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

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Highlights:

Light and electron microscopic evidences have been provided for the identical intracellular localization of NADPH-diaphorase reactive and NOS immunolabeled elements in the snail CNS.

For the first time, we have shown that NADPH-diaphorase reactivity and NOS immunoreactivity are bound to transmitter vesicle membranes in varicosities of ganglionic neuropil of an invertebrate.

The presence of NOS in certain synaptic configurations of the snail CNS suggests a role of NO in fast and target specific interneuronal signaling processes.

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.

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 haemodynamics 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 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.

MATERIALS AND METHODS

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

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.

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 dimethyl-formamide, then it was carefully added to the Tris-HCl buffer during continuous shaking. All chemicals were purchased from Sigma-Aldrich.

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 high homology in sequenced invertebrate NOS genes (Table 1). For specificity control, incubation of the antibody with a recombinant mammalian nNOS protein (360870, Cayman Chemicals, Ann Arbor, MI) was carried out in the *Helix* CNS and described in detail recently (Nacsa et al., 2012). Negative control experiments were performed by omitting the primary antibody. To accelerate tissue penetration, 0.1% TX, or 0.05% saponin (Serva Electrophoresis) was used. Endogenous peroxidase activity was blocked by 1% H_2O_2 diluted in PBS at room temperature for 1-2 h, and the non-specific binding sites by 0.25% BSA diluted in TX/saponin-PBS solution (antibody-dilution buffer) for 1 h. The NOS antibody was applied at 1:200 dilution in the antibody-dilution buffer at 4 °C overnight, followed by a secondary labeling with the SuperSensitive Polymer-horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody solution (BioGenex) in darkness at room temperature for 2 h. The enzyme-linked immunoreaction was developed in Tris-HCl (0.05 M, pH 7.5) containing 0.05% DAB and 0.001% H_2O_2.

Alternatively, for light microscopy, an alkaline-phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich) diluted to 1:200 was also applied as secondary antibody. In this case the enzyme reaction was developed for 5-10 min in darkness in the following solution: 50 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Roche), 100 mg/ml NBT, 10 mM $MgCl_2$ diluted in Tris-HCl (0.2 M, pH 9.5). Cryostat sections were covered and investigated similarly as in NADPH-d histochemistry.

Sample preparation for electron microscopy

Intensively labeled areas of the *Helix* metacerebrum and procerebrum, and *Lymnaea* cerebral and pedal ganglia were cut under a stereomicroscope, post-fixed with 1% osmium-tetroxide diluted in Na-cacodylate buffer (0.1 M, pH 7.2) for 1 h at room temperature. After dehydration in graded ethanol and propylene oxide, the samples were embedded in Araldite (Durcupan, Fluka). Fifty nm ultrathin sections were cut on an LKB ultramicrotome, stained

with uranyl acetate and lead citrate, and examined in a JEOL 1200EX electron microscope. In order to unequivocally identify the immunopositive membrane labeling, unstained ultrathin sections were also analyzed.

RESULTS

Correlation of the NADPH-d histochemistry and NOS immunolabeling in the snail CNS

Light microscopy

Prior to electron microscopy, both NADPH-d histochemistry and NOS immunohistochemistry was carried out at the light microscopic level, to compare the pattern of labeling of the two methods and select the best labeled regions for ultrastructural investigations. In sections taken from the *Helix* cerebral ganglia and treated by NADPH-d histochemistry and NOS immunohistochemistry, respectively, the same anatomical units were found to be labeled, indicating the target specificity and the complete overlapping of labeling resulted in the two techniques applied (Fig. 1A, B). NADPH-d/NOS containing neural perikarya were found in the cerebral ganglion, including small size (5-10 μm) globuli cells of the procerebrum and large size (60-80 μm) mesocerebral neurons (Fig. 1A, B). Discrete neuropil areas such as the internal and intermediate neuropils of the procerebrum displayed intensive labeling (Fig. 1A, B). In the *Lymnaea* CNS, extensive NOS activity appeared in fiber tracts interconnecting the ganglia, originating from peripheral sensory organs (Serfőző et al., 2002) and terminating in the ganglionic neuropils (Fig. 1C). Sensory elements in the head area and their central projections revealed a very similar pattern of NADPH-d labeling in *Helix* (Serfőző et al., 2008) and other molluscan species (Moroz and Gilette, 1995). NADPH-d/NOS also appeared in discrete groups of neurons of the *Lymnaea* buccal ganglia, including the identified B2 giant cell (Fig. 1C insert, D). The homologue of *Lymnaea* B2 cell and neuronal clusters of similar distribution possessed also NOS immunoreactivity (Fig. 1E), and NADPH-d reactivity (Serfőző et al., 2008) in *Helix* buccal ganglion. In the buccal ganglia NO was shown to play a key role in regulating the feeding motor pattern (Moroz et al., 1993). Based on our light microspcopic observations, the *Helix* cerebral ganglia, including the cell body layer and neuropil regions of the *Helix* procerebrum which is abundant in synaptic contacts, as well as the *Lymnaea* buccal, cerebral, and pedal ganglia were selected for subsequent ultrastructural investigations to analyze the subcellular distribution of NADPH-d/ NOS.

Electron microscopy

In NADPH-d reactive neural perikarya of the *Helix* mesocerebrum, NADPH-d reactive material could be detected along the nuclear envelope and rER and sER elements in the cytoplasm (Fig. 2A), appearing as fine NADPH-d reactive electron dense deposits on membrane segments (Fig. 2A). In control, NADPH-d reaction-free samples no staining could be observed along the intracellular membranes (Fig. 2B). When NADPH-d treated samples were not stained with uranyl acetate and lead citrate the NADPH-d reactive membrane segments emerged as more electron dense structures from the background, clearly supporting the specificity of the membrane labeling observed (Fig. 2C). In *Lymnaea*, NADPH-d reactive membrane segments of the nuclear membrane of the B2 giant cell were also found, characterized by massive folding of the nucleus-cytoplasm interface ~~is characteristic~~ because of the intensive protein synthesis (Fig. 2D). At ultrastructural level, the localization of NOS

immunoreactivity seemed to be similar to that found following the NADPH-d reaction (Fig. 2E). Fine immunoprecipitate of increased electron density was observed on the rER elements in the cell body layer of the *Helix* procerebrum (Fig. 2E). On the other hand, in control sections not incubated with the primary antibody, only a moderate electron density of the membranes was seen due to the contrast staining (Fig. 2F).

Localization of NADPH-d reactivity in the axon profiles

Numerous axon profiles displayed NADPH-d reactivity in discrete areas of the ganglionic neuropils (Fig. 3). In *Lymnaea*, thick neural tracts entering neuropil areas were filled with many similarly shaped, small diameter (1-3 nm) electron lucent axons ~~bunches~~ with segmental NADPH reactivity on the axolemma (Fig. 3A). In *Helix*, NADPH-d reactive varicosities were often characterized also by electron-lucent axoplasm, containing small size round or pleomorph, agranular (clear) vesicles of 50-200 nm diameter (Fig. 3B, C), and rarely 50-100 nm size granular vesicles (Fig. 3D). NADPH-d reaction was confined to segments of the axolemma (Fig. 3B-D), axoplasmic sER fragments (Fig. 3B, C), and vesicle membranes (Fig. 3B-D). Along the axolemma NADPH-d reaction often appeared as folded amorphous structure (Fig. 3C, D). In certain axon profiles the extensive NADPH-d reactive material seemed to overlap the axolemmal segments, sER, and vesicles (Fig. 3C).

NADPH-d reactivity and NOS immunoreactivity at the site of interneuronal communication

In the neuropil studied (*Helix* metacerebrum, *Lymnaea* pedal ganglion) NADPH-d reactive varicosities contacted neighboring axons with non-synaptic, unspecialized membrane segments (Fig. 4A-C). NADPH-d reactive vesicles were seen mostly in varicosities containing only agranular vesicles (Fig. 4A, B). This vesicle type however occurred in other varicosities too, mixed with granular and dense-core vesicles (Fig. 4C). In these varicosities a low number of NADPH-d reactive agranular vesicles were only present (Fig. 3 C, 4B). NADPH-d reactivity was seen associated with vesicle membranes partly or entirely covering the vesicles (Fig. 4 A, B). In varicosities containing only clear vesicles in the ganglia investigated,16% of the vesicles displayed NADPH-d reactivity (total number of the analyzed NADPH-d reactive varicosities was 126, ~~that was ultrastructural preparations of~~ obtained from both species). In NADPH-d reactive varicosities vesicles situated near to the axolemma frequently seemed to face with their NADPH-d reactive part to the axolemma (Fig. 4C). Also, opposite to these vesicles the axolemma displayed NADPH-d reactivity (Fig. 4C).

Following the application of NOS immunohistochemistry the enzyme could be detected on vesicles in the varicosities that formed either connection with other unlabeled axon profiles with closely (16-20 nm) apposed unspecialized membrane segments, or they occurred at the appositions of specialized contacts in the procerebrum, where synaptic membranes also displayed NOS immunoreactivity (Fig. 4D, E). In the synapse rich internal neuropil of the procerebrum, NOS-IR agranular vesicles were mostly found to be the only vesicular component of the varicosities, without intermingling with other types (i.e. dense-core) of vesicles. The NOS-IR material on the vesicle membranes was not evenly distributed, but often confined to shorter membrane segments, or showed a dot-like appearance (Fig. 4D, E).

DISCUSSION

Specificity and identity of the demonstration of NADPH-d and NOS in the snail CNS

At both light and ultrastructural level similar structures displayed the NADPH-d histochemical and the immunohistochemical reactions visualizing NOS in the snail CNS, which refers to the identical localization of the two types of labeling. It corresponds well to earlier findings on different mollusks (Moroz, 2000), although there are also a number of observations, describing differences between the results of the two staining techniques in the CNS of some molluscan species (Cooke et al., 1994; Huang et al., 1997; Pisu et al., 1999; Di Cosmo et al., 2000). Presumably, different anti-NOS antibodies used and also different experimental circumstances (e.g. seasonal influence on NOS distribution [Pisu et al., 1999]) have a significant impact on the NOS immunostaining pattern. Nevertheless, in our present study, the procerebral lobe of *Helix*, and the sensory afferents projecting to central ganglionic neuropils of *Helix* and *Lymnaea* were consequently and reliably labeled both by the NADPH-d reaction and NOS immunoreaction. The presence and action of NO in these structures have also been confirmed by biochemical and physiological experiments (Gelperin, 1994; Moroz, 2000, Watanabe et al, 2010). Hence, our light microscopic data could serve a basis indeed for the localization of NOS at ultrastructural level in the ganglionic neuropil of the *Helix* cerebral ganglion. At the ultrastructural level both NADPH-d histochemistry and NOS immunohistochemistry visualized NOS on membrane structures. Control experiments indicated that the membrane labeling did not derive from the background of the histochemical techniques applied or unspecific secondary antibody binding. However, in case of membrane labeling, especially when the labeling is weak, it is difficult to distinguish between the positive signal and the electron dense contrast of the membranes by the heavy metal (uranyl and lead) staining. We have demonstrated that when omitting the conventional lipid contrasting heavy metal salts, the specific histo- or immunohistochemical staining pattern was even more pronounced in the nerve cells, even if the general ultrastructural organization could not clearly be distinguished. Consequently, the NADPH-d reaction on the axolemma and different intracellular membranes can be considered to be specific. A similar ultrastructural localization of NADPH-d at membrane level has been reported in the nervous system of mammals (Wolf et al., 1992; Rothe et al., 1998), and crayfish (Johansson et al., 1996), supporting our observations. A negative aspect of the pre-embedding labeling of the enzyme reactions in electron microscopic preparations is that the formation of the polymer precipitate can mask the exact location of the target molecule. We also found in some preparations that the NADPH-d reactive product displayed a folded precipitate which overlapped different membranous elements, making difficult to decide the exact site of the NADPH-d reaction product (see Fig. 3B). This is probably due to the crystallization of the polymer endproduct of the substrate BSPT at the site of NADPH oxidation, as it was suggested earlier (Rothe et al., 1998).

NOS was detected at subcellular level in the cytoplasm of cortical NOS-IR neurons (Rothe et al., 1998), but was also found at the postsynaptic complex of glutamatergic synapses in the hippocampus (Szabadits et al., 2010). According to enzyme activity measurements, NOS has also been demonstrated both in cytosolic and particulate fractions of the mammalian (Hecker et al., 1994) and the snail (Huang et al., 1997) CNS. These studies support our findings concerning the membrane association of this enzyme. However, the lack of detection of cytoplasmic NOS in our preparations at ultrastructural level is hard to explain. One possibility is that NOS was translocated from the cytosol to the membranes during preparation of the sample. It might be supported by the observation that NOS is carried by adapter proteins between these compartments after distinct stimuli (Jaffrey et al., 2002; Ohnishi et al., 2009; Rőszer et al., 2010). On the other hand, due to the intensive DAB reaction distributed in

the entire axoplasm, the association of NOS to vesicle membranes in presynaptic profiles was not excluded either by the authors describing the cytoplasmic location of NOS (Aoki et al., 1993; Atkinson et al., 2003). Others have emphasized that in preparations of high quality the cytoplasmic NOS-IR material was clearly located around synaptic vesicles (Rodrigo et al., 1997; Sancesario et al., 2000). In accordance with the aforementioned literature data our findings ~~with those from the literature would~~ suggest the association of NOS with the vesicle membranes,.In addition, according to their location and the ultrastructural appearance of the labeled vesicles, the NADPH-d reactive and NOS-IR vesicles seem to represent identical types.

Possible functional consequences of the distribution of NADPH-d reactive and NOS-IR elements

Although earlier reports showed that NADPH-d reaction was bound to sER elements in axon terminals (Wang et al., 1995; Yeo et al., 1997), to our knowledge, the present findings are the first reporting on the route of NADPH-d reactive/NOS-IR membranes from the neuronal perikarya along the axon processes to the varicosities, and revealing there the labeling of the membrane of agranular vesicles. In the neuropil NADPH-d reactive and NOS-IR materials occurred more frequently on the axolemma than on intracellular elements, meanwhile near to the site of axo-axonic contacts the positive labeling was mainly found on vesicle membranes. Similarly to our results obtained in the snail CNS, NOS was observed in axon terminals containing small size ovoid or pleomorph agranular vesicles in the brain of the Atlantic salmon (Holmqvist and Ekström, 1997), the rat spinal trigeminal nucleus (Yeo et al., 1997), the monkey visual cortex (Aoki et al., 1993), as well as in neural components of the guinea-pig intestine (Wang et al., 1995). According to the vesicle morphology in insect and vertebrate neurons, these vesicles contain presumably an amino acid or acetylcholine (Watson, 1988; Holmqvist and Ekström, 1997; Helfert et al., 1992). Evidences for GABA or acetylcholine as a primary transmitter in NADPH-d reactive/NOS-IR terminals have been presented in insect sensory interneurons (Seidel and Bicker, 1997) and the identified B2 giant cell of the buccal ganglion of the snail, *Lymnaea* (Moroz 2000), as well as in the mammalian aspiny striatal interneurons and peduncolopontine tegmental neurons projecting to the thalamus (seen in: Vincent, 2010). However, in the snail cerebral ganglia, the localization map of NADPH-d/NOS does not match with the distribution of other amino acid transmitter candidates such as GABA in *Helix* (Hernádi, 1994) and glutamate in *Lymnaea* (Hatakeyama et al., 2007).

Our ultrastructural observations showed that both NADPH-d and NOS was also localized at the site of the axo-axonic connections, regardless whether they were close membrane contacts characterized by unspecialized membrane appositions or by synaptic specializations, suggesting that NO acts as an anterograde signal molecule in the snail CNS. In insects and a number of marine invertebrates it has already been demonstrated that NO behaves as an anterograde signal molecule (Bicker, 2001; Palumbo, 2005), similar to vertebrates (Garthwaite, 2008). On the other hand, although the retrograde signal function of NO is well demonstrated in mammalian cortical and hippocampal spiny neurons (Garthwaite, 2008), and in lower vertebrates (Holmqvist and Ekström, 1997), it might be absent in invertebrates (Vincent, 2010), which is also suggested by the present study.

In the neuropil regions of the snail CNS, whereto NADPH-d reactive and NOS-IR sensory axons project NADPH-d reactive varicosities formed exclusively close membrane contacts with neighboring axon profiles without showing any membrane specialization. In the gastropod CNS, specialized synaptic contacts can rarely be found, although there are neuropils such as in the *Helix* procerebrum, where synapses are relatively abundant (Ratté and

Chase, 2000, Elekes et al., 2013). In this area which is responsible for central processing of odor information, and proved to play a significant role in olfactory learning (Watanabe et al., 2010), we have found NADPH-d/NOS labeled synapses, suggesting NOergic events. Another site of NO release might be the varicosities displaying extensive NADPH-d reactivity and NOS immunoreactivity, respectively, along axolemma segments through which NO could influence by volume transmission on the neighboring axon profiles. In summary, the presence of NADPH-d and NOS at different, specialized or unspecialized neural connections suggests different form of action in different information processes elicited by NO in the gastropod CNS.

Both NADPH-d reactive and NOS-IR material seemed to be accumulated between vesicles and the varicosity membrane (see Fig. 4C-E), which is a very narrow space, suggesting that NO has some implication in vesicle trafficking. Interestingly, in a number of NADPH-d reactive/NOS-IR vesicles only a part of the vesicle membrane displayed NADPH-d reactivity/NOS-immunoreactivity. It suggests that the vesicle surface serves for NOS as a carrier, and the NO production is partly independent from transmitter release, similarly to that described in the vertebrate gastrointestinal tract (Olgart et al., 2000). On the other hand, it cannot be excluded either that in NOergic varicosities NO may influence transmitter function by facilitating its release, as it has been described at the neuromuscular junctions of the *Drosophila* embryo (Wildemann and Bicker, 1999), and/or by modulating transmitter vesicle docking/fusion (Meffert et al., 1996; Sporns and Jenkinson 1997) and turnover (Tegenge et al., 2009), similarly as it has been described in different vertebrate models (Garthwaite, 2008).

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TABLE

Table 1. Comparison of the amino acid sequence recognized by the applied rat anti-nNOS antibody and the equivalent NOS amino acid sequences identified in *Drosophila* (Stasiv, et al., 2001) and some mollusks (*Aplysia* [Moroz, et al., 2006]; *Lymnaea* [Korneev et al., 1998, 2005]). Identical sequences are highlighted. *The whole genome of *Lottia gigantea* has recently been determined (Simakov et al., 2012) wherein a similar decoded sequence was found within a protein putatively identical to NOS.

FIGURE LEGENDS

Fig. 1. Light microscopic evidences for the co-localization of NADPH-d reactive (A, D) and NOS-IR (B, C, E) elements in the snail CNS (A-B, E from *Helix*, C-D from *Lymnaea*)
A-B. Cryostat sections taken from the cerebral ganglia of *Helix* showing intensive NADPH-d reactivity in discrete (internal [in] and intermediate [imn]) neuropil areas of the procerebrum (PC). The cellular zone (cz) of the PC and some cell bodies (arrowheads) in the mesocerebrum (MC) also display reactivity. **B.** Detail (left PC) of an alternate section taken from the same cerebral ganglion showing intensive immunostaining with the anti-NOS antibody in identical areas of the PC as seen in **A** following NADPH-d reaction. HRP-DAB staining. MTC: metacerebrum; ln: lateral neuropil, tn: terminal neuropil of the PC. cc: cerebro-cerebral commissure; ct: connective tissue. **C.** NOS immunoreactivity in the tracts (arrows) connecting the ganglia which display widespread arborization (asterisks) in the neuropils, as seen in an overview of the whole *Lymnaea* CNS. Cryostat section. BCIP/NBT staining. **Insert**: Enlarged view of a NOS-IR cell cluster (arrow) and the NOergic identified B2 neuron (arrowhead, Moroz, 2000) in the left buccal ganglion (lBG). Abbreviations of the ganglia: rBG, rCG, rPeG, rPlG, rPag are the right, whereas lCG, lPeG, lPlG, lPag are the left cerebral, pedal, pleural, parietal ganglia. VG: visceral ganglion. **D.** A whole-mount preparation of *Lymnaea* buccal ganglia displaying an intensive NADPH-d reaction in the tracts (cerebro-buccal connective [cbc], buccal commissure [bc]) and cell clusters (arrows). Note the identified B2 neuron in the left ganglion (arrowhead), and the similar location of the cell cluster (arrow) immunolabeled with NOS in **C**. bn: buccal nerve; pbn: postbuccal nerve. **E**. B2 giant cell (arrowhead) and clusters of neurons (arrows) in the anterior medial part of the *Helix* left buccal ganglion show NOS immunoreactivity. The neuropil (asterisk) is rich with NOS-IR varicosities. Open arrowheads: unlabeled cells. cbc: cerebro-buccal connective. cryostat section Scale bars: 200 μm in A, B, D, E, and C insert, and 500 μm in C.

Fig. 2. Localization of NADPH-d (A-D) and NOS-IR (D, E) labeling in neural perikarya (A-C, E, F from *Helix*, D from *Lymnaea*)
NADPH-d reactive electron dense precipitate (arrows) is located along segments of the nuclear envelope (ne), elements of rough (rER) endoplasmic reticulum, and the cell membrane (cm) in a large (80 μm diameter) neuron of a ~~*Helix*~~ cerebral ganglion. Open arowheads: unlabeled membranes. Gb: Golgi body; m: mitochondrium; N: nucleus. **B.** Detail of a control perikaryon of the cerebral ganglion from a preparation incubated in NADPH-free medium. In contrast to a similar type of labeled perikaryon seen in **A**, only a moderate electron density of the membranes (open arrowheads) originating from staining with heavy metal salts can be seen. Gb: Golgi body; m: mitochondrium; N. nucleus; rER: rough endoplasmic reticulum. **C.** Nuclear envelope (ne), smooth endoplasmic reticulum elements (sER), and the cell membrane (cm) are labeled by NADPH-d reaction (arrows) in a small (8 μm diameter) globulus cell of the *Helix* procerebrum seen in an unstained (no uranyl and lead salts) section. **D**. Detail of the nuclear-cytoplasmic interface of the giant (120 μm diameter) B2 cell perikaryon in *Lymnaea*. Note NADPH-d reactivity (arrows) at segments of the massively folded nuclear envelope (ne). Arrowheads: unlabeled membrane segments. c: cytoplasm; N: nucleus; **E.** NOS-IR segments (arrows) of the membrane system of the rough endoplasmic reticulum (rER) in a globulus cell. N: nucleus. **F.** A control globulus cell incubated without the anti-NOS antibody. Note the low electron density of the rough endoplasmic reticulum (rER) membranes (open arrowheads) compared to A. N: nucleus; m: mitochondrium. Scale bars: 1 μm in C, E, F, and 5 μm in A, B, D.

Fig. 3. NADPH-d reactive structures in fiber tracts and neuropil areas (A from *Lymnaea*, B- D from *Helix*)

A. Cross section of a thick fiber tract (large arrows) entering the *Lymnaea* cerebral ganglion neuropil. Insert: enlarged view of axons (asterisks) with NADPH-d reactive membrane segments (arrowheads). **B.** Oblique section of the neuropil taken from the metacerebrum of the *Helix* cerebral ganglion, showing NADPH-d reactive axolemma segments (arrows), as well as reactive membrane structures in the axoplasm (short arrow), and vesicle membranes (arrowheads) in the axon profiles. m: mitochondrium. **C.** Varicosities (asterisks) in the *Helix* pedal ganglion, with folded axolemma displaying NADPH-d reaction (arrows). Labeled axolemma segments are embedded into the cytosol, interconnected with labeled sER elements (short arrows). Arrowheads: labeled vesicles. Note NADPH-d reactivity is associated to axon profiles containing small (100 nm diameter) round or pleomorph agranular vesicles. **D.** Sample from the procerebrum internal neuropil showing intensive NADPH-d reaction along the axolemma (arrows) of profiles containing a mixed (agranular and granular) population of vesicles. Arrowheads: labeled agranular vesicles; m: mitochondrium. The section, similar to that seen in Fig. 2C, was not stained with lead and uranyl salts. Scale bars: 250 nm in B, C, D, 1 μm in A insert, and 50 μm in A.

Fig. 4. NADPH-d reactivity and NOS immunoreactivity at interneuronal connections (A, C-E from *Helix*, B from *Lymnaea*)

A. Axo-axonic unspecialized connections (arrows) between varicosities containing NADPH-d reactive (T1) and negative (T2) agranular vesicles and an electron lucent large axon profile (asterisk) in the *Helix* metacerebrum. Note that only a small number of vesicles are labeled in T1 (arrowheads). m: mitochondrium. **B.** The membrane of agranular vesicles is coated partly or entirely by NADPH-d reactivity (arrowheads) in two varicosities (T1, T2) located in the neuropil of the *Lymnaea* pedal ganglion. Short arrow: coated pit. **C**. A thin NADPH-d reactive axon process (asterisk) is located between two axon profiles (T1, T2) in a *Helix* metacerebrum. NADPH-d reactivity is seen both on the surface of some vesicles facing the axolemma (arrowheads), and along the axolemma segments facing the vesicles (arrows). **D-E.** NOS-IR labeling on the surface of agranular synaptic vesicles (arrowheads) in the internal neuropil of the *Helix* procerebrum. Arrows: symmetric NOS-IR membrane appositions. Note enhanced immunoreactivity on vesicles situated at the presynaptic membrane. m: mitochondrium. Scale bars: 100 nm.