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Ecdysteroid coordinates optic lobe neurogenesis via a nitric oxide signaling pathway

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SUMMARY

Proliferation of neural precursors in the optic lobe of *Manduca sexta* is controlled by circulating steroids and by local production of nitric oxide (NO). Diaphorase staining, anti-NO synthase (NOS) immunocytochemistry and the NO-indicator, DAF-2, show that cells throughout the optic anlage contain NOS and produce NO. Signaling via NO inhibits proliferation in the anlage. When exposed to low levels of ecdysteroid, NO production is stimulated and proliferation ceases. When steroid levels are increased, NO production begins to decrease within 15 minutes independent of RNA or protein synthesis and cells rapidly resume proliferation. Resumption of proliferation is not due simply to the removal of NO repression though, but also requires an ecdysteroid stimulatory pathway. The consequence of these opposing pathways is a sharpening of the responsiveness to the steroid, thereby facilitating a tight coordination between development of the different elements of the adult visual system.

Key words: Ecdysteroid, Nitric oxide, Cell cycle, Optic lobe neuroblast, *Manduca sexta*

INTRODUCTION

A frequent theme in the development of complex nervous systems is the parallel development of repeated units. A particularly striking example of this is provided by the visual system of insects. The visual system is organized into layers; the compound eye, followed successively by the lamina, medulla and lobula neuropils in the optic lobe of the brain. Development proceeds in parallel both within and between these layers. For example, the repeating units of the eye (the ommatidia) are created in rows along the dorsal-ventral axis of the imaginal eye disc (Wolff and Ready, 1993). In concert with this, sets of optic lobe neurons are produced by proliferation of neuroblasts in the optic anlage (OA) (Meinertzhagen and Hanson, 1993). Formation of ommatidia progresses sequentially in the anterior-posterior axis so that rows of more anterior photoreceptors become connected with sets of later born optic lobe neurons. The strict registry of these events leads to a retinotopic organization of the optic lobe neuropils (Meinertzhagen and Hanson, 1993).

Ingrowing photoreceptor axons provide a coordinating link between development of the eye and the optic lobe (Selleck and Steller, 1991; Monsma and Booker, 1996b). Hedgehog protein released from the incoming photoreceptor axons is required for the cell divisions in the OA that produce the lamina neurons (Huang and Kunes, 1996, 1998). During metamorphosis of the moth *Manduca sexta*, circulating steroid hormones, the ecdysteroids, provide a second coordinating link between development of the eye and optic lobe. Ecdysteroid levels must be maintained above a sharp threshold concentration for the progressive formation of ommatidia in cultured eye discs (Champlin and Truman, 1998b). Similarly, ecdysteroid levels must be above this same threshold concentration to support optic lobe neurogenesis in cultured brains (Champlin and Truman, 1998a). If steroid levels fall below this threshold, the neural precursors undergo proliferative arrest late in the G_2 phase of the cell cycle (Champlin and Truman, 1998a). When tissues arrested in low levels of ecdysteroid are returned to suprathreshold levels, development of the eye disc and brain rapidly resume. An interesting feature of this control is that the neural precursors within each OA appeared to respond to ecdysteroid as a unit (Champlin and Truman, 1998a), suggesting local coordination of the proliferative response within the anlage.

Nitric oxide (NO) is a short-lived diffusable signaling factor (Bredt and Snyder, 1994) that is a candidate for coordinating ecdysteroid-dependent proliferation within the OA. NO is produced from arginine by nitric oxide synthase (NOS). Insects appear to have a single NOS gene and insect NOS most closely resembles the neuronal NOS of vertebrates (Regulski and Tully, 1995; Nighorn et al., 1998). In vertebrates, NO inhibits proliferation of a variety of cell types (see Heller et al., 1999, for examples). Similarly, in *Drosophila*, NO can inhibit proliferation both during embryogenesis (Wingrove and O'Farrell, 1999) and during metamorphosis (Kuzin et al., 1996). NO also inhibits proliferation of neural precursors in the OA of *Manduca*. In this case, the NO signaling is involved in the proliferative response to ecdysteroid because increases in the steroid result in a rapid decrease in NO production. This interplay between ecdysteroid and NO signaling leads to a

refinement in the coordination of visual system development by the steroid.

MATERIALS AND METHODS

Experimental animals and tissue culture conditions

Larvae of the tobacco hornworm, *Manduca sexta* (L.), were reared in individual containers on an artificial diet (Bell and Joachim, 1976) at 26°C under long-day conditions (17 hours light/7 hours dark). The age of animals was referenced to developmental transitions: ecdysis to the final larval instar or pupa (V+0 and P+0, respectively). For example, P+1 day animals had pupated approximately 24 hours earlier.

Brains were dissected from staged animals and cultured as has been described by Champlin and Truman (1998a). Briefly, brains were isolated, manually desheathed and cultured individually at 26°C in a 95% O_2-5% CO_2 atmosphere in 0.2 ml of Grace's medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated hemolymph from diapausing pupae plus various levels of 20E (20-hydroxyecdysone). 20E (a gift from Dr Takeshi Matsumoto, Daicel Chemical Co., Japan) was dissolved in ethanol and the concentration determined spectrophotometrically (ε_{240}=12,677; Lafont and Wilson, 1992).

Detection of NOS and modifiers of NO signaling

NADPH-diaphorase histochemistry was performed as described by Hope et al. (1991); Gibbs and Truman (1998). Briefly, brains were fixed 30 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0) and then 100 μm horizontal sections were cut using a Vibratome 1000 (TPI, St. Louis, MO). Sections were mounted onto pig skin gelatin-coated coverslips and fixed for an additional 30 minutes. Samples were then incubated with 1 mM NADPH and 0.2 mM nitroblue tetrazolium (Sigma, St. Louis,MO) in PBS-TX (PBS plus 0.1% Triton X-100) for 2 hours in the dark at room temperature, rinsed in PBS and mounted in 80% glycerol.

NOS was identified by immunocytochemistry with an anti-NOS polyclonal antibody (Affinity Bioreagents, Golden, CO). Brains were fixed overnight at 4°C with 4% paraformaldehyde in PBS at pH 7.0. Brains were then incubated sequentially at 4°C in PBS-TX (PBS with 0.3% Triton X-100) plus each of the following with extensive washes in PBS-TX between each: 2% normal donkey serum overnight; a 1:200 dilution of anti-NOS antibody for 24 hours; a 1:1,000 dilution of FITC-conjugated donkey anti-rabbit antibody (Jackson Labs, West Grove, PA) for 24 hours; and 2 μg propidium iodide/ml for 10 minutes. Brains were then dehydrated through an ethanol series, cleared in xylene, mounted in DPX (Fluka, Buchs, Switzerland), and examined using a BioRad MRC600 confocal microscope (BioRad, Hercules, CA).

The following modifiers of NO signaling were solublized according to the supplier's recommendations and used at the final concentrations indicated in the text: N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-D-arginine methyl ester (D-NAME), and 8-bromoguanosine 3′:5′-cyclic monophosphate (8-bromo-cGMP) (Sigma, St. Louis, MO); N^G-nitro-L-arginine (L-NNA), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), and 1H-[1,2,4]oxadiazolo[4,3,-alpha]quinoxalin-1-one (ODQ), (CalBiochem, La Jolla, CA); S-nitroso-N-acetylpenicillamine (SNAP) and spermine NONOate (Cayman, Ann Arbor, MI).

Analysis of the cell cycle

Cells in S phase were identified by the incorporation of 5-bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) followed by immunocytochemistry using an anti-BrdU monoclonal antibody (Gratzner, 1982). Cultured brains were labeled by addition of 15 μg BrdU/ml to the medium for 2 hours. Brains were fixed overnight at 4°C with 4% paraformaldehyde in PBS and then treated in 2 N HCl for 90 minutes to expose the BrdU epitope, as described previously (Champlin and Truman, 1998a). Brains were then incubated as above for NOS staining except that a 1:200 dilution of anti-BrdU monoclonal antibody (Becton-Dickinson, San Jose, CA) was used followed by a 1:1,000 dilution of horseradish peroxidase-conjugated donkey anti-mouse antibody (Jackson Labs, West Grove, PA). To detect the antibody, brains were then incubated in PBS-TX containing 0.5 mg diaminobenzedine/ml, 2 mg β-D-glucose/ml, 0.4 mg ammonium chloride/ml, and 0.2 units glucose oxidase. Brains were dehydrated through an ethanol series, cleared in xylene, and mounted in DPX (Fluka, Buchs, Switzerland). BrdU-labeled cells in the outer OA were counted at 200× magnification using a camera lucida.

Cells in M phase were identified with an antibody to phosphorylated histone H3 (Hendzel et al., 1997). Brains were fixed and processed for immunocytochemistry as above for NOS staining except that a 1:4,000 dilution of an anti-phospho-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY) was used.

NO histochemistry

NO production was measured in live cells using 5 μM DAF-2 DA (Kojima et al., 1998a,b) (Cal Biochem, La Jolla, CA). Cultured brains were placed in modified Miyazaki's saline (140 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 28 mM D-glucose, 5 mM Hepes, pH 7.4 (Trimmer and Weeks, 1989) plus 20 ng 20E/ml, and tungsten needles were used to cut and peel the outer OA off the optic lobe. The strip of cells was then mounted with the outer side down on a poly-L-lysine-coated slide. The tungsten needles were used to attach the edges of the strip to the slide, thereby flattening the preparation. Samples were examined using a BioRad MRC600 confocal microscope with the aperture fully open (BioRad, Hercules, CA). A 40× water immersion objective was used and solutions were exchanged without interrupting microscopy by withdrawing the solution from the slide using a drawn-out pipet and replacing it with 500 μl of the new solution. Evaporation was reduced by covering the objective and solution with a plastic cowling. Fluorescent images were collected at the intervals described in the text. Similar high levels of fluorescence were induced at the end of each experiment by addition of an exogenous NO donor (100 μM SNAP). To avoid experimenter bias, this final time point was used to select the appropriate region to be quantified for each sample. Analysis was restricted to regions along the center of the strip of cells because the signal along the edges was variable, most likely due to slight curling of the preparations. Fluorescent intensity was quantified using NIH Image (available online at http://rsb.info.nih.gov/nih-image). Regions containing about fifty cells were highlighted using the lasso tool and the mean density of fluorescence within this region was quantified at each time point using the Measure tool. Values were subtracted from background fluorescence over an area that had no cells.

RESULTS

Effect of NOS inhibitors on optic lobe proliferation

The continuing presence of ecdysteroid is needed for neurogenesis in the OA of *Manduca sexta*, and the proliferative response to ecdysteroid is regulated in an all-or-none fashion within each OA (Champlin and Truman, 1998a). Brains isolated from P+1 day pupae were cultured with various levels of 20E for 24 hours and then proliferating cells in S phase were identified by labeling with BrdU for 2 hours prior to fixation and anti-BrdU immunocytochemistry. BrdU-labeled cells were counted in the outer OA of each optic lobe (Fig. 1C, closed circles). A given optic lobe showed one of two proliferative

states: either very few (<26) or many (244-444) BrdU-labeled cells. With increasing levels of 20E, the optic lobe abruptly jumped from one state to the other. This transition began at about 60 ng 20E/ml and all optic lobes had high numbers of S phase cells at 100 ng 20E/ml. At a concentration of 60 ng 20E/ml, a given brain could show one optic lobe fully proliferating and the other quiescent (e.g., Fig. 1A). The all-or-none nature of this response suggested that local signaling plays a role in the decision of whether or not to proliferate.

As shown below, the OA contains high levels of NOS. We examined a possible role for NO signaling in coordinating the proliferative response to ecdysteroid by culturing brains in various concentrations of 20E and 1 mM L-NAME, an arginine analog that inhibits production of NO by NOS (Bredt and Snyder, 1994). In the presence of L-NAME, proliferation was still ecdysteroid-dependent (Fig. 1C), but a scattering of BrdU-labeled cells was now observed throughout each OA at 60 ng 20E/ml rather than the all-or-none response seen without NO inhibition (Fig. 1B,C). In addition, concentrations of 20 and 40 ng 20E/ml, which were subthreshold in control cultures, now stimulated proliferation in a dose-dependent fashion (Fig. 1C). Similar results were obtained when brains were cultured at 40 ng 20E/ml with a different NOS inhibitor, 1 mM L-NNA (84±24 BrdU-labeled cells/optic lobe compared with 8±5 in the absence of inhibitor and 11±6 when cultured with 1 mM D-NAME, the biologically inactive isomer of L-NAME; n=8, 10, and 10 optic lobes, respectively). These results suggest that NO is normally involved in coordinating the proliferative response to ecdysteroid in the optic lobe and that NO may act to inhibit proliferation.

NOS expression in the optic lobe

Since proliferation within the OA showed a coordinated response to ecdysteroid even when small pieces of the OA were cultured in isolation (Champlin and Truman, 1998a), we reasoned that the source of NO may be cells within the OA itself. NO is generated by NOS using NADPH as a cofactor. This “diaphorasing” activity is insensitive to fixation, allowing cells containing NOS to be identified (Hope et al., 1991). We observed strong diaphorase staining of the inner and outer OA in brains from P+1 day pupae (Fig. 2A,B). The levels of diaphorase staining were indistinguishable whether brains were cultured under conditions that either supported proliferation (100 ng 20E/ml) or led to proliferative arrest (20 ng 20E/ml). A similar distribution was observed by immunocytochemistry using an antibody directed against a region of NOS conserved between vertebrates and invertebrates, including *Manduca* (Nighorn et al., 1998). High levels of NOS immunoreactivity were present throughout both the inner and outer OA. The amount of NOS immunoreactivity appeared to be independent of the proliferative state of the tissue (Fig. 2C,D).

During early larval stages, neuroblasts in the OA undergo symmetric divisions to increase the number of neuroblasts. At the outset of the final (fifth) larval instar, the neuroblasts switch to the asymmetric divisions that lead to production of neurons (Monsma and Booker, 1996a). Proliferation of cells within the OA then becomes ecdysteroid-dependent 4 days later at the start of wandering (Champlin and Truman, 1998a). NOS was not detected in the OA late in the fourth larval instar (Fig. 3A,C) but NOS immunoreactivity was evident by day 1 of the fifth larval instar (Fig. 3B,D). Thus, NOS appears in the OA when the neuroblasts shift to the asymmetric divisions that produce the optic lobe neurons.

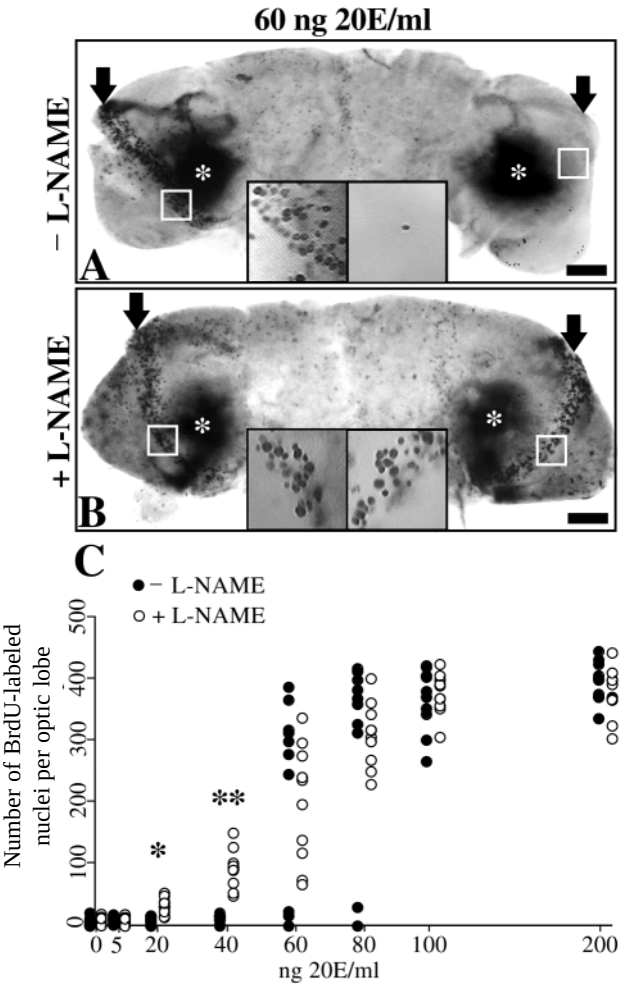

Fig. 1. Ecdysteroid-dependent proliferation in the presence and absence of an inhibitor of NOS. Brains were isolated from P+1 day pupae, desheathed and cultured for 24 hours in various concentrations of 20E. 15 μg BrdU/ml was added at 24 hours and cultures continued for 2 hours. Brains were then fixed and stained with an antibody against BrdU to identify cells that had incorporated BrdU. (A,B) Brains cultured with 60 ng 20E/ml. In B, 1 mM L-NAME was included from the start of culture. BrdU-labeled nuclei are black. Arrows point to the outer OA. Asterisks mark a pigmented region that is present in each optic lobe. In A one optic lobe shows full proliferation while the other is inactive. Scale bars, 100 μm. Insets show higher magnification views of the white-boxed region in each optic lobe. (C) The number of BrdU-labeled nuclei in the outer OA after various treatments. Each point represents BrdU-labeled cells from one optic lobe. Twenty optic lobes were counted at each concentration of 20E, ten cultured with L-NAME (open circles) and ten without (closed circles). *, P<0.05, and **, P<0.005, unpaired Student’s t-test comparing brains cultured with and without L-NAME.

Effects of NO on proliferation in the OA

When brains are cultured in subthreshold ecdysteroid, proliferating cells in the OA arrest late in the G_2 phase of the cell cycle, but they begin entering mitosis within 2 hours after

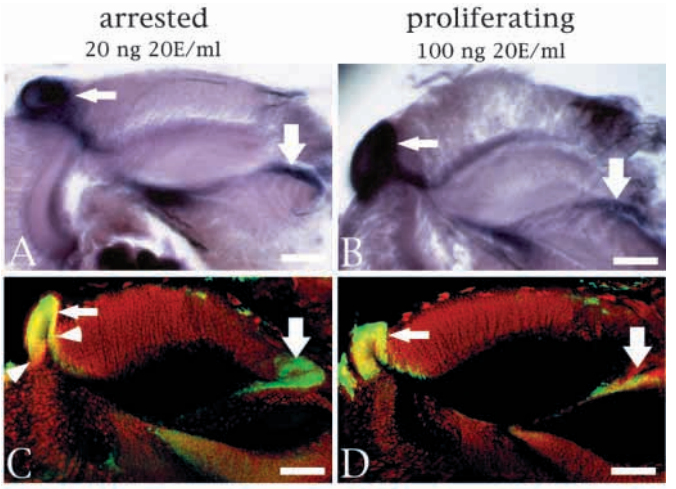

Fig. 2. NOS expression in the optic lobe. P+1 day pupal brains were isolated and cultured for 24 hours in the indicated concentration of 20E. Brains were then fixed and sectioned (100 μM horizontal Vibratome sections) to show cross sections through the outer OA (narrow arrows) and inner OA (wide arrows). (A,B) Diaphorase staining of the outer and inner OA. (C,D) Confocal images of sections stained with a NOS antibody (yellow-green signal). Red signals are propidium iodide-stained nuclei. Arrowheads in C show the approximate positions of cuts used to remove the outer OA in later experiments. Each panel is typical of *n*=5. Scale bars, 50 μm.

being shifted to suprathreshold levels of the steroid (Champlin and Truman, 1998a). This rapid response allowed us to examine the effect of NO donors on ecdysteroid-dependent entry into mitosis. Proliferation was first arrested by culturing brains from P+1 day pupae for 24 hours in subthreshold ecdysteroid (20 ng 20E/ml). Brains were then switched to high levels of 20E (500 ng/ml). To assess the cumulative number of cells that entered mitosis following this increase, we included the mitotic inhibitor, colcemid (30 μg/ml), which blocks the cells in prometaphase (Champlin and Truman, 1998a). Many cells in the outer OA had entered mitosis within 4 hours following the switch to high levels of 20E as evidenced by staining with the mitotic marker, anti-phospho-histone H3 antibody (Hendzel et al., 1997) (Fig. 4A). In contrast, few mitotic cells were observed when arrested brains were pre-incubated for 10 minutes with an NO donor, (10 μM of either SNAP or spermine NONOate), and then shifted to high levels of 20E plus colcemid and the NO donor (Fig. 4B). The repressive effect of SNAP was eliminated when 1 mM PTIO,

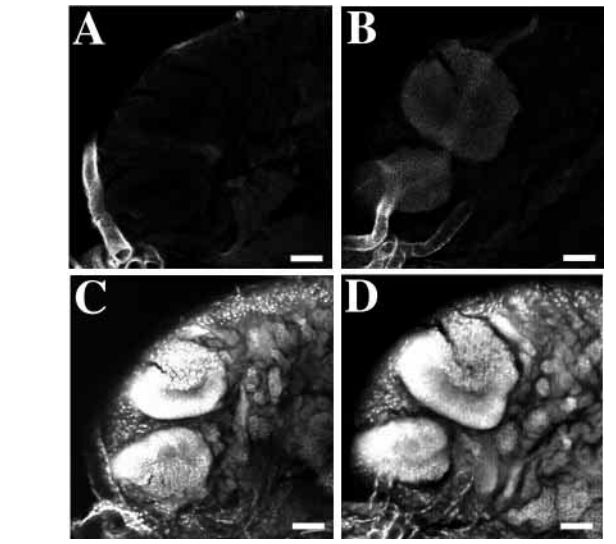

Fig. 3. Confocal images showing immunostaining for NOS (A,B) and propidium iodide-labeled nuclei (C,D) in the growing OA of larval brains. **(**A,C) Day 3 4th instar larval brain just prior to the molt to the 5th instar shows no NOS immunostaining. (B,D) In day 1 5th instar larval brains, NOS antibody staining is now evident in the OA. Each panel is typical of *n*=5. Fluorescence of trachea in (A,B) is due to autofluorescence of the cuticle. Lateral is to the left. Scale bars, 40 μm.

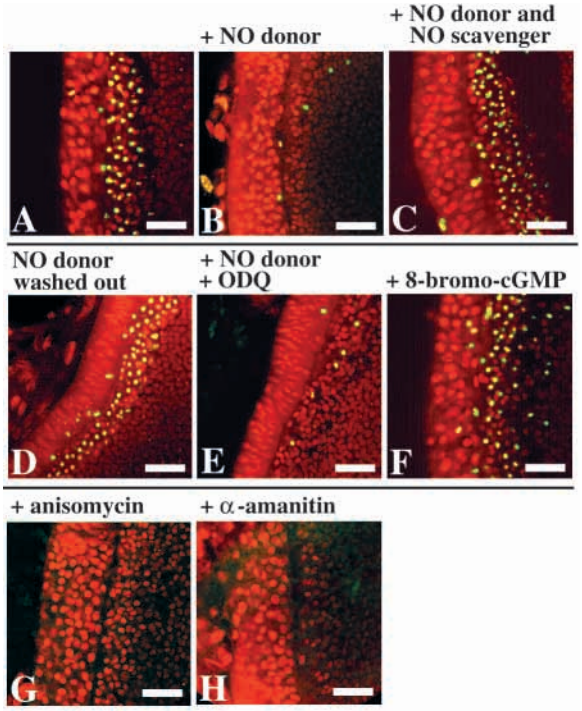

Fig. 4. Regulation of ecdysteroid-dependent entry into mitosis. Brains were isolated from P+1 day pupae and cultured for 24 hours in 20 ng 20E/ml to allow cells to arrest in G_2. Brains were then shifted to 500 ng 20E/ml, to stimulate entry into mitosis, plus 30 μg/ml colcemid to prevent cells from passing beyond prometaphase. After 4 hours, brains were fixed and stained with antibody to phospho-histone H3 (yellow-green signals). Red signals are propidium iodide nuclei. High magnification confocal views are shown of the outer OA perpendicular to the orientation shown in Fig. 2 and focused on neural precursors along the medial edge of the outer OA. (A) High numbers of cells have entered mitosis by 4 hours after the increase to 500 ng 20E/ml. (B) As A, exept 10 μM SNAP was added 10 minutes before 20E levels were increased. (C) As B, except 1 mM of the NO scavenger PTIO was added with the SNAP. (D) As B, except brains were rinsed repeatedly to wash out the SNAP and then maintained for an additional 4 hours in 500 ng 20E/ml plus colcemid. (E) As B, except that 100 μM ODQ was added with the SNAP. F) As A except 10 μM 8-bromo-cGMP was added 1 hour before 20E levels were increased. (G) As A except 5 μg anisomycin/ml was added 30 minutes before 20E levels were increased. (H) As A, except 30 μg α-amanitin/ml was added 30 minutes before 20E levels were increased. Each panel is typical of *n*=5. Scale bars, 20 μm.

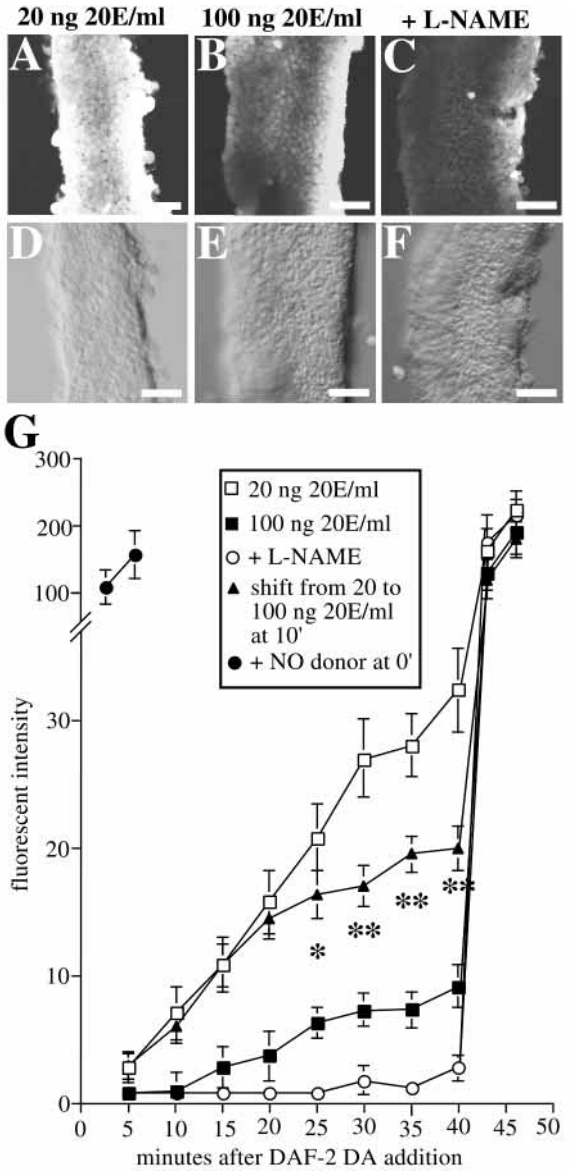

Fig. 5. Ecdysteroid represses NO production by cells in the outer OA. Brains from P+1 day pupae were desheathed and cultured 24 hours in 20 ng 20E/ml to allow proliferation to cease. The outer OA were then microdissected, mounted onto coverslips and incubated with various concentrations of 20E or L-NAME. NO production was assessed by adding the NO indicator, DAF-2 DA, and measuring fluorescence intensity in arbitrary machine units at 5 minute intervals using confocal microscopy. 40 minutes later, high levels of an exogenous NO donor (100 μM SNAP) were added as a positive control to show that the fluorescent marker was present in cells. Fluorescent intensity was measured at 3 minutes and 6 minutes after adding SNAP. (A-C) Fluorescence was measured 40 minutes after addition of DAF-2 DA to the outer OA, (A) maintained in 20 ng 20E/ml, (B) switched to 100 ng 20E/ml 1 hour prior to adding DAF-2 DA, and (C) switched to 20 ng 20E/ml plus 1 mM L-NAME 1 hour prior to adding DAF-2 DA. D-F) Differential contrast images of the outer OA shown in A-C, respectively. Scale bars, 20 μm. (G) Fluorescence intensity of outer OA over time in response to different treatments. DAF-2 DA was added at time 0. In each case, 100 μM SNAP was added at 40 minutes and fluorescence measured 3 minutes and 6 minutes later. Closed circles = DAF-2 DA and SNAP were added simultaneously and fluorescence measured 3 minutes and 6 minutes later. Open squares=outer OA maintained in 20 ng 20E/ml. Open circles=1 mM L-NAME added 1 hour prior to adding DAF-2 DA. Closed squares = outer OA shifted to 100 ng 20E/ml 1 hour prior to adding DAF-2 DA. Closed triangles = outer OA shifted to 100 ng 20E/ml 10 minutes after adding DAF-2 DA. *n*=5 outer OA from independent brains for each point. Error bars are s.d.

a scavenger of NO, was included in the incubation (Fig. 4C). The repressive effect of NO was not simply due to general toxicity because the cells entered mitosis within 4 hours after the SNAP was washed out (Fig. 4D).

A common mechanism of NO action on target cells is through the activation of soluble guanylate cyclase leading to an increase in cGMP levels (Bredt and Snyder, 1994). However, cGMP was not detected in the OA at any stage of optic lobe development by staining with an antibody to cGMP (Schactner et al., 1998; J. Schactner and J. W. T., unpublished results). Also, inclusion of 100 μM ODQ, an inhibitor of soluble guanylate cyclase (Garthwaite et al., 1995), did not overcome the block caused by 10 μM SNAP (Fig. 4E). Furthermore, culturing arrested brains with the membrane permeable cGMP analog, 8-bromo-cGMP, at 10 μM, did not block ecdysteroid-dependent entry into mitosis (Fig. 4F).

Effects of ecdysteroid on NO production in the OA

Since an inhibitor of NO production allowed proliferation to occur at reduced levels of 20E (Fig. 1) and since exogenous NO inhibited entry into mitosis even in the presence of high levels of 20E, we reasoned that 20E might act to inhibit NO production by cells of the OA. This inhibition would have to be rapid since cells begin to enter mitosis by 2 hours after a shift to suprathreshold levels of the steroid. We used the intracellular fluorescent NO-indicator, DAF-2 (Kojima et al., 1998a,b; Zayas et al., 1999), to measure ongoing NO production by cells of the OA. DAF-2 DA diffuses into cells and is cleaved by esterases to DAF-2, trapping it in the cell. Within the cell, reaction of DAF-2 to form the triazolofluorescein, DAF-2T, yields more than a 180-fold increase in fluorescence (Kojima et al., 1998a). To facilitate rapid loading of DAF-2 DA into the cells of the OA, the outer OA was microdissected from the optic lobe using tungsten needles and then mounted as a flat preparation on a microscope slide. The resulting strip of cells contains most of the cells within the outer OA that stained with the anti-NOS antibody (see arrowheads in Fig. 2C for approximate locations of the cuts). Following addition of 5 μM DAF-2 DA, fluorescence was monitored at intervals by confocal microscopy. Rapid loading of DAF-2 DA into the cells was confirmed following addition of an NO donor (100 μM SNAP). Similar high levels of DAF-2 fluorescence were detected 3 minutes after addition of SNAP whether it was added at the same time as DAF-2 DA or 40 minutes after DAF-2 DA (Fig. 5G).

To examine the effect of ecdysteroid on NO production, brains from P+1 day pupae were first arrested by culture in 20 ng 20E/ml for 24 hours. The outer OAs were then microdissected, maintained in 20 ng 20E/ml and 5 μM DAF-2 DA was added. Samples showed a steady increase in fluorescence over at least 40 minutes following addition of DAF-2 DA (Fig. 5A,D,G; open squares). The DAF-2T produced from the reaction of DAF-2 is quite stable (Kojima

et al., 1998a). In our preparations, the addition of the NOS inhibitor, L-NAME at 1 mM, 10 minutes after the DAF-2 DA resulted in a leveling out of the fluorescence that then remained stable over the next 40 minutes (n=3; not shown). Our schedule of repeated scanning of the OA also did not appear to have a deleterious effect on the DAF-2T or the cells because similar levels of fluorescence were obtained 40 minutes after addition of DAF-2 DA whether samples were examined every five minutes or just after 40 minutes.

When arrested OAs were shifted to a suprathreshold concentration of 20E (100 ng/ml) 1 hour prior to adding DAF-2 DA, the subsequent increase in fluorescence was again linear but was decreased four fold relative to 20 ng 20E/ml (Fig. 5B,E,G; closed squares). This reduced signal still appeared to reflect specific accumulation of NO, however, because it was significantly higher than that seen when NO production was blocked by incubating with 1 mM L-NAME for 1 hour prior to adding DAF-2 DA and maintaining the cells in L-NAME (Fig. 5C,F,G; open circles). The time-course by which suprathreshold 20E levels could suppress NO production was determined by loading DAF-2 DA into isolated outer OA arrested in 20 ng 20E/ml and then, 10 minutes later, raising the concentration of 20E to 100 ng/ml (Fig. 5G; triangles).

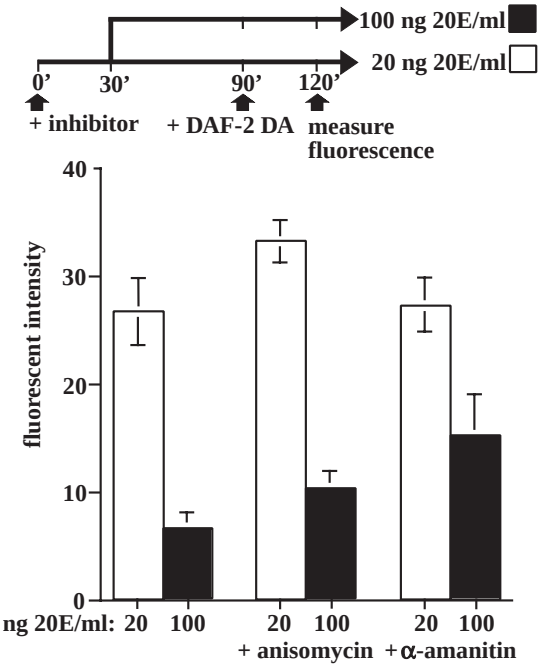

Fig. 6. Effect of 20E on NO levels in the presence of RNA or protein synthesis inhibitors. Brains were isolated from P+1 day pupae, desheathed and cultured 24 hours in 20 ng 20E/ml to allow proliferation to cease. Outer OAs were then microdissected and mounted onto slides. The time line summarizes the timing of subsequent treatment. At time 0, either a protein synthesis inhibitor (5 μg anisomycin/ml), or an RNA synthesis inhibitor (30 μg α-amanitin), or nothing was added. 10 OAs were used for each treatment. At 30 minutes, half the OAs from each group were shifted to 100 ng 20E/ml plus the inhibitor while the rest were maintained at 20 ng 20E/ml. DAF-2 DA was added 1 hour later and fluorescence intensity measured after 30 minutes. Average values for each treatment are plotted. n=5 outer OA from independent brains for each treatment. Error bars are s.d.

Fluorescence was significantly reduced within 15 minutes compared to samples maintained in 20 ng 20E/ml (P<0.05, Student's t-test). Thus, suprathreshold levels of 20E cause a rapid decrease in NO production well before the cells would normally enter mitosis.

The effect of 20E on NO production was so rapid that it suggested the steroid might be acting through a non-genomic signaling mechanism. To test this, we incubated outer OA with 30 μg α-amanitin/ml or 5 μg anisomycin/ml to inhibit RNA or protein synthesis, respectively. Culturing intact brains with this level of anisomycin blocked 95% of protein synthesis and this level of α-amanitin was sufficient to block induction of mRNA for the ecdysteroid-inducible early gene, *MHR3* (unpublished results). Neither inhibitor, though, caused toxicity as judged by the ability of cells in the OA to resume proliferation when the inhibitors were washed out after 6 hours of treatment. Outer OAs were isolated from arrested brains as above and maintained in 20 ng 20E/ml. One of the inhibitors was added and, 30 minutes later, 20E levels were either raised to 100 ng/ml or maintained at 20 ng/ml. DAF-2 DA was added 1 hour later and the amount of NO produced over the next 30 minutes was measured by DAF-2 fluorescence. Although neither inhibitor by itself caused a significant decrease in NO production, a decrease in NO production occurred when steroid levels were increased to 100 ng 20E/ml in the presence of either inhibitor (Fig. 6). Thus, the rapid decrease in NO production caused by increased levels of the steroid does not appear to require either RNA or protein synthesis.

Although inhibitors of protein and RNA synthesis did not prevent 20E from supressing NO production, these inhibitors nevertheless prevented entry of the cells into mitosis. This effect was demonstrated using brains from P+1 day pupae that had been cultured for 24 hours in 20 ng 20E/ml. The arrested brains were pretreated with either 30 μg α-amanitin/ml or 5 μg anisomycin/ml for 30 minutes followed by a shift to 500 ng 20E/ml plus 30 μg/ml colcemid and the inhibitor. No cells had entered mitosis by 4 hours after the increase in steroid as judged by the absence of phosphohistone H3 staining in the OA (Fig. 4G,H).

DISCUSSION

Proliferation of neural precursors in the optic lobe of *Manduca* is regulated by both endocrine and local signals. Ecdysteroids circulating in the hemolymph are required to stimulate entry into mitosis (Champlin and Truman, 1998a). However, there is a second, local control over proliferation that is exerted by NO. Diaphorase staining, anti-NOS immunocytochemistry and the NO-indicator, DAF-2, show that cells throughout the OA contain high levels of NOS and produce NO. Signaling via NO suppresses proliferation in the OA as indicated both by the decrease in the threshold amount of steroid needed for proliferation in the presence of the NOS inhibitor, L-NAME, and by the ability of exogenous NO to block the proliferative response to ecdysteroid.

We interpret the scattered BrdU incorporation seen at low levels of 20E, when NO production was blocked with L-NAME, as showing cells that were above their steroid threshold needed to enter mitosis. The number of these "competent" cells increased progressively as 20E was

increased from 20 ng to 100 ng/ml in the presence of L-NAME. This same shift in steroid concentration brought about a reciprocal decline in NO production in the OA as measured by the NO-indicator, DAF-2 (Fig. 5). Based on these observations, we propose that at low steroid levels (e.g., 20 ng and 40 ng/ml), some cells in the OA have been exposed to sufficient ecdysteroid to initiate proliferation, but NO levels are high enough to inhibit their entry into mitosis. At 60 ng 20E/ml, the number of these competent cells has increased and NO production has now been suppressed by ecdysteroid to low enough levels in some OAs that these cells are allowed to enter mitosis. Considering that NO diffuses readily through tissue, we expect that a given cell is influenced by NO produced by its neighbors as well as by itself. Hence, NO would establish a "majority rules" situation in which the response to the steroid within a given OA would essentially be all or none.

In both *Drosophila* and *Manduca*, ingrowing photoreceptor axons stimulate proliferation in a subset of OA cells and provide one way of coordinating development of the eye with the optic lobe (see Introduction). In addition, ecdysteroids provide a means to coordinate development throughout the entire visual system including developing layers that are not in direct contact. For example, ecdysteroid is required to sustain the movement of the morphogenetic furrow and, hence, the progressive formation of rows of photoreceptors in the imaginal eye disc (Brennan et al., 1998; Champlin and Truman, 1998b). The same ecdysteroid requirements were found for proliferation of precursors for the medulla and lobula as well as the lamina optic lobe neurons (Champlin and Truman, 1998a). In this context, the function of NO signaling within the OA appears to be to sharpen the proliferative responsiveness of cells to the steroid so that optic lobe neuron production is tightly coordinated along the entire length of the OA and progresses in unison with photoreceptor production in the eye disc. When NO signaling is inhibited, a five-fold increase in the 20E titer (20 to 100 ng/ml) is needed to go from the first signs of proliferation to full proliferation. In the presence of NO signaling, this range is reduced to just two-fold (see Fig. 1C). This may be of particular importance in *Manduca* since metamorphic development of the visual system is reversibly interrupted during pupal diapause when environmental cues act to suppress the ecdysteroid titer (Champlin and Truman, 1998a).

In controlling proliferation within the OA, the ecdysteroid and NO pathways intersect at two levels (Fig. 7). They both appear to act at the target cell level in controlling entry into mitosis with ecdysteroid promoting entry and NO inhibiting it. However, ecdysteroid also acts upstream by suppressing NO synthesis. NO production decreases rapidly (within 15 minutes) in response to an increase in 20E and even if RNA or protein synthesis is blocked. Ecdysteroid suppression of NO production, therefore, appears to occur through a rapid, non-genomic signaling mechanism.

Regulation of proliferation by NO and steroid

NO has been shown to inhibit proliferation in a variety of cell types, the best characterized of which are the endothelium and smooth muscle of vertebrate blood vessels. NO produced by endothelial cells inhibits proliferation both in the endothelium itself (Yang et al., 1994; Sarkar et al., 1995) and also the overlying smooth muscle cells (Garg and Hassid, 1989; Ishida et al., 1999). Although it is well established that endothelial-derived NO acts as a vasodilator on smooth muscle cells via a cGMP-mediated pathway (Bredt and Snyder, 1994), conflicting reports have been published for both endothelial cells and smooth muscle cells as to whether NO also acts through cGMP to inhibit proliferation (Heller et al., 1999; Ishida et al., 1999 and references therein). In the OA, we found no evidence for an involvement of cGMP in the inhibition of proliferation by NO. Ecdysteroid-dependent entry into mitosis was blocked by incubation with an NO donor even when the soluble guanylate cyclase inhibitor, ODQ, was included to block production of cGMP. Furthermore, the cGMP analog, 8-bromo-cGMP, was not able to mimic the inhibitory effect of NO. Consistent with this, cGMP has not been detected in the OA during development or in response to NO treatment. An alternative target for NO signaling within the OA may be through the nitrosylation of target cell proteins (Stamler, et al., 1997).

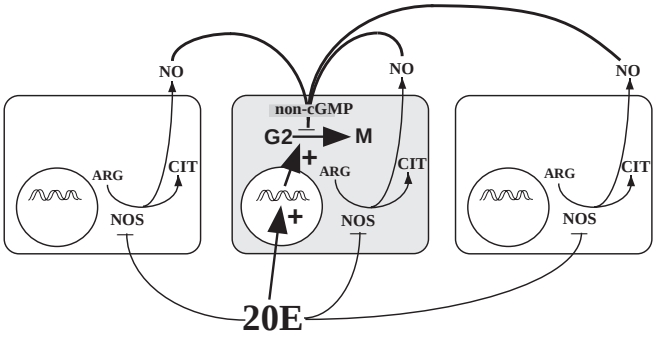

Fig. 7. Summary of regulation of proliferation within the outer OA by ecdysteroid and NO. The schematic depicts three neural precursor cells within the OA, focusing on the factors that regulate entry into mitosis of the central, shaded cell. 20E acts via a non-genomic pathway to repress NOS activity. Since NOS is expressed throughout the outer OA, the NO levels for a given cell are likely the product of itself and neighboring neural precursors. NO can repress entry into mitosis even in the presence of high levels of 20E via a pathway independent of cGMP. On the other hand, ecdysteroid is required to stimulate entry into mitosis, even when NO production is reduced using an inhibitor of NOS. Ecdysteroid-dependent entry into mitosis requires RNA and protein synthesis suggesting that this stimulatory pathway acts through a genomic mechanism, presumably involving the nuclear receptor for 20E.

The exact relationship between NO repression and steroid regulation of the cell cycle is not yet known in either vertebrates or invertebrates. In principle, these opposing signals could interact at any level between the steroid receptor and the regulated cell cycle factors. In any case, NO repression in the OA of *Manduca* does differ from that seen in vertebrate in at least one respect. In the latter, the arrest typically occurs during the G_1 phase of the cell cycle (e.g., Gansauge et al., 1998; Ishida et al., 1999) while in the OA it is late in the G_2 phase.

Regulation of NO production by steroids

The classical pathway for steroid hormone signaling in both vertebrates and invertebrates is through receptors that are hormone-dependent transcription factors, all of which are members of the highly conserved nuclear receptor gene family

(Mangelsdorf et al., 1995). Vertebrate steroid hormones have been shown to modulate the levels of NOS protein or mRNA in a variety of cell types (Wang and Morris, 1998; Warembourg et al., 1999, for recent examples), but since the activity of some forms of NOS are Ca^{2+}/calmodulin-dependent and regulated by co- and post-translational modifications (Bredt and Snyder, 1994), increases in NOS protein would not necessarily lead to increased NO production. Recent experiments, though, show that steroids can also control NOS activity. For example, treatment of endothelial cells with estrogen causes rapid increases in DAF-2 fluorescence (Goetz et al., 1999) and citrulline production (NOS converts arginine to NO and citrulline) via a non-genomic pathway (Shaul, 1999). A plasma membrane-localized form of ERα, one of the nuclear receptors for estrogen, has been identified in cell lines (Watson et al., 1999) and has been implicated in this non-genomic signaling between estrogen and NOS in endothelial cells (Kim et al., 1999; Shaul, 1999).

While signaling via non-genomic pathways is well established for several of the vertebrate steroids (Wehling, 1997), little is known about the potential for non-genomic signaling by ecdysteroid in invertebrates (reviewed by Tomaschko, 1999). Our findings that L-NAME released a block to proliferation at low ecdysteroid levels while exogenous NO could block entry into mitosis at high levels of the steroid provided indirect evidence that ecdysteroid inhibits NO production. Use of the NO indicator, DAF-2, allowed us to show directly that NO production decreased when cells of the outer OA were shifted to elevated levels of 20E, and furthermore, this appears to occur via a non-genomic signaling pathway. The similarities between our findings and those reported for estrogen on endotheilial cell proliferation suggest that non-genomic signaling may be a conserved feature of steroid action between vertebrates and invertebrates. Interestingly, while increasing estrogen levels increased NO production (Shaul, 1999), we found that increases in ecdysteroid caused a decrease in NO production in the OA.

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