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Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development

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SUMMARY

In insects, the ecdysteroids act to transform the CNS from its larval to its adult form. A key gene in this response is the *ecdysone receptor* (*EcR*), which has been shown in *Drosophila* to code for 3 protein isoforms. Two of these isoforms, EcR-A and EcR-B1, are prominently expressed in the CNS and we have used isoform-specific antibodies to examine their fluctuations through postembryonic life. EcR expression at the onset of metamorphosis is extremely diverse but specific patterns of EcR expression correlate with distinct patterns of steroid response. Most larval neurons show high levels of EcR-B1 at the start of metamorphosis, a time when they lose larval features in response to ecdysteroids. Earlier, during the larval molts, the same cells have no detectable receptors and show no response to circulating ecdysteroids; later, during the pupal-adult transformation, they switch to EcR-A expression and respond by maturing to their adult form. During the latter period, a subset of the larval neurons hyperexpress EcR-A and these cells are fated to die after the emergence of the adult.

The stem cells for the imaginal neurons show prominent EcR-B1 expression during the last larval stage correlated with their main proliferative period. Most imaginal neurons, by contrast, express only EcR-A when they subsequently initiate maturation at the start of metamorphosis. The imaginal neurons of the mushroom bodies are unusual amongst imaginal neurons in expressing the B1 isoform at the start of metamorphosis but they also show regressive changes at this time as they lose their larval axons. Imaginal neurons of the optic lobe show a delayed expression of EcR-B1 through the period when cell-cell interactions are important for establishing connections within this region of the CNS.

Overall, the appearance of the two receptor isoforms in cells correlates with different types of steroid responses: EcR-A predominates when cells are undergoing maturational responses whereas EcR-B1 predominates during proliferative activity or regressive responses. The heterogeneity of EcR expression at the start of metamorphosis presumably reflects the diverse origins and requirements of the neurons that nevertheless are all exposed to a common hormonal signal.

Key words: ecdysone receptor, *Drosophila*, *Manduca*, metamorphosis, CNS, hormone action

INTRODUCTION

In insects that undergo complete metamorphosis, the adult is constructed from a mixture of larval- and adult-specific tissues. This is especially true in the CNS where most larval neurons persist through metamorphosis but are 'remodeled' to conform with the behavioral requirements of the adult (Truman, 1988, 1990; Levine et al., 1991). The adult CNS also contains adult-specific ('imaginal') neurons that are generated during larval life but do not mature until metamorphosis. Large number of these neurons make up the optic and olfactory lobes that process information from the adult compound eyes and antennae. Similarly, many new neurons are added to the thoracic CNS to accommodate the sensory and motor demands imposed by the adult legs and wings. The areas of the brain most associated with learning, the mushroom bodies, become greatly elaborated (Technau and Heisenberg, 1982), reflecting the richness of new adult behaviors.

The steroid hormones, the ecdysteroids, control the transformation of the larval CNS into that of the adult (Fig. 1; see Truman, 1988; Truman et al., 1993; Levine et al., 1991 for reviews). Larval neurons, however, vary markedly in how they respond to ecdysteroid challenges. During early larval stages, they appear aloof to the ecdysteroid surges that cause larval molting. During the larval-pupal transition, by contrast, most respond to ecdysteroids by synapse elimination and loss of dendritic and axonal arbors as their larval specializations are removed. Ecdysteroids during the pupal-adult molt then induce these cells to show process outgrowth and synaptogenesis. Not only does a given cell show stage-specific differences in its

steroid responses, different cells may respond to the same steroid signal in different ways. For example, the prepupal ecdysteroid peak induces regressive changes in most larval neurons, maturational changes in imaginal neurons (Booker and Truman, 1987) and no morphological responses in the intersegmental muscle motoneurons (Levine and Truman, 1985). Recent studies of cultured *Manduca* motoneurons of known identity (Witten and Levine, 1991; Prugh et al., 1992) demonstrated that these isolated cells show responses to ecdysteroids that reflect the stage and cell specificities characteristic of these cells in vivo. Hence, these variations in neuronal response appear to reflect intrinsic differences in the target cells themselves.

These issues of stage and cell specificity in the CNS are part of the larger issue of how a steroid hormone coordinates diverse tissue responses within the animal. Studies on the puffing response of the giant salivary gland chromosomes of *Drosophila* to 20-hydroxyecdysone (20E) identified a genetic regulatory hierarchy which is activated by the primary response of a half-dozen early genes whose expression is required for a much larger number of late genes(Ashburner et al., 1974). Three of the early genes have been cloned and found to code for transcription factors (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991), some of which have been shown to regulate late genes in the network (Guay and Guild, 1991). These early genes also exhibit a primary response to 20E in other tissues. Tissue- or cell-specific differential activation of these early gene sets therefore provides an attractive model for explaining how differences in hormone response might be determined (Burtis et al., 1990; Thummel et al., 1990).

The cloning and characterization of the *EcR* gene of *Drosophila* has extended the molecular definition of the hierarchy to its key player, the ecdysone receptor (EcR; Koelle et al. 1991; Talbot et al., 1993). This gene encodes three protein isoforms (EcR-A, EcR-B1 and EcR-B2) that possess the same DNA- and hormone-binding domains but are distinguished by different N-terminal regions. Using antibodies specific for EcR-A and EcR-B1, Talbot et al. (1993) found that these two major isoforms exhibit quite different tissue distributions at the onset of metamorphosis. Since tissues showing different metamorphic responses to ecdysteroids (e.g., imaginal discs versus larval tissues) also differed in which EcR isoform was most abundant, the divergence in the ecdysteroid response pathways may therefore begin at its very first step.

EcR proteins by themselves are not active ecdysone receptors; rather they are activated by forming heterodimers with USP, another member of the steroid receptor superfamily encoded by the *Drosophila* gene *ultraspiracle* (*usp*, Koelle, 1992; Yao et al., 1992; Koelle et al., 1993; Thomas et al., 1993). EcR isoform distributions do not, therefore, necessarily represent distributions of active receptors. EcR isoform expression at the onset of metamorphosis (Talbot et al., 1993) is overlapped by USP expression which appears to be ubiquitous at this stage of development (W. S. T., unpublished data). Furthermore, all three EcR isoforms form active receptors when combined with USP (Koelle, 1992; Koelle et al., 1993). Hence, it is likely that variation in active receptor complexes is due to variation in the respective EcR component.

This paper focuses on EcR expression in the CNS of *Manduca sexta* and *Drosophila melanogaster*. We find that neurons show qualitative and quantitative changes in EcR expression during their life history and that these differences correlate with distinct patterns of ecdysteroid response.

MATERIALS AND METHODS

Experimental animals

Drosophila melanogaster were of the Canton-S wild-type strain raised

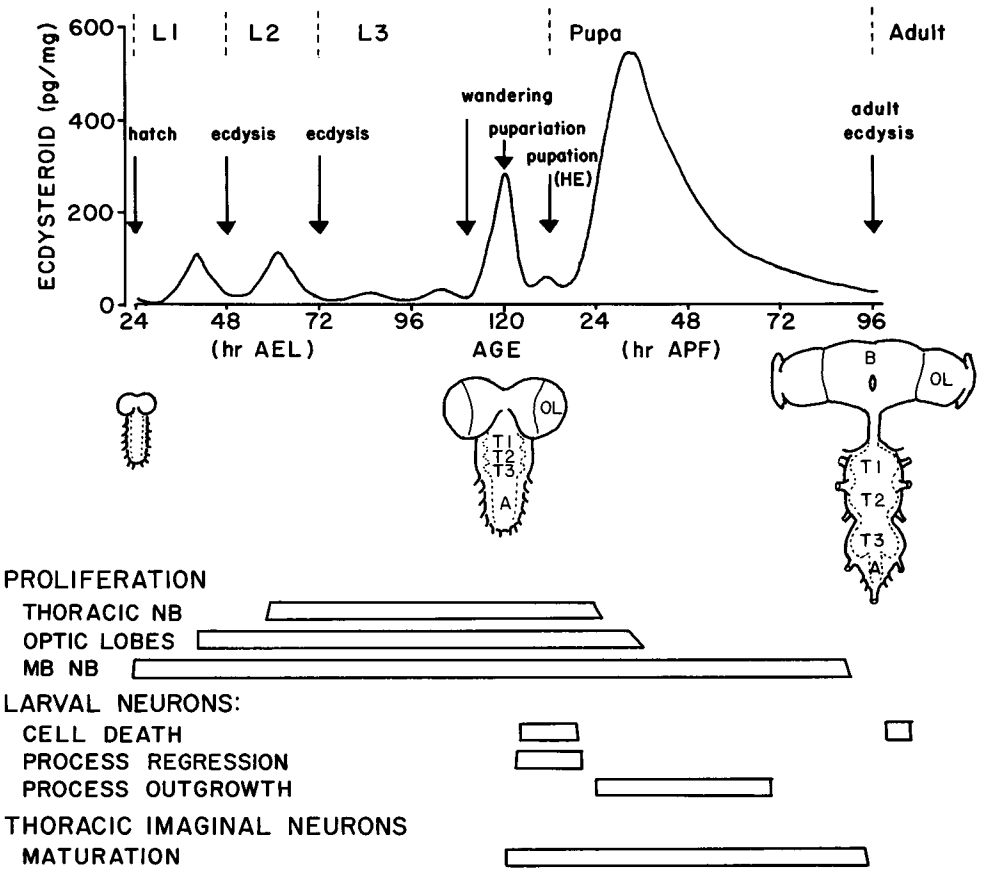


Fig. 1. Summary of the main developmental changes in the *Drosophila* CNS during postembryonic life. Ecdysteroid titers from various sources as summarized in Riddiford (1993). A, abdominal neuromeres; B, brain; Ln, larval instars; MB, mushroom bodies; NB, neuroblasts; OL, optic lobe; T, thoracic neuromeres.

at 25°C on standard medium. For early larval stages, eggs were collected on agar plates supplemented with yeast paste. Larval ages are given in hours after egg laying (AEL), but larvae within a given instar were aged as a synchronous cohort that ecdysed to that stage within a 1 hour window. Animals were also resynchronized at wandering and at the white puparium stage. Under our conditions hatching occurred at 24 hours AEL, ecdysis to L2 at 48 hours, ecdysis to L3 at 72 hours, wandering at 112 hours and pupariation at 120 hours. During metamorphosis animals were staged by hours after puparium formation (APF). Nervous systems were examined at 6 to 8 hour intervals through larval life, at 3 hour intervals through the first 50 hours after pupariation and at approximately 6 hour intervals thereafter. Overall, the analysis involved dissected and stained nervous systems from over 1700 staged animals.

Larvae of *Manduca sexta* were raised on artificial diet at 26°C. Their progression of development relative to the endocrine events of the larval and pupal molts is as given in Curtis et al. (1984) and Wolfgang and Riddiford (1986).

Antibodies and immunohistochemistry

Immunocytochemistry was performed using antibodies that recognized all forms of *Drosophila* EcR and others that were selective for specific isoforms. Antibodies directed against epitopes in the common region of the EcR isoforms included a rabbit polyclonal antiserum (Koelle at al., 1991) and monoclonal antibodies (mAbs) DDA2.7, IID9.6, AC12.4, JG6.2 and GGD11.6 (Talbot et al., 1993). Antibodies specific to EcR-A included a rabbit polyclonal antiserum raised against an EcR-A-specific fusion protein and the mAbs 15G1a, 18F6 and 12H4 (Talbot et al., 1993). EcR-B1 was specifically recognized by mAb AD4.4 (Talbot et al., 1993).

For *Drosophila*, we stained complete developmental series with the common region antibodies DDA2.7 and IID9.6, and the polyclonal anti-EcR, with the EcR-A-specific antibodies 15G1a and the polyclonal anti-EcR-A, and with the EcR-B1-specific mAb AD4.4. Other mAbs were used only on one or two key developmental stages. EcR-staining in *Manduca sexta* was with the polyclonal anti-EcR and with the mAb JG6.2. The identity of glial cells in *Drosophila* was confirmed by their failure to stain with rat antibodies against the neuron-specific protein ELAV (Robinow and White, 1991).

Drosophila nervous systems were fixed in 4% paraformaldehyde in (0.01 M) phosphate-buffered saline pH 7.4 for 2 hours at room temperature (RT) or overnight at 4°C. Immunostaining for EcR and double-staining procedures for EcR and ELAV were as given in Robinow et al. (1993). After treatment with biotinylated secondary antibodies, the complexes were detected using fluorescence (avidin-FITC or avidin-RITC; Vector Labs, Burlingame, CA) or enzyme-linked (ABC Kits, Vector Labs) detection systems. The mAbs were used at a dilution of 1:20, the polyclonal antisera at 1:2000.

Manduca ganglia were fixed for 1 hour. After rinsing tissues were incubated in 0.5 mg/ml collagenase (Type IV, Sigma) in PBS for 1 hour to aid penetration. Incubations with primary antisera were for 36 to 60 hours. Incubations with secondary and tertiary reagents were 1 to 2 hours each.

Preblocking experiments were performed for the anti-EcR. A 1:1000 dilution of the affinity-purified antibody was incubated overnight at 4°C with various concentrations of the TrpE-EcR fusion protein and control TrpE protein (Koelle et al., 1991). Each mixture was then used for immunostaining of *Manduca* ganglia as above.

Quantification of the immunosignal

Measurement of levels of EcR immunostaining employed a BioRad MRC600 scanning confocal system using a 60× oil immersion objective. *Drosophila* tissues in a given developmental series (24-110 hours AEL; 72-120 AEL hours AEL, and 0 to 91 hours APF) were split into replicate groups and stained for EcR-A or EcR-B1. The final step involved avidin-FITC and a counterstain for 5 minutes in 4 μg/ml propidium iodide (PI; Sigma). Each developmental series was scanned during a single session using the same gain and background settings. Files were collected as 'Kalmann' averaged images and stored as split-screen, digital files containing both PI and immunostained frames. During data analysis, the PI image was used to determine cell type and to select nuclei for measurement. The main criterion for selection was the sharpness of nuclear outline, suggesting that the optical section was cut through its center. The nuclear positions were marked on the screen and the PI image replaced with the FITC-stained section for measurement of the average intensity of fluorescence in the selected nuclei. An 'Area' program was used to measure average pixel intensity in each nucleus using a scale that extended from 0 to 256. The measurements obtained by this method are not completely linear because of systematic deviations in the detector at the high and low limits of its range. In preparations without primary antiserum, neuronal nuclei showed background fluorescence values of 5 to 10 units.

The thickness of the *Manduca* ganglia required that a baseline be established for each optical section. Sensitivity was adjusted so that neighboring neuropil showed an average pixel intensity of about 30 units. This background value was then subtracted from the average pixel intensity measured in the nucleus of each D-IV motoneuron.

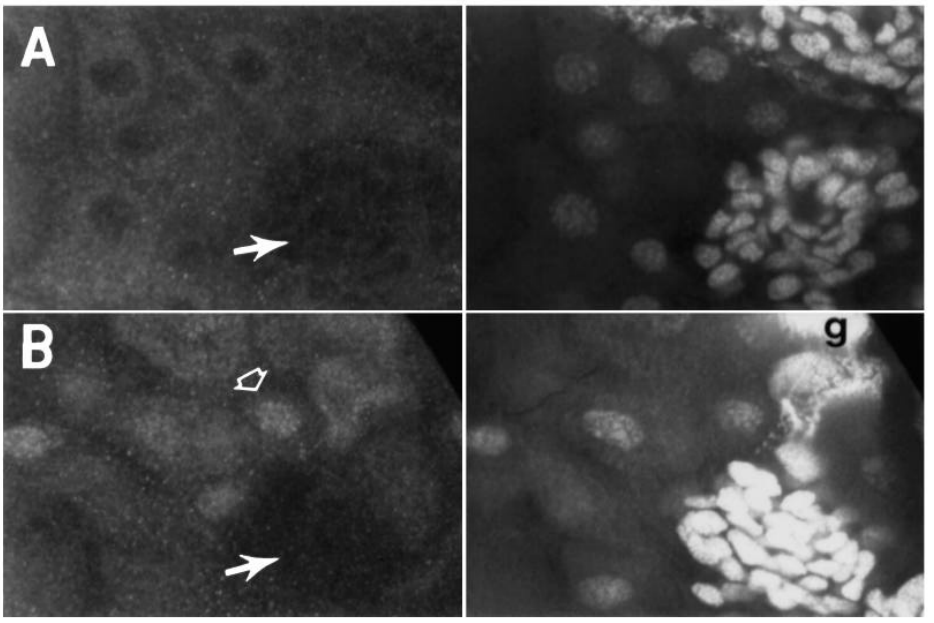


Fig. 2. Optical section through a thoracic ganglia from wandering stage *Manduca* larvae that were immunostained with anti-EcR (left) and propidium iodide (right). (A) Antibody preblocked with TrpE-EcR fusion protein; (B) antibody preblocked with 4× higher concentration of control TrpE protein. In B, larval neurons show nuclear EcR staining (open arrow) but the immature, imaginal neurons (arrow) are negative. g, giant glia.

Combined autoradiography and receptor immunocytochemistry

Autoradiographic procedures were modified from those described previously (Fahrbach and Truman, 1989; Fahrbach, 1992). Dissected *Manduca* ganglia were rinsed in saline at RT for 1 hour to remove endogenous ecdysteroid and then incubated in saline containing 4-5 nM ^{125}I-ponasterone A (Cherbas et al., 1988) for 1 hour at RT. The ganglia were then rinsed (3× 5 minutes in ice-cold saline) and immediately frozen in a drop of Polyfreeze freezing medium (Polysciences, Warrington, PA) onto cryostat chucks using powdered dry ice. Under safelight conditions, cryostat sections (6 μm) were thaw-mounted directly onto dry slides previously coated with Kodak NTB-3 nuclear track emulsion. The sections were exposed for 45 days at 4°C in light-tight boxes packed with desiccant.

To preserve the receptor antigenicity through the photodeveloping procedure, we modified the protocol from Morrell and Pfaff (1983). After warming to RT, the slides were fixed for 10 minutes at 16°C in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4), rinsed in PBS (3× 1 minute at 16°C), and photodeveloped for 6 minutes in freshly prepared Kodak D-170 (Amidol) developer at 16°C. Developing was stopped in PBS (1 minutes, 18°C), followed by two baths of Kodak Fixer (2 and 4 minutes, 18°C), and rinses in PBS (10 minutes). Immunostaining involved a nickel sulfate-diaminobenzidine intensification procedure modified from Hancock (1982), using 25 mM KPBS (pH 7.6) throughout. Sections were preblocked for 1 hour at RT with 10% normal goat serum and 5% Carnation non-fat dry milk in KPBS with 0.3% Triton X-100. The slides were then incubated with mAb JG6.2 (1:5 dilution) in a humid container for 48 hours at 4°C. They were subsequently warmed to RT, rinsed in KPBS (3× 15 minutes), and incubated overnight at 4°C with a peroxidase-labeled goat anti-mouse IgG (1:400, Sigma). Sections were rinsed in KPBS (2× 10 minutes), followed by 2× 10 minute rinses in acetate-imidazole buffer (175 mM acetate, 10 mM imidazole, pH adjusted to 7.2-7.4 with glacial acetic acid), and then incubated for 4-5 minutes in a freshly prepared chromagen solution (33 ml dH_2O, 5 ml 1 M sodium acetate, 2 ml 0.2 M imidazole, 1.05 g nickel (II) sulfate hexahydrate (Fluka 72280), 20 mg DAB (Sigma) and 40 μl 30% H_2O_2). No staining was seen when any of the immune reagents were omitted.

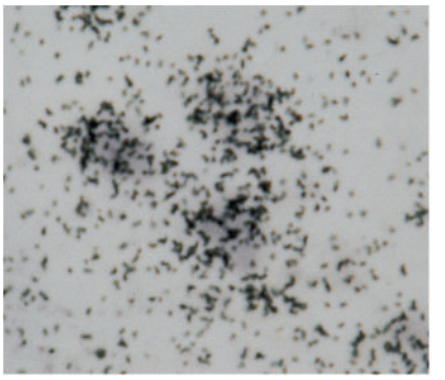


Fig. 3. Neurons from a wandering stage *Manduca* showing co-localization of EcR staining using mAb JG6.6 (pale violet staining in nuclei) with binding of ^{125}I-ponasterone A (clusters of silver grains).

RESULTS

General aspects of EcR antibody staining

Distinct patterns of immunostaining were observed when CNSs were stained using antibodies specific to EcR-A and EcR-B1 (e.g., Figs 5, 7), but antibodies directed against different epitopes of the same isoform gave identical patterns of staining. The EcR-B2 isoform has a very short N-terminal-specific region (17 residues), and no antibodies specific to this isoform have yet been obtained (Talbot et al., 1993). However, all of the cell types that stained with antibodies against common region epitopes also stained positive for EcR-A, EcR-B1, or both. Thus, there appears to be no cell types in the CNS that express only EcR-B2, although it may be expressed in combination with EcR-A and/or EcR-B1.

A nuclear antigen in *Manduca* was recognized by two mAbs (JG6.2 and GGD11.6) directed against epitopes in the C region of *Drosophila* EcR (Talbot et al., 1993) and by a polyclonal antiserum raised against the D region (Koelle et al., 1991). This nuclear staining was completely blocked by preincubation of

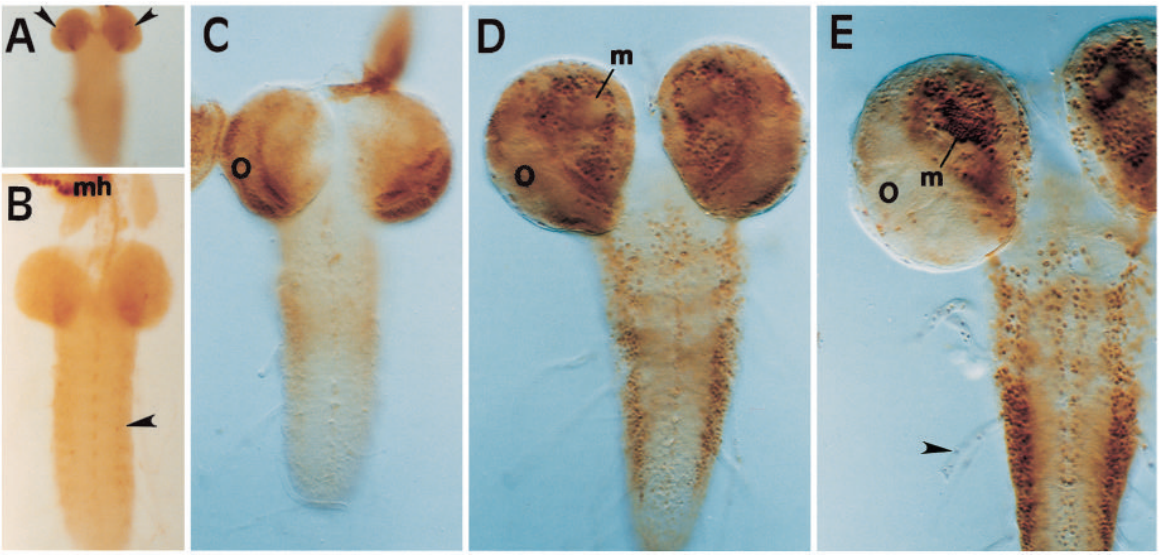


Fig. 4. CNSs of larval *Drosophila* immunostained with mAb AD4.4 to show the distribution of EcR-B1. (A) 10 hour 1st instar larva (34 hours AEL); arrowheads: a pair of weakly staining, brain neurons. (B) 12 hours 2nd instar larva (60 hours AEL) with weak staining in tracheal nuclei (arrow); mouth hooks (mh) show strong nuclear staining. (C-E) 3rd instar larvae at 14 (C, 86 hours AEL), 30 (D, 102 hours AEL) and 40 (E, 112 hours AEL) hours postecdysis. As larvae aged, B1 staining was lost from the OL proliferation zones (O) but appeared in larval neurons (D) and mushroom body neurons (E; m). Arrowhead, peripheral glia.

the antiserum with a fusion construct containing the 113 residue D region noted above (Fig. 2). Recent cloning of the *EcR* gene from *Manduca* show that the C regions are highly conserved between the moth and the fly (95% identity; R. Palli, R. Newitt, and L. M. Riddiford, unpublished data), and hence it is likely that *Manduca* EcR is being recognized by the *Drosophila* antibodies.

At the two stages examined, larvae at wandering (W) and day W+1, neurons whose nuclei were immunopositive for mAb JG6.2 also showed nuclear binding of ^{125}I-ponasterone as evidenced by accumulations of reduced silver grains (Fig. 3). By contrast, clusters of imaginal neurons in the same sections had very low or no immunostaining and had no grain counts above the background (data not shown). The colocalization of immunostaining and ^{125}I-ponasterone binding indicate that the *Manduca* EcR detected by antibodies is part of an active receptor complex since in *Drosophila*, and presumably in *Manduca*, hormone binding also requires the association of EcR with USP (Koelle, 1991; Koelle et al., 1993). Since the antibodies that reacted with the *Manduca* EcR recognize common regions of the receptor, they provide data about changes in total levels of receptor but not about which isoforms are responsible for these changes.

We often found a fluorescent signal in the cytoplasm, but this remained when the primary antibody was omitted or was pre-absorbed with EcR protein (Fig. 2). Hence, it appears to be due to cytoplasmic autofluorescence. Also, no cytoplasmic staining was evident when we used peroxidase/DAB detection systems. This nuclear localization of EcR is in accord with autoradiographic evidence showing accumulation of significant radiolabeled ecdysteroids only in nuclei of *Manduca* neurons (Fahrbach and Truman 1989; Fahrbach, 1992).

Time course of EcR expression in larval neurons

Drosophila neurons

Newly hatched larva showed only a single pair of ventrolateral brain neurons that weakly expressed EcR-B1 (Fig. 4A). These cells expressed this isoform throughout larval life. All other larval neurons were devoid of detectable immunoreactivity to antibodies against common or isoform-specific regions of EcR (e.g., Figs 4A,B, 6). Hence, during the first and second larval instars, neurons appear to lack all EcR isoforms. The same was true for glial cells. In contrast to neurons and glia, the tracheal cells within the CNS expressed both EcR-B1 (Fig. 4B) and EcR-A with the B1 staining being stronger. Peripheral larval tissues such as muscle and epidermis were similar to trachea in their EcR staining (Fig. 4B).

EcR-B1 staining was marginal at the start of the third (last) instar (72 hours AEL) but became unmistakable by 80 to 88 hours AEL (Figs 4, 6, 7). It then increased after 96 hours and reached its highest levels at pupariation (Figs 4, 5, 6A). EcR-A also increased through this period but to more modest levels. After pupariation, the levels of EcR-B1 declined precipitously, with most of the decline occurring during the first 3 hours APF (data not shown). In contrast to EcR-B1, the A isoform underwent only a minor decline during this period.

Larval neurons showed three major patterns of EcR expression during metamorphosis. Most neurons showed the *type I* pattern summarized in Fig. 6A. After its decline at

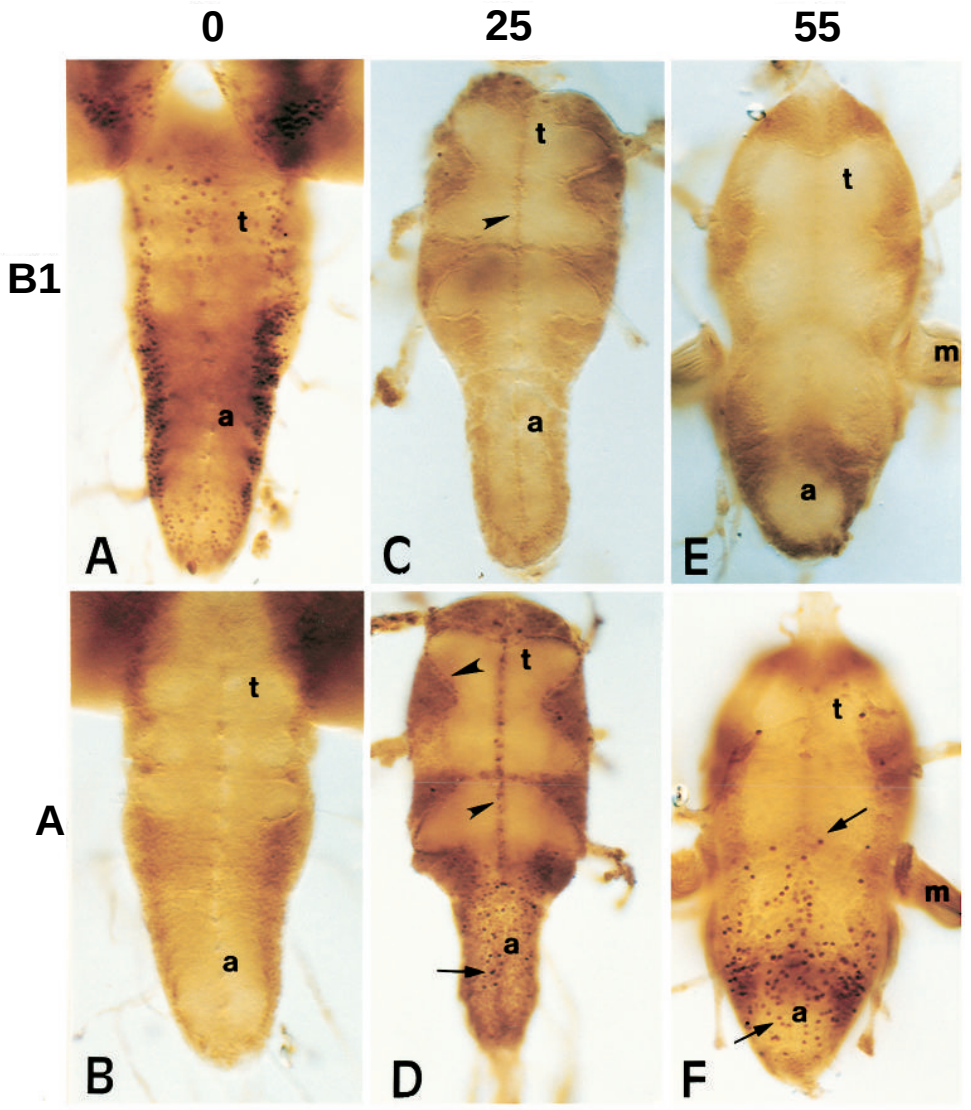


Fig. 5. Immunostained ventral CNS from *Drosophila* during the first half of metamorphosis showing distribution of staining for EcR-B1 (top; mAb AD4.4) and EcR-A (bottom; mAb 15G1a). (A,B) White puparium stage; (C,D) 25 hours APF, small arrowhead, midline glia; large arrowhead, perineuropilar glia; arrow, type II larval neurons. E,F) 55 hours APF; arrows, type II neuron; m, muscle. a, abdominal neuromeres; t, thoracic neuromeres.

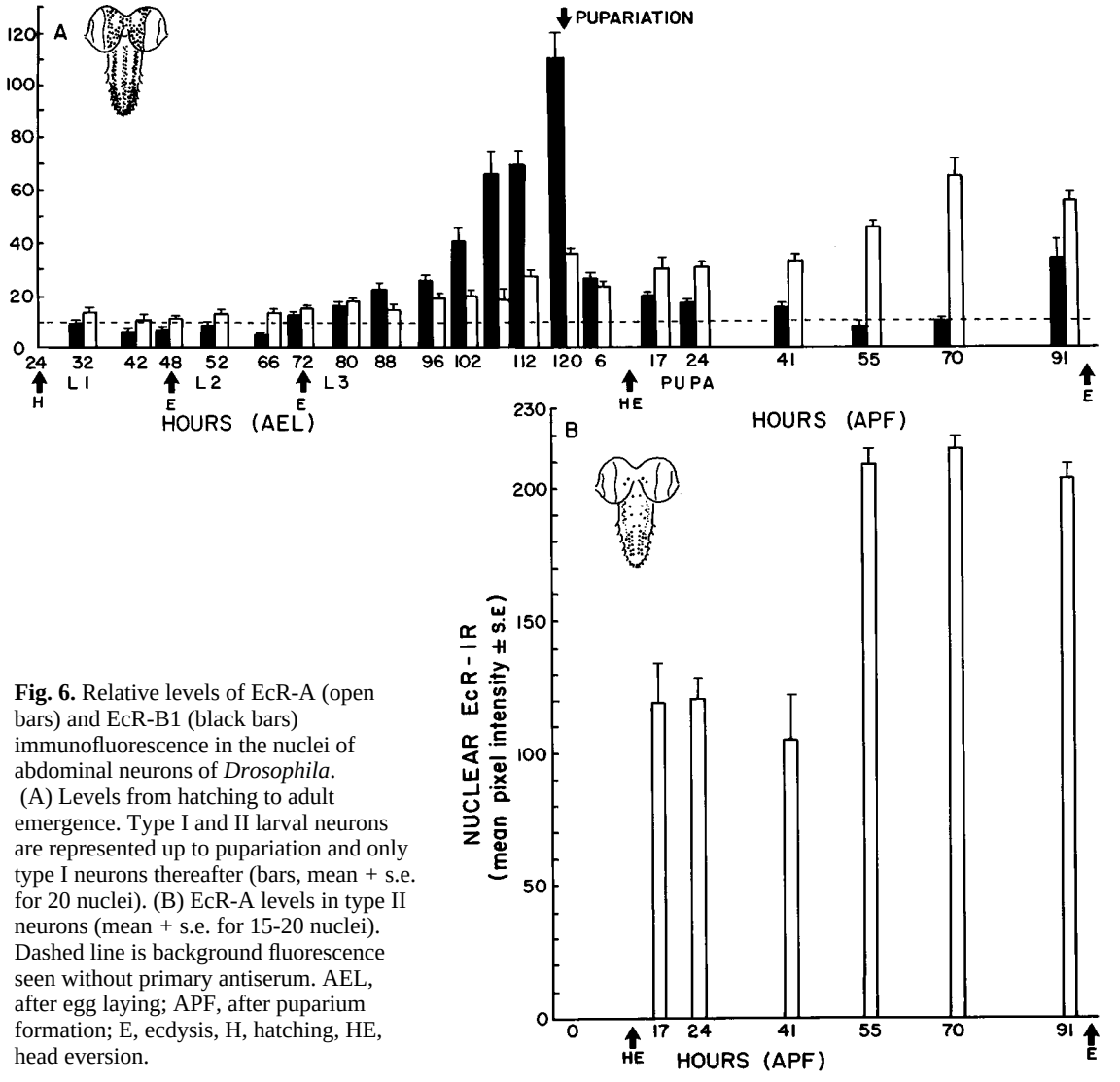


Fig. 6. Relative levels of EcR-A (open bars) and EcR-B1 (black bars) immunofluorescence in the nuclei of abdominal neurons of *Drosophila*. (A) Levels from hatching to adult emergence. Type I and II larval neurons are represented up to pupariation and only type I neurons thereafter (bars, mean + s.e. for 20 nuclei). (B) EcR-A levels in type II neurons (mean + s.e. for 15-20 nuclei). Dashed line is background fluorescence seen without primary antiserum. AEL, after egg laying; APF, after puparium formation; E, ecdysis, H, hatching, HE, head eversion.

pupariation, the EcR-B1 isoform persisted at low levels until finally disappearing at about 40 hours APF. It later reappeared toward the end of metamorphosis (85 hours APF) and persisted into the adult stage (data not shown). With the decline of EcR-B1 after pupariation, EcR-A became the major isoform during the pupal-adult transformation. It was present at moderate levels through the first two-thirds of this period with an up-regulation at about 70 hours APF.

Fig. 8 follows EcR expression in one type I neuron, the motoneuron MN5. It innervates body wall muscles in the larva but is then remodeled to supply flight muscles in the adult (C. M. Bate, unpublished). MN5's size and location on the dorsal T1-T2 boundary allowed it to be identified through most of metamorphosis. During the larval-pupal transition (at pupariation), it showed higher levels of EcR-B1 than EcR-A, but the subsequent dramatic loss of EcR-B1 from these cells left EcR-A as the major isoform during the pupal-adult transition.

Approximately 350 neurons in the brain and ventral CNS showed a *type II* pattern of EcR expression (Fig. 6B; Robinow et al., 1993). These neurons could be distinguished from the type I cells at about 12 hours APF and were characterized by expressing EcR-A at about 10-fold higher levels than in other neurons. These high levels were maintained through the remainder of metamorphosis (Figs 5F, 6B). As with the type I neurons, the type II neurons lost EcR-B1 staining during the middle of metamorphosis, but it reappeared 10-15 hours before adult ecdysis. Double-labeling experiments showed that the levels of B1 expressed by type I and type II neurons during this time were not correlated with their levels of EcR-A (Fig. 9). All neurons showing the type II pattern of EcR expression died after the emergence of the adult (Robinow et al., 1993).

A few larval neurons in each abdominal neuromere had no detectable EcR-B1 at the time of pupariation (Fig. 10), although they exhibited moderate levels of the EcR-A (data not shown). These cells were also B1 negative at wandering but their condition earlier in the third instar is unknown. These are designated as *type III* neurons. Since all neurons severely down-regulate EcR-B1 after pupariation, we could not follow their subsequent EcR expression through metamorphosis but it is most likely similar to the type I cells.

Manduca neurons

Manduca has large and readily identifiable neurons whose developmental responses to ecdysteroids have been well char-

acterized. Consequently, we used *Manduca* to explore the relationship of EcR expression to the ecdysteroid surges that cause the larval and pupal molts. For example, Fig. 11 follows changes in EcR levels in the D-IV motoneurons that innervate the ventral, abdominal intersegmental muscles. Prior to the initiation of the larval molt, the D-IV neurons showed no detectable EcR immunoreactivity (Figs 11, 12A). Their levels of EcR immunostaining continued to be low or non-detectable during most of the ecdysteroid surge, but as steroid titers were declining these cells showed a transient expression of moderate levels of EcR. EcR immunoreactivity then receded to low levels through the early part of the last instar but became elevated on day 3 of the 5th instar at the time of the small 'commitment peak' of ecdysteroid that triggers wandering behavior. EcR levels continued high through wandering (Fig. 12B) and peaked at W+1 prior to the prepupal ecdysteroid peak. Receptor levels in the D-IV cells were then reduced for the remainder of the pupal molt with a slight rebound just prior to pupal ecdysis (Fig. 11).

Most abdominal neurons showed patterns of EcR induction that were similar to that seen for the D-IV cells. Neurons differed, however, in when they subsequently lost their EcR immunoreactivity. Some had already lost all immunostaining by day W+2 (Fig. 12D) while most cells declined on day W+3. Importantly, the overall spatial and temporal patterns of receptor abundance provided by the antibody staining corresponded to those determined by binding of ^{125}I-ponasterone (Fahrbach, 1992).

EcR expression in neuroblasts and imaginal neurons

The isolated neuroblasts

In *Drosophila*, most of the adult-specific neurons are produced by isolated neuroblasts (NBs) situated in stereotyped locations in the thoracic neuromeres and central brain (e.g., Truman and Bate, 1988). Each NB undergoes an extended series of asymmetric divisions to produce a series of ganglion mother cells (GMCs). Each GMC then divides once to yield two imaginal neurons. Depending on location, the NBs start dividing during the first or second larval instar and produce neurons until about 24 hours APF (Fig. 1; Truman and Bate, 1988; Ito and Hotta, 1992). Irrespective of the time of their birth, the imaginal neurons remain arrested as immature cells until the start of metamorphosis.

The isolated NBs in the thorax and medial brain showed detectable EcR-B1 midway through the 2nd larval stage, a time coinciding with their resumption of proliferative activity (Truman and Bate, 1988). Their B1 levels subsequently peaked by the middle of the third instar (Fig. 13A), began to drop after 102 hours AEL and reached background levels by pupariation. We found no EcR-A staining in these cells. EcR was not found in either the GMCs or the arrested imaginal neurons (Fig. 14A). This lack of EcR staining was also seen for the arrested imaginal neurons in larval *Manduca* (Fig. 12C,D).

Imaginal neurons began to express EcR at pupariation but only the A isoform (Fig. 13B). EcR-A was then expressed at moderate levels through metamorphosis with an up-regulation at about 70 hours APF. EcR-B1 finally appeared in these cells at 85-90 hours APF.

The mushroom body neurons

The four mushroom body NBs in each brain hemisphere are unique in that they generate neurons throughout metamorphosis until about 10 hours before adult emergence (Fig. 1; Ito and Hotta, 1992). Throughout this period, the younger neurons can be readily identified because they form a compact column that extends centripetally from each neuroblast down towards the mushroom body neuropil. To follow receptor expression in mushroom body neurons that were born during larval stages, we selected cells that had a superficial location and were distant from the NB. EcR-B1 was first detected in these cells at 108 hours AEL (Figs 4D,E, 13C), and rose to peak levels by pupariation. EcR-A staining was not evident through this period (Fig. 14B). After pupariation, EcR-B1 then rapidly declined to moderate levels that were maintained until it finally disappeared at 50 hours APF. As with other neurons, EcR-B1 later reappeared at 85 hours APF. EcR-A was first detected at moderate levels shortly after pupariation and persisted until adult emergence (Fig. 13C).

During the middle and later stages of metamorphosis, when EcR-B1 had disappeared from the superficial mushroom body neurons, we nevertheless always saw 4 small, deep clusters of EcR-B1-positive cells in each mushroom body. Each cluster was located in a column of young neurons that extended down from the NB (Fig. 14C). The most superficial cells (youngest) within a column showed no receptor expression, farther down they expressed EcR-B1 and the deepest cells (oldest) expressed only EcR-A. This distribution was seen within the columns during the latter half of metamorphosis irrespective of when we looked. Thus, there appears to be an age-related shift in EcR expression with a new neuron first showing no receptors, and then EcR-B1, and finally EcR-A.

Imaginal neurons: optic lobes

Each optic lobe (OL) has two major proliferation zones. As with the isolated NBs, the proliferation zones began EcR expression in the late second instar and expressed only the B1 isoform (Figs 4C, 15A). EcR-B1 levels peaked by about 96 hours AEL but by pupariation the OL was devoid of EcR-B1 staining. The only positive nuclei (Fig. 7A) belong to 3 visual interneurons that are the only larval neurons in the OL (Tix et al., 1989).

EcR levels in the optic lobes were either low (EcR-A) or undetectable (EcR-B1) through 6 hours APF (Figs 7A,C, 15B). EcR-B1 staining reappeared at 9 hours (data not shown), but in the OL neurons rather than the stem cells. High levels of this isoform as well as EcR-A were then maintained until about 50 hours APF after which B1 disappeared (Figs 7, 15B). EcR-B1 reappeared at the end of metamorphosis.

Patterns of expression in glia

The patterns of expression for some of the major glial types are summarized in a qualitative fashion in Fig. 16. The cortical glia (Fig. 17A) are presumably functional in the larval stage and are found superficially in the ventral CNS along with the lineages of imaginal neurons. These glia showed high levels of EcR-B1 through the larval-pupal transition and into the early phases of adult differentiation. Their eventual fate is unknown.

The midline glia (T. Awad and J. W. T., unpublished) and the OL glia situated at the border between the lamina and

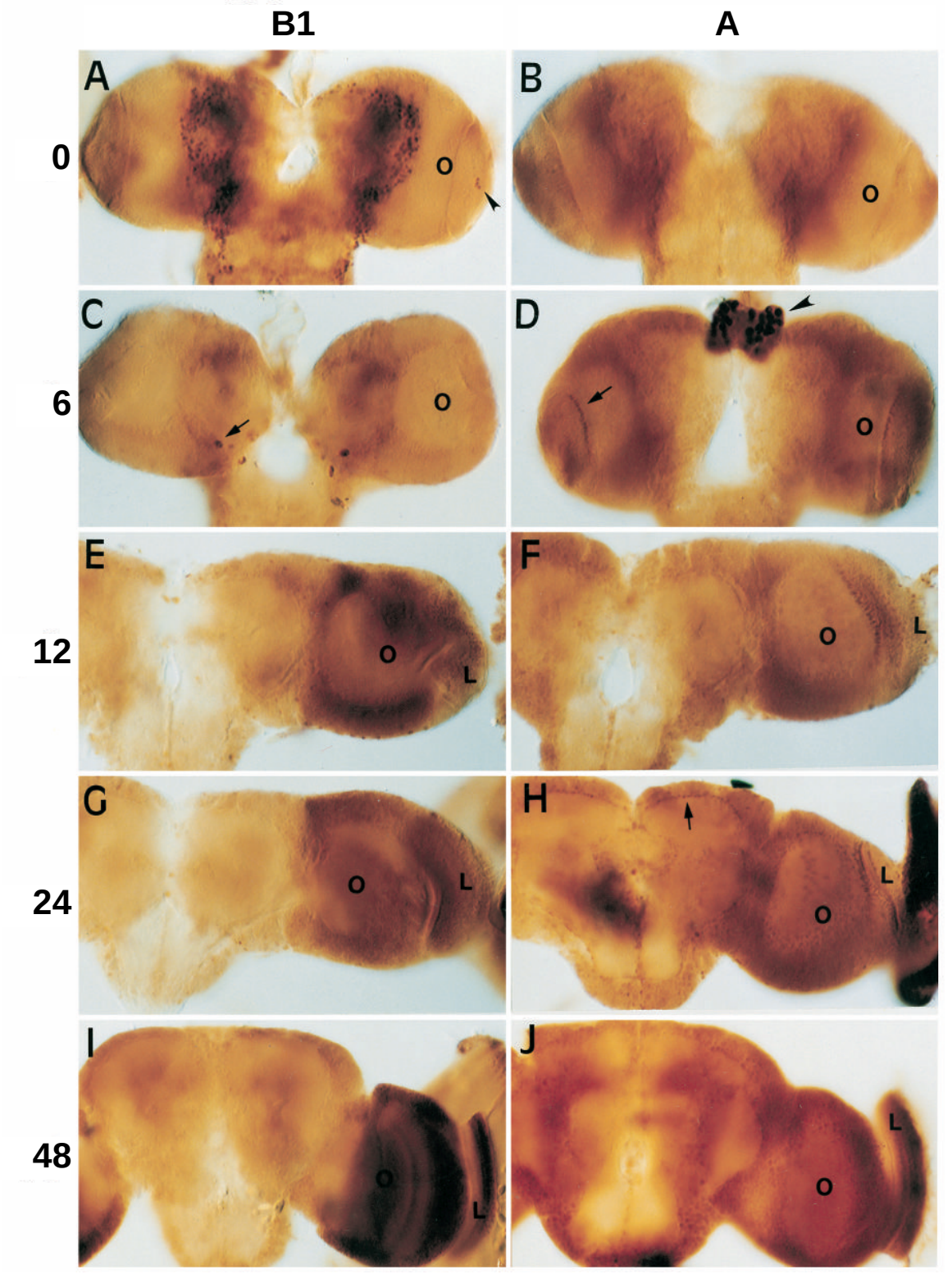


Fig. 7. Immunostained brains of *Drosophila* during the first half of metamorphosis showing distribution of EcR-B1 (left; mAb AD4.4) and EcR-A (right; mAB 15G1a). (A,B) White puparium stage showing prominent EcR-B1 staining and weak EcR-A staining in larval neurons. Arrow, larval visual interneurons. (C,D) 6 hours APF showing loss of EcR-B1 in neurons but prominent tracheal staining (arrow, C). Lamina glia (arrow, D) and ring gland (arrowhead) are positive for EcR-A. (E,F) 12 hours APF. (G,H) 24 hours APF. Prominent EcR-A staining in perineuropilar glia (arrow). (I,J) 48 hours APF. L, lamina of optic lobes; O, optic lobe.

medulla (Winberg et al., 1992) undergo proliferation during the last larval instar. During this time the midline glia expressed EcR-B1 but not EcR-A (Fig. 17C,D). The OL glia also did not express EcR-A but their possible EcR-B1 expression is uncertain because the OL NBs showed strong EcR-B1 expression at this time and we could not readily distinguish the young glial cells from the NBs and their progeny. After the start of metamorphosis both types of glial cells switched to expressing EcR-A (Figs 5D, 7H, 17B).

The peripheral glia and perineuropilar glia proliferate during the first day of metamorphosis (T. Awad and J. W. T., unpublished). Some of the peripheral glia on the segmental nerves

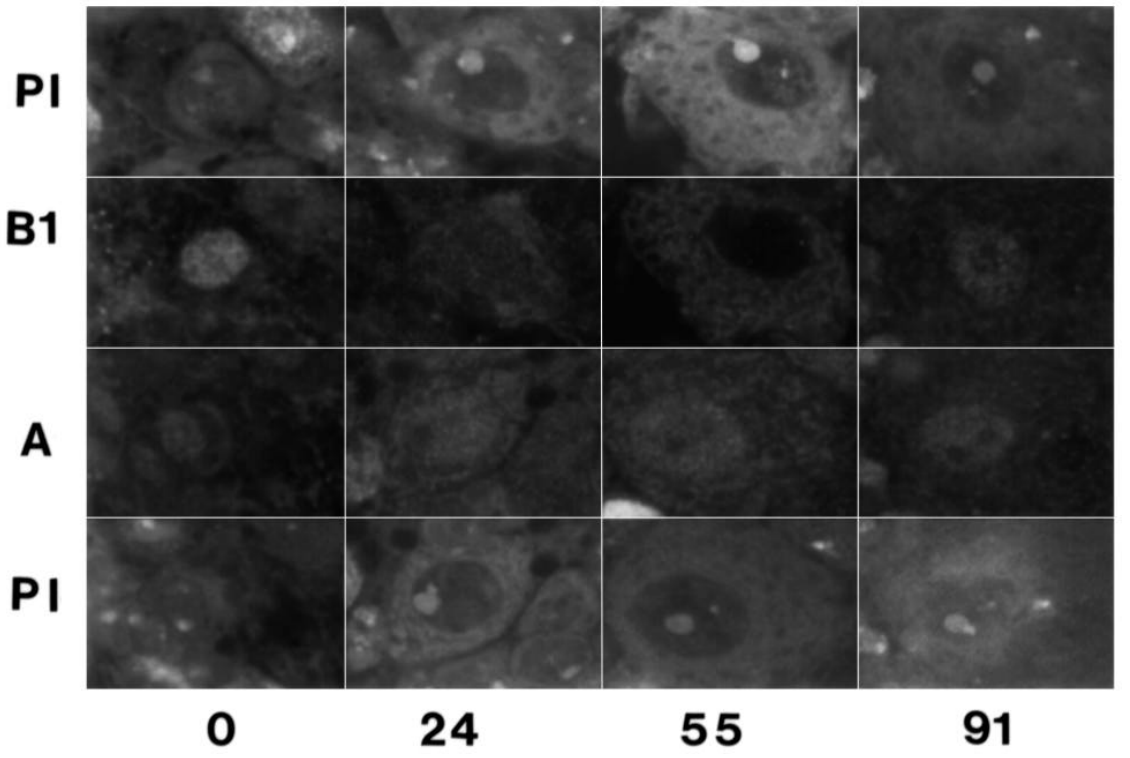


Fig. 8. Confocal optical sections showing the nuclear expression of EcR isoforms in the *Drosophila* motoneuron MN5 through metamorphosis. Top two rows are paired images showing propidium iodide (PI) and EcR-B1 staining (B1) at various hours after pupariation (numbers). Bottom two rows are paired images showing EcR-A (A) and propidium iodide (PI) of MN5 in another set of preparations.

showed transient expression of EcR-B1 late in larval life (Fig. 4E) and a subsequent expression early in their proliferative period. EcR-A expression followed that of EcR-B1 (Fig. 16). The perineuropilar glia, by contrast, showed prominent expression of EcR-A while they were proliferating.

By the middle of metamorphosis, EcR-A was the sole isoform expressed in all of these glia. We did not analyse glial expression beyond this period.

DISCUSSION

Although ecdysteroids initiate and coordinate insect metamorphosis, the complexity of the tissue-specific responses to these fluctuations seems at odds with the simple nature of the signal. This difficulty of interpretation is particularly acute in the CNS which contains many cellular elements that persist during metamorphosis. These neurons, glia and imaginal cells show a variety of specific responses to the same set of endocrine signals. The present results show that this complexity in hormone responses is matched by a complex and cell-specific pattern of EcR expression.

The existence of multiple isoforms is a feature of certain members of the steroid hormone receptor superfamily such as the thyroid hormone receptors (e.g., Chinn, 1991) and the retinoid receptors (Chambon et al., 1991). These receptors show complex spatial and temporal patterns of expression during development and these patterns are typically most complex in the CNS (Bradley et al., 1992; Rees et al., 1989; Ruberte et al., 1990). Also, metamorphosis in amphibians has interesting parallels with that of insects since the former is accompanied by complex patterns of thyroid hormone receptor expression (Kawahara et al., 1991; Yaoita and Brown, 1992). Although the stage and tissue specificities

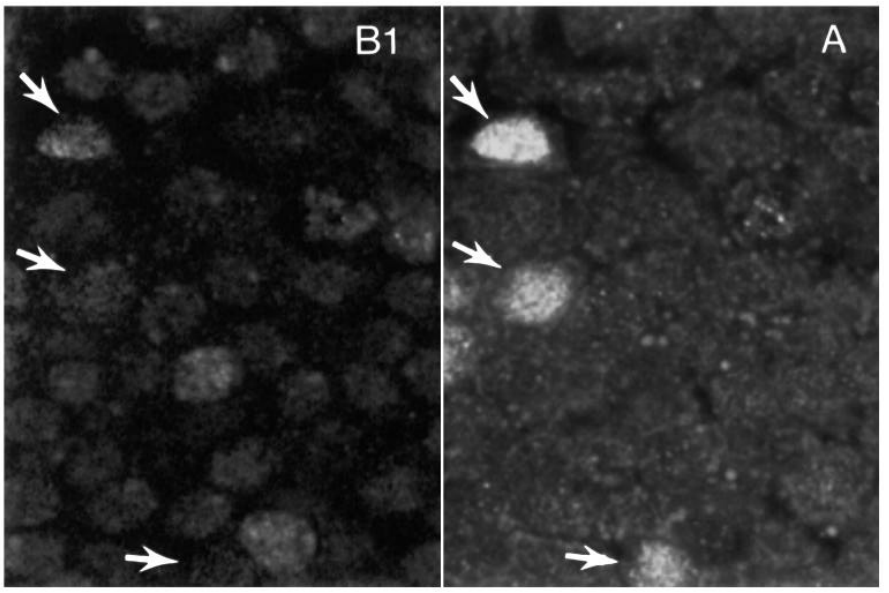


Fig. 9. Confocal image of an EcR-A/EcR-B1 double-labeled nervous system from *Drosophila* showing that neurons independently regulate expression of the 2 isoforms. The images are of dorsal abdominal neurons at 95 hours APF. Left, EcR-B1; right, EcR-A. Arrows identify three type II neurons that vary in their expression of EcR-B1. The remaining nuclei are type I neurons showing low EcR-A and variable amounts of EcR-B1.

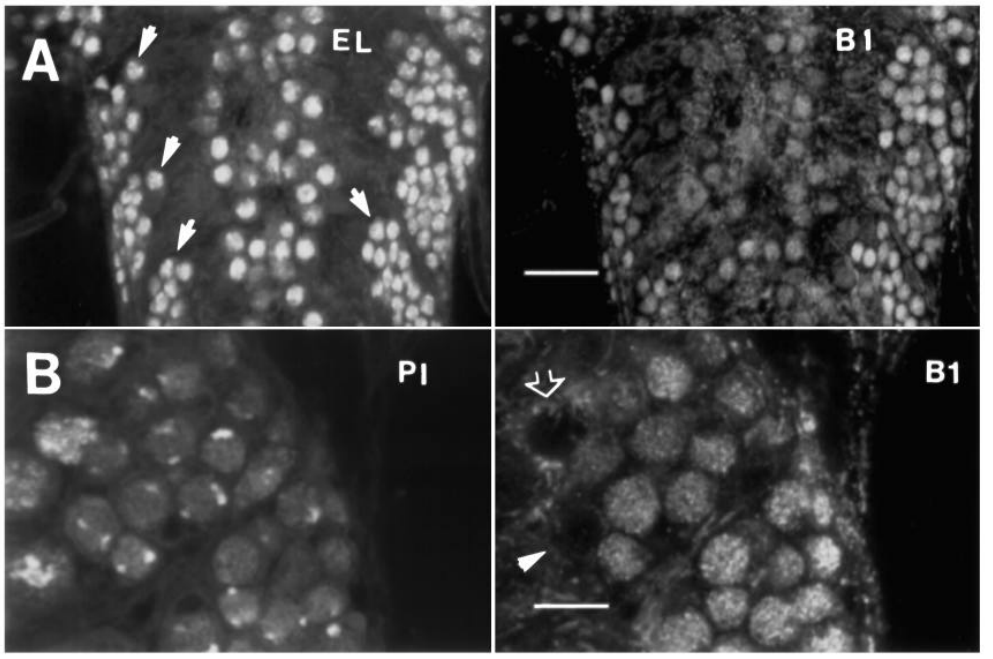


Fig. 10. Confocal images of the dorsal abdominal nervous system from a white puparium stage of *Drosophila* showing type III neurons that are EcR-B1 negative. (A) Low-power view of a preparation double-stained preparation for ELAV (EL) and EcR-B1 (B1); arrows show ELAV-positive neurons that are negative for EcR-B1. Scale bar, 25 μm. (B) High-magnification image of an abdomen stained for EcR-B1 (B1) and counterstained with propidium iodide (PI); both type III neurons (arrowhead) and some glial cells (open arrow) are negative for EcR-B1. The polyploid glial cells have enhanced PI staining. Scale bar, 10 μm.

of receptor expression are intriguing, the roles of these multiple receptor isoforms are unclear.

EcR expression and its relationship to the patterns of ecdysteroid response in the CNS

Larval neurons

Larval neurons in *Manduca* (Fig. 11) and *Drosophila* (Fig. 8) show dramatic shifts in EcR expression and these shifts are correlated with how the neurons respond to ecdysteroids. They show very low (*Manduca*) or undetectable (*Drosophila*) levels of EcR through most of larval life and they appear to 'ignore' the ecdysteroid surges that cause the larval molting. During the last instar, though, larval neurons begin to express high levels of EcR and the next ecdysteroid surge causes them lose their larval specializations. While we see similar quantitative changes in EcR levels in both *Manduca* and *Drosophila*, we have information on isoforms only from the latter. In the fly, this early metamorphic increase is due mainly to accumulation of the EcR-B1 isoform (Fig. 6A). As metamorphosis proceeds, though, the B1 isoform is lost leaving the cells with primarily EcR-A. They then respond to the ecdysteroid surge that causes the formation of the adult with sprouting and synaptogenesis.

Although the above pattern (type I) was the one observed for most cells, larval neurons showed two other major patterns of EcR expression. The type II pattern was characterized by exceptionally high levels of EcR-A expression through metamorphosis (Robinow et al., 1993; Figs 5F, 6B). In *Manduca*, a subset of abdominal neurons likewise show high levels of ponasterone A binding at the end of metamorphosis (Fahrbach and Truman, 1989). In both species, the cells expressing high EcR undergo programmed death after the emergence of the adult, a fate that is dependent on the withdrawal of ecdysteroids (Fahrbach and Truman, 1989; Robinow et al., 1993). *Drosophila* neurons that show the type III pattern lack detectable EcR-B1 at pupariation and have only moderate levels of EcR-A. These cells are most numerous in the thoracic neuromeres, in the areas occupied by the leg motoneurons. The latter larval neurons are of interest because they do not have peripheral targets in the larva but first extend axons into the periphery at the onset of metamorphosis to innervate the forming imaginal leg muscles (C. M. Bate, unpublished data). The possibility that the leg motoneurons express only EcR-A early in metamorphosis is intriguing because they respond by axon outgrowth at the same time that the type I neurons (that had high EcR-B1) are pruning back.

Imaginal cells of the CNS

For imaginal tissues, postembryonic life is divided between

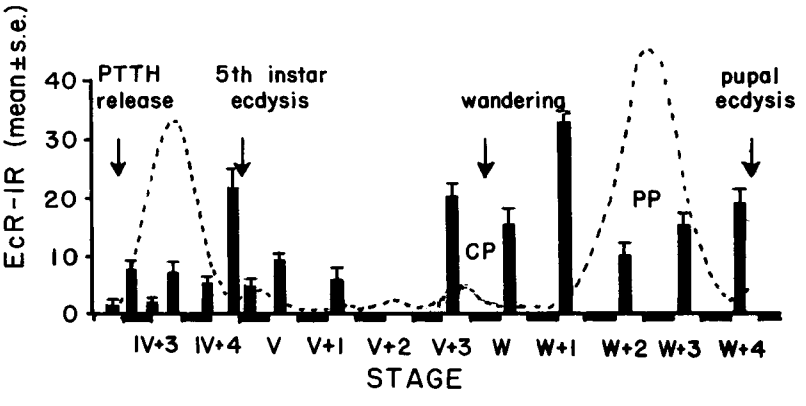


Fig. 11. Relative levels of EcR immunoreactivity in the nuclei of the abdominal D-IV motoneurons of *Manduca* during the last larval and the pupal molts. Dashed line gives the relative ecdysteroid titer. Black bars denote the dark period of each day. CP, commitment peak of ecdysteroids, PP, prepupal peak, IV, 4th larval instar, V, 5th larval instar; W, wandering stage. Values are mean + s.e. values for 10-15 neurons per point.

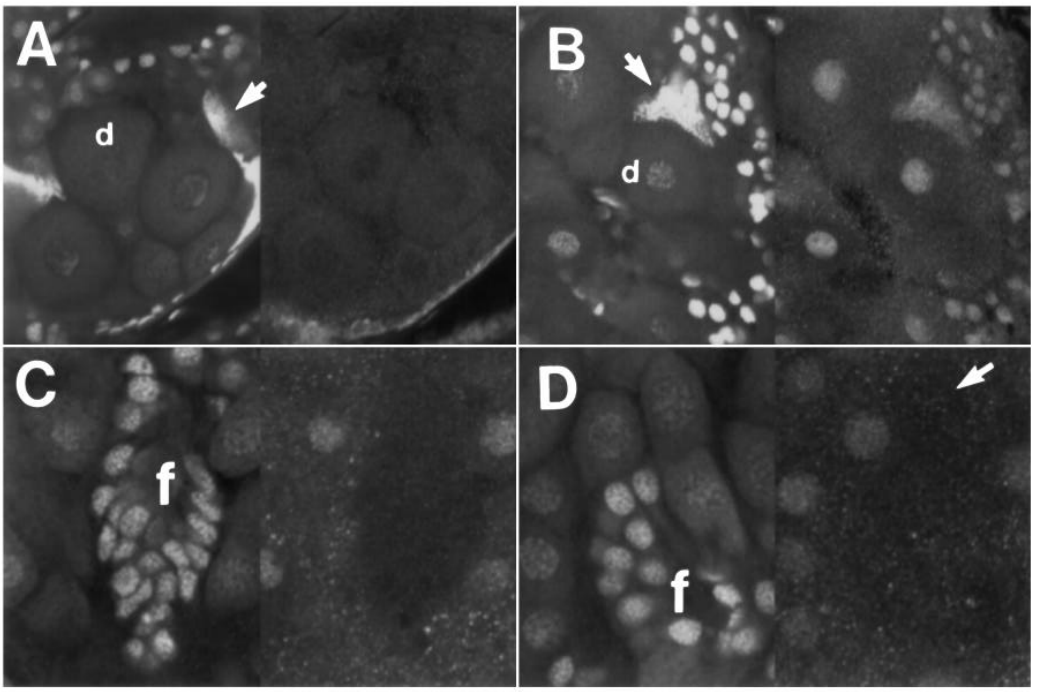
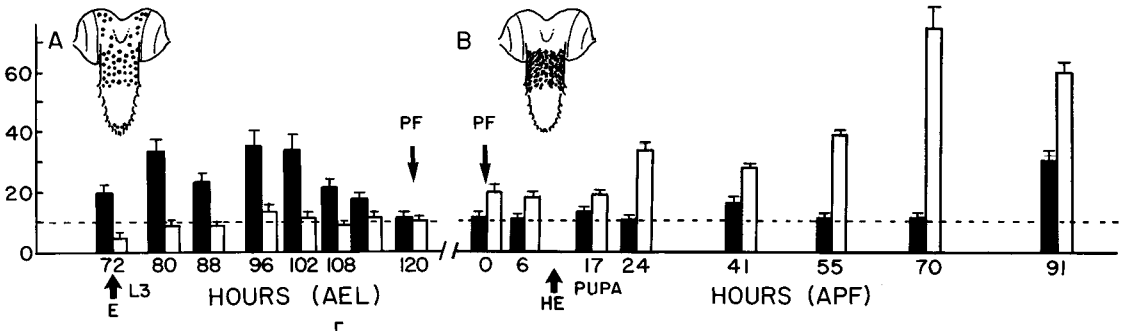


Fig. 12. Pairs of confocal images showing levels of EcR-immunoreactivity in the CNS of *Manduca*. For each pair, left = propidium iodide; right = EcR staining. (A,B) The D-IV motoneurons at: (A) the start of the molt to the 5th instar and (B) a wandering stage larva. Arrow, nucleus of giant glial cell; d, a D-IV motoneuron; small nuclei are glia associated with the perineurium. (C,D) Ventral aspect of a thoracic ganglion showing the F group of imaginal neurons (f) and surrounding larval neurons; (C) wandering stage and (D) 2 days later. Arrow in D shows a larval neuron that has lost its EcR.



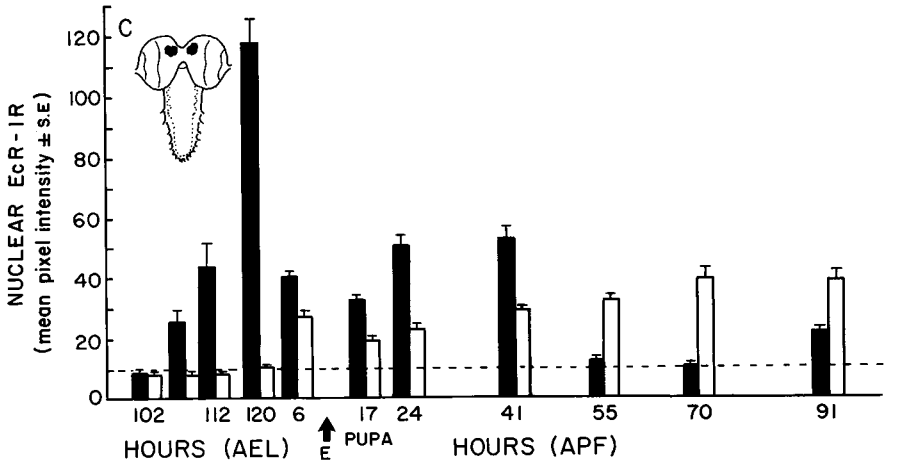


Fig. 13. Relative levels of immunofluorescence due to EcR-A (open bars) and EcR-B1 (black bars) in the nuclei of (A) the isolated neuroblasts in the central brain and thoracic ganglia, (B) the imaginal neurons in the ventral thoracic area and (C) superficial neurons of the mushroom bodies. PF, puparium formation; other abbreviations as in Fig. 6. Each value is the mean + s.e. of measurements on 10 (A) or 20 (B,C) nuclei.

proliferative and maturational periods. For the isolated NBs and OL proliferation zones, if they express any EcR, it is only the B1 isoform (Figs 4C, 13A). Likewise, the midline and peripheral glia express EcR-B1 during their proliferative phase (Figs 16, 17C,D). The latter is interesting because these glia proliferate at different times: the midline glia during larval life and the peripheral glia during early metamorphosis. Hence, the B1 isoform seems to be associated with proliferative activity irrespective of stage. The only exception that we found were the perineuropilar glia that expressed EcR-A when they were dividing (Fig. 16). Whether these cells represent a stem cell population or are already functioning glia at the time that they divide is unknown.

The early to mid-third instar peak of EcR-B1 observed in NBs (Figs 13A, 15A) and midline glia (Fig. 16) occurs during a time of low ecdysteroid titer. Studies of the rates of neuro-

genesis in cultured *Drosophila* CNSs (T. Awad and J. W. T., unpublished data) and of proliferation in *Drosophila* cell lines (Wyss, 1976; Cherbas and Cherbas, 1981; Peel and Milner, 1992) show that these low, intermolt levels of 20E (1-10 ng/ml) stimulate division. The elevated levels of EcR-B1 in these dividing cells may be important for responding to these low levels of ecdysteroid.

EcR-A was expressed when imaginal neurons started their maturational phase. For imaginal neurons in the thorax and central brain (Fig. 13B), the A isoform was the only one detected. These cells respond to the ecdysteroid peak that causes the larval-pupal transition by enlargement and enhanced expression of regulatory genes such as *Ultrabithorax* (Glicksman and Truman, 1990).

The OL and the mushroom bodies are exceptional because their imaginal neurons express high levels of EcR-B1 in addition to the moderate levels of EcR-A (Fig. 13C). The mushroom body neurons are interesting because they are the only postembryonic neurons that prune back their axons at the start of metamorphosis before they start their adult growth (Technau and Heisenberg, 1982). Their B1 expression occurs through this period of axon pruning.

The anomalous EcR-B1 expression in the OL is intriguing since these imaginal neurons are not used during larval life. It may be related to the unique development of this brain region which is coordinated with that of the compound eye. During the latter part of the 3rd instar, retinal cells differentiate in the wake of the morphogenetic furrow that moves across the eye imaginal disc. The ingrowing retinal axons then induce proliferation in the lamina, the first layer of the OL (Selleck and Steller, 1991). This gradient of axon ingrowth, which ends about 10 hours APF, thereby sets up in the lamina a corresponding gradient of differentiation that is evident through at least the next 30-40 hours (Hoffbauer and Campos-Ortega, 1990). This asynchrony in lamina development is likely also reflected in developmental gradients in the deeper layers of the OL.

EcR-B1 occurs prominently during this period of asynchronous development (10-50 hours APF; Figs 7, 15B). Since early EcR-B1 expression in other neurons is associated with regressive changes, we speculate that the presence of EcR-B1 in the OL neurons may maintain these imaginal neurons in an immature, plastic state even though they are faced with rising ecdysteroid titers that cause rapid maturation in other imaginal neurons. Such an extended period of plasticity would allow these neurons to participate in the inductive interactions that are required for establishing connections in the highly-ordered OL.

EcR-B1 at the end of metamorphosis

The reappearance of EcR-B1 in virtually all neurons at the end of metamorphosis (Figs 6A, 13B,C, 15B) suggests a novel function for this isoform as the ecdysteroid titers decline. In both beetles (Slama, 1980) and *Manduca* (Schwartz and Truman, 1983), treatment with ecdysteroids late in metamorphosis slows the rate of maturation. This inhibitory action is thought to insure developmental synchrony of tissues as metamorphosis is completed (Schwartz and Truman, 1983). Perhaps the reappearance of EcR-B1 mediates this suppressive action.

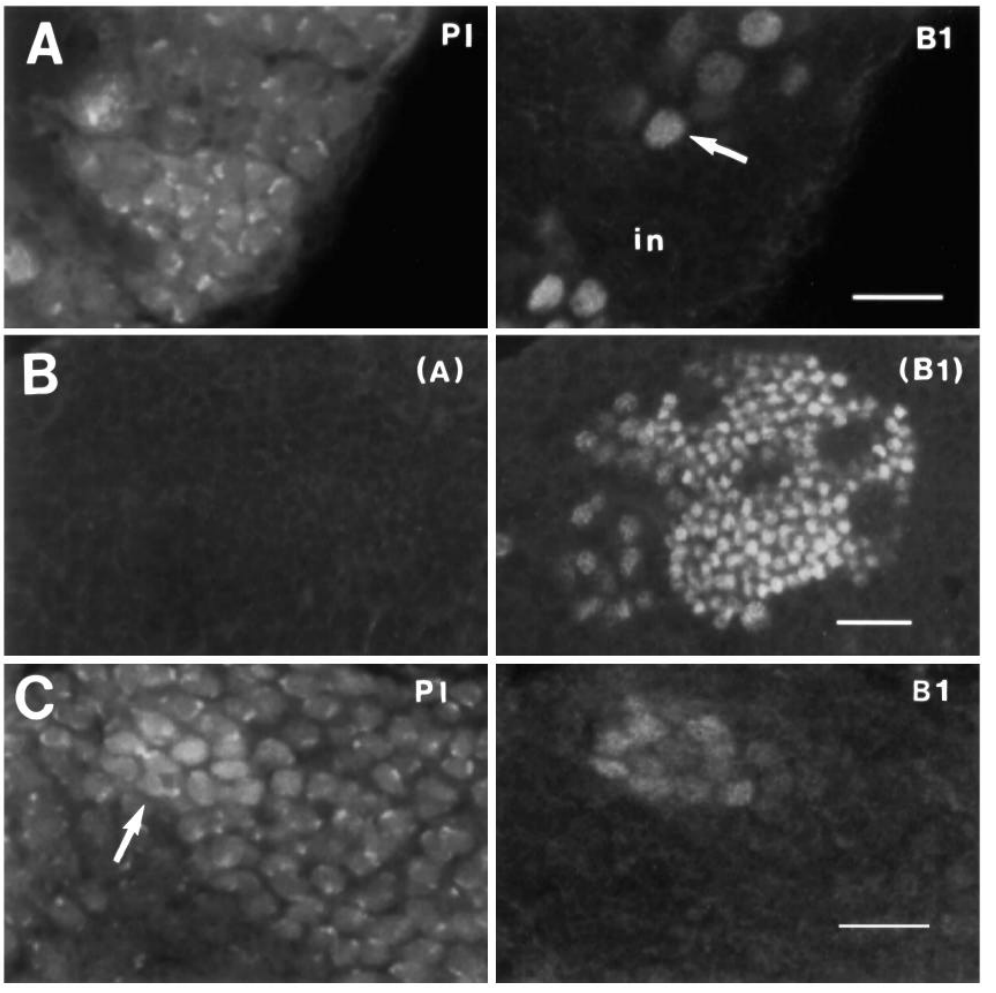


Fig. 14. Confocal optical sections showing EcR-B1 expression in imaginal neurons. (A) Lateral region of a thoracic neuromere of a 0 hour puparium stained for EcR-B1 (B1) and counterstained with propidium iodide (PI) showing that the clusters of imaginal neurons (in) are immunonegative while the surrounding larval neurons (arrow) are positive. (B) Superficial regions of the mushroom bodies of a white puparium showing cells that are EcR-B1 (B1) positive but EcR-A (A) negative. (C) Deep section through a mushroom body at 55 hours APF. Propidium iodide (PI) staining shows a tightly packed cluster of young neurons (arrow) which also stain for EcR-B1 (B1). Scale, 10 μm (A,C), 25 μm (B).

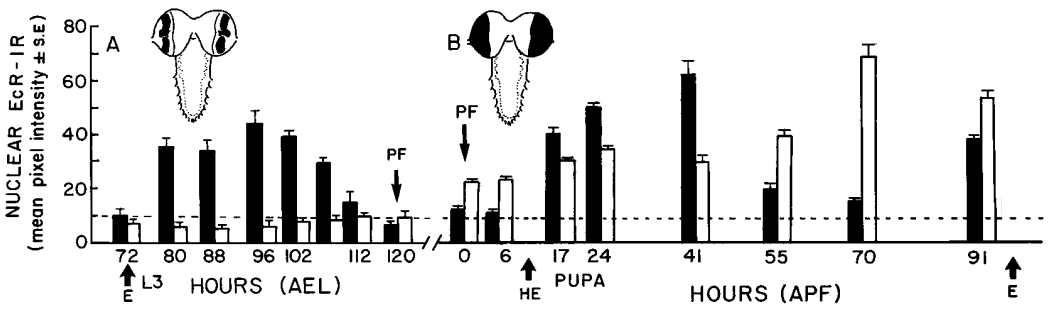


Fig. 15. Relative levels of immunofluorescence due to EcR-A (open bars) and EcR-B1 (black bars) in the nuclei of (A) stem cells in the optic lobe proliferation zone and (B) postmitotic optic lobe neurons. PF, puparium formation; other abbreviations as in Fig. 6. Each value is the mean + s.e. of measurements on 20 nuclei.

EcR isoforms and developmental switches

Talbot et al. (1993) showed that imaginal tissues reported two different patterns of EcR expression at pupariation: EcR-A was the major isoform in the imaginal discs and imaginal rings, whereas the abdominal histoblast nests and midgut imaginal islands expressed only EcR-B1. This difference was correlated with the observation that the former were entering their differentiative phase whereas the latter were preparing to begin extensive proliferation (Talbot et al., 1993). Interestingly, earlier in larval life, when imaginal disc cells are rapidly proliferating, they too express mainly EcR-B1 (J. W. T., W. S. T., and D. S. H., unpublished data). Hence, the association of EcR-B1 with active proliferation is a characteristic of peripheral tissues as well as the CNS.

The expression of EcR-B1, though, is not confined to dividing cells. It is expressed at characteristic times in numerous postmitotic cells as well: in larval neurons and mushroom body neurons when they are showing regressive changes and in OL neurons during their period of asynchronous development. It also reappears in all neurons at the end of metamorphosis when ecdysteroids act to suppress the rate of development. These steroid responses seen when EcR-B1 is present are in marked contrast to those seen when EcR-A is present alone: the maturation of imaginal neurons and, possibly, the type III larval neurons during the larval-pupal transition and the maturation of type I larval cells during the pupal-adult transformation.

These correlations, while being consistent with the hypothesis that EcR-A and EcR-B1 each oversees its own unique set of cellular responses, do not, of course, prove its validity. Recent mutational analyses of the *Drosophila EcR* gene have, however, provided evidence that the EcR isoforms are functionally distinct. For example, the non-pupariating lethality of B1-specific mutations can be rescued by the ubiquitous expression of EcR-B1 from heat-shock transgenes, but not by the expression of equivalent EcR-A or EcR-B2 constructs (M. T. Bender, W. S. T. and D. S. H., unpublished). These results argue that there are some functions that are unique to EcR-B1 and cannot be fulfilled by the other receptor isoforms.

The recent findings noted in the Introduction that EcR isoforms act as heterodimers with USP could clearly affect the functional interpretation of the EcR isoform expression patterns. Preliminary results examining the distribution of USP protein in the CNS of *Drosophila* suggests that USP expression generally overlaps the expression of the EcR isoforms (J. W. T., D. S. King, F. C. Kafatos, unpublished). This suggests that most, if not all, of the temporal and spatial variation in the active receptor complex is due to variations in EcR. The other possible complication, of course, is the unknown role and distribution of EcR-B2.

Shifts in receptor isoforms as cells move from a proliferative to a differentiative state are also seen in *Xenopus* during the androgen-induced growth of the larynx muscle. This muscle expresses mRNAs for two androgen receptors (AR) isoforms, a constitutive form (AR_α) and a regulated form (AR_β). The mRNA for AR_β is temporally and spatially corre-

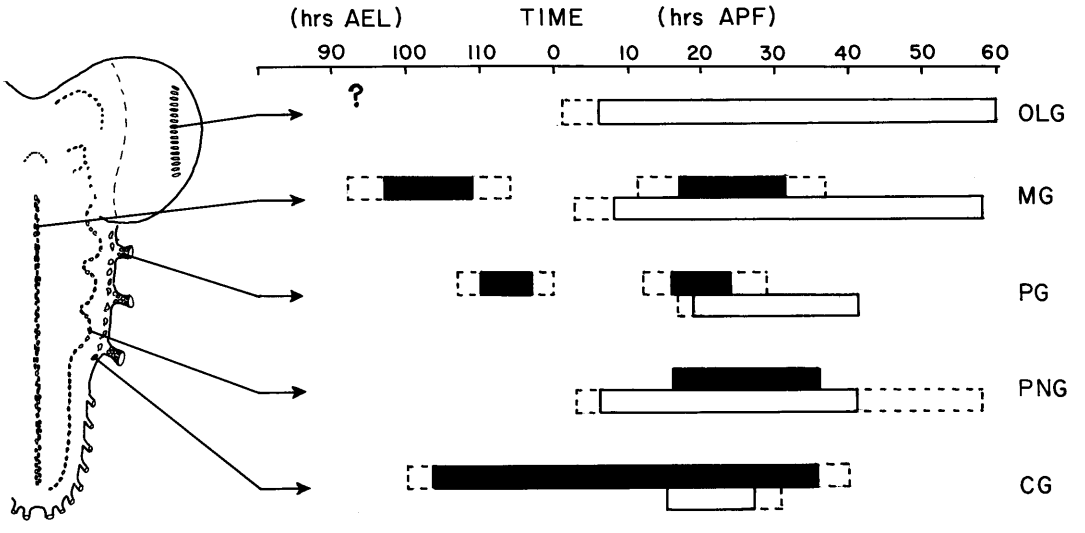


Fig. 16. Qualitative summary of expression of EcR isoforms in glial cells in *Drosophila* through late larval life and the first half of metamorphosis. Black bars, expression of EcR-B1; white bars, expression of EcR-A. Dashed boxes indicate periods of weak expression. CG, cortical glia; MG, midline glia; OLG, optic lobe glia; PG, peripheral glia; PNG, perineuropilar glia.

lated with hormone-induced proliferation, but once the cells begin to differentiate the mRNA for this receptor form disappears. (Fischer et al., 1993).

Regulation of isoform expression

What is responsible for the complex spatial and temporal fluctuations that are seen in EcR? In considering this question, it is important to realize that the A and B1 isoforms are encoded by overlapping transcription units that have different promotors and can be separately controlled (Talbot et al., 1993). By contrast, the B1 and B2 isoforms are encoded by mRNAs that derive from the *EcR-B* primary transcript by alternate splicing.

For many insects, juvenile hormone (JH) may be a key player in regulating EcR expression. In *Manduca* the enhanced neuronal expression of EcR during the last instar (Fig. 11) occurs after a normal decline in JH and can be prevented by treatment with JH mimics (M. Renucci and J. W. T., unpublished). More intriguing is the possible role of JH in selecting the types of isoforms that are present. Our data on larval neurons in *Drosophila* clearly show that stage-specific patterns of ecdysteroid response in these cells are correlated with unique patterns of isoform expression. Since the classic function of JH is in controlling such stage-specific responses (Riddiford, 1993), it is possible that a principle mode of action of JH is through controlling EcR isoform expression. This hypothesis cannot be tested at present because the JH responses of *Drosophila* are poor and multiple EcR isoforms have not yet been found in *Manduca*.

Another factor involved in the regulation of *EcR* appears to be the ecdysteroids themselves. For example, EcR mRNA is rapidly induced in isolated larval organs by low concentrations of 20E (Karim and Thummel, 1992). This action of ecdysteroid is likely responsible for the appearance of EcR-B1 protein in larval neurons during the mid-third instar (Fig. 6A).

It must be cautioned though that the relationship of EcR expression to the ecdysteroid titer cannot be a simple one. For example, late in larval life, when EcR-B1 is being induced in some cells, it is disappearing from others (Fig. 13A). Moreover, in cells that show the EcR-B1 induction, the timing of it varies: it appears in larval neurons in the middle of the third instar, in mushroom body neurons at the onset of the large pupariation peak, and in the OL during the small ecdysteroid peak at 10 hours APF. Moreover, in some cells EcR induction is not correlated with any specific steroid peak: for the mushroom body neurons that are born during metamorphosis, their EcR isoform expression appears locked to their developmental time table rather than to the overall state of the animal or the ecdysteroid titers.

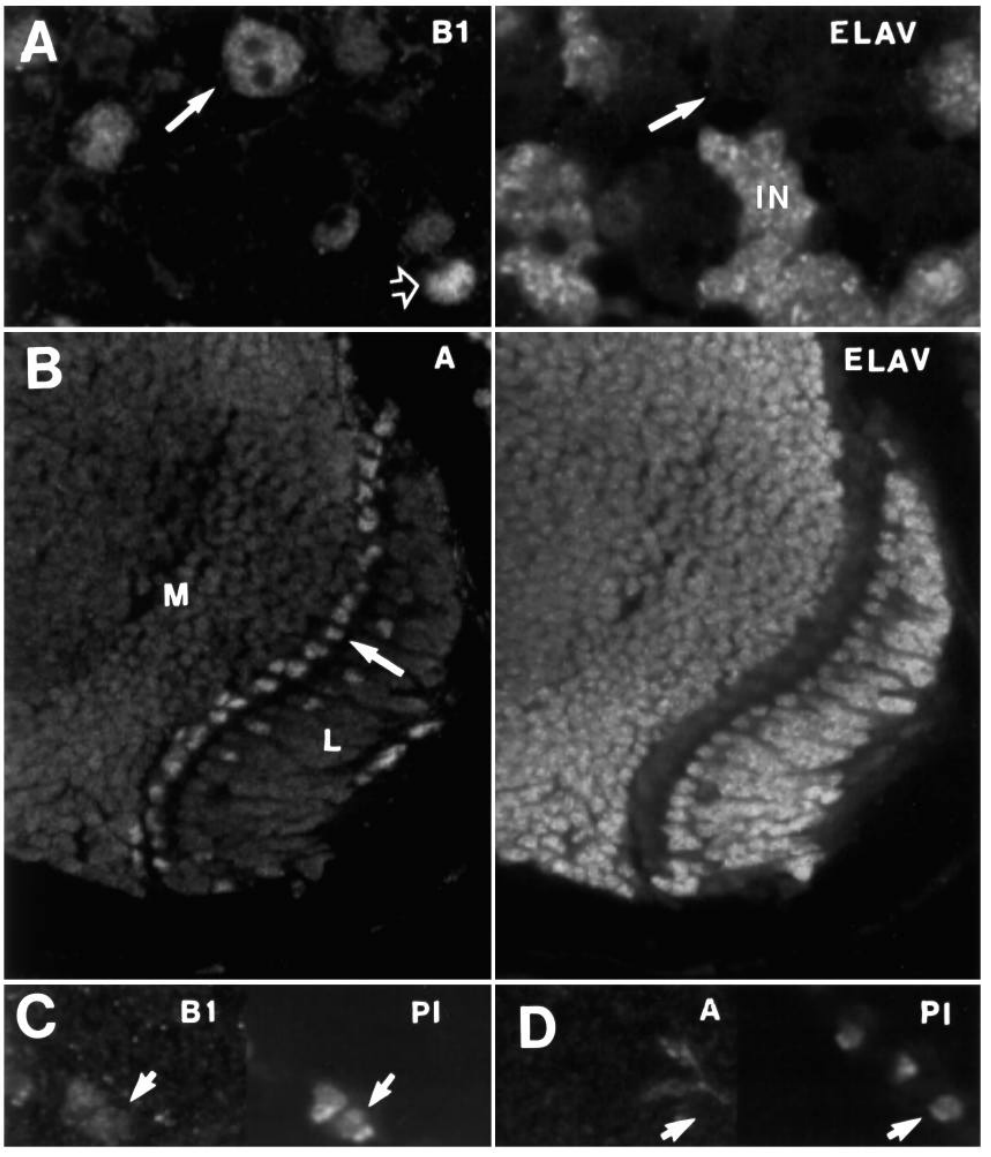


Fig. 17. Confocal optical sections showing glial expression of EcR isoforms in *Drosophila*. (A) High-magnification image of the ventral nervous system of a white puparium stage stained with EcR-B1 (B1) and ELAV (ELAV) showing the large nuclei of cortical glia (arrow) which are EcR-B1 positive but which lack ELAV; (open arrow = type I neuron; IN, tightly packed nuclei of imaginal neurons that are ELAV positive but B1 negative. (B) Double-stained preparation of the optic lobes at 18 hours APF showing strong EcR-A staining (A) in a band of glia (arrow) that are negative for ELAV (ELAV). L, lamina; M, medulla. (C,D) High-magnification image of midline glia from 3rd instar larvae (104 hours AEL). Their nuclei (arrow) are positive for EcR-B1 (B1) but negative for EcR-A (A). Each is presented as a pair with EcR staining and propidium iodide (PI) counterstain. For each pair, the arrows are at comparable positions.

The diverse regulation of *EcR* results in highly individualized patterns of receptor expression at the start of metamorphosis. This initial receptor heterogeneity presumably reflects the diverse origins and requirements of the neurons that nevertheless are all exposed to a common soup of circulating hormones. By the end of metamorphosis, this complex ensemble of cells has been melded into a unified structure in which all neurons are involved in similar processes associated with the final maturation of their synaptic connections. The uniformity in EcR expression during this late period presumably reflects this convergence of developmental programs.

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