Induction of Metamorphosis Decreases Nitric Oxide Synthase Gene Expression in Larvae of the Marine Mollusc *Ilyanassa obsoleta* (Say)

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Abstract:

Many marine organisms spend the early part of their lives as larvae suspended in the water column before metamorphosing into benthic reproductive adults. Metamorphosis does not occur until a larva has become competent to respond to appropriate stimuli and after a suitable habitat for the young juvenile has been encountered. The gaseous neurotransmitter nitric oxide is thought to be important in the regulation of metamorphosis by holding the organism in the larval state. We have investigated expression of the neuronal nitric oxide synthase (nNOS) gene in larval and metamorphosing individuals of the marine mud snail Ilyanassa obsoleta. Our results indicate that nNOS is expressed at constant levels throughout larval development In contrast, expression of nNOS decreases markedly during the first 24 h of metamorphosis. Our observations support previous findings that demonstrate that nitric oxide is present in larvae though competence. The decrease in nNOS gene expression that occurs during metamorphosis corresponds with a previously described reduction in nNOS activity.

Abbreviations. CNS, central nervous system; cDNA, complementary DNA; 5-HT, serotonin; NO, mtnc oxide; NOS, nitric oxide synthase; nNOS, neuronal nitnc oxide synthase; RT-PCR, reverse transcnption based-polymerase chain reaction.

Article:

Most animals on Earth reach adulthood only after completing a complex life cycle marked by the process of indirect development. After fertilization, embryos progress to a feeding or nonfeeding larval stage. During this stage of development, larvae disperse and eventually metamorphose into juvenile or miniature adult forms once a suitable environment has been encountered. The amount of time any individual remains in the larval stage varies and is determined largely by the animal's genetic program. The length of larval life can be modified by temperature, resource availability, and ecological interactions. In the case of the marine caenogastropod *Ilyanassa obsoleta*, larvae from adult individuals collected along the coast of North Carolina become physiologically competent to metamorphose in the laboratory, usually between 16 and 21 days after hatching, at 23-26 °C. However, during competence, the larval state can be maintained, often for several days or weeks, until a suitable metamorphic environment is found (1). Although much has been learned in recent years about the regulation of metamorphosis, our understanding of the mechanism by which competent larvae can stave off this transformation remains incomplete.

Several recent studies provide compelling evidence that nitric oxide (NO) is an important regulatory molecule that maintains the larval state in marine gastropods and other invertebrates (2-4). Nitric oxide synthase (NOS) is the enzyme responsible for producing NO through the conversion of the amino acid L-arginine to L-citrulline. NOS activity, as detected by NADPH diaphorase activity (5), is present throughout larval development in *Ilyanassa* and is highest in metamorphically competent larvae, but it decreases dramatically once metamorphosis begins (6). Immunolabeling of competent larvae with antibodies raised against mammalian NOS reveals several populations of cells in *Ilyanassa*, including a group of cells located in the central nervous

system (CNS) and called the apical ganglion. The amount of immunoreactivity and number of cells labeled increase throughout the planktonic phase and are maximal in competent larvae (7). In the laboratory, larval metamorphosis in *Ilyanassa* can be induced by the NOS inhibitor 7-nitroindazole (8) and by the neurotransmitter serotonin (5-HT) (9, 10). When competent larvae are placed in a solution containing 5-HT for 12 to 48 h, 75%-100% of them undergo metamorphosis (8, 10). However, the rate of serotonergically induced metamorphosis decreases significantly when larvae are treated simultaneously with exogenous NO from NO-donors such as *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) or 3-morpholino-sydnonimine (SlN-1) (2). In the same study, Froggett and Leise (2) discovered that metamorphosis can be triggered in the absence of inducers by treating larvae with NOS inhibitors. During metamorphosis, NADPH diaphorase activity in the neuropil of the apical ganglion decreases dramatically. Taken together, these observations suggest that NO acts as a negative regulator of larval metamorphosis.

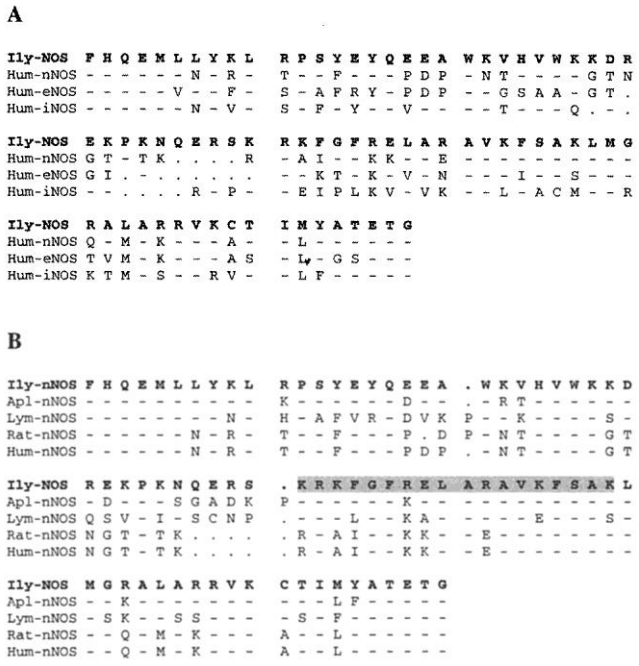

Figure 1. Partial amino acid sequence of *Ilyanassa obsoleta* nNOS. (A) The deduced amino acid sequence of the *Ilyanassa* NOS cDNA obtained by PCR (GenBank accession #AY763405) was aligned with those of the human neuronal NOS (accession #L02881), endothelial NOS (#M93718), and inducible NOS (#L09210) isoforms. This *Ilyanassa* NOS amino acid sequence shares 70% similarity and 60% identity with the human neuronal NOS sequence. Degrees of similarity and identity between the *Ilyanassa* amino acid sequence and those of the endothelial and inducible isoforms were not as high (60% similar/46% identical and 64% similar/48% identical, respectively), indicating that our cDNA encodes part of the *Ilyanassa* neuronal NOS ortholog. (B) Alignment of the deduced amino acid sequence of the *Ilyanassa* nNOS cDNA with other nNOS orthologs demonstrates 94% similarity and 80% identity to *Aplysia californica* nNOS (accession #AF288780), 91% similarity and 80% identity to *Lymnaea stagnalis* nNOS (accession #AF373019), 71% similarity and 59% identity to *Rattus norvegicus* nNOS (accession #NP434686), and 70% similarity and 60% identity to human nNOS (accession #L02881). Dashes (−) indicate identical residues. Dots (.) indicate missing residues. The shaded box indicates the Ca^{++}/calmodulin binding domain. Methods: Total RNA was isolated and purified from metamorphically competent larval snails with average shell lengths of 596 mm, using the Qiagen RNeasy system. cDNAs were generated by reverse transcription of 2.5 mg of purified total RNA and used as templates in homology PCR to obtain a cDNA encoding a portion of the *Ilyanassa* NOS gene. Degenerate primers corresponding to FHQEM (forward: 5′-TT(TC)CA(TC)CA(AG)GA(GA)ATG-3′) and YATETG (reverse: 5′-ICCIGT(CT)TCIGTIGC(AG)TA-3′) were designed on the basis of the sequences of previously described molluscan and mammalian NOS orthologs (14, 19, 20). PCR was carried out under the following conditions: 94 °C for 45 s, 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2.5 min, followed by a 7-min extension at 72 °C. A MegaBACE automated sequencer was used to obtain nucleotide sequence data from the amplified cDNA, and the Basic Local Alignment Search Tool (BLAST) software available through the National Center for Biotechnology (www.ncbi.nlm.nih.gov) was used to analyze and compare the sequence to other known NOS gene products.

The apical ganglion in the CNS of the gastropod larva appears to be a sensorimotor structure, responsible for the regulation of larval behaviors, including feeding and locomotion (11), and is involved in the detection and processing of environmental metamorphic signals in at least one species (12). In *Ilyanassa,* this larval structure is lost within 3 days after metamorphic induction and is not present in the brain of the adult snail. Gifondorwa and Leise (8) demonstrated that the disappearance of the apical ganglion during metamorphosis is the result of programmed cell death, which is detectable by cellular degeneration and nuclear condensation that begins within 12 h of induction. They also showed that programmed cell death in the apical ganglion can be artificially triggered simply by inhibiting NOS activity.

If NO signaling is vital to the retention of the larval state at competence, then the NOS gene is likely to be expressed at high levels during larval development, particularly once larvae become competent. Likewise, the level of NOS gene expression is expected to decrease following metamorphic induction. To learn more about the role of NO in metamorphosis, we isolated and cloned a complementary DNA (cDNA) encoding a portion of

the *Ilyanassa* ortholog of the neuronal NOS (nNOS) gene. We used this cDNA as a tool to investigate the expression of the nNOS gene during larval development and during serotonergically induced metamorphosis.

Adult specimens of *Ilyanassa obsoleta* (Say, 1822) were gathered from intertidal mudflats off the coast of Wilmington, North Carolina, at the University of North Carolina Wilmington Center for Marine Sciences, and were maintained in the laboratory in aerated seawater aquaria. Egg capsules were removed from the tanks several times a week and kept in fresh 0.2-μm filtered Instant Ocean or Reef Crystals (FI0). Veliger larvae were cultured as described by Couper and Leise (10) in 50% FIO/50% 0.2-μm filtered seawater containing streptomycin sulfate and penicillin G at 50 μg/m1 each. Larvae were fed an algal combination of *Isochrysis galbana* and *Dunaliella tertiolecta* daily until collected for experimental use. Larval snails were decalcified prior to use in room temperature Tris-buffered seawater at a pH of 6 (13).

Neuronal NOS gene expression during larval development and the 24 h following metamorphic induction was assessed by reverse transcription based—polymerase chain reaction (RT-PCR). Exact match deoxynucleotide primers based on the nucleotide sequence of a partial *Ilyanassa* nNOS cDNA generated by RT-PCR were designed for this investigation. The amplified cDNA was cloned, and its identity as a nitric oxide synthase was confirmed by DNA nucleotide and deduced amino acid sequence analyses. A comparison between the deduced amino acid sequence from the cloned *Ilyanassa* cDNA and the sequences of human orthologs of neuronal NOS, endothelial NOS, and inducible NOS indicated that the *Ilyanassa* sequence was far more similar to the human neuronal NOS than to either of the other two isoforms (Fig. 1A). On the basis of this finding and on the high degree of shared identity with other molluscan neuronal NOS sequences (Fig. 1B), we determined that our cDNA is part of the *Ilyanassa* neuronal NOS coding region and corresponds to residues 358-436 of *Lymnaea* neuronal NOS (14).

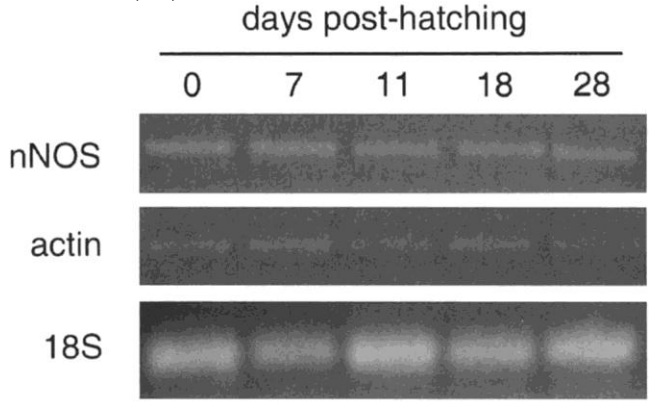

Figure 2. RT-PCR time-course analysis of nNOS gene expression during larval development. cDNAs used as templates in the PCRs were prepared by reverse transcription of 1.0 mg of total RNA purified from larvae collected at 0, 7, 11, 18, and 28 days post-hatching. Amplified nNOS and actin cDNAs were electrophoresed on a 2% agarose gel and stained with ethidium bromide. No significant changes in nNOS or actin (as a control) gene expression across developmental time-points were observed. 18S ribosomal RNA resolved by formaldehyde-agarose gel electrophoresis of 1.0 mg of total larval RNA and visualized by staining with ethidium bromide confirms that equal amounts of RNA were used in preparing the template cDNAs used in the RT-PCR. Exact match primers for *Ilyanassa* NOS were synthesized using the sequence data reported in Figure 1. PCR was carried out as follows: 94 °C for 45 s, 22 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2.5 min, followed by a 7-min extension at 72 °C.

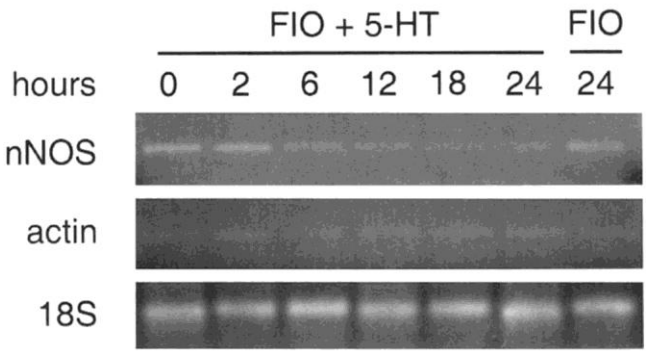

Figure 3. RT-PCR analysis of nNOS gene expression following induction of metamorphosis by 5-HT. cDNAs used as templates in the PCRs were prepared by reverse transcription of 1.0 μg of total RNA purified from larvae after culturing for 0, 2, 6, 12, 18, and 24 h in the presence of 0.1 mmol l^{-1} 5-HT or after 24 h in 5-HT-free filtered Instant Ocean (FIO). NOS gene expression decreased during the 24-h period after the induction of metamorphosis. Larvae bathed simultaneously for 24 h in FIO without 5-HT showed no decrease in NOS expression levels. Actin expression remained relatively unchanged following metamorphic induction by 5-HT. 18S ribosomal RNA resolved by formaldehyde-agarose gel electrophoresis of 1.0 mg of total larval RNA confirms that equal amounts of RNA were used to prepare the template cDNAs. PCRs were carried out under the conditions described in Figure 2.

The results of previous studies demonstrate that NOS activity increases during larval development in *Ilyanassa* and decreases in correspondence with the onset of metamorphosis. To see if these changes in NOS activity are due to changes in nNOS gene expression, we assessed the levels of nNOS mRNA during larval development and after the induction of metamorphosis. RT-PCR analyses of cDNAs prepared from RNA isolated from larvae collected at the time of hatching and from four time points thereafter failed to reveal any detectable change in nNOS gene expression during larval development up to the time of competence (Fig. 2). To investigate nNOS

gene expression following metamorphic induction, cDNAs were prepared from competent larvae that had been maintained in culture for up to 24 h in the presence of 0.1 mmol 1^{-1} 5-HT. RT-PCR analyses revealed a conspicuous decrease in nNOS transcript levels after induction of metamorphosis by 5-HT. Neuronal NOS transcript levels remained unchanged in sibling larvae cultured for the same amount of time in the absence of 5-HT (Fig. 3).

A NO signaling pathway that retains the larval state has been identified in several marine taxa (2-4, 15). Our observations provide further support for this mechanism. We have demonstrated that nNOS transcripts are present in *Ilyanassa* throughout larval development. Under appropriate regulation, their presence would allow for the observed increase in NADPH diaphorase activity during larval development (6) and account for the strong NOS immunoreactivity observed in the cell bodies of the apical ganglion (7). Whether the changes in diaphorase activity are due to differences in translation or post-translational modifications is not yet known. Although similar modifications could be responsible for the dramatic drop in NADPH diaphorase, and thus for the NOS activity that follows metamorphic induction, our findings clearly indicate that the availability of neuronal NOS mRNAs decreases. It is interesting that larval nNOS transcript levels are greatly reduced by 12 h after metamorphic induction, by which time cellular degeneration and nuclear condensation are visible in the apical ganglion (8).

Considering previously published findings and those re-ported here, we expect that in response to a naturally occurring metamorphic cue, 5-HT will be released in the larval CNS and will lead to the down-regulation of nNOS gene expression, in turn leading to diminished amounts of NO in the apical ganglion. NO can inhibit programmed cell death (16 -18), and Gifondorwa and Leise (8) demonstrated recently that NO functions in this capacity in cells of the apical ganglion. Thus, our current model states that the down-regulation of larval nNOS gene expression that follows metamorphic induction reduces NO levels and brings about the disinhibition of programmed cell death, which leads to the demise of the apical ganglion in the larval CNS.

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