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Cell-Autonomous Requirement of the USP/EcR-B Ecdysone Receptor for Mushroom Body Neuronal Remodeling in *Drosophila*

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Summary

Neuronal process remodeling occurs widely in the construction of both invertebrate and vertebrate nervous systems. During *Drosophila* metamorphosis, γ neurons of the mushroom bodies (MBs), the center for olfactory learning in insects, undergo pruning of larval-specific dendrites and axons followed by outgrowth of adult-specific processes. To elucidate the underlying molecular mechanisms, we conducted a genetic mosaic screen and identified one *ultraspiracle* (*usp*) allele defective in larval process pruning. Consistent with the notion that USP forms a heterodimer with the ecdysone receptor (EcR), we found that the EcR-B1 isoform is specifically expressed in the MB γ neurons, and is required for the pruning of larval processes. Surprisingly, most identified primary EcR/USP targets are dispensable for MB neuronal remodeling. Our study demonstrates cell-autonomous roles for EcR/USP in controlling neuronal remodeling, potentially through novel downstream targets.

Introduction

Neurons need to make specific connections with their targets by elaborating their dendrites and projecting their axons through defined paths. These projections are subject to a wide range of modifications during development and in adult life. For instance, neurons often send out exuberant processes in early stages and later undergo selective pruning to eliminate extra branches (Hubel et al., 1977; O'Leary and Koester, 1993). This developmentally regulated remodeling of neuronal projections has been observed in various mammalian neurons, including most layer 5 pyramidal neurons projecting to subcortical areas (Stanfield et al., 1982); neurons constituting the callosal cortical connections between the two hemispheres (Innocenti, 1981; O'Leary et al., 1981); and layer 2/3 local interneurons of the visual cortex (Katz and Callaway, 1992). It is possible that analogous mechanisms may be used for reorganization of neuronal connections in adult animals as a consequence of learning and experience. The molecules important for neuronal process remodeling have just begun to be identified (e.g., see Weimann et al., 1999); however, the mechanisms underlying how maturing neurons reorganize their projections remain to be elucidated.

Holometabolous insects, which undergo complete metamorphosis, offer a model system for studying neuronal remodeling, because formation of the adult central nervous system (CNS) during metamorphosis involves reorganization of larval neural circuits (reviewed in Truman, 1990). In the holometabola, the adult CNS is more complex and composed of many more neurons than the larval CNS. Whereas the majority of adult neurons are born after the establishment of the larval CNS, most neurons constituting the larval neural circuits also persist into the adult stage (Truman, 1990). Following identified larval neurons through metamorphosis has revealed that these neurons undergo extensive remodeling in order to acquire the adult pattern of projections. Such remodeling involves loss of larval axonal and dendritic branches followed by outgrowth of adult-specific projections (e.g., Truman and Reiss, 1976; Technau and Heisenberg, 1982; Levine and Truman, 1985; Weeks and Truman, 1985; Lee et al., 1999).

Previous studies have implicated the steroid molting hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) as a regulator of insect neuronal remodeling (reviewed in Levine et al., 1995). In insects, both larval development and metamorphosis are under the control of the ecdysteroid pulses (reviewed in Thummel, 1996). Like the metamorphosis of other larval tissues, the remodeling of larval neurons depends on the prepupal ecdysone peak (Truman, 1990) and functional ecdysone receptors (Schubiger et al., 1998). Attempts have been made to determine whether ecdysone acts directly on individual neurons to mediate the remodeling process. For instance, regression of identifiable motoneuron dendrites during *Manduca* metamorphosis, although regulated by ecdysone, appears to be independent of their muscle targets or input from some sensory neurons (Weeks and Truman, 1985; Jacobs and Weeks, 1990). Further cell culture studies indicate that application of ecdysone results in significant morphological changes in dissociated neurons of *Manduca* and *Drosophila* (Prugh et al., 1992; Kraft et al., 1998). Although these experiments suggest that ecdysone could act directly on neurons, definitive evidence is lacking that individual remodeling neurons are direct targets for ecdysone in vivo.

Studies from *Drosophila* have revealed a transcriptional regulatory hierarchy that mediates diverse ecdysone-dependent biological activities (reviewed in Thummel, 1996). When *Drosophila* larval salivary glands are exposed to ecdysone, a series of transcriptional changes are induced, as revealed by the sequential appearance of characteristic chromosome puffs in the polytene chromosomes (Ashburner et al., 1974). The binding of ecdysone to heterodimeric receptors, composed of the nuclear receptor superfamily members ecdysone receptor (EcR) and Ultraspiracle (USP) (Yao et al., 1992; Yao et al., 1993; Thomas et al., 1993), upregulates the

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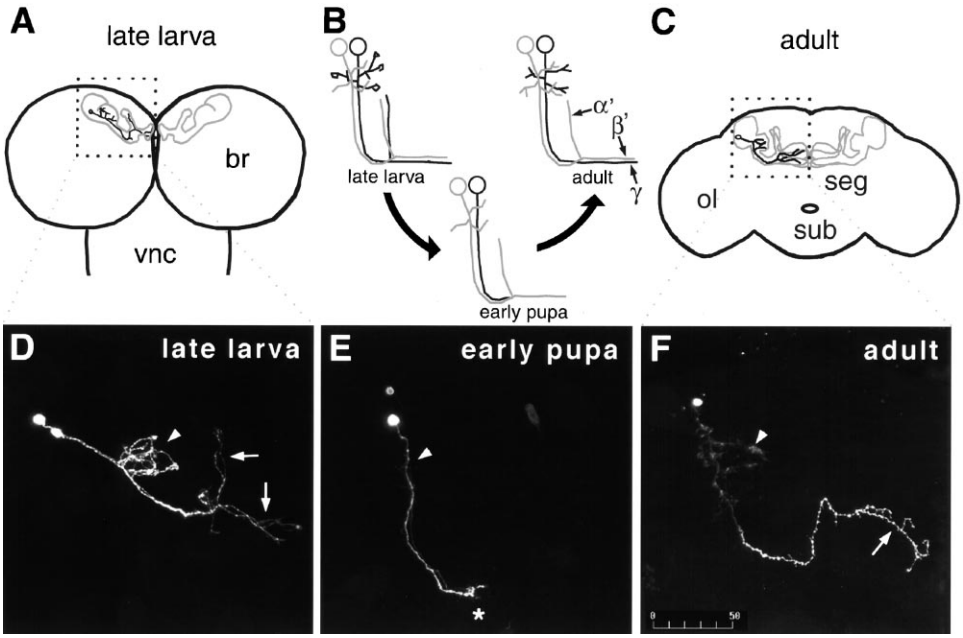

Figure 1. Remodeling of MB γ Neurons

In all figures, the unit of the scale bar is μm; the left brain hemispheres are shown; midline is toward right and dorsal is up. br, brain; vnc,ventral nerve cord; seg, supraesophageal ganglion; sub, subesophageal ganglion; ol, optic lobe.

(A and C) Schematic drawing of *Drosophila* brains at late larval (A) and adult (C) stages, not to scale. One MB neuroblast clone (one quarter of the entire MB) in each brain lobe is outlined, and one representative γ neuron is drawn at the left brain hemisphere. At the larval stage, every MB γ axon bifurcates into the dorsal and medial lobes. In adults all γ neurons project axons medially toward the midline without dorsal branching.

(B) Schematic drawing of a single γ (black) and α′/β′ (gray) neurons in late larval, 18-hr APF, and adult stages, showing that axonal and dendritic reorganization occurs in γ but not in α′/β′ neurons (Lee et al., 1999).

(D–F) Composite confocal images of single-cell/two-cell clones of MB γ neurons at late larval (D), 18-hr APF (E), and adult (F) stages. These MARCM clones were generated in newly hatched larvae; tissues were processed for immunofluorescence (see Experimental Procedures) at the indicated stages. Note that the larval dendrites undergo extensive pruning at the early pupal stage (arrowheads in [D] and [E]) and the larva-specific axonal branches (arrows in [D]) were completely pruned (star in E) before being replaced by the processes without dorsal branching (arrow in [F]). Genotype: *hs-FLP/Y;FRTG13,UAS-mCD8GFP,GAL4–201Y/FRTG13,tubP-GAL80*.

transcription of a small number of early puff (primary response) genes. These encode transcription factors (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991) that in turn activate the expression of a large number of late puff genes (e.g., Walker and Ashburner, 1981; Huet et al., 1993). It has been generally thought that the combined actions of various late puff genes finally mediate distinct tissue-specific biological responses, including neuronal remodeling (reviewed in Levine et al., 1995; Thummel, 1996).

The recently established MARCM (for *m*osaic *a*nalysis with a *r*epressible *c*ell *m*arker) genetic mosaic system has allowed the study of functions of genes in various neural developmental processes in the *Drosophila* brain (Lee and Luo, 1999; Awasaki et al., 2000; Lee et al., 2000; Liu et al., 2000; Martini et al., 2000). The MARCM system allows unique labeling of homozygous mutant cells in a mosaic tissue, which is important for phenotypic analysis of individual mutant neurons in the complex brain. Because typical neurogenesis involves the generation of ganglion mother cells (GMCs) from neuroblasts followed by the formation of two postmitotic neurons from each GMC, the MARCM system can be used to mark the entire axonal and dendritic projections of single neurons if mitotic recombination occurs during GMC division (Lee et al., 1999; Lee and Luo, 1999; see also Figure 1).

The MARCM-based analysis has elucidated the cellular basis for the development of the mushroom bodies (MBs), including neuronal reorganization of the MB during metamorphosis (Technau and Heisenberg, 1982) at a single cell level (Lee et al., 1999). The MBs are prominent neuropils of the central brain that are essential for several forms of learning and memory (Heisenberg et al., 1985; Davis, 1993; de Belle and Heisenberg, 1994; Liu et al., 1999). Each MB is composed of approximately 2,500 neurons, which are derived from four neuroblasts that undergo hundreds of asymmetric divisions through embryonic, larval, and pupal stages (Ito and Hotta, 1992; Ito et al., 1997). Unlike most larval-born neurons, which are arrested as immature neurons until the pupal stage, MB neurons elaborate axonal and dendritic projections shortly after mitosis (Lee et al., 1999). In the larval brain, every MB neuron extends a single process from which dendrites branch out into the calyx. The axon extends further and then bifurcates into two major branches, one projecting medially and the other projecting dorsally (Figures 1A and 1D; Lee et al., 1999). Interestingly, MB neurons generated prior to the mid-third instar stage, named γ neurons (Crittenden et al., 1998; Lee et al., 1999), prune the medial and dorsal branches during early metamorphosis and subsequently project axons only into the medial γ lobe of the adult MB (Figures 1C and 1F). In contrast, the α′/β′ MB neurons that are born after

the mid-third instar stage retain their larval projections during metamorphosis (gray neuron in Figure 1B; see also Lee et al., 1999).

Because the MARCM system further allows one to generate clones homozygous for any mutation of interest only in the uniquely labeled γ neurons, we decided to use the MB γ neuron as a genetic model system to investigate the molecular mechanisms of neuronal remodeling. We report here that both a forward genetic screen and a candidate gene approach have indicated that USP, the *Drosophila* homolog of the vertebrate retinoid X receptor (RXR) that is a component of the functional ecdysone receptor complex, is essential for MB γ neuron remodeling. We further show that the EcR-B1 isoform is specifically expressed in the MB neurons destined for remodeling, and that it mediates the axonal pruning of MB γ neurons independent of the surrounding cells. Finally, we tested the individual functions of several ecdysone primary response genes, including *Broad-Complex (BR-C)*, *E74*, and *E75*, and found that none of them are essential for the EcR/USP-mediated MB remodeling. This study demonstrates cell-autonomous roles for EcR/USP in controlling MB neuronal remodeling, potentially through novel downstream targets.

Results

Remodeling of MB γ Neurons

Throughout this study, the MARCM system was used to generate clones of uniquely labeled MB neurons by inducing mitotic recombination in newly hatched larvae (NHL). All single-cell/two-cell MB clones induced in NHL were γ neurons, and their axon projection patterns underwent reorganization during the pupal stage (Lee et al., 1999). Following the remodeling of MARCM-labeled single γ neurons at different stages revealed how the bifurcated larval axons were converted into adult processes lacking dorsal branching (Figures 1D–1F). Shortly after puparium formation, both dorsal and medial branches started to degenerate (Figure 1E; data not shown). It remains to be determined whether the degenerating axons are pruned by fragmentation or retraction. Interestingly, the axon pruning stopped at the bifurcation point (Figure 1E). Later, the pruned axons extended growth cones and projected new processes toward the midline without dorsal branching (Figure 1F). In addition, the dendrites of γ neurons underwent extensive degeneration followed by re-elaboration during metamorphosis (arrowheads in Figures 1D–1F).

Mosaic Screen for Mutations Defective in MB Axon Remodeling

Genetic screens in mosaic organisms are effective methods for identifying pleiotropic genes required for a late developmental process (e.g., Xu and Rubin, 1993; Liu and Montell, 1999; Newsome et al., 2000). In mosaic screens, only cells of interest are made homozygous for a random mutation and their phenotypes are examined in an otherwise phenotypically wild-type background. Using the MARCM system, we could selectively and efficiently generate specifically labeled clones of MB neurons that were homozygous for a random mutation. We reasoned that if a gene is required in MB γ neurons for pruning their larval-specific axons, the loss of such a gene in MB γ neurons would be expected to result in a phenotype in which adult γ neurons retain the larval dorsal branches, a readily identifiable phenotype. We therefore conducted a genetic mosaic screen and searched for novel mutations having abnormal axon projections in the uniquely labeled homozygous mutant γ neurons in the adult. From 750 independent ethyl methanesulfonate (EMS)-induced X chromosome-linked lethal mutant lines (see Experimental Procedures; Figure 2A), we identified two, *l(X)48* and *l(X)101*, that retained the larval type of axon projections in mutant γ neurons at the adult stage. The phenotypes were indistinguishable in both lines. Mutant neurons possessed bifurcated axons, mimicking the larval type of projections (Figures 2B and 2C) instead of having all axons projecting toward the midline as in wild type (Figure 1F).

To determine the cause of such abnormal axon projections, we examined γ neurons homozygous mutant for *l(X)48* or *l(X)101* lines (hereafter referred to as mutant neurons) through different developmental stages. In larvae, mutant neurons acquired the wild-type pattern of dendrites and axons (data not shown). However, pruning of the larval dendrites and axons was not observed during early metamorphosis (data not shown). By 18 hr after puparium formation (APF), larval-specific axonal branches and most dendrites had been pruned in wild-type γ neurons (Figure 2E), but mutant γ neurons retained larval dendrites and axonal branches (Figure 2D). These experiments indicated that the larval axon projection pattern persisted in the adult *l(X)48* and *l(X)101* mutant γ neurons as a result of a failure in axon pruning.

Cell-Autonomous Requirement of USP for the MB Remodeling

In parallel with the mosaic genetic screen, we took a candidate gene approach to test whether mutations in known genes would affect MB remodeling. Because the timing of pruning coincides with the onset of metamorphosis, and because ecdysone has been implicated in mediating the metamorphosis of neurons (see Introduction) and changes of MB neuronal morphology in culture (Kraft et al., 1998), we decided to test the involvement of the ecdysone receptor in MB neuronal remodeling. The functional ecdysone receptor is a heterodimeric complex composed of EcR and USP subunits (Yao et al., 1993). Because the *EcR* locus is centromeric to all available FRT sites on the 2R chromosomal arm, we decided to perform mosaic analysis of *usp* in MB metamorphosis. MB neurons born in newly hatched larvae were made homozygous for a loss-of-function *usp* mutation (usp^3) (Oro et al., 1992; Henrich et al., 1994; Zelhof et al., 1997) using MARCM, and their projection patterns were analyzed at several later stages. We found that in larval brains, usp^3 mutant γ neurons acquired axonal and dendritic projections indistinguishable from wild type (Figure 3A), suggesting that normal morphogenesis of MB γ neurons does not require USP activity (see Discussion). However, during metamorphosis, pruning of larval dendrites and axons was not observed in usp^3 mutant single-cell or two-cell clones (Figure 3B), and 100% usp^3 mutant γ neurons ($n > 20$) retained their larval-type bifurcation of axons into the adult stage (Figure 3C). This failure in axon and dendrite remodeling was completely rescued by a transgene containing the usp^+ genomic

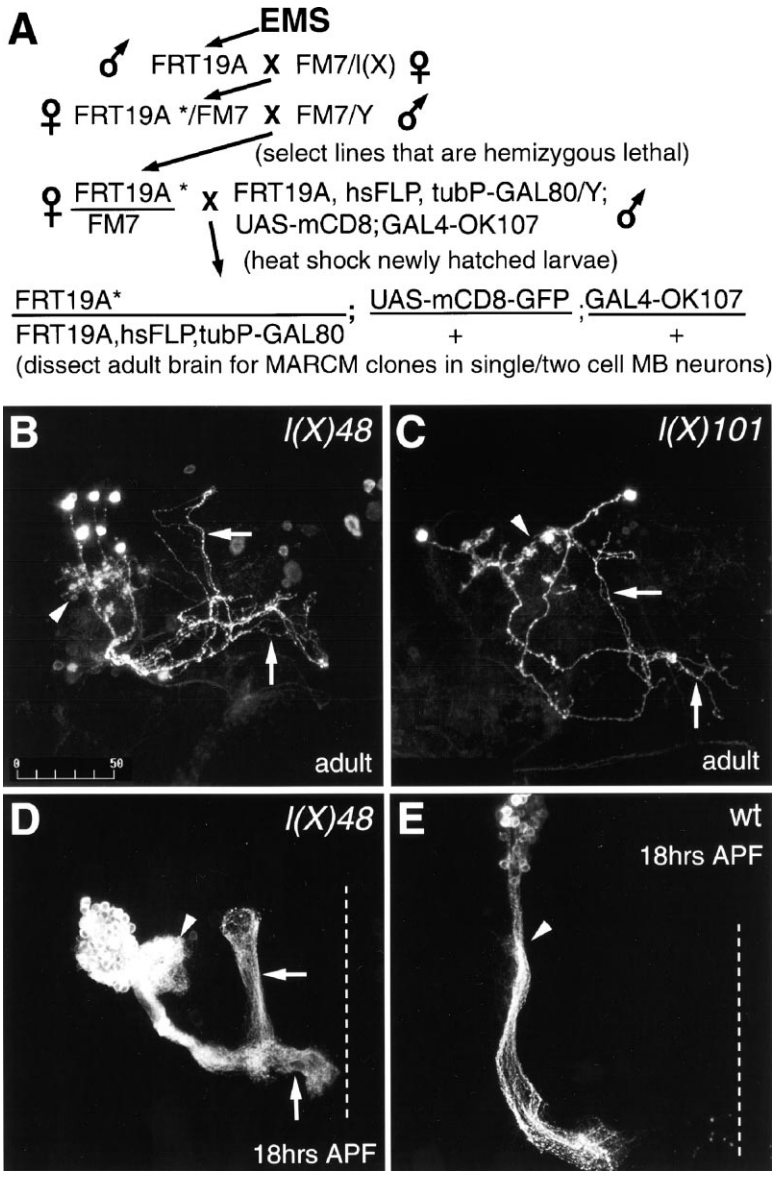

Figure 2. Identification of the *l(X)48* and *l(X)101* Mutants Defective in the Pruning of Larval-Specific Axons and Dendrites

(A) Summary of the genetic crosses for the MARCM-based genetic screen. The asterisk represents a mutagenized chromosome.

(B and C) Composite confocal images of multiple single-cell/two-cell MARCM clones of γ neurons at the adult stage. They are homozygous for the *l(X)48* (B) and *l(X)101* (C) mutations, respectively. Note that the larval type of bifurcated axons (horizontal and vertical arrows represent dorsal and medial lobes, respectively, in this and all subsequent images) persisted into the adult stage. Genotypes: (B) *FRT^{19A},l(X)48/FRT^{19A},hs-FLP,tubP-GAL80; UAS-mCD8GFP/+;GAL4-OK107/+*; and (C) *FRT^{19A},l(X)101/FRT^{19A},hs-FLP,tubP-GAL80; UAS-mCD8GFP/+;GAL4-OK107/+*.

(D and E) Composite confocal images of the MARCM neuroblast clones (Lee et al., 1999) composed of MB γ neurons labeled with *GAL4–201Y*. The *l(X)48* homozygous mutant clone (D) and the wild-type clone (E) were both generated in newly hatched larvae and examined at 18-hr APF. In contrast to the pruning of both larval dendrites and the larval-specific axonal branches in wild type (E), the *l(X)48* mutant MB clone retained a prominent calyx composed of MB dendrites (arrowhead in [D], compared with the arrowhead in [E]) and two axonal lobes (arrows in [D]). The broken lines indicate the brain midline. Genotypes: (D) *FRT^{19A},l(X)48/FRT^{19A},hs-FLP,tubP-GAL80;UAS-mCD8GFP,GAL4–201Y/+*; and (E) *hs-FLP/X;FRTG13,UAS-mCD8GFP,GAL4–201Y/FRTG13,tubP-GAL80*.

DNA (Zelhof et al., 1997; data not shown). These experiments indicated that loss of USP activity, like the *l(X)48* and *l(X)101* mutations, makes MB γ neurons resistant to the pruning of larval processes. In addition, the pruning defects were observed in isolated single mutant neurons in otherwise phenotypically wild-type organisms, demonstrating a cell-autonomous requirement of USP for MB neuronal remodeling.

Because USP regulates biological activities through transcriptional control, one possible interpretation is that lack of USP activity alters the cell fate of γ neurons, which indirectly perturbs normal process remodeling. In particular, the later-born α′/β′ MB neurons, although derived from the same neuroblasts as the γ neurons, do not prune their bifurcated axonal branches during the pupal stage (Figure 1B; Lee et al., 1999). Lack of USP may simply transform γ neurons into α′/β′ neurons. However, three lines of evidence strongly argue against this possibility. First, at the wandering third instar larval stage, the morphology of γ neurons lacking USP appears much more like wild-type γ neurons than α′/β′ neurons, as evidenced by the clawlike dendritic structures characteristic of mature γ neurons (Figures 1D and 3A; Lee et al., 1999). Second, *usp^3* γ neuron axonal projections are distinct from those of α′/β′ neurons in adult (data not shown). Third, two markers that are expressed in wild-type γ but not α′/β′ neurons, GAL4–201Y and FasII (Yang et al., 1995; Crittenden et al., 1998; Lee et al., 1999), are still expressed in the *usp^3* mutant γ neurons (Figure 3; data not shown), demonstrating that the mutant neurons retain γ-like characteristics. These observations strongly suggest that USP-mediated transcriptional regulation directly orchestrates remodeling without affecting the identity of the MB γ neurons.

l(X)48 and *l(X)101* Contain *usp* Lethal Mutations

The fact that the *l(X)48*, *l(X)101*, and *usp* mutations all share the same phenotypes and are located on the X

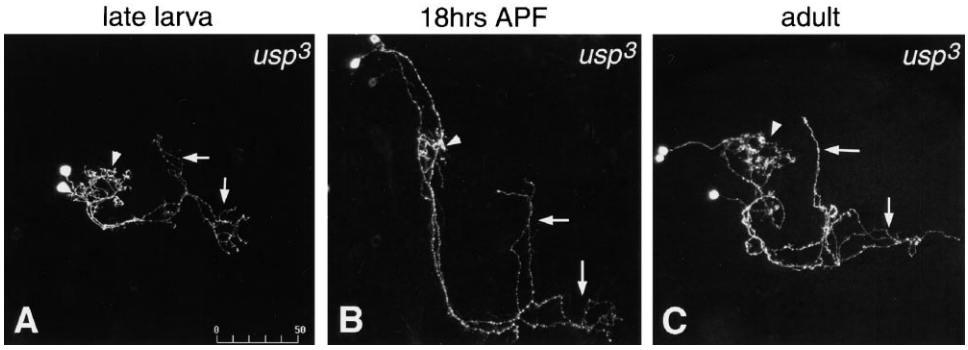

Figure 3. Lack of Axonal and Dendritic Pruning in *usp*^{3} Mutant MB γ Neurons

Composite confocal images of single-cell/two-cell MARCM clones of *usp*^{3} mutant γ neurons generated in newly hatched larvae and examined in late larva (A), 18-hr APF (B), and adult (C) stages, respectively. Note the persistence of bifurcated axons in pupae and adults (arrows in [B] and [C]), and the lack of obvious pruning of larval dendrites in pupae (arrowhead in [B]). Genotype: *FRT*^{19A}*,usp*^{3}*/FRT*^{19A}*,hs-FLP,tubP-GAL80; UAS-mCD8GFP,GAL4–201Y/+;+/TM3*.

chromosome prompted us to test whether the *l(X)48* and *l(X)101* chromosomes carry mutations in the *usp* gene. Because normal male flies are hemizygous for the X chromosome, lethal complementation tests were done by crossing the heterozygous *l(X)48* or *l(X)101* female and the hemizygous *usp*^{3} male bearing a genomic *usp*$^+$ transgene located on one marked third chromosome (Zelhof et al., 1997; Schubiger and Truman, 2000). Three observations indicated that both *l(X)48* and *l(X)101* carry *usp* lethal mutations. First, both *l(X)48* and *l(X)101* mutant X chromosomes failed to complement the *usp*^{3} mutation. Second, the *usp*$^+$ genomic transgene fully rescued the transheterozygotes of *l(X)48* or *l(X)101* and *usp*^{3}. Third, the hemizygous *l(X)48* and *l(X)101* male progeny survived in the presence of the *usp*$^+$ transgene. However, only about 10% of the *l(X)101* hemizygous males could be rescued to adulthood as compared with 100% of *l(X)48* hemizygous males or *l(X)101/usp*^{3} females, suggesting that a second-site mutation on the *l(X)101* chromosome is responsible for the semilethal phenotype.

To determine the molecular identity of *usp* mutations on the *l(X)48* and *l(X)101* chromosomes, the *usp* open reading frames (ORFs) from both mutant chromosomes were sequenced. Surprisingly, we found the same single nucleotide change in both *l(X)48* and *l(X)101* in the entire ORF of the *usp* gene. This was not likely due to a polymerase chain reaction (PCR) cross-contamination (see Experimental Procedures). Nor was it likely due to mutant stock contamination, as *l(X)48* and *l(X)101*were identified from two different rounds of EMS mutagenesis several months apart, and they are genetically distinct as *l(X)101* apparently carries an additional semilethal mutation (see above). Because both alleles we isolated resulted in the same base change, we named this allele *usp*^{5}. This nucleotide change in *usp*^{5} results in an Arg to Lys change in the second zinc finger of the DNA binding domain of USP (Figure 4). This Arg makes direct contact with the phosphate group in target DNA and is invariant among all nuclear hormone receptors (Rastinejad et al., 1995). Interestingly, two previously identified *usp* alleles also change two other phosphate-contacting arginine residues in the DNA binding domain (Figure 4; Henrich et al., 1994).

EcR-B1 Is Expressed in MB γ but not α′/β′ Neurons

A functional ecdysone receptor is a heterodimeric complex composed of USP and EcR (Yao et al., 1993; Hall and Thummel, 1998). There are three known EcR isoforms, EcR-A, EcR-B1, and EcR-B2 (Talbot et al., 1993), that are differentially expressed in neurons undergoing different developmental changes during metamorphosis (Robinow et al., 1993; Truman et al., 1994). EcR-A predominates in adult-specific neurons undergoing maturational processes, whereas EcR-B1 predominates in functional larval neurons that reorganize their projection patterns during metamorphosis. Because γ neurons, but not α′/β′ neurons, are subject to process remodeling (Figure 1B; Lee et al., 1999), we tested whether the EcR-B1 is specifically expressed in the MB γ neurons. To distinguish γ neurons from α′/β′ neurons unequivocally, we used *GAL4–201Y* to drive expression of a GFP (green fluorescent protein) marker in γ neurons but not in α′/β′ neurons (Lee et al., 1999). We found that in the region of MB cell bodies, the EcR-B1 expression pattern perfectly matched the *GAL4–201Y*-driven GFP expression (Figure 5), indicating that all γ neurons and only γ neurons in the larval MB express EcR-B1. This observation supports the notion that EcR-B1 expression is enriched in neurons destined for remodeling during metamorphosis (Truman et al., 1994).

Requirement of EcR-B Isoforms for the MB Remodeling

To test directly whether EcR-B1 activity is essential for MB metamorphosis, we asked whether the MB γ neurons reorganize their projections during the pupal stage in *EcR-B* mutants. Because of the difficulty in collecting enough homozygous mutant "pupae" (see Experimental Procedures), these experiments were performed using a combination of two EcR-B mutant alleles. The *EcR*^{W53st} allele has a nonsense mutation in a B1-specific exon (Bender et al., 1997), and the *EcR*^{31} allele is derived from P-element imprecise excision-removing DNA near the transcription start site shared by both EcR-B isoforms (Schubiger et al., 1998). In *EcR*^{W53st}*/EcR*^{31} (*EcR-B1/EcR-B)* mutant pupae, the projections of the MB γ neurons were visualized by expressing a membrane-targeted GFP selectively in γ neurons using the *GAL4–201Y*

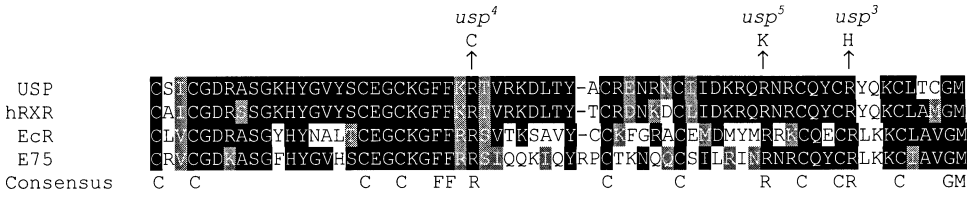

Figure 4. Molecular Nature of *usp* Mutant Alleles

Sequence alignment of the DNA binding domain of USP, its human homolog hRXR, and two other *Drosophila* nuclear hormone receptors investigated in this study, EcR and E75. The amino acid changes of *usp*^{5} identified in this study, as well as two previously identified *usp* alleles are shown. All three alleles result in changes of invariant arginines that contact phosphates in target DNA. The consensus sequence is derived from sequence analysis of the C4-type zinc fingers (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00105).

driver. The projections of GFP-positive neurons were examined at 24-hr APF (see Experimental Procedures), when these MB γ neurons should have already lost their larval-specific dorsal projections and the dendrites of the larval calyx in normal pupae. Instead, we found that MB γ neurons in *EcR*^{W53st}/*EcR*^{31} "pupae" still retained their larval characteristics (n = 18 brain lobes), including both dorsal and medial lobes and a prominent calyx (Figure 6A). This result indicated that MB reorganization was blocked in the *EcR-B1*/*EcR-B* mutant "pupae."

As non-mosaic *EcR-B* mutants are predicted to have widespread defects during metamorphosis (Bender et al., 1997; Schubiger et al., 1998), MB remodeling defects could be a secondary consequence of a general delay or disruption in brain development. Indeed, we observed gross abnormalities in brain structure in these mutant "pupae" (data not shown). To determine whether EcR-B1 is indeed required in remodeling neurons for the reorganization process, we attempted to rescue the MB metamorphosis defect in the *EcR-B1*/*EcR-B* mutant "pupae" by targeting the expression of various EcR isoform-specific trangenes to MB γ neurons using the *GAL4–201Y* driver. Expression of *UAS-EcR-A*, *EcR-B1*, or *EcR-B2* under *GAL4–201Y* in a wild-type background did not result in any detectable abnormalities (data not shown). In *EcR*^{W53st}/*EcR*^{31} mutant brains at 24-hr APF, we observed normal pruning of both larval dendrites and axons in MB γ neurons expressing the EcR-B1 (Figure 6C; n = 30 brain lobes) or EcR-B2 (Figure 6D; n = 24 brain lobes) isoform, indicating that γ neuron-specific expression of EcR-B1 or EcR-B2 is sufficient for rescuing the MB remodeling. In contrast, expression of the EcR-A isoform did not rescue axonal pruning in *EcR*^{W53st}/*EcR*^{31} mutants (Figure 6B; n = 38 brain lobes). Interestingly, some degree of dendritic pruning was observed in EcR-A expressing MB γ neurons in *EcR*^{W53st}/*EcR*^{31} background, as evidenced by the lack of strong calyx staining (arrowhead in Figure 6B, compared with Figure 6A), suggesting differential requirements for the remodeling of MB dendrites versus axons. Taken together with the wild-type EcR-B1 expression pattern, these results indicate that the EcR-B1/USP heterodimer controls MB γ neuronal remodeling during metamorphosis.

MB Neuronal Remodeling in *BR-C*, *E75*, and *E74* Mutants

A transcriptional regulatory hierarchy involving the primary response genes *BR-C*, *E74*, and *E75* has been shown to mediate EcR/USP-dependent ecdysone signaling in diverse tissues (Thummel, 1996) (Figure 7F). In order to gain further insights into the molecular mechanisms underlying EcR-B/USP-dependent neuronal remodeling, we tested whether the primary response genes *BR-C*, *E74*, and *E75* are required for MB metamor-

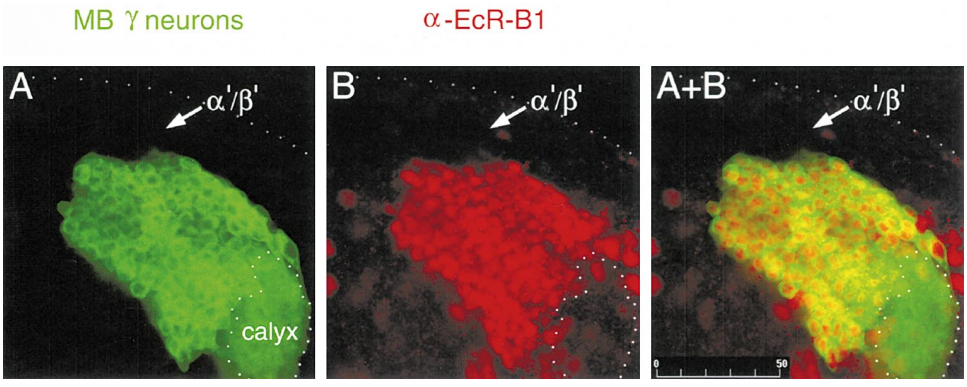

Figure 5. Expression of EcR-B1 in MB γ Neurons

A single-section confocal image of the MB cell body region in a wandering third instar larval brain. The brain was immunostained for both the mCD8GFP expression ([A], green) and the EcR-B1 expression ([B], red). Merging (A) and (B) revealed that all γ neurons marked by the mCD8GFP are positive for EcR-B1 (A + B). Note that EcR-B1 expression is not present in the GFP-negative α′/β′ cell bodies (arrows) that are located superficial to the GFP-positive γ neurons. The calyx composed of dendrites is outlined by white dots and the brain margin is labeled by gray dots. Genotype: *UAS-mCD8GFP/GAL4–201Y*.

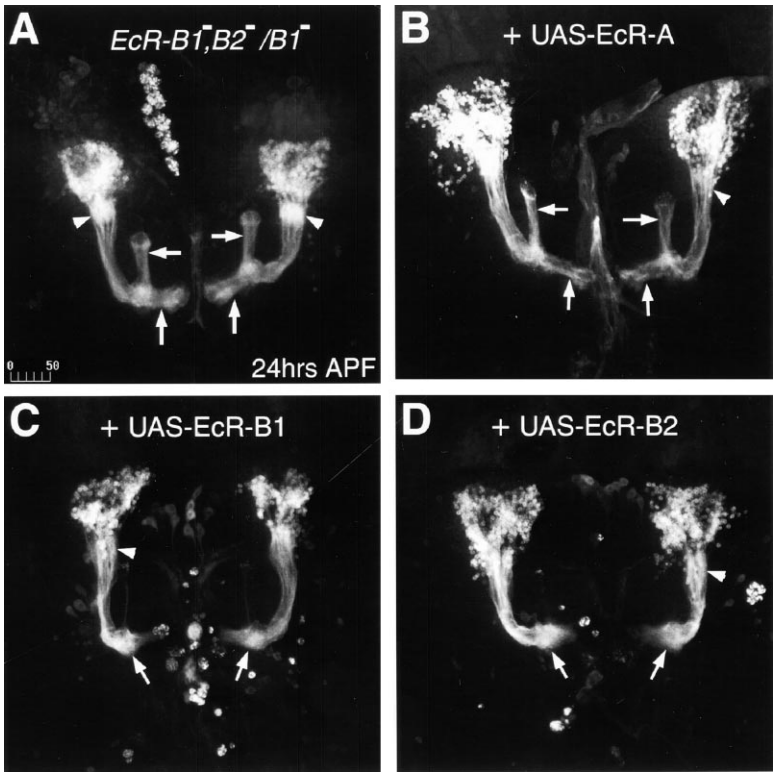

Figure 6. Rescue of the MB γ Neuron Pruning in the *EcR-B1*/*EcR-B* Mutants

Composite confocal images of the paired MBs in the EcR^{W53st}/EcR^{31} mutant "pupal" brains in the absence of any EcR transgenes (A) and in the presence of *UAS-EcR-A* (B), *UAS-EcR-B1* (C), and *UAS-EcR-B2* (D). *GAL4–201Y* was used to restrict the expression of mCD8GFP to all γ neurons.

(A) In the EcR^{W53st}/EcR^{31} mutant background, the MB γ neurons still retained the larval calyces (the MB dendritic field indicated by arrowheads) and two axonal lobes (arrows) even at 24-hr APF, indicating a lack of pruning of larval-specific processes. Genotype: EcR^{W53St}, *UAS-mCD8GFP, GAL4–201Y*/EcR^{31}.

(B) The larval-specific axonal lobes (arrows) still persisted after expressing EcR-A specifically in EcR^{W53st}/EcR^{31} mutant MB γ neurons (100% penetrance, n = 38 brain lobes). In contrast, the calyces (arrowhead) became less prominent, indicating some degree of pruning of larval dendrites. Genotype: EcR^{W53St},*UAS-mCD8GFP,GAL4–201Y*/EcR^{31}; *UAS-EcR-A*/+.

(C and D) After expressing EcR-B1 (C) or EcR-B2 (D) specifically in EcR^{W53st}/EcR^{31} mutant γ neurons, pruning of larval-specific axonal branches was rescued and re-extension of new processes was observed (arrows) (n = 30 and 24 brain lobes for EcR-B1 and EcR-B2, respectively). In addition, the larval calyces (arrowheads) were barely detectable. Genotypes: (C) EcR^{W53St},*UAS-mCD8GFP,GAL4–201Y*/EcR^{31};*UAS-EcR-B1*/+; and (D) EcR^{W53St}, *UAS-mCD8GFP,GAL4–201Y*/EcR^{31};*UAS-EcR-B2*/+.

phosis. *BR-C* encodes a family of four zinc finger-containing transcription factors (DiBello et al., 1991). The *E74* gene consists of two isoforms of an ETS domain-containing transcription factor (E74A and E74B) (Burtis et al., 1990), and the two E75 isoforms (E75A and E75B) are members of the nuclear hormone receptor family (Segraves and Hogness, 1990).

We tested four representative mutations, $npr1^3$, $E75^{e213}$, $E74^{P[neo]}$, and $E74^{DL-1}$ by generating homozygous mutant MB γ neurons in otherwise phenotypically wild-type brains. $npr1^3$ disrupts the functions of all BR-C isoforms (Kiss et al., 1988); $E75^{e213}$ is a strong loss-of-function or null mutation affecting both E75A and E75B (Buszczak et al., 1999; M. Buszczak, personal communication); and $E74^{P[neo]}$ and $E74^{DL-1}$ specifically disrupt E74A and E74B, respectively (Fletcher et al., 1995). Using the MARCM strategy, we generated multiple uniquely labeled single-cell/two-cell clones of γ neurons homozygous for each of the four selected mutations. To study the pruning of both dendrites and axons, the projections of homozygous mutant neurons were examined around 18-hr APF, when both larval-specific dendrites and axons are completely pruned in wild type (Figure 1E). All mutant γ neurons except those homozygous for the $E74^{DL-1}$ mutation were well labeled, and we observed no evidence for any delay or defect in dendritic or axonal pruning (Figures 7A–7C). Accordingly, no defect in MB reorganization was detected in the adult stage (data not shown). In the case of the $E74^{DL-1}$ mutant (disrupting the E74B isoform), we encountered difficulty in following γ neuron remodeling through the pupal stage. The frequency of obtaining single-cell clones was drastically reduced. In the occasional single-cell clones we did obtain, MB γ neurons were weakly and unevenly labeled so that we could not determine whether pruning had occurred normally (Figure 7D). However, when examined at the adult stage, $E74^{DL-1}$ mutant γ neurons appeared to complete the reorganization and project axons only to the medial γ lobe (Figure 7E). Taken together, our MARCM analyses did not reveal specific functions for BR-C, E74, and E75, three well-characterized EcR/USP downstream targets, in MB γ neuronal remodeling.

Discussion

Deciphering the mechanisms by which neurons reorganize their existing processes and create new projection patterns is important for understanding how neuronal networks are modulated. Our study demonstrates that the remodeling of MB neurons is dependent on the activities of nuclear hormone receptors USP and EcR-B1. The cell-autonomous requirements of USP and EcR-B1 revealed by the studies in mosaic organisms strongly suggest that nuclear hormone ecdysone works directly on target neurons to orchestrate the neuronal process reorganization program.

In insects, the steroid hormone ecdysone initiates and coordinates diverse tissue-specific developmental programs at different developmental stages, especially during metamorphosis (Thummel, 1996). Specifically, work

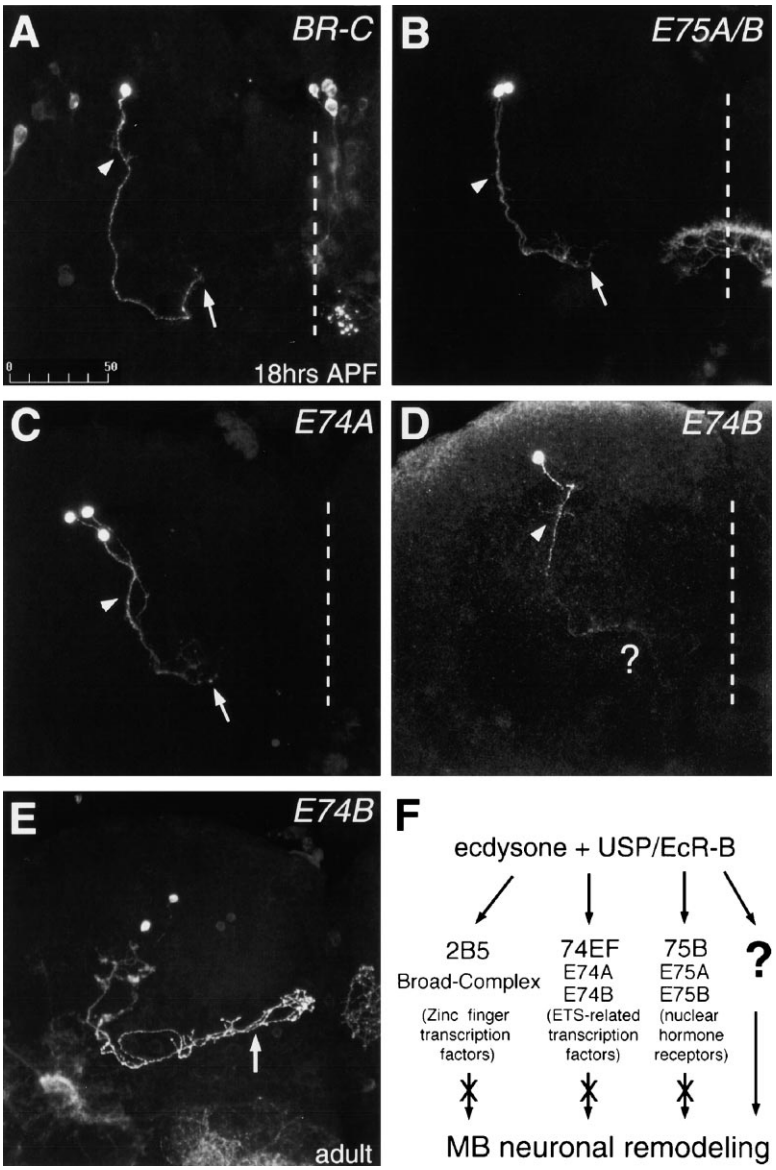

Figure 7. Normal MB γ Neuron Pruning in *BR-C*, *E75*, and *E74* Mutants

(A–D) Composite confocal images of single-cell/two-cell MARCM clones of γ neurons examined around 18-hr APF. (A–C) In mosaic brains, γ neurons homozygous for $npr1^{3}$ (A), $E75^{e213}$ (B), or $E74^{P[neo]}$ (C) lost most larval dendrites (arrowheads), and their axonal termini (arrows) were far away from the midlines (broken lines) and without bifurcation, indicating normal pruning of larva-specific processes. (D) The $E74^{DL\text{-}1}$ mutant γ neuron was weakly and unevenly labeled. This is an example of the strongest labeling. The weak labeling prevented a detailed analysis of the dendritic elaboration (arrowhead) and the axon projection (question mark). Genotypes: (A) *FRT*^{19A}, *npr1*^{3}/*FRT*^{19A},*hs-FLP,tubP-GAL80;UAS-mCD8 GFP/+;GAL4-OK107/+;* (B) *hs-FLP,UAS-mCD8 GFP/X;FRT2A,E75*^{e213}/*FRT2A,tubP-GAL80; GAL4-OK107/+;* (C) *hs-FLP,UAS-mCD8GFP/ X;FRT2A,E74*$^{P[neo]}$/*FRT2A,tubP-GAL80;GAL4-OK107/+;* and (D) *hs-FLP,UAS-mCD8GFP/X; FRT2A,E74*$^{DL\text{-}1}$/*FRT2A,tubP-GAL80;GAL4-OK107/+*. Consistent results were seen for at least 10 samples for each genotype.

(E) Composite confocal images of MARCM-labeled adult γ neurons homozygous for $E74^{DL\text{-}1}$. Note their axons (arrow) only projected medially toward the midline. Genotype: *hs-FLP,UAS-mCD8GFP/X;FRT2A,E74*$^{DL\text{-}1}$/*FRT2A,tubP-GAL80;GAL4-OK107/+*. Consistent results were seen for at least 10 samples of this genotype.

(F) A summary illustrating that the well-characterized BR-C, E74, E75 primary response genes for ecdysone are not individually essential for MB neuronal remodeling, and that the USP/EcR-B heterodimer probably mediates the ecdysone-dependent MB neuronal remodeling through other target genes.

in both *Manduca* and *Drosophila* has implicated the involvement of ecdysone in neuronal process reorganization (Weeks and Truman, 1985; Prugh et al., 1992; Kraft et al., 1998; Schubiger et al., 1998). Our finding that the ecdysone receptor subunit USP is essential for MB neuronal process pruning provides direct genetic evidence to support the importance of ecdysone in orchestrating neuronal process reorganization. USP activity has also been shown to be required for the suppression of both precocious photoreceptor differentiation in eye discs and premature neuronal morphogenesis in wing discs (Zelhof et al., 1997; Schubiger and Truman, 2000), suggesting that the EcR/USP may also regulate neuronal development before the prepupal ecdysone peak. We found that USP is dispensable for the normal morphogenesis of MB γ neurons before puparium formation. However, we cannot rule out the possibility that the USP protein inherited from heterozygous precursors at the time of clone generation may be adequate for larval MB development.

CNS metamorphosis involves two distinct types of cellular changes. Most neurons constituting the larval neural circuits reorganize their projections, while adult-specific neurons begin differentiation (Truman, 1990). There are three different isoforms of the ecdysone receptor, EcR-A, EcR-B1, and EcR-B2, that share common DNA binding and hormone binding domains, but differ in the N-terminal portion. These isoforms are generated by alternative use of promoters (EcR-A versus EcR-B) and alternative splicing (EcR-B1 versus EcR-B2), and exhibit distinct patterns of tissue-specific expression (Talbot et al., 1993). Interestingly, EcR-B1 is expressed in neurons that undergo reorganization, whereas EcR-A is expressed in differentiating neurons (Robinow et al., 1993; Truman et al., 1994). We found that EcR-B1 is abundantly present in the remodeling MB γ neurons but absent in α′/β′ neurons, corroborating previous findings (Truman et al., 1994). Because of the lack of antibodies against the EcR-B2-specific 17 amino acid residues, we have no knowledge regarding EcR-

B2 expression in MB neurons. The lack of MB neuronal remodeling in the *EcR-B1/EcR-B* genotype (i.e., a complete loss of the EcR-B1 activity and one half of the EcR-B2 activity, but wild-type level of the EcR-A activity) indicates that EcR-B1 is necessary for MB neuronal remodeling. Remarkably, this remodeling defect can be rescued equally well by both EcR-B1 and EcR-B2 isoforms, suggesting that the difference between the EcR-B1-specific 226 residues and the EcR-B2-specific 17 residues may not be of functional significance in this context. In contrast, having the EcR-A isoform-specific N-terminal domain cripples EcR's function in mediating axonal pruning. Independent studies by Truman and colleagues have shown that in the EcR-B mutant background, selective expression of EcR-B2 and B1 in the thoracic FMRFamide cells rescued pruning, whereas EcR-A was ineffective (M. Schubiger, S. R., and J. Truman, unpublished results).

During metamorphosis, different neurons in the CNS undergo distinct morphological changes while integrating into the final adult neural networks. It is conceivable that neurons may rely on correct inputs and outputs to orchestrate their morphogenesis. Our study strongly suggests that individual neurons undergo remodeling independent of the surrounding neurons and any other cells. In the MARCM analysis, isolated single *usp* mutant MB γ neurons failed to reorganize their processes in an otherwise phenotypically wild-type brain, while their sibling MB γ neurons and upstream/downstream neurons presumably acquired adult-type projections after normal remodeling. Conversely, in the *EcR-B1/EcR-B* mutant brains expressing *EcR-B* transgenes only in MB γ neurons, the axonal and dendritic pruning can be rescued without the reorganization of other components of the circuit. These experiments imply that ecdysone works directly on MB γ neurons through USP/EcR-B to orchestrate the neuronal reorganization. The fact that neurons are independent functional units during the neural circuit reorganization may have some bearing on developing strategies for neuron replacement therapies.

Ecdysone primary response genes *BR-C*, *E74*, and *E75* are essential for the transcriptional hierarchy mediating the metamorphosis of many larval tissues, including the nervous system (Kiss et al., 1988; Restifo and White, 1991; Fletcher et al., 1995; Fletcher and Thummel, 1995a; Fletcher and Thummel, 1995b; Segraves and Hogness, 1990). To our surprise, our study did not reveal any function for these genes in MB neuronal reorganization. Given that the BR-C, E74, and E75 protein products belong to entirely different classes of transcription factors, it is unlikely that there is significant functional redundancy among these proteins. However, several potential caveats exist regarding E74. First of all, although γ neurons homozygous for the $E74^{DL\text{-}1}$ mutant (for the E74B isoform) chromosome acquired the normal adult type of projections, remodeling of their neuronal processes could not be examined in detail because of weak labeling of mutant neurons at the early pupal stage that might be due to transient downregulation of the *GAL4-OK107* driver expression in the mutant background. Second, because no well-documented mutations inactivate both E74A and E74B, it remains unclear whether there is any functional redundancy between E74A and E74B. We attempted to address this question by performing MARCM analysis using the $E74^{v4}$ allele (FlyBase) that failed to complement both *E74A* and *E74B* specific alleles (our unpublished observations). Unfortunately, MB neurons homozygous for the chromosome arm containing the $E74^{v4}$ mutation were rarely found in mosaic larval brains and had very faint processes even before puparium formation, which prevented us from analyzing the remodeling of these dying neurons; and no mutant neurons could be detected after adult eclosion. There is a tightly linked vtd^4 mutation associated with the $E74^{V4}$ allele (FlyBase), so we could not rule out the possibility that vtd^4 or other background mutation(s) are responsible for the cell lethal phenotype. Identifying more *E74* mutations should help elucidate further E74 functions in neuronal development. The negative results of our *BR-C*, *E74*, and *E75* mosaic studies suggest that the USP/EcR-B1 probably induces a different transcriptional hierarchy to mediate neuronal remodeling.

Like insect ecdysone, vertebrate steroid hormones can significantly influence the development and function of diverse tissues, including the nervous system. For instance, it has been shown that gonadal steroids have remarkable and persistent effects on neuronal survival and dendritic elaboration in particular regions of the brain and spinal cord during specific critical periods of their early development (Arnold and Gorski, 1984). In rats, thyroid hormone has been implicated in regulating the pruning of spinal cord collaterals of certain cortical neurons (Li et al., 1995). The key role for USP in MB neuronal process remodeling raises the possibility that its mammalian homolog, the RXR, may participate in similar processes.

Although regulated pruning of axons and dendrites has long been appreciated as an important cellular mechanism for eliminating exuberant processes, very little is known about the underlying molecular mechanisms. Recently, the homeodomain transcription factor Otx1 has been found to be required for the pruning of spinal cord collaterals of visual cortical pyramidal neurons in mouse (Weimann et al., 1999). However, how nuclear proteins, such as the EcR-B1/USP heterodimer and Otx1, mediate this selective pruning of neuronal processes remains to be elucidated. Transcription factors alone cannot regulate the precise spatial control as to which branches are to be eliminated and to what extent. They are likely to allow neurons to be "competent" for reorganization by regulating the expression of essential components of the pruning machinery or a receptor that receives spatially regulated pruning signal to ensure precisely controlled process pruning. The ability to perform mosaic genetic screens using the *Drosophila* MB γ neuron as a paradigm may allow us to identify these additional components essential for neuronal process pruning in both invertebrates and vertebrates.

Experimental Procedures

Fly Strains and Genetic Crosses

For MARCM analysis, FRT^{19A},*tubP-GAL80,hs-FLP;UAS-mCD8GFP; GAL4-OK107* and FRT^{19A},*tubP-GAL80,hs-FLP;UAS-mCD8GFP, GAL4–201Y* were used for the mutations on the X chromosome; *UAS-mCD8GFP,hs-FLP;FRT2A,tubP-GAL80;GAL4-OK107* was used for the mutations on the chromosome arm 3L. In the MB region, GAL4–

201Y is expressed in all γ neurons and a small subset of pupal-born MB neurons, and GAL4-OK107 is expressed in all MB neurons (Lee et al., 1999).

The mutant alleles of known genes collected for this study include *usp*^{3} (Oro et al., 1992; Henrich et al., 1994; Zelhof et al., 1997), *EcR*^{W53St} (Bender et al., 1997), *EcR*^{31} (Schubiger et al., 1998), *npr1*^{3} (Kiss et al., 1988), *E74*$^{P[neo]}$ (E74A-specific; Fletcher et al., 1995), *E74*$^{DL-1}$ (E74B-specific; Fletcher et al., 1995), *E74*^{v4} (FlyBase), and *E75*^{e213} (Buszczak et al., 1999). For MARCM analysis, *usp*^{3} and *npr1*^{3} were recombined with *FRT*^{19A}*(X)*, and the *FRT*^{19A}*,usp*^{3} *;* λ*10Tb/TM3* stock was generated (λ*10* is a transgene containing genomic DNA of the *usp* locus). The *E74* mutations were recombined with *FRT2A (3L)*. To identify *npr1*^{3} recombinants, hemizygous lethality was used as a criterion. In addition, similar morphologically characteristic dead larvae were found on the wall of vials both before and after recombination. Recombinant chromosomes containing *E74* mutations were selected based on lack of complementation between the *E74*^{v4} allele and *E74*$^{P[neo]}$ or *E74*$^{DL-1}$. The *FRT2A,E75*^{e213} recombinant stock had been used for mosaic analysis recently, which gave a strong oogenesis phenotype (Buszczak et al., 1999).

For marking γ neurons in the *EcR-B* mutants, the *EcR*^{W53St}*,UAS-mCD8GFP,GAL4–201Y* and *EcR*^{31}*,UAS-mCD8GFP,GAL4–201Y* recombinant chromosomes were generated. For expressing various EcR isoforms in the *EcR-B1/EcR-B* mutants, the *UAS-EcR-A*, *UAS-EcR-B1*, and *UAS-EcR-B2* on the third chromosomes were individually put together *with EcR*^{W53St}*,UAS-mCD8GFP,GAL4–201Y* or *EcR*^{31}*,UAS-mCD8GFP,GAL4–201Y*.

UAS-EcR Transgenic Flies

A 3.26 kb EcoRI fragment from pWT57 containing a cDNA of the EcR-A ORF (Talbot et al., 1993) was cloned into the EcoRI site of the pUAST vector (Brand and Perrimon, 1993) generating pUAS-EcR-A. NotI linkers were ligated to a blunt-ended 3.11 kb FspI/HindIII restriction fragment from the plasmid pMK1 that contained a cDNA of the EcR-B1 ORF (Koelle et al., 1991). This NotI fragment was cloned into the NotI site of pUAST (Brand and Perrimon, 1993) generating pUAS-EcR-B1. A 3.13 kb EcoRI restriction fragment from pWT56 containing a cDNA of the EcR-B2 ORF (Talbot et al., 1993) was cloned into the EcoRI site of the pUAST vector (Brand and Perrimon, 1993) generating pUAS-EcR-B2. UAS-EcR-A, UAS-EcR-B1, and UAS-EcR-B2 transgenic lines were generated by P-element mediated transformation (Spradling and Rubin, 1982).

EMS Mutagenesis

Standard procedures (Lewis and Bacher, 1968) were used to perform chemical mutagenesis with the EMS concentration of 25 mM on isogenized *y,w,FRT*^{19A}-containing X chromosomes. 750 X chromosomes carrying independent lethal mutations were isolated over the *FM7* balancer and proceeded for MARCM analysis (Figure 2A).

MARCM Analysis of MB γ Neurons

For using the MARCM system to generate clones of MB γ neurons, embryos of the appropriate genotypes (see Figure legends) were collected over a 4-hr window and newly hatched larvae were heat shocked at 37°C for one hour. In the genetic screen, three to six non-*FM7* female adult flies were dissected in cold phosphate-buffered saline and their brains were immediately examined for the projections of GFP-expressing γ neurons under a compound fluorescent microscope. For phenotypic analysis of known mutations, mosaic brains were dissected and fixed at various stages, and the mCD8GFP-labeled clones were detected by immunofluorescence, as previously described (Lee et al., 1999; Liu et al., 2000).

Phenotypic Analysis of *GAL4–201Y*-Positive MB Neurons

To determine the expression of EcR-B1 in γ neurons, *UAS-mCD8GFP/GAL4–201Y* wandering third instar larval brains were immunostained with the rat mAb anti-mCD8 α chain (1:10; Caltag) and the AD4.4 mAb (1:20; Talbot et al., 1993). Fluorescein isothiocyanate-conjugated goat anti-rat secondary Ab and Cy3-conjugated goat anti-mouse secondary Ab (Jackson) were used to detect the anti-mCD8 mAb and the AD4.4 mAb, respectively. Confocal images were collected and processed, as previously described (Lee et al., 1999).

To examine the projections of γ neurons in the *EcR-B1/EcR-B* mutant "pupae," *y,w;EcR*^{W53St}*,UAS-mCD8GFP,GAL4–201Y/Cyo,P[y*$^{+}$*]* was crossed with *y,w;EcR*^{31}*,UAS-mCD8GFP,GAL4–201Y/Cyo,P[y*$^{+}$*]*, and third instar *yellow*$^{-}$ larvae were transferred to new vials. After transferring to new vials, significant numbers of transheterozygotes wandered out of the fly food. These mutants then acquired certain pupal characteristics. For instance, they stopped moving, turned yellow, and hardened their cuticles. Their brains were dissected and fixed about 24-hr APF. GFP-positive γ neurons were examined using confocal microscopy.

Molecular Characterization of the *l(X)48* and *l(X)101* Mutations

Standard procedures were used to purify genomic DNA from female flies heterozygous for the *l(X)48*, *l(X)101*, and another independent, X chromosome, lethal mutation as a control. The *usp* ORF is contained within a single exon, which was PCR amplified by either primer set #1 (GTTCCTCCAATATACCCAG and TTTTTCGGATGGAGAACGG), or primer set #2 (AAGAAGAAACCGGTAGGCG and AGGGATAGA GAGGAGAAATG), both flanking the entire ORF. At least two independent PCR products amplified from each genomic DNA preparation were cycle sequenced by the ABI sequencing system. The natures of the *l(X)48* and *l(X)101* mutations were revealed as double peaks in sequence (mutant chromosome over the *FM7* balancer) that are absent from control strains. The identical results for *l(X)48* and *l(X)101* are extremely unlikely to be derived from cross-contamination during the PCR amplification, because *l(X)48* genomic DNA was obtained a few months before *l(X)101* and was isolated using primer set #1. Primer set #2 used to amplify *l(X)101* genomic DNA has binding sites that are outside the primer set #1 amplicon, so primer set #2 should not be able to amplify any contaminant caused by amplified *l(X)48* genomic DNA. All PCR experiments were performed from at least three independent genomic DNA isolations.

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