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INDUCTION OF METAMORPHOSIS IN THE MARINE GASTROPOD *ILYANASSA OBSOLETA:* 5HT, NO AND PROGRAMMED CELL DEATH*

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The central nervous system (CNS) of a metamorphically competent larva of the caenogastropod *Ilyanassa obsoleta* contains a medial, unpaired apical ganglion (AG) of approximately 25 neurons that lies above the commissure connecting the paired cerebral ganglia. The AG, also known as the cephalic or apical sensory organ (ASO), contains numerous sensory neurons and innervates the ciliated velar lobes, the larval swimming and feeding structures. Before metamorphosis, the AG contains 5 serotonergic neurons and exogenous serotonin can induce metamorphosis in competent larvae. The AG appears to be a purely larval structure as it disappears within 3 days of metamorphic induction. In competent larvae, most neurons of the AG display nitric oxide synthase (NOS)-like immunoreactivity and inhibition of NOS activity can induce larval metamorphose. Because nitric oxide (NO) can prevent cells from undergoing apoptosis, a form of programmed cell death (PCD), we hypothesize that inhibition of NOS activity triggers the loss of the AG at the beginning of the metamorphic process. Within 24 hours of metamorphic induction, cellular changes that are typical of the early stages of PCD are visible in histological sections and results of a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in metamorphosing larvae show AG nuclei containing fragmented DNA, supporting our hypothesis.

Keywords: Apoptosis – caenogastropod – mollusc – nitric oxide – serotonin

INTRODUCTION

Larval *Ilyanassa obsoleta*, like most marine molluscs, undergo a series of physiological and anatomical transformations at metamorphosis which allow them to begin their juvenile life history phase. The most obvious external change is the loss of the ciliated larval feeding organ or velum [39], which, in the laboratory, occurs with a delay of some 12–36 hours after exposure to an inducing substance. In addition to loss of the velum, within 48 hours of metamorphic induction, internal transformations include rearrangements within the digestive tract and nervous system [13, 14, 27]. However, until recently, few changes in larval physiology or morphology during the 12–36 hour delay period in *I. obsoleta* had been described. In this species, by

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about 83% of larval development [27, 42], the CNS contains rudiments of all of the adult ganglia along with a medial, unpaired apical ganglion (AG). Typically, the larval AG innervates the muscular and ciliary components of the velar lobes [20, 29–31, 34, 35]. The AG is an outgrowth of the trochophore apical tuft and has long been postulated to have a sensory function [6, 34, 35, 38]. Recent experiments on a nudibranch provide evidence that the AG can detect a metamorphic cue [17], but a review of literature about molluscan AGs suggests that they are sensorimotor, coordinating the functions of the velar lobes [34, 35] and sensing inductive and other stimuli [34]. Currently, the AG appears to be the only part of the larval CNS that is lost at metamorphosis. Some evidence from *I. obsoleta* and other species suggests that the AG is lost apoptotically [1, 27, 30] but where identification of AG neurons has depended upon immunocytochemical methods, it becomes unclear whether loss of the AG occurs by a form of programmed cell death (PCD), a respecification of neuronal neurotransmitter composition, or a migration of AG neurons into the adjacent cerebral ganglia.

In the Leise laboratory, we have been studying the regulation and events of the metamorphic process in *I. obsoleta* [11, 15, 23–25, 28, 42] and in this paper, we describe the current state of our knowledge and briefly review recent results that shed light on the fate of the AG and events that precede velar loss.

Serotonin and induction of metamorphosis in Ilyanassa

Adult *I. obsoleta* are facultative carnivores [19], but at sizes suggesting they are several months past metamorphosis, small *Ilyanassa* grow successfully on diets of benthic diatoms [8]. Our personal observations have also revealed that fish carrion is unattractive to juveniles at 4 days after metamorphic induction, when the proboscis and radula appear to be functional. Among molluscs, metamorphic inducers can be associated with algal food sources [5, 21, 32, 41], so we tested acellular extracts from cultures of several species of benthic diatoms to determine if any might be a source of a natural metamorphic inducer for *I. obsoleta.* Cultures were derived from clones of isolated single cells and one species of *Coscinodiscus* induced a significant number, about 60% of competent larvae, to metamorphose [24]. However, at best, these diatom extracts induced somewhat less metamorphosis than our typical positive control solution of 0.1 mM serotonin (5HT, Fig. 1), suggesting either that the extract we used is only part of a complex, natural odorant mixture to which competent larvae can respond, or that 5HT triggers a stronger response than natural inducers. Furthermore, the cultures we used were not axenic, so the inducer may arise from associated marine bacteria. More experiments are needed to determine the cellular source of this inductive substance.

In laboratory experiments that run for 48 hours, we can routinely induce metamorphosis in 75–100% of competent larvae with 5HT (Fig. 1) [11, 15, 26]. This response was initially described by Levantine and Bonar [26], but they did not determine the active site for bath-applied 5HT. To distinguish between the two most com-

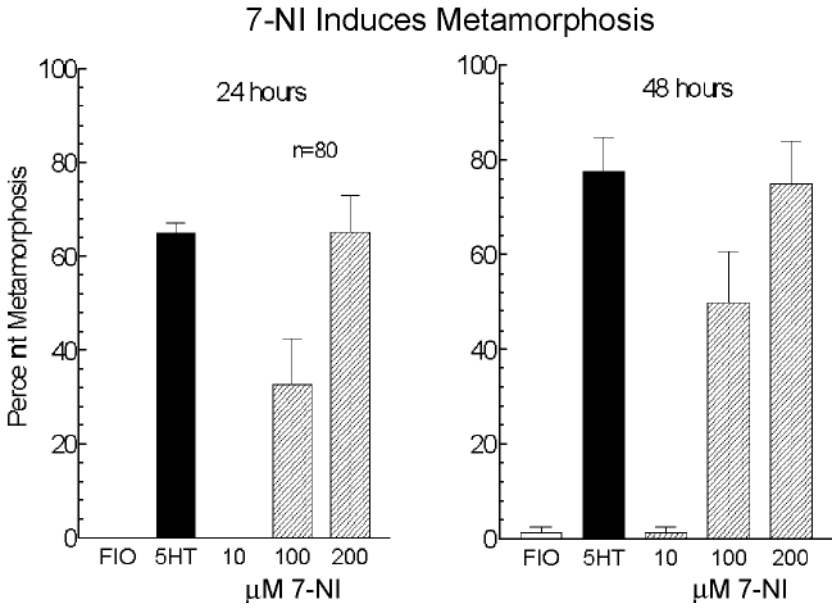

Fig. 1. Bath application [15] of the NOS inhibitor 7-Nitroindazole (7-NI) induces metamorphosis in competent larvae at rates similar to 5HT. 7-NI was obtained from Alexis Biochemicals and retains activity for approximately one year only if stored at –20 °C
(other manufacturers have different recommendations)

mon possibilities, that 5HT was interacting with membrane receptor proteins on epithelial chemosensory neurons or that 5HT was being taken up by larvae to act internally in the metamorphic pathway as a neurotransmitter or modulator, we conducted a series of injection experiments using several serotonergic reagents. Results with injections of 5HT, α-methyl-5HT, a 5HT agonist, fluoxetine, a 5HT reuptake inhibitor, and gramine, a $5HT_3$ receptor blocker, were all consistent with the idea that 5HT acts internally [11]. However, our results did not eliminate the possibilities that 5HT might be modified after uptake or that it could bind to external chemoreceptors, as GABA does in mimicking the action of an algal ligand that induces metamorphosis in the abalone *Haliotis rufescens* [32]. Our experiments also did not reveal the types of 5HT receptors that might be active in this pathway. Further experiments are necessary to resolve these issues.

Serotonergic neurons are conserved in AGs or ASOs across animal phyla [20, 22, 34, 35]. In *I. obsoleta*, as in other molluscs [20, 30, 33–35], the AG contains 5 serotonergic neurons (Fig. 2). Serotonin is an effective inducer of metamorphosis in *Ilyanassa,* but 5HT does not act as a universal inducer of metamorphosis, even though serotonergic neurons are highly conserved. As an example contrary to our experience with *Ilyanassa,* in the oyster *Crassostrea gigas*, 5HT is only a weak inducer of larval attachment [2], with little or no effect on metamorphosis [2]. Thus, while AGs (or ASOs) and their serotonergic components appear to be phylogeneti-

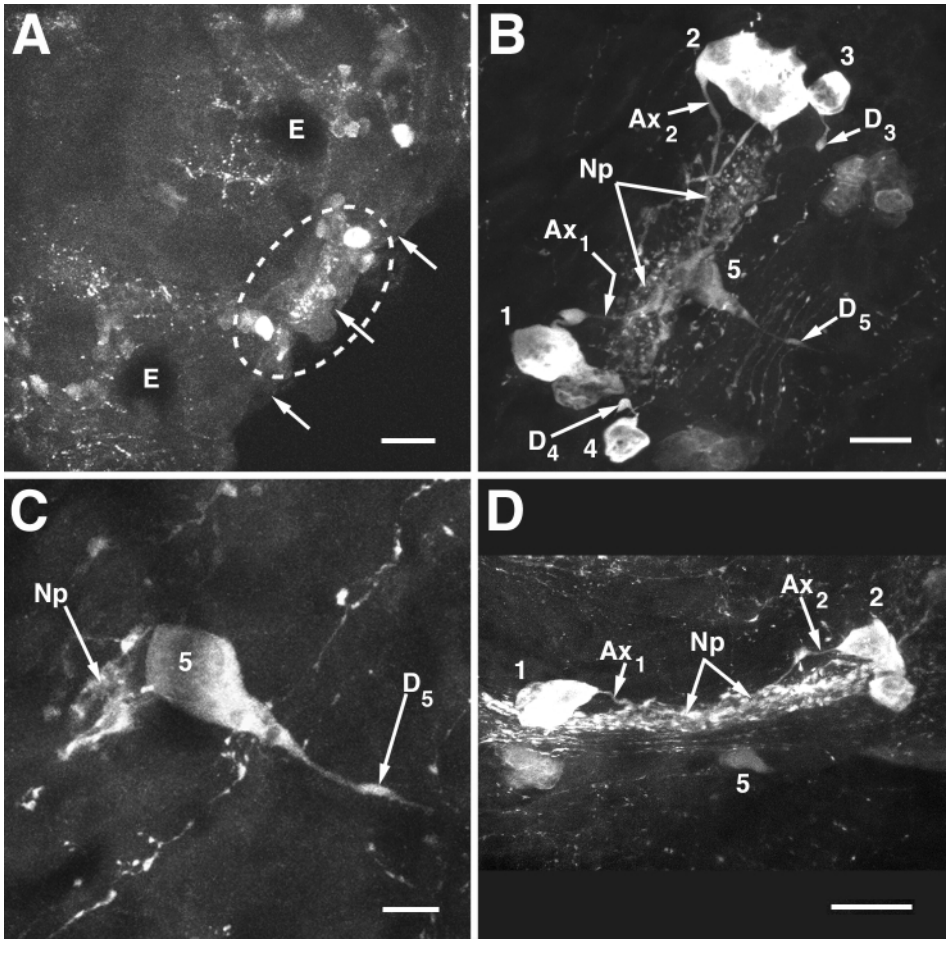

Fig. 2. Confocal maximum intensity projections of immunolabeled serotonergic components of the AG prepared as described previously by Kempf et al. [20]. A. Oblique frontal view at low magnification showing location (dashed oval) of AG. Arrows, pre-trochal surface, E, eyespot, scale bar = 20 μm. B. Orientation as in A. High magnification image of five 5HT neurons (1–5) and associated axons and dendrites in the AG. The 2 more posterior, lateral neurons (1 and 2) are large and irregular in shape and each neuron projects an axon (Ax1, Ax2) into the subjacent neuropil (Np). These neurons lack dendrites. The 2 anterior lateral neurons (3 and 4) and the medial neuron (5) have dendrites (D3-D5) extending to the pre-trochal surface. Neuron 5 lies above the AG neuropil, but in this view the underlying neuropil axons disguise this fact. Dendrites of neurons 3 and 4 have been digitally brightened for easier viewing. Scale bar = 10 μm. C. Medial neuron (5) as in B. If the apical serotonergic complex of *I. obsoleta* is homologous to that of nudibranch larvae [20], the axons crossing diagonally originate from the AG neuropil and innervate both velar lobes. Scale bar = 5 μm. D. Image of AG neurons illustrating the flattened nature of the AG neuropil atop the cerebral commissure. Neurons 1, 2 and part of 5 are visible. Axons from neurons 1 and 2 are seen entering the neuropil. Scale bar = 10 μm

cally ancient and important brain components, their specific cellular functions and interactions have responded to selective pressures in difference ways.

Nitric oxide inhibition of metamorphosis

Unlike 5HT, nitric oxide (NO) is produced upon demand by the enzyme nitric oxide synthase (NOS), whereupon it diffuses into adjoining cells to interact with intracellular targets. The most common target for NO is the enzyme guanylyl cyclase [43], although NO has other direct actions, including the modification of ion channels [9], inhibition of apoptotic caspase activity [7] and suppression of cellular proliferation by interference with DNA synthesis [37]. After NADPH diaphorase (NADPHd) was demonstrated to be a form of NOS [18], and because staining for this enzyme had illuminated sensory neurons in adult molluscs [10], we used this histochemical method in an attempt to locate larval chemosensory neurons. We stained no epithelial neurons, but discovered NADPHd activity in all ganglionic neuropils. Staining intensity increased throughout larval development, with the neuropil of the AG displaying the strongest NADPHd activity in competent larvae [28]. Once metamorphosis was initiated, staining intensity dropped dramatically [28]. With immunocytochemical methods that incorporated the use of mammalian anti-neuronal NOS antibodies, we determined that the majority of neurons in the AG of competent larvae displayed NOS-like immunoreactivity (NOS-IR) [42]. These anatomical findings raised questions concerning both the role of NO in the metamorphic process and in its cellular actions in neurons of the AG.

To determine if NO promotes or inhibits metamorphosis, we conducted a series of experiments in which we treated larvae with a variety of nitrergic reagents. We applied NO donors to larvae in bath solution and injected NOS inhibitors directly into larvae. Alone, NO-donors had no effect on larvae; in combination with 5HT, NO reduced rates of serotonergically-induced metamorphosis. Although this reduction may have resulted from direct interactions between NO and 5HT in solution, injections of NOS inhibitors confirmed that a decrease in NOS activity induced metamorphosis in significant numbers of larvae [15]. To date, the only NOS inhibitor that has been effective in bath application is 7-Nitroindazole (7-NI) (Fig. 1).

Among its various actions in developing nervous systems, NO regulates cellular proliferation [37] and apoptosis [7], although most of its endogenous actions in this latter case are inhibitory. Because (1) the AG disappears during metamorphosis, (2) most neurons in the AG display NOS-IR, and (3) the results of our experiments with nitrergic reagents were all consistent with the idea that NO inhibited metamorphosis, we hypothesized that NO's major cellular activity as an inhibitor of metamorphosis is to suppress PCD in the AG. To first confirm that the AG is indeed lost by a form of PCD, we examined it within 24 hours of metamorphic initiation. Induction by either 5HT or 7-NI yielded signs of cellular degeneration, condensation and loss of nuclei, and evidence of phagocytic activity in histological sections [16]. In the early stages of PCD, endonucleases cleave nuclear DNA into fragments that are multiples

of 180–200 base pairs in length [40]. Results of a TUNEL assay, which identifies such fragmented DNA, confirmed that cells of the AG are lost by a form of PCD [16]. We also determined that within 60 hours of induction, cells of the AG exist only as cytoplasmic remnants, which are then lost by 3 days after induction.

CONCLUSIONS

Together, results of our experiments with *I. obsoleta* suggest that in their natural environment, a mixture of diatom or associated bacterial exudates, including that from a species of *Coscinodiscus,* induce competent larvae to metamorphose. Some epithelial chemosensory neurons may be serotonergic, but we expect that the chemosensory process activates serotonergic neurons in the AG. Presumably, the addition of 5HT to the bath seawater mimics the activation of such neurons. Through a process that has yet to be discovered, perhaps by inducing changes in membrane Ca^{++} currents, 5HT inhibits the activity of endogenous NOS throughout the AG. The resulting decrease in NO production allows for activation of the programmed cell death pathway. We also have some evidence which suggests that nitrergic inhibition of PCD occurs through a cGMP-dependent pathway [12].

Our results allow us to infer possible mechanisms underlying metamorphic control, but they raise as many questions as they answer. Clearly, during the initial delay period of some 12–36 hours, the AG is undergoing genetically programmed destruction. We expect this loss to be common among molluscs with AGs. To determine if this loss is conserved more broadly throughout the animal kingdom, more experimentation is required. In *Ilyanassa,* whether or not subsequent tissue losses and metamorphic remodeling require AG degeneration or are merely triggered concurrently with this process, is unknown. Functions of the AG serotonergic neurons also remain enigmatic, with interactions between serotonergic neurons and NOS activity needing further clarification. And, while nitric oxide has been implicated in metamorphic regulation in the echinoderms and ascidians [3, 4], whether or not it also acts in an anti-apoptotic fashion in these species is as yet unknown.

Finally, larval *Ilyanassa* retain their ability to metamorphose for several weeks in culture and will begin to lose specificity and metamorphose spontaneously as they age. Our research points towards an explanation for this loss of specificity. Gifondorwa [16] discovered that in such larvae the AG is undergoing spontaneous destruction. Like Pechenik [36], we suspect, but have not yet confirmed, that such larvae show a decrease in NOS activity. How this might occur, at the translational or transcriptional levels, is likewise unclear.

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