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Ultrastructural localization of NADPH diaphorase and nitric oxide synthase in the neuropils of the snail CNS

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ABSTRACT

Comparative studies on the nervous system revealed that nitric oxide (NO) retains its function through the evolution. In vertebrates NO can act in different ways: it is released solely or as a co-transmitter, released from presynaptic or postsynaptic site, spreads as a volumetric signal or targets synaptic proteins. In invertebrates, however, the possible sites of NO release have not yet been identified. Therefore, in the present study, the subcellular distribution of the NO synthase (NOS) was examined in the central nervous system (CNS) of two gastropod species, the terrestrial snail, *Helix pomatia* and the pond snail, *Lymnaea stagnalis*, which are model species in comparative neurobiology. For the visualization of NOS NADPH-diaphorase histochemistry and an immunohistochemical procedure using a universal anti-NOS antibody were applied. At light microscopic level both techniques labeled identical structures in sensory tracts ramifying in the neuropils of central ganglia and cell bodies of the *Lymnaea* and *Helix* CNS. At ultrastructural level NADPH-d reactive/NOS-immunoreactive materials were localized on the nuclear envelope and membrane segments of the rough and smooth endoplasmic reticulum, as well as the cell membrane and axolemma of positive perikarya. NADPH-d reactive and NOS-immunoreactive varicosities connected to neighboring neurons with both unspecialized and specialized synaptic contacts. In the varicosities, the majority of the NADPH-d reactive/NOS-immunoreactive membrane segments were detected in round and pleomorph agranular vesicles of small size (50–200 nm). However, only a small portion (16%) of the vesicles displayed the NADPH-d reactivity/NOS-immunoreactivity. No evidence for the postsynaptic location of NOS was found. Our results suggest that the localization of NADPH-diaphorase and NOS is identical in the snail nervous system. In contrast to vertebrates, however, NO seems to act exclusively in an anterograde way possibly released from membrane segments of the presynaptic transmitter vesicle surface. Based on the subcellular distribution of NOS, NO could be both a volume and a synaptic mediator, in addition NO may function as a co-transmitter.

1. Introduction

Nitric oxide (NO) is a prominent gaseous signal molecule playing a particular role in neurotransmission (Garthwaite, 2008). In the nervous system NO is synthetized by the neuronal form of nitric oxide synthase (nNOS), and exerts its effect mainly by inducing cyclic guanosine monophosphate (cGMP) synthesis through the activation of its receptor, soluble guanylate cyclase (sGC), or influences protein activity via S-nitrosylation of defined cysteine residues. In the past twenty years the involvement of NO in different processes of the mammalian nervous system, such as regulation of hemodynamics in the CNS, neuronal development, memory formation and consolidation, neuroprotection as well as neurodegeneration, has been well documented (Zhou and Zhu, 2009; Vincent, 2010). NO is considered to be a transmitter or modulator acting alone or as a co-transmitter in anterograde way in the cerebral cortex, cerebellum, brainstem, and the peripheral nervous system. It also can be released from the postsynaptic sites of spiny neurons, acting in a retrograde way in the cerebral cortex and hippocampus (Garthwaite, 2008). Because of the physico-chemical properties of NO it is not surprising that NO behaves as a classical neurotransmitter both at the synapses and neural appositions without membrane specializations, moreover, NO is proposed to act also in a different, less target specific way as a volume signal molecule (Garthwaite, 2008; Vincent, 2010). By volume release NO was shown to modulate the rhythmic activity in a broad tissue area, affecting a number of neurons located even far from the release site (Kiss and Vizi, 2001; Philippides et al., 2005; Ott et al., 2007; Münch et al., 2010). The volumetric mode of NO action has already

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been implicated the modulation of transmitter release and global neuronal activity in vertebrates (Kiss and Vizi, 2001; Steinert et al., 2008), as well as central decoding of olfactory, mechanosensory, and visual perception (Gelperin, 1994; Bicker, 2001), maintaining arousal (Susswein and Chiel, 2012), and the NO-dependent phase of memory formation and consolidation (Kemenes et al., 2002; Yabumoto et al., 2008) in invertebrates.

Since NO is an evolutionary conserved molecule (Bicker, 2001; Palumbo, 2005; Moroz and Kohn, 2011) invertebrate species have been favorite subjects for studies on the general principles of NO signaling. From this point of view it is rather strange why only a single study has dealt until now with fine details of the localization of NOS (Johansson et al., 1996), in contrast to vertebrates (Rodrigo et al., 1997; Aoki et al., 1998; Rothe et al., 1998; Sancesario et al., 2000; Seress et al., 2005). At light microscopic level NOS was found mainly in sensory- and interneurons, and less in motor neurons of invertebrates, and it seemed that NO mainly acts in an anterograde way (Park et al., 1998; Wildemann and Bicker, 1999; Serfőző et al., 2008). At the ultrastructural level, Johansson et al. (1996) showed that NADPH-diaphorase (NADPH-d) reaction, which is used for the histochemical demonstration of NOS, labeled different types of membrane fragments in the neural perikarya and fibers in the crayfish, *Pacifastacus leniusculus*, CNS (Johansson et al., 1996). However, this study has not provided any data for the localization of NOS in axon terminals and varicosities that would help identify the possible way(s) of NO-signaling, providing also a basis for further interpretation of NOergic regulation of different physiological and behavioral processes in invertebrates.

A possible reason why our knowledge about NO is poor might be the uncertainty in specificity of the applied histochemical methods. In the vertebrate nervous system, after aldehyde fixation, only NOS was able to retain its dehydrogenase (NADPH-diaphorase [NADPH-d]) activity (Blottner et al., 1995), and in addition, non-NOS dehydrogenases such as cytochrome p450 reductase (Norris et al., 1994) and glucose-6-phosphate dehydrogenase (Ferria et al., 2005) were not found to be co-localized with NADPH-d. However, in the CNS of insects, both the presence of formaldehyde insensitive NADPH-d activity, which did not correspond to NOS according to immunohistochemical and biochemical experiments (Gibson and Nighorn, 2000), and poor quality of NOS visualization by aldehyde fixation have been reported (Ott and Burrows, 1999). Moreover, mostly due to using different and less characterized antisera which were raised against mammalian NOS sequences, light microscopic detection of the NOS immunoreaction and therefore comparison of neuronal targets labeled by the NADPH-d reaction and NOS immunoreaction were carried out with varying success in invertebrates. Therefore, NADPH-d staining pattern and its relation to NOS were required interpretation with caution and only following profound immunobiological and/or biochemical control experiments (Ott and Elphick, 2002). It also concerns the immuno-electron microscopic visualization of NOS since ultrastructural and biochemical studies suggested that a significant amount and activity of NOS are of cytosolic location (Hecker et al., 1994; Huang et al., 1997; Zhou and Zhu, 2009). Hence the exact identity of the ultrastructural NADPH-d reaction product, which is only membrane-bound, remains for further discussion (Rothe et al., 1998).

In order to gain a more precise insight into the subcellular distribution of NOS here we carried out an electron microscopic study, localizing the NADPH-d histochemical reaction and NOS immunolabeling, respectively, in the nervous system of two gastropod mollusks, *Helix pomatia* and *Lymnaea stagnalis*. In a preliminary study we have demonstrated that an antibody raised against the conserved isoform-independent site of the mammalian NOS unequivocally detected the molluscan NOS in *Helix* CNS extract and tissue samples (Nacsa et al., 2012). This antibody was used in the present study parallel with the NADPH-d reaction, to localize NOS in both the *Helix* and *Lymnaea* CNS. It has been shown that at the ultrastructural level NADPH-d reactive/NOS-immunoreactive (NOS-IR) elements were confined to identical membrane structures which were the axolemma, elements of the endoplasmic reticulum, and membranes of agranular vesicles. Varicosities containing NADPH-d reactive or NOS-IR vesicles were found mostly in non-synaptic but close membrane contact position, and much rarely, at presynaptic apposition. Hence both modulatory and classical neurotransmitter function of NO in the snail CNS is suggested.

2. Materials and methods

Adult, fully active specimens of the terrestrial snail, *H. pomatia*, and the pond snail, *L. stagnalis* were collected locally from late spring to early autumn, kept under laboratory conditions and fed on lettuce.

2.1. Tissue preparation

For light microscopy the CNS was dissected and fixed in 4% paraformaldehyde (PFA) diluted in phosphate buffer saline (PBS, 0.1 M, pH 7.4) solution for 3 h at 4 °C. After cryoprotection in PBS containing 20% sucrose at 4 °C overnight, specimens were embedded and cut with a cryostat (Leica) in 15 μm sections, placed on sylane-coated slides and processed for histo- or immunohistochemistry. Some CNS specimens were used as whole mount preparations.

For electron microscopy the CNS was fixed in a mixture of 4% PFA and 0.1% glutaraldehyde diluted in PBS for 3 h at 4 °C, then, the CNS were embedded in PBS containing 30% egg albumin, and 4.5% gelatin (both from Sigma–Aldrich, Budapest, Hungary), post-fixed in the same fixative overnight at 4 °C, and cut into 50 μm thick sections on a Vibratome (Pelco, CA). Altogether, 20 animals of each species were used in the study.

2.2. NADPH-diaphorase histochemistry

For light microscopy the method of Scherer-Singler et al. (1983), for electron microscopy the method of Wolf et al. (1992) was applied with slight modification. Samples for light microscopy were incubated in 1 mM reduced NADPH, 0.2 mM nitro blue tetrazolium chloride (NBT), and 0.1% Triton-X 100 (TX) containing Tris–HCl buffer (0.1 M, pH 8.1) in dark at room temperature until the blue formazan precipitate appeared (approx. 5–30 min). Cryostat sections were covered with a glycerol-PBS (1:1) solution, and then viewed in a Zeiss Axioplan microscope equipped with a Canon PS G5 digital camera. In case of Vibratome sections processed for electron microscopy, the NADPH concentration was elevated to 1.2 mM, the formazan dye was changed to the osmiophilic 2-(2′-benzothiazolyl)-5-styryl-3(4′-phthalhydrazidyl) tetrazolium chloride (BSPT, 1.2 mM), and the reaction was stopped after 30 min. BSPT was dissolved first in a small amount (5–10 μl) of dimethylformamide, then it was carefully added to the Tris–HCl buffer during continuous shaking. All chemicals were purchased from Sigma–Aldrich.

2.3. NOS immunohistochemistry

A conventional immunohistochemical procedure was carried out for the light and electron microscopic visualization of NOS. As a primary antibody a rabbit polyclonal antibody (Sigma–Aldrich, N-217) raised against the synthetic peptide (Asp-Gln-Lys-Arg-Tyr-His-Glu-Asp-Ile-Phe-Gly), derived from amino acids 1113–1123 of the N-terminal of NOS, was used. It is a conserved sequence found in all mammalian NOS isoforms, and was also found with