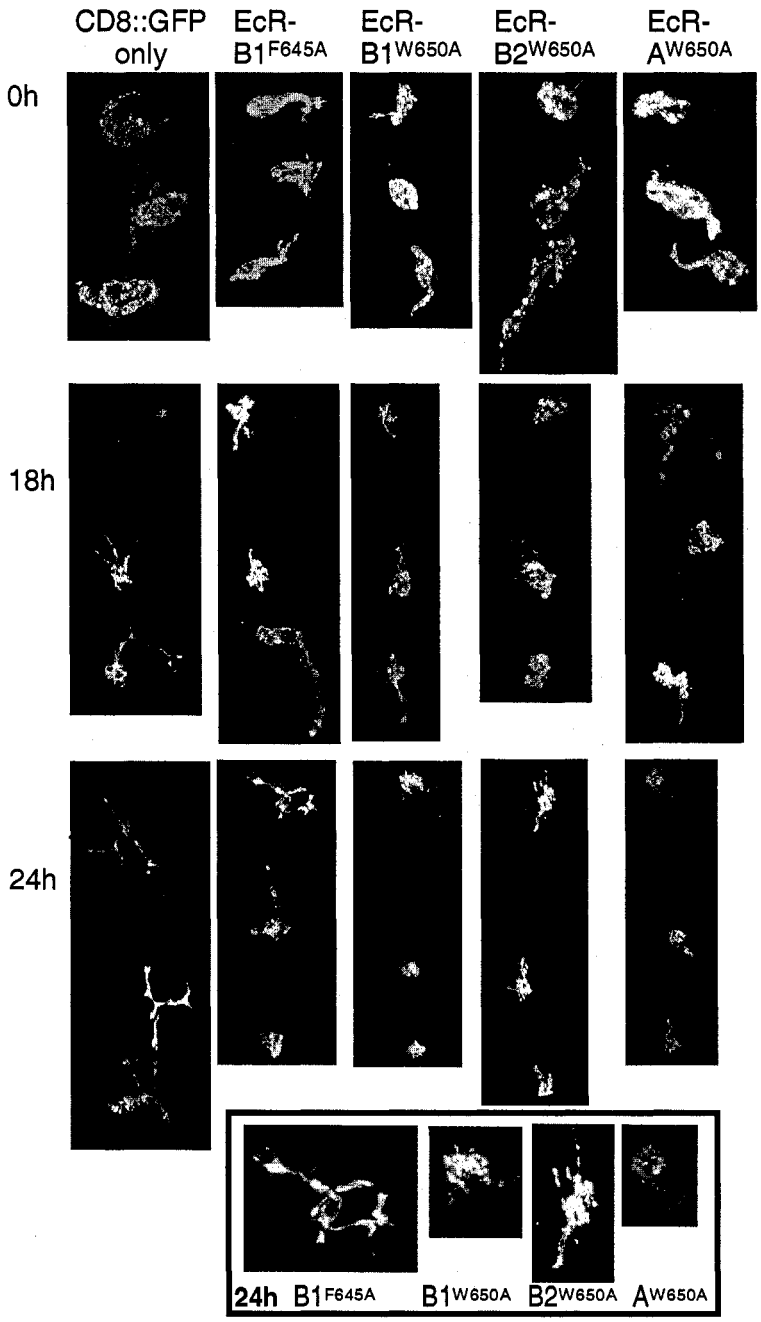


Figure 2.3. *Immunocytochemistry (α-SCP) of Tv cell axon arbors during the pruning phase of remodeling, for control cells and cells expressing EcR-DN.* Variability in nervous system size and spacing of Tv cell neurohemal organs was seen in all treatments and did not appear to be correlated with any particular treatment. Inset: magnification of the T1 axon arbor at 24 hours APF of cells expressing EcR-DN's.

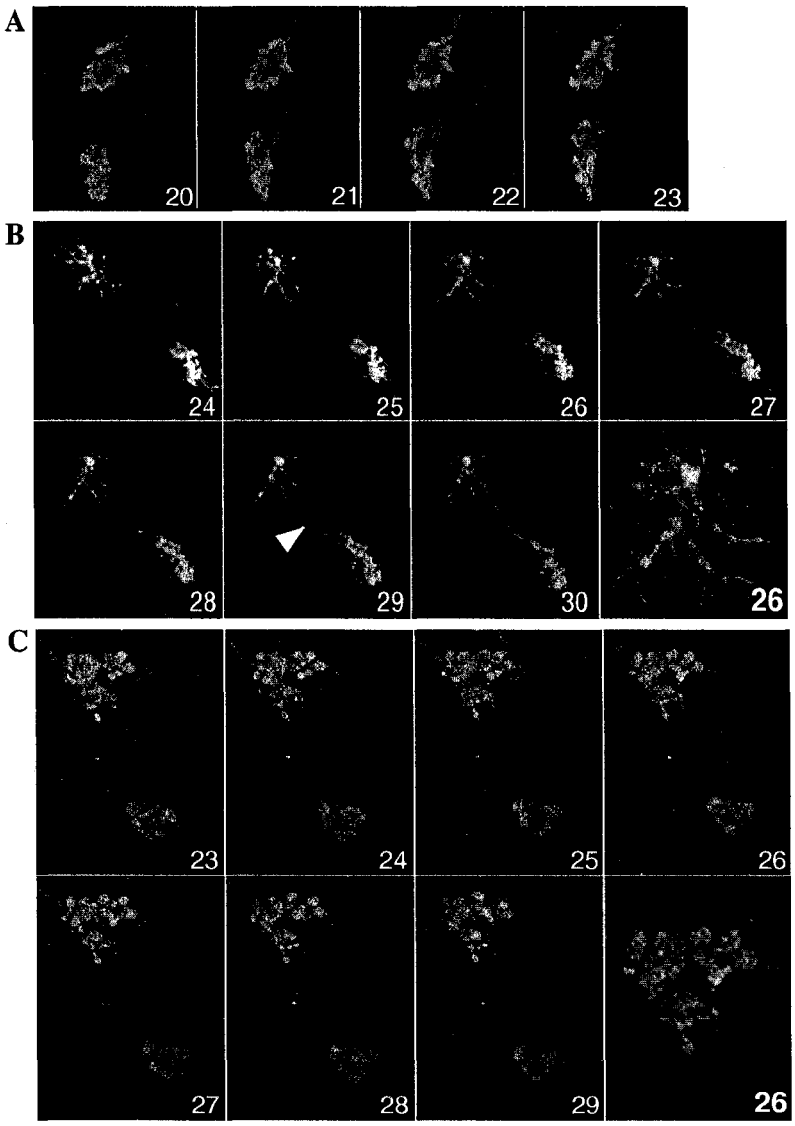


Figure 2.4. *Z-stack projections from time-lapse videos of axons from Tv cells expressing EcR-B1^{F645A} or EcR-B1^{W650A}.*
A) Z-stack time-lapse projection of 2 neurohemal organs (T2 above, T3 below) from cells expressingEcR-B1^{F645A} from 20 to 23 hours APF, 60 minutes apart. No filopodia are visible during this time period. B) Z-stack projections of remodeling axon arbors from cells expressing EcR-B1^{F645A}. From 24 to 30 hours APF, filopodia from the partially pruned T1 (above) fasciculate and stabilize with filopodia from T2 (arrowhead). Last panel is an enlarged still of T1 at 26 hours APF, showing filopodia. C) Z-stack time-lapse projections of axon arbors (T3 above, T2 below) from cells expressing EcR-B1^{W650A}, from 23 to 29 hours APF, with an enlarged still at 26 hours APF, showing no filopodia.

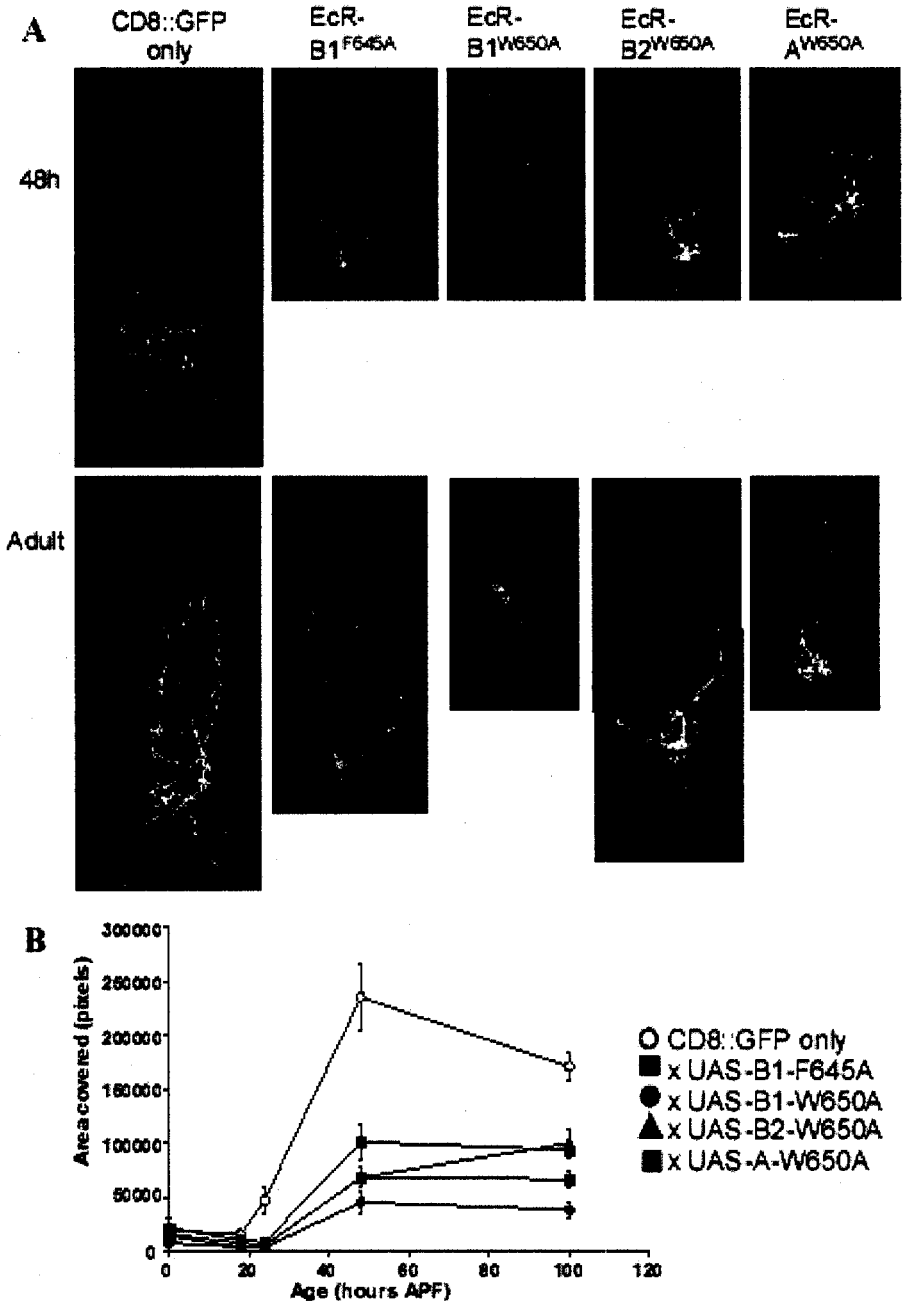


Figure 2.5. *Outgrowth in Tv axon arbors of cells expressing EcR-DN.*
A) Immunocytochemistry (α-SCP) of Tv cell axon arbors during outgrowth, for control cells and cells expressing EcR-DN. B) Comparison of arbor footprints from pupariation to adult. Arbor footprint was measured by taking a pixel count of the polygon created by connecting the outermost spread of the axon arbor.

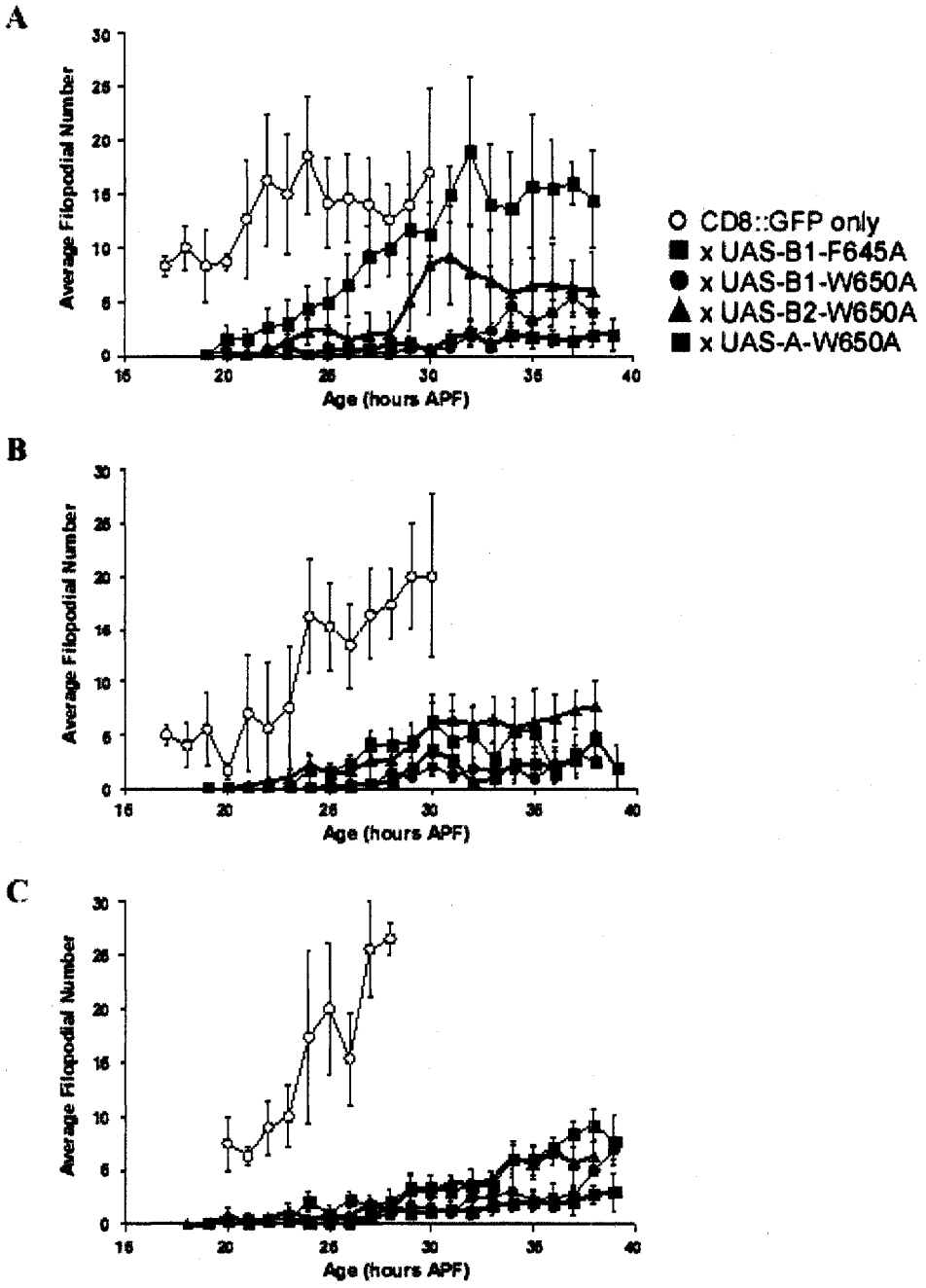


Figure 2.6. *Quantitative analysis of filopodial activity for Tv cell axons.* Average filopodial number per hour for control cells and cells expressing EcR-DN was calculated from live imaging data for T1 (A), T2 (B) and T3 (C).

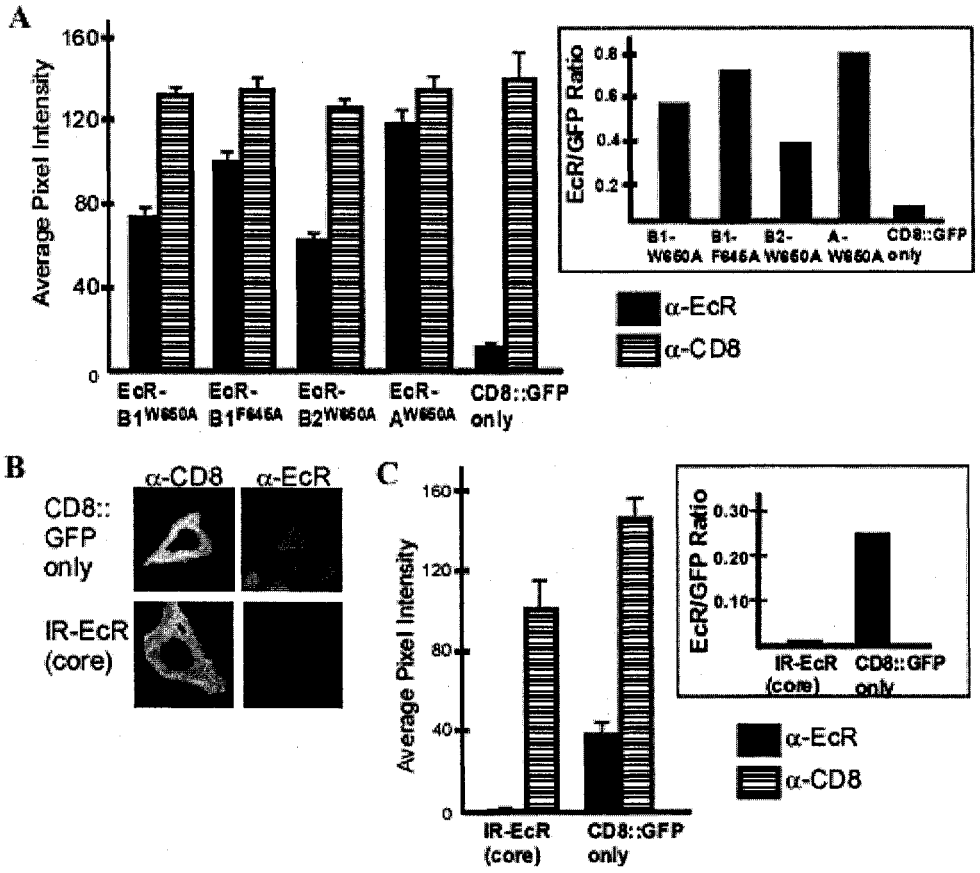


Figure 2.7. *Quantification of EcR (common) and CD8 immunoreactivity in Tv cell bodies.*

A) Cells expressing dominant negative, 24 hours APF. Inset shows the EcR to CD8 ratio for each genotype. B) Tv cell bodies, stained with EcR (common) antibody (right) and CD8 antibody (left). C) IR-EcR (core) expressing cells, late 3rd instar. Inset shows the EcR to CD8 ratio for each.

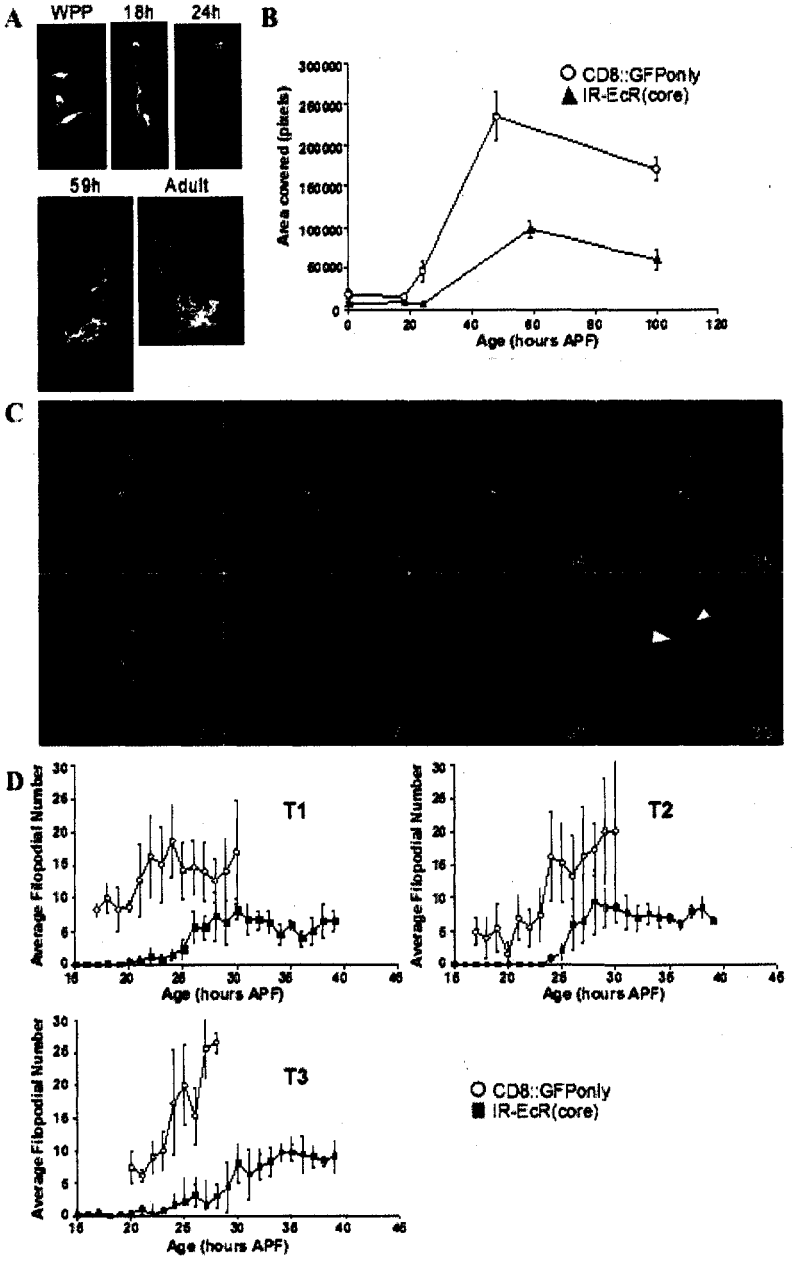
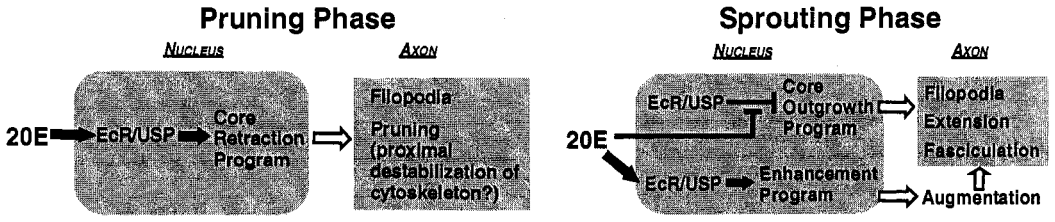


Figure 2.8. Pruning *and outgrowth in Tv cells expressing IR-EcR(core).*
A) SCP-labeled, fixed Tv cell axons from cells expressing IR-EcR(core)
B) Arbor footprint, from pupariation to adult for control cells and cells expressing IR-EcR(core). C) Live imaging montage of Tv axons (T1, T2 and T3; T1 at top) of UAS-IR-EcR(core) expressing cells, from 32 to 39 hours APF, 60 minutes apart. Filopodia are present and stabilize into adult-type branches (arrowhead), but the arbor retains a larval-like dense core (arrow). D) Quantification of filopodia on control cells and cells expressing IR-EcR(core).



	Filopodia	Pruning
EcR-RNAi	−	−
EcR-B1^{F645A}	−	−
EcR-B2^{W650A}	−	−
EcR-B1^{W650A}	−	−
EcR-A^{W650A}	−	−

	Core Program	Enhancement Program
EcR-RNAi	+	−
EcR-B1^{F645A}	+	−
EcR-B2^{W650A}	+	−
EcR-B1^{W650A}	−	−
EcR-A^{W650A}	−	−

Figure 2.9. *Model of EcR action and summary of the effects of EcR dominant negative and RNAi treatments during the pruning and sprouting phases of the Tv cell axons.*

Table 2.1. *Average pixel counts and s.e.m. for arbor area and arbor footprint measurements of cells expressing EcR-DN at 25° and 29°.*

	25°			29°		
Construct expressed	n	Arbor area ($x10^3$)	Arbor footprint ($x10^3$)	n	Arbor area ($x10^3$)	Arbor footprint ($x10^3$)
EcR-B1^{F645}	11	6.1±0.5	93.9±7.0	7	7.0±0.8	71.5±10.3
EcR-B1^{W650}	8	3.3±0.5	38.6±7.6	10	3.8±0.3	12.4±2.1
EcR-A^{W650A} TP5	16	5.5±0.5	66.4±6.7	7	4.1±0.6	44.2±6.0
EcR-A^{W650A} TP3	–	–	–	8	3.8±0.4	43.1±5.7
EcR-B2^{W650A} TP1	6	7.3±0.9	99.7±12.5	9	6.7±0.5	97.9±6.6
EcR-B2^{W650A} TP5	–	–	–	12	7.3±0.4	83.1±7.7

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Chapter 3

Altering the blend of EcR isoforms modifies pruning and outgrowth phases during neuronal remodeling

Introduction

Nuclear receptors play a vital role in regulating signaling pathways during development. Critical developmental triggers, such as steroids, are translated into cellular responses via binding of a ligand-specific nuclear receptor. For a given steroid, there may be multiple receptors coded by different genes and/or many receptor isoforms that act in quantifiably different ways. For example, thyroid hormone binds with high affinity to TR1 and TR2, both of which undergo alternative splicing to produce five different TR isoforms. Although coexpressed in some tissues, the TRα and TRβ genes are not functionally redundant. In mice, TRα is important in cardiac function, while TRβ is necessary for proper auditory development and neither can compensate for the loss of the other receptor (Zhang and Lazar, 2000). Additionally, TRβ2 can mediate transcriptional activation with or without hormone, while the other TRβ isoform requires hormone binding for activation (Yang and Privalsky, 2001). Differential expression of TRα mRNA and TRβ mRNA in the chick brain suggests that TRβ regulates early nervous system development and TRα regulates the later development of the nervous system. TRα is present in the cerebellum after the neuronal migratory phase, while TRβ is transcribed earlier in proliferating and migrating granule cells (Forrest et al., 1991). Receptor and receptor isoform specificity for particular cells, tissues and developmental processes is seen in many other members

of the nuclear receptor superfamily, making an investigation of isoform-specific functions critical to our understanding of how steroid hormones regulate development.

The primary regulators of post-embryonic neuronal development in *Drosophila* are the steroid hormone family of ecdysteroids. Ecdysone is produced by the ring gland, secreted into the hemolymph and converted into 20-hydroxyecdysone (20E) by peripheral tissues. In the metamorphosing nervous system, this ubiquitous signal may trigger neuronal cell death, arbor remodeling, or sprouting and outgrowth of branches depending on the type of neuron and the time of development. These cell-specific responses to 20E are driven by the ecdysone receptor complex.

The ecdysone receptor, EcR, has the same structure as the other receptors in the nuclear receptor superfamily. Nuclear receptors typically have an E domain that binds ligand, and contains a ligand-dependent activational region (AF2). At the N-terminus, nuclear receptors have a variable A/B domain with potential ligand-independent activation region, AF1 (Robinson-Rechavi et al., 2003). This AF1 region can selectively interact with cofactors to induce transcriptional activation. Additionally, the A/B regions of some nuclear receptors also contain a repressive domain that can suppress transcription (Lavery and McEwan, 2005; Mouillet et al., 2001). EcR forms a heterodimer with its partner, ultraspiracle (USP), and binds to ecdysone response elements (EcRE) located in regulatory sequences of DNA (Cherbas et al., 1991; Koelle et al., 1991; Yao et al., 1992). EcR, like many other nuclear receptors, functions as a transcription factor to regulate gene transcription via repression and activation. In the absence of ligand, EcR represses transcription of some genes, via binding of co-

repressors (Dressel et al., 1999; Schubiger and Truman, 2000; Tsai et al., 1999). After ecdysteroids bind to EcR, it undergoes a conformational change, releasing bound co-repressors and exposing co-activator docking sites that, when bound to co-activators, enhance transcription (Bai et al., 2000; Sedkov et al., 2003).

Translation of the 20E signal into cell specific responses is not only affected by the presence or absence of EcR, but also by the type of EcR isoform present in the cell. In *Drosophila melanogaster,* there are three EcR isoforms (A, B1 and B2) which differ in their N-terminal A/B domains (Talbot et al., 1993). In vitro studies show that EcR-B1 and EcR-B2 have strong activational functions in their A/B domains, while A may have a repressive function (Mouillet et al., 2001). In vivo, the presence of a particular EcR isoform in a cell directly impacts its response to 20E. Nuclear EcR-B1 is high preceding pruning of remodeling neurons, while EcR-A steadily increases as neurons sprout (Truman et al., 1994). In both the mushroom body (Lee et al., 2000) and the Thoracic-ventral neurosecretory neurons (Tv neurons) (Schubiger et al., 2003; Schubiger et al., 1998), dendritic remodeling is affected cell-autonomously by the particular EcR isoforms present in the cell. Eliminating EcR-B isoforms results in deficient pruning of these neurons; pruning can be effectively rescued by cell-autonomous expression of EcR-B1 or EcR-B2 but not EcR-A.

In a previous study, I expressed various dominant negative constructs of the EcR isoforms in the Tv neurons (Brown et al., 2006). Within each dominant negative, the AF2 domain was disabled (W650A mutation) while the A/B region was untouched and functional (Cherbas et al., 2003). Individual expression of the EcR-A^{W650A}, EcR-

B1^{W650A} and EcR-B2^{W650A} constructs resulted in severe pruning defects, indicating that activation via the ligand-dependent AF2 region is necessary for completion of pruning. However, outgrowth phenotypes were variable, with expression of EcR-A^{W650A} or EcR-B1^{W650A} resulting in deficient outgrowth and EcR-B2^{W650A} giving only moderate defects in sprouting and outgrowth (Brown et al., 2006). In addition to suggesting that the EcR AF1 region impacts the neuron's remodeling response, these results specifically implicate the EcR-B2 AF1 region in affecting sprouting and outgrowth as well as its previously known role in pruning.

In this study, I set out to directly assess the affects of each of the EcR isoforms on neuronal remodeling, using both overexpression and knockdown of specific isoforms in the Tv neurons. I found that the timing of pruning can be accelerated by overexpression of each EcR isoform, while the ability to change the timing of outgrowth varied amongst the isoforms. Additionally, results suggested a possible role for EcR-B1 in maintaining Tv cell plasticity during remodeling, and a new function for EcR-B2 in regulating transcriptional repression.

Materials and Methods

Fly Stocks

The UAS-[IR]EcR-B1 construct was made by amplifying cDNA fragments, then cloning in the pUAST vector in both reverse and direct orientations. The construct consisted of a 597-bp fragment between positions 1110 and 1707 relative to the EcR-B1 cDNA sequence repeated in a head-to-head arrangement. Transgenic flies were

created using a w^{1118} recipient stock (Roignant et al., 2003). The UAS-EcR-A, UAS-EcR-B1 and UAS-EcR-B2 transgenic flies were made by cloning cDNA of each isoform into the pUAST vector, followed by P-element mediated transformation (Lee et al., 2000). For each experiment detailed above, one UAS-EcR construct was co-expressed cell-autonomously with membrane bound GFP in the Tv neurosecretory cells using the Gal4-UAS system (Brand and Perrimon, 1993). This was accomplished by crossing the UAS-EcR construct stock with ywUASmCD8::GFP; FG10, a stock containing a membrane-targeted UAS-GFP insert (Lee and Luo, 1999) and a FMRF-amide promoter driving GAL4 (Schubiger et al., 2003; Suster et al., 2003).

Immunocytochemistry

Antibody staining procedures were described in Brown et al, 2006. Central nervous systems were dissected and fixed for 30 minutes in 4% paraformaldehyde, rinsed several times in phosphate buffered saline with 1% Triton X-100, and blocked with 5% normal donkey serum for 15 minutes. Tissue was incubated in primary antibody overnight; antibodies used were monoclonal mouse anti-SCP (Masinovsky et al., 1988), rat anti-mCD8 (1:200, CALTAG), mouse anti-EcR (1:100; IID9.6, common) mouse anti-EcR-A (1:100; 15G1A ascites), and mouse anti-EcR-B1 (1:100; AD4.4) (Talbot et al., 1993). Secondary antibody was used 1:200 in PBS-Tx 1% (Texas red conjugated donkey anti-mouse IgG or FITC conjugated donkey anti-rat IgG from Jackson Immunoresearch, West Grove, PA). Nervous systems were rinsed several times in PBS and attached to polylysine-coated coverslips, then dehydrated, cleared in xylene and mounted in DPX (Fluka, Seelze, Germany). To quantify levels

of EcR or mCD8 immunoreactivity, all genotypes were dissected, antibody stained and imaged as one batch.

Staging and Imaging

Animals were collected in the white puparium stage and maintained at 29° C. After staining and mounting, slides were imaged on Bio-Rad Radiance 2000 confocal microscope.

Analysis

Larval neurohemal site footprint, axon tip number, larval neurohemal site presence and quantification of EcR and mCD8 immunoreactivity were calculated using NIH ImageJ, and all analysis (except for EcR quantification) was done double-blind. EcR quantification was calculated as described in Brown et al, 2006. Larval footprint was calculated similarly to the whole arbor footprint described in Brown et al, 2006, but the polygon formed encased the remaining larval arbor, excluding the extended neurites. Larval arbor was determined by the appearance of a dense bundle of branches wound together and located immediately dorsal of the axon stalk emerging from the neuropil, rather than long, single branches that were characteristic of the adult arbor. Often, single neurites characterized as adult branches extended from densely bundled central larval arbor. These branches were not included in the larval arbor measurement as a polygon was drawn encompassing only the dense central arbor.

Axon tip number was calculated by counting only the free ends of neurites on each nervous system preparation. Branch intersections at 24 hours AFP were calculated in the illustrator program Canvas 9.0 by overlaying a set grid over the axonal arbor and

counting the number of times gridlines were crossed by branches. Branch nodes were calculated by counting the number of branch bifurcations or branch junctions (Fig. 3.5). At 48 hours and in the adult, branches exiting a rectangular box of set size were counted, as were the number of branch nodes contained inside the box. This box was centered over the Tv axon stalk in the T2 neuromere (Fig. 3.6). All error bars on graphs show standard error. To calculate statistical significance for the analyses above, a non-paired t-test (α=0.05) was run using SigmaStat and SPSS software.

Results

Axonal pruning and outgrowth in the Tv neurons

The Thoracic-ventral (Tv) neurosecretory neurons are a paired set of neurosecretory cells located in each thoracic neuromere of the *Drosophila* central nervous system (Nassel et al., 1988; Taghert and Schneider, 1990). Each neuron has a sparse dendritic projection in the neuropil, and an axonal projection that extends dorsally from the dendrites and wraps around two support cells, forming one globular neurohemal site per thoracic neuromere in the larva (Nassel et al., 1988). During metamorphosis, these neurohemal structures prune via retraction of the terminal axonal arbor, and are replaced by a more extensive, branched, adult axonal arbor. Pruning of the larval arbor begins around 10 hours after puparium formation (APF; at 29° C), starting with the most anterior neurohemal site and finishes around 24 hours APF with complete pruning of the most posterior neurohemal site (Brown et al., 2006). New branches form from a new growth zone below the pruning larval arbor at

approximately 18 hours APF. These branches extend and fasciculate, eventually becoming the adult axonal network that stretches across the dorsal surface of the CNS (Fig. 3.1B). After 48 hours APF the adult axon structure is essentially formed, changing little before eclosion. In the previous study, live imaging of the remodeling Tv axons revealed a high amount of filopodial activity beginning around 5 hours APF and continuing through pruning and branching phases until approximately 56 hours APF (Brown et al., 2006).

Quantification of EcR protein levels

Expression of EcR-A, EcR-B1 and EcR-B2 constructs using the Gal4-UAS system resulted in increased levels of EcR protein relative to control animals at developmentally relevant times in the Tv cell nucleus. Since I intended to change the proportions of EcR isoform within the cell (raising the levels of each isoform when they would normally be low), I needed to confirm that the isoform constructs were highly expressed at these times. Using an EcR common antibody, I examined expression of the EcR-B1 and EcR-B2 constructs at 20 hours APF at 29°C. At this time, the endogenous levels of the EcR-B1 isoform in the Tv neurons were very low and the axons were almost completely pruned (Truman et al., 1994). Expression of either the EcR-B1 or the EcR-B2 constructs gave 8 to 10 times the relative total EcR levels in the nucleus at 20 hours APF (Fig. 3.2A). At pupariation, endogenous EcR-A levels are low, but expression of the EcR-A construct resulted in approximately 12 times the normal EcR-A levels in the nucleus at pupariation when examined using the EcR-A antibody (Fig 3.2B). Measurements of EcR-B1 levels in the nucleus just before

pupariation when endogenous EcR-B1 levels should be high showed that expression of the inverted repeat EcR-B1 construct (IR-EcR-B1) reduced EcR-B1 to a quarter of its normal expression (Fig. 3.2C). Since the EcR isoforms have been reported to affect each others' transcription, I also measured the EcR-B1 level in cells overexpressing EcR-A and found that EcR-B1 immunoreactivity in the nucleus was not different from control animals at pupariation (not shown).

Effects of EcR isoform overexpression on pruning of Tv axons

As larval neurons progress through pruning and outgrowth phases during metamorphosis, the blend of EcR isoforms present in the nucleus changes. At pupariation, just prior to the pruning phase, the Tv neurons show high levels of EcR-B1, and low EcR-A. EcR-B1 levels then drop sharply, followed by a slow increase of EcR-A starting at around 24 hours APF (Truman et al., 1994). Because of these very specific changes in the type of EcR isoforms present during remodeling, I wanted to alter the proportions of the isoforms and examine the effects on the timing and final result of pruning and outgrowth phases. To do this, I used the Gal4-UAS system to separately express each EcR isoform construct along with membrane bound GFP specifically in the Tv neurons. Each isoform construct was expressed throughout larval life, but no effects were seen prior to the start of pruning (Fig. 3.3, 0 hours APF). During the pruning phase, however, overexpression of either EcR-B1 or EcR-B2 resulted in accelerated pruning. At 18 and 20.5 hours APF, most of the larval neurohemal sites were pruned in neurons overexpressing either B isoform, while remnants of larval arbors still remained in the controls (Fig. 3.3). This accelerated

pruning was quantified by blind scoring of axon terminals with a larval neurohemal site. Neurons overexpressing either EcR-B1 or EcR-B2 had a much lower percentage of remaining larval arbors from 15.5 to 24 hours APF than neurons of control animals (Fig. 3.4A). Larval arbor area as calculated by measuring the footprint of the persisting neurohemal site was also consistently and significantly smaller than those of control animals (Fig. 3.4B). Overexpression of EcR-A also resulted in somewhat accelerated loss of the larval arbors (Fig. 3.4A, although its effect was less than that seen with expression of the EcR-B constructs (Fig. 3.4B). All constructs expressed resulted in complete pruning of the Tv cell axons and was fastest in cells overexpressing EcR-B1 and EcR-B2.

Effects of EcR isoform overexpression on outgrowth of Tv axons

After pruning, the Tv neurons shift over to a primarily outgrowth-oriented remodeling phase, during which EcR-B1 isoform is low and EcR-A is steadily increasing (Truman, 1990; Truman et al., 1994). Expression of the EcR isoform constructs altered the timing of outgrowth. Overexpression of EcR-B2 resulted in the early appearance of adult branches starting at 15.5 hours APF. This is reflected in significantly more branch tips per nervous system, from 15.5 to 24 hours APF (Fig. 3.5B). When a grid analysis was done (Fig. 3.5A) branch nodes at 24 hours APF were also higher, as were the number of branches intersecting the grid (Fig. 3.5B). The branch-grid intersections were significantly higher for animals overexpressing EcR-B2 than control animals or animals overexpressing EcR-B1 or EcR-A (Fig. 3.5A). However, this accelerated sprouting of branches and increased branch size during

outgrowth did not affect the final adult arbor (Fig. 3.6). At 48 hours APF and in post-eclosion adults, I counted the number of branches crossing the T2 positioned box (Fig. 3.6B). Overexpression of EcR-B2 gave no significant differences from controls for the total branches exiting the box at 48 hours (Fig. 3.6C) or in the adult (Fig. 3.6D), and number of nodes in the box in the adult (Fig. 3.6D). At 48 hours APF, the number of nodes within the box for neurons overexpressing EcR-B2 was lower (Fig. 3.6C).

Unlike EcR-B2, overexpression of EcR-B1 or EcR-A did not result in any noticeable sprouting of branches at early time points (Fig. 3.3). At 15.5 and 18 hours APF, the average number branch tips were not significantly different from control animals (Fig. 3.5B). At 20.5 and 24 hours though, overexpression of either EcR-B1 or EcR-A gave a higher average number of branch tips (Fig. 3.5B). Both overexpression of EcR-B1 and EcR-A resulted in a higher number of branch nodes at 24 hours APF, but had a normal number of branch-grid intersections, indicating that although sprouting of new branches was increased, branches formed were not extending more than control animals (Fig. 3.5C). Adult axon arbors of Tv neurons overexpressing EcR-A had normal numbers of branches exiting the box at 48 hours APF and in the adult, and had normal numbers of nodes at 48 hours APF, but lowered numbers of nodes in the adult (Fig. 3.6D). However, overexpression of EcR-B1 resulted in a lower number of nodes at 48 hours APF in the adult, and fewer branches exiting the box in the adult, indicating the formation of a less complex adult arbor (Fig. 3.6D).

Effects of EcR-B1 knockdown on pruning and outgrowth

Since overexpression of EcR-B1 resulted in both a temporal change in pruning and a less complex adult arbor, I decided to examine axonal pruning and outgrowth in Tv neurons with reduced EcR-B1. I used the Gal4-UAS system to express an inverted repeat construct of EcR-B1; this construct specifically knocked down EcR-B1 levels in the cell (Roignant et al., 2003). The expression of the UAS-IR-EcR-B1 construct resulted in a 75% decrease in levels of EcR-B1 immunoreactivity in the nucleus of the Tv neurons (Fig. 3.2C). In Tv neurons with reduced EcR-B1, larval neurohemal sites abnormally persisted past 24 hours APF, indicating a delay in pruning (Fig. 3.7A). The percentage of axon terminals with larval arbors was much higher in neurons expressing the EcR-B1 inverted repeat construct, and the average larval arbor size was increased relative to controls, although only significantly at 15.5 hours APF (Fig. 3.7B, C). Despite the persistent larval neurohemal sites, the timing of sprouting in EcR-B1-deficient Tv axons appeared relatively normal, with branches starting to appear around 18 hours APF. At 24 hours however, arbors showed increased nodes and branch-grid intersections (Fig. 3.7D). These increases in branching and extension did not continue into adult maturation of the arbor, since adult arbors were heavily branched (Fig. 3.8A), and showed no effects when total branches exiting the box and total nodes in the box were measured (Fig. 3.8B). However, nodes could not be counted in several adult arbors due to a concentration of branches in the central area of the arbor and these clusters appeared to be a remnant of the unpruned larval arbor.

Discussion

The N-terminal region of nuclear receptors can be highly variable, resulting in multiple isoforms due to alternative splicing or separate promoter regions. This A/B domain can have both activation and repression domains, interacting with specific cofactors to regulate transcription (Lavery and McEwan, 2005). Differential expression of nuclear receptor isoforms combined with isoform-specific cofactors can translate ubiquitous hormone signals into very specific tissue and cell specific responses. This mode of action is seen in both vertebrate and invertebrate nervous system development (Mangelsdorf et al., 1995). The common structure of nuclear receptors in both groups may allow application of invertebrate studies to inform the direction of further research on vertebrate receptors.

In earlier studies of the EcR isoforms, expression of EcR-B1 and EcR-B2 most effectively rescued pruning defects in both the mushroom body axons and the Tv cell dendrites of EcR-B mutant animals, with expression of EcR-B2 giving the greatest response and EcR-A giving the least (Lee et al., 2000; Schubiger et al., 2003). Expression of EcR-B2 was able to rescue the Tv dendrite pruning defects in 66% of the animals, while EcR-B1 was able to rescue pruning defects only 38% of the time and expression of EcR-A rescued 1 in 20 animals (Schubiger et al., 2003). The ability of both EcR-B isoforms to effectively rescue pruning suggested that EcR-B1 and EcR-B2 may function redundantly in this capacity. Expression of EcR-A resulted in limited rescue, indicating it may not play a role in pruning. This data correlates with the

increased EcR-B1 levels in remodeling neurons just prior to the pruning phase (Truman et al., 1994).

EcR AF2 domain and remodeling

The ability of the EcR-B isoforms to drive pruning in remodeling neurons was seen in this study as well. In control animals, the Tv axons began to prune around 10 hours APF at 29°C, and were almost finished by 24 hours APF. While overexpression of each of the isoforms was able to accelerate the pruning program in these cells, overepression of EcR-B2 resulted in the strongest pruning increase, followed by EcR-B1. Since expression of EcR-A did accelerate pruning, albeit to a lesser extent than the B isoforms, I conclude that the ligand-dependent activation region (AF2) common to all the EcR isforms is essential for directing pruning. Prior research expressing EcR dominant negative constructs in the Tv neurons showed that expression of EcR-DN constructs with a poisoned AF2 domain effectively blocked most pruning in Tv cell axons (Brown et al., 2006). However, overexpression of the EcR-B isoforms, particularly EcR-B2, was more effective than expression of EcR-A in driving axonal pruning. This difference in efficacy between the isoforms underlines the isoform-specific requirements for pruning seen in previous studies. Therefore, I propose that the AF1 region of EcR-B1 and EcR-B2 act in concert with the AF2 region to drive correct and timely pruning of the Tv axons.

Role of EcR-B isoforms in pruning

In contrast to the earlier rescue experiments by Lee et al., 2000, and Schubiger et al., 2003, our experiments reducing EcR-B1 levels with the inverted repeat EcR-B1

construct may point to an overlapping, but not entirely redundant role for the B isoforms in pruning. In neurons that had reduced EcR-B1 levels, axons did undergo some pruning, but it was delayed and incomplete. The adult arbor appeared to retain some remnants of the larval neurohemal sites. At 20.5 and 24 hours APF, adult branches emerged from the retained larval arbor rather than below in the growth zone, indicating that the intensely immunostained central arbor clumps seen at 48 hours APF and in adults were arising from incompletely pruned larval arbors. This may indicate that the presence of EcR-B1 itself is necessary for complete pruning, and that EcR-B2 alone cannot direct complete pruning. However, eliminating EcR-B1 did not eliminate pruning altogether. Endogenous EcR-B2 must be able to drive some pruning by itself. This is supported by the ability of EcR-B2 to elicit the strongest pruning response in the Tv axons out of the EcR isoforms expressed, as well as the rescue data from the studies on Tv dendrites and mushroom body axons (Lee et al., 2000; Schubiger et al., 2003).

Role of EcR isoforms in Tv axon outgrowth

Effects of isoform overexpression on the outgrowth phase of remodeling were more complex. Overexpression of EcR-A or EcR-B1 caused early branch formation, but only after 20.5 hours APF. At 15.5 and 18 hours APF, when the axons are still undergoing pruning, excess EcR-A and EcR-B1 in the cell could not force early entrance into the outgrowth phase. This may mean that once the neuron switches over to a primarily outgrowth oriented phase after 20 hours APF, a nuclear increase in EcR can enhance branch formation. Interestingly, this coincides approximately with the

beginning of the large ecdysteriod pulse during metamorphosis, triggering maturation in remodeling neurons (Riddiford, 1993; Truman, 1990). The switch to the outgrowth phase may be partially regulated via derepression of EcR. Our prior experiments reducing total EcR in the cell via an EcR-RNAi construct suggested that EcR derepression is sufficient for moderate outgrowth in the Tv neurons (Brown et al., 2006). When the inverted repeat construct of the EcR common region was expressed in the Tv neurons, moderate filopodial activity and adult branch formation was seen, although it was reduced relative to controls. This moderate phenotype was also seen with expression of the EcR-B2 dominant negative construct (EcR-B2^{W650A}), but expression of the EcR-B1 dominant negative construct (EcR-B1^{W650A}) severely retarded outgrowth (Brown et al., 2006). From an in vitro study by Mouillet et al., 2001, EcR-A is known to have an inhibitory function in it's A/B region. Based on our dominant negative expression data I suggested that the N-terminus of EcR-B1 may likewise have a repressive domain as well as an AF1 activation domain. Before 20 hours APF, the repressive domain of EcR-A and B1 may function to regulate the beginning of the outgrowth phase by suppressing branch formation until the ecdysteroid titer begins to rise and bind to the receptor, both relieving repression and triggering activation through the ligand-dependent activation domain.

Axonal arbors of neurons overexpressing EcR-B1 were abnormal at 48 hours and in the adult, showing fewer branches and branch nodes. It was suggested previously that EcR-B1 may be associated with maintaining neurons in an immature, plastic state (Truman et al., 1994). The imaginal neurons of the optic lobes express high levels of

EcR-B1 starting early in metamorphosis and continuing until approximately 50 hours APF. These are the only neurons that do not prune but nevertheless express EcR-B1. It is thought that EcR-B1 expression in these neurons may be related to the progressive development of the retina. As the optic lobe develops, the morphogenetic furrow induces differentiation of the retina, followed by asynchronous innervation of the lamina by the retinal axons (Selleck and Steller, 1991). This developmental period extends from 10 to 50 hours APF, during which these neurons are establishing their connections within the optic lobe. However, the asynchronous development of the retina means that optic lobe neurons must delay maturation despite increasing ecdysteroid levels. High EcR-B1 in these neurons may help maintain their plasticity regardless of the hormonal signal (Truman et al., 1994). In the Tv neurons, the axons are still undergoing pruning of larval arbors at 18 hours APF as new branches are beginning to sprout. This overlap of pruning and outgrowth phases may reflect temporary plasticity of the Tv neurons, as its axonal cytoskeleton is at once being disassembled and reassembled. The decrease in complexity as seen by lowered nodes and branches in adult neurons with excess EcR-B1 may be due to B1 extending this immature state past its normal developmental time, resulting in instability of adult branches (Fig. 3.9).

Unlike EcR-B1 or EcR-A, overexpression of EcR-B2 shifted the outgrowth phase to the earliest time point measured. At 15.5 hours APF, axonal branching from the growth zone at the base of the larval neurohemal site was clearly seen in immunostained nervous systems overexpressing EcR-B2, and there were significantly

more branch tips than in controls. Why is the overexpression EcR-B2 more effective than the other EcR isoforms at inducing early outgrowth? One possibility is the strength of the EcR-B2 AF1 activation domain. However, expression of EcR A/B regions in yeast cells showed that the EcR-B1 and EcR-B2 A/B regions were similar in their activation potentials, with EcR-B1 being a slightly more effective activator (Mouillet et al., 2001). Furthermore, the A/B region of EcR-B2 showed a much reduced activation potential when expressed in HeLa cells. Similar results were found when the A/B regions of EcR were tested in L57-3-11 cells, a *Drosophila* cultured cell line lacking EcR (Hu et al., 2003). An increased activation function for the AF1 region of EcR-B2 also does not account for the results seen in our previous experiments with EcR dominant negative. Dominant negative constructs made in EcR-A or EcR-B1 (EcR-A^{W650A} and EcR-$B1^{W650A}$) suppressed most outgrowth, whereas EcR-$B2^{W650A}$ did not (Brown et al., 2006). Since the EcR W650A amino acid substitution results in a receptor that cannot bind ligand and therefore remains bound to corepressors, it follows that the EcR-$B2W^{650A}$ construct cannot bind corepressors initially, resulting in a receptor that does not repress. This hypothesis is supported by the similar phenotype of neurons expressing EcR-$B2^{W650}$ to those expressing EcR-RNAi, and also fits with the data from Tv neurons overexpressing EcR-B2. Therefore, a lack of repression due to an excess of EcR-B2 may allow early initiation of the outgrowth program in the Tv axons, while overexpression of EcR-A or EcR-B1 continue to suppress outgrowth until the 20E titer increases to relieve repression.

In our model, I propose that activation through the ligand-binding domain of EcR is necessary for pruning, acting in concert with the AF1 regions of EcR-B1 and EcR-B2 (Fig. 3.9). EcR-B1 promotes an immature, plastic state of the Tv cell axons, which normally occurs during development as the pruning and outgrowth phases overlap. The outgrowth phase can be triggered by derepression of EcR and augmented by EcR activation; expression of EcR-B2 may cause loss of repression and an early entry into the outgrowth phase. These in vivo experiments indicate that the AF1 region of nuclear receptors can be essential in regulation of both the timing and outcome of developmental events in the nervous system, and argue for a closer examination of this complex region in both EcR and vertebrate nuclear receptors.

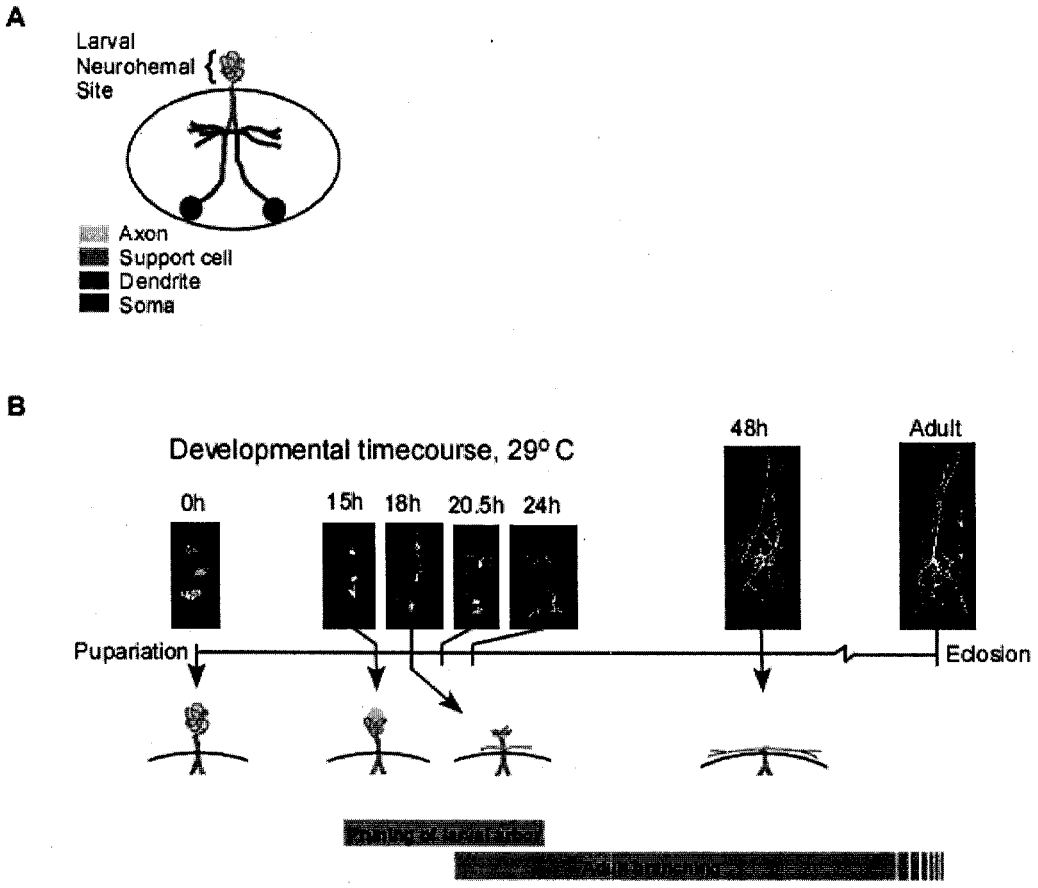


Figure 3.1. *Remodeling of the Tv neurosecretory neurons.*
(A) Schematic transverse section of the nervous system, showing the axons, dendrites and cell bodies of a pair of Tv neurons, before metamorphosis. The axons extend above the dorsal surface of the CNS and wrap around a pair of support cells to form the larval neurohemal organ. 1A modified from Brown et al., 2006. (B) Dorsal view of the Tv axons as they undergo remodeling during metamorphosis, progressing first through a pruning phase, then an outgrowth phase. Nervous systems were immmunostained with anti-SCP. The lower schematic is a transverse section of the Tv axons, showing the retraction of the larval axonal arbor and sprouting of the adult axonal arbor from the growth zone under the pruning larval arbor.

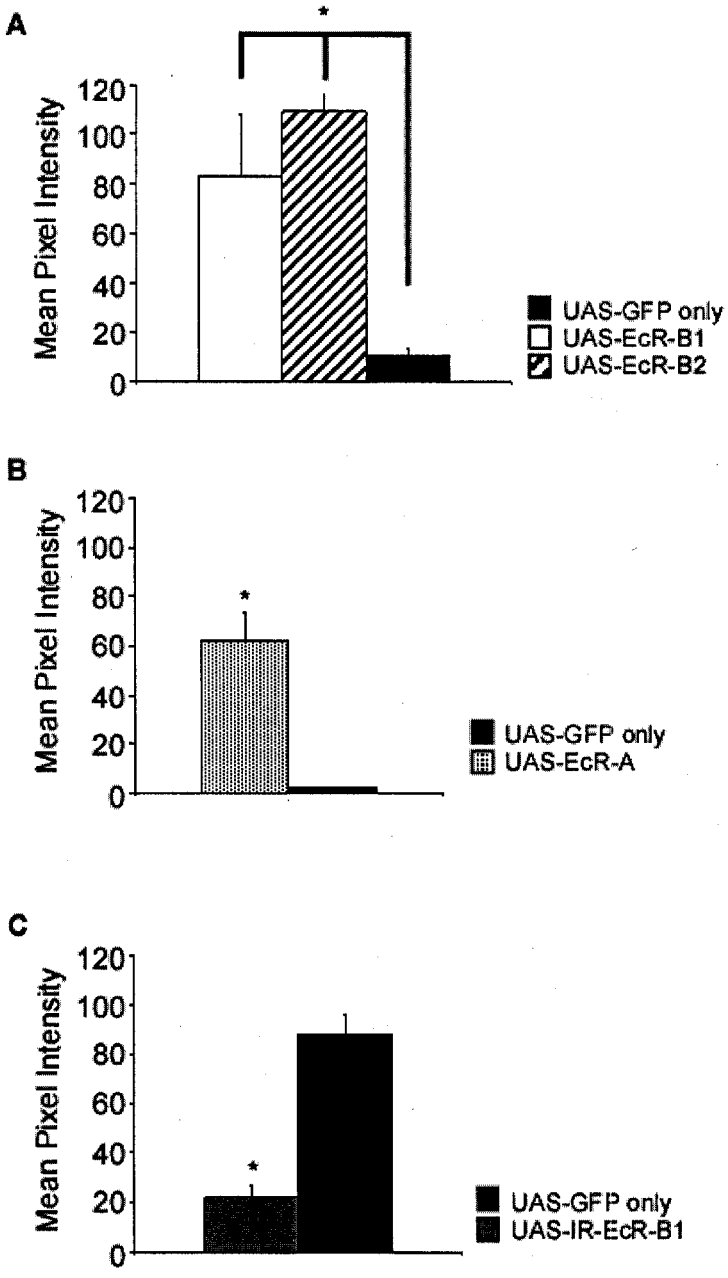


Figure 3.2. *Quantification of EcR levels in nuclei of Tv neurons.*
(A) anti-EcR common immunoreactivity of control Tv cells and Tv cells overexpressing either EcR-B1 or EcR-B2, measured at 20 hours APF. (B) anti-EcR-A immunoreactivity of control Tv cells and Tv cells overexpressing EcR-A, measured at pupariation. (C) anti-EcR-B1 immunoreactivity of control Tv cells and Tv cells expressing IR-EcR-B1, measured just before pupariation at the enlarged salivary gland stage. Asterisks indicate statistical significance.

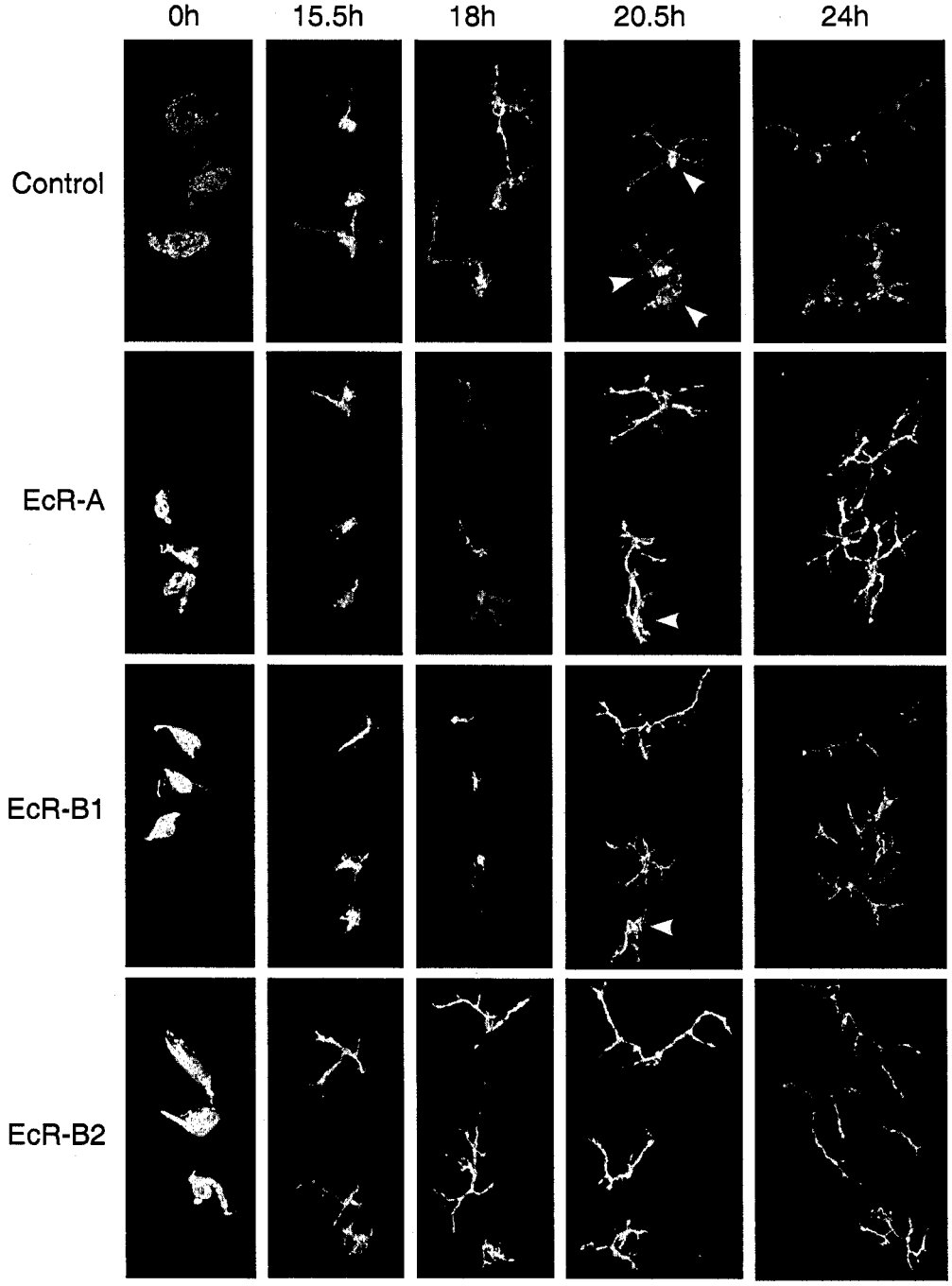


Figure 3.3. *Immunocytochemistry of Tv cell axons for control neurons and neurons overexpressing EcR-A, EcR-B1 or EcR-B2 from 0 hours APF to 24 hours APF.* Fixed and immunostained (anti-SCP) nervous systems shown as Z-projections, oriented anterior up. Arrowheads at 20.5h point to remaining larval neurohemal sites.

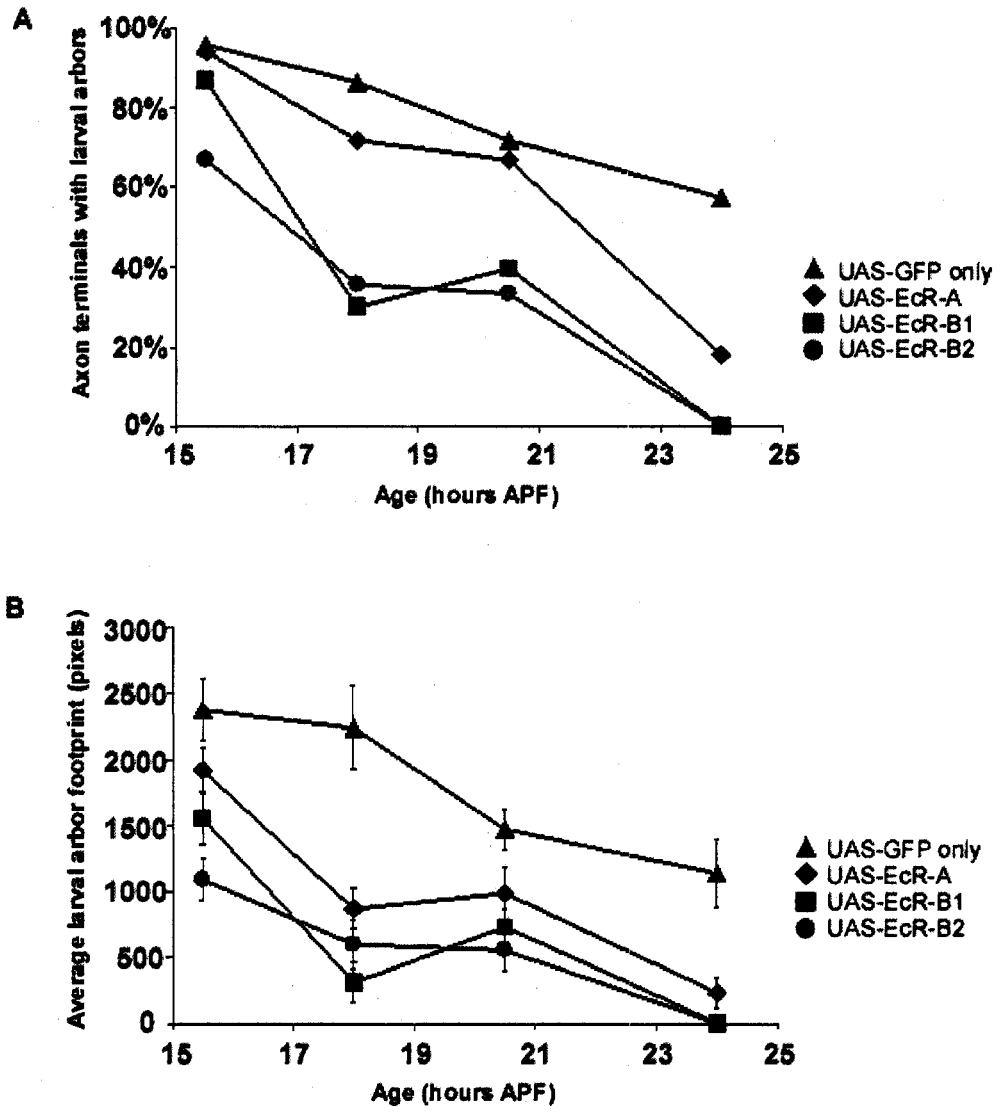
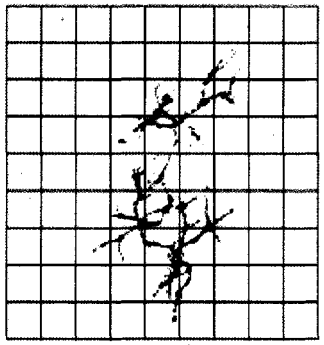


Figure 3.4. *Analysis of pruning in Tv neurons overexpressing EcR isoforms.* (A) The percentage of Tv axon terminals that contain a complete or partial larval neurohemal arbor. (B) Average larval neurohemal site size as calculated by measuring the footprint of the larval arbor. All treatments yield statistically significantly lower footprints than control animals, except Tv neurons expressing EcR-A at 15.5 hours APF.

Figure 3.5. *Analysis of outgrowth in Tv neurons overexpressing EcR isoforms.* (A) To analyze Tv cell axons at 24 hours APF, a grid was placed over the confocal image. Branches intersecting the vertical and horizontal gridlines were counted. (B) Average number of axon branch tips per nervous system. Tv neurons overexpressing EcR-B2 have significantly higher numbers of tips at all time points, while neurons overexpressing EcR-B1 have significantly higher tip numbers at 15.5, 20.5 and 24 hours APF. Neurons overexpressing EcR-A have significantly higher tip numbers at only 20.5 and 24 hours APF. (C) Average number of total nodes per nervous system and total branch-grid intersections at 24 hours APF. Asterisks indicate statistical significance.

A



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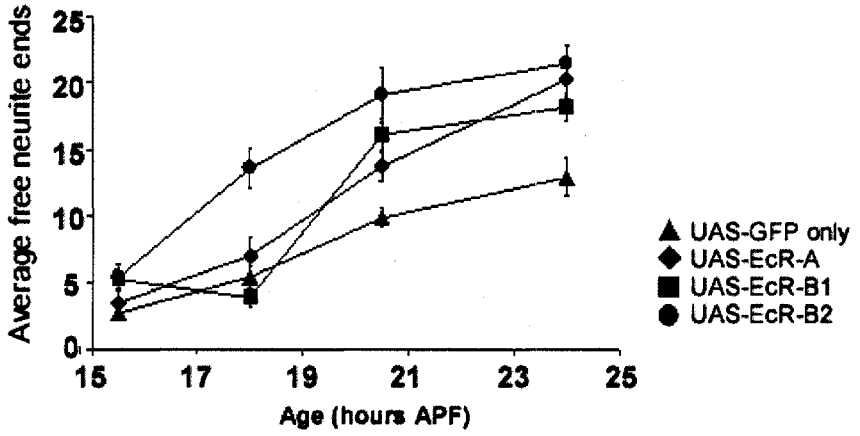
Average free neurite ends
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10
5
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Age (hours APF)
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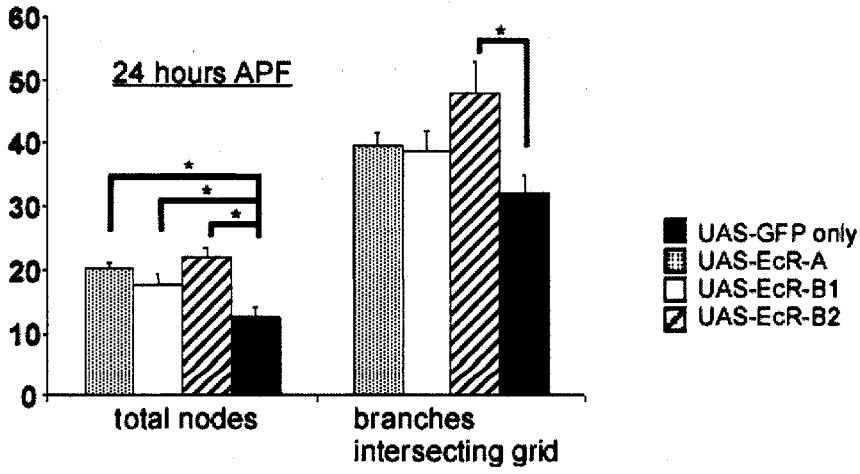
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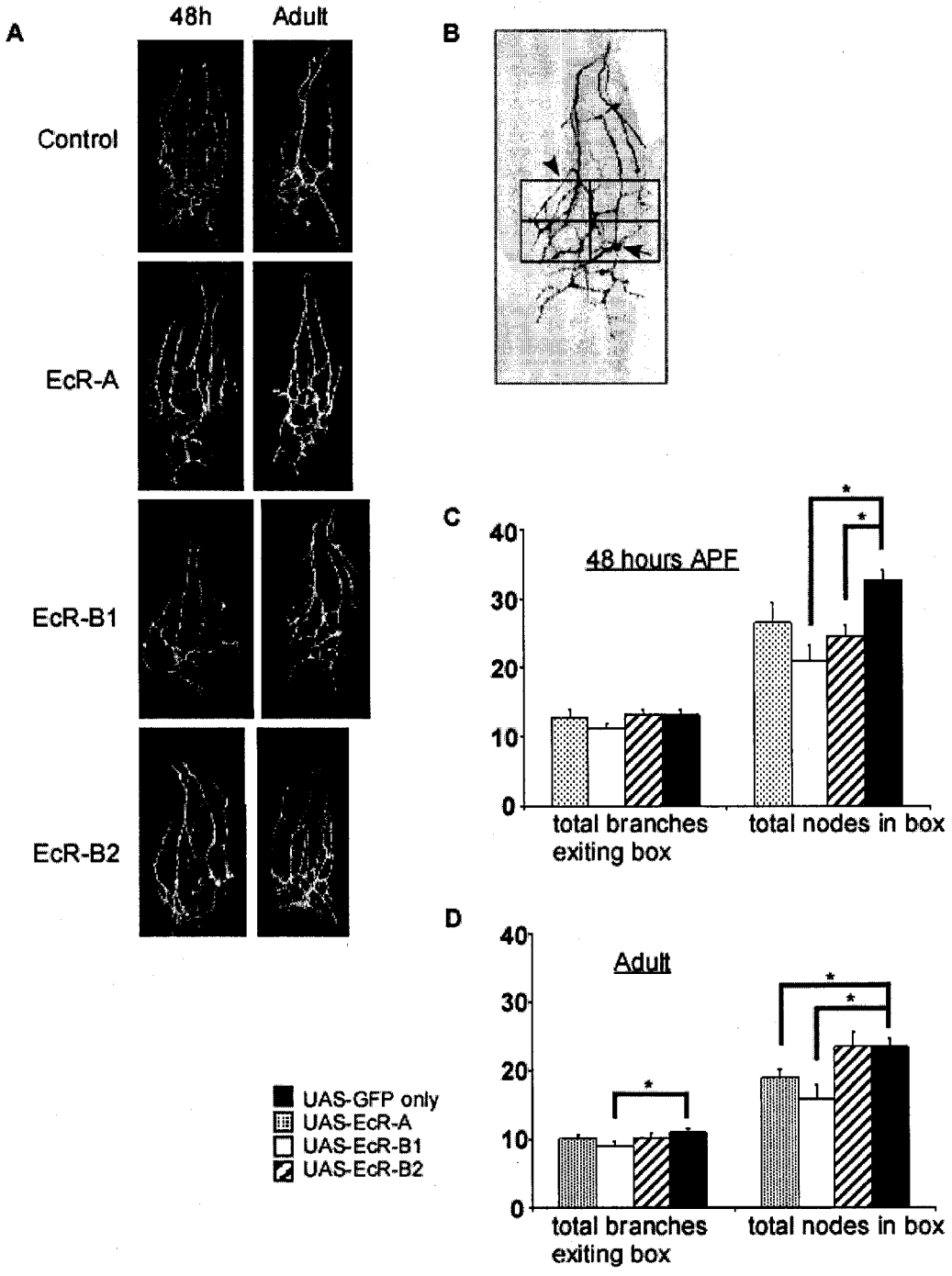
UAS-EcR-B1
UAS-EcR-B2

Figure 3.6. *Analysis of outgrowth in Tv neurons at 48 hours APF and in the adult.* (A) Fixed and immunostained (anti-SCP) nervous systems for controls and animals expressing EcR-A, EcR-B1 and EcR-B2. (B) To analyze Tv cell axons at 48 hours APF and in the adult, a box of set size was centered above the T2 axon stalk, and nodes inside the box (arrow) as well as branches crossing the perimeter of the box (arrowhead) were counted. (C) Average number of branches exiting the box and average number of branch nodes within the box at 48 hours APF. (D) Average number of branches exiting the box and average number of branch nodes within the box in the post-eclosion adult. Asterisks indicate statistical significance.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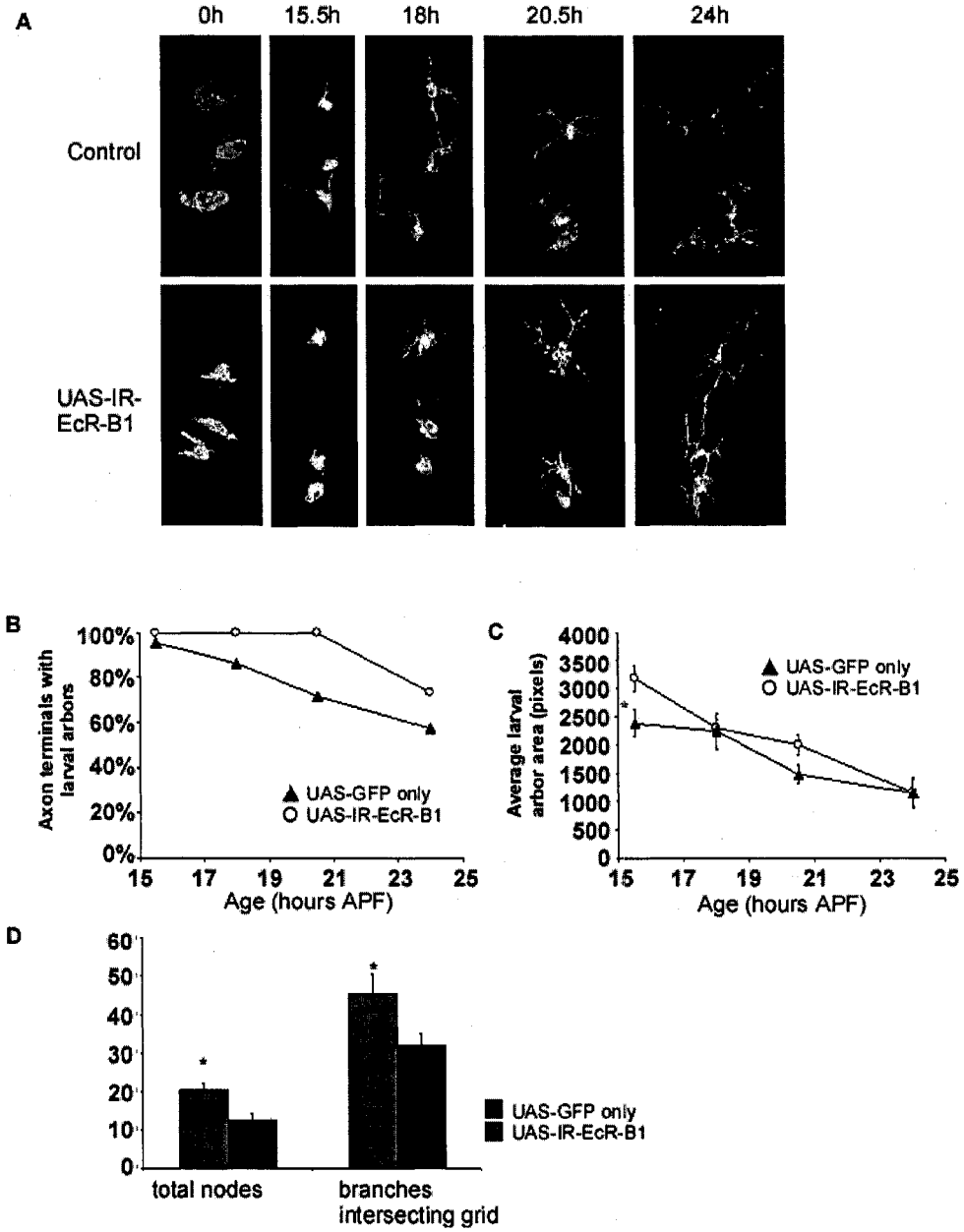

Figure 3.7. *Axons of Tv neurons expressing the inverted repeat EcR-B1 construct (IR-EcR-B1), imaged from 0 to 24 hours APF.*
(A) Anti-SCP immunostained Tv axons from animals with control Tv neurons and neurons expressing IR-EcR-B1. (B) The percentage of Tv axon terminals that contain a complete or partial larval neurohemal arbor, for control and IR-EcR-B1 Tv neurons. (C) Average larval neurohemal site size as calculated by measuring the footprint of the larval arbor. (D) Average number of total nodes per nervous system and total branch-grid intersections at 24 hours APF. Asterisks indicate statistical significance.

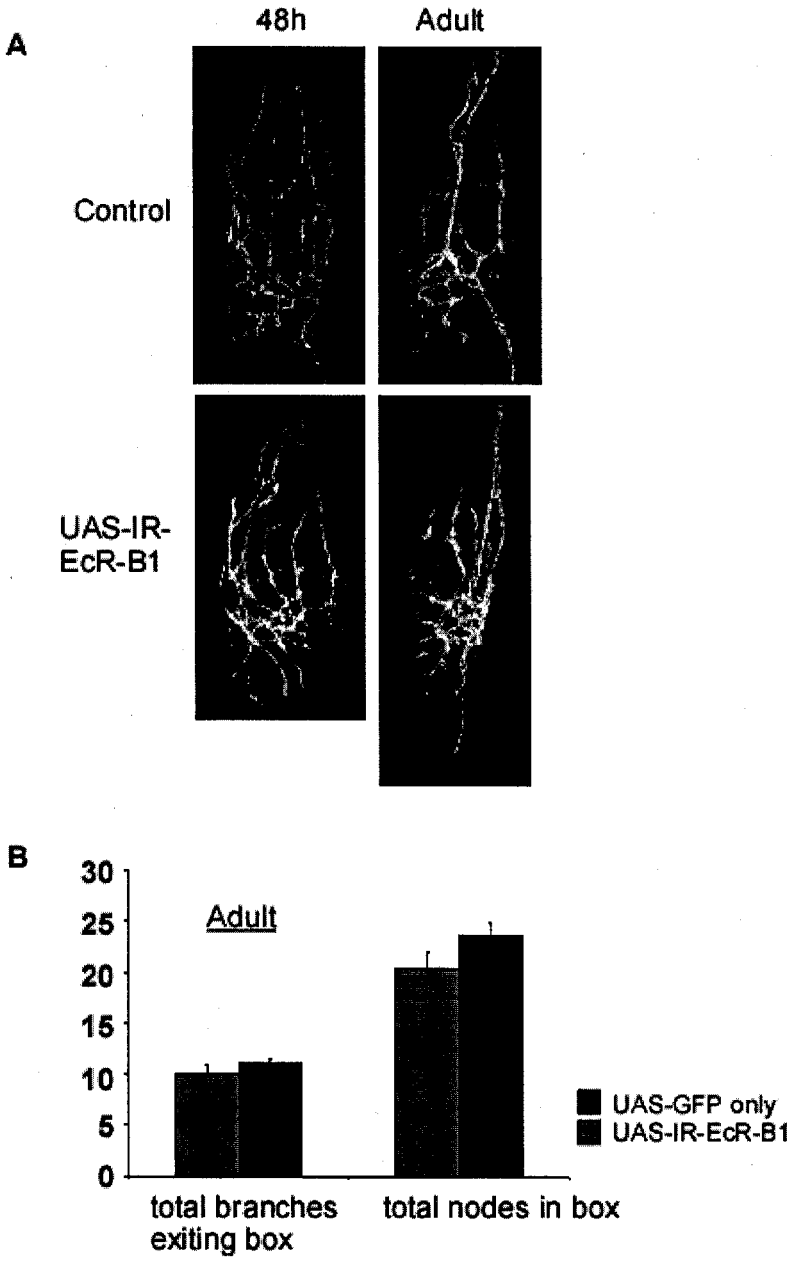

Figure 3.8. *Analysis of outgrowth in Tv neurons expressing IR-EcR-B1.*
(A) Anti-SCP immunostained Tv axons from controls and neurons expressing IR-EcR-B1, at 48 hours APF and in the adult. (B) Average number of branches exiting the box and average number of branch nodes within the box in the post-eclosion adult.

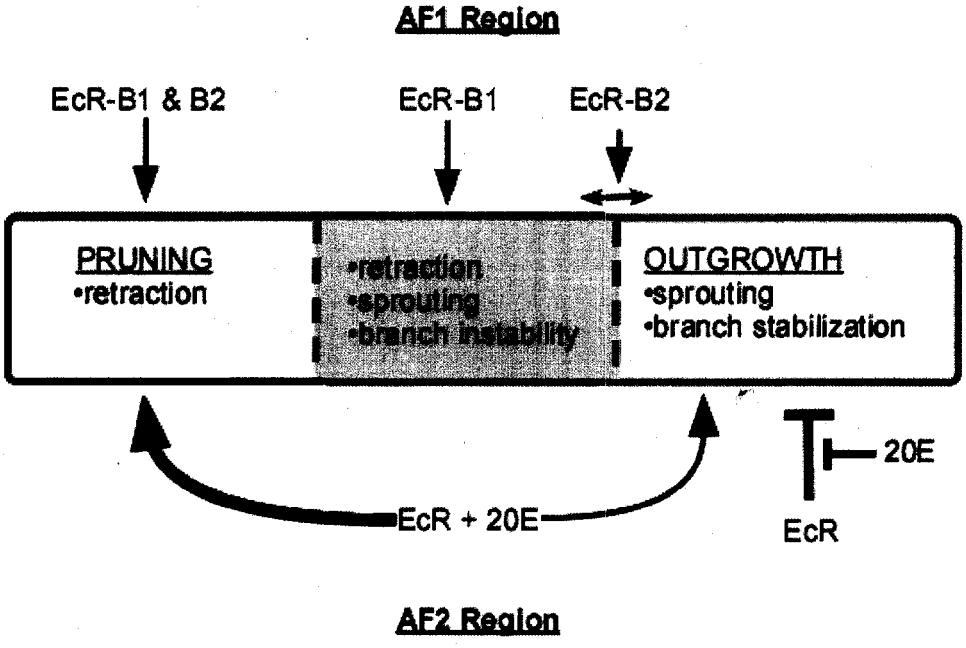

Figure 3.9. *Model of EcR isoform action on pruning and outgrowth phases of remodeling Tv cell axons.*
Both EcR activation through the ligand-dependent activation region (AF2) and the ligand-independent activation region (AF1) of the EcR-B isoforms are necessary for complete and timely pruning of larval Tv axons. EcR-B1 is hypothesized to promote a plastic state as pruning and outgrowth occur simultaneously. EcR-B2 can shift branch stabilization to earlier time points. Outgrowth of Tv cell axons can be directed via derepression of EcR and augmented through EcR ligand activation.

Notes to Chapter 3

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Chapter 4

The role of EcR in directing development of the adult-specific neuron lineages in the *Drosophila* ventral CNS

Introduction

Complete metamorphosis in insects dictates the formation of two different nervous systems to meet the specialized needs of the animal during both larval and adult life. Although some larval neurons are recycled for new adult usage, the majority of the central nervous system is made up of neurons born from reactivated embryonic neuroblasts (NBs) during the larval stages (Truman, 1990). Each NB divides to give rise to a cluster of progeny, or neuron lineage. These neuron lineages have been identified and their projections mapped in the *Drosophila melanogaster* larval brain (Pereanu and Hartenstein, 2006) and thoracic CNS (Truman et al., 2004). Each neuron in a lineage sends out a primary projection to an initial target, then arrests until metamorphosis, when secondary outgrowth establishes their adult connections (Truman and Bate, 1988).

The post-embryonic development of the *Drosophila* nervous system is coordinated by the steroid hormone ecdysone. Ecdysone is converted into 20-hydroxyecdysone (20E), a potent metabolite, and is translated into cellular responses by the ecdysone receptor, a heterodimer made of USP and EcR (Koelle et al., 1991; Yao et al., 1992). EcR binds to an ecdysone response element on DNA to regulate transcription of target genes via repression or activation (Cherbas et al., 1991). When 20E is absent, corepressors present in the cell may bind to EcR to shut down transcription. Binding of ligand then releases corepressors and recruits coactivators to promote transcriptional

activation (Bai et al., 2000; Dressel et al., 1999; Schubiger et al., 2005; Schubiger and Truman, 2000; Tran et al., 2001; Tsai et al., 1999). Cellular responses may also be mediated by the specific blend of EcR isoforms present in the nucleus (Talbot et al., 1993; Truman et al., 1994). The N-terminal regions of EcR-B1 and EcR-B2 have been shown to have strong ligand-independent activational functions (Hu et al., 2003; Mouillet et al., 2001), and to promote pruning in remodeling neurons (Schubiger et al., 1998), while EcR-A has an inhibitory domain at its N-terminus (Mouillet et al., 2001).

Both neuronal remodeling of larval neurons and maturation of adult-specific neurons are driven by the 20E titer during metamorphosis. When ecdysone action is blocked using a dominant negative form of EcR (EcR-DN), pruning of the peripheral neurons is blocked (Williams and Truman, 2005). Remodeling of the mushroom body axons is also inhibited in animals that lack USP, effectively blocking ecdysone signaling (Lee et al., 2000). The mushroom body axons specifically require EcR-B isoforms in order to prune, as do other central neurons (Lee et al., 2000; Schubiger et al., 1998). Although many studies have looked at the role of EcR in directing remodeling, little is known about how EcR directs the maturation of other adult-specific neurons.

Previously, I examined the role of EcR in neuronal remodeling using EcR-dominant negative constructs. When EcR-DN was expressed in a set of remodeling neurons, pruning of larval axons was severely disrupted and outgrowth of adult branches was reduced (Brown et al., 2006). However, retaining a larval arbor during the normal outgrowth phase may also disrupt a neuron's sprouting and branching

ability, so defects in outgrowth may not be due solely to disrupting ecdysone signaling during this phase. In this study, I expressed EcR-DN in the adult-specific neurons and examined the requirement for EcR during outgrowth in immature neurons that had not previously experienced pruning. I found that expression of EcR-DN in the adult-specific neuron lineages resulted in variable defects in secondary branching depending on the lineage identity, and this branching defect was also seen in the motorneuron axonal arbor in the leg.

Materials and Methods

Fly Stocks

Generation of the EcR dominant negative construct UAS-EcR-B1-d655-W650A (referred to in this paper as UAS-EcR-B1^{W650A}) was described in Hu et al., 2003 and Cherbas et al., 2003, and generation of UAS-EcR-A^{W650A} was described in Brown et al., 2006. To induce randomly-generated neuroblast clones expressing both an EcR-DN construct and membrane-bound GFP, the MARCM technique was employed using GAL4 driven by Elav (Lee and Luo, 1999). The following genotypes were generated.

Control (GFP only) clones:

GAL4^{C155}, hsFLP, UAS-mCD8::GFP; FRT^{2A}, tubP-GAL80/FRT^{2A}

EcR-DN clones (EcR-B1^{W650A}):

GAL4^{C155}, hsFLP, UAS-mCD8::GFP/UAS-EcR-B1^{W650A}; FRT^{2A}, tubP-GAL80/FRT^{2A}

EcR-DN clones (EcR-A^{W650A}):

GAL4^{C155}, hsFLP, UAS-mCD8::GFP; UAS-EcR-A^{W650A}/+; FRT^{2A}, tubP-GAL80/FRT^{2A}

MARCM Clone Generation

Animals from the above crosses were allowed to lay eggs in fly food vials for 2-4 hours. Vials with eggs were then maintained at 25° for 25-28 hours, followed by either a single heat shock (45-60 minutes) or a double heat shock (30 minutes, 30 minute rest, 45 minutes) in a 37° water bath. Therefore, larvae were heat shocked in the early L1 stage, between 25 and 34 hours after egg laying (AEL). After heat shock, larvae were maintained at 29°. For pupal staging, animals were collected at the white puparia stage and kept at 29°C for the indicated times. Staging was designated as hours after puparium formation (APF). Adults were dissected 0.5-3 days after eclosion.

Immunocytochemistry and Imaging

Nervous systems were fixed for 30 minutes in 4% paraformaldehyde, rinsed several times in phosphate buffered saline with 1% Triton X-100 (PBS-Tx), and blocked with 5% normal donkey serum for 15 minutes. After blocking, nervous systems were incubated overnight in some of the following primary antibodies: rat anti-mCD8 (1:100; CALTAG), mouse anti-EcR-A (1:100; 15G1A ascites), mouse anti-EcR-B1 (1:100; AD4.4) and mouse anti-EcR (Talbot et al., 1993). After several rinses with PBS-Tx, the tissues were incubated overnight in secondary antibody (1:200 in PBS-Tx of Cy5 conjugated donkey anti-mouse IgG and FITC conjugated donkey anti-rat IgG; Jackson Immunoresearch, West Grove, PA, and 1:100 Texas Red phalloidin; Molecular Probes, Carlsbad, CA). Nervous systems were rinsed several times, attached to polylysine-coated coverslips, dehydrated through an alcohol series, cleared in xylene, and mounted in DPX (Fluka, Seelze, Germany).

Legs were dissected from adults and fixed overnight in 4% paraformaldehyde, rinsed several times in phosphate buffered saline with Trition-X and blocked with 5% normal donkey serum for 15 minutes. Primary antibodies were added (rat anti-mCD8, diluted 1:100 from CALTAG stock) and left on for several days. After removal of primary antibody, legs were rinsed and secondary was added (1:200 FITC conjugated donkey anti-rat IgG; Jackson Immunoresearch). After several days, legs were rinsed and mounted in Fluoromount (Southern Biotech, Birmingham, AL). All experiments were imaged on a Bio-Rad Radiance 2000 confocal microscope.

Analysis

Analysis of lineage clones was performed in ImageJ (http://rsb.info.nih.gov/ij/). Serial Z-series confocal images were reconstructed into stacks, and then analyzed. To create isolated images of a particular clone, surrounding clones were removed by hand using either ImageJ or Adobe Photoshop CS 8.0. To determine if NBs were present, clones were scored blind for the presence of a large cell located at the clone's apex and having a diameter much larger than the neurons surrounding it. For analysis of the projection of lineage 15 into the femur, images were scored blind. Endplates were counted manually, and images were scored on the presence or absence of branches and if the projection pattern appeared abnormal. Statistical significance for number of neuron endplates in the femur was determined using the statistical analysis program SPSS (www.spss.com).

Results

Identification of adult neuroblast lineages

The postembryonic neural lineages in the thoracic neuromeres were previously identified and mapped in the pre-metamorphic CNS (Truman et al., 2004). Each lineage is associated with a neuroblast (NB) and its progeny remains in a discrete cluster through larval life (Fig. 4.1A), but as the CNS expands during metamorphosis, the lineage clusters are pushed laterally and dorsally within the cortical rind. Dividing NBs were evident at pupariation, but by 24 hours after puparium formation (APF; at 29°C) thoracic neurogenesis was complete and no NBs could be found. Since the position of the lineage clusters changed from their previously mapped larval positions, I could not rely on location of the cell cluster in the hemisegment alone for identification. I could, however, identify a number of the lineages in the pupal and adult central nervous systems based on the projection of their primary neurite bundles that exited the cluster. Neurons in a lineage sent out an initial stereotyped neurite during larval life (Truman et al., 2004). This initial projection remained through metamorphosis, and aided in identification of the lineages in the adult.

I was able to identify many of the lineages at 24 hours APF and in the adult CNS and will briefly outline the normal development of two lineages. Lineage 6 is made of two classes of neurons, one class that sends their initial neurite across the posterior dorsal commissure and a second class whose neurites travel through the posterior intermediate commissure (Truman et al., 2004). By 24 hours APF, both sets of neurons showed strong terminal sprouting and also an interstitial sprouting zone that

was ipsilateral to the cell bodies (Fig. 4.1B, arrowheads and arrows). These terminal and interstitial arbors continued to expand within the central neuropil, so that in the adult, the neurons in both clusters had well developed ipsilateral and contralateral arbors (Fig. 4.1B", arrow and arrowhead). The neurons in the dorsal commissure were interesting because their targets appear to be in the T2 neuromere. Consequently, they projected anteriorly in T3 (Fig. 4.1B) but posteriorly in T1 (Fig. 4.1B'', arrowhead). The cell bodies of the two groups of neurons from lineage 6 often ended up as distinct, but adjacent cell clusters.

Lineage 18 is simpler than lineage 6 because in the larva it has only one primary neurite bundle that crosses to the contralateral side via the anterior intermediate commissure (Truman et al., 2004). By 24 hours APF at 29°C, the terminal arbor had enlarged and extended dorsally, and a dorsally projecting interstitial arbor was also evident ipsilateral to the cell cluster. Both arbors were much enlarged in the adult and there was also a small branch extended into the ventral portion of the contralateral leg neuropil (seen in 3 of 3 preparations; Fig. 4.1C", arrow).

Expression of EcR-DN in NB lineages

To test the role of EcR in the development of the adult-specific neurons, I used the MARCM system to express both CD8::GFP and EcR-DN constructs. I expressed several different EcR-DN constructs in the lineages. I tested the expression levels of EcR-B1^{W650A} by examining EcR-B1 immunoreactivity within the cells of the clone at 24 hours APF, 29°C. Since the adult-specific neurons do not normally express EcR-B1 at this time, any EcR-B1 immunoreactivity within a clone was due to expression of

EcR-B1^{W650A} (Fig. 4.2A-A'). Two other insertion lines of EcR-B1^{W650A} were tested and showed similar upregulation of EcR-B1 (not shown). Because the adult-specific neurons do express endogenous EcR-A, expression of EcR-A^{W650A} would lead to an increase in EcR-A immunoreactivity within the clone. Therefore, the increased EcR-A observed within a clone as compared to the surrounding lineages was due to expression of EcR-A^{W650A} (Fig. 4.2B-B'). Since all dominant negatives tested were expressed in clones and gave similar phenotypes in the lineages (not shown), I will focus on results using the UAS-EcR-B1^{W650A} construct and refer to it as EcR-DN unless otherwise specified.

Clones expressing EcR-DN have persisting NBs

Neuroblast MARCM clones expressing EcR-DN retained NBs much longer than control clones (Fig. 4.3). NBs were seen in control animals at pupariation, but never at 18 or 24 hours APF at 29°C. However, at 24 hours APF, 18 of 101 clones expressing an EcR-DN still had a large NB at the apex of the clone (Fig. 4.3, arrow; Table 4.1). I observed 3 examples of persisting NBs in lineage 3, two examples in lineage 6 clones, two in lineage 1 clones, and one clone each in lineages 5, 8, 12, 16, 18, 19 and 21. Four clones with persisting NBs could not be identified. NBs were never seen in the adult nervous system for control clones or those expressing EcR-DN. Hence, the persistence of the NB was temporary. I did not see a dramatic increase in the number of neurons found in the adult clones that expressed EcR-DN, so the impact of the delayed disappearance of the NB is not known.

Effects of EcR-DN expression in the the adult CNS lineages

Of the 24 lineages in the thoracic ganglia of the adult nervous system, I was able to identify and analyze multiple examples of seven of these lineages in both control animals and animals expressing EcR-DN. The secondary projections of the NB lineages were much expanded in the adult central nervous system of control animals. When EcR-DN was expressed in NB clones, the adult arbors showed a range of phenotypes. The primary targeting of all lineages was unchanged in all clones that expressed EcR-DN. However, the secondary sprouting of clones expressing EcR-DN was variable across the lineages in the adult CNS, showing varying degrees of clumped, reduced, and misrouted arbors. Since this is the first time the lineages of the thoracic ganglia have been identified in adult nervous systems, I described the adult morphology of the controls as well as the phenotype of clones expressing EcR-DN.

Lineage 1

At pupariation, the cell cluster of lineage 1 is located along the ventrolateral anterior edge of the neuromere, and has two neurite bundles. The bundle from one group of neurons projects across the anterior ventral (aV) commissure to the contralateral leg neuropil, and the other projects anteriorly to the ipsilateral leg neuropil of the next neuromere. This anterior projection is absent in T1 (Truman et al., 2004). I examined four lineage 1 clones located in T2 and T3 in adult control animals. In the adult, the cell cluster projecting contralaterally was located dorsolaterally along the anterior side of the neuromere. The neurons had grown an ipsilateral arbor that extended through the ventral region of the leg neuropil (Fig. 4.4A, arrow). The

contralateral projection retained the characteristic "hook" in the medial region of the leg neuropil (Fig. 4.4A, arrowhead) and projected a diffuse arbor into the lateral region of the neuropil. The cell bodies of the neurons with the ipsilateral projection were pulled anteriorly to the ventral posterior side of the adjacent neuromere, and were no longer tightly clustered together. Their neurites stayed confined to the ventral region of that leg neuropil. Expression of EcR-DN in lineage 1 resulted in no discernable changes in morphology of either class of neurons. I examined 12 examples of lineage 1 in T2 and T3, and saw no reduction in terminal or interstitial arbor area or changes in projection (Fig. 4.4B).

Lineage 2

The neurons in lineage 2 project a single neurite bundle dorsally from a cell cluster located ventrally along the anterior, medial margin of the neuromere. The bundle projects to the dorsal surface of the neuropil and then turns sharply to end at the dorsolateral margin of the ipsilateral neuropil (Truman et al., 2004). I examined a total of 7 adult control clones with examples in each thoracic neuromere. In T1-T3, the cell cluster was in the same location as before metamorphosis, and the terminal arbor had expanded to form a fan-shaped, diffuse projection within the ipsilateral dorsal neuropil (Fig. 4.5A). A smaller interstitial arbor extended ipsilaterally from midway along the dorsally extending bundle in the second and third neuromeres. In eleven of the thirteen lineage 2 clones expressing EcR-DN, no disruptions in initial or secondary projections were found (Fig. 4.5B). One out of the ten lineage 2 clones in T2 or T3 had a reduced interstitial arbor, and one of the two examples in T1 had a reduced terminal arbor.

Lineage 6

The adult anatomy of lineage 6 clones is described above (Fig. 4.1B"; Fig. 4.6A). When EcR-DN was expressed in lineage 6, five of the seven clones examined were normal, while two showed dorsal projecting neurons with a reduced ipsilateral arbor (Fig. 4.6B, arrow).

Lineage 8

At pupariation, the cell cluster for lineage 8 is located along the ventrolateral region of the neuromere and it has two neurite bundles. The neurite bundle from one set of neurons projects into the ipsilateral leg neuropil and that from the other set projects across the anterior intermediate (aI) commissure and terminates just after crossing the midline (Truman et al., 2004). I examined four lineage 8 clones in adult control animals. The cell bodies separated into two clusters, located next to each other on the ventrolateral anterior edge of the neuromere. The ipsilateral bundle projected dorsally up through the leg neuropil, establishing a diffuse arbor terminating on the dorsolateral region (Fig. 4.7A, arrowhead). The contralateral bundle had several interstitial arbors branching off the primary neurite. Ipsilateral to the cell bodies, an arbor extended across the ventral portion of the ipsilateral leg neuropil (Fig. 4.7A, arrow). Multiple interstitial branches extended off the primary neurite as it crossed the aI commissure, extending dorsally in both the anterior and posterior directions with a major arbor extending anteriorly just before the midline (Fig. 4.7A, asterisk). In the contralateral neuropil, the primary projection split and extended branches along the A-P axis at the level of the intermediate commissure. In three of the six clones expressing EcR-DN,

the intersitital arbors appeared to be clustered in dense strands, rather than the diffuse pattern seen in control clones (Fig. 4.7B).

Lineage 9

Prior to metamorphosis, Lineage 9 sends an ipsilateral neurite bundle to the dorsal leg neuropil and a thin contralateral bundle across the aV commissure that stopped just before the contralateral leg neuropil (Truman et al., 2004). The cell cluster is located dorsally in the anterior region of the neuromere. In the adult CNS, the lineage 9 cell cluster remained in a dorsal position and its neurite bundle extended ventrally along the lateral edge of the leg neuropil, forming a dense finely branched arbor that stretched from the anterior midline, across the ventral side of the leg neuropil to its posterior margin (Fig. 4.8). A diffuse ipsilateral arbor was seen in the ventrolateral leg neuropil. The contralateral branch of lineage 9 was composed of small, diffuse neurites that extended across the aV commissure to the anterior ventral margin of the leg neuropil in all nine clones of lineage 9 examined (Fig. 4.8A, arrow). When EcR-DN was expressed in lineage 9, both the ipsilateral and contralateral terminal arbors appeared normal. However, six of the fourteen clones examined showed defasciculation of the primary bundle as it curved ventrally around the leg neuropil (Fig. 4.8B, arrow).

Lineage 18

The adult form of lineage 18 is also described above and this morphology was evident in three of three control examples (Fig. 4.9A). I obtained three examples of lineage 18 in animals expressing EcR-DN and these were indistinguishable from lineage 18 in control animals (Fig. 4.9B).

Unidentified interneuron lineage

Of the seven lineages I analyzed in this study, one could not be related to its larval morphology. However, its adult morphology was easily and repeatedly recognized by its distinctive projection. In T1, this unknown lineage was located on the medial anterior border of the neuromere, and sent its primary neurite bundle dorsally, turning sharply to cross the midline at the level of the pI commissure and extending dorsally into the contralateral flight neuropil. On either side of the midline, large diffuse arbors extended dorsally into the flight neuropil (Fig. 4.10A). When EcR-DN was expressed in this lineage, defects in both the initial and secondary projections were seen. In nine of the eleven clones analyzed, the ipsilateral dorsally projecting arbor was not composed of evenly spaced fine branches, but clumped together to produce dense strands interspersed with areas with no branches. The contralateral arbor was also compressed, but in a longitudinal manner, extending anterior and posterior to form a T shape (Fig. 4.10B, arrowhead). Additionally, as the primary bundle crossed the midline, it frayed and defasciculated in 5 clones (Fig. 4.10B, arrow).

I also examined a set of recognizable clones that appear to be the T3 homologs of the unidentified clone in T1. The neurons in this lineage also crossed the pI commissure, extending a large dorsal ipsilateral arbor prior to crossing. Contralaterally, the bundle turned anteriorly and sent the main arbor into a dorsal longitudinal tract, while sending a smaller arbor posterior into the leg neuropil contralateral to the cell cluster. The cell cluster was located laterally in the neuromere, but its position varied somewhat in the 5 clones examined (Fig. 4.10C). The defects

produced by expression of EcR-DN were less severe than in T1. Only one clone in seven showed a clumped ipsilateral arbor (Fig. 4.10D, arrow), one had a reduced ipsilateral arbor, and one had a clumped contralateral arbor, while the remaining four appeared normal.

Lineage 15

Lineage 15 is a motor lineage whose neurons project out of the CNS to the leg. In the larva, the laterally located cell cluster extends axons dorsally through the leg neuropil and out to a leg imaginal disc in the periphery. At this stage, the bundle partially defasciculates as it passes through the neuropil (Truman et al., 2004). During metamorphosis, the lineage 15 neurons grew their dendritic arbors at the site of defasciculation and these extended through the dorsal regions of the leg neuropil, producing an arbor of fine, evenly spaced branches in the adult (Fig. 4.11A, C and E). A small branch extended toward the midline, barely crossing the pI commissure in 13 of 14 clones before terminating immediately. In clones expressing EcR-DN, the secondary projection within the leg neuropil was clumped and reduced in all 16 clones examined. The fine branches appeared to be compacted together, and left large spaces devoid of dendrites within the leg neuropil (Fig. 4.11B, D and E). However, all but one still had the contralaterally projecting branch.

The axons from the motoneurons in lineage 15 exited the CNS and projected down into the leg, terminating in the tibia. They remained tightly fasciculated until reaching the femur, where they branch to establish neuromuscular junctions (NMJs) both proximally and distally (Fig. 4.12, arrows). These two branch points were predictable;

in 15 of 16 clones there were both a proximal arbor and a smaller distal arbor spreading over the muscles (Fig. 4.13B). These arbors were characteristic in their morphology, extending multiple branches longitudinally along the muscle fibers (Fig. 4.12A and D). After entering the tibia, lineage 15 branched in a much more disorganized manner, but the arbor was distributed fairly evenly and densely to innervate the tarsus levator and depressor muscles of the tibia (Fig. 4.12B). Lineage 15 ends in the tibia, reflecting the lack of musculature within the tarsal segments (Soler et al., 2004).

The peripheral projection of lineage 15 was disrupted in clones expressing either EcR-B1^{W650A} or EcR-A^{W650A}. Expression of either DN resulted in missing arbors in the femur and tibia, and misaligned and clumped NMJs. Tibial projections were reduced in all clones expressing either EcR-B1^{W650A} or EcR-A^{W650A} (Fig. 4.12C). Total endplates within the femur were reduced by half in clones expressing either EcR-B1^{W650A} or EcR-A^{W650A} (Fig. 4.13A). Almost all had proximal femoral branches. Half of the fifteen clones expressing EcR-B1^{W650A} had a clumped or misaligned proximal branch, while all seven clones expressing EcR-A^{W650A} had clumped or misaligned branches (Fig. 4.12C, Fig. 4.13C). Clumped branches resulted in arbors that were highly compacted, resulting in fewer endplates in the femur (Fig. 4.12F). Misaligned branches were characterized by arbors that were not longitudinally oriented along the muscle fibers, but projected relatively perpendicularly away from the primary tract, producing a star-shaped morphology (Fig. 4.12E). In clones expressing EcR-B1^{W650A} or EcR-A^{W650A}, distal femoral arbors were missing from approximately half of the legs examined. Most of the distal arbors present in clones expressing EcR-B1^{W650A} had a

misaligned phenotype, while clones expressing EcR-A^{W650A} were split between the misaligned and clumped phenotypes (Fig. 4.13C).

Discussion

Steroid signaling and neurogenesis

Postembryonic neurogenesis is initiated by the reactivation of NBs in the late 1st instar, and terminates during the 24 hours after pupariation (Truman, 1990). Reactivation of quiescent NBs requires growth of the 1st instar larva (Britton and Edgar, 1998). While 20E has been implicated in regulation of NB cell cycle speed, it is not sufficient to initiate NB divisions in starved larvae (Britton and Edgar, 1998; Truman et al., 1993). It is unknown what signals terminate NB division. NBs could be predetermined to stop cycling after a specific number of divisions, or an external cue could trigger termination. Previous studies suggested that cessation of postembryonic NB activity is not dependent on ecdysone. In *Manduca sexta*, transplantation of ganglia from 4th instar larvae into wandering 5th instar larvae resulted in continuing neurogenesis in the implanted ganglia well after neurogenesis had terminated in the host. This suggests that young NBs can ignore later hormonal cues, and cannot be shut off early by metamorphic signals (J.Witten and J. Truman, unpublished). Additionally, *Drosophila* larval central nervous systems cultured without 20E do stop divisions, but it is unknown if they make the correct number of progeny (Truman et al., 1993). In this study I found that lineage clones expressing EcR-DN had NBs that persisted longer than usual. In 20% of clones expressing EcR-DN, I observed NBs at 24 hours APF in

animals raised at 29°C. This would correspond to a developmental time point of approximately 29 hours APF at 25°C (Brown et al., 2006; Powsner, 1935), well past the time when neurogenesis ceases in the ventral CNS. However, the number of neurons in clones expressing EcR-DN was not grossly increased (data not shown). Delaying death in the thoracic NBs through expression of EcR-DN may extend the neurogenic period, but the NB's life may only be prolonged for a short time, resulting in clones with only slightly more neurons. Taken together with previous studies of 20E and NB termination, this data suggests that the number of NB division may be largely regulated endogenously, but precise timing of NB termination may be fine-tuned through 20E signaling.

Development of the adult specific lineages

After each neuron is born during the larval instars, it sends out an initial process before arresting. Each neuron within a cluster sends out the same projection as the previously born neuron from the same sibling class. If a lineage has more than one neurite bundle (for example, Lineage 1 projects to both the contralateral leg neuropil and the neighboring ipsilateral leg neuropil; Fig. 4.4A), one neuron from the division of the ganglion mother cell (GMC) projects to one of the initial targets, while its sibling projects to the other (Truman et al, 2004). Typically, the projections from a lineage make contact with the neurite bundle from another lineage, potentially setting up a future synaptic connection. Before metamorphosis, these contacts are limited and the neuropil remains partitioned into domains allotted to each lineage (Truman et al., 2004). In this study, the final adult arbor morphology of 8 of the 24 thoracic lineages

was described. After metamorphosis was complete, all lineages examined showed intense sprouting and elaboration. Within the CNS, this entailed formation of finely branching terminal and interstitial arbors, many located within the leg or flight neuropil of the CNS. The prior partitioning of the CNS was dissolved during metamorphosis, as the neurons in each lineage grew their complex arbors and established their final connections with neurons from other lineages. The high connectivity seen within the CNS is undoubtedly a response to the increased behavioral and sensory demands on the adult nervous system.

Effects of ecdysone signaling on neuronal outgrowth and sprouting

In centrally located remodeling neurons, expression of EcR-DN results in defects in both pruning and outgrowth. Pruning is slow and incomplete in Tv neurosecretory neurons expressing EcR-DN, while sprouting and outgrowth is severely stunted, with only a few adult-like branches emerging from a larval-like axon arbor (Brown et al., 2006). Remodeling neurons in the periphery show similar pruning defects when EcR-DN is expressed (Williams and Truman, 2005). In this study, expression of EcR-DN in adult-specific neurons resulted in varied and less severe phenotypes when compared to remodeling neurons. None of the lineages examined had defects in their initial projections. As these initial projections are established before pupariation, it is not surprising that EcR is not involved in their development. The adult-specific neurons have very low levels of EcR prior to pupariation, with moderate levels of EcR-A becoming evident after pupariation (Truman et al., 1994). The secondary outgrowth of the adult-specific neurons was reduced or clumped in some lineages, but many

appeared normal despite expression of EcR-DN. In Tv neurons expressing EcR-DN (EcR-B1^{W650A}), sprouting and outgrowth was almost completely absent (Brown et al., 2006). This may reflect the environment in which neurite growth is occurring. The axons of the Tv neurons are situated on the dorsal surface of the CNS, embedded in the basal lamina (Nassel et al., 1988). They are neurosecretory cells, and do not make axonal synaptic connections with other neurons. The lack of surrounding neurons and glia may mean that the Tv neurons are dependent upon hormone as their only cue to initiate outgrowth during metamorphosis. Additionally, pruning prior to outgrowth in the Tv neurons is suppressed by EcR-DN. This retention of the larval arbor may also influence the outgrowth phenotype in the Tv neurons.

Although expression of EcR-DN in the adult-specific neurons was not as strong as that seen in the remodeling Tv neurons (Brown et al., 2006), defective outgrowth was seen in multiple lineages. The cell clusters of clones could be picked out based on their increased EcR immunoreactivity (Fig. 4.2A' and B'). Additionally, the phenotypes seen in all EcR-DN constructs and instertion lines tested were similar, despite appearing to have varied EcR-DN expression levels. Severity of phenotype was not correlated with EcR protein levels.

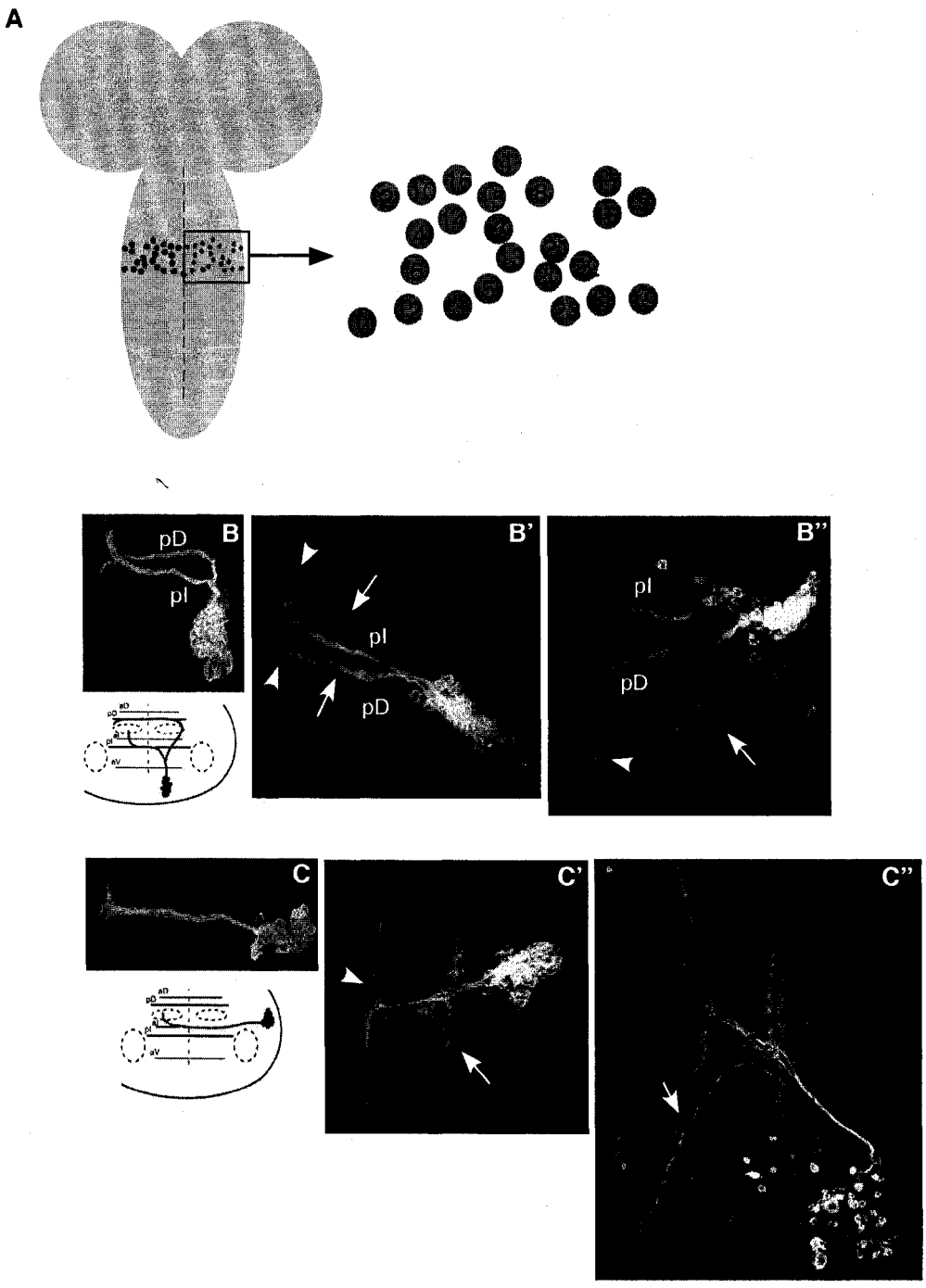
Expression of EcR-DN in the adult-specific neurons resulted in variable phenotypes, ranging from normal in appearance to reduced and clumped terminal and interstitial arbors. Lineage 15 was by far the most affected, in both its central and peripheral projections. The unidentified interneuron lineage also had relatively consistent deficiencies in its interstitial and terminal arbors. Lineages 1 and 18

expressing EcR-DN were indistinguishable from controls, while lineages 2, 6, 8, and 9 had relatively minor defects when EcR-DN was expressed.

Why are some lineages more affected by EcR-DN expression than others? Interpretation of the effects of EcR-DN on the adult-specific neuron lineages is hindered by the lack of information on the functions of the lineages examined. Lineage 15 is the only lineage in which the neuron functions are known, as it is composed of only leg motorneurons. In response to expression of EcR-DN, its dendritic arbor within the central nervous system is much reduced. In comparison, lineage 9 shows little effect of EcR-DN expression and the specific function of neurons within lineage 9 is unknown. However, the major arbor of lineage 9 ramifies through the ventral-medial leg neuropil which receives the projections of the leg mechanosensory neurons (Shepherd and Smith, 1996), possibly implicating them as primary mechanosensory interneurons. These two lineages would therefore receive their presynaptic inputs at very different times during metamorphosis. The motorneurons of lineage 15 contact their potential synaptic partners in the CNS early in metamorphosis as they begin sprouting, while the neurons of lineage 9 would not see presynaptic afferents until after 44 hours APF when the sensory axons begin navigating in from the periphery (Shepherd and Smith, 1996). If synaptic connections are established late in metamorphosis, the sprouting and outgrowth of the adult-specific neurons may be directed via contact with the synaptic partner rather than through the waning 20E signal.

The interplay between a neuron's extrinstic environment and intrinsic regulatory mechanisms often results in the formation of a stereotyped projection pattern. In this study, I have explored one aspect of the intrinsically regulated EcR pathway in adult-specific neurons. Not only does disruption of EcR signaling result in variable phenotypes across the complement of neuronal lineages, but the lack of visible phenotypes in many lineages suggests that extrinsic factors such as local signals from surrounding neurons and glia may play a large role in regulation of outgrowth.

Figure 4.1 *Development of the postembryonic neuron lineages of the CNS.* (A) Neuroblasts are arranged in a stereotyped pattern in each hemisegment of the larval ventral CNS. (B) At 0 hours after pupariation, the lineage of neuroblast 6 in T3 projects primary neurites across the posterior intermediate and posterior dorsal commissures (from Truman et al., 2004). The posterior intermediate (pI) and posterior dorsal (pD) bundles are labeled. Below is a cross-section schematic of the CNS showing the major commissures. (B') Lineage 6 began to sprout terminal (arrowheads) and interstitial (arrows) arbors from both neurite bundles 24 hours APF (raised at 29°C, in T2). (B'') Lineage 6's complete final adult arbor in the post-eclosion adult (raised at 29°C, in T1). Note the posterior projecting terminal arbor on the pD-crossing bundle (arrow). (C) At 0 hours after pupariation, the lineage of neuroblast 18 projects across the posterior intermediate commissure in T2 (from Truman et al., 2004). (C') At 24 hours APF, lineage 18 began to sprout A-P projecting terminal (arrowhead) and interstitial (arrow) arbors (raised at 29°C, in T2). (C'') Lineage 18's arbor was expanded in the adult CNS (raised at 29°C, in T3). It also extended a small branch into the contralateral leg neuropil (arrow).


A
B
pD
pl
B'
pl
pD
B''
pl
pD
C
C'
C''

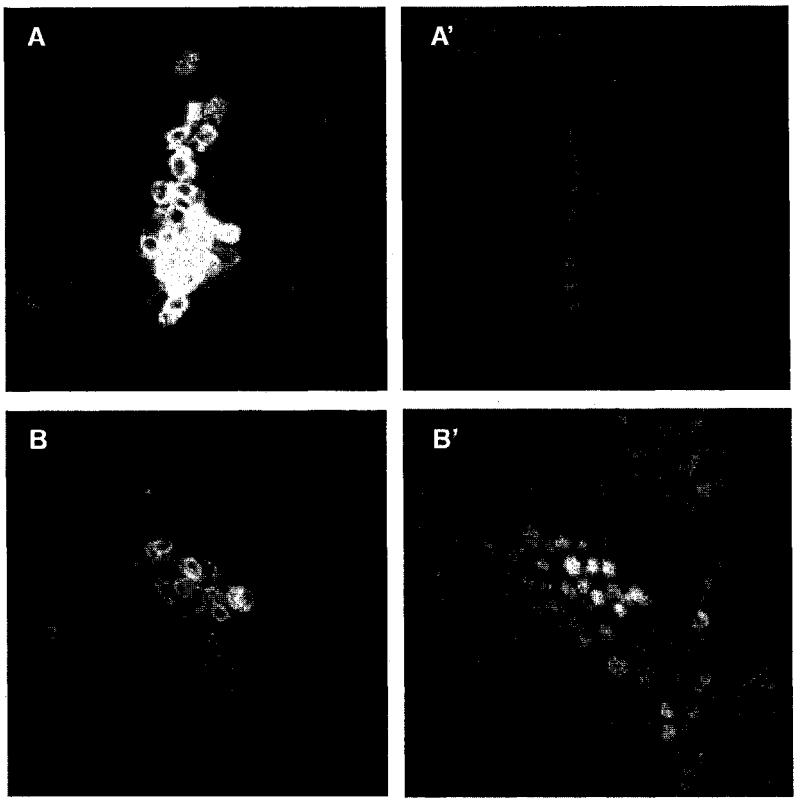


Figure 4.2 *Expression levels of EcR-DN constructs.*
Anti-CD8 expression (**A**) and anti-EcR-B1 expression (A') in a MARCM neuroblast clone expressing CD8-GFP and EcR-B1^{W650A}. Anti-CD8 expression (B) and anti-EcR-A expression (B') in a MARCM neuroblast clone expressing CD8-GFP and EcR-A^{W650A}. EcR-A immunoreactivity in cells surrounding the clone is due to endogenous EcR-A in the adult-specific neurons. Both A and B are from animals at 24 hours APF, 29°C.

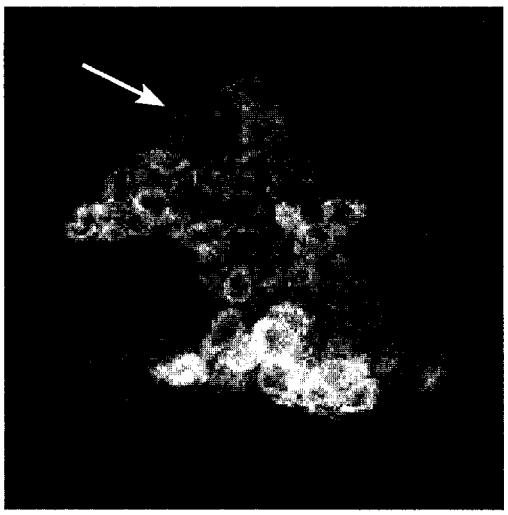


Figure 4.3 *A clone (lineage 16) at 24 hours APF (29°C) expressing EcR-DN with a persistant neuroblast.*
Arrow points to the neuroblast.

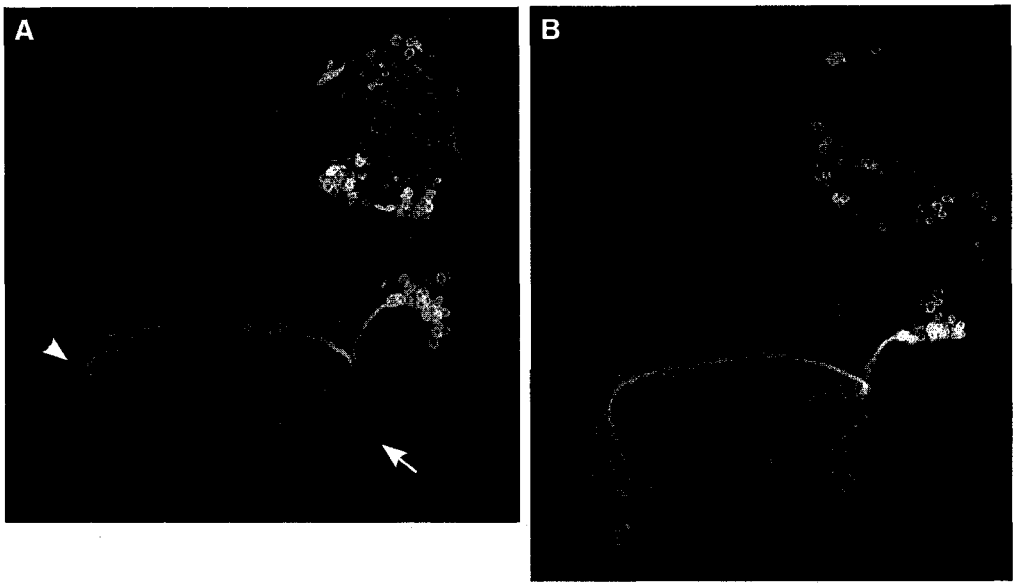


Figure 4.4 *Lineage 1 in the adult CNS.*
(A) A ventral projection of T2 lineage clone in the adult CNS. The clone has resolved into two cell clusters. One has a contralateral projection in T2 with an ipsilateral arbor (arrow) and a contralateral terminal arbor with a characteristic "hook" (arrowhead). The other cluster is shifted anteriorly into T1 and projects throughout the ipsilateral neuropil. (B) A ventral projection of T2 lineage 1 expressing EcR-DN looks similar to the control.

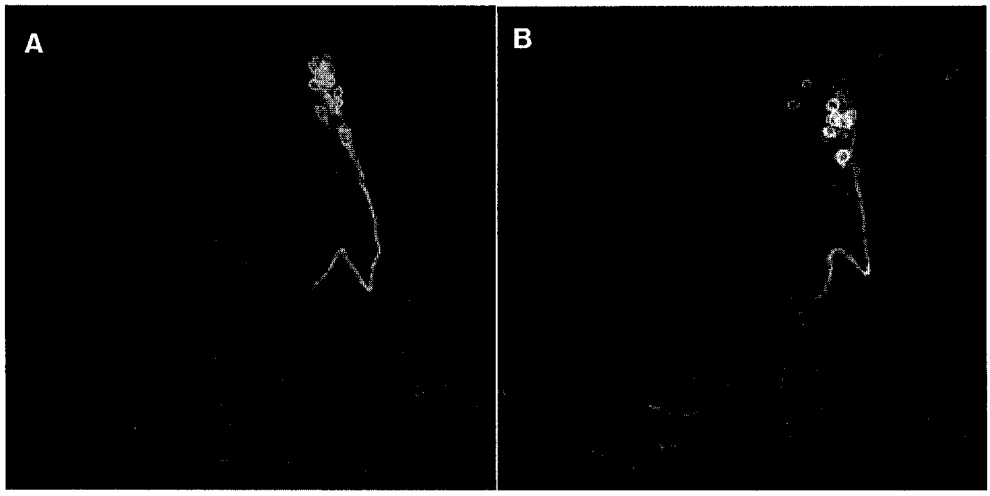


Figure 4.5 *Lineage 2 in the adult CNS.*
(A) A ventral projection of lineage 2 in control animals, in the first thoracic neuromere. Lineage 2 has a characteristic "jog" as it extends dorsally through the neuropil before forming a diffuse ipsilateral arbor in the flight neuropil. (B) A ventral projection of lineage 2 expressing EcR-DN, in the first thoracic neuromere. Two of the thirteen clones examined had a reduced arbor.

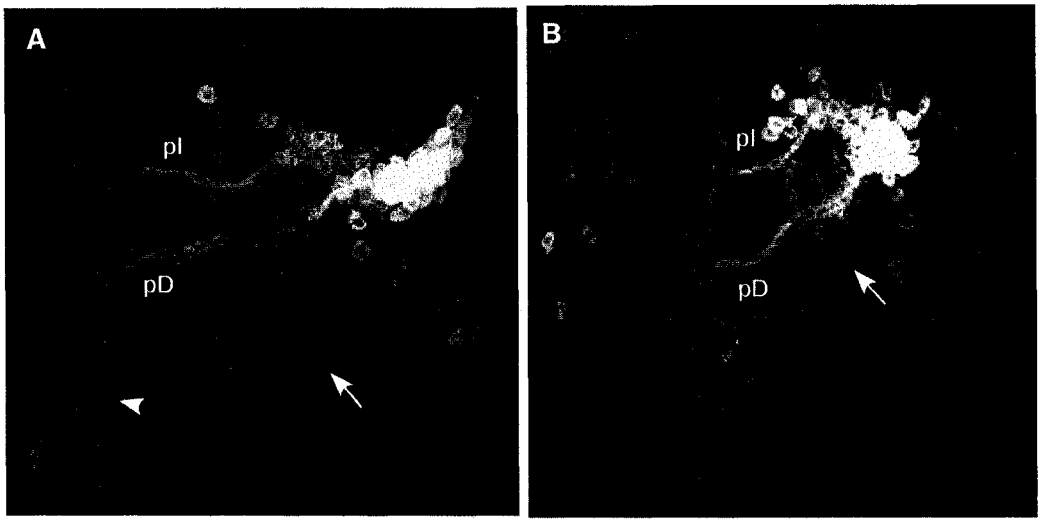


Figure 4.6 *Lineage 6 in the adult CNS.* (A) A ventral projection of lineage 6 in control animals, in the first thoracic neuromere. The more dorsal bundle extends a large ipsilateral arbor (arrow) before crossing at the pD commissure, while the other bundle crosses at the pI commissure. Both extend a terminal arbor along the dorsal longitudinal tract (arrowhead). (B) A ventral projection of lineage 6 expressing EcR-DN, in the first thoracic neuromere. The ipsilateral arbor is reduced (arrow) and the contralateral arbor is more diffuse. This phenotype was seen in two of seven lineage 2 clones.

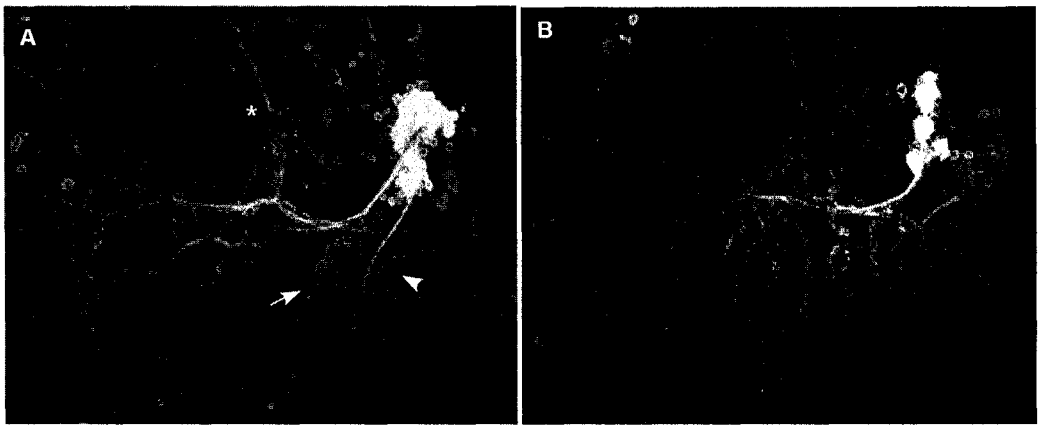


Figure 4.7 *Lineage 8 in the adult CNS.*
(A) A ventral projection of lineage 8 in control animals, in the second thoracic neuromere. One cluster projects to the ipsilateral leg neuropil (arrowhead), while the other projects across the aI commissure. The contralaterally projecting bundle extends an ipsilateral arbor into the leg neuropil (arrow) and a major ipsilateral arbor anteriorly (asterisk). (B) A ventral projection of lineage 8 expressing EcR-DN, in the second thoracic neuromere, showing dense strands of secondary arbor.

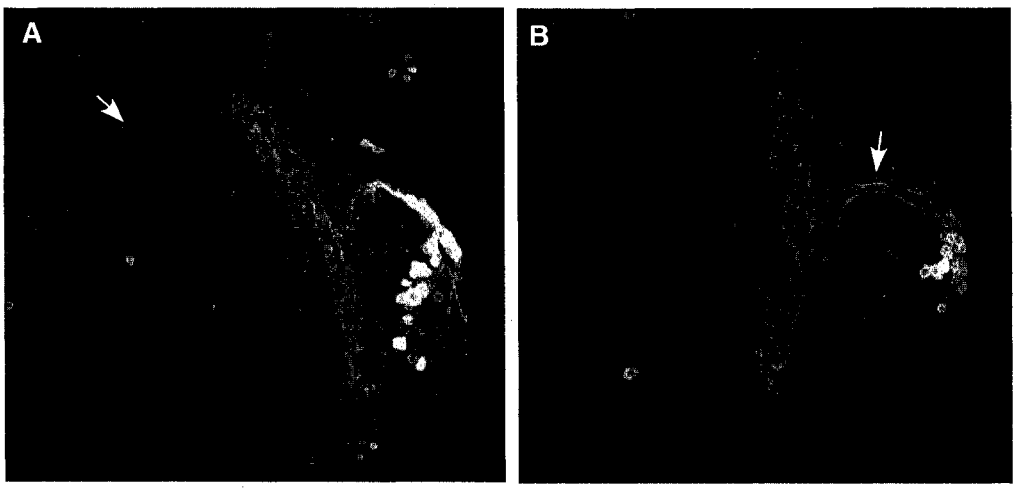


Figure 4.8 *Lineage 9 in the adult CNS.*
(A) A ventral projection of lineage 9 in control animals, in the third thoracic neuromere. Lineage 9 projects around the anterior edge of the leg neuropil to form an arbor along the ventral side. A small arbor extends contralaterally across the aV commissure (arrow). (B) A ventral projection of lineage 9 expressing EcR-DN, in the third thoracic neuromere. Note the defasciculation of the primary bundle (arrow). This was seen in six of fourteen clones examined.

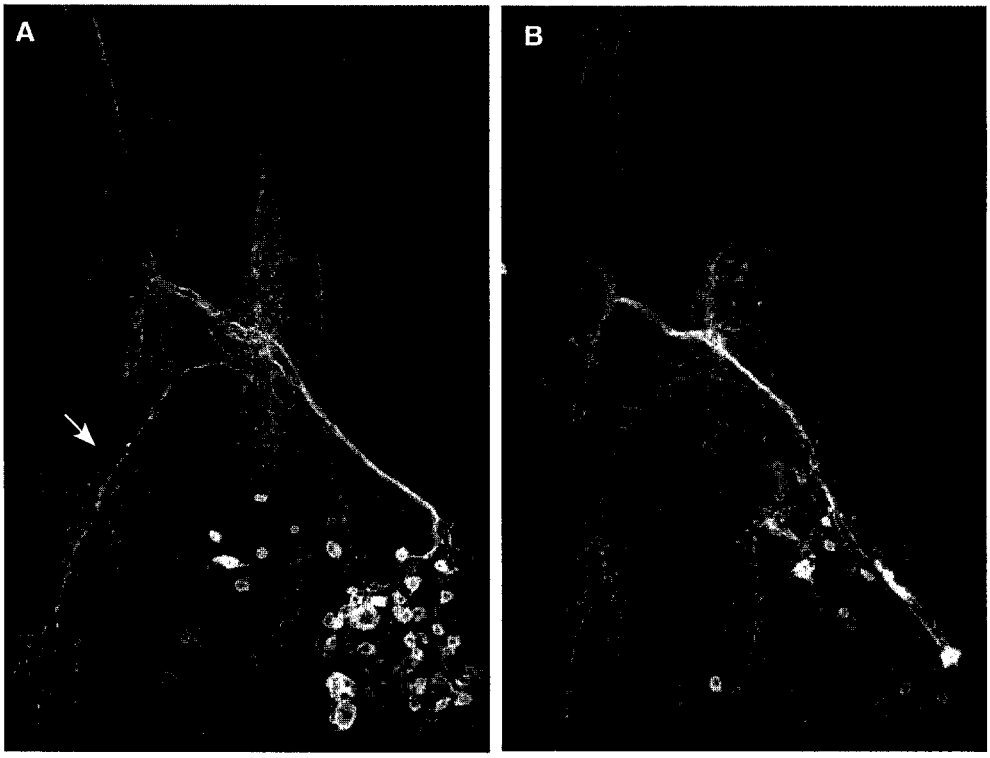


Figure 4.9 *Lineage 18 in the adult CNS.*
(A) A ventral projection of lineage 18 in control animals, in the third thoracic neuromere. It extends across the aI commissure, with dorsally projecting arbors on either side of the midline, and a smaller arbor projecting into the contralateral leg neuropil (arrow). (B) A ventral projection of lineage 18 expressing EcR-DN, in the third thoracic neuromere, appears similar to the control.

Figure 4.10 *Unidentified lineage in the adult CNS.*
(A) A ventral projection of the repeatedly recognizable lineage of unknown origin, in the first thoracic neuromere of control animals. It extends across the pI commissure, forming dorsally projecting arbors on both sides of the midline. (B) A ventral projection of the unidentified lineage expressing EcR-DN, in the first thoracic neuromere. The ipsilateral arbor is clustered in dense strands, the contralateral arbor is longitudinally compressed and projects anteriorly (arrowhead), and the primary neurite bundle is defasciculated (arrow). (C) A ventral projection of the unidentified lineage in the third thoracic neuromere of control animals. (D) A ventral projection of the unidentified lineage expressing EcR-DN in the third thoracic neuromere. The ipsilateral arbor is compressed (arrow). C and D are thought to be from the same homologous neuroblast as A and B, but in T3 instead of T1.

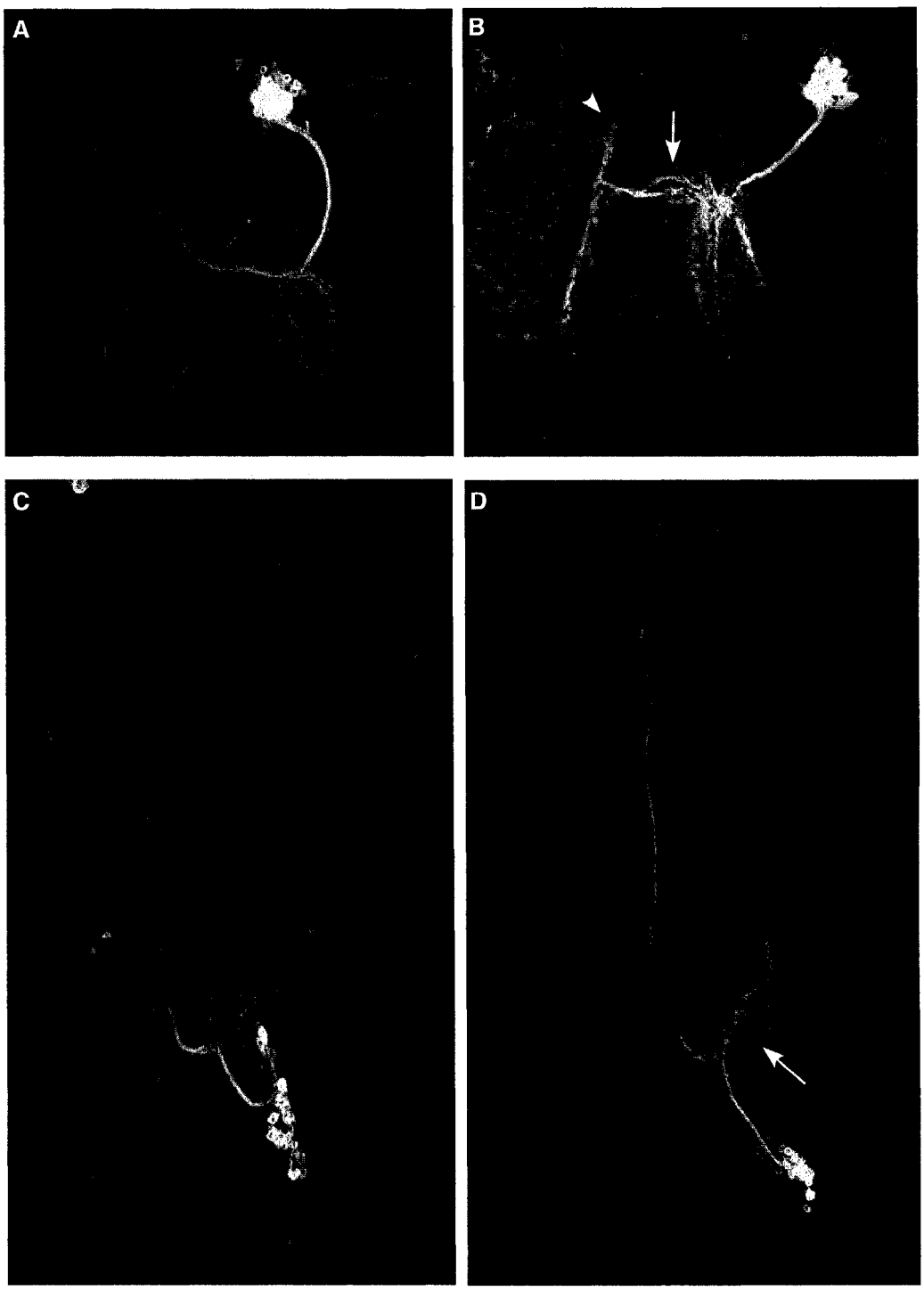

A
B
C
D

Figure 4.11 *Lineage 15 in the adult CNS.*
Ventral projections of lineage 15 in control animals, in the first (A), second (C), and third (E) thoracic neuromeres. Lineage 15 expressing EcR-DN, in the first (B), second (D) and third (F) thoracic neuromeres. Clones of lineage 15 expressing EcR-DN have reduced and clumped dendritic arbors.

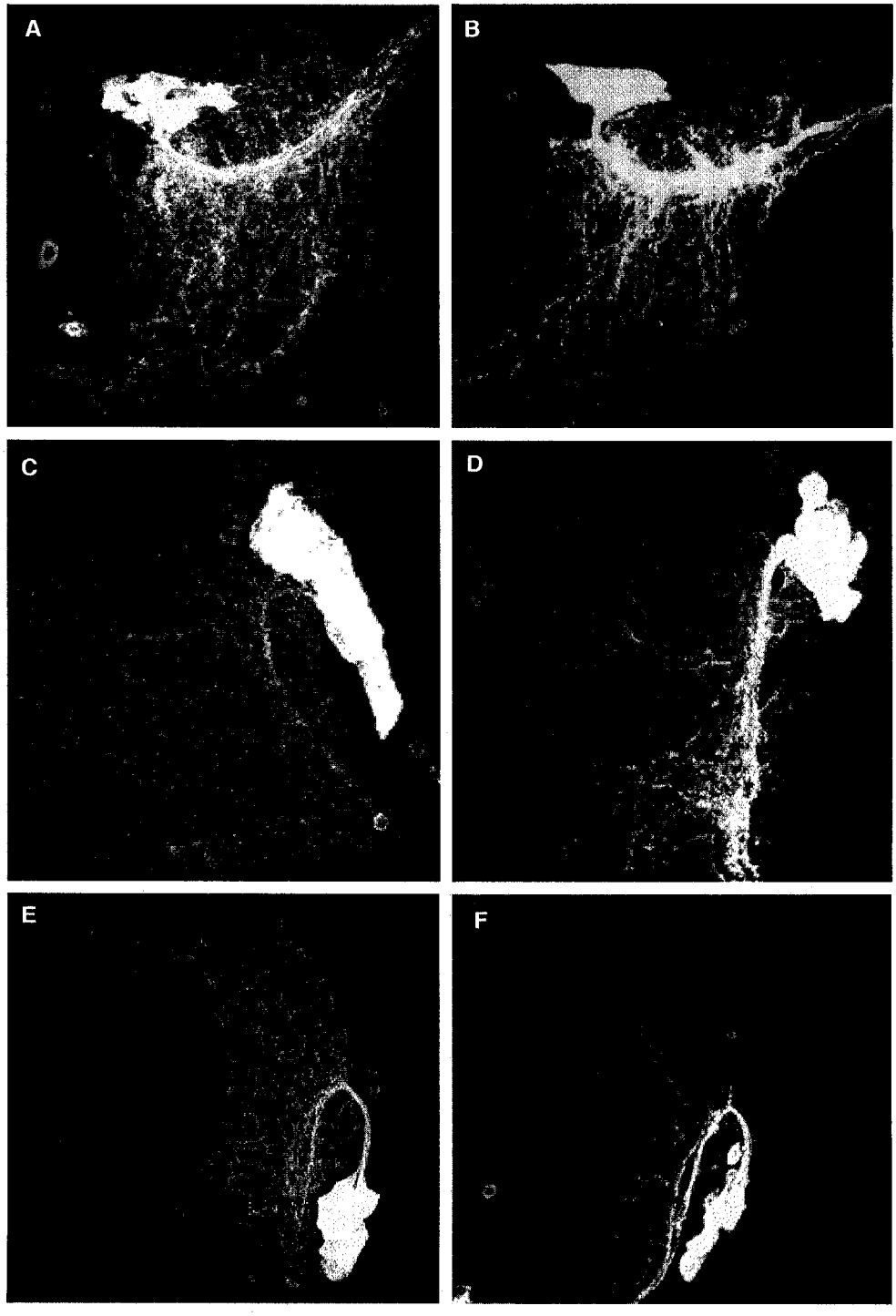

A
B
C
D
E
F

Figure 4.12 *Lineage 15 projections into the adult leg.*
(A) Lineage 15 peripheral projections into the adult leg of control animals. Arrows point to the two major branch elaboration sites, proximally and distally within the femur. (B) Lineage 15 peripheral projections from clones expressing the EcR-DN $B1^{W650A}$. (C) Lineage 15 peripheral projections from clones expressing the EcR-DN A^{W650A}. (D) Enlarged distal arbor in the femur of a control animal, showing longitudinally oriented endplates along the muscle fibers. (E) The distal arbor in the femur of an animal expressing EcR-$B1^{W650A}$ in lineage 15 is misaligned and star-shaped, lacking parallel end plates that run along the muscle fibers. (F) The proximal arbor in the femur of an animal expressing EcR-$B1^{W650A}$ in lineage 15 is reduced, compacted and clumped together, so that the neuronal endplates do not spread out along the femoral muscle.

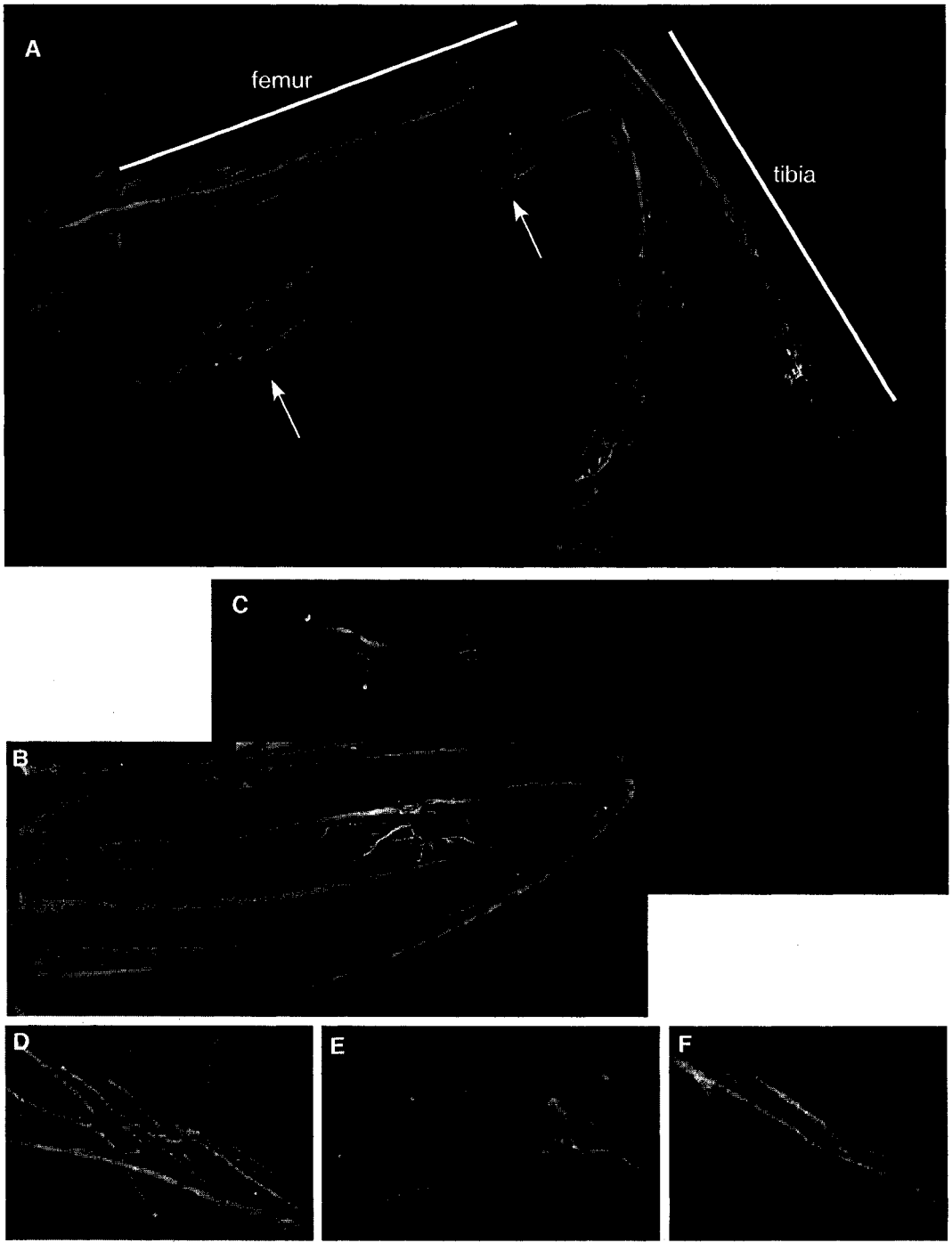
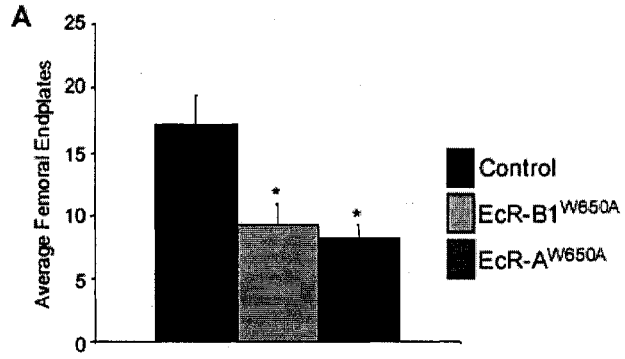
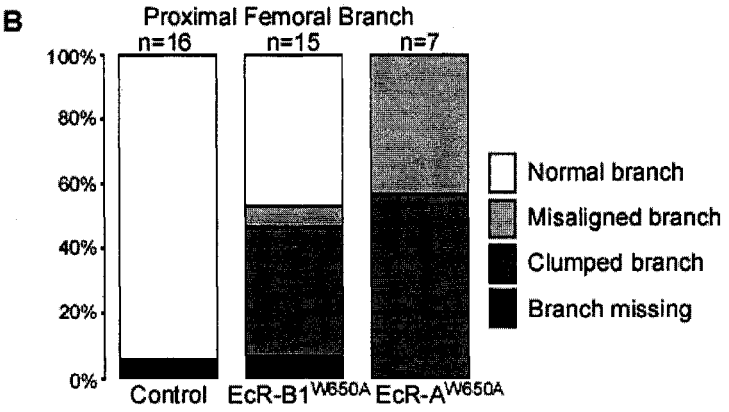

A
femur
tibia
C
B
D
E
F

Figure 4.13 *Quantification of lineage 15 secondary branch defects in the femur.* (A) Control clones have significantly more axonal endplates in the femur than clones expressing $B1^{W650A}$ or A^{W650A}. (B) Clones expressing EcR-DN have normal numbers of proximal femoral arbors, but half of those expressing EcR-$B1^{W650A}$ have clumped or misaligned proximal branches, and all expressing EcR-A^{W650A} have clumped or misaligned branches. (C) Clones expressing EcR-DN have half the number of distal femoral arbors. Expression of EcR-$B1^{W650A}$ results in many arbors with a misaligned phenotype while expression of EcR-A^{W650A} results in either clumped or misaligned arbors. Asterisks indicate statistical significance.


A
Average Femoral Endplates
25
20
15
10
5
0
*
*
Control
EcR-B1W650A
EcR-AW650A


B
Proximal Femoral Branch
n=16
n=15
n=7
100%
80%
60%
40%
20%
0%
Control
EcR-B1W650A
EcR-AW650A
Normal branch
Misaligned branch
Clumped branch
Branch missing

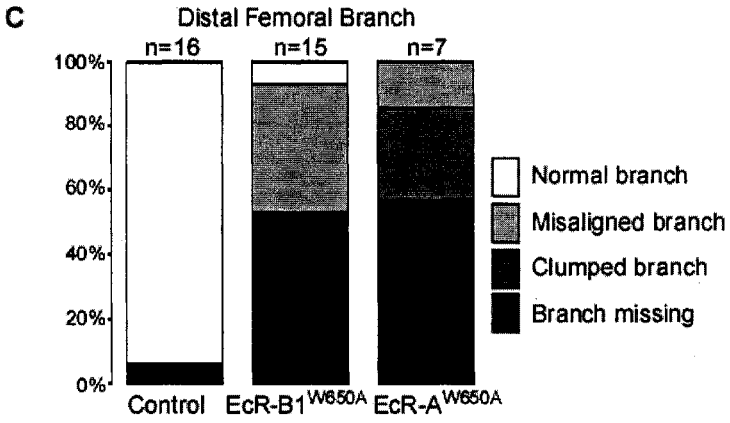

C
Distal Femoral Branch
n=16
n=15
n=7
100%
80%
60%
40%
20%
0%
Control
EcR-B1W650A
EcR-AW650A
Normal branch
Misaligned branch
Clumped branch
Branch missing

Table 4.1 *Quantification of clones containing a neuroblast at 24 hours APF.*

	Total clones analyzed	Clones with NBs
Control	47	0
EcR-B1^{W650A}	101	18
EcR-A^{W650A}	12	3

Notes to Chapter 4

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CURRICULUM VITAE

Education

Ph.D, Molecular and Cellular Biology Program, University of Washington, 2001-2007. Principal Advisor: Dr. James W. Truman, Department of Biology

B.S., Zoology, minors in Biology and Ecology. Washington State University, 1994-1998.

Research

Doctoral Graduate Student, Fall 2001 to 2007.

Steroid hormone receptor regulation of neuronal pruning and outgrowth in the *Drosophila* central nervous system. Funded from 2003 to 2006 by the NIH Developmental Biology Training Grant.

Undergraduate Research, Washington State University, 1997-1998

Principal Advisor: Dr. Lisa Shipley, Department of Natural Resource Sciences. Funded by the Howard Hughes Undergraduate Research Fellowship.

Honors and Awards

NIH Developmental Biology Training Grant, 2003-2006
Howard Hughes Undergraduate Research Fellowship, 1997-1998
Phi Beta Kappa, 1998
S. Town Stephenson Scholar, Honors College Award, 1998
Outstanding Zoology Senior, 1998
Mortar Board Member and Officer, 1997-1998
Washington Scholar, 1994-1998
Glen Terrell Presidential Scholar, 1994-1998
Washington State University Honor Roll, 1994-1998

Teaching and Outreach

Undergraduate Research Mentor, 2007 to present.

Mentoring of an undergraduate neurobiology student in the development and implementation of a *Drosophila* neurodevelopment research project.

Greenhouse Docent, UW Department of Biology, 2003 to present.

Docent for groups of all ages touring the botanical collections.

Teaching Assistant, Marine Biological Laboratory, Woods Hole, MA Summer 2006 and 2007. Neural Systems and Behavior Summer Course.

Teaching Assistant, University of Washington.
Entomology, Winter Quarter 2007 and Foundations in Molecular and Cellular Biology, Winter Quarter 2003.

SEP Teaching Assistant, Fred Hutchinson Cancer Research Center Science Education Partnership Program, Summer 2002. Included public scientific outreach activities and laboratory mentoring of high school teachers, along with training workshops in scientific communication.

Bioscience Careers Seminar Committee Member, 2005 to present.

Campfire USA Science Day Group Leader, July 2006

UWSOM Bioscience Experience Session Leader, July 2005.

Professional Meetings and Activities

Northwest Neuroscience Meeting, Seattle WA, April 2007.
40th Northwest Regional Developmental Biology Conference, Friday Harbor Laboratories, WA, March 2007. Poster presented.
47th Annual Drosophila Research Conference, Houston TX, March 2006
Poster and Ecdysone workshop talk.
Neurobiology of Drosophila, Cold Spring Harbor Laboratories NY, October 2003.
Poster presented.
Northwest Neuroscience Meeting, Seattle WA, April 2005
Poster presented.
45th Annual Drosophila Research Conference, Washington DC, March 2004.
Poster and Ecdysone Workshop talk.
36th Northwest Regional Developmental Biology Conference, Friday Harbor Laboratories WA, March 2003. Platform talk.
Developmental Biology Training Grant Retreats, 2003-2006.
Talks given each year.

Publications

Brown H.L., Cherbas L., Cherbas P., and J.W. Truman. Use of time-lapse imaging and dominant negative receptors to dissect the steroid receptor control of neuronal remodeling in Drosophila. *Development*, 2006. Jan; 133(2): 275-85.