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Identifiable Nitrergic Neurons in the Central Nervous System of the Nudibranch *Melibe leonina* Localized with NADPH-Diaphorase Histochemistry and Nitric Oxide Synthase Immunoreactivity

JAMES M. NEWCOMB[1,2]* AND WINSOR H. WATSON III[1,2]
[1]Zoology Department and Center for Marine Biology, University of New Hampshire, Durham, New Hampshire 03824
[2]Friday Harbor Laboratory, University of Washington, Friday Harbor, Washington 98250

ABSTRACT

Nitric oxide (NO) is a gaseous intercellular messenger produced by the enzyme nitric oxide synthase (NOS). In this study, we used two different techniques—nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry and NOS immunocytochemistry—to demonstrate that NOS is present in a pair of identifiable cells in the central nervous system of the nudibranch *Melibe leonina*. In the *Melibe* brain, NADPH-d histochemistry revealed only a single pair of bilaterally symmetrical cells in the cerebropleural ganglia. NOS activity also was found in the neuropil of the cerebral, pedal, and buccal ganglia; in the tentacles of the oral hood; in the sensory end of the rhinophores; and in the epithelial tissue of the mouth, preputium, and glans penis. Immunocytochemistry using NOS antisera corroborated the results of the NADPH-d histochemistry by staining the same two cells in the cerebropleural ganglia. Each of these identifiable nitrergic neurons projects into the ipsilateral pedal ganglion. Because the pedal ganglia play a critical role in the control of locomotion, our results provide morphological evidence suggesting that NO may influence swimming or crawling in *Melibe leonina*. J. Comp. Neurol. 437: 70–78, 2001.

Indexing terms: nitrergic cells; immunocytochemistry; gastropod; mollusk

Nitric oxide (NO) is a gaseous molecule that is used as an intercellular messenger in the central nervous system (CNS; Garthwaite et al., 1988). It is synthesized by the enzyme nitric oxide synthase (NOS), which converts L-arginine to L-citrulline, releasing NO in the process. There are several different isoforms of NOS, but the neuronal form is calcium/calmodulin-dependent and requires oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) as cosubstrates (Bredt and Snyder, 1992).

NO appears to serve as a neurotransmitter or neuromodulator in the molluscan class Gastropoda. There is evidence that NO plays a role in feeding and locomotion in the mollusk *Clione limacina* (Moroz et al., 2000), regulation of feeding in *Aplysia californica* (Lovell et al., 2000), food-attraction conditioning in *Helix pomatia* (Teyke, 1996), chemosensory activation of feeding in *Lymnaea stagnalis* (Moroz et al., 1993; Elphick et al., 1995), and oscillation of olfactory neurons in the procerebral lobe in *Limax maximus* (Gelperin, 1994). Most of these functions involve modulation of rhythmic cells or circuits. This nitrergic modulation of rhythmic activity may be a common function of NO in animals other than gastropods as well. For example, in the crustacean *Cancer productus*, NO appears to modulate the stomatogastric ganglion (Scholz et al., 2001) and the cardiac ganglion (Scholz, personal communication). There also is evidence that NO controls oscillatory activity in mammalian thalamocortical neu-

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*Correspondence to: James M. Newcomb, Georgia State University, Biology Department, P.O. Box 4010, Atlanta, GA 30302.
E-mail: bnewcomb@juno.com

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rons (Pape and Mager, 1992). Because gastropods express a number of rhythmic behaviors (e.g., feeding, breathing, crawling, and swimming) that are controlled by relatively simple central pattern generators, they offer very suitable model systems for investigating how NO modulates the rhythmic circuits underlying specific behaviors.

Due to the short half-life of NO in biological systems (about 3–5 seconds; Palmer et al., 1987), it is very difficult to stain for NO in histological preparations. Therefore, the presence or activity of NOS, as determined by NADPH-diaphorase (NADPH-d) histochemistry or immunocytochemistry, often is used as an indirect indicator of NO. NOS is an enzyme that catalyzes the NADPH-dependent reduction of a tetrazolium salt to an insoluble blue formazan; however, unlike other NADPH-dependent enzymes, such as cytochrome p-450, it is resistant to aldehyde fixation (Matsumoto et al., 1993). Therefore, NADPH-d histochemistry can be used in conjunction with aldehyde fixation to locate areas of NOS activity (Dawson et al., 1991; Hope et al., 1991), and it has been applied successfully in both vertebrates and invertebrates (Elofsson et al., 1993; Garthwaite and Boulton, 1995).

There have been a number of studies involving NADPH-d histochemistry in gastropod nervous systems (Elofsson et al., 1993; Jacklet and Gruhn, 1994; Jacklet, 1995; Moroz and Gillette, 1995; Moroz et al., 1996; Moroz, 2000), and there appears to be large variation in NOS localization between species. However, Moroz and Gillette (1995) noticed several general trends in NOS activity in the nervous systems of gastropods. First, there seems to be an evolutionary trend of nitrergic neurons moving from the peripheral nervous system (PNS) into the CNS. Second, predatory species tend to have more staining in the CNS as opposed to herbivorous species. Finally, NOS activity often is present in feeding-related parts of the nervous system (e.g., buccal ganglia, olfactory neurons, etc.).

NADPH-d reactivity has been shown to correlate with NOS immunocytochemistry (Bredt et al., 1991), but often only after fixation time is adjusted to eliminate the NADPH activity of enzymes other than NOS (Matsumoto et al., 1993). There are only two published accounts of successful NOS immunocytochemistry with gastropods (*Helix aspersa*: Cooke et al., 1994; *Lymnaea stagnalis*: Moroz et al., 1994), largely because the only commercially available NOS antibodies are derived from mammals. Korneev and colleagues (1998) successfully cloned neuronal NOS (nNOS) from the snail *Lymnaea stagnalis* and found only 50% homology with the mammalian (murine) form of NOS. It is interesting to note that those authors found only 47% homology with the nNOS in *Drosophila melanogaster* (Regulski and Tully, 1995). Likewise, Sadreyev et al. (2000) cloned nNOS in *Aplysia californica* and also found only 48–52% homology with other nNOS isoforms. This suggests that there is high variability in the sequence of nNOS between species, even among invertebrates. This problem has been addressed by the creation of a universal NOS antibody (Affinity Bioreagents, Inc., Golden, CO) that binds to a highly conserved region of the enzyme, is specific for NOS, and has been used successfully in other immunocytochemical studies (Scholz et al., 1998, 2001; Zayas et al., 2000).

In this paper, we report results of NOS immunocytochemistry in the CNS of *Melibe* using this universal NOS antibody. These results are correlated with NADPH-d histochemistry, confirming the presence of two identifiable nitrergic neurons in the cerebropleural ganglia.

MATERIALS AND METHODS

Animal collection and maintenance

Adult *Melibe leonina* were collected from subtidal eelgrass beds near the University of Washington's Friday Harbor Laboratories (FHL) on San Juan Island, Washington by the authors and David Duggins and also from Patricia Bay in Saanich Inlet, British Columbia by WestWind SeaLab Supplies (Victoria, British Columbia, Canada). Experiments were carried out at FHL, where *Melibe* were kept in flow-through seawater tables at ambient temperatures (≈10°C), and at the University of New Hampshire, where they were maintained in recirculating seawater tanks at 10°C. All *Melibe* were anesthetized by chilling prior to dissection.

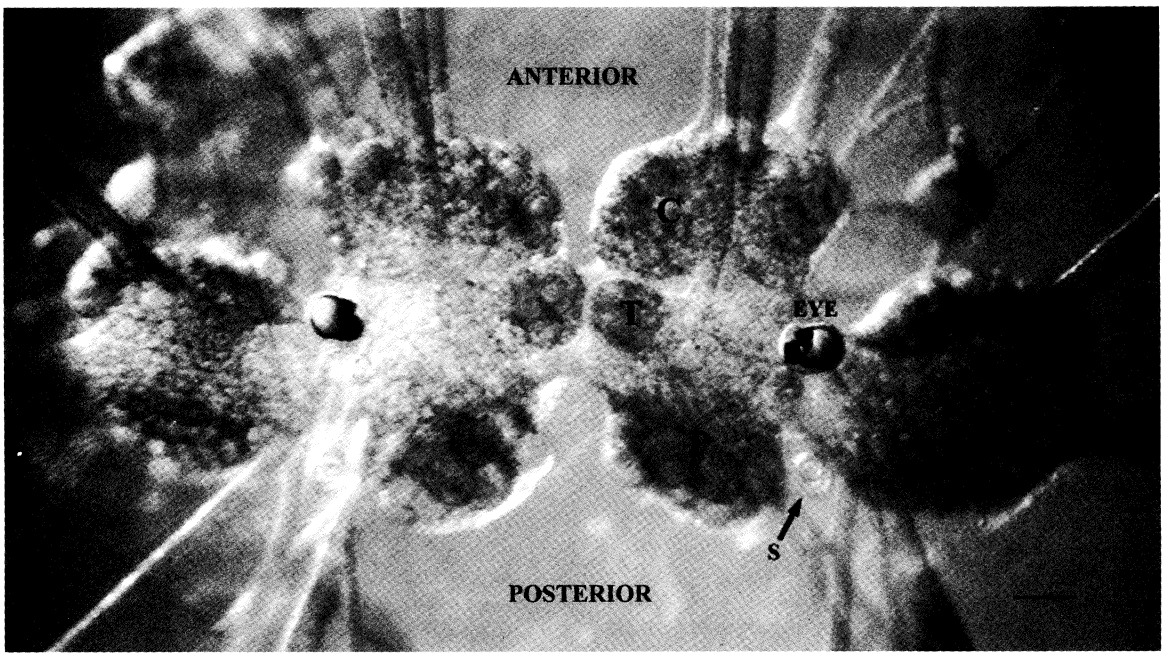
NADPH-d histochemistry

The CNS (including the cerebral, pleural, pedal, and buccal ganglia) was removed from each animal (n = 35) and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer solution (PB), pH 7.6, for 15 minutes at 4°C. They were then washed three times for 5 minutes each in 0.5 M Tris buffer, pH 8.0. Staining of wholemount preparations was carried out in the dark overnight at 4°C in a 2-ml solution consisting of 0.11 mM NADPH, 0.02 mM nitro blue tetrazolium, 0.11 μM dicumarol, 0.41 M Tris, and 0.28% Triton X-100. The next day, the tissue was washed twice for 5 minutes each in 0.5 M Tris and then postfixed for 1 hour in 4% paraformaldehyde in methanol. Preparations were dehydrated in an alcohol series to 100% EtOH, cleared in methyl salicylate, and mounted in Permount for viewing and photography on a Zeiss Axiophot microscope (Zeiss, Thornwood, NY). Nicotinamide adenine dinucleotide (NAD+) was substituted for NADPH (n = 3) as a control. Adobe Photoshop software (version 5.5; Adobe Systems, Mountain View, CA) was used to compile photomicrographs into composites for the figures. The photomicrographs were converted to gray scale, and minor adjustments were made in overall brightness and contrast levels to highlight important aspects of the figures.

Peripheral organs also were removed (n = 3) and processed for NADPH-d histochemistry to examine possible non-CNS areas of NOS activity. These organs included the tentacles, rhinophores, mouth, salivary glands, esophagus, stomach, intestine, foot, skin, heart, preputium, glans penis, and ovotestis.

NOS immunocytochemistry

The immunocytochemical methods described below were adapted from Watson and Willows (1992). Antiuniversal NOS antibody was acquired from Affinity Bioreagents, Inc. with a peptide sequence of QKRYHEDIFG, representing amino acids 1,113–1,122 of murine bNOS. All steps were carried out at 4–6°C. The CNS was removed from each animal (n = 7) and fixed overnight in 4% paraformaldehyde in 0.1 M PB. Wholemount preparations were then rinsed in 0.1 M PB four times over the course of 1 hour and treated with 0.1% trypsin for 15 minutes and 4% Triton X-100 in 0.1 M PB for 1 hour. They were incubated overnight in 0.4% Triton X-100 and 0.1% so-



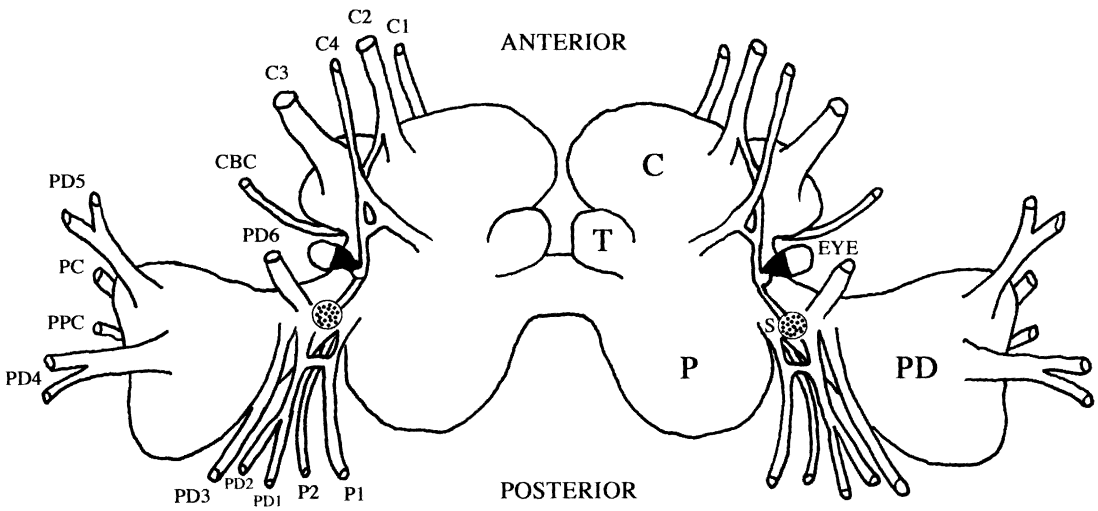


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Fig. 1. **A,B:** Anatomy of the *Melibe* brain. **A:** Photomicrograph of an isolated central nervous system (CNS). The two fused ganglia that constitute most of the brain are the cerebral (C) and pleural (P) ganglia (also referred to jointly as the cerebropleural ganglia). Near the large commissure that connects both halves of the brain, there are the paired tentacular lobes (T). The left and right pedal ganglia (PD) are lateral to the cerebropleural ganglia. Note that the eyes are located in very close association with the brain. The statocysts (S) also are located in close association with the brain, nestled between the pleural and pedal ganglia. Also seen in this photomicrograph is one of the buccal ganglia (B), which is connected to the cerebral ganglia by the cerebral-buccal connective (CBC). Normally, they are located ventral to the brain on either side of the esophagus, near the pedal connectives. **B:** Drawing of the *Melibe* brain with abbreviations for each of the nerves. In vivo, the pedal-pedal connectives (PPC and PC) connect both pedal ganglia, encircling the esophagus. Scale bar = 200 μm.

dium azide in 0.1 M PB (PTA), followed by 24 hours in 6% goat serum (GS) in PTA (PTA-GS). This was followed by incubation in primary antibodies (antiuniversal NOS diluted 1:100 in PTA-GS) for 48 hours and a 24-hour rinse in PTA. Brains were then incubated for 24 hours in goat anti-rabbit secondary antibodies conjugated to fluorescein (diluted 1:100 in PTA-GS) and finally rinsed in several changes of PB over 24 hours. Preparations were dehy-

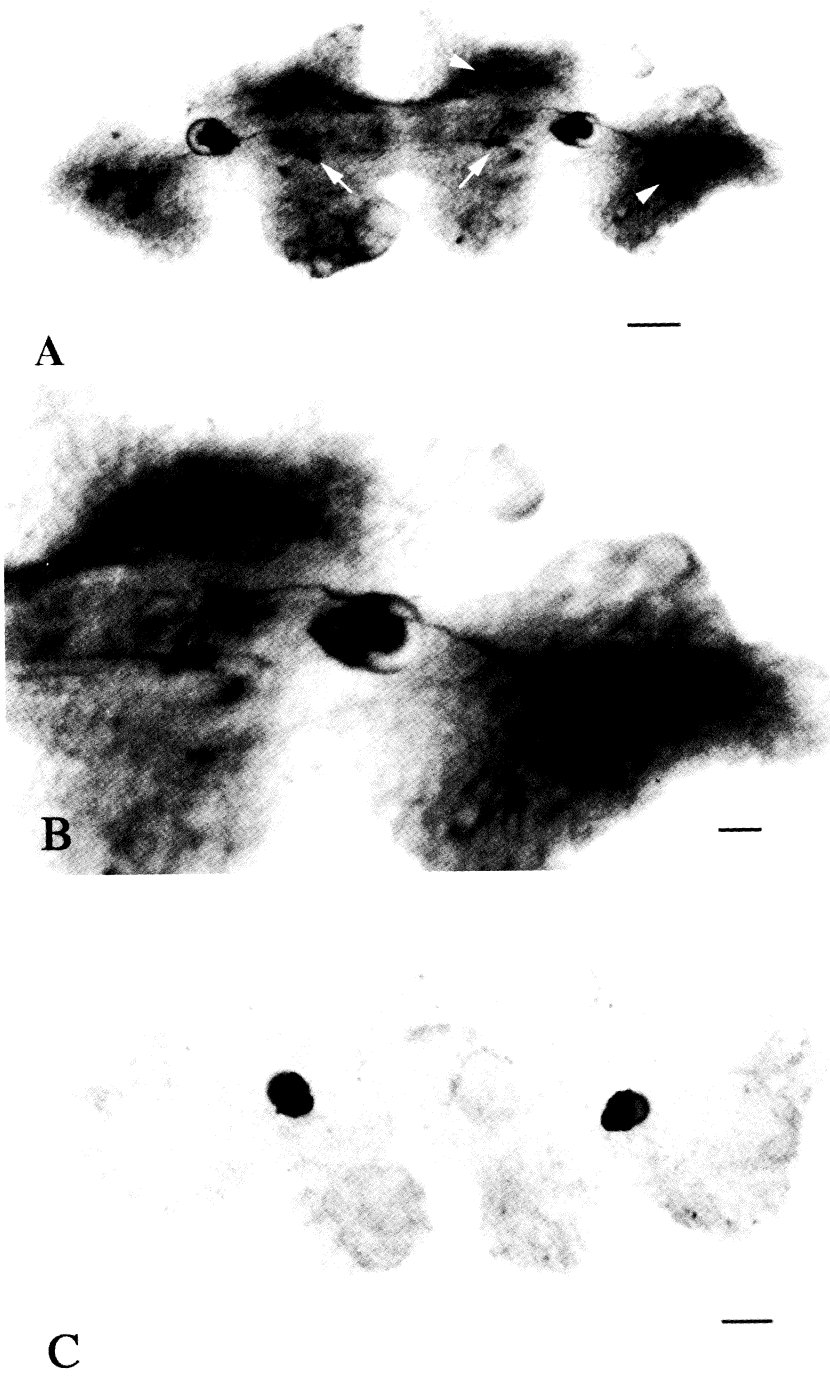

Fig. 2. Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry in the CNS of *Melibe*. **A:** NADPH-d reactivity. There was intense staining of two bilaterally symmetrical cells in the cerebropleural ganglia (arrows). These neurons each projected into the ipsilateral pedal ganglion. There was also nitric oxide synthase (NOS) activity in the neuropil in the cerebral and pedal ganglia (arrowheads). **B:** High-magnification photomicrograph of the NADPH-d reactivity shown in A. **C:** NADPH-d histochemistry control. There was no staining when NAD+ was substituted for NADPH in control preparations. Scale bars = 250 μm in A,C, 100 μm in B.

drated in an alcohol series to 100% EtOH, cleared in xylene and methyl salicylate, and mounted in DPX for viewing and photography on a Bio-Rad MRC-600 confocal microscope (Bio-Rad, Cambridge, MA). Controls (n = 3) were carried out using the same methods described above, except that primary antibodies were omitted. Adobe Pho-

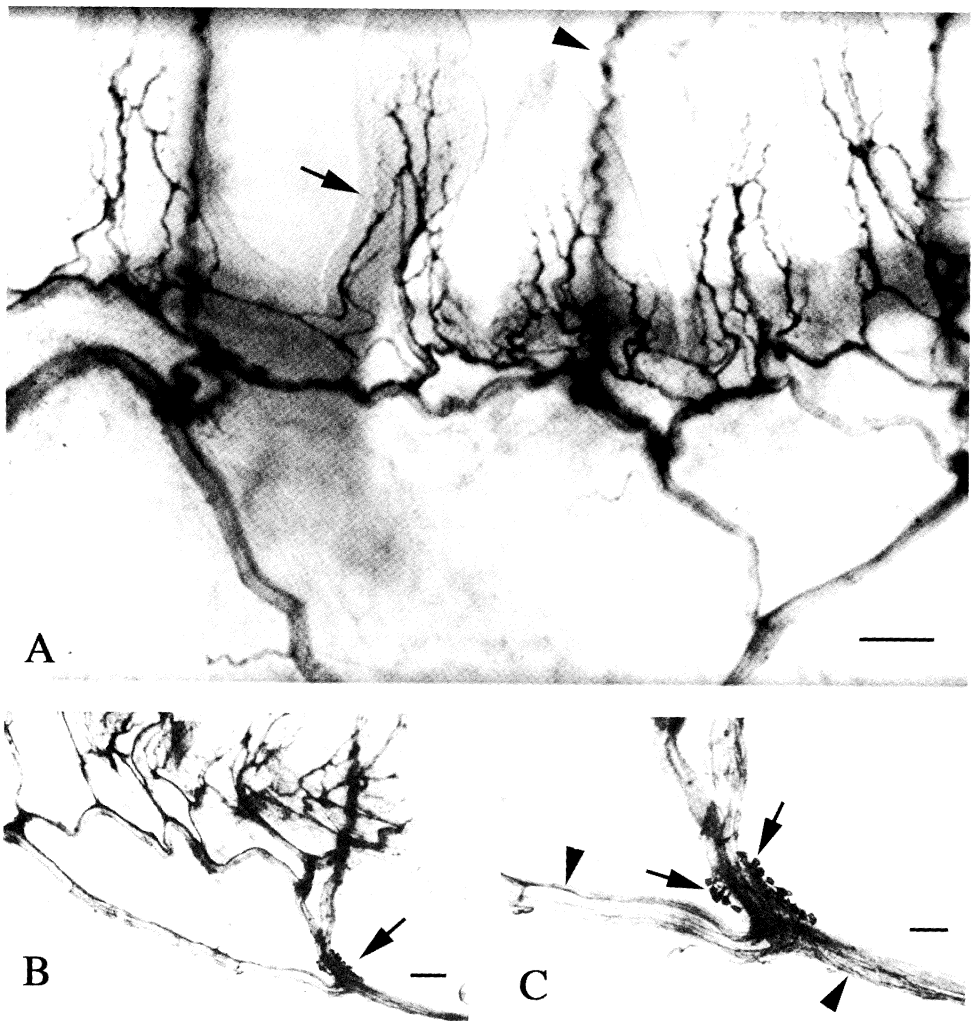

Fig. 3. NADPH-d reactivity in tentacles. **A:** NOS activity was seen in both the inner (arrow) and outer (arrowhead) rows of tentacles. **B:** NADPH-d reactivity in another set of tentacles showing the presence of sensory cell bodies that stained for NOS activity (arrow). **C:** High-magnification photomicrograph of the cell bodies in B (arrows). Axons from the sensory neurons also show NOS activity (arrowheads). Scale bars = 500 μm in A,B, 250 μm in C.

toshop software was used as described above to compile the immunocytochemistry figure.

RESULTS

The CNS of *Melibe* consists of four ganglia, two cerebropleural ganglia and two lateral pedal ganglia (Fig. 1A,B), which are situated just above the dorsal surface of the esophagus (Hurst, 1968; Trimarchi and Watson, 1992). Two pedal connectives pass underneath the esophagus, connecting the pedal ganglia. The buccal ganglia are situated just anterior to the CNS and are connected to the cerebral ganglia by the cerebral-buccal connectives.

NADPH-d histochemistry

NADPH-d histochemistry of the CNS revealed only two bilaterally symmetrical cells in the cerebropleural ganglia (Fig. 2A, B). Each cell has an axon that projects through the cerebral-pedal connective to the ipsilateral pedal ganglion. Once in the pedal ganglion, the axon bifurcates and surrounds much of the pedal neuropil. Neuropil staining also was present in the anterior portion of the cerebral ganglia and in the pedal ganglia (Fig. 2A,B). Somata and neuropil staining were eliminated in control preparations (Fig. 2C).

NADPH-d reactivity varied with fixation time. Fixation for less than 15 minutes often resulted in intense general staining throughout the CNS. Fixation for periods longer than 15 minutes, however, did not result in much variation in staining pattern and were typically like the preparation shown in Figure 2A. NADPH-d reactivity also was temperature-dependent. At room temperature, the entire CNS stained regardless of fixation time, whereas performing the procedure at 4–15°C eliminated this nonspecific staining.

NADPH-d histochemistry also was carried out on the PNS and selected organs. NOS activity was intense in both the inner and outer rows of tentacles on the oral hood (Fig. 3A) as well as in cell bodies and axons of sensory neurons associated with the tentacles (Fig. 3B,C). NOS

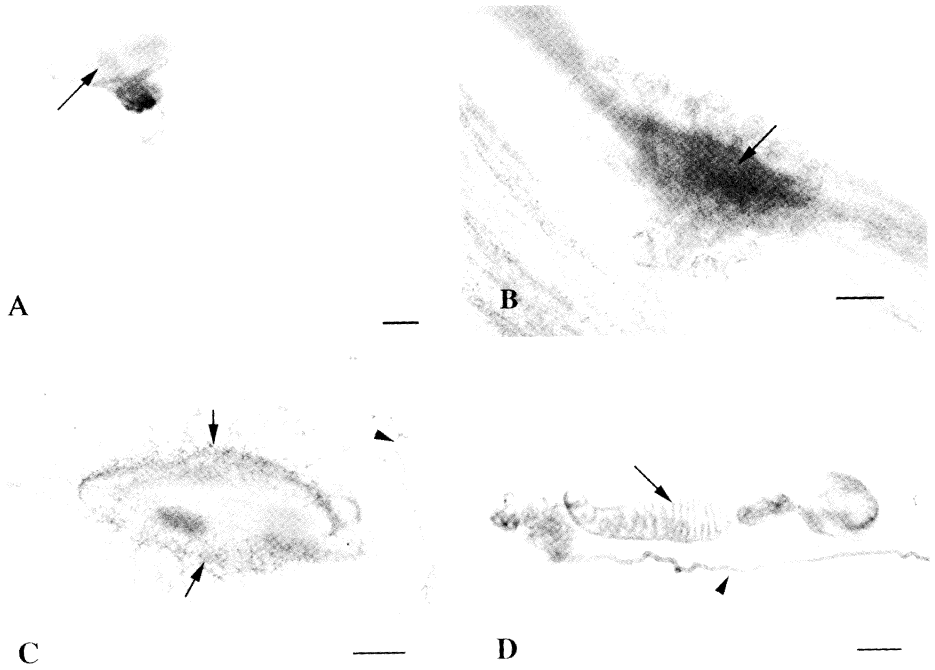

Fig. 4. NADPH-d reactivity in various peripheral organs and ganglia. **A:** Rhinophore. NOS activity was most prominent in the distal, sensory end of the rhinophore (arrow) (rhinophore tissue is not visible due to clearing with methyl salicylate). **B:** Buccal ganglia. NOS activity was apparent in the central neuropil region (arrow) but not in any of the cell bodies of the buccal ganglion. **C:** Mouth. Numerous fine neuronal processes around the mouth (arrows) are stained for NOS activity. Staining also can be seen at the base of the tentacles (arrowhead) in this view. **D:** Preputium and glans penis. NOS activity also was seen both in the circular muscle bands of the preputium (arrow) and in the core of the glans penis (arrowhead). Scale bars = 150 μm in A, 50 μm in B, 1 mm in C, 500 μm in D.

activity was seen in the sensory ends of the rhinophores (Fig. 4A) and in the neuropil of the buccal ganglia (Fig. 4B). Epithelial tissue lining the mouth contained numerous NADPH-d-positive projections (Fig. 4C) as well as the preputium and glans penis (Fig. 4D). NOS activity was not observed in the salivary glands, heart, esophagus, digestive system, foot, skin, body wall, or ovotestis (not shown). Control preparations with NAD+ showed no staining (not shown).

NOS immunocytochemistry

Immunocytochemical staining with universal NOS antisera revealed two bilaterally symmetrical cells in the cerebropleural ganglia with the same morphology as those stained with NADPH-d histochemistry (Fig. 5A). Double-labeling experiments with both NOS immunocytochemistry and NADPH-d histochemistry indicated that the two techniques, in fact, were labeling the same cells (not shown). The fluorescent labeling with the NOS antisera facilitated greater morphological resolution of these nitrergic cells than was possible with NADPH-d histochemistry and showed a number of fine axonal processes extending anteriorly into the cerebral ganglia (Fig. 5B). However, immunocytochemistry did not show any neuropil staining in either the cerebral, pedal, or buccal ganglia. No immunoreactivity was evident in control preparations (not shown). NOS immunocytochemistry also was carried out on peripheral structures but never resulted in any staining (not shown).

DISCUSSION

In this study, both NADPH-d histochemistry and NOS immunocytochemistry were used to identify two bilaterally symmetrical cells in the cerebropleural ganglia of the CNS of *Melibe* that contain NOS (Fig. 2A,B). Each of these cells is located at the base of a rhinophore nerve, and their axons project into the ipsilateral pedal ganglion, with other fine processes also projecting into the ipsilateral cerebral ganglion.

NADPH-d activity also was present in the neuropil of the anterior portion of the cerebral ganglia and the center of the pedal ganglia. The neuropil staining was not sensitive to long-term fixation, which is a criterion of NADPH-d activity specific to NOS (Matsumoto et al., 1993), suggesting that the neuropil staining seen with NADPH-d histochemistry, in fact, was due to NOS rather than other NADPH-d enzymes. The staining in the cerebral neuropil may have been due to NOS activity from the two nitrergic cells or from sensory cells in the tentacles and hood that project back to the cerebral ganglia. The NADPH-d reactivity in the pedal neuropil most likely was due to projections from the two nitrergic cells, because there was no staining of afferent fibers projecting into the pedal ganglia from the periphery.

NOS immunocytochemistry corroborated the NADPH-d results in the CNS by staining the same two cells in the cerebropleural ganglia (Fig. 5A,B). However, there was no neuropil staining in the immunocytochemical preparations. Perhaps the concentration of NOS in the neuropil

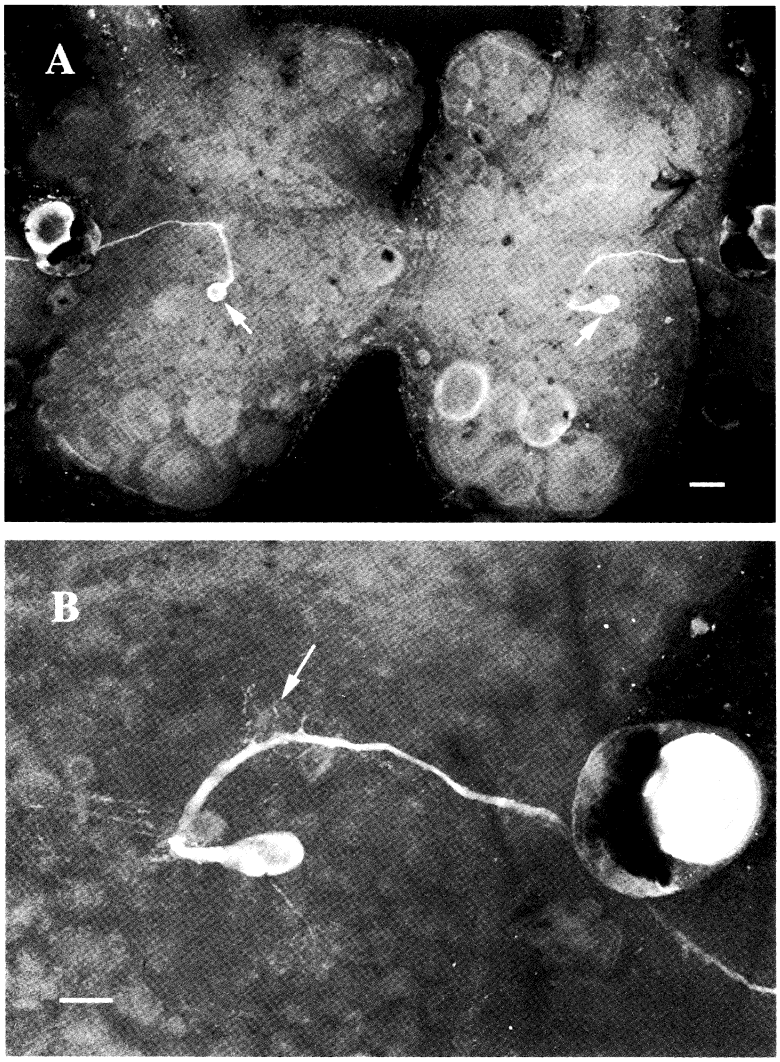

Fig. 5. NOS immunocytochemistry. **A:** Arrows indicate the same two nitrergic cells that are shown in Figure 2A with NADPH-d histochemistry. The eyes can be seen as well, because they are autofluorescent (as seen in controls). **B:** High-magnification photomicrograph of a nitrergic cell. Arrow indicates fine processes that branch off of the axon and project into the cerebral neuropil. Scale bars = 100 μm in A, = 50 μm in B.

was too low to register a signal with this technique, or it is possible that the neuropil staining resulting from the NADPH-d histochemistry was due to another NOS-related enzyme despite the resistance to long-term fixation. In peripheral structures, NOS immunocytochemistry did not result in any staining, suggesting that the NADPH-d labeling of these peripheral organs may have been due to an isoform of NOS different from that found in the CNS.

There are only two other published accounts of NOS immunocytochemistry in gastropods, as stated above. In the CNS of *Lymnaea stagnalis*, Moroz and colleagues (1994) found the highest concentration of NOS-immunoreactive cells in the buccal ganglia, although there also were cells in other areas of the CNS. In *Helix aspersa*, Cooke et al. (1994) found a small number of nitrergic cells in both the cerebral and pedal ganglia. Our results, which show the presence of only two NOS-positive cells in the cerebropleural ganglia of *Melibe*, do not resemble the patterns reported in either of these previous studies. This is not too surprising, however, because both *Lymnaea* and *Helix* belong to the gastropod subclass Pulmonata, whereas *Melibe* belongs to the subclass Opisthobranchia.

NADPH-d reactivity in the CNS of other gastropods has proven to be quite variable. Moroz and Gillette (1995) found that basal lineages of gastropods had little NOS activity in the CNS and more extensive activity in the PNS. In more derived lineages, this pattern of NADPH-d reactivity was reversed. Nudibranchs were an exception to this pattern, however, because none of them produced much NADPH-d reactivity in the CNS, even though they are considered to be more derived than other gastropods, such as polyplacophorans, monoplacophorans, aplacophorans, anaspids, and saccoglossids (Schmekel, 1985;

Salvini-Plawen and Steiner, 1996). It is possible that the use of NO in the periphery may have been evolved secondarily in nudibranchs, but the reasons remain unclear.

There may be some behavioral and ecological factors that explain more readily how NO is used in the nervous system of gastropods. It has been hypothesized that NADPH-d activity is more pronounced in the CNS of invertebrates with a predatory feeding style compared with herbivorous grazers (Moroz and Gillette, 1995). *Melibe*, although it is carnivorous, exhibits a grazing mode of feeding and, thus, would be classified as an herbivorous grazer (Agersborg, 1919, 1921; Hurst, 1968; Watson and Trimarchi, 1992; Watson and Chester, 1993). Localization of NOS in *Melibe* supports the aforementioned hypothesis due to the fact that only two cells in the entire CNS contain NOS compared with a predatory opisthobranch, such as *Pleurobranchaea californica*, which has a high number of putative nitrergic cells in the CNS (Moroz and Gillette, 1996). Moroz and Gillette (1995) also found that grazers had a relatively higher amount of NADPH-d activity in the PNS and in the neuropil of the CNS compared with predators. Once again, these findings correlate well with our results showing extensive NADPH-d reactivity in the PNS of *Melibe* (Figs. 3, 4) and a large amount of neuropil staining in the CNS (Fig. 2A).

NADPH-d histochemistry in the PNS and peripheral organs and tissues of *Melibe* showed staining in the buccal ganglia as well as the tentacles, rhinophores, and mouth (Figs. 3, 4). This staining suggests a chemosensory role for NO. This was not surprising, because NO has been implicated in chemoreception and feeding in a number of gastropods (Moroz et al., 1993, 2000; Gelperin, 1994; Elphick et al., 1995; Teyke, 1996; Lovell et al., 2000). However, the localization of two putative NO-releasing cells in the cerebropleural ganglia of the CNS with axons that extend into the pedal ganglia suggests a role for NO beyond just chemoreception or activation of a feeding central pattern generator (CPG). Although most, if not all, swim CPGs (sCPGs) usually reside in the cerebral ganglia of gastropods, the swim motoneurons usually are situated in the pedal ganglia and receive input from interneurons in the cerebral ganglia (Willows, 1973; Getting et al., 1980; Lennard et al., 1980; Getting, 1983a,b; Arshavsky et al., 1985; Satterlie, 1985, 1991; Satterlie et al., 1985; McPherson and Blankenship, 1991; Gamkrelidze et al., 1995; Jing and Gillette, 1995, 1999). In *Melibe*, the pedal ganglia contain two of the four identified sCPG interneurons as well as all of the motoneurons involved in swimming (Lawrence, 1997; Newcomb and Watson, 2000; Watson et al., 2001). Because the NOS-containing cells project into the pedal ganglia in *Melibe*, this is suggestive of a role for NO in locomotion, possibly as a modulator of swimming.

When they are feeding, *Melibe* typically stop crawling and swim less often (personal observation). Therefore, it is possible that NO is being used as a neuromodulator to inhibit swimming in the presence of food. Recent pharmacological work in our laboratory supports this (Newcomb and Watson, 2000; Watson et al., 2001). Moroz et al. (2000) also recently found evidence suggesting that NO is used as a neuromodulator in both the swimming and feeding networks of the pteropod mollusk *Clione limacina*. In this case, NO seems to play an excitatory role by activating the swimming and feeding CPGs. However, unlike *Melibe*, which need to stop swimming to feed, *Clione* feed in the water column and, thus, need to stimulate swimming in order to feed.

In summary, both NADPH-d histochemistry and NOS immunocytochemistry stain the same two bilaterally symmetrical cells in the CNS of the nudibranch *Melibe leonina*. These nitrergic neurons each project into the ipsilateral pedal ganglion. Because the pedal ganglia largely control locomotion, our results provide morphological evidence suggesting a possible role for NO in the modulation of swimming or crawling in *Melibe leonina*. In addition, because there are only two easily identifiable nitrergic cells in the CNS, this may be an effective model system for investigating many fundamental aspects of the function of nitrergic neurons.

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