THE JOURNAL OF COMPARATIVE NEUROLOGY 437:70–78 (2001)

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-

Grant sponsor: National Institutes of Health; Grant number NS36411.

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

Received 23 January 2001; Revised 15 May 2001; Accepted 23 May 2001

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-