Selective Retention of the Fluorescent Dye DASPEI in a Larval Gastropod Mollusc After Paraformaldehyde Fixation

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Abstract:

In the vertebrates, the vital mitochondrial dye DASPEI (2-(4-dimethylaminostyryl)-N-ethylpyridinium iodide) has been used for the rapid visualization of several distinct classes of epidermal cells in vivo and in vitro: epidermal electroreceptors, mechanoreceptors, and chloride cells in teleosts, and mechanoreceptors in amphibians. I used DASPEI in an attempt to locate a different type of sensory cell, namely, the chemosensory neurons that mediate the initiation of metamorphosis in veliger larvae of the prosobranch gastropod *Ilyanassa obsoleta*.

In vivo, bath-applied DASPEI stains entire larvae in a relatively non-specific fashion. After fixation in phosphate-buffered paraformaldehyde, most of the cells in these animals retained relatively little stain, with some exceptions. Significant DASPEI staining was maintained in approximately nine neurons in the apical ganglion, a temporary cephalic structure that is lost at metamorphosis. Little staining was observed in the rest of the larval central nervous system (CNS), nor could any peripheral sensory neurons be definitively identified. DASPEI was also retained by cells in other larval organs such as the velum and buccal mass, and in two acellular structures, the radula and operculum.

Key words: Ganglion, Neuroanatomy, Nervous system, Prosobranch, Veliger

Article:

INTRODUCTION

DASPEI, a fluorescent dye that is selectively taken up by mitochondria (Bereiter-Hahn, 1976), has been used to identify the mitochondrial-rich chloride cells in teleost skin (Kiiltz et al., 1992; Marshall and Nishioka, 1980; Marshall et al., 1992; Yoshikawa et al., 1993), endothelial cells from tadpole hearts (Bereiter-Hahn, 1976), amphibian and teleost hair cells (Balak et al., 1990; Jørgensen, 1989), and teleost epidermal electroreceptors (Jørgensen, 1992). Because this dye stained several types of sensory cells, I used it in an attempt to identify the sensory neurons likely to be responsible for transducing the chemical cue that triggers metamorphosis in larvae of the marine snail *Hyanassa obsoleta.* I applied this dye to competent larvae and found that after fixation, DASPEI was selectively and routinely retained by a limited number of cells within these animals. The most conspicuous cellular staining occurred in part of the larval brain.

The "brain" or anterior ganglia of a metamorphically competent larva of *Ryanassa obsoleta* is similar to those of other prosobranch gastropods at this stage (D'Asaro, 1969; Delsman, 1914; Demian and Yousif, 1975). It consists of five pairs of ganglia, the cerebrals, buccals, pleurals, pedals, and intestinals (Lin, 1995), plus a recently recognized unpaired apical ganglion (Marois et al., 1993) that lies above the dorsal commissure connecting the cerebral ganglia (Fig. 1). The apical ganglion appears to develop as an outgrowth of the apical plate, which has historically been thought to contain a central sensory region (D'Asaro, 1969; Raven, 1966; Tardy, 1970). Ultrastructural examinations of the region above the dorsal cerebral commissure in larval opisthobranch gastropods have revealed putative sensory neurons in what has been called the "cephalic sensory

organ" (Bonar, 1978a,b; Chia and Koss, 1984; Page, 1993). The physiology of these cells remains unexplored. In the sea hare *Aplysia californica,* the apical ganglion is a purely larvae structure which is lost at metamorphosis (Marois et al., 1993). My use of the styryl dye DASPEI allowed me to find a similar ganglion in larval *Ilyanassa.*

DASPEI has previously been used as a vital dye. I examined larvae in vivo and as preserved specimens by epifluorescence microscopy. In this paper I focus on the ability of this dye to illuminate a specific population of neurons in the central nervous system (CNS) of paraformaldehyde-fixed larvae and briefly describe other regions that retain DASPEI in these preserved specimens.

MATERIALS AND METHODS

Adult and larval culture methods for *Ilyanassa obsoleta* (Say, 1922) are well known (Collier, 1981). Briefly, larval *Ilyanassa* were obtained from egg capsules laid in laboratory aquaria by two populations of adult snails. Newly hatched larvae were cultured at room temperature in air-lift containers (Miller and Hadfield, 1986; Pires and Hadfield, 1991) in a 1:1 mixture of 0.2 μm filtered natural seawater and filtered Instant Ocean (FI0). Larvae were fed a combination of laboratory cultures of the algae *Nannochloropsis sp., Isochrysis galbana,* and *Monochrysis sp.* Larvae became competent to metamorphose in 3-6 weeks, depending upon culture density. Shell lengths of competent larvae were typically >600 p.m. Twenty to thirty representative larvae from cultures of large (>600 μm) larvae were tested for metamorphic competence by exposure for 48 hours to 10^{-4}M serotonin (5-HT) (Levantine and Bonar, 1986). Normally, over 90% will metamorphose in response to 5-HT with less than 10% metamorphosing in FIO controls. Larvae from cultures that yielded positive results or that contained spontaneously metamorphosed juveniles were deemed competent. To facilitate microscopic observations and sectioning, all larvae were decalcified by overnight immersion in a low pH (7.0) and calcium-free artificial seawater (Pires and Hadfield, 1993). Larvae were then reacclimated to normal seawater (pH 7.9) using several changes of FIO.

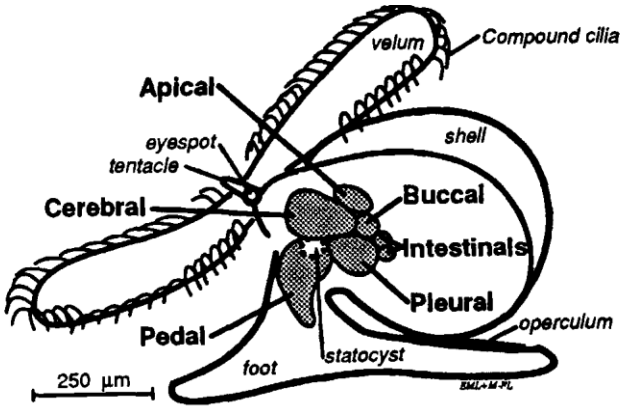

Fig. 1. Diagrammatic lateral view of a competent larva showing approximate locations of the ganglia of the CNS. Reconstructed from serial sections (after Lin, 1995).

Intact larvae and isolated larval head-foot preparations were stained for 20 minutes in 1 mM DASPEI (2-(4-dimethylaminostyry1)-N-ethylpyridinium iodide (Molecular Probes, Inc., Eugene, OR) in 90% FIO. DASPEI was dissolved by heating a 10^{-2}M distilled water solution which was then diluted to 1 mM with FIO. Head-foot preparations were isolated from visceral masses by cutting across a larva with a piece of broken razor blade (Leise and Hadfield, 1991; Pires and Hadfield, 1993). Larvae were anesthetized in ice-cold FIO before surgery. Head-foot preparations were initially used to avoid potential penetration problems. This method proved to be unnecessary and was later discontinued. All specimens were rinsed for approximately 15 minutes in 8-10 changes of full-strength FIO. Specimens were anesthetized in ice-cold FIO before fixation. Fixation was conducted on ice in chilled 4% paraformaldehyde in Millonig's phosphate buffer (Cloney and Florey, 1968) for 5 minutes and then continued in a 7°C refrigerator overnight. A graded ethanol series was used for dehydration.

For epifluorescence microscopy, most live whole mounts were viewed in FIO. Additional specimens were observed after being cleared in a mixture of 80% glycerol in FIO (modified from Beltz and Kravitz, 1983).

Preserved and dehydrated specimens were transferred to acetone, embedded in soft Spurr's resin, which has low autofluorescence (Spurr, 1969), sectioned at 8 μm, arid mounted in Cytoseal 280 (Electron Microscopy Sciences, Fort Washington, PA). Sections were viewed on an Olympus BH-2 compound micro-scope equipped with a 100 W epifluorescence unit and a Lucifer yellow cube (excitation filter 430 nm, barrier filter 540 nm). While not designed specifically for DASPEI, which has absorption and emission peaks at 459 and 584 nm, respectively, this filter set provided sufficiently bright yellow images. Larvae displayed a pale green background fluorescence which was useful for determining the relative positions of DASPEI-staining cells within larval structures. This autofluorescence appears dark grey on the fluorescence photo-micrographs.

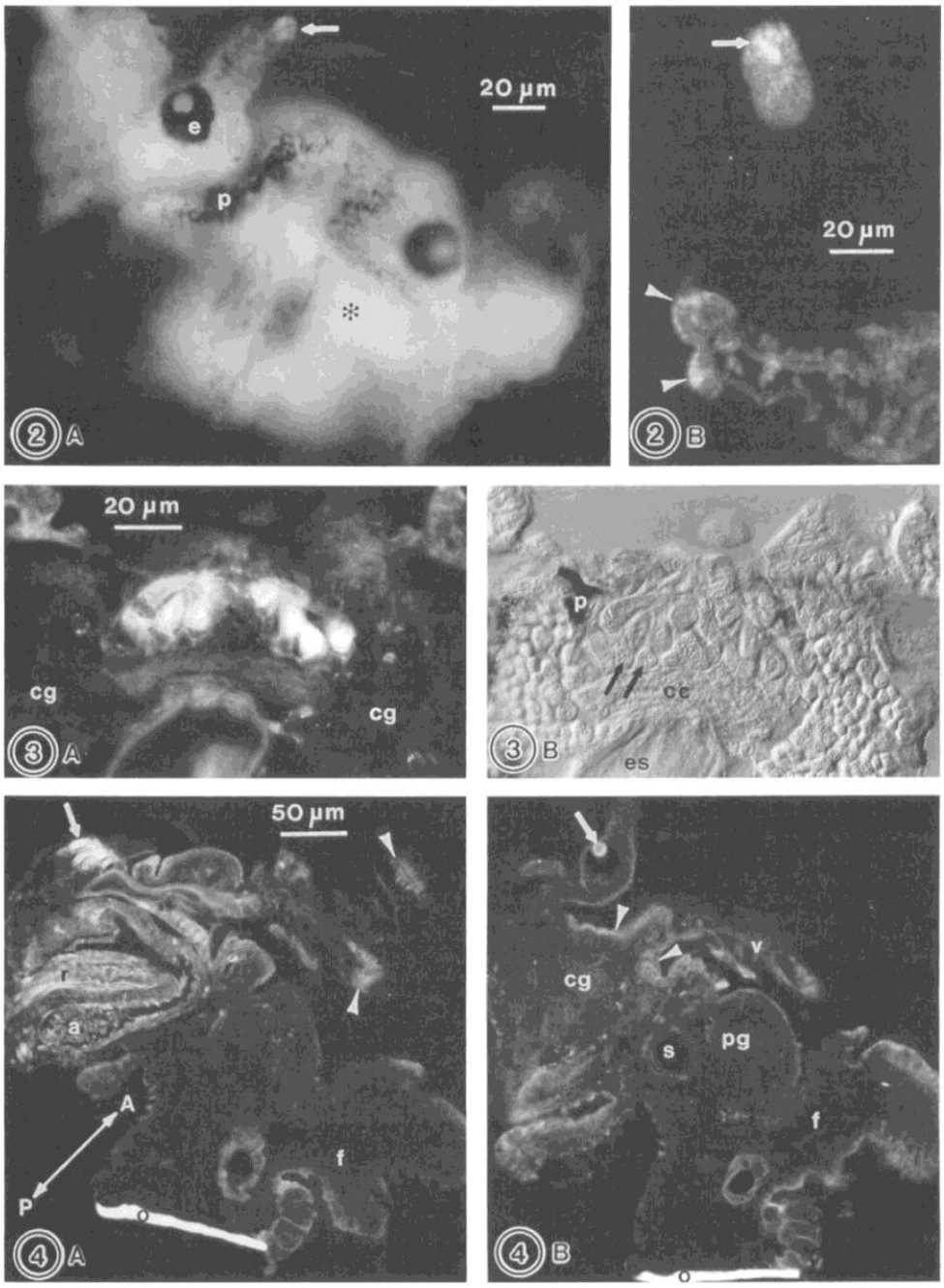

Fig. 2. **A:** In vivo fluorescence micrograph of a whole mount of a larval head from a competent 80-day-old larva. Note stained cells at tip (arrow) of tentacle and bright fluorescent glow from the entire head (asterisk). **B:** Sagittal plastic section through the tip of a tentacle from a 62-day-old larva. Note single stained cell (arrow). Ciliated cells of the velar edge (arrowheads) also retain some DASPEI. e, eyespot; p, epidermal pigment.

Fig. 3. Plastic 8 μm transverse section through an apical ganglion in a 50-day-old competent larva. **A:** Fluorescence image of stained neurons. Only large cells in the apical ganglion retain DASPEI. Note lack of staining in the adjacent cerebral ganglia (cg). **B:** Nomarski image of section in A. Notice small somata (arrows) just above the cerebral commissure (cc) that were unstained in A. es, esophagus; p, epidermal pigment.

Fig. 4. A,B: Plastic 8 μm sagittal sections through a head-foot preparation from a 56-day-old competent larva. Visceral mass was to the left of the individual. **A:** Midline sagittal section includes apical ganglion with brightly fluorescing neurons (arrow). Stain is also retained by radula (r) and surrounding cells of the buccal mass, ciliated cells of the velar edge (arrowheads), regions of the foot (f), by the operculum (o), and by algal cells (a) in the gut. Anterior-posterior axis indicated by double-headed arrow. **B:** More lateral section includes eyespot with its stained lens (arrow). Some stain is also retained in various regions of the skin (arrowheads). Note lack of stained cells in cerebral (cg) and pedal (pg) ganglia. s, statocyst; v, velum.

RESULTS

In vivo, DASPEI appears to stain most of the cells in the animal in a relatively non-selective fashion, yielding a brightly fluorescing specimen (Fig. 2A). Internal structures could not be discerned, even in glycerol-cleared specimens. Epidermal pigment (Figs. 2A, 3B) also tended to obstruct views of the larval interior. One to four cells at the tips of one or both tentacles were often stained (Fig. 2). To determine if these were sensory neurons, stained specimens were fixed, embedded in Spurr's resin, and sectioned (n = 20). Small neural processes, those under 1.0 μm in diameter, that may be indistinguishable in whole mounts, can often be visualized in sections (Leise et al., 1986). However, in these specimens, no axons were seen connecting the tentacular cells to the CNS (Fig. 2B).

Specimens lost most of their staining during the fixation and embedding procedures. However, some cells and structures routinely retained the dye. In sectioned material, DASPEI was regularly retained in an average of nine (s.d. ± 1.9, n = 8) neurons in the apical ganglion (Figs. 3,4). Only somata were stained; no projecting axons or dendritic processes were visible. Especially when viewed with Nomarski optics, additional smaller, unstained neurons could be seen in this ganglion (Fig. 3). Neurons of the remaining central ganglia, the cerebral, pedal, pleural, buccal, and intestinal ganglia were generally unstained (Fig. 4, buccals, pleurals, and intestinals not shown).

In addition to the large neurons of the apical ganglion, other stained cells include a few around the mouth and esophagus (Fig. 5A) and some of the cells of the ciliated bands of the velar edge (Figs. 2B, 4A, 5B). The large prototrochal cells (Fig. 5B, left arrow) bear compound cilia and provide the propulsive force for swimming (Carter, 1926). In all specimens examined (n = 20), large segments, but not the entire length, of the velar edge were usually stained. In addition to these cellular structures, the radula and operculum remained brightly stained after fixation (Figs. 4, 5A).

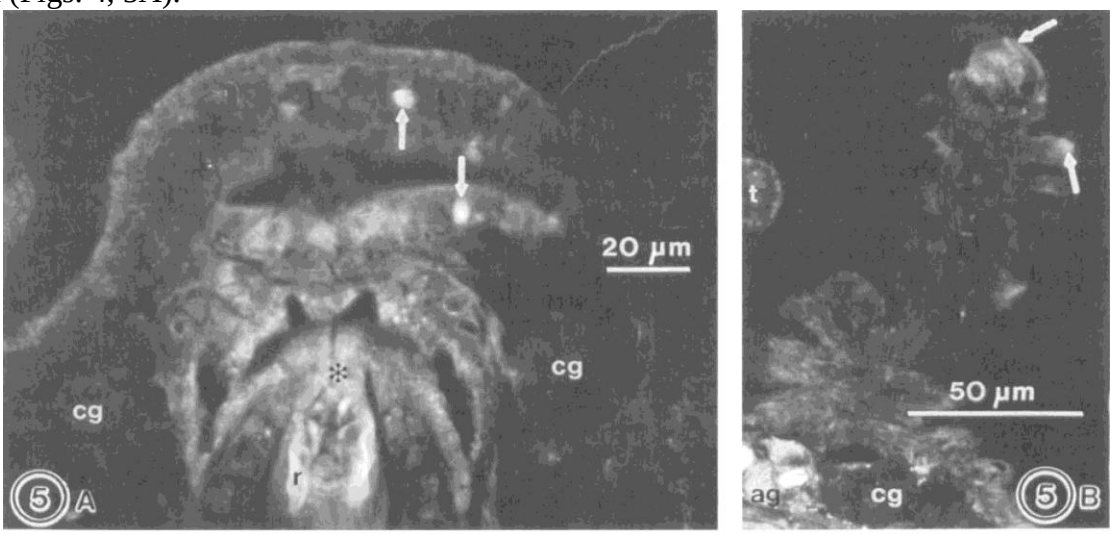

Fig. 5. Horizontal plastic sections through a 50-day-old competent larva. **A:** Stained cells occur in the oral epithelium (arrows). Cells of the buccal mass are lightly stained (asterisk) while parts of the radula (r) are intensely fluorescent. **B:** Ciliated cells of the velar edge (arrows) stain less intensely than cells of the apical ganglion (ag). cg, cerebral ganglion; t, tentacle.

DISCUSSION

The fluorescent dye DASPEI is known to be taken up specifically by mitochondria (Bereiter-Hahn, 1976) and has been used to stain metabolically active cells in both neural and non-neural tissues (Balak et al., 1990; Jørgensen, 1989; Killtz et al., 1992; Marshall and Nishioka, 1980; Marshall et al., 1992; Yoshikawa et al., 1993). Previous work with DASPEI appears to have been limited to in vivo usages. The results described here extend its usefulness to the study of fixed material. In larval *Ilyanassa obsoleta,* DASPEI was selectively retained by certain cells and acellular structures throughout paraformaldehyde fixation, ethanolic and acetone dehydration, resin infiltration, and the final curing procedure. The reason for dye retention in these specific sets of cells is unclear, although one can speculate that the cells retaining the dye may have been highly active, containing large numbers of mitochondria. For any group of cells, the intensity of DASPEI staining depends upon the physiological state of the mitochondria and is influenced by mitochondrial membrane potential, the

energetic state of the mitochondria, oxygen levels, and any mitochondrial substrate deficiencies (Bereiter-Hahn, 1976). In *Ilyanassa* larvae, the ciliated cells of the prototrochal and metatrochal bands are continuously active during larval swimming and feeding. Cells of the prototroch are known to contain numerous mitochondria (Mackie et al., 1976) so their retention of DASPEI during fixation is not surprising. The non-uniform staining characteristics may reflect the discontinuous swimming behavior of larvae during the staining procedure. The mitochondrial con-tent of the neurons of the apical ganglion, the most intensely staining cells, is unknown. The affinity of DASPEI for the radula and operculum, two chitinous structures, may depend upon some ionic or covalent interactions between the dye and their organic matrices.

In gastropod larvae, chemosensory neurons have been described in the apical organs of the nudibranchs *Phestilla sibogae* (Bonar, 1978a,b), *Rostanga pulchra* (Chia and Koss, 1984; Page, 1993), and *Doridella steinbergae* (Page, 1993), on the side of the foot in *Onchidoris bilamellata* (Arkett et al., 1989; Chia and Koss, 1989), and in the rhinophores of *Rostanga pulchra* (Chia and Koss, 1982). These putative chemosensory neurons typically have epidermal or subepidermal somata with dendritic ends that are exposed to the environment and basal axons leading into the CNS. I ob-served no cells with such characteristics in living or fixed *Ilyanassa obsoleta.* The stained cells at the tips of the tentacles could be sensory, but a definitive functional identification of these cells awaits other neuroanatomical and/or physiological methods. Similarly, a functional identification of the stained cells of the apical ganglion requires further study.

Like the apical ganglion of *Aplysia* (Marois et al., 1993) and *Berghia verrucicornis* (Kempf et al., 1991), the apical ganglion in *Ilyanassa* contains five to six serotonergic neurons that innervate the velum and foot (Kempf, personal communication), suggesting that this ganglion plays a role in larval swimming, feeding, and crawling behaviors. The relationship of the DASPEI stained neurons to these serotonergic ones is unknown. DASPEI appears to be retained by more than half of the 14-18 large neurons (Lin, 1995) present in this ganglion. Whether or not the dye is retained by the same subset of large neurons in each animal is not yet known.

My use of DASPEI resulted in a novel view of both neural and non-neural structures in this invertebrate larva. The conspicuous staining of a subset of the neurons in the apical ganglion suggests a compartmentalization of function even within this small component of the nervous system. Further study with other neuroanatomical and physiological techniques should allow my co-workers and I to understand the functional significance of this unusual staining pattern.

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