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Fine-tuning of secondary arbor development: the effects of the ecdysone receptor on the adult neuronal lineages of the *Drosophila* thoracic CNS

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The adult central nervous system (CNS) of *Drosophila* is largely composed of relatively homogenous neuronal classes born during larval life. These adult-specific neuron lineages send out initial projections and then arrest development until metamorphosis, when intense sprouting occurs to establish the massive synaptic connections necessary for the behavior and function of the adult fly. In this study, we identified and characterized specific lineages in the adult CNS and described their secondary branch patterns. Because prior studies show that the outgrowth of incumbent remodeling neurons in the CNS is highly dependent on the ecdysone pathway, we investigated the role of ecdysone in the development of the adult-specific neuronal lineages using a dominant-negative construct of the ecdysone receptor (EcR-DN). When EcR-DN was expressed in clones of the adult-specific lineages, neuroblasts persisted longer, but we saw no alteration in the initial projections of the lineages. Defects were observed in secondary arbors of adult neurons, including clumping and cohesion of fine branches, misrouting, smaller arbors and some defasciculation. The defects varied across the multiple neuron lineages in both appearance and severity. These results indicate that the ecdysone receptor complex influences the fine-tuning of connectivity between neuronal circuits, in conjunction with other factors driving outgrowth and synaptic partnering.

KEY WORDS: *Drosophila*, Metamorphosis, Ecdysone, EcR, Axon outgrowth

INTRODUCTION

Complete metamorphosis in insects dictates the sequential 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 (CNS) in the adult is made up of neurons born from persisting embryonic neuroblasts (NBs) that are reactivated during the larval stages (Truman, 1990). Each NB gives rise to a cluster of progeny, which is its neuronal lineage. These 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 its adult connections. Unlike the high diversity of neurons generated by a given NB during embryogenesis (Schmid et al., 1999), the larval phase of neurogenesis generates relatively homogeneous neuronal classes that remain clustered together, projecting towards a common initial target (Truman et al., 2004).

The post-embryonic development of *Drosophila* is coordinated by the steroid hormone ecdysone. To drive these metamorphic changes, ecdysone is converted into its active metabolite, 20-hydroxyecdysone (20E), and is translated into cellular responses by the ecdysone receptor complex, a heterodimer consisting of ultraspiracle (USP) and ecdysone receptor (EcR) (Koelle et al., 1991; Yao et al., 1992). The EcR/USP heterodimer binds to a response element to regulate the transcription of target genes via repression or activation (Cherbas et al., 1991). When 20E is absent, co-repressors may bind to EcR/USP to shut down transcription (Dressel et al., 1999; Tsai et al., 1999). The binding of 20E releases co-repressors, after which co-activators are recruited to promote transcriptional activation (Bai et al., 2000; Dressel et al., 1999; Tran et al., 2001; Tsai et al., 1999). Ecdysone responses are also modulated by the specific blending of the three different EcR isoforms expressed (Talbot et al., 1993; Truman et al., 1994). The N-terminal regions of EcR-B1 and EcR-B2 have a strong ligand-independent activational function (Hu et al., 2003; Mouillet et al., 2001), and these isoforms promote pruning in remodeling neurons (Lee et al., 2000; Schubiger et al., 1998). EcR-A has an inhibitory domain at its N terminus (Mouillet et al., 2001), and is the most prevalent isoform in maturing neurons. As a neuron progresses through specific developmental phases, the levels of the different EcR isoforms within the neuron fluctuate (Truman et al., 1994), thereby influencing the response of the neuron to ecdysone during metamorphosis (H. L. D. Brown, PhD thesis, University of Washington, 2007).

The remodeling of larval neurons requires exposure to 20E at the outset of metamorphosis. The mushroom body axons, along with other central neurons, need ecdysone signaling through the EcR-B isoforms in order to prune correctly (Lee et al., 2000; Schubiger et al., 1998). Previously, we examined the role of EcR in neuronal remodeling using EcR dominant-negative (EcR-DN) constructs. When 20E action was blocked in a set of remodeling neurons by expression of an EcR-DN that could not bind 20E, pruning was severely disrupted and outgrowth was reduced (Brown et al., 2006). EcR-DN expression in peripheral neurons also impedes pruning during remodeling (Williams and Truman, 2005).

Although multiple studies have looked at the role of EcR in directing remodeling, little is known about how EcR affects the maturation of the adult-specific neurons that compose the majority of the adult ventral CNS. In this study, we identified and

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characterized the adult morphology and development of seven of the 25 neuron lineages in the thoracic CNS, including the motoneuron innervation of the adult *Drosophila* leg. To test the requirement for EcR during outgrowth in neurons that had not previously experienced pruning, we expressed EcR-DN in the adult-specific neuron lineages. We found that expression of EcR-DN resulted in variable defects in secondary branching amongst the lineages, with severe disruption of the axonal and dendritic arbors of the motoneuron lineages.

MATERIALS AND METHODS

Fly stocks

Generation of the EcR dominant-negative construct UAS-EcR-B1-Δ655-W650A (UAS-EcR-B1^{W650A}) was described by Hu et al. (Hu et al., 2003) and Cherbas et al. (Cherbas et al., 2003); and generation of UAS-EcR-A^{W650A} was described by Brown et al. (Brown et al., 2006). To induce randomly generated clones expressing both EcR-DN construct and membrane-bound mCD8::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

Flies laid eggs in fly food vials for 2-4 hours, and the eggs were then maintained at 25°C for 25-28 hours. Between the ages of 25 and 32 hours after egg laying (AEL), larvae received either a single heat shock (45-60 minutes) or a double heat shock (30 minutes, 30-minute rest, 45 minutes) at 37°C, and were then maintained at 29°C. For pupal staging (hours after puparium formation, or APF), animals were collected as white puparia and kept at 29°C for the indicated times. Adults were dissected 0.5-3 days after eclosion. Because clone generation could not be targeted to a specific lineage, the number of clones obtained varied amongst the lineages.

Immunocytochemistry and imaging

CNSs were fixed for 30 minutes in 4% buffered formaldehyde, blocked in 2% donkey serum in phosphate buffered saline with 1% Triton X-100 (PBS-TX), and incubated in primary antibody overnight as described by Brown et al. (Brown et al., 2006). The following primary antibodies were made up in PBS-TX: rat anti-mCD8 (1:100; CALTAG), and mouse monoclonal antibodies anti-EcR-A (1:10,000; 15G1A ascites), anti-EcR-B1 (1:100; AD4.4) and anti-EcR-common (1:100; IID9.6) (Talbot et al., 1993). After rinsing, 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, USA). CNSs were attached to polylysine-coated coverslips, dehydrated and cleared, then mounted in DPX (Fluka, Seelze, Germany).

Legs were dissected from adults and fixed overnight in 4% paraformaldehyde, rinsed, blocked and incubated in primary antibody for several days at 4°C (rat anti-mCD8, diluted 1:100; Caltag). After removal of primary antibody, legs were rinsed and secondary antibody was added (1:200 FITC-conjugated donkey anti-rat IgG; Jackson ImmunoResearch) for several days; legs were then mounted in Fluoromount (Southern Biotech, Birmingham, AL, USA). All experiments were imaged on a Bio-Rad Radiance 2000 confocal microscope.

Analysis

Confocal image stacks were reconstructed and analyzed using ImageJ (http://rsb.info.nih.gov/ij/). Some images were adjusted for brightness. To determine whether neuroblasts (NBs) were present, clones were scored blind for the presence of a large cell located at the apex of the clone. In order to show the innervation of the musculature, cuticular autofluorescence was removed from image stacks of the adult leg. Leg motor endplates were counted manually and images were scored blind on branch presence and abnormality of projection pattern. Statistical significance for femoral motor endplate number was determined using the statistical analysis program SPSS (www.spss.com).

RESULTS

Identification of adult neuron lineages

In this study, we identified the adult version of seven of the postembryonic neuron lineages found in the thoracic CNS. The postembryonic neuron lineages in the thoracic neuromeres were previously identified and mapped in the larval CNS (Truman et al., 2004). Each lineage is associated with a NB, the progeny of which remain in a discrete cluster throughout larval life (Truman et al., 2004). In lineages that have two initial targets, a neuron born from a ganglion mother cell (GMC) sends a single projection to one of the targets, while its sibling neuron projects to the other (J.W.T. and D. W. Williams, unpublished). As the CNS expands during metamorphosis, the lineage clusters are pushed laterally and dorsally within the cortical rind, sometimes separating sibling clusters from the same lineage. Dividing NBs were evident at pupariation, but by 24 hours after puparium formation (APF; at 29°C) neurogenesis was complete and no NBs were found. Because the position of the lineage clusters changed from their previously mapped larval positions, we could not rely on location of the cell cluster alone for identification. We were, however, able to identify a number of the lineages in the pupal and adult central nervous systems based on the persisting tracts and commissures through which their primary neurite bundle extended (Fig. 1B-B″).

We identified some of the lineages at both 24 hours APF and in the adult CNS, and will briefly outline the normal development of one such lineage. Lineage 6 is composed of two classes of neuron; one class sends their initial neurite across the posterior dorsal (pD) commissure, whereas the second class projects neurites across the posterior intermediate (pI) commissure (Truman et al., 2004). By 24 hours APF, both sets of neurons showed an interstitial sprouting zone ipsilateral to the cell bodies and a terminal sprouting zone contralateral to the cell bodies (Fig. 1B′). These arbors continued to expand within the central neuropil, becoming large and diffuse in the adult (Fig. 1B″).

Expression of EcR-DN in NB lineages

To test the role of EcR in the development of the adult-specific neurons, we used the MARCM system to express both CD8::GFP and two different EcR-DN constructs in the lineages. The dominant-negative constructs have a point mutation in helix 12 of the ligand-binding domain of EcR that prevents steroid binding, resulting in competitive inhibition of all endogenous EcR isoforms (Brown et al., 2006; Cherbas et al., 2003). We tested the expression levels of EcR-B1^{W650A} by examining EcR-B1 immunoreactivity within the cells of the clone at 24 hours APF, at 29°C. Because the adult-specific neurons do not normally express EcR-B1 at this time, the EcR-B1 immunoreactivity we saw within a clone was due to expression of EcR-B1^{W650A} (Fig. 2A,A′). Two other insertion lines of EcR-B1^{W650A} were tested and showed similar expression of EcR-B1 (not shown). Because the adult-specific neurons normally express EcR-A, expression of EcR-A^{W650A} lead to an observable increase of EcR-A in the clone above that seen in neurons outside of the clone (Fig. 2B,B′). As all of the dominant negatives we tested were well expressed in

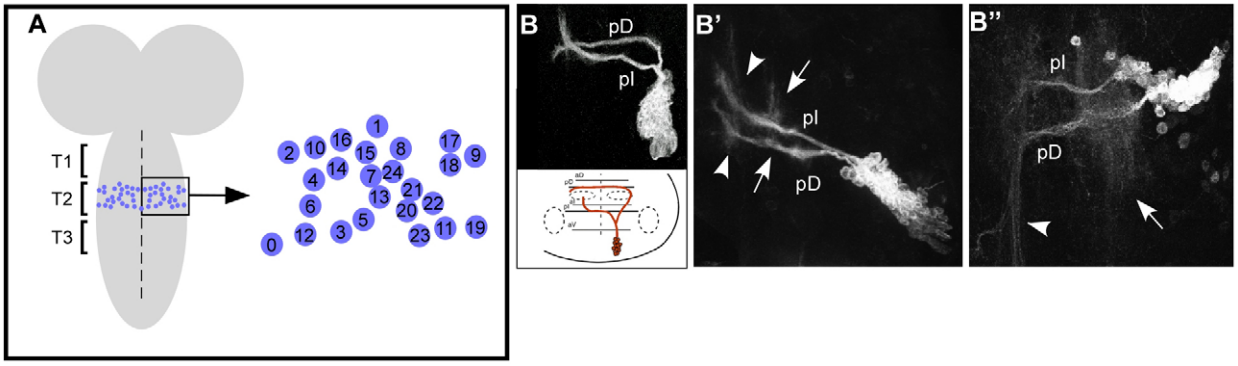

Fig. 1. Postembryonic neuron lineage development in the CNS. (**A**) Neuroblast (NB) map of the second thoracic (T2) hemineuromere. Thoracic neuromeres T1 and T3 lack one or two of the NBs present in the T2 set. (**B-B″**) Examples of confocal projections of MARCM clones showing the time course of development of neurons produced by NB 6. (B) A lineage 6 clone in T3 at pupariation, showing the two axon bundles emerging from the cell cluster; one projects across the posterior intermediate (pI) commissure and the other across the posterior dorsal (pD) commissure. Below is a cross-section schematic of the thoracic segmental neuropil showing the bundle trajectories relative to the major commissures (see Truman et al., 2004). (B′) A lineage 6 clone in T2 at 24 hours APF (raised at 29°C) showing sprouting of terminal (arrowheads) and interstitial (arrows) arbors from both bundles. (B″) Both hemilineages of lineage 6 have well-developed ipsilateral arbors (arrow) and contralateral projections in the adult. Contralateral axons that cross in the pD commissure have posterior projecting terminal arbors (arrowhead).

clones and gave similar phenotypes in the lineages (not shown), in all of the following examples the UAS-EcR-B1^{W650A} construct (referred to as EcR-DN) was used, unless otherwise indicated.

NBs persist in clones expressing EcR-DN

Neuroblast MARCM clones expressing EcR-DN retained NBs longer than did control clones (Fig. 3). NBs were seen in control animals at pupariation, but never at 24 hours APF at 29°C (Fig. 3A; Table 1). However, at 24 hours APF, 18 out of 101 clones that expressed EcR-DN still had a large NB at the apex of the clone (Fig. 3B, arrow; Table 1). Of the 18 clones containing NBs, we observed three examples of NBs persisting in lineage 3, two examples in lineage 6, two in lineage 1, and one clone each in lineages 5, 7, 8, 12, 16, 19 and 21. Four clones with persisting NBs could not be definitively identified. NBs were never seen in the adult CNS for either control clones or clones expressing EcR-DN, indicating that the persistence of the NB was temporary. We did not observe an obvious increase in the number of neurons found in the adult clones that expressed EcR-DN, so either the delay in NB inactivation did not result in extensive numbers of new neurons, or any neurons born from persisting NBs were then cleared by cell death.

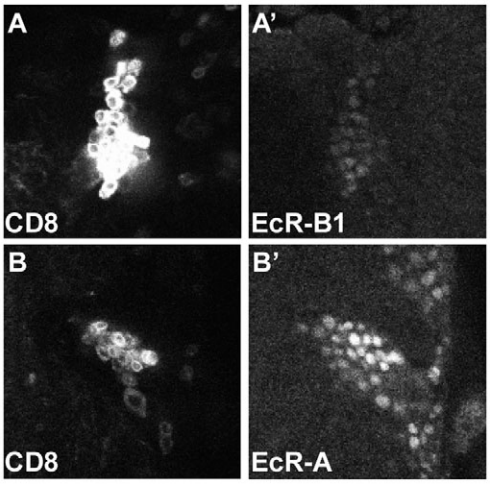

Fig. 2. Expression levels of EcR-DN constructs in MARCM neuroblast clones from pupae at 24 hours APF (raised at 29°C). (**A**,**A′**) CD8::GFP expression (A) and EcR-B1 immunoreactivity (A′) in a MARCM clone expressing CD8::GFP and EcR-B1^{W650A}. (**B**,**B′**) CD8::GFP expression (B) and EcR-A immunoreactivity (B′) in a MARCM neuroblast clone expressing CD8::GFP and EcR-A^{W650A}. EcR-A immunoreactivity is seen in all adult-specific neurons, but it is enhanced in the neurons within the clone.

Effects of EcR-DN expression on outgrowth of the adult CNS lineages

Of the 25 lineages in the thoracic ganglia of the adult nervous system, we were able to identify and analyze multiple examples of seven in both control animals and animals expressing EcR-DN. In control animals, the neurons from the various lineages elaborated extensive dendritic and axonal arbors. When EcR-DN was expressed in clones, the primary targeting of all lineages examined was unchanged, and all underwent sprouting of secondary arbors. However, the secondary arbors of clones expressing EcR-DN showed differing degrees of clumped, reduced and misrouted arbors, varying across the lineages. Because this is the first time the lineages of the thoracic ganglia have been identified in adult nervous systems, we will describe the adult morphology of the controls as well as the phenotype of clones expressing EcR-DN.

Motoneuron lineages

Lineage 15 and lineage 24 are made entirely of motoneurons that project out of the CNS into the periphery. In the larva, the laterally located lineage 15 cell cluster extends axons dorsally through the leg neuropil and out to the leg imaginal disc. At this stage, the bundle

Table 1. Quantification of clones containing neuroblasts at 24 hours APF*

	Total clones analyzed	Clones with NBs
Control	98	0
EcR-B1^{W650A}	101	18
EcR-A^{W650A}	12	3

*Raised at 29°C.

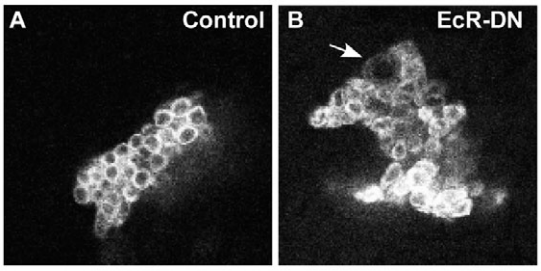

Fig. 3. Neuroblast persistence in MARCM clones expressing EcR-DN. (**A**) A MARCM clone from a control pupa at 24 hours APF (raised at 29°C). No neuroblast is present. (**B**) A MARCM clone expressing EcR-DN at 24 hours APF (raised at 29°C), showing a persistent neuroblast (arrow), identified by its large size and position at the apex of the cell cluster.

partially defasciculates as it passes through the leg neuropil (Truman et al., 2004). During metamorphosis, the lineage 15 neurons sprouted dendritic arbors at this site of defasciculation to produce an arbor of fine, highly dispersed branches in the dorsal region of the adult leg neuropil (Fig. 4A-C). A small branch extended out of this dendritic cloud toward the midline, barely crossing the pI commissure in 13 out of 14 clones (Fig. 4A, arrow). When we expressed EcR-DN in lineage 15, we observed a clumped and reduced dendritic arbor in all 14 clones examined. The fine branches within the leg neuropil were compacted, leaving large spaces devoid of dendrites (Fig. 4D-F). All but one retained the contralaterally projecting branch.

All of the axons from the lineage 15 motoneurons project to the leg disc, and we used the expression of membrane-bound GFP to track the targets of lineage 15 in the adult leg. The axons from lineage 15 motoneurons remained tightly fasciculated until reaching the femur, where some neurons branched to establish neuromuscular junctions (NMJs) both proximally and distally on femur muscles, while other axons extended into the tibia (Fig. 5A). These two femoral branch points were stereotyped; 15 out of 16 control clones had both a proximal arbor and a smaller distal arbor spreading over the muscles (Fig. 5A, arrows). These arbors were characteristic in their morphology, extending multiple branches longitudinally along the muscle fibers (Fig. 5C). After entering the tibia, lineage 15 axons branched to innervate the tarsus levator and reductor muscles (Baek and Mann, 2009; 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 EcR-DN resulted in missing arbors in the femur and tibia, and misaligned and clumped NMJs (Fig. 5B,D,E). Tibial projections were reduced in all clones expressing either EcR-B1^{W650A} or EcR-A^{W650A}. Endplates within the femur were reduced in number by approximately half in clones expressing either EcR-DN construct (Fig. 6A). Almost all had proximal femoral branches, but they were clumped or misaligned in seven out of the 15 clones expressing EcR-B1^{W650A} and in all seven clones expressing EcR-A^{W650A} (Fig. 5; Fig. 6B). Clumped branches resulted in arbors that were highly compacted, resulting in fewer endplates in the femur (Fig. 5E; Fig. 6A). Misaligned branches were characterized by endplates that were not longitudinally oriented along the muscle fibers, but which projected away from the primary tract, producing a star-shaped morphology (Fig. 5D). In clones expressing EcR-DN, distal femoral arbors were completely missing from approximately half of the legs examined. Of the distal arbors that were present in clones expressing EcR-B1^{W650A}, most had a misaligned phenotype, whereas clones expressing EcR-A^{W650A} were split between the misaligned and the clumped phenotypes (Fig. 6C).

Lineage 24 was the only other lineage in the CNS that consisted entirely of motoneurons. It was made up of fewer cells (less than 10) than lineage 15, and projected through the dorsolateral leg neuropil before exiting the CNS. Dendrites from these motoneurons were concentrated in the dorsolateral region of the leg neuropil (Fig. 7A,A′). A recent study reports that these motoneurons innervate proximal leg muscles (Baek and Mann, 2009). Expression of EcR-DN in lineage 24 resulted in a similar phenotype to that arising following the expression of EcR-DN in lineage 15, with a severe reduction in the extent of the dendritic arbor and clumping of the normally finely interspersed branches. (Fig. 7B,B′).

Interneuron lineages

Interestingly, expression of EcR-DN in interneuron clones gave varied phenotypes across the different lineages examined, ranging from clumping of the arbors and defasciculation of the primary

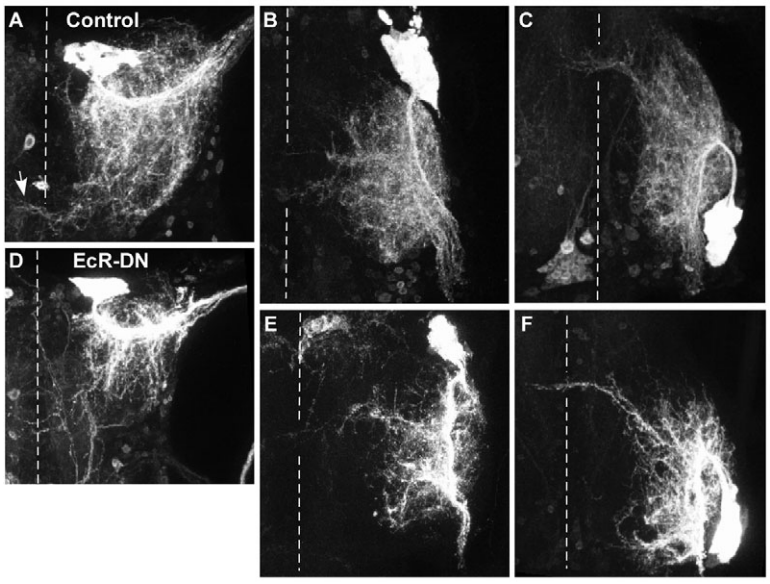

Fig. 4. Expression of EcR-DN severely affects the dendrites of lineage 15 in the adult CNS. (**A-C**) Dorsal projections of confocal stacks showing the adult morphology of lineage 15 motoneurons in T1 (A), T2 (B) and T3 (C) of control animals. Arrow indicates small contralateral branch. (**D-F**) Projections of lineage 15 clones expressing EcR-DN, in T1 (D), T2 (E) and T3 (F). Clones of lineage 15 expressing EcR-DN have reduced and clumped dendritic arbors. The dashed line indicates the midline in this and subsequent figures.

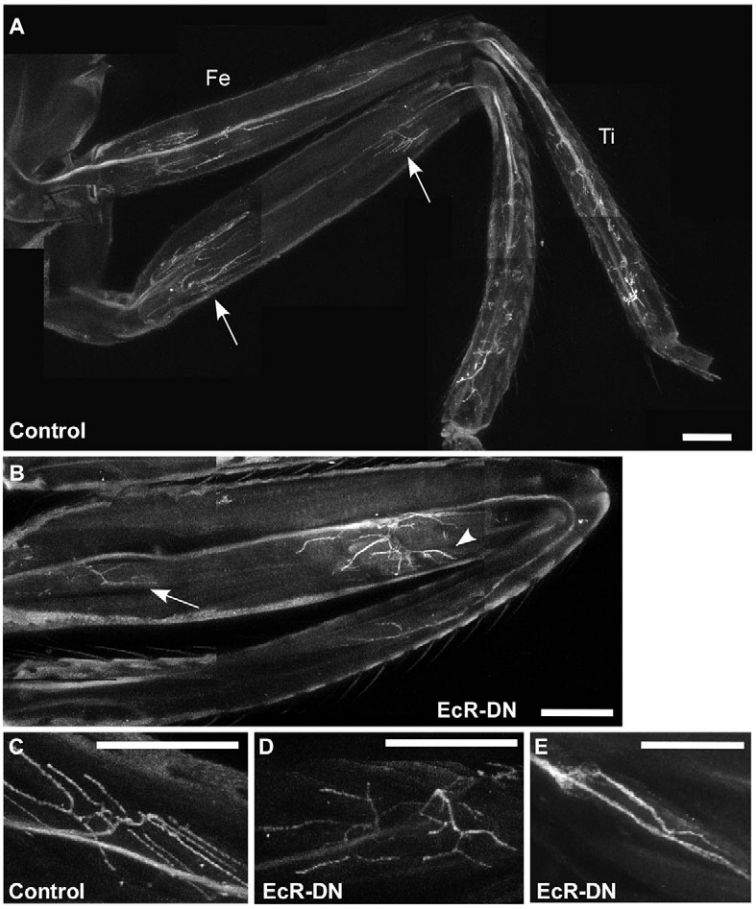

Fig. 5. Expression of EcR-DN results in disrupted peripheral projections of lineage 15 in the leg. (**A**) Legs from control adults showing branching of GFP-labeled axons from lineage 15 MARCM clones in T2 and T3. Arrows point to the two major branch elaboration sites, proximally and distally within the femur (Fe). Axon branches in the tibia (Ti) are less stereotyped than those in the femur. (**B**) A lineage 15 clone expressing EcR-DN B1^{W650A} has a greatly reduced proximal branch (arrow) and a misaligned distal branch in the femur (arrowhead). (**C**) Enlarged distal arbor of lineage 15 in the femur of a control animal, showing parallel endplates oriented along the muscle fibers. (**D**) The distal arbor in the femur of an animal expressing EcR-B1^{W650A} in lineage 15 shows a star-shaped arbor that is not oriented properly along the muscle fibers. (**E**) The proximal arbor in a femur of an animal expressing EcR-B1^{W650A} in lineage 15 shows reduced, clumped processes. Scale bars: 0.1 mm.

bundle to no discernible phenotype relative to controls. The arborization of lineage 7 in the first thoracic (T1) neuromere was the most severe effect observed. In the larva, lineage 7 neurons project from the cell cluster located ventrolaterally in the anterior of the neuromere. The single bundle crosses the midline in the anterior intermediate (aI) commissure before turning anteriorly and inserting into a dorsal tract (Truman et al., 2004). During metamorphosis, the cell body cluster of lineage 7 in T1 is translocated to the anterior border of the neuromere. Hence, in the adult, its primary neurite bundle extends posteriorly but then turns sharply to cross the midline in the aI commissure before extending dorsally. On either side of the midline, large diffuse arbors extended dorsally into the flight neuropil (Fig. 8A). Expression of EcR-DN caused extensive defects in the secondary projections in this lineage. In nine of the 11 T1 clones analyzed, the ipsilateral arbor was not composed of evenly spaced fine branches as seen in controls, instead branches were clumped together to produce dense strands interspersed with areas containing no branches (Fig. 8B, arrowhead). Additionally, as the primary bundle crossed the midline, it frayed and defasciculated in five out of the 11 clones (Fig. 8B, arrow). The contralateral arbor also showed clumping of neurites.

The adult projections of lineage 7 in T3 differed in appearance from its homolog in T1, although the structure of the primary neurite bundles established in the larva were similar. The mature neurons of

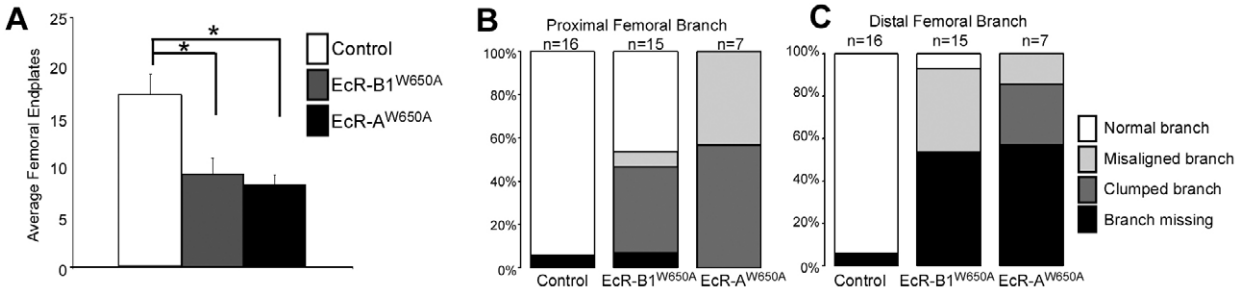

Fig. 6. Quantification of lineage 15 secondary branch defects in the femur. (**A**) The average number of femoral endplates in controls (white), clones expressing B1^{W650A} (gray) and clones expressing A^{W650A} (black). (**B**,**C**) Percentage of femurs that are missing branches (black), have clumped branches (dark gray), have misaligned branches (light gray), or have normal branches (white) in lineage 15 controls, and in clones expressing B1^{W650A} or A^{W650A}. (B) Analysis of femoral proximal branches; (C) analysis of femoral distal branches.

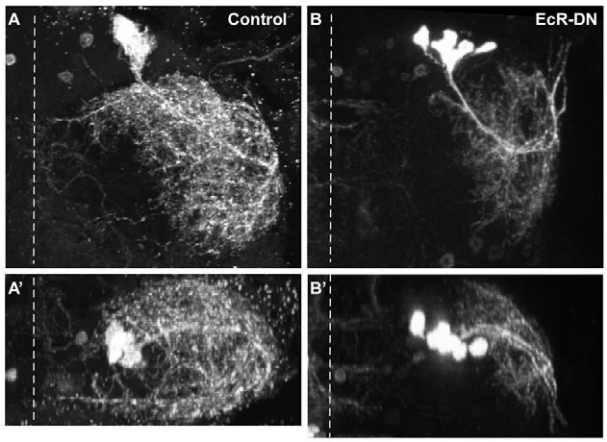

Fig. 7. Expression of EcR-DN severely affects the dendrites of lineage 24 in the adult CNS. Dorsal (top) and transverse (bottom) projections of lineage 24 MARCM clones. (**A**,**A′**) A control clone in T1 has a diffuse dendritic arbor innervating the leg neuropil. (**B**,**B′**) A lineage 24 clone expressing EcR-DN has stunted dendritic arbors.

the cluster produced a large, diffuse ipsilateral arbor that expanded dorsally and a contralateral, terminal arbor that extended anterodorsally. There was also an apparent single axon that diverged from the main neurite bundle before crossing the midline and terminating in the contralateral leg neuropil. This morphology was evident in all six control examples (Fig. 8C). Although the phenotype of T3 lineage 7 clones expressing EcR-DN was not as extreme as that seen in T1, they showed some abnormal fasciculation of ipsilateral and contralateral arbors (Fig. 8D, arrow). The leg neuropil axon bundle was unaffected.

In the larva, lineage 6 extends two primary neurite bundles, one crossing in the pI commisure and one in the pD commissure (Truman et al., 2004). After metamorphosis, the cell bodies of the two groups of neurons from lineage 6 often ended up as distinct but adjacent cell clusters, and terminal and interstitial arbors extended from both the pI and the pD bundles (Fig. 9A,A′). The terminal arbors of the dorsal bundles were interesting because their targets appeared to be in the T2 neuromere. Consequently, they projected anteriorly in T3 (not shown) but posteriorly in T1 (Fig. 9A, arrowhead). Lineage 6 was moderately affected by expression of EcR-DN. When EcR-DN was expressed in lineage 6, one of the five clones examined appeared normal, one had a clumped ipsilateral arbor and two showed dorsal projecting neurons with both reduced and clumped ipsilateral arbors (Fig. 9B,B′, arrow).

Another moderately affected interneuron lineage was lineage 18. This dorsally located lineage extends only one primary neurite bundle that crosses to the contralateral side via the anterior intermediate commissure in the larva (Truman et al., 2004). In the adult, the cell bodies occupied a dorsolateral position in the anterior portion of the neuromere. The projections of lineage 18 extended a large dorsal ipsilateral arbor, continued through the aI commissure, then turned anteriorly into a dorsal longitudinal tract (Fig. 10A,A′). A minor axon bundle projected into the leg neuropil contralateral to the cell cluster (Fig. 10A, arrowhead). Expression of EcR-DN in T3 lineage 18 clones resulted in a moderate phenotype. Three out of four clones had a reduced ipsilateral arbor (Fig. 10B, arrow), with two of these showing clumping of the terminal branching on the ipsilateral arbor.

Lineage 9 was mildly affected by expression of EcR-DN. Prior to metamorphosis, lineage 9 sends an ipsilateral neurite bundle to the dorsal leg neuropil and a thin contralateral bundle across the anterior ventral (aV) commissure that stops just before the contralateral leg neuropil. The cell cluster is located dorsally in the anterior region of the neuromere (Truman et al., 2004). In the adult, the cell cluster remained in a dorsal position with its neurite bundle extending ventrally along the anterior edge of the leg neuropil, forming a dense finely branched arbor that stretched from the anterior midline across the ventral side of the ipsilateral leg neuropil to its posterior margin (Fig. 11A). The contralateral branch was composed of small, diffuse

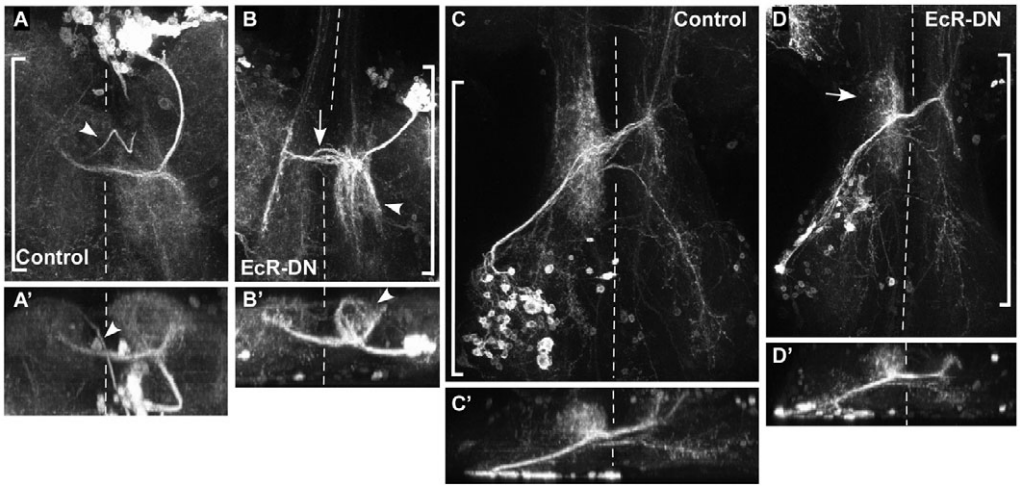

Fig. 8. Expression of EcR-DN causes severe effects on lineage 7 arbors in the adult CNS. Dorsal (top) and transverse (bottom) projections of lineage 7 MARCM clones. Brackets indicate the portion of the confocal stack used to create the transverse projection in this and subsequent figures. (**A**,**A′**) A control clone in T1 extends across the al (anterior intermediate) commissure, forming dorsally projecting arbors on both sides of the midline. Arrowhead points to the axon bundle from an adjacent lineage 2 clone. (**B**,**B′**) A clone of lineage 7 expressing EcR-DN has a clustered ipsilateral arbor in dense strands (arrowhead), a contralateral arbor that is longitudinally compressed and projects anteriorly, and a defasciculated primary neurite bundle (arrow). (**C**,**C′**) A T3 lineage 7 clone in a control animal. (**D**,**D′**) A T3 lineage 7 clone expressing EcR-DN. The ipsilateral arbor is compressed (arrow). The posterior contralateral axon appears unaffected by EcR-DN.

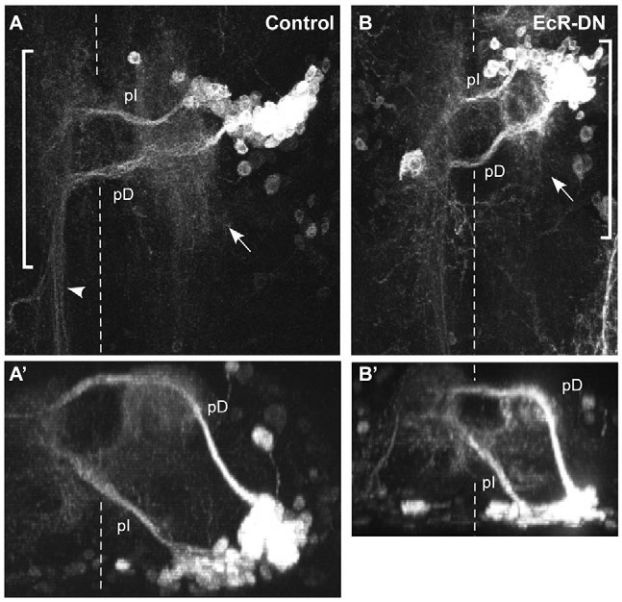

Fig. 9. EcR-DN reduces lineage 6 arbors in the adult CNS. Dorsal (top) and transverse (bottom) projections of lineage 6 MARCM clones. (**A**,**A′**) A T1 control clone showing the two hemilineage clusters. One sends a dorsal axon bundle with a large ipsilateral arbor (arrow) before crossing the pD commissure and extending a compact bundle into the T2 neuromere (arrowhead). The other cluster has a smaller ipsilateral arbor and a more diffuse contralateral projection that is primarily in T1. (**B**,**B′**) A T1 lineage 6 clone expressing EcR-DN. The ipsilateral arbors of both clusters are reduced and somewhat clumped (arrow), while the contralateral projection shows a high degree of disorganization of the T2 projection.

neurites that extended across the aV commissure to the anterior ventral margin of the leg neuropil in all nine control clones examined (Fig. 11A, arrow). When EcR-DN was expressed in lineage 9, both the ipsilateral and the contralateral terminal arbors appeared normal. However, seven out of 14 clones examined showed defasciculation of the primary bundle as it curved ventrally around the leg neuropil (Fig. 11B, arrowhead), compared with one out of six controls.

Lineage 2 appeared to not be affected by EcR-DN expression. In the larva, lineage 2 neurons project a single bundle dorsally from a cell cluster located ventrally at the anteromedial margin of the neuromere. After reaching the dorsal surface of the neuropil, the bundle then turns sharply to end at the dorsolateral margin of the ipsilateral neuropil (Truman et al., 2004). In all seven adult control clone examples, the cell cluster was located in the same relative position as before metamorphosis, and the terminal arbor had expanded to form a fan-shaped, diffuse projection within the ipsilateral dorsal neuropil (Fig. 12A,C). Interestingly, the neurons from the clusters in T2 and T3 also had a prominent interstitial arbor at the level of the intermediate commissure (Fig. 12C,C′, arrow), but this arbor was missing in the T1 cluster (Fig. 12A). We did not find any significant disruptions in the interstitial or terminal arbors of the 15 lineage 2 clones that expressed EcR-DN (Fig. 12B,D).

DISCUSSION

Steroid signaling and neurogenesis

The majority of the adult CNS of *Drosophila* is composed of adult-specific neurons born during larval life. Proper neuronal connectivity and function of the nervous system rely on both regulation of neurogenesis to produce correct neuron number, and establishment of appropriate synaptic connections by these neurons. In the thorax, postembryonic neurogenesis is initiated by the reactivation of NBs in the late first instar larva, and terminates within 24 hours after pupariation (Truman and Bate, 1988). This reactivation of quiescent NBs requires growth of the first instar larva (Britton and Edgar, 1998). Although 20E has been implicated in the 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), and the signals that terminate NB division are unknown. NBs could be predetermined to stop cycling after a specific number of divisions, or an external cue could trigger termination. Previous studies in *Manduca sexta* showed that transplantation of ganglia from fourth instar larvae into wandering fifth instar larvae resulted in continuing neurogenesis in the implant well after neurogenesis had terminated in the host. This suggests that young NBs cannot be shut off early by the host's metamorphic signals (J. Witten and J.W.T., unpublished). Temperature-sensitive ecdysone-mutant larvae that wander permanently rather than undergoing pupariation have NBs that cycle more slowly, but apparently stop divisions in the same spatiotemporal pattern as in normal metamorphosing flies (Berreur et al., 1984; Garen et al., 1977). Additionally, *Drosophila* larval CNSs cultured without 20E divide at a slower rate, but stop dividing when approximately the correct number of neurons has been produced (Truman et al., 1993).

In this study, blocking the ecdysone signal through the expression of EcR-DN resulted in lineage clones with NBs that persisted longer than usual. In 20% of clones expressing EcR-DN, we observed NBs at 24 hours APF in animals raised at 29°C, whereas no NBs were seen in control animals at the same stage. The developmental state of these pupae corresponds to a developmental time 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

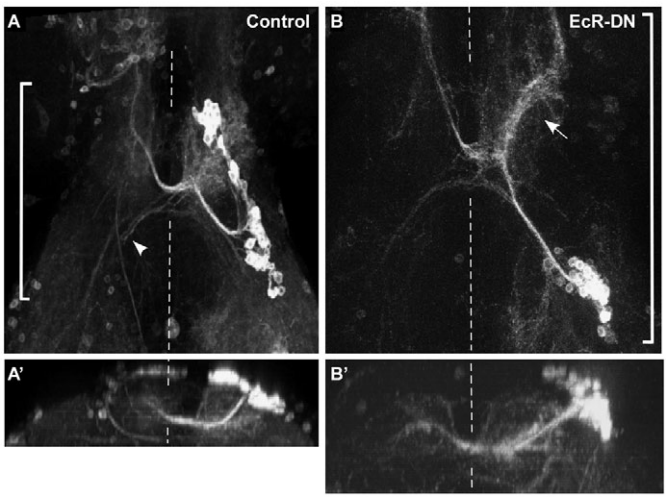

Fig. 10. EcR-DN expression affects the dendritic arbor of lineage 18 in the adult CNS. Dorsal (top) and transverse (bottom) projections of lineage 18 MARCM clones. (**A**,**A′**) A ventral projection of a T3 lineage 18 clone in a control CNS that extends across the al commissure, and then extends dorsally projecting arbors on either side of the midline and a smaller arbor into the contralateral leg neuropil (arrowhead). The cell bodies have been pushed apart during metamorphosis. (**B**,**B′**) A ventral projection of a T3 lineage 18 clone expressing EcR-DN. Arrow indicates the reduced and compacted ipsilateral arbor.

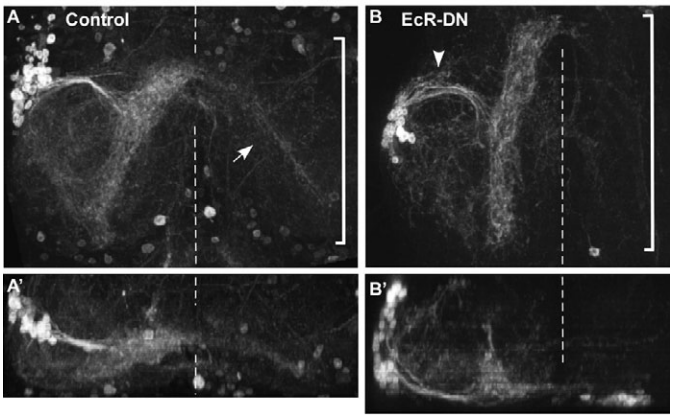

Fig. 11. EcR-DN expression in lineage 9 results in minor defasciculation of the primary bundle. Dorsal (top) and transverse (bottom) projections of lineage 9 MARCM clones. (**A**,**A′**) A T3 clone of lineage 9 in a control CNS. Lineage 9 projects around the anterior edge of the leg neuropil to form an arbor along the ventral side. A small arbor extends contralaterally across the aV commissure (arrow). (**B**,**B′**) A T3 clone of lineage 9 expressing EcR-DN, in which the primary bundle is defasciculated (arrowhead).

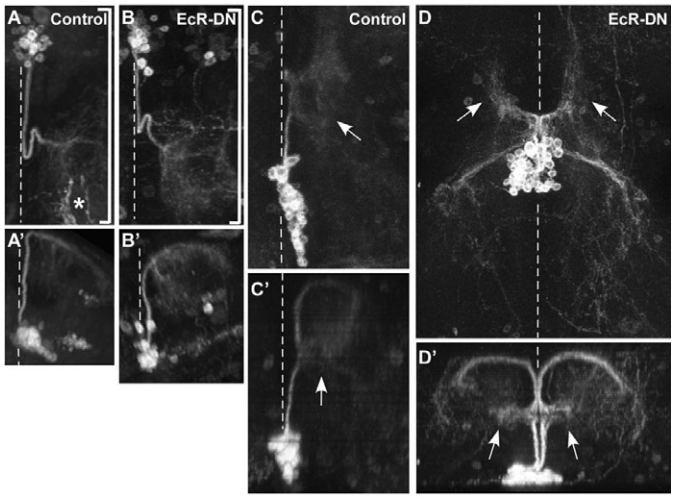

Fig. 12. EcR-DN expression does not affect lineage 2 arborizations in the adult CNS. Dorsal (top) and transverse (bottom) projections of lineage 2 MARCM clones. (**A**,**A′**) A T1 clone of lineage 2 in control animals. Lineage 2 has a characteristic 'jog' as it extends dorsally through the neuropil before forming a diffuse ipsilateral arbor in the flight neuropil. Asterisk marks the projection of an afferent peripheral neuron clone not associated with lineage 2. (**B**,**B′**) A T1 clone of lineage 2 expressing EcR-DN appears to be unaffected by EcR-DN expression. (**C**,**C′**) A T2 clone of lineage 2 in a control animal. Unlike in T1, lineage 2 in T2 and T3 shows interstitial sprouting (arrow) in addition to its terminal arbor. (**D**,**D′**) T2 clones of lineage 2 expressing EcR-DN. Lineage 2 in both hemineuromeres of T2 is marked, and results in identical lineage 2 clones on either side of the midline. Arrows indicate the interstitial arbors of both clones.

EcR-DN was not grossly increased (data not shown). Thus, delaying death in the thoracic NBs through expression of EcR-DN either fails to extend the neurogenic period, or extends it for just a short time, resulting in clones with only slightly more neurons. Taken together with previous studies examining 20E and NB termination, these data suggest that the number of NB divisions might be approximated through endogenous mechanisms, but that the precise timing of NB termination might be fine-tuned through 20E signaling.

Development of the adult-specific lineages

After an adult-specific neuron is born in the larva, it sends out an initial process before arresting its development. Each neuron born from the same neuroblast sends its projection along the same path as previously born neurons from the same sibling cluster, or hemilineage. If a lineage has more than one neurite bundle (for example, lineage 6 projects across both the pD and the pI commissures; Fig. 1B), one of the two neurons born from a GMC projects to one initial target, while its sibling projects to the other (Truman et al., 2004). Typically, the projections from a hemilineage make contact with the neurite bundle from a different hemilineage, possibly setting up future synaptic connections. Before metamorphosis, these contacts are limited and the neuropil remains partitioned into domains allotted to each hemilineage (Truman et al., 2004). We described here the final adult arbor morphology of seven of the 25 thoracic lineages. After metamorphosis was complete, all lineages examined showed intense sprouting and elaboration to form finely branched terminal and interstitial arbors, many located within the leg or flight neuropils. The mature neurons within a given hemilineage typically shared similar domains of dendritic and axonal arborization. There are exceptions, though, such as in lineages 7 (Fig. 8) and 18 (Fig. 10), in which a few neurons in a hemilineage took a trajectory that differed from that of the rest of the group. The peripheral projections of lineage 15 defasciculated and extended over the femoral and tibial muscles of the leg, forming neuromuscular junctions throughout (Fig. 5). Overall, the early partitioning of the immature neuropils by the hemilineages is dissolved during metamorphosis as complex arbors develop, overlapping with those from other lineages to establish the final pattern of synaptic connections.

Ecdysone signaling affects neuron outgrowth and sprouting

Previously, we examined the role of EcR in *Drosophila* neuronal development by expressing EcR-DN in remodeling neurons. When ecdysone signaling through EcR is blocked, pruning is slow and incomplete in both peripherally and centrally located neurons (Brown et al., 2006; Williams and Truman, 2005). In the neurosecretory Tv neurons, filipodial activity, axonal sprouting and growth is also limited, resulting in a much reduced, misshapen axonal arbor (Brown et al., 2006). By comparison, expression of EcR-DN in the adult-specific neurons examined in this study resulted in less severe phenotypes, with no defects in initial projections or in ability to sprout secondary arbors. Initial projections are established before pupariation when EcR levels are low (Truman et al., 2004; Truman et al., 1994), so it is not surprising that EcR-DN expression does not affect this initial phase of growth. However, because all lineages examined still showed some secondary sprouting despite expression of EcR-DN, sprouting initiation might be prompted by signals other than the rising ecdysone titer, such as by cell-cell interactions. The lineage arbors exist within a complex neuropil in the vicinity of future synaptic partners, making dependence on local cues likely. By contrast, the Tv neuron axon arbors are embedded within the basal lamina on the dorsal surface of the CNS, away from other neurons (Nassel et al., 1988), thereby increasing their reliance on hormonal signals rather

than on local cues to initiate growth. The increased specific synaptic connectivity among the interneuron lineages might result in a decreased reliance on circulating factors, and place greater importance on the surrounding environment to correctly pattern the complicated neural circuitry of the CNS. Interestingly, EcR modulation of growth but not sprouting is also seen in the dendritic arborizing (da) sensory neurons during larval growth. The elimination of ecdysone signaling in da neurons via *usp*[2] or EcR-DN MARCM clones causes reduced numbers of branches, but growth is still initiated (Ou et al., 2008). The reduction in complexity but not the footprint of the da neurons might reflect their establishment of an early dendritic scaffold to tile the body wall, followed by elaboration of higher order branches (Williams and Truman, 2004). Unlike the da neurons in the larva, we find that blocking EcR signaling during metamorphosis reduces the overall arbor area in some of the lineages.

Expression of EcR-DN in the adult neuron lineages resulted in varying phenotypes amongst lineages, ranging from normal in appearance to having reduced and clumped arbors. This variability was not correlated with the expression level of EcR-DN within the MARCM clone (data not shown). Also, the abnormalities were likely not to be due to adding more EcR to the system, but rather to the suppression of ecdysone signaling by the dominant-negative receptor. This conclusion is based on expressing wild-type EcR-A or EcR-B pan-neuronally using the *elav* driver through metamorphosis and finding no effect on behavior or the size or structure of the adult CNS. However, expression of EcR-DN with the same driver resulted in flies that were unable to eclose, their CNSs were reduced in size, and a few immature neurotactin-positive bundles were still evident (data not shown).

Lineages 15 and 24 were by far the most affected, with both the central and peripheral projections of lineage 15 showing drastically reduced and compacted arbors. Cell-cell cohesion factors often function in bundling neurites together (Yu et al., 2000); misexpression of these factors could contribute to the compacted arbor phenotype. We examined the expression of neurotactin, shown to be a cell adhesion molecule involved in axon guidance (Speicher et al., 1998), but found no difference between control clones and those expressing EcR-DN (data not shown). Another factor that might affect the size and density of arbors is the extent and quality of filopodia during sprouting and outgrowth (Brown et al., 2006; Evers et al., 2006; Williams and Truman, 2005). Unfortunately, the location of the motoneuron dendritic arbors within the central neuropil negatively impacted our ability to use live imaging techniques, so the state of filopodial activity could not be ascertained.

In contrast to the motor lineages, the interneuron lineages were less severely affected by expression of EcR-DN. Lineages 6, 7 and 18 had moderate defects, whereas lineage 9 was mildly affected by expression of EcR-DN and lineage 2 cells expressing EcR-DN were indistinguishable from controls. Why are some lineages more affected by expression of EcR-DN than others? Our interpretation of the effects of EcR-DN on the adult-specific interneuron lineages is hindered by the lack of information on their neural function. However, if synaptic partners specific to each interneuron lineage help to direct arbor formation, variability between different lineages could be expected owing to differences in local cues. The extent of input from local cues might modulate the developmental response of a particular lineage to ecdysone, leading to variability across the lineages. Not only does the disruption of EcR signaling give different phenotypes across the complement of neuronal lineages, but the consistent lack of visible phenotypes in some lineages suggests that the extent of the effects of EcR on fine-tuning the connectivity of a neuron within the CNS might depend on the identity of its synaptic partners and the timing of their connections. The reasons for the differential effects seen with EcR-DN expression may become clear as the circuitry and functions of the CNS neuron lineages are revealed.

How might altering the morphology of the *Drosophila* adult lineages through expression of EcR-DN impact the functionality of the nervous system? Reduced or compacted secondary arbors could result in either absent, decreased or misaligned connections with synaptic partners, or changes in autonomous neuron function. In vertebrates, small changes in arbor morphology and size can have substantial effects on neuronal processing, even if the correct synaptic partners are established. Pyramidal neurons have been shown to have non-linear operations based on morphology, and branch points and varicosities can affect signal propagation in axons (Debanne, 2004; Mel, 1993). Wiring optimization also predicts that axons and dendrites have specific dimensions to minimize metabolic material, signaling delay and space costs to the cell, while keeping a fixed functionality (Chklovskii, 2004). This means that a reduction in the secondary arbor of a neuron lineage might impair its processing ability. However, given the current lack of specific labeling or drivers for individual neuron lineages, the particular functions of the *Drosophila* CNS lineages are still unknown, complicating our ability to test functional impairment.

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