Localization of nitric oxide synthase-like immunoreactivity in the developing nervous system of the snail *Ilyanassa obsoleta*

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Abstract:

Production of nitric oxide (NO), an evolutionarily conserved, intercellular signaling molecule, appears to be required for the maintenance of the larval state in the gastropod mollusc *Ilyanassa obsoleta*. Pharmacological inactivation of endogenous nitric oxide synthase (NOS), the enzyme that generates NO, can trigger metamorphosis in physiologically competent larvae of this species. Neuropils in the brains of these competent larvae display histochemical reactivity for NADPH diaphorase (NADPHd), an indication of neuronal NOS activity. The intensity of NADPHd staining is greatest in the neuropil of the apical ganglion (AG), a region of the brain that contains the apical sensory organ and that innervates the bilobed ciliated velum, the larval swimming and feeding organ. Once metamorphosis is initiated, the intensity of NADPHd staining in the AG and presumably, concomitant NO production, decline. The AG is finally lost by the end of larval metamorphosis, some 4 days after induction. To determine if the neurons of the AG are a source of larval NO, we conducted immunocytochemical studies on larval *Ilyanassa* with commercially available antibodies to mammalian neuronal NOS. We localized NOS-like immunoreactivity (NOS-IR) to 3 populations of cells in competent larvae: somata of the AG and putative sensory neurons in the edge of the mantle and foot. Immunocytochemistry on pre-competent larvae demonstrated that numbers of NOS-IR cells in the AG increase throughout the planktonic larval stage.

Article:

Introduction

Investigations into the actions of NO during organismal development have begun to reveal some broad patterns of nitrergic activity in developing nervous systems. NO production is often transient (Bredt & Snyder, 1994; Wu *et al.*, 1994; Truman *et al.*, 1996; Scholz *et al.*, 1998; Cramer & Sur, 1999) and can control aspects of cellular proliferation in arthropod and vertebrate nervous systems (Peunova & Enikolopov, 1995; Kuzin *et al.*, 1996, 2000; Gibbs & Truman, 1998; Enikolopov *et al.*, 1999), most likely through its ability to regulate gene expression (Haby *et al.*, 1994; Morris, 1995; Peunova & Enikolopov, 1995; Pilz *et al.*, 1995; Enikolopov *et al.*, 1999; Kuzin *et al.*, 2000). NO can inhibit DNA synthesis, altering rates of cellular passage through mitosis (Sarkar *et al.*, 1995, 1997a,b; Bundy *et al.*, 2000; Chen *et al.*, 2000) and inhibit forms of programmed cell death (PCD) (Farinelli *et al.*, 1996; Kim *et al.*, 1997; Mohr *et al.*, 1997; Thippeswamy & Morris, 1997; Estévez *et al.*, 1998), actions which, if they occur during neural development, can alter organ size and patterns of neural connectivity (Kuzin *et al.*, 1996,2000;Gibbs &Truman, 1998; Wright *et al.*, 1998; Enikolopov *et al.*, 1999; Schachtner *et al.*, 1999; Cogen & Cohen-Cory, 2000; Bicker, 2001). As implied, studies on developmental actions of NO have largely been conducted on arthropod and vertebrate model organisms, with relatively few studies focusing on nitrergic actions in the development of other animal phyla (Jacklet, 1997; Bishop *et al.*, 2001). In particular, in the Mollusca, NOS activity, as demonstrated by NADPHd histochemistry, occurs in the central nervous systems (CNSs) of larval and juvenile *Ilyanassa obsoleta* (Lin & Leise, 1996b), in metamorphosing and post-metamorphic encapsulated embryos of the pond snail *Lymnaea stagnalis* (Serfözö *et al.*, 1998) and in larvae of the nudibranch *Phestilla sibogae* (Meleshkevitch *et al.*, 1997).

In the developing nervous system of *Ilyanassa*, NAD-PHd staining is apparent in all ganglionic neuropils before metamorphosis and decreases dramatically once metamorphosis begins (Lin & Leise, 1996b). In larvae, staining is especially pronounced in the AG, a ganglion of some 24–26 neurons that has been shown, in related gastropods, to innervate the velum, the larval feeding and swimming organ (Kempf *et al.*, 1997; Marois & Carew, 1997a–c). In *Ilyanassa*, as in other gastropod molluscs, the AG houses the apical sensory organ (ASO), which contains a number of sensory neurons, including 3–6 that are serotonergic (Kempf *et al.*, 1997; Marois & Carew, 1997a; Page & Parries, 2000). Recent experiments strongly suggest that sensory neurons in the ASO of *P. sibogae* can detect metamorphic cues (Hadfield *et al.*, 2000), a function that has long been postulated for the ASO (Bonar, 1978; Chia & Koss, 1982,1984). The ASO is apparently an ontogenetic outgrowth of the trochophore apical tuft, which has also been postulated to have a sensory function (Raven, 1966), although little physiological work exists to support this idea. In juvenile *Ilyanassa*, the AG is lost by the 4th day after metamorphic induction (Lin & Leise, 1996a) and in the abalone *Haliotis rufescens* and the sea hare *Aplysia californica*, its neurons are presumed to disappear through a form of programmed cell death (PCD) rather than by incorporation into the subjacent cerebral ganglia (Barlow & Truman, 1992; Marois & Carew, 1997b).

Experimental application of NO-donors and pharmacological manipulation of endogenous NO levels in competent larvae of *Ilyanassa* have demonstrated that NO can inhibit serotonergically induced metamorphosis, supporting the idea that NO is needed to maintain the larval state (Froggett & Leise, 1999). Presumably, this nitrergic action is reflected in the rise of NADPHd activity seen during larval development in the neuropil of the AG and in the decline of staining intensity that occurs during metamorphosis. However, the cellular source of larval NO has been unknown; NADPHd staining in the nervous systems of competent *Ilyanassa* failed to reveal any neuronal somata (Lin & Leise, 1996b). To determine if neurons of the AG might be nitrergic, we used commercially available mammalian anti-neuronal NOS antibodies to study NOS expression in larval *Ilyanassa*. Results of our immunocytochemical procedures revealed NOS-IR cells in the AG and in 2 additional sites in the larval epithelium.

Methods

Adult *Ilyanassa* were collected from coastal mudflats in Wilmington, NC and maintained in the laboratory in aerated tanks. Animals were fed daily, which promoted spawning for most of the year. Detailed descriptions of larval culture methods have been published previously (Couper & Leise, 1996; Leise, 1996; Froggett & Leise, 1999; Leise *et al.*, 2001). In the laboratory, at 24–25°C, larvae require at least 16–18 days of culture to become competent (Scheltema, 1961, 1962; Leise, 1996) and they continue to grow during this competent period. To estimate competence, we measure shell lengths on 10–15 animals, randomly selected from each culture. At shell lengths between 560–650 μm, over85% of larvae will typically metamorphosis in response to 10^{-4} M serotonin. We now use a shell length of 600 μm as a benchmark of 100% of larval development. However, because larvae grow during competence and because larvae within cultures vary in size, we use animals above 100% of development in our experiments. Several stages were used for immunocytochemistry, including larvae at 6 days after hatching (about 380 μm shell length or 63% of development), at 12 days after hatching (about 550 μm shell length or 92% of development) and at competence (93–108 % of development). These stages were chosen because their larvae display significant neuroanatomical differences from younger and older animals (Lin & Leise, 1996a).

IMMUNOCYTOCHEMICAL PROCEDURES

Larvae were decalcified overnight at room temperature in calcium-free or low pH (6.0) artificial seawater (Cavanaugh, 1956), rinsed in 0.2 μm filtered Instant Ocean (FIO) for 1 h, then fixed in a chilled vial containing a cold mixture of 4% paraformaldehyde in 0.2 M MPB (Millonig's phosphate buffer with 0.14 M sodium chloride; Kempf *et al.*, 1997). Larvae were immediately transferred to a refrigerator at 7C for 2 h, then inspected for autofluorescence. Specimens with high background were discarded. Others were washed twice with ice cold 0.2 M MPB at 20 minute intervals. After being washed, specimens were made permeable by dehydration in an ethanol series to xylene, on ice, and then were rehydrated. Specimens were then rinsed twice at 10 min intervals in 20 mM phosphate buffer solution with 0.1% Triton X-100, 0.1% sodium azide, and 0.14 M sodium chloride (PBS+), as this reduced background fluorescence more than typical rinses in blocking

medium (BM, 3% goat serum and PBS+; Kempf *et al.*, 1997). Specimens were then transferred to a 24-well plastic tissue culture plate and left in BM overnight, in the dark, on a shaker, at 6°C. All subsequent incubations occurred in the dark, shaken, and cold. The BM was then removed, replaced by a solution of the primary antibody, whole IgG, mammalian anti-neuronal NOS (BIOMOL Research Laboratories, Inc., #SA-202) at 1: 400, in BM, and incubated overnight. A control group of specimens containing only the secondary antibody was also produced, which showed no non-specific binding of the secondary antibody.

Following incubation with the primary antibody, specimens were washed for 6, 1 h rinses with PBS+ to reduce background and remove excess primary antibody. A final rinse occurred overnight in PBS+. Specimens were incubated in a goat-anti-rabbit secondary antibody conjugated to either fluorescein or Texas Red (Vector Laboratories) that was diluted in BM at 6.5 μl/ml. Finally, specimens were rinsed in PBS+ 5 to 8 times at 45 min intervals then kept overnight in PBS+ in the refrigerator.

PREADSORPTION CONTROL

A control peptide solution (500 μg/mL of control peptide and a 1: 400 dilution of the BIOMOL #SA-202 anti-neuronal NOS primary antibody in BM) was incubated overnight in the dark at 6°C. After incubation, the solution was centrifuged at 100,000× g for 30 min at 4°C in a Beckman TL-100 Tabletop Ultracentrifuge with a Beckman TLA-100.2 fixed angle rotor. The supernatant was added to the specimens during the primary antibody incubation step and the remainder of the protocol was conducted as described above.

WHOLE MOUNT PREPARATION

Specimens to be viewed were placed in mixtures of 20 %, 50 % and 80% glycerol in FIO for 15 minutes each. They were then mounted on a slide in 80% glycerol in FIO under a coverslip supported by plasticine feet and viewed under a fluorescence microscope equipped with a 470–490 nm exciter filter, 500 nm beam splitter and 515 nm emission filter for fluorescein isothiocyanate or with a 533–590 nm exciter filter for Texas Red specimens. Specimens were photographed with a Kodak DCS 420c digital camera, images were adjusted with Adobe Photo-shop, stored in digital format on compact disks, and printed with a Kodak DS 8650 color dye sublimation printer.

SECTION PREPARATION

Specimens to be sectioned were embedded in Spurr's (1969) resin after immunocytochemistry (ICC) was completed and sectioned at 10 μm on a Reichert-Jung 1040 Autocut microtome. Sections were mounted in Fluoromount (BDH Laboratories Supply), a non-fluorescing mounting medium and viewed under Differential Interference Contrast and fluorescence optics as appropriate, then photographed. Files were handled as described above.

SPECIFICITY

Specificity of NOS staining was tested by comparing specimens prepared with the BIOMOL primary antibody (#SA-202) to specimens prepared with similar mammalian anti-NOS antibodies from Affinity BioReagents, Inc., Chemicon Int'l., Inc., and Calbiochem-Novabiochem Corp. Similar results were detected with the 3 additional antibodies, supporting the idea that the antibodies were binding to neuronal NOS proteins.

Results

Whole mounts of metamorphically competent larvae displayed 3 major populations of NOS-IR cells (Fig. 1). In the anterior portion of the larval head, 2 clusters of neuronal somata, each with about 13 cells, were revealed with the BIOMOL antibody (Fig. 1A). In sections, these clusters of immunoreactive neurons were found to lie in the apical ganglion, above the cerebral commissure (Fig. 2). Few neural processes were immunoreactive. In addition to the NOS-IR somata of the AG, we routinely detected 6–8 putative sensory neurons along the edge of the mantle (Fig. 1B). Each cell had a process that extented to the surface of the epithelium, but no axonal projections could be discerned. A similar set of 4–5 putative sensory neurons occurred in the edge of the propodium, the anterior portion of the foot (Fig. 1C). Again, cells displayed dendritic processes but no projections into the CNS. Pre-absorption of the primary antibody with the NOS control peptide extinguished all

immunoreactivity (data not shown). Because results with all other commercial primary antibodies displayed only subsets of the neurons we observed with the BIOMOL antibody, but never additional immunoreactivity, we did not pursue results with these other antibodies. As examples, ICC with an anti-neuronal NOS antibody obtained from Calbiochem-Novabiochem revealed low levels of NOS-IR in 8–10 cells of the apical ganglion, while ICC with the Chemicon primary antibody revealed only 3 or 4 punctate structures in the mantle edge, instead of the 6–8 cells typically seen with the BIOMOL anti-neuronal NOS antibody.

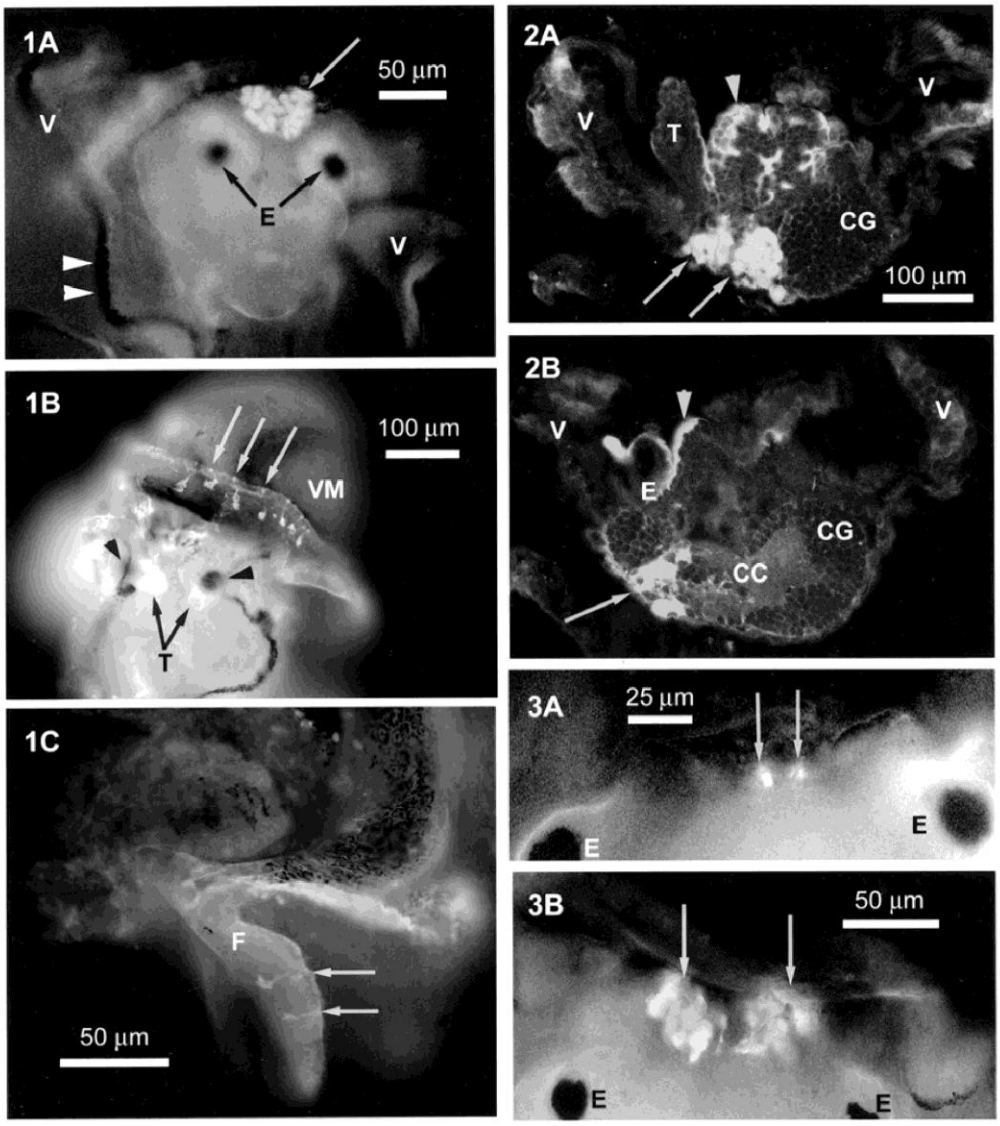

Fig. 1. Whole mounts of competent larvae displaying NOS-IR cells. (A, B) Ventral views of the head, anterior is up. (A) Immunoreactive neurons of the AG (white arrow) occur in 2 clusters. Edge of the velum (V) is pigmented (white arrowheads). E, eyespot. ×230. (B) Putative sensory neurons in the edge of the mantle have apical dendrites (white arrows) that extend to the surface of epithelium. One eyespot (arrowheads) occurs at the base of each tentacle (T, black arrows). VM, visceral mass. ×125. (C) Putative sensory neurons display NOS-IR in the edge of the foot (F) and have apical dendrites that extend to the surface of the epithelium (white arrows). ×375.

Fig. 2. Adjacent, oblique frontal sections through the head of a competent larva. Anterior is up. (A) Two clusters of NOS-IR somata (arrows), each with approximately 13 cells, occur in the AG. ×155. (B) An adjacent section displays fewer NOS-IR neurons (arrow) but larger portions of the right cerebral ganglion (CG) and its internal neuropil. Non-specific staining occurred in some regions of the specimen (arrowhead, also in A). ×150. CC, cerebral commissure; E, eyespot; T, tentacle; V, velum.

Fig. 3. Whole mount preparations of NOS-IR cells in the apical ganglia of two stages of pre-competent larvae. Ventral views, anterior is up. (A) At 63% of development (6 days after hatching), the AG contains only 2 NOS-IR neurons (arrows). ×450 (B) At 92% of development (12 days after hatching), the AG contains two distinct clusters of NOS-IR neurons (arrows). Each cluster contains approximately 8 somata. ×325. E, eyespot.

An examination of NOS activity in younger larvae indicated that the number of NOS-IR cells increases in the AG throughout development (Fig. 3). In whole mounts of 6 day-old larvae, two somata were NOS immunoreactive. (Fig. 3A). The number of NOS-IR cells increases to approximately 7–10 in each cluster in 12 day-old larvae (Fig. 3B) and to about 13 per cluster in competent larvae (Fig. 1A).

In sections, we detected non-specific staining in some regions of the head (Fig. 2, white arrowheads). This staining was seen inconsistently from larva to larva and we have no explanation for it, other than perhaps some specimens were insufficiently rinsed after application of the primary antibody solution.

Discussion

In the CNS of larval *Ilyanassa*, NOS immunoreactivity occurred in 2 clusters of somata. ICC with anti-NOS antibodies from Affinity BioReagents, Chemicon and Calbiochem displayed subsets of the cells detected with the BIOMOL antibody, but no additional neurons or cellular processes. We have no explanation for the difference in results with these antibodies, except that perhaps the residues (#724–739) used to produce the #SA-202 antibody resulted in an antibody with more affinity for the molluscan protein than the other commercial antibodies. NOS activity in the periphery occurred in putative sensory neurons with apical dendrites that are similar to sensory neurons described for congeneric adults (Crisp, 1971). We detected no centrally projecting axons. Earlier studies with NADPHd histochemistry displayed staining only in neuropilar arborizations (Lin & Leise, 1996b). These results suggest either a localization of NOS to neuronal somata, or the presence of NOS in the neurites that contains different sequences or post-translational modifications that interfered with antibody binding sites. Further investigations with molluscan probes may be the only way to resolve this apparent paradox, as the BIOMOL SA-202 antibody is no longer being produced. In preliminary experiments, Meleshkevitch *et al.* (1997) demonstrated NADPHd activity in putative sensory neurons of the ASO in larval *P. sibogae*, but we are unable to infer specific functions for the NOS-IR neurons in the AG of *Ilyanassa*.

Preadsorption of the BIOMOL #SA-202 anti-NOS antibody did indeed extinguish staining, but we are unable to include these data as images were not recorded from our initial control procedures. As we are now unable to obtain this antibody, we cannot recreate these investigations.

Our immunocytochemical results indicate that neurons of the AG gain NOS activity as they develop. At 63% of development, when the AG contains about 8 neurons (Lin & Leise, 1996a), only two cells displayed NOS activity. By 92% of development, shortly before competence, the AG contains some 14-18 neurons (Lin & Leise, 1996a), and our results showed NOS activity in most, if not all of them. ICC on competent larvae revealed approximately 26 IR cells. Thus, the initial appearance of NOS immunoreactivity is delayed relative to the births of AG neurons, but by competence, nearly all somata of the AG displayed NOS-IR. We had anticipated conducting an examination into levels of NOS-IR in cells of the AG during metamorphosis, but because our supply of the BIOMOL antibody was limited, we were unable to conduct this investigation. We expect NO production to rapidly cease after metamorphosis is triggered, but we do not yet know if this occurs through a destruction of NOS or alterations in its activity, perhaps by a decrease in its binding to Ca^{+2}/calmodulin.

Overnight decalcification in low Ca^{+2} seawater often resulted in a high percentage of metamorphosis, even in the absence of an inducer substance, so we discontinued this treatment in favor of low pH decalcification. This procedure resulted in fewer metamorphosing individuals. However, it is worth noting that we do not know if either procedure affected binding of the anti-body to the NOS protein.

The cellular function for NO in larval *I. obsoleta* is still unclear and we are considering several possibilities. Cells of the AG innervate the velum (Kempf *et al.*, 1997; Marois & Carew, 1997a—c), so NO could be involved in modulating circuits controlling larval swimming or feeding. These activities cease during metamorphosis, which parallels the metamorphic decrease in NOS activity detected with NADPHd histochemistry (Lin & Leise, 1996b). While further investigations are needed to determine if NO modulates any of these larval activities, NO is known to be active in adult feeding and locomotory circuits. For example, NO is a neurotransmitter in chemosensory and feeding neurons in *L. stagnalis*, where it can initiate and regulate aspects of feeding (Elphick *et al.*, 1995; Park *et al.*, 1998; Sadamoto *et al.*, 1998). NO acts as a co-transmitter in the feeding circuit of the sea hare *Aplysia californica* (Koh & Jacklet, 1999) and can modulate activity of cells

in feeding and locomotory circuits of the pteropod *Clione limacina* (Moroz *et al.*, 2000). While it is not unreasonable for NO to be active in similar larval circuits, the production of NO by such a large proportion of cells in the AG suggests a broader action.

NO is an important mediator of olfactory processing in the pulmonate *Limax maximus* (Gelperin, 1999; Gelperin *et al.*, 2000, 2001) and we have considered a similar role for NO in larval *Ilyanassa*. Recent experiments on larvae of *P. sibogae* indicate that the ASO is required for larval perception of a natural metamorphic cue (Hadfield *et al.*, 2000). Arkett *et al.* (1989) had previously suggested that sensory neurons of the foot in the nudibranch *Onchidoris bilamellata* might also be chemosensory. We detected NOS-IR cells in both locations, so NO may be involved in processing information about metamorphic cues in *Ilyanassa*. The metamorphic decline in NOS activity, as indicated by a decrease in NADPHd staining (Lin & Leise, 1996b), might reflect a reduction in chemosensory activity during metamorphosis, but such correlations fail to explain why injections of NOS inhibitors induce metamorphosis (Froggett & Leise, 1999), unless NO plays multiple roles in competent larvae.

As mentioned earlier, NOS is often expressed transiently during development and NO has been found to be involved in regulating synaptogenesis (Wu *et al.*, 1994; Wang *et al.*, 1995; Truman *et al.*, 1996; Gibbs & Truman, 1998; Scholz *et al.*, 1998; Wright *et al.*, 1998; Cramer & Sur, 1999; Posada & Clarke, 1999; Schachtner *et al.*, 1999; Cogen & Cohen-Cory, 2000; Gibbs, 2001) and controlling the interplay between cellular proliferation, growth arrest, cell death, and differentiation, processes fundamental to correct organogenesis (Peunova & Enikolopov, 1995; Sarkar *et al.*, 1995,1997a,b; Kuzin *et al.*, 1996, 2000; Ogura *et al.*, 1996; Enikolopov *et al.*, 1999; Wildemann & Bicker, 1999). NADPHd histochemistry revealed NOS activity in the neuropils of ganglia in competent *I. obsoleta* (Lin & Leise, 1996b), all of which, except for the AG, are retained in adults. NO may regulate some crucial step in the formation of adult circuitry, but again, this idea does not suggest a mechanism whereby NOS inhibition promotes metamorphosis.

NO has been identified as both an inducer and an inhibitor of apoptosis, a form of PCD, but its ability to induce apoptosis is most often the result of tissue stress or disease (Nicotera *et al.*, 1997; Brüne *et al.*, 1998, 1999; Liu & Stamler, 1999; Murphy, 1999). Where NO protects cells from undergoing PCD, it can do so by directly binding to and inactivating cellular caspases (Kim *et al.*, 1997) and by a cGMP-dependent pathway (Farinelli *et al.*, 1996; Mohr *et al.*, 1997; Thippeswamy & Morris, 1997; Estévez *et al.*, 1998). Because of the long duration of NO production in larval neurons and its nearly ubiquitous occurrence in cells of the AG, we favor the hypothesis that NO is protecting these cells from PCD. A nitrergic inhibition of premature PCD would also explain why the inactivation of NOS induces metamorphosis. Loss of the velum, one of the first and most obvious morphological change of metamorphosis, includes loss of epithelial, muscle and nervous tissue (Fretter, 1967; Fretter, 1969; Bonar & Hadfield, 1974; Mackie *et al.*, 1976). After exposure to a metamorphic inducer, the delay to velar loss can vary, and in gastropods like *Ilyanassa*, may be 12 to 24 hours. The reason for this long delay has prompted much speculation, but if cell death of neurons within the apical ganglion must be initiated for velar loss to occur, then the time lag be-comes understandable. The initiation of PCD can take several hours from the application of an appropriate stimulus (Locksin & Williams, 1965; Dimmeler *et al.*, 1998), but may only be morphologically recognizable after one or more days (Kerr *et al.*, 1972; Streichert *et al.*, 1997). The delay from stimulus to visible metamorphic changes in *Ilyanassa* certainly falls with this time period. Experimental tests of this idea are currently in progress.

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