

# Ecdysteroid control of cell proliferation during optic lobe neurogenesis in the moth *Manduca sexta*

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## SUMMARY

Cell proliferation within the optic lobe anlagen is dependent on ecdysteroids during metamorphosis of the moth *Manduca sexta*. We use cultured tissues to show that ecdysteroids must be maintained above a sharp threshold concentration to sustain proliferation. Proliferation can be turned on and off repeatedly simply by shifting the ecdysteroid concentration to above or below this threshold. In subthreshold hormone, cells arrest in the G<sub>2</sub> phase of the cell cycle. Ecdysteroid control of proliferation is distinguished from differentiative and maturational responses to ecdysteroids by requiring tonic exposure to the

hormone and lower levels of 20-hydroxyecdysone, and by being sensitive to either 20-hydroxyecdysone or its precursor, ecdysone. These characteristics allow optic lobe development to be divided into two ecdysteroid-dependent phases. Initially, moderate levels of ecdysteroid stimulate proliferation. Later, high levels of 20-hydroxyecdysone trigger a wave of apoptosis within the anlage that marks completion of its proliferative phase.

Key words: Ecdysteroid, 20-hydroxyecdysone, Ecdysone, G<sub>2</sub>, Cell cycle, *Manduca sexta*, Optic lobe, Neuroblast

## INTRODUCTION

Proper development of spatially complex tissues such as the nervous system necessitates precise temporal coordination of proliferation, patterning and differentiation. Development of the visual system of adult holometabolous insects is a particularly tractable system for identifying mechanisms that govern these events. It consists of the compound eyes and the underlying optic lobes of the brain, which develop during metamorphosis from the eye discs and the optic lobe anlagen (OA), respectively.

The optic lobe is composed of three ganglia, the lamina, medulla and lobula (Meinertzhagen and Hanson, 1993). The neurons of the optic ganglia are progeny of neuroblasts in the OA. The organization of the OA has been examined in several insects and is similar (Nordlander and Edwards, 1969; White and Kankel, 1978; Hofbauer and Campos-Ortega, 1990; Monsma and Booker, 1996a). In *Manduca sexta*, the neuroblasts are arranged in a double-banded bracelet that comprises the inner and outer OA. During early larval stages, expansion of the neuroblast population occurs by symmetric cell divisions. In the final larval instar, the neuroblasts switch to the asymmetric divisions that lead to neuron production. The smaller daughter of each asymmetric division, the ganglion mother cell (GMC), divides to produce neurons. GMCs of the inner OA produce the neurons of the lobula. The medial margin of the outer OA is composed of the neuroblasts and GMCs that produce the neurons of the medulla (medulla precursor cells, MPC), while the lateral margin is composed of the neuroblasts and GMCs that produce the neurons of the lamina (lamina precursor cells, LPC).

Proliferation of the LPC has been shown to be regulated by incoming retinal afferents. In eyeless *Drosophila* mutants, LPCs arrest in the G<sub>1</sub> phase of the cell cycle (Selleck et al., 1992; Huang and Kunes, 1996). Transection of the optic nerve in *Manduca* also leads to disruption of LPC proliferation (Monsma and Booker, 1996b). Here we examine the role of the ecdysteroids, the steroids that cause metamorphosis, in controlling proliferation within the OA of *Manduca*. Relatively little is known about ecdysteroid control of cell proliferation. Instead, studies of ecdysteroid action have focused on differentiative and maturational responses such as cuticle synthesis by the epidermis, imaginal disc eversion, salivary gland chromosome puffing and process elongation by the Kc cell line (e.g., Riddiford, 1978; Fristrom and Yund, 1976; Ashburner et al., 1974; Cherbas and Cherbas, 1981). Nevertheless, increased rates of proliferation in a variety of tissues have been correlated with increases in blood ecdysteroid titers and ecdysteroids have been shown to stimulate proliferation in culture (see Kirschenbaum et al., 1995; Luedeman and Levine, 1996, for recent examples). Only rarely, however, has stimulation of proliferation by ecdysteroids been examined at the level of the cell cycle. Dumser (1980) found an ecdysteroid-dependent increase in the mitotic index of spermatagonia in cultured locust testes and suggested that the hormone removed a block between G<sub>2</sub> and mitosis. Similarly, ecdysteroids were shown to stimulate passage through G<sub>2</sub> in cultured accessory glands of *Tenebrio* (Yaginuma et al., 1988). Here we show that proliferating cells throughout the OA of *Manduca* arrest reversibly in G<sub>2</sub> whenever the concentration of ecdysteroid drops below a critical threshold. Furthermore, this proliferative response to ecdysteroid exhibits

several characteristics that distinguish it from differentiative and maturational responses, thereby allowing optic lobe neurogenesis to be divided into distinct ecdysteroid-dependent phases.

## MATERIALS AND METHODS

### Experimental animals

Larvae of the tobacco hornworm *Manduca sexta* (L.) were reared in individual containers on an artificial diet at 26°C under long-day conditions (17/7 hours light/dark) for continuous development or under short-day conditions (12/12 hours light/dark) to induce diapause (Bell and Joachim, 1976). Animals were chilled on ice prior to injection or dissection.

The age of animals was with reference to developmental transitions: ecdysis to the final larval instar or pupa (V+0 and P+0, respectively), and onset of wandering behavior (W+0). For example, P+1 day animals had pupated approximately 24 hours earlier.

### Ecdysteroids

20-hydroxyecdysone (20E; Sigma, St Louis, MO) and ecdysone (E; Fluka, Buchs, Switzerland) were dissolved in 10% isopropanol and 100% ethanol, respectively, and their concentrations measured spectrophotometrically ( $E_{240}=12,670$  and  $E_{242}=12,388$ , respectively). This source of E is contaminated with less than 1% 20E (R. Lafont, personal communication).

### Culturing of *Manduca* tissues

Animals were surface-sterilized in 95% ethanol and dissected in cold Levine-modified Weever's saline (Levine and Truman, 1985). Tissues were rinsed twice in Grace's medium (Grace, 1962). A few crystals of phenylthiourea were included in the second rinse to inhibit tyrosinase activity (Riddiford et al., 1979). Individual brains were cultured in Linbro Dispotrays (ICN Biomedicals, Horsham, PA) at 26°C in a 95% O<sub>2</sub>, 5% CO<sub>2</sub> atmosphere in 0.2 ml of Grace's medium supplemented with 10% heat-inactivated hemolymph from diapausing pupae. We found the addition of hemolymph to the medium as suggested by Grace (1962) led to increased viability when tissues were cultured for an extended time. Penicillin, streptomycin, and amphotericin were included (Sigma, St Louis, MO). Tissues were transferred to fresh medium every 4 days. Hormone washouts were done by transferring tissues twice for 5 minutes each through a large volume of medium.

### Detection of patterns of DNA synthesis

Cells in S phase were identified by the incorporation of 5-bromodeoxyuridine (BUdR) (Sigma, St Louis, MO) (Gratzner, 1982). Tissues were pulsed with BUdR for 6 hours, except where noted, either by injection (250 µg BUdR/g body weight) or by addition to the culture medium (15 µg BUdR/ml). Tissues were fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline, pH 7.0 (PBS; 130 mM NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>). Brains to be sectioned were then embedded in 8% low-melting point agarose (AT Biochem, Malvern, PA) in HistoPrep molds (Fisher, Pittsburgh, PA). 100 µm horizontal sections were cut using a Vibratome 1000 (TPI, St Louis, MO) and mounted onto pig skin gelatin-coated coverslips. Fixed tissues were processed for immunocytochemistry as described previously (Hegstrom and Truman, 1996a,b).

### TUNEL labeling

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) was used to identify apoptotic nuclei (Gavrielli et al., 1992). Brains were fixed as above with the addition of 1% Triton X-100. Brains were then incubated for 3 hours at 37°C in TdT buffer containing 66 µM dUTP, 33 µM biotinylated-dUTP (Boehringer Mannheim, Indianapolis, IN), 0.1% Triton X-100 and 0.3 units/µl TdT (Gibco/BRL, Gaithersburg, MD). Samples were processed as above and the brains were stained with a 1:500 dilution of avidin-FITC (Jackson Labs, West Grove, PA).

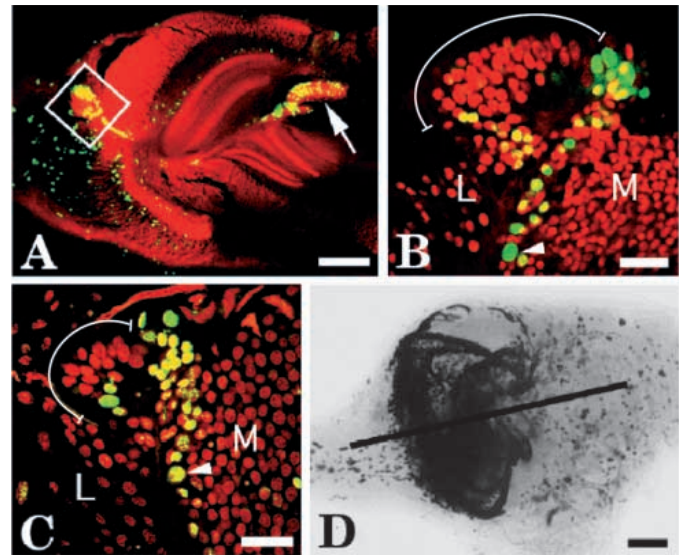
## RESULTS

### Proliferation during optic lobe development

We used incorporation of BUdR during S phase to follow cell proliferation in the OA. We found patterns of BUdR incorporation similar to those described recently for the outer OA (Monsma and Booker, 1996a). The MPC and LPC of the outer OA are readily identified in pupae because they are separated by a band of neuroblasts that divide very rarely (Fig. 1). The number of these 'quiescent' neuroblasts decreases over time (Fig. 1B,C), suggesting that they are progressively recruited to initiate rapid asymmetric divisions at the margins of the outer OA. Neuroblasts, GMCs and neurons have progressively smaller nuclei. We found large BUdR-labeled nuclei scattered throughout the region containing the MPCs, suggesting that proliferating neuroblasts move with their progeny away from the band of neuroblasts (Fig. 1). It is not known, though, how many neurons a given OA neuroblast ultimately generates. High levels of BUdR incorporation were seen in the OA throughout the final (5th) larval instar and continued in the pupa until P+7 days. The numbers of BUdR-labeled nuclei subsequently decreased and few or no BUdR-labeled nuclei were detected in this region by P+10 days.

### Relationship of cell proliferation to ecdysteroid concentration in culture

To define the role of ecdysteroids in regulating proliferation

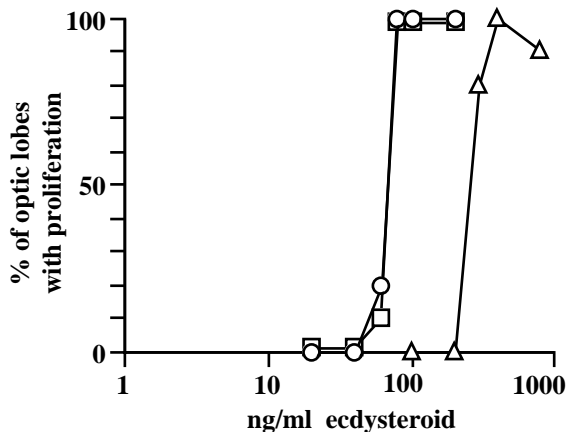


**Fig. 1.** BUdR incorporation in the optic lobe. (A-C) Horizontal vibratome sections were made following a 6-hour BUdR pulse. (A) P+1-day optic lobe. The outer OA is boxed and the arrow points to the inner OA. (B) Higher magnification view of the outer OA at P+1-days. (C) The outer OA at P+7-days. Nuclei stained with propidium iodide are red. BUdR-labeled nuclei are yellow-green. M, region of medulla neurons; L, region of lamina neurons. The white line approximately brackets the large, unlabeled neuroblasts that separate the MPC and LPC of the outer OA. Arrowheads point to MPC nuclei with sizes similar to neuroblasts but which have presumably moved away from the main band of neuroblasts. (D) Frontal view of a P+1-day optic lobe showing BUdR incorporation. The line shows the orientation and position of the sections shown in A, B and C. Nuclei that have incorporated BUdR are black. Lateral is to the left. Bars, 100 µm (A,D); 20 µm (B,C).

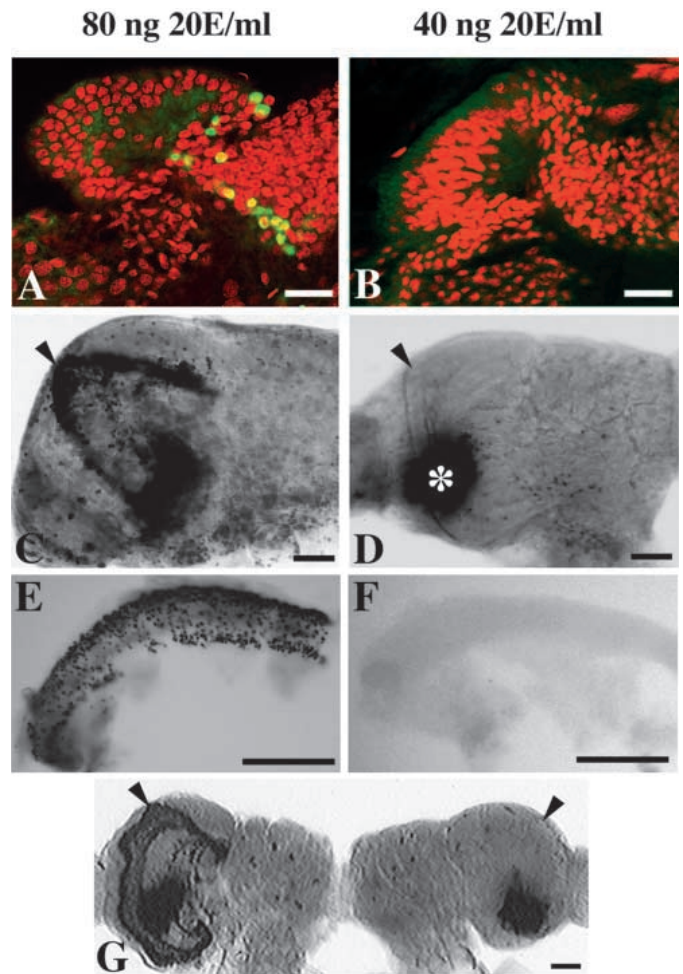
within the optic lobe, we utilized a tissue culture system similar to that used for *Manduca* epidermis (Riddiford et al., 1979). Brains from P+1 day animals were cultured in medium supplemented with various concentrations of 20E and the state of OA proliferation was then assessed by BUdR incorporation. Fig. 2A shows the dependence of BUdR incorporation in the OA on varying concentrations of ecdysteroid. High levels of BUdR incorporation were seen in the MPC when brains were cultured with 80 ng 20E/ml and above (Fig. 3A). At 80 ng 20E/ml, the brains kept their pupal appearance throughout the culture period and high levels of BUdR incorporation continued for as long as cultures were maintained (up to 14 days) (Fig. 3C). In contrast, when brains were exposed to 20E concentrations of 40 ng/ml or lower, BUdR incorporation ceased within 24 hours (Fig. 3B) even when the BUdR labeling period was extended through 6 days (Fig. 3D). At any time through this period, though, we could reinitiate BUdR incorporation by raising the 20E concentration to 80 ng/ml. BUdR incorporation in cells of the inner OA exhibited the same ecdysteroid requirements as did those of the MPC.

In contrast to the MPC and the inner OA, BUdR incorporation within the LPC ceased within 18 hours of initiating the cultures, irrespective of hormone concentration (e.g. Fig. 3A). This result is not surprising since LPC proliferation is known to be dependent on input from photoreceptor axons (Monsma and Booker, 1996b), which are severed when the brains are explanted.

The outer OA is an identifiable lip of tissue that can be microdissected. BUdR incorporation occurred in the same ecdysteroid-dependent fashion in isolated outer OAs as in intact brains (Fig. 3E,F). Incorporation again appeared to be confined to the medulla side of the outer OA. Thus, regulation of MPC proliferation appears to reside within the outer OA itself, rather than being dependent on signals from the developing eye, as are the LPC, or from other parts of the brain.



**Fig. 2.** Dose-response dependence of BUdR incorporation on different concentrations of ecdysteroids. Brains were cultured for 24 hours and then pulsed with BUdR. Circles, brains from P+1-day animals cultured in 20E; triangles, brains from P+1-day animals cultured in E; squares, brains from newly diapausing pupae cultured in 20E. Optic lobes were scored as not proliferating if the region of the outer OA contained fewer than 10 BUdR-labeled nuclei. All other optic lobes had greater than 100 BUdR-labeled nuclei labeled in this region and were scored as positive for proliferation. 20 optic lobes scored/point.



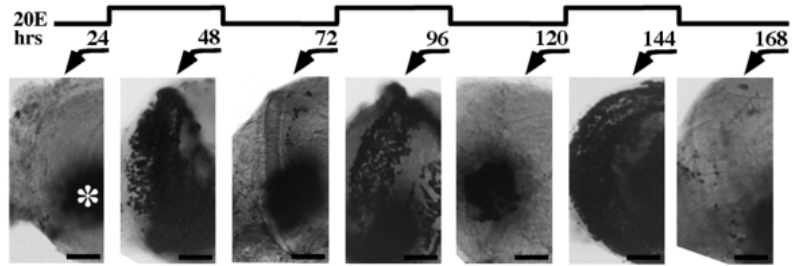
**Fig. 3.** Effects of 20E concentration on BUdR incorporation in cultured tissues. Brains or microdissected outer OAs from P+1-day animals were cultured in 80 ng 20E/ml (A,C,E), 40 ng 20E/ml (B,D,F) or 60 ng 20E/ml (G) for 24 hours (except for 10 days in C). Brains were then pulsed with BUdR for 6 hours (except for 6 days in D). (A,B) Outer OA of sectioned brains. Nuclei stained with propidium iodide are red; BUdR-labeled nuclei are yellow-green. Orientation is as in Fig. 1B. (C-G). BUdR-labeled nuclei are black. Arrowheads point to the outer OA. \* marks the larval optic pigments present in the brain. Lateral is to the left. Bars, 20 µm (A,B); 100 µm (C-G).

At intermediate 20E concentrations between 40 ng/ml and 80 ng/ml, cultured brains showed a mixed response. Some brains showed no BUdR incorporation in the OA and others showed full incorporation in both OA and in a few brains ( $n=3$  of 20); the OA in one optic lobe showed no incorporation while the OA in the other optic lobe showed a full response (Fig. 3G). In every case, the response within a given optic lobe appeared uniform. In other words, the outer OA appeared to respond to ecdysteroids as a unit.

The proliferative response to moderate levels of ecdysteroid was repeatedly reversible. This was shown by switching brains back and forth between subthreshold and suprathreshold 20E at 24 hour intervals for 7 days. Proliferation in the OA was assessed each day by pulsing a subset of the brains with BUdR. BUdR incorporation ceased within 24 hours after each shift to subthreshold 20E, but then resumed within 24 hours of each



**Fig. 4.** Reversible stimulation of BUdR incorporation by ecdysteroids in the OA. Brains isolated from P+1-day animals were alternately cultured in 20 ng 20E/ml or 100 ng 20E/ml for 24 hour intervals over 7 days. Every 24 hours, proliferation was assessed in a subset of the brains ( $n=5$ ) by adding BUdR to the culture medium and then culturing for an additional 10 hours without switching the concentration of 20E followed by fixation. Arrowheads point to the outer OA. \* marks the larval optic pigments present in the brain. Lateral is to the left and dorsal is up. Bars, 100  $\mu$ m.



shift back to suprathreshold 20E (Fig. 4). The levels of BUdR incorporation appeared similar each time proliferation was switched on.

Ecdysone (E), the precursor of 20E, was also effective in stimulating BUdR incorporation within the OA in cultured brains, although at a concentration about fourfold higher than 20E (Fig. 2). When we combined subthreshold concentrations of E and 20E (200 ng E/ml and 40 ng 20E/ml), the mixture was ineffective in stimulating BUdR incorporation. Consequently, these two steroids appear to be neither additive nor synergistic in their control over proliferation.

#### Development of ecdysteroid sensitivity in the OA

Proliferation in the OA begins in the larva, initially in the form of symmetric divisions to increase the number of neuroblasts, and then switching during the final larval instar to the asymmetric divisions that produce neurons (Monsma and Booker, 1996a). We cultured brains from animals on each day of the final larval instar to determine when OA proliferation became sensitive to ecdysteroid. Fig. 5 shows that the switch occurs abruptly on the day of wandering. Prior to this stage, high levels of BUdR incorporation continued when ecdysteroid levels were subthreshold or absent. By contrast, beginning on W+0, a minimum of 80 ng 20E/ml was required to maintain high levels of BUdR incorporation in the OA in culture. This switch to steroid dependence coincides with the time that asymmetric divisions begin in the LPC (Monsma and Booker, 1996a). In the MPC, asymmetric divisions begin prior to this time, but Monsma and Booker (1996a) suggest that W+0 corresponds to a switch from production of tangential cells to medullar cortex neurons by the MPC.

#### Proliferative arrest associated with diapause

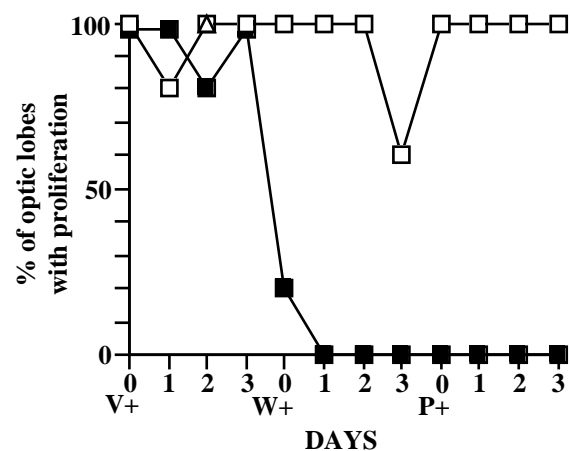
The only time proliferation in the OA is obviously interrupted in vivo is during pupal diapause. *Manduca* larvae can be programmed by short-day length to enter diapause shortly after pupation (Bell et al., 1975). Animals destined to diapause are indistinguishable from those programmed by long-day length for continuous development until P+2 days (Bowen et al., 1985), and high levels of BUdR incorporation were seen in the OA of both long-day and short-day reared animals through this time (Fig. 6A,B). On P+2 days, prothoracicotrophic hormone, the neuropeptide that stimulates ecdysteroid synthesis, fails to be released in short-day animals, the ecdysteroid titer drops to very low levels, and diapause ensues (Bowen et al., 1984). By day P+3, while high levels of BUdR incorporation continue in long-day animals, incorporation has ceased in short-day animals (Fig. 6C,D). Cells in the OA remain in this state of proliferative arrest during the months that the pupae are in diapause even though the animals may remain at the normal

rearing temperature. Diapausing pupae can be induced to resume development at any time by injection of physiological doses of ecdysteroid (Bradfield and Denlinger, 1980). In such animals, high levels of BUdR incorporation are evident in the OA within 18 hours of injection (data not shown).

The level of 20E needed to stimulate resumption of BUdR incorporation in the OA of brains isolated from short-day reared animals that had just entered diapause (4 days after pupation) were identical to those needed to maintain BUdR incorporation in brains from long-day animals (Fig. 2). Similar requirements were also seen for brains from animals that had been in diapause 1 month (data not shown). In both cases, the threshold for BUdR incorporation in the OA was about 60 ng 20E/ml. Consequently, the proliferative arrest seen during diapause appears to be maintained solely by the lack of ecdysteroid. This tonic dependence on ecdysteroid may have been exploited in the evolution of pupal diapause to provide a simple mechanism for interrupting metamorphic development.

#### Relationship of ecdysteroid treatments to cell cycle events

The ability to add and remove hormone in culture allowed us to explore the relationship between ecdysteroid exposure and events of the cell cycle. Brains from P+1 day animals were first cultured



**Fig. 5.** Ontogeny of ecdysteroid-dependent BUdR incorporation in the OA. Brains were isolated from larvae and pupae at the stages indicated and cultured for 24 hours with 80 ng 20E/ml (open squares), 40 ng 20E/ml (filled squares) or no hormone (triangle) and then pulsed with BUdR. Optic lobes were scored as not proliferating if the region of the outer OA contained fewer than 10 BUdR-labeled nuclei. All other optic lobes had greater than 100 BUdR-labeled nuclei labeled in this region and were scored as positive for proliferation. 10 optic lobes/point.

in subthreshold ecdysteroid (20 ng 20E/ml) for 24 hours to allow proliferation to cease. Brains were then shifted to suprathreshold 20E plus BUdR and a subset of the brains were fixed at 2 hour intervals. To ensure that we determined the minimum latency, we used 500 ng 20E/ml, one of the highest 20E concentrations that supports continuous proliferation. No BUdR incorporation was observed in the OA within the first 8 hours after the shift (Fig. 7A). By 10 hours, scattered incorporation was evident in the MPC, primarily in large neuroblast nuclei (Fig. 7B), and by 12 hours, high levels of incorporation were seen throughout the wide band of MPC (Fig. 7C).

Although there was more than an 8 hour delay between the shift to suprathreshold levels of 20E and the onset of BUdR incorporation, hormone was not required throughout this entire period. This was shown by washing out the hormone after varying lengths of time followed by maintenance for an additional 48 hours in subthreshold 20E plus BUdR. No BUdR incorporation occurred following a 30 minute exposure to 20E (Fig. 7D). A 1 hour pulse stimulated BUdR incorporation in a subset of the MPC (Fig. 7E). BUdR-labeled nuclei typically occurred in pairs, although single nuclei were also observed. Pairs of labeled nuclei were found that were both equal (42 of 65 pairs) and unequal (23 of 65 pairs) in size (e.g. Fig. 7E). Besides 20E, a 1 hour pulse of E (500 ng/ml) was also sufficient to trigger scattered BUdR incorporation in the OA (data not shown). Exposure of arrested brains to 500 ng 20E/ml for 2 hours stimulated BUdR incorporation in a large number of nuclei throughout the wide band of MPCs (Fig. 7F).

A pulse of 20E appeared to be as effective as continuous exposure in initiating high levels of BUdR incorporation, but the response was transient. Arrested brains were exposed to suprathreshold 20E for 2 hours and then maintained in subthreshold 20E. At 2 hour intervals, a subset of the brains were

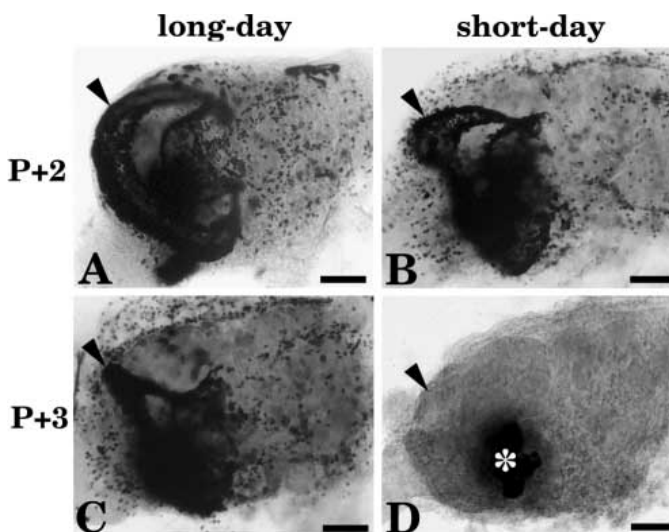
pulsed with BUdR for 2 hours and then fixed. BUdR incorporation was first detected in the large MPC neuroblasts by 10 hours after the switch to suprathreshold hormone and continued until 16 hours. BUdR labeling of these large nuclei was reduced by 18 hours and absent after that time. For the smaller MPC nuclei, that were presumably GMCs, BUdR incorporation was evident in a few nuclei by 10 hours, was at high levels by 12 hours and continued at high levels until 20 hours. BUdR incorporation declined after 20 hours but could still be detected in a few of these nuclei as late as 24 hours. Thus, for both classes of nuclei, a 2 hour exposure to 20E supported a period of BUdR incorporation that lasted about 8-10 hours.

The timing and nature of BUdR incorporation in the OA of brains isolated from pupae that had just entered diapause were the same in every way as those from long-day animals that had been arrested in low hormone in culture (data not shown).

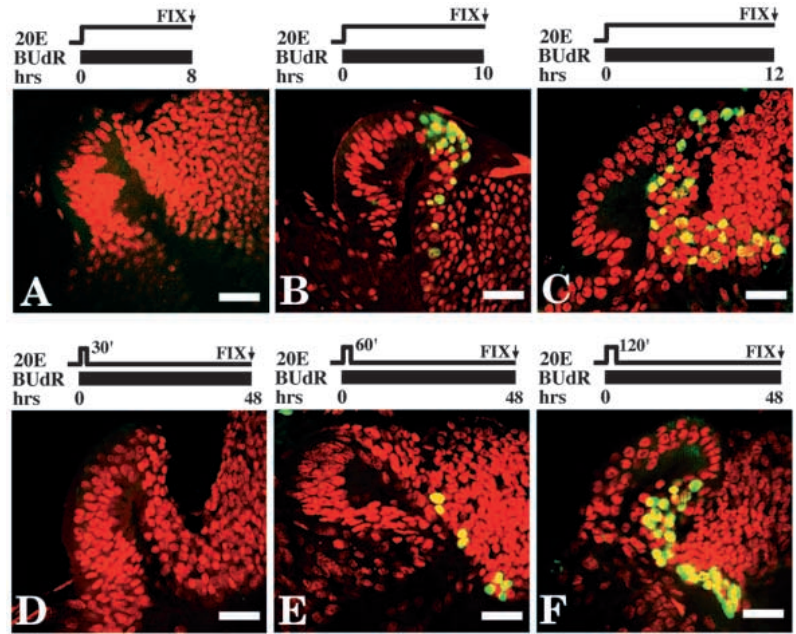
An intriguing aspect of the response to either pulsed or continuous 20E is the 8 hour latency between the start of steroid exposure and entry into S phase. We examined the basis for this delay using the mitotic inhibitor colcemid. When arrested brains or brains from pupae that had just entered diapause were cultured with colcemid along with BUdR and 500 ng 20E/ml, no BUdR incorporation was observed in the OA even after 24 hours (Fig. 8A). After the colcemid was washed out, a burst of BUdR incorporation followed, even though the brains were cultured in subthreshold 20E following colcemid washout (Fig. 8B). These results suggest that ecdysteroid-stimulated cells must first pass through mitosis before they enter S phase. Thus, proliferating cells in the OA appear to arrest in G<sub>2</sub> in response to subthreshold ecdysteroid either when the animal enters diapause or when tissues from developing animals are experimentally subjected to ecdysteroid withdrawal. Consistent with this interpretation, we find that many cells in the OA show chromosomal condensation by 2.5 hours after exposing brains from diapause animals to suprathreshold 20E (Fig. 8C,D).

### Effect of high ecdysteroid concentrations on proliferation and apoptosis in the OA

As described above, BUdR incorporation continued in the OA for a least 14 days when brains were cultured with moderate levels of ecdysteroid. By contrast, high levels of 20E caused a proliferative response that was transient. As seen in Fig. 9, BUdR incorporation in the region of the OA had stopped after 4 days exposure to 1.5  $\mu$ g 20E/ml, whereas high levels of incorporation were still found after 8 days in 600 ng 20E/ml. This abrupt termination of proliferation was correlated with high levels of apoptosis within the OA. 24 hours after shifting P+1 day brains to 1.5  $\mu$ g 20E/ml, we found many apoptotic nuclei in the OA, as detected using the TUNEL-labeling method (Fig. 10B). The apoptotic cells were distributed throughout the inner and outer OA including the MPC, LPC and the band of neuroblasts that separates them. In brains cultured in lower levels of 20E (600 ng/ml), apoptotic cells were also evident but at a lower frequency and these deaths did not involve the cells of the OA itself (Fig. 10A). Rather, death was confined to the region of young neurons just flanking the OA. This latter pattern is similar to that found during the early phases of metamorphosis when a low level of apoptosis occurs continuously in this region OA (Monsma and Booker, 1996a). When high concentrations of E were used (up to 20  $\mu$ g/ml, fivefold higher than the maximum titer observed in the animal; Warren and



**Fig. 6.** BUdR incorporation in the optic lobe of P+2-day and P+3-day animals. Animals were reared under long-day conditions to program continuous development or short-day conditions to program entry into diapause. Animals were injected with BUdR and brains dissected 6 hours later. (A) P+2, long-day. (B) P+2, short-day. (C) P+3, long-day. (D) P+3, short-day. Arrowheads point to the outer OA. \* marks the larval optic pigments present in the brain. Lateral is to the left and dorsal is up. Bars, 100  $\mu$ m.



**Fig. 7.** BUdR incorporation in the outer OA following ecdysteroid treatments. Proliferation was first arrested by culturing P+1-day brains in 20 ng 20E/ml for 24 hours. Brains were then exposed to 500 ng 20E/ml plus BUdR for (A) 8 hours, (B) 10 hours and (C) 12 hours. Alternatively, arrested brains were exposed to 500 ng 20E/ml plus BUdR for (D) 30 minutes, (E) 1 hour or (F) 2 hours, and then the hormone was washed and brains maintained in subthreshold ecdysteroid (20 ng 20E/ml) plus BUdR for an additional 48 hours. Propidium iodide-stained nuclei are red. Nuclei that have incorporated BUdR are yellow-green. Orientation is as in Fig. 1B. Bars, 20  $\mu$ m.

Gilbert, 1986), BUdR incorporation was still high after 8 days in culture and apoptosis was not detected within the OA.

In vivo, extensive apoptosis begins within the OA on day P+8 (Fig. 10C,D and data not shown). The timing of this death correlates with decreasing BUdR incorporation that begins on P+7 days. By P+10 days, little or no BUdR incorporation or apoptosis was detected in this region of the optic lobe and cells with large nuclei, the size of neuroblasts, were no longer present.

## DISCUSSION

Cultured brains of *Manduca* must be maintained within a precise concentration range of ecdysteroid for proliferation of neural precursors in the optic lobe to be sustained. If the concentration of ecdysteroid drops below about 60 ng 20E/ml or about 250 ng E/ml, the cells arrest in the G<sub>2</sub> phase of the cell cycle. Concentrations above this threshold stimulate proliferation, but if the level of 20E rises above about 1  $\mu$ g/ml, the neuroblasts undergo apoptosis and neurogenesis is terminated.

The ecdysteroid requirements defined in culture fit the proliferation pattern that is observed in vivo. Proliferation becomes sensitive to ecdysteroids at the time the larva begins wandering and proliferation continues at an essentially constant rate until about P+7 days. Blood ecdysteroid titers range widely during this period but stay above the 'proliferation threshold' that we defined in culture (Fig. 11). Since the rate of proliferation does not vary significantly through these peaks and valleys of ecdysteroid titer (Monsma and Booker, 1996a, and our unpublished results), the proliferative response to ecdysteroids appears to be 'all or none'. The only time during this period that the blood ecdysteroid titer drops below the proliferative threshold is in animals that enter diapause. In response to this drop, proliferating cells in the OA arrest in G<sub>2</sub> and remain arrested until rising ecdysteroid titers terminate diapause months later.

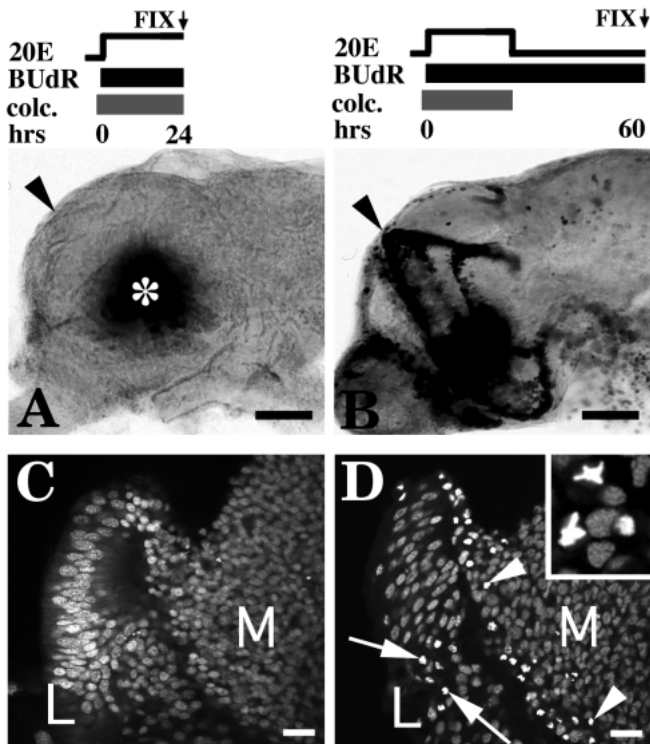
On day P+7, neurogenesis begins to wane and the neuroblasts in the OA undergo apoptosis. At this time the levels of 20E in

the animal exceed 1  $\mu$ g 20E/ml (Fig. 11) and our culture data show these levels of 20E induce apoptosis of the neuroblasts. Consequently, it appears the OA have more neuroblasts than are normally needed, and neurogenesis is terminated by an extrinsic signal (high 20E) that induces the death of these cells. This conclusion is supported by results showing we can induce early neuroblast death and termination of neurogenesis by premature exposure to high 20E. Conversely, long-term maintenance of brains in E or moderate levels of 20E extends neurogenesis beyond the time it would normally stop.

## Ecdysteroid control of the cell cycle

Cells begin incorporating BUdR fairly synchronously after switching arrested brains to suprathreshold ecdysteroid. There is, however, an 8–10 hour lag between the shift to high hormone and entry into S phase. Early in this interval, many cells within the LPC and MPC show chromosome condensation as they enter mitosis. This observation, along with the results from the use of the mitotic inhibitor colcemid, suggest that proliferating cells within the OA arrest in G<sub>2</sub> in subthreshold ecdysteroid.

Although we have not established the length of the cell cycle for the different cell types present in the OA, we interpret the duration of BUdR incorporation following a 2 hour pulse of suprathreshold ecdysteroid to be indicative that this exposure is sufficient to stimulate cells to pass through a single round of the cell cycle, but they arrest again in the next G<sub>2</sub>. This interpretation is supported by the observation that BUdR-labeled nuclei often occurred in isolated pairs when arrested brains were subjected to a brief pulse of suprathreshold ecdysteroid and then maintained for 48 hours in subthreshold ecdysteroid (Fig. 7E). The finding that cells reach S phase at the same time whether they are cultured briefly or continuously in suprathreshold ecdysteroid is also consistent with the interpretation that ecdysteroids are not required at other points in the cell cycle. Ecdysteroid regulation of the mitotic cell cycle in G<sub>2</sub> rather than G<sub>1</sub> is in striking contrast to other extracellular growth factors, including the steroid hormones of vertebrates (Murray and Hunt, 1993).

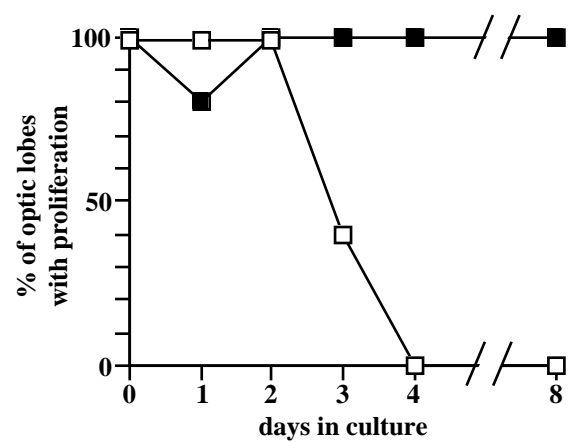


**Fig. 8.** Effect of mitotic block by colcemid on the ecdysteroid-induced proliferation in the OA. Brains from animals that had been in diapause for 1 day were cultured in 20  $\mu$ g colcemid/ml for 1 hour prior to adding 500 ng 20E/ml plus BuDR. (A) BuDR incorporation was assessed following 25 hours in culture. (B) Brains as in A except colcemid was washed out at 25 hours and then the brains were maintained an additional 36 hours in subthreshold ecdysteroid (20 ng 20E/ml) plus BuDR. Black arrowheads point to the outer OA. \* marks the larval optic pigments present in the brain. (C,D) Confocal images of the outer OA after exposure to 500 ng 20E/ml for (C) 1.5 hours and (D) 2.5 hours. The DNA was stained with propidium iodide to show condensed chromosomes of cells entering mitosis. White arrows and arrowheads point to examples of LPCs and MPCs, respectively, entering mitosis. M, medulla neurons; L, lamina neurons. Inset shows a higher magnification view. Orientation as in Fig 1. Bars, 100  $\mu$ m (A,B); 20  $\mu$ m (C,D).

Overall, the effects of ecdysteroids on proliferation in insects are complex in that ecdysteroids stimulate proliferation in some cell types but inhibit proliferation in others. The ecdysteroid-dependent stimulation of proliferation that we have described here is not limited to the OA. Myogenic cells of the ventral diaphragm of *Manduca* exhibit the same ecdysteroid requirements for proliferation as do the neurogenic cells of the optic lobe (our unpublished results). In both cases, ecdysteroids stimulate cells to pass through G<sub>2</sub>. The opposite response is seen in the epidermis of *Tenebrio* (Besson-Lavoignet and Delachambre, 1981) and in the *Drosophila* Kc cell line (Stevens et al., 1980). In these cells, ecdysteroids cause cycling cells to arrest in G<sub>2</sub>.

#### Ecdysteroid requirements for proliferation in the OA

The threshold concentration needed to support proliferation remains at about 60 ng 20E/ml for at least 8 days following the time the cells become dependent on ecdysteroid at wandering. Thus, despite the ongoing development of the optic lobes and



**Fig. 9.** Effect of high levels of 20E on BuDR incorporation in the OA. Brains were cultured in 600 ng 20E/ml (filled squares) or 1.5  $\mu$ g 20E/ml (open squares) and then pulsed for 6 hours with BuDR. Optic lobes were scored as not proliferating if the region of the outer OA contained fewer than 10 BuDR-labeled nuclei. All other optic lobes had more than 100 BuDR-labeled nuclei labeled in this region and were scored as positive for proliferation. 10 optic lobes scored/point.

the major fluctuations in the endogenous ecdysteroid titers, cells within the OA maintain a constant level of sensitivity to ecdysteroid during this period.

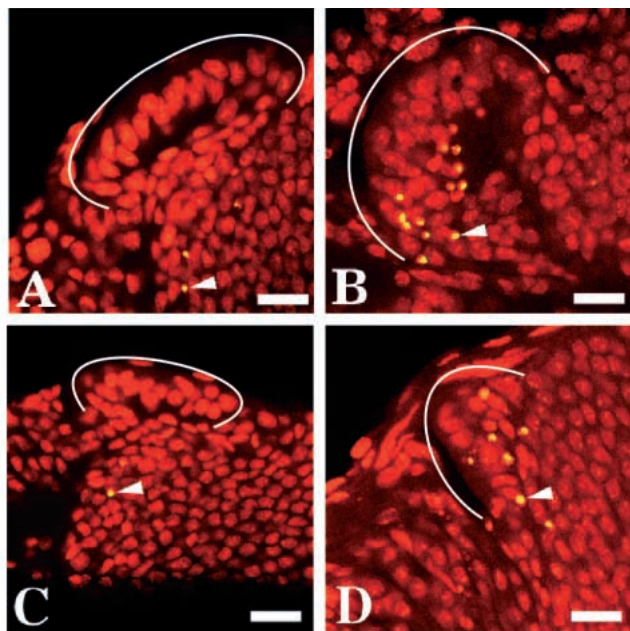
An important feature of the proliferative response is that it is sensitive to moderate levels of E as well as 20E. Ecdysteroids are secreted into the blood as 3-dehydroecdysone and E and are subsequently hydroxylated to 20E (Warren et al., 1988). The finding that E is a hundredfold to a thousandfold less effective than 20E in triggering differentiative and maturational responses such as salivary gland puffing (Ashburner et al., 1974) and imaginal disc development (Fristrom and Yund, 1976) has led to the general belief that it is an inactive prohormone (reviewed in Smith, 1985; Grieneisen, 1994). Early studies, though, suggested E may be active in stimulating proliferation (reviewed by Oberlander, 1972). Our results support the latter conclusion.

In our culture system, E was ineffective in promoting the maturation of the optic lobes, as measured by apoptosis in the OA, even at levels 20-fold higher than the effective 20E concentration. Under the same conditions, though, E was only about four times less effective than 20E in supporting proliferation. We think it unlikely that the proliferative effects of E are due to conversion to 20E. Firstly, the nervous system lacks the appropriate enzyme (Smith, 1985). Secondly, the same brief exposure (1 hour) to either E or 20E was sufficient to stimulate proliferation, yet when brains were maintained in 1  $\mu$ g E/ml for 24 hours and then fresh brains added, the new brains showed only a proliferative response and no induction of the early genes E75 and MHR3, whose expression is sensitive to 20E but not E (our unpublished results). Thirdly, the lack of an additive response with a combination of just subthreshold concentrations of E and 20E argues that little E is being converted to 20E. Finally, E is essentially the only ecdysteroid present in the early pupa (Warren and Gilbert, 1986), yet high levels of proliferation are maintained through this period.

#### Organization of the outer OA

Communication via retinal afferents provides a coordinating





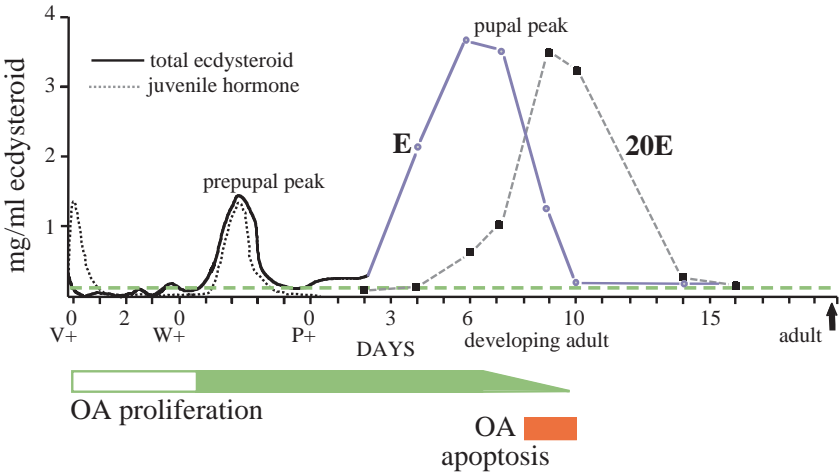
**Fig. 10.** Effect of high levels of 20E on apoptosis within the outer OA. Brains were cultured in 600 ng 20E/ml (A) or 1.5 µg 20E/ml (B) for 24 hours, fixed and nuclei of apoptotic cells labeled by the TUNEL method. Brains from P+7-day (C) and P+8-day (D) animals were fixed and labeled by the TUNEL method. Propidium iodide-stained nuclei are red. TUNEL-labeled nuclei are yellow-green and are limited to areas flanking the outer OA (A,C) or are found throughout the outer OA (B,D). Arrowheads point to an example of TUNEL-labeled nuclei in each panel. The white lines approximately outline the band of outer OA neuroblasts. Orientation as in Fig. 1B. Bars, 20 µm.

link between the developing retina and production of lamina neurons (see Introduction). Ecdysteroids, by contrast, control production of neurons throughout the optic lobe. The precursors for the medullar and lobular neurons both require ecdysteroids for proliferation. The optic nerve is severed in our cultures making it difficult to determine if the same is true for the LPCs. Even under these conditions, though, arrested LPCs enter mitosis in response to 20E (Fig. 9D). It appears that neural precursors throughout the optic lobe have a similar

ecdysteroid-dependent G<sub>2</sub> control point. The failure of LPCs in cultured brains to incorporate BUdR suggests that 20E allows the cells to divide but they then arrest in G<sub>1</sub> in the absence of innervation. This interpretation is consistent with data from *Drosophila* that LPCs arrest in G<sub>1</sub> in mutants lacking proper retinal innervation (Selleck et al., 1992). Thus, the cell cycle of LPCs in *Manduca* may have two developmentally regulated control points, stimulation by retinal afferents being required for progression through G<sub>1</sub> and stimulation by ecdysteroids being required for progression through G<sub>2</sub>.

Manipulation of the cell cycle with ecdysteroid has allowed us to begin to assess the organization of the developing optic lobe. Even though proliferation within the optic lobe has been examined in several insects (see Introduction), the pattern of cell division of individual neuroblasts is not certain. Elsewhere in the CNS, individual neuroblasts and their progeny are physically isolated which has allowed their stereotyped pattern of cell divisions to be established; a neuroblast divides asymmetrically and the smaller progeny (the GMC) divides once to produce two daughter neurons. The presence of distinct sizes of nuclei has led some authors to conclude that the optic lobes adhere to the proliferative pattern shown by these other neuroblasts. When arrested brains of *Manduca* were exposed to ecdysteroids, BUdR incorporation appeared fairly synchronously throughout the MPCs in a pattern similar to that seen in normally cycling brains (Fig. 7). Apparently, most or all of the cell division steps in medullar neuron production are interrupted in subthreshold steroid, not just division of the neuroblasts. Since G<sub>2</sub>-arrested cells must go through mitosis before they enter S, the pairs of BUdR-labeled cells found following a brief pulse of ecdysteroid (Fig. 7E) are likely to be progeny of a single arrested precursor. Pairs of BUdR-labeled nuclei were found that were of unequal size; this situation is expected when an arrested neuroblast is stimulated to divide. Importantly, we also found pairs of labeled nuclei of equal size. These could not be derived from a ‘classic’ GMC arrested in G<sub>2</sub> because the daughters of this cell are neurons that do not go through an S phase. Paired nuclei of equal size could arise from a neuroblast undergoing a symmetric terminal division, but the number of neuroblasts in this area is low (about 5% of the total cells, Monsma and Booker, 1996b). A more likely explanation is that medulla GMC undergo more than one division. This situation is also thought to exist in the LPC of *Drosophila* (Selleck and Steller, 1991).

**Fig. 11.** Ecdysteroid requirements for optic lobe development in culture relative to blood hormone titers. The dotted green line marks the threshold concentration of 20E required to maintain high levels of BUdR incorporation in the OA in culture. OA proliferation: the open bar marks the period of ecdysteroid-independent proliferation early in the final larval instar and the closed bar marks the period of ecdysteroid-dependent proliferation. OA apoptosis: The bar marks the period during which apoptosis occurs in cells within the OA. Hormone levels adapted from Riddiford (1993). Titers of individual ecdysteroids have been reported during the pupal-adult transition (Warren and Gilbert, 1986) and the levels of E (circles) and 20E (squares) are shown instead of the total ecdysteroid titer.





MPCs were found to proliferate in an ecdysteroid-dependent manner even in small pieces of the outer OA, clearly indicating that other long-range signals are not required for these cells. Cells throughout the OA, including the MPCs, express the ecdysteroid receptor (D. T. Champlin, M. Jindra, L. M. Riddiford, and J. W. Truman, unpublished results) and so these cells could be responding directly to ecdysteroids. An important issue is whether the ecdysteroid-dependent proliferation of the MPC is a direct response to the steroid or due to short-range signals from neighboring cells. Evidence for communication between surrounding glia and neuroblasts is seen for the protein product of the *anachronism* locus, which is secreted by nearby glial cells and inhibits proliferation of optic lobe neuroblasts in *Drosophila* (Ebens et al., 1993). The fact that the MPCs appear to respond to ecdysteroid as a unit (Fig. 4G) suggests that there may be coordinating signals within the OA of *Manduca*.

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