Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform

Steven Robinow[1],*, William S. Talbot[2],†, David S. Hogness[2] and James W. Truman[1]

[1]Department of Zoology NJ-15, University of Washington, Seattle, WA 98195, USA
[2]Department of Biochemistry and Department of Developmental Biology, Stanford University Medical Center, Stanford, CA. 94305, USA

*Author for correspondence
†Present address: Institute of Neuroscience, University of Oregon, Eugene, OR. 97403, USA

SUMMARY

At adult emergence, the ventral CNS of *Drosophila* shows a group of approximately 300 neurons, which are unique in that they express 10-fold higher levels of the A isoform of the ecdysone receptor (EcR-A) than do other central neurons. This expression pattern is established early in metamorphosis and persists throughout the remainder of the pupal stage. Although these cells represent a heterogeneous group of neurons, they all share the same fate of undergoing rapid degeneration after the adult emerges from the pupal case. One prerequisite for this death is the decline of ecdysteroids at the end of metamorphosis. Treatment of flies with 20-hydroxyecdysone blocks the death of the cells, but only if given at least 3 hours before the normal time of degeneration. The correlation of a unique pattern of receptor isoform expression with a particular steroid-regulated fate suggests that variations in the pattern of receptor isoform expression may serve as important switches during development.

Key words: neuronal development, hormonal regulation, neurobiology, ecdysteroid, 20-hydroxyecdysone, neuronal death

INTRODUCTION

Steroid hormones, thyroid hormones and retinoids are involved in the coordination of complex developmental processes, and are themselves subject to developmental regulation. Although cells within a tissue may be exposed to identical hormonal environments, they can, nevertheless, adopt different fates, which are dependent upon this hormonal exposure. From this apparent contradiction, it is clear that the history of hormonal exposure alone does not control cell fate and that the complexity of hormonally regulated developmental processes is not fully reflected by the fluctuating hormonal titers. This complexity must therefore be integrated into other aspects of the hormonally regulated responses. Since these hormones mediate their genomic actions through nuclear hormone receptors that act as ligand-regulated transcription factors, it is possible that differential regulation of receptor expression may provide a mechanism by which similar cells respond differentially to the same hormonal cues.

These receptors belong to the steroid receptor superfamily whose members are characterized by a number of features including a variable amino-terminal region, a highly conserved DNA-binding domain containing two zinc fingers, and a less-well-conserved hormone (ligand)-binding domain (reviewed in Evans, 1988; Green and Chambon,

1988; Beato, 1989). Receptors for a given hormone are often represented by multiple forms that display different temporal and spatial patterns of expression, as is the case for the four thyroid hormone receptor isoforms of rat (Wills et al., 1991; Cook et al., 1992; Bradley et al., 1992; Jannini et al., 1992; reviewed in Chin, 1991), and the multiple forms of the mammalian retinoic acid receptor (reviewed in Chambon et al., 1991; and in Mangelsdorf and Evans, 1992; Ruberte et al., 1993). While the developmental implications of this complexity are undoubtedly significant, they are, nonetheless, poorly understood.

Holometabolous insects offer a simpler system in which to study the hormonal regulation of development. The ecdysteroids, a class of polyhydroxylated steroids, play a major role in virtually all aspects of their development, including the reorganization of the central nervous system (CNS) during metamorphosis. Appropriately, the ecdysteroid-dependent responses within the CNS are heterogeneous; for example, adult-specific neurons that had arrested their development shortly after their postembryonic birth undergo maturation. By contrast, most embryonically derived neurons that functioned in the larva are remodeled and are incorporated into the forming adult nervous system, while others degenerate (Truman, 1988; Weeks and Levine, 1990). Interestingly, at least some of the neurons that are fated to die appear to contain higher levels of an ecdysone

receptor than those that survive (Fahrbach and Truman, 1989). Could quantitative differences in receptor levels control the hormonally regulated aspects of this developmental decision?

To address this question, we have focused on the ecdysone receptors that are encoded by the *Drosophila EcR* gene (Koelle et al., 1991). Three of these receptors have been identified and characterized. The isoforms EcR-A, EcR-B1 and EcR-B2 (Talbot et al., 1993) share common DNA and ligand-binding domains and are distinguished by their different amino-terminal regions. Using a panel of anti-EcR monoclonal antibodies that includes those specific for the EcR-A and EcR-B1 isoforms, Talbot et al. (1993) showed that these isoforms display distinctive patterns of tissue expression at the onset of metamorphosis (i.e., at pupariation). They also observed, however, that tissues belonging to a given metamorphic class (e.g., the imaginal discs) exhibit strikingly similar expression patterns, suggesting that particular EcR isoforms, or combinations thereof, are required for a particular ecdysone response.

In this paper, we use the panel of anti-EcR antibodies to examine the expression pattern of the EcR isoforms in the CNS during its reorganization within the pupa and shortly after emergence. In particular, we focus on a unique pattern of EcR-A expression and show that it is highly correlated with a unique developmental fate, i.e., degeneration after the emergence of the adult.

MATERIALS AND METHODS

Drosophila stocks

All experiments described here have used the Canton-S wild-type strain of *Drosophila melanogaster*. Animals were raised at room temperature on standard agar/cornmeal/yeast/molasses medium unless otherwise specified.

Antibodies

Most of the immunocytochemistry was performed using the monoclonal antibody 15G1a, which was raised against a fusion peptide containing the EcR-A-specific amino-terminus of the ecdysone receptor (Talbot et al., 1993). Identical staining patterns were also obtained using three other EcR-A-specific monoclonal antibodies and also a rabbit polyclonal antiserum raised against the EcR-A-specific fusion peptide.

Antibodies specific to the common regions of EcR included a rabbit polyclonal anti-EcR (Koelle et al., 1991) and the monoclonal antibodies DDA2.7; IID9.6; AC12.4 (Talbot et al., 1993). mAb AD4.4 is specific for the EcR-B1 isoform (Talbot et al., 1993).

Immunohistochemistry

Ventral nervous systems were dissected from staged or treated animals in ice-cold Ca^{2+}-free Ikeda's Ringer solution (Budnik et al., 1989). Tissue was fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline pH 7.0 (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4). After rinsing three times in PBS/TX (PBS+0.3% Triton X-100) for a total of 30 minutes, samples were incubated with the monoclonal anti-EcR-A antibody designated mAb15G1a (1:20 in PBS/TX), unless otherwise specified, for a minimum of 6 hours at room temperature or overnight at 4°C. After repeated washes, samples were incubated in biotinylated anti-mouse IgG for a minimum of 2.5 hours at room temperature. Samples were washed again in PBS/TX as above prior to incubation with either an avidin-peroxidase complex (ABC kit;

Vector Laboratories) or FITC-avidin (Vector Laboratories) for a minimum of 2.5 hours at room temperature. Samples were rinsed in PBS/TX and then in PBS. After rinsing, samples incubated in the avidin-peroxidase reagent were further incubated for 15 minutes in PBS+0.5 mg/ml diaminobenzidine (DAB), and then transferred to a fresh solution of DAB containing 0.003% H_2O_2. The reaction was stopped by transferring the tissue to 30% ethanol. After mounting on a poly-lysine-coated coverslip, the samples were dehydrated and mounted in DPX (Fluka). Samples incubated in FITC-avidin were stained with propidium iodide (4 ug/ml) for 2-4 minutes and mounted in 80% glycerol, 20% PBS and 2% (w/v) propyl gallate prior to viewing on a BioRad MRC600 confocal microscope.

Tissue, which was double labeled for both EcR-A and the ELAV protein, was dissected and fixed as described above. Tissue was incubated in rat anti-ELAV affinity-purified antibodies (Robinow and White, 1991) for 6 hours at room temperature, washed in PBS/TX, incubated in tetramethylrhodamine-conjugated sheep anti-rat IgG (Sigma) overnight at 4°C, washed again and incubated in polyclonal rabbit anti-EcR-A (1:500). After washing, the tissue was processed as described above using a biotinylated goat anti-rabbit IgG, followed by FITC-conjugated avidin. Alternatively, after incubation in anti-ELAV antibodies, tissue was sequentially treated with the following reagents with washes of PBS/TX between reagents as described above: FITC anti-rat IgG; 1% rat serum; mAb15G1a (1:20) in 1% rat serum; biotinylated anti-mouse IgG pre-incubated with 2% rat serum; Texas Red-avidin.

20-HE injections

At half hour intervals after eclosion, animals were anesthetized on ice and injected with approximately 0.1 μl of a 3 μg/ml solution of 20-hydroxyecdysone (Sigma) in Ca^{2+}-free Ringer's solution (Budnik et al., 1989). The apparatus used for injection was driven with air pressure and did not deliver measured volumes. All animals were slightly distended by the injection to approximately the same degree. We used a system to code samples without the knowledge of the investigator so as not to bias our scoring of these samples.

RESULTS

EcR isoform expression at adult emergence

At the completion of metamorphosis, cells in the ventral CNS showed heterogeneous expression of ecdysone receptors (Fig. 1). Most nuclei showed a moderate level of receptors, but a scattered collection of cells showed enhanced levels. This pattern was seen using four monoclonal antibodies (mAbs) and a polyclonal antiserum all of which were specific for the unique amino-terminal region of the EcR-A protein. It was also seen using a polyclonal antiserum and three mAbs that recognized various epitopes that were common to all EcR isoforms. By contrast, a mAb that recognized an EcR-B1-specific epitope failed to show this heterogeneity in the ventral nervous system. Consequently, the enhanced expression of EcR in the scattered cells appears to be due to a higher abundance of EcR-A in these cells.

An average of 284 cells in the ventral ganglia showed high levels of EcR-A immunoreactivity. The majority of these cells were situated in the abdominal and third thoracic (T3) neuromeres with only about 24 cells in neuromeres T1 and T2. Comparison of the EcR-A immunoreactivity in the thoracic neuromeres from a number of individuals showed
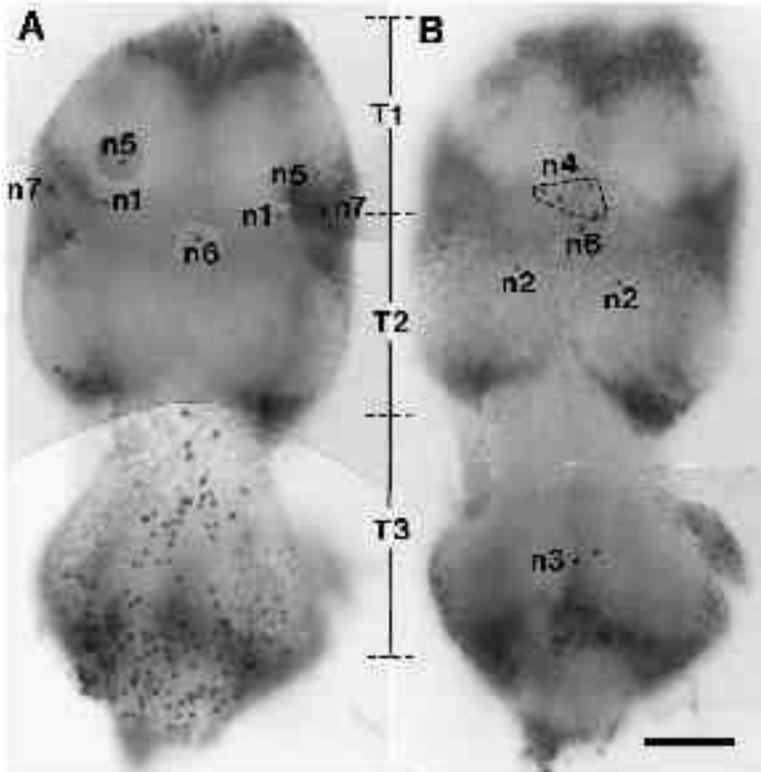
Fig. 1. EcR-A is expressed heterogeneously in the ventral nervous system of newly eclosed adults. (A) A montage of the dorsal view of an immunoperoxidase-stained whole mount of the ventral CNS showing the EcR-A immunoreactive cells at the time of adult emergence. (B) Ventral view as in A. Cells expressing high levels of EcR-A, which can be reliably identified based on their position within the cortex, have been given an alphanumerical designation. T1, T2 and T3 designate the approximate extents of the pro-, meso-, and meta- thoracic neuromeres. Due to the fusion of the abdominal neuromeres and the compression with T3 during metamorphosis, we have not attempted to identify specifically the abdominal neuromere, and refer to the cells of this region generally as T3/abdominal cells. Scale bar, 50 µm.

that the number and position of these cells was highly stereotyped, a feature that allowed us to give unique designations to seven sets of these cells (n1-n7; Fig. 1). The anterodorsal-most pair, n5, is located just anterior of one of the largest neurons in *Drosophila*, MN-5 (Ikeda and Koenig, 1988). The n1 pair is located posterior and ventral to n5. The n6 pair is located along the medial dorsoventral tract near the T1/T2 border. Both n6 cell bodies may be found dorsal, or both may be ventral, or one may be dorsal and the other ventral. n7 is a large cell located in posterior T1 towards the ventral surface at the cortex/neuropile boundary. The quartet of n4 cells are located at the midline, on the ventral surface near the T1/T2 border. Posterior and lateral to the n4 cluster is the n2 pair.

Presumably, the cells that express high levels of EcR-A in the T3 and abdominal neuromeres also represent a set of uniquely identifiable cells, but the compressed nature of this region of the nervous system made discrimination by position alone difficult. However, we were able to identify a single pair, n3, which is located ventromedially in an anterior position.

To quantify the difference in EcR-A protein expression in the two types of cells, we used an FITC detection system and measured the mean levels of immunofluorescence in individual nuclei using a confocal microscope. The nuclei of the cells expressing high levels of EcR-A showed an average pixel intensity of 134 ± 19 ($n=13$) as compared to a value of 12 ± 4 ($n=29$) for the other cells, yielding a ratio of about 10:1 for the average abundance of the EcR-A isoform in the two cell populations.

The cortical position and nuclear morphology of these cells was consistent with them being neurons. This suppo-

sition was tested by double labeling ventral ganglia with antibodies against EcR-A and ELAV, a protein expressed exclusively in neurons of *Drosophila* (Robinow and White, 1991). All of the cells that expressed high levels of EcR-A co-expressed ELAV, as shown for the n4 neurons in Fig. 2, confirming that they are indeed neurons.

EcR-A expression during metamorphosis

The difference in levels of EcR-A expression that is evident at adult emergence is established during the initial stages of adult differentiation. Fig. 3 shows that neurons first begin to diverge in their EcR content by 12 hours after puparium formation (APF), when approximately 29 neurons showed elevated levels of EcR-A. These early cells included the n5 and n1 neurons in T1 and T2, a set of ventromedial neurons repeated in abdominal segments A2 to A6, and six lateral neurons at the thoracic/abdominal boundary. The number of neurons hyperexpressing EcR-A increased to about 95 by 18 hours. The n4 cells were now evident, as were a pair of neurons located in the midline glial investiture, which are likely to be the n6 neurons. The T2 and T3 neuromeres showed cells that are segmental homologues of n5 and n1 based on their size and position. By 28 hours, the number of neurons expressing high levels of EcR-A reached about 240, primarily through additional abdominal neurons increasing their expression of EcR-A. Interestingly, the n6 cells no longer expressed high levels of EcR-A and were indistinguishable from the majority of neurons, which showed low, basal levels of EcR-A protein. By 42 hours, the number of neurons showing elevated levels of EcR-A plateaued at approximately 300, a number that was then maintained through the rest of metamorphosis (Fig. 3). The
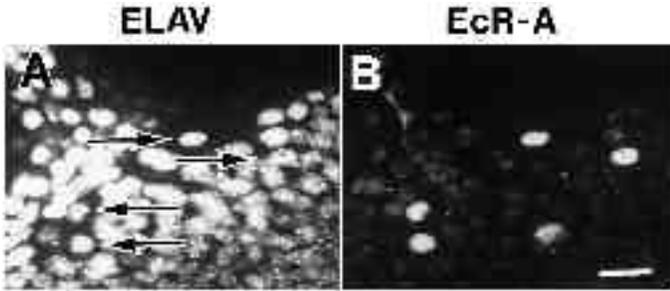
ELAV   **EcR-A**

Fig. 2. The cells that express high levels of EcR-A are also ELAV positive. An optical section through a ventral nervous system of a newly eclosed adult that was immunostained for both ELAV (A) and EcR-A (B) and analyzed using the confocal microscope. The n4 cells were examined to determine whether they co-express ELAV. The four ELAV-positive nuclei in A (arrows) are those of the EcR-A immunoreactive n4 cells evident in B. The fifth cell that displays some EcR-A immunoreactivity is a glancing section through an n6 neuron. Scale bar, 10 µm.

n7 neurons only began to show high levels of EcR-A between 79 and 89 hours APF. The n6 neurons resumed expression of high levels of EcR-A during this same period.

With the exception of the n6 cells, once an identified neuron established elevated levels of EcR-A, these levels were maintained through the remainder of metamorphosis. Compression and distortion within the abdominal and T3 neuromeres through metamorphosis made it impossible to keep track of individual cells, but the constant pattern and number of expressing cells in these regions (Fig. 3) suggests

that this sustained high expression of EcR-A was a universal feature of these neurons.

Thus, there are two general patterns of EcR-A expression during metamorphosis. Most neurons expressed low levels of EcR-A throughout adult development and have been designated as type I neurons. By contrast, approximately 300 neurons express high levels of EcR-A protein starting early in the pupal stage and they sustain these levels for the remainder of metamorphosis (Figs 1, 3). These have been designated as type II neurons. A few neurons conformed to neither pattern; the notable examples being the n6 and n7 neurons.

Fate of the type II neurons

The distribution of type II neurons is similar to the distribution of cells that die within the first 24 hours after adult eclosion (Kimura and Truman, 1990). Also, type II cells were not evident in the nervous systems from 24 hours old flies. To determine if type II cells did indeed die, we examined the nervous systems from flies at various intervals after adult emergence. Fig. 4 shows that during the first 24 hours after eclosion, there is a dramatic reduction in the number of neurons expressing high levels of EcR-A. Fig. 5 follows the fate of the four neurons of the n4 group. During the first 2 hours after eclosion, the nuclei of these cells had a normal appearance with dispersed chromatin throughout. By 4 hours, however, their DNA had condensed and appeared as a densely stained sphere at the margin of the nucleus. Interestingly, EcR-A staining appears to be largely excluded from this body. At 6 hours, some n4 cells had already disappeared and the ones that remained showed
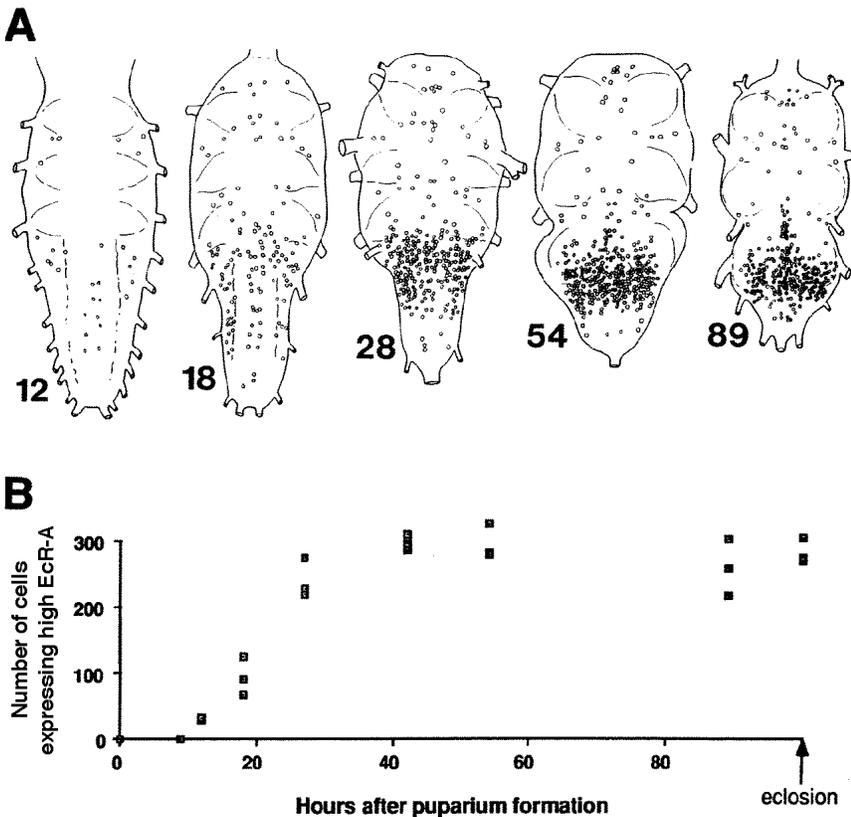

Fig. 3. Time course of appearance of neurons expressing high levels of EcR-A immunoreactivity through metamorphosis as estimated from immunoperoxidase-stained ventral nervous systems as in Fig. 1. (A) Camera-lucida drawings of ventral nervous systems showing the appearance and distribution of neurons that express high levels of EcR-A through metamorphosis. Numbers indicate hours after puparium formation. (B) Counts of total numbers of cells in the ventral CNS expressing high levels of EcR-A during adult development. Each symbol represents counts from a single CNS.
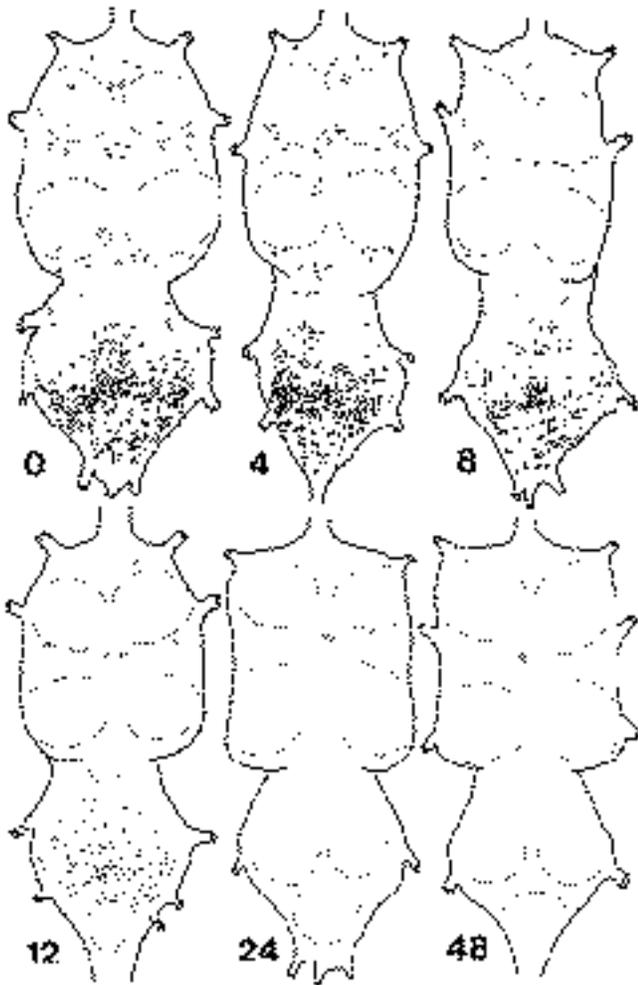
Fig. 4. Loss of the type II neurons during the first 48 hours after adult emergence. Camera-lucida drawings of immunoperoxidase-stained nervous systems showing the decline in the number of neurons that express high levels of EcR-A after adult emergence. Numbers indicate hours after eclosion.

cytoplasmic degeneration as well as nuclear shrinkage. By 8 hours, we could no longer detect EcR-A immunoreactivity typical of type II cells in the region appropriate for the n4 cluster. With the exception of the n6 and n7 neurons, all cells expressing high levels of EcR-A at the time of adult emergence showed this pattern of degeneration.

The n6 and n7 neurons are interesting in that they expressed high levels of EcR-A but did not die. The n6 cells continued to express high levels of EcR-A through the latest day that we examined (7 days after emergence). The n7 pair, on the other hand, down-regulated EcR-A expression during the first day and a half of adult life, so that by approximately 36 hours after emergence the EcR-A expression in these cells was indistinguishable from type I neurons. During this 36 hour period, while they were still identifiable based on their elevated levels of EcR-A, the n7 neurons appeared healthy. Their ultimate fate is unknown, but their persistence well beyond the known period of cell death suggests that they survive through adult life.

20-hydroxyecdysone (20-HE) delays the death of the type II cells

By the time of adult emergence, circulating ecdysteroid titers have dropped to low levels (Richards, 1981; Bainbridge and Bownes, 1988), yet the doomed neurons continued to express high levels of EcR-A. In *Manduca*, exogenous application of ecdysteroids at this time prevents the death of most of the doomed neurons (Schwartz and Truman, 1983). We have tested whether the death of the type II neurons in *Drosophila* is sensitive to ecdysteroid titers at eclosion by injecting newly eclosed adults with 20-HE, which is considered to be the active ecdysteroid in *Drosophila*. In animals treated with 20-HE, all of the identifiable type II neurons were still evident at 12 hours, whereas in control animals, these cells had all degenerated (Fig. 6). The n6 and n7 neurons, which do not die, were identifiable in both 20-HE-treated and control animals (Fig. 6). The sparing of the type II neurons caused by the 20-HE treatment was, however, only temporary. By 24 hours after steroid treatment, these cells had finally degenerated.

To determine the time at which death could no longer be affected by ecdysteroids, 20-HE was injected at various times after eclosion and the ventral nervous system was assessed for EcR-A expression 12 hours post-emergence. Fig. 7 shows the response of the n4 neurons to such treatments. The commitment point for the n4 neurons, the time when 50% of these neurons became ecdysteroid independent, was approximately 1.25 hours after eclosion. The commitment point for the other identified type II neurons was in a narrow range from 30 minutes to about 1.75 hours post-emergence.

DISCUSSION

Many holometabolous insects such as *Drosophila* (Kimura and Truman, 1990) and *Manduca* (Truman, 1983) display a bout of neuronal death soon after the emergence of the adult. The cells that die are larval neurons that persist through metamorphosis to be used for the final time during adult emergence. In *Manduca*, many of the doomed neurons have been identified as motoneurons (Levine and Truman, 1985) that innervate larval muscles that degenerate after eclosion (Schwartz and Truman, 1983). Postmetamorphic death of larval muscles also occurs in *Drosophila* (Miller, 1950; Kimura and Truman, 1990), and is accompanied by death of central neurons (Kimura and Truman, 1990; this paper). Although the identity of these neurons has not been determined, we presume that they include the motoneurons that innervate the doomed muscles. Indeed, backfilling of abdominal motoneurons from cut nerve roots and subsequent processing for EcR-A immunoreactivity has demonstrated that some of the type II cells are, in fact, motoneurons (M. Schubiger, personal communication).

A key factor that regulates the death of the doomed neurons is the changing titer of ecdysteroids. In *Drosophila*, this titer increases at the beginning of the pupal stage with kinetics that are similar to the rise in the number of type II neurons shown in Fig. 3 (Handler, 1982; Bainbridge and Bownes, 1988). While the type II cell number plateaus at
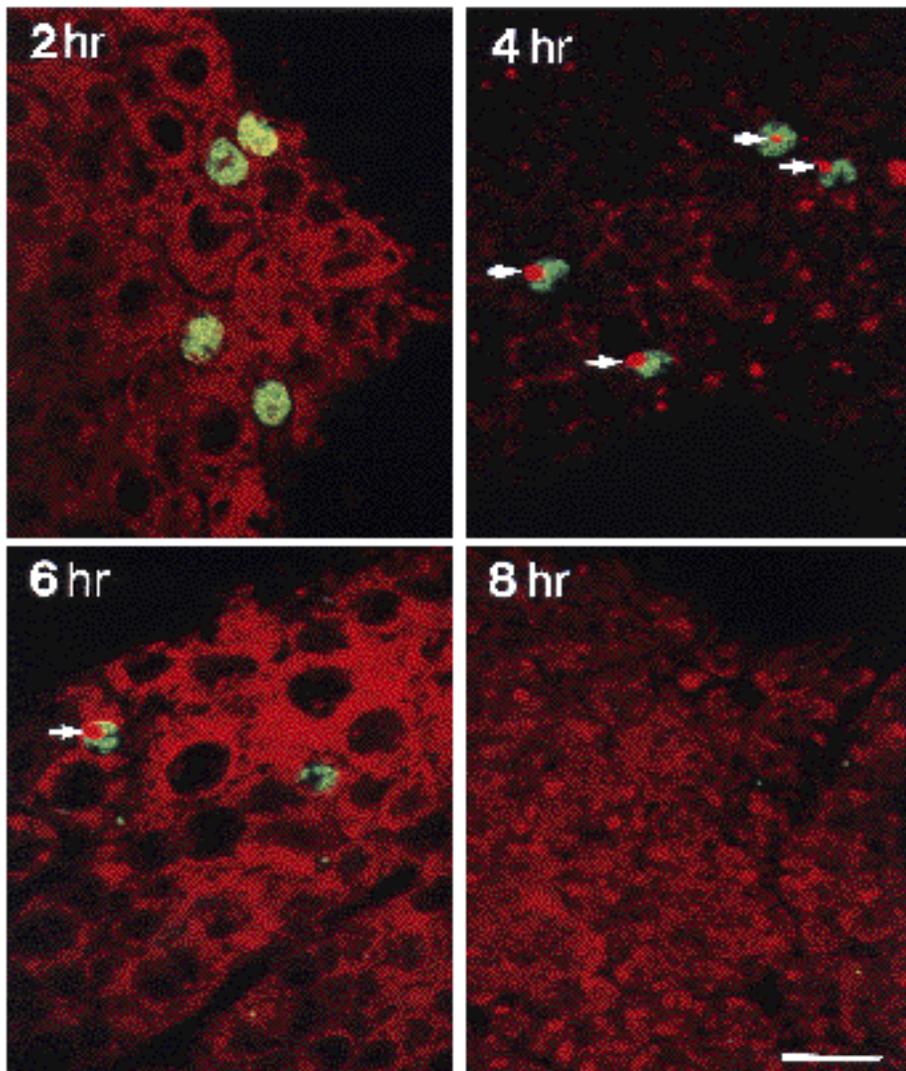
Fig. 5. Merged confocal images showing the degeneration of identified type II neurons. The n4 neurons are shown at various times after adult emergence. Red represents propidium iodide staining of nucleic acids; green is EcR-A immunoreactivity. (2 hr) 2 hours after eclosion showing EcR-A immunoreactivity distributed throughout the nuclei of the n4 cells. (4 hr) At 4 hours after eclosion, the nucleic acid appears as a condensed ball (arrows) which excludes the EcR-A immunostaining which is still nuclear. (6 hr) At 6 hours, only 2 of the n4 cells are still evident and are identified by their condensed nucleic acid and fading EcR-A immunoreactivity. (8 hr) By 8 hours, no remains of the n4 cells are evident.

~40 hours APF, however, the ecdysteroid titer begins to decrease at about this time, dropping to quite low levels by eclosion. It is this withdrawal of the hormone that is the proximal event required for cell death and degeneration (Figs 6, 7). This is also the case in *Manduca* (Truman and Schwartz, 1984).

Since all central neurons are presumed to be exposed to the same change in titer of the circulating hormone, why do some cells die while their neighbors do not? The two most striking characteristics of the doomed neurons revealed in the present study are that they exhibit approximately 10-fold higher EcR levels than do other central neurons, and that this elevated expression is due to a single receptor isoform, EcR-A. This result is consistent with autoradiographic studies of the binding of radiolabeled ecdysteroids in *Manduca*, which showed that doomed neurons had greater steroid-binding capacity than did other neurons (Fahrbach and Truman, 1989). The potential importance of the finding that doomed neurons exhibit a high abundance of EcR-A is highlighted by the observation that the pupal-adult molt represents the only stage in the life of these cells when EcR-A is the predominant isoform (J. W. T., W. S. T., and D. S. H., unpub-

lished data). This temporal specificity of EcR-A expression in the doomed neurons, which are embryonic in origin, would also provide an explanation for why these cells do not die after the ecdysteroid pulses that mark the molts at earlier stages of development (Richards, 1981).

Another important question is why do the doomed neurons not die during the last hours of the pupal stage, just before adult emergence, since ecdysteroid titers have dropped to quite low levels by this time (Handler, 1982; Bainbridge and Bownes, 1988). A simple answer would be that these titers are still not low enough to trigger cell death, and that adult emergence is coupled with a further decrease in titer and, indeed, Handler (1982) has reported a further decrease in ecdysteroid titer associated with emergence. An alternative is that steroid withdrawal prepares the cells for degeneration but there is an additional signal that actually triggers the death. In either situation, it is clear that the adult ecdysteroid titers must remain low until the doomed neurons pass a commitment point when their fate to degenerate becomes independent of the ecdysteroid titer (Fig. 7). However, in *Manduca* (and presumably in *Drosophila*) death can be blocked even after the commitment point by
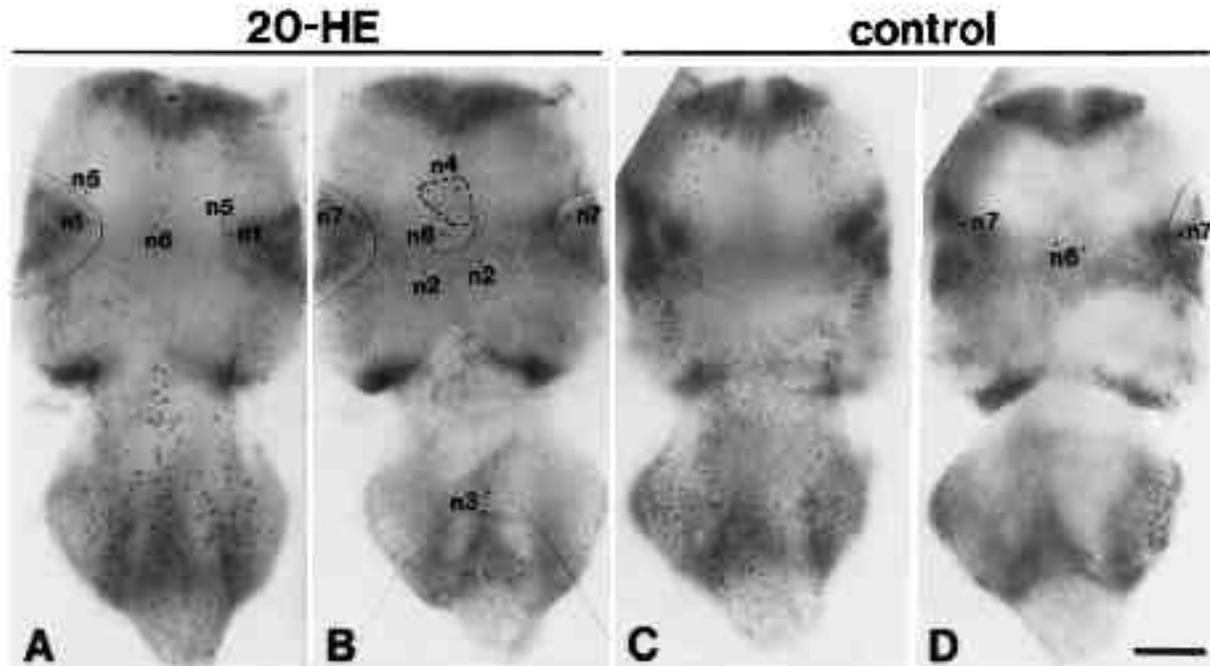
Fig. 6. 20-HE treatment blocks the death of the type II neurons. (A,C) A montage of the dorsal aspect of immunoperoxidase-stained nervous systems showing the effects of 20-hydroxyecdysone (20-HE) treatment on the survival of the EcR-A hyperexpressing cells. (B,D) Ventral aspect of samples as described above. Adult flies were injected with 20-HE at eclosion and aged at 25°C. 12 hours later, the ventral nervous system was processed for EcR-A immunoreactivity. Control animals were injected with Ikeda's Ringer. (A,B) A CNS from an animal injected with 20-HE showing survival of all of the identified type II neurons at this time. (C,D) A control nervous system of the same age (12 hours) showing the presence of only the n6 and n7 neurons. Scale bar, 50 µm.
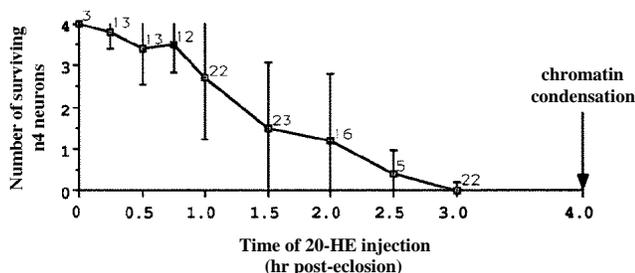

Fig. 7. Death of the n4 cells becomes steroid independent after adult emergence. Adults were injected with 20-HE at various times after eclosion, and aged at 25°C until 12 hours after eclosion, when their ventral CNS was removed and processed for EcR-A immunoreactivity using the immunoperoxidase method. The commitment point, the time when only half of the n4 cells could be saved by the steroid treatment, was 1.25 hours after eclosion. Numbers refer to the number of samples per time point. Error bars indicate the standard deviation. Condensation of the nucleic acid is normally observed in these cells at 4 hours (arrow).

inhibitors of RNA or protein synthesis (Fahrbach and Truman, 1987) suggesting that the death of these cells is genetically regulated.

Because the decision to live or die can be controlled by manipulating the ecdysteroid levels immediately after adult emergence (Fig. 6), we suppose that the presence of the EcR-A isoform in the doomed cells at this time is also critical to that decision. This supposition raises the question

of whether the earlier high-level EcR-A expression during the terminal three days of the pupal period (Fig. 3) is also critical to the doomed fate of the type II neurons. It may be instructive in this regard to consider the EcR-A expression schedules for the n6 and n7 neurons, both of which exhibit high-level EcR-A expression just before and during the critical period after adult emergence, but neither of which die. Their persistence suggests that high-level EcR-A expression only at this critical time is not sufficient for the life-or-death decision. While the n7 pair does not exhibit earlier high-level EcR-A expression, the n6 pair initiates such expression at about the same time as the type II neurons (Fig. 3). However, by 28 hours APF, the n6 neurons cease to exhibit high levels of EcR-A but then reinitiate expression between 79 and 89 hours APF, i.e., when the n7 pair first initiates high level EcR-A expression. The n6 pattern thus suggests the possibility that the period during metamorphosisbounded by these two times identifies another stage when high-level EcR-A expression is critical to establish the doomed fate of the type II neurons.

In constructing a working model for the role of ecdysteroids and the EcR-A isoform in the decision of type II neurons to live or die, we have borrowed from a hierarchical model originally developed by Ashburner et al. (1974) to explain the puff response to ecdysteroids in larval salivary glands. Subsequently, this model has been confirmed in many regards and extended to other target tissues by molecular genetic analysis. In this model, ecdysteroid/EcR complexes have two functions. One is to induce the transcription of a small set of early genes, which induce the tran-

scription of a larger set of late genes (Guay and Guild, 1991). Three early genes have, in fact, been shown to encode transcription factors (Segraves and Hogness, 1990; Burtis et al., 1990; DiBello et al., 1991). These transcription factors also feedback negatively to turn off early gene transcription in the continued presence of ecdysteroids. This feedback does not, however, appear to operate in pupae, thus allowing early gene transcription to continue for long periods in the continued presence of ecdysteroids (Thummel et al., 1990). The second function of the ecdysteroid/EcR complex is to repress the transcription of late genes so as to delay their expression after exposure to ecdysteroids until that repression is overcome by the rising concentration of early gene transcription factors. This function was postulated to account for the observation that the artificial withdrawal of ecdysteroids, at a time when early gene puffs are only partially induced and late gene puffs are not yet induced, results in the premature induction of late puffs (Ashburner et al., 1974). This is consistent with the recent finding that EcR proteins bind to both the early and late puff sites in salivary gland polytene chromosomes (Talbot, 1993).

In our working model, the pre-emergence and post-emergence critical periods are the respective times when 'death-related' early and late genes are expressed; these late genes presumably encode products that are required for and intimately involved in the death of the type II neurons. While these late genes are assumed to fit the criteria of being repressed by ecdysteroid/EcR-A complexes and of being induced by proteins encoded by the early genes, the relationship among the early and late components of the model is assumed to be atypical. That is, we assume that the concentration of the early gene protein(s) produced during the first critical period is insufficient to overcome the repression of the late genes by the exceptionally high levels of ecdysteroid/EcR-A complex found in these cells. The withdrawal of ecdysteroids during the second critical period removes the repression and finally allows the death-related genes to be expressed. Artificially increasing the ecdysteroid titer during the second period would then prevent the induction of the late genes and rescue the type II cells from death.

This is the first demonstration in identified cells that a developmental fate can be correlated with a specific pattern of steroid receptor expression. These data support the notion that the regulation of receptor isoform expression allows different cell types and tissues to respond divergently to the same hormonal cues.

REFERENCES

Ashburner, M., Chihara, C., Meltzer, P. and Richards, G. (1974). Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **38**, 655-662.

Bainbridge, S. P. and Bownes, M. (1988). Ecdysteroid titers during Drosophila metamorphosis. *Insect Biochem.* **18**, 185-197.

Beato, M. (1989). Gene regulation by steroid hormones. *Cell* **56**, 335-344.

Bradley, D. J., Towle, H. C. and Young, W. S. (1992). Spatial and temporal expression of alpha- and beta-thyroid hormone receptor mRNAs, including the beta 2-subtype, in the developing mammalian nervous system. *J. Neurosci.* **12**, 2288-2302.

Budnik, V., Wu, C.-F. and White, K. (1989). Altered branching of serotonin-containing neurons in Drosophila mutants unable to synthesize serotonin and dopamine. *J. Neurosci.* **9**, 2866-2877.

Burtis, K., Thummel, C. S., Jones, C. W., Karim, F. D. and Hogness, D. S. (1990). The Drosophila E74EF early puff contains *E74,* a complex ecdysone-inducible gene that encodes two ets-related proteins. *Cell* **61**, 85-99.

Chambon, P., Zelent, A., Petkovich, M., Mendelsohn, C., Leroy, P., Krust, A., Kastner, P. and Brand, N. (1991). The family of retinoic acid nuclear receptors. In *Retinoids, 10 Years On.* (ed. J.-H. Saurat) pp. 10-27. Basel: S. Karger.

Chin, W. W. (1991). Nuclear Thyroid Hormone Receptors. In *Nuclear Hormone Receptors.* (ed. M. G. Parker) pp. 79-102. San Diego: Academic Press.

Cook, C. B., Kakucska, I., Lechan, R. M. and Koenig, R. J. (1992). Expression of thyroid hormone receptor beta 2 in rat hypothalamus. *Endocrin.* **130**, 1077-1079.

DiBello, P. R., Withers, D. A., Bayer, C. A., Fristrom, J. W. and Guild, G. M. (1991). The Drosophila *Broad-Complex* encodes a family of related proteins containing zinc fingers. *Genetics* **129**, 385-397.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889-895.

Fahrbach, S. E. and Truman, J. W. (1987). Mechanisms for programmed cell death in the nervous system of a moth. In *Selective Neuronal Death.* (eds. G. Bock and M. O'Connor) pp. 65-81. New York: John Wiley and Sons.

Fahrbach, S. E. and Truman, J. W. (1989). Autoradiographic identification of ecdysteroid-binding cells in the nervous system of the moth Manduca sexta. *J. Neurobiol.* **20**, 681-702.

Guay, P. S. and Guild, G. M. (1991). The ecdysone-induced puffing cascade in Drosophila salivary glands: a *Broad-Complex* early gene regulates intermolt and late gene transcription. *Genetics* **129**, 169-175.

Green, S. and Chambon, P. (1988). Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet.* **4**, 309-314.

Handler, A. M. (1982). Ecdysteroid titers during pupal and adult development in Drosophila melanogaster. *Dev. Biol.* **93**, 73-82.

Ikeda, K. and Koenig, J. H. (1988). Morphological identification of the motor neurons innervating the dorsal longitudinal flight muscle of Drosophila melanogaster. *J. Comp. Neurol.* **273**, 436-444.

Jannini, E. A., Mitsuhashi, T. and Nikodem, V. M. (1992). Developmental expression of mRNAs from a rat *C-erbA* genomic locus. *Biochem. Biophys. Res. Comm.* **184**, 739-745.

Kimura, K. I. and Truman, J. W. (1990). Postmetamorphic cell death in the nervous and muscular systems of Drosophila melanogaster. *J. Neurosci.* **10**, 403-411.

Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P. and Hogness, D. S. (1991). The Drosophila *EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67**, 59-77.

Levine, R. B. and Truman, J. W. (1985). Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, Manduca sexta. *J. Neurosci.* **5**, 2424-2431.

Mangelsdorf, D. J. and Evans, R. M. (1992). Retinoid receptors as transcription factors. In *Transcriptional Regulation*. (eds. K. Yamamoto and S. L. McKnight) in press. Plainview: Cold Spring Harbor Press.

Miller, A. (1950). The internal anatomy and histology of the imago of *Drosophila melanogaster*. In *Biology of* Drosophila melanogaster. (ed. M. Demerec) pp. 420-534. New York: John Wiley and Sons.

Richards, G. (1981). The radioimmune assay of ecdysteroid titres in Drosophila melanogaster. *Mol. Cell. Endocrin.* **21**, 181-197.

Robinow, S. and White, K. (1991). Characterization and spatial distribution of the ELAV protein during Drosophila melanogaster development. *J. Neurobiol.* **22**, 443-461.

Ruberte, E., Friederich, V., Chambon, P and Morriss-Kay, G. (1993). Retinoic acid receptors and cellular retinoid binding proteins. *Development* **118**, 267-282.

Schwartz, L. M. and Truman, J. W. (1983). Hormonal control of rates of metamorphic development in the tobacco hornworm Manduca sexta. *Dev. Biol.* **99**, 103-114.

Segraves, W. A. and Hogness, D. S. (1990). The *E75* ecdysone-inducible

gene responsible for the 75B early puff in Drosophila encodes two new members of the steroid receptor superfamily. *Genes Dev.* **4**, 204-219.

Talbot, W. S. (1993). Structure, expression and function of ecdysone receptor isoforms in Drosophila. Ph.D. thesis, Stanford, University, California.

Talbot, W. S., Swyryd, E. A. and Hogness, D. S. (1993). Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell*, **73**, 1323-1337.

Thummel, C. S., Burtis, K. C. and Hogness, D. S. (1990). Spatial and temporal patterns of *E74* transcription during Drosophila development. *Cell* **61**, 101-111.

Truman, J. W. (1983). Programmed cell death in the nervous system of an adult insect. *J. Comp. Neurol.* **216**, 445-452.

Truman, J. W. (1988). Hormonal approaches for studying nervous system development. *Adv. Insect Phys.* **21**, 1-34.

Truman, J. W. and Schwartz, L. M. (1984). Steroid regulation of neuronal death in the moth nervous system. *J. Neurosci.* **4**, 274-280.

Weeks, J. C. and Levine, R. B. (1990). Postembryonic neuronal plasticity and its hormonal control during insect metamorphosis. *Ann. Rev. Neurosci.* **13**, 183-194.

Wills, K. N., Zhang, X. K. and Pfahl, M. (1991). Coordinate expression of functionally distinct thyroid hormone receptor alpha isoforms during neonatal brain development. *Mol. Endocrin.* **5**, 1109-1119.