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NO nerves in trematodes, too! NADPH-diaphorase activity in adult *Fasciolopsis buski*

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Abstract

The free radical nitric oxide (NO) is a unique molecule with an avidity to react with other molecules and is known to function as a neuronal messenger. This nitrergic transmitter with diverse functions in signal transduction, being a gas, is not stored in synaptic vesicles but is generated in various neuronal cells by a family of nitric oxide synthases (NOSs). The NADPH-d histochemical reaction is regarded as a selective marker for NOS in the neuronal tissue. With histochemical detection of NADPH-d, the presence of NOS is demonstrated in the digenetic trematode, *Fasciolopsis buski*. Strong NADPH-d staining was observed in the neuronal cell bodies in the two cerebral ganglia, the brain commissure and the nerve fibers in the main nerve cords. NADPH-d staining was also detectable in the innervation of the pharynx, the cirrus sac and the ventral sucker besides being observable sporadically in the nerve tributaries in the general parenchyma. NO released by the whole worm kept in PBS at 37°C could also be measured biochemically. The NOS activity was assayed in the whole worm homogenate and also in the tissue homogenate containing only the anterior pre-acetabular part of the parasite body. The presence of NOS in this digenean parasite confirms that a nitrergic innervation occurs in the trematode group also as in other groups of exclusively parasitic helminths and that NO represents an old signal molecule in evolutionary scale.

Keywords: Trematode; Parasite; *Fasciolopsis buski*; Nitric oxide; NADPH-diaphorase; Nitric oxide synthase; Nitrergic transmitter

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1. Introduction

Nitric oxide is the most current and markedly uncommon neuronal messenger [1,2] and the smallest cum lightest molecule (the first gas) known to act as a neuronal transmitter [2,3]. The molecule has one unpaired electron making it a free radical that avidly reacts with other molecules [4]. The nature of the nitrergic transmitter has been discussed by Rand and Li [5]. Neurotransmitters generally are small hydrophobic molecules, which are stored in and secreted from synaptic vesicles. Due to the water-soluble gaseous nature of NO, it is not stored in the synaptic vesicles; instead NO is produced when required by a family of nitric oxide synthase enzymes (NOSs), which produces NO from L-arginine. NOS exists in a split form — one constitutive, occurring mainly in the nervous or endothelial tissue and the other inducible that occurs mainly in the macrophages [6]. Neuronal NOS requires the presence of nicotinamide adenine dinucleotide phosphate (NADPH), which acts as a donor of electrons, and also a higher level of intracellular Ca^{2+} to become activated [6]. According to Bredt and co-workers [7,8] and Hope et al. [9] neuronal NOS and NADPH-diaphorase (NADPH-d) are identical molecules. The NADPH-d histochemical reaction is regarded as a selective marker for NOS in neuronal tissue.

NOS has been detected by histochemical and immunocytochemical methods in the CNS and PNS of vertebrates [3]. In invertebrates NOS has been detected in the nervous system of molluscs [10–12], annelids [10,13], crustaceans [14,15], insects [16] and a nematode [17], though in the free living flatworms and coelenterates no NOS was detectable [10]. NADPH-d activity was also demonstrated in the adult pseudophyllidean and cyclophyllidean cestodes [18–20] as well as in tetrathyridia of *Mesocestoides vogae* and free living turbellaria [21,22], and recently in adult *Fasciola hepatica* [23].

In the giant intestinal fluke, *Fasciolopsis buski*, of swine host, the organization of the cholinergic nervous system has been described (Kar and Tandon, unpublished observations). In the present study, the NADPH-d histochemical reaction was applied to longitudinal/sagittal sections of this trematode, so as to demonstrate the NADPH-d reactivity in the nervous system of the parasite. In addition, biochemical demonstration of NO in the effluent of the in vitro kept worm and NOS activity in the tissue homogenates form the basis of this communication.

2. Materials and methods

Live specimens of adult *Fasciolopsis buski* were obtained from freshly slaughtered pigs at the local abattoirs in Shillong.

2.1. NADPH-d histochemistry

The worms were immersion-fixed in freshly made 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4, overnight at 4°C. After fixation the worms were transferred to PBS with 10% sucrose for a few days at 4°C. The frozen material was sectioned frontally and sagittally at 20 μm on a SLEE HR cryostat at −15 to −20°C. The sections collected on chrome-alum gelatine/poly-L-lysine coated glass slides were dried for 2 h at room temperature and stained directly or kept at −70°C.

NADPH-d histochemical staining was performed following the protocol as detailed by Gustafsson et al. and Lindholm et al. [18,19]. The concentration of β-NADPH in the incubation medium was 2 mg ml^{-1}. After incubating at 37°C for 1–4 h, the sections were rinsed in 0.01 M PBS with 0.2% Triton X-100 thrice for 15 min each, rinsed in distilled water and mounted in 50% glycerol in PBS.

For controls, β-NADPH was substituted with β-NADH in the same concentration and the incubation was performed for 1 h at 37°C.

2.2. NOS assay

The NOS activity was assayed separately in the whole worm tissue and also in the pre-acetabular cut portion of the parasite; the latter contains the cephalic neuronal tissue in the form of brain ganglia and the transverse commissure. A 10%

homogenate of the freshly collected worm was prepared in a homogenizing buffer containing 20 mM HEPES buffer (pH 7.2), 300 mM mannitol, 1 mM ethylenediamine-tetra-acetic acid (EDTA), 1 mM dithiothritol (DTT), using a motor-driven Potter-Elvehjem glass homogenizer with a Teflon pestle. Phenylmethylsulfonyl fluoride (PMSF) (10 mg ml^{-1}) was also added in the homogenate. The homogenate was treated with 0.5% Triton X-100 in a 1:1 ratio for 30 min, followed by a mild sonication for proper breakage of mitochondria and centrifuged at $10\,000 \times g$ for 10 min. The supernatant was then used for assaying the NOS activity. All the steps were carried out at 4°C.

The NOS activity was assayed following the method of Salter and Knowles [24] with certain modifications. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.2), 50 mM L-arginine, 1.2 mM $MgCl_2$, 0.24 mM $CaCl_2$, 0.12 mM NADPH and 0.1 ml of tissue homogenate (prepared as above) in a final volume of 1 ml. The reaction mixture also contained 20 units of urease, sufficient enough to convert all the urea formed (due to the reaction of arginase) to ammonia and carbon dioxide, so that it would not interfere with the estimation of citrulline. The reaction mixture was incubated at 37°C for 15 min and the reaction was stopped by adding 1 ml of 10% perchloric acid (PCA), followed by centrifugation to precipitate out the protein. Citrulline so formed as the reaction product was estimated in the supernatant spectrophotometrically (Beckman DU 640) at 490 nm following the method of Moore and Kauffman [25] against a reagent blank, where 10% PCA was added prior to the addition of tissue homogenate, and expressed as enzyme activity. One unit of enzyme activity is defined as that amount that catalyses 1 μmol of citrulline formed per hour at 37°C.

2.3. NOs estimation in the culture medium

Pre-weighed live worms were kept in different petri dishes each containing 15 ml of PBS and incubated at 37°C for 12 h. The medium was saturated with oxygen at 1 h intervals by administering oxygen into the solution. Sample (0.9 ml) was taken out from each petri dish for estimation of NO released by the parasite. NO in oxygenated aqueous solution in the absence of oxyhemoglobin is oxidized primarily to nitrite (NO_2^-) with little or no formation of NO_3^- [26]. NO_2^- concentration in the incubation medium was determined spectrophotometrically at 540 nm following the Griess reaction as described by Sessa et al. [27], and was equivalent to NO released by the parasite. A standard curve was prepared with sodium nitrite to calculate the NO concentration in the medium.

Values of NOS activity and NO release are expressed as mean ± S.E.M.

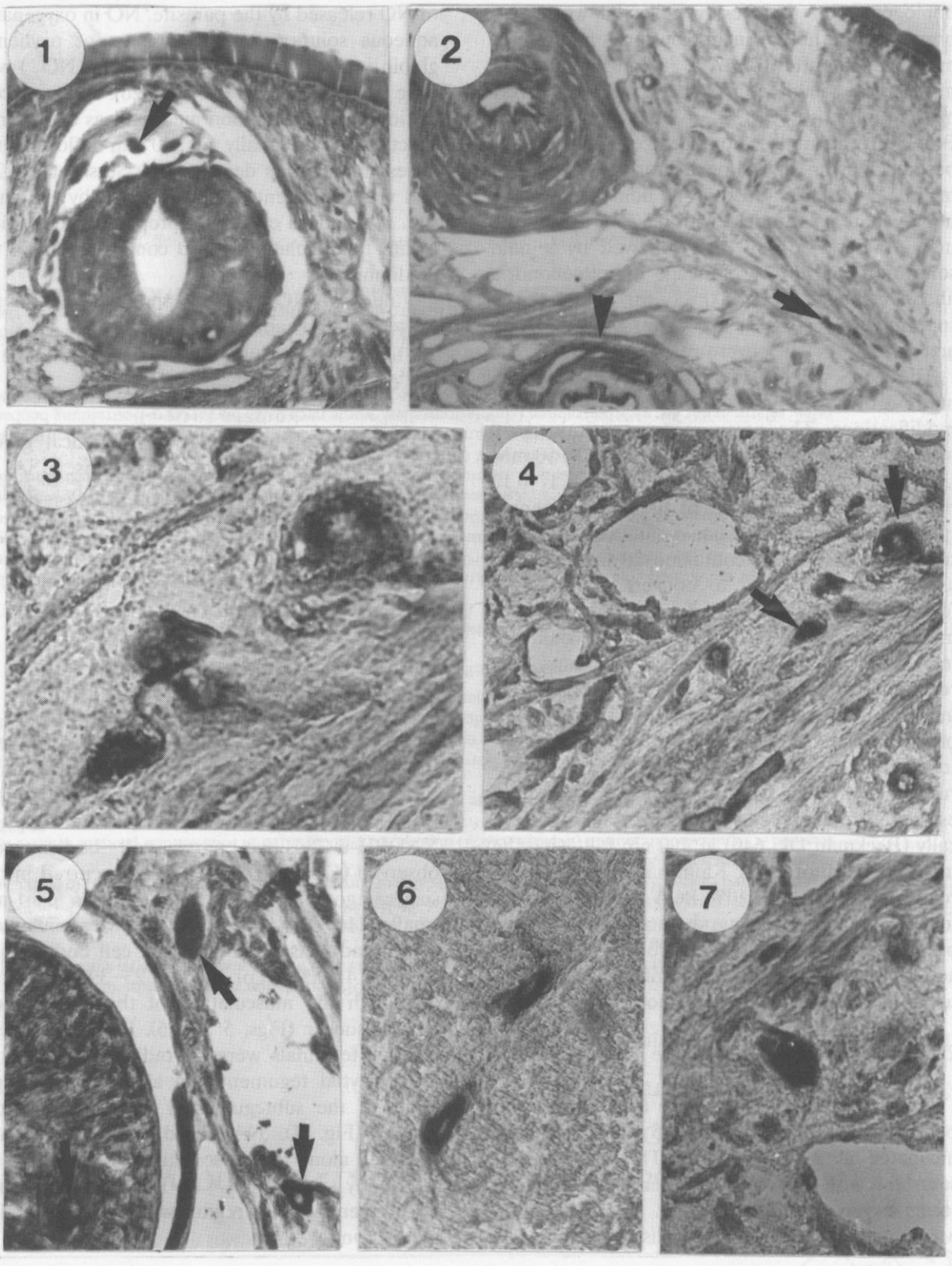
2.4. Chemicals

All chemicals used were of analytical grades and obtained from Sigma (St. Louis, USA) or Sisco Research Laboratories (Mumbai, India). Deionized, double distilled water was used for all preparations.

3. Results

3.1. NADPH-d activity

Strong NADPH-d staining was demonstrable in the neuropile of the transverse cerebral commissure, cerebral ganglia and the main nerve cords (MCs), wherein rounded, oval or elliptical NADPH-d positive nerve cell bodies were observed (Figs. 1–4). These cells occurred in two size categories: larger (36–69 × 16–36 μm) and smaller (16–34 × 9–23 μm). Positive staining was also found in isolated neuronal cell bodies (size: 59–80 × 41–59 μm or 34–57 × 23–39 μm) associated with the musculature of the pharynx and ventral sucker (Figs. 5 and 6). Small NADPH-d positive terminals were sporadically observed in the syncytial tegument and also as the components of the subtegumental peripheral nervous system (Fig. 7). NADPH-d staining was also observed along the musculature of the cirrus sac (cell body size: 20–46 × 11–23 μm) (Fig. 2) and circum-genital pore region where it was moderately strong.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3.2. NO release by the whole worm and NOS activity

There was a continuous release of NO by the live worms kept in vitro in the PBS at 37°C for 12 h at uniform rate. The rate of release of NO by the worm was found to be 9.96 ± 0.37 nmol g^{-1} body wt h^{-1} ($n = 12$).

NOS enzyme activity was also measured in the parasite. It was found to be 19.21 ± 0.4 ($n = 3$) units g^{-1} wet wt. in the pre-acetabular cephalic region and 5.33 ± 0.31 ($n = 3$) units g^{-1} wet wt. in the whole body.

4. Discussion

The NADPH-d staining method is considered to be a selective marker for neuronal NOS [28]. The nerve cells of *F. buski* may thus be regarded as capable of synthesizing NO. In the nervous system of this digenetic fluke, cholinergic nerve fibers and nerve cells have been detected (Kar and Tandon, unpublished results). Through this study the presence of nitrergic innervation is also demonstrated. Among the parasitic helminths, NOS has been detected in the nematode, *Ascaris suum* [17], the cestodes, *Diphyllobothrium dendriticum* and *Hymenolepis diminuta* [22] and *Mesocestoides* tetrathyridia [21], and in the digenetic liver fluke, *Fasciola hepatica* [23]. The presence of NOS in *F. buski* and *F. hepatica* clearly indicates that nitrergic innervation occurs in the trematode group also as in other groups of exclusively parasitic helminths.

NADPH-d staining was detected along muscle fibers in the pharynx, ventral sucker around the genital atrium and in the sensory endings in the tegumental and subtegumental regions in *F. buski* resembling the distribution pattern as indicated in *F. hepatica* [23], as well as in cestode and free living flatworms [22]. NADPH-d positive neurons in two sizes (large and small) are known to occur in platyhelminth parasites [18,19]. The presence of NADPH-d activity along the muscle fibers is suggestive of a myoinhibitory role for NO [23]. In *A. suum* it has been suggested that NO has a potential role as a neurotransmitter at the neuromuscular junctions [17]. In the mammalian system also, NO has been shown to be involved in diverse functions, e.g. food intake, muscle relaxation (through increasing cyclic GMP levels), etc. [29,30]. Among the lowly organized metazoans, involvement of NO in feeding response of *Hydra* has been shown [31]. The NOS activity in the pharyngeal musculature may be suggestive of a similar function in the orally feeding trematodes.

The free radical NO is synthesized from L-arginine and molecular oxygen by NOS; in the synthesis NO and L-citrulline are produced [32,33]. In the present study NO released by the parasite maintained in PBS at 37°C could be measured for 12 h. The NO activity was analyzed in *H. diminuta* by measuring the formation of L-citrulline after incubation with L-[^{3}H]arginine [20]. The demonstration of the NO release in *F. buski* corroborates the presence of NOS in digenean parasites. Biochemically also, the NOS activity was demonstrable in the fluke. It seems evident that NO, synthesized in the parasitic platyhelminths also, represents an old signal molecule that has been conserved through evolution.

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Fig. 1. NADPH-d positive cells in the transverse cerebral commissure (arrow) anterior to the pharynx (48 ×).
Fig. 2. Neuronal cells (arrow) in the MC anterior and lateral to the ventral sucker. NADPH-d staining is evident in the cirrus pouch region as well (arrow head) (48 ×).
Fig. 3. A closer view of the NADPH-d positive cells in the pre-pharyngeal region (120 ×).
Fig. 4. NADPH-d active cells (arrows) in the cerebral ganglion (120 ×).
Fig. 5. Strong enzyme activity depicted in the neuronal cells along the nerve beside pharynx and a cell associated with the musculature of the pharynx (arrows) (300 ×).
Fig. 6. Strong NADPH-d staining in the cells along the musculature of the ventral sucker (300 ×).
Fig. 7. A single NADPH-d positive cell in the subtegumental parenchyma (120 ×).

References

[1] Garthwaite J. Glutamate, nitric oxide and cell–cell signalling in the nervous system. Trends Neruosci 1991; 14:60–67.

[2] Snyder SH. Nitric oxide: first in a new class of neurotransmitters? Science 1995;257:494–496.

[3] Vincent SR. Localization of nitric oxide neurons in the central nervous system. In: Vincent S, editor. Nitric oxide in the nervous system. London: Academic Press, 1995:83–102.

[4] Culotta E, Koshland Jr. DE. NO news is good news. Science 1992;258:1862–1865.

[5] Rand MJ, Li CG. Nitric oxide in the autonomic and enteric nervous system. In: Vincent S, editor. Nitric oxide in the nervous system. London: Academic Press, 1995:228–279.

[6] Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 1991;43:109–142.

[7] Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature (London) 1991;351:714–718.

[8] Bredt DS, Glatt CE, Hwang PM, Fothui M, Dawson TM, Snyder SH. Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuron 1991;7:615–624.

[9] Hope BT, Michael GJ, Knippe KM, Vincent SR. Neuronal NADPH-diaphorase is a nitric oxide synthase. Proc Natl Acad Sci USA 1991;88:2811–2814.

[10] Elofsson R, Carlberg M, Moroz L, Nezlin L, Sakharov D. Is nitric oxide (NO) produced by invertebrate neurones? Neuroreport 1993;4:279–282.

[11] Moroz LL, Gillete R. From Polyplacophora to Cephalopoda: comparative analysis of nitric oxide signalling in the mollusca. ISIN, Tihany, Proc. 1995:46.

[12] Moroz LL, Winlow W. Nitric oxide signalling in the gastropod mollusc, *Lymnaea stagnalis* (L). ISIN, Tihany, Proc. 1995:47.

[13] Leake LD, Moroz LL. Nitric oxide in the CNS of the leech *Hirudo medicinalis*. ISIN, Tihany, Proc. 1995:42.

[14] Johansson KUI, Carlberg M. NADPH-diaphorase histochemistry and nitric oxide synthase activity in deutocerebrum of the cray fish, *Pacifastacus leniusculus* (Crustacea, Decapoda). Brain Res. 1994;649:36–42.

[15] Johansson KUI, Hallberg E. Fine structural localization of NADPH-diaphorase in the crayfish olfactory lobe. ISIN, Tihany, Proc. 1995:34.

[16] Elphick M, Green IC, O'shea M. Nitric oxide synthesis and action in an invertebrate. Brain Res. 1993;619: 344–346.

[17] Bascal ZA, Montgommery A, Holden-dye L, Williams RG, Walker RJ. Histochemical mapping of NADPH-diaphorase in the nervous system of the parasitic nematode *Ascaris suum*. Parasitology 1995;110:625–637.

[18] Gustafsson MKS, Lindholm AM, Terenina NB, Reuter M. NO nerves in tapeworm. NADPH-diaphorase histochemistry in adult *Hymenolepis diminuta*. Parasitology 1996;113:559–565.

[19] Lindholm AM, Reuter M, Gustafsson MKS. The NADPH-diaphorase staining reaction in relation to the aminergic and peptidergic nervous system and the musculature of adult *Diphyllobothrium dendriticum*. Parasitology 1998;117:283–292.

[20] Terenina NB, Onufriev MV, Gulyaeva NV, Lindholm AM, Gustafsson MKS. A radiometric analysis of nitric oxide synthase activity in *Hymenolepis diminuta*. Parasitology 2000;120:91–95.

[21] Terenina NB, Reuter M, Gustafsson MKS. An experimental, NADPH-diaphorase histochemical and immnocytochemical study of *Mesocestoides vogae* tetrathyridia. Int J Parasitol 1999;29:787–793.

[22] Gustafsson MKS, Lindholm AM, Mäntylä K, Reuter M, Lundström CA, Terenina N. No news on the flatworm front! Nitric oxide synthase in parasitic and free living flatworms. Hydrobiologia 1998;383:161–166.

[23] Gustafsson MKS, Terenina N, Kreshchenko ND, Reuter M, Maule AG, Halton DW. Comparative study of the spatial relationship between nicotinamide adenine dinucleotide phosphate diaphorase activity, serotonin immunoreactivity and the musculature of the adult liver fluke, *Fasciola hepatica* (Digenea, Fascioloidae). J Comp Neurol 2001;429:71–79.

[24] Salter M, Knowles RG. Measurement of NOS activity by conversion of radiolabelled arginine to citrulline using ion-exchange separation. In: Titheradge. MA, editor. Nitric oxide protocols. New Jersey, USA: Human Press, 1998:67–74.

[25] Moore RB, Kauffman NG. Simultaneous determination of citrulline and urea using diacetylmonoxime. Anal Biochem 1970;33:263–272.

[26] Ignarro LJ, Fokoto JM, Griscarage JM, Rogers NE, Byrns RE. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison of enzymatically formed nitric oxide from L-arginine. Proc Natl Acad Sci USA 1993;90:8103–8107.

[27] Sessa WC, Pritchart K, Seyedi N, Wang J, Hintz TH. Chronic exercise in dogs increase coronary vascular nitric oxide production and endothelial cell nitric oxide gene expression. Circulation Res. 1994;74:349–353.

[28] Bredt DS. Molecular characterization of nitric oxide synthase. In: Vincent. S, editor. Nitric oxide in the nervous system. London: Academic Press, 1995:1–19.

[29] Garthwaite J, Boulton CL. Nitric oxide signalling in the central nervous system. Ann Rev Physiol 1995;57: 683–706.

[30] Koesling D, Humbert P, Schultz G. The NO receptor: characterization and regulation of soluble guanylyl cy-

clase. In: Vincent S, editor. Nitric oxide in the nervous system. London: Academic Press, 1995:43–50.
[31] Colasanti M, Lauro GM, Venturini G. NO in hydra feeding response. Nature (London) 1995;374:505.
[32] Knowles RG, Palacios M, Palmer RMJ, Moncada S. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. Proc Natl Acad Sci USA 1989;86:5159–5162.
[33] Stuehr DJ, Griffith OW. Mammalian nitric oxide synthases. Adv Enzymol 1992;65:287–346.