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Distribution of NADPH-diaphorase in rat mesencephalon: A light and electron microscopical study

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ABSTRACT: NADPH-diaphorase is a useful technique to reveal NO producing neurons at light microscopic level (LM). A modification of the technique using the tetrazolium salt BSPT as susbtrate, is useful to study the ultrastructure of NO neurons. The aim of this work was to perform a detailed analysis of NADPH-diaphorase reactive neurons in rat mesencephalon both at light and electron microscopic levels.

NADPH-diaphorase reactive neurons were observed in superior colliculus, in central gray matter, in dorsal and medial raphe and in the pedunculopontine tegmental nucleus using two histochemical techniques at LM. Electron microscopy showed deposits on membranes of the endoplasmic reticulum, Golgi apparatus and nuclear envelope of dorsal raphe neurons. Presynaptic and postsynaptic terminals showed deposits on membranous elements but postsynaptic terminals also showed deposits on the inner surface of their membranes.

Further physiological studies are needed to clarify the meaning of the ultrastructural findings such as the putative interaction of NOS with postsynaptic proteins, receptors or membranous channels.

Introduction

Nitric oxide (NO) is a gas involved in blood vessel relaxation, cytotoxicity of macrophages and neurotransmission. In the nervous system, NO is synthesized from arginine by a calmodulin dependent enzyme, the neuronal isoform of nitric oxide synthase (nNOS) (Bredt and Snyder, 1990; Garthwaite, 1991; Snyder *et al*., 1998). Intracellular calcium level increments, ie after NMDA receptor stimulation, triggers nNOS activation generating NO. As nitric oxide is a highly diffusible molecule, it may act not only in the postsynapsis but in the presynapsis as a retrograde messenger as well. The target of NO is the heme group of different enzymes being soluble guanylate cyclase the most important target enzyme (Dawson and Dawson, 1994; Dawson and Snyder, 1994; Snyder *et al*., 1998). NO has diverse functions in the nervous system from the morphogenesis to synaptic plasticity, regulation of gene expression, long term potentiation (memory), pain transmission (thermal hyperalgesia and chronic pain), etc (Dawson and

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Dawson, 1994; Garthwaite, 1991). Besides, excesive release of NO in certain oxidative states of the molecule is related with neurotoxicity and may be involved in diseases such as Parkinson, Hungtinton, Alzheimer, and ischemia (Dawson and Dawson, 1994; Capani *et al*., 1997; Wolf, 1997).

The histochemical NADPH-diaphorase (NADPH-d) technique has been used to demonstrate NO producing neurons in the nervous system (Bredt *et al*., 1990, 1991; Hope *et al*., 1991; Vincent and Kimura, 1992). Although localization of NADPH-d and NOS was performed in mesencephalon, little is known about the ultrastructural distribution of diaphorase activity in the region. The substrate employed in the diaphorase reaction (nitroblue tetrazolium [NBT]) renders a formazan precipitate which is soluble in organic solvents so that ultrastructural studies cannot be carried out using this histochemical technique. The introduction of a different tetrazolium salt (2-(2´-benzothiazolyl)-5-styryl-3-(4´-phthalhydrazidyl)-tetrazolium chloride [BSPT]) enabled to carry out ultrastructural studies in different areas of the nervous system as the formazan precipitate is osmiophilic and solvent resistant (Darius *et al*., 1995; Faber-Zuschratter and Wolf, 1994; Rothe *et al*., 1997; Wolf *et al*., 1992; Würdig and Wolf, 1994).

The aims of this work were to study the distribution of NADPH-d neurons in the rat mesencephalon with both histochemical techniques (using either NBT or BSPT as substrates) at the light microscope (LM) to validate the use of BSPT in the area, and to make a detailed ultrastructural description of NO synthesizing neurons in the region using the BSPT/NADPH-d technique.

Material and Methods

Wistar rats were anesthetized and perfused with a fixative solution containing 4% paraformaldehyde and 0.25% glutaraldehyde. Vibratome mesencephalic sections (thickness: 50 μm) were incubated in a solution containing either 1.2 mM β-NADPH and 0.8 mM BSPT in 0.1 M phosphate buffer (PB) (pH :8) or 1.2 mM β-NADPH and 0.25 mM NBT in 0.1 M PB (pH:7.4) at 37ºC for 1hr in a humid chamber. Before preparing solution, BSPT was dissolved in 200 μl of N,N-dimethylformamide. Sections were mounted with PBS:glycerol (1:3) or Histomount depending on the histochemical procedure (NBT or BSPT substrates respectively). Later slides were observed and photographed using a light microscope (Zeiss Axiophot). Most of the sections stained using the BSPT substrate were osmicated, dehydrated and flat embedded in Durcupan. Ultrathin sections from selected areas corresponding to dorsal raphe were contrasted and observed at the electron microscope (Zeiss 109). Negative control sections were performed omitting NADPH in the incubation mixtures.

Results

Sections stained using both NBT and BSPT tetrazolium salts showed faint stained neurons and fibres in superior colliculus, small reactive mainly bipolar/fusiform and triangular neurons in dorsal and lateral regions of central gray matter (CG), medium bipolar reactive neurons in dorsal raphe (DR), weakly reactive neurons in medial raphe and strong reactive bipolar and multipolar neurons in the pedunculopontine tegmental nucleus (PPTg). Although both techniques showed the same neuronal populations, the histochemical technique using NBT demonstrated a higher extension of dendritic arborizations at LM (Fig. 1 A-F).

Electron microscopy of DR neurons showed deposits on membranes of the endoplasmic reticulum, nuclear envelope, Golgi apparatus and outer mitochondrial membranes (Fig. 2A, B).

NADPH diaphorase reactivity was observed in both, pre- and postsynaptic terminals, although mainly presynaptic terminals showed staining (Fig. 2 C-F). In presynaptic terminals reactive deposits were observed in small ovoid membranous elements slightly bigger than synaptic vesicles (Fig. 2 C, D). In postsynaptic terminals reactive deposits were observed in the inner surface of terminal membranes, on outer mitochondrial membranes and on the surface of small membranous and non-membranous postsynaptic structures that resemble cytoskeletal microtubules and filaments (Fig. 2 E, F).

Control sections, in which NADPH was omitted in the incubation mixture, neither showed reactivity at LM nor at EM level.

Discussion

This work provides a detailed and complete analysis of the NADPH-d reactivity in the mesencephalon using two histochemical techniques both at LM and EM levels. A previous LM description of NADPH-d reactivity distribution in mesencephalon was included in a full NADPH-d mapping in rat brain using only NBT/NADPH-d technique (Vincent and Kimura, 1992). Other

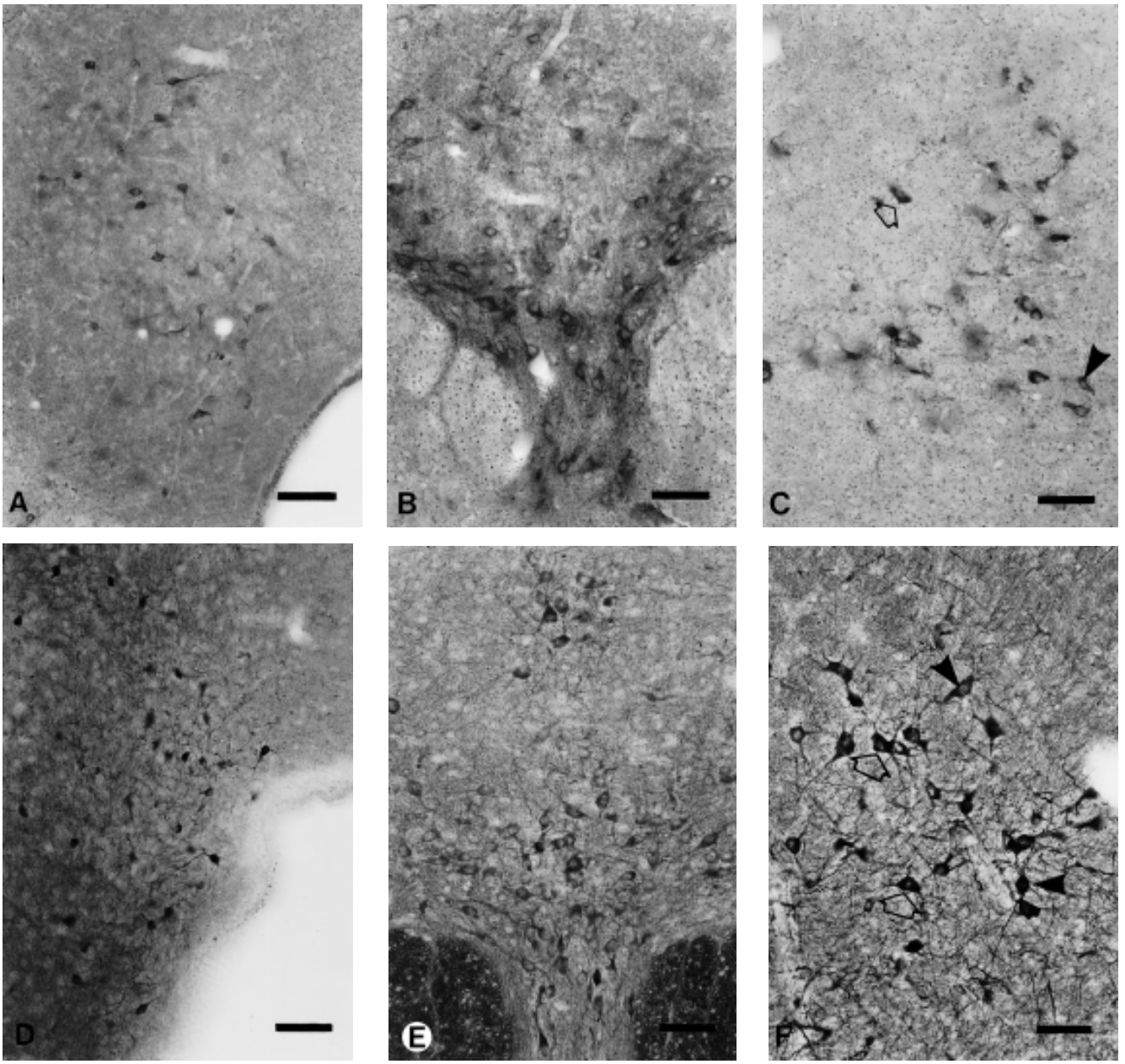

FIGURE 1. Top row : Mesencephalic sections stained with the NADPH-diaphorase technique using BSPT as substrate. Bottom row : Mesencephalic sections stained with the NADPH-diaphorase technique using NBT as substrate. **A and D.** Small reactive bipolar and triangular neurons from dorsal and lateral regions of central gray matter (CG). Reactivity is concentrated in cell somas and dendrites while nuclei are not stained. Both BSPT and NBT histochemical methods show the same neuronal populations. Scale bar: 70 μm.
B and E. Bipolar neurons from dorsal raphe (DR). Neuronal somas and processes are reactive while nuclei are not stained. As in the other regions, both techniques showed the same neuronal populations. Reactivity is not as strong as that observed in the PPTg nucleus. Scale bar: 70 μm.
C and F. High magnification of pedunculopontine tegmental nucleus (PPTg). Observe the presence of strong reactive fusiform/bipolar (head arrow) and multipolar (hollow arrow) neurons. Both BSPT and NBT histochemical methods show the same neuronal populations. NBT histochemistry seems to demonstrate neuronal processes with a higher detail at LM. Scale bar: 70 μm.

LM and EM studies using both NBT/NADPH-d and BSPT/NADPH-d techniques described similar results to our findings but in different areas of the CNS or in some cases, only the most evident nucleus, the pontine laterodorsal tegmental (LDT), which we properly named pedunculo pontine tegmental nucleus (PPTg), was studied (Darius *et al*., 1995; Faber–Zuschratter and Wolf, 1994; Rothe *et al*., 1997; Wolf *et al.*, 1992; Würdig and Wolf, 1994).

NADPH-d reaction is a simple histochemical method that has been widely used to demonstrate NOS containing neurons. Although a group of NADPH-dependent oxidoreductases may be evidenced by the NADPH-d reaction only NOS retains its enzymatic activity after aldehyde fixation (Rothe *et al*., 1997; Wolf, 1997). In addition, NADPH-d and NOS share similar physical and enzymatic activities (Hope *et al*., 1991; Bredt *et al*., 1990) and NADPH-d labeling and NOS immunoreactivity are co-localized in the same neuronal populations confirming that the histochemical method labels nitrergic neurons (Bredt *et al*., 1991). However, there are a few exceptions to co-localization in nervous system but none is found at mesencephalic level (olfatory bulb, rat and mouse suprachiasmatic nuclei and some neurons in the cerebral cortex, Rothe *et al*., 1997).

The described localization of NADPH-d employing the substrate BSPT in the mesencephalon is the same that the distribution of NBT/NADPH-d at LM, as observed in Fig. 1 A-F, validating the use of the BSPT diaphorase technique in the area. Besides NADPH-d distribution reported here is in agreement with previous mapping in the same area using NBT/NADPH-d technique (Vincent and Kimura, 1992).

Some of the reported mesencephalic diaphorase reactive neuronal populations do not only contain NO but other neurotransmitters as well. That is the case of dorsal raphe nuclei which contain NOS/NADPH-d reactivity and serotonin (5HT) and pedunculopontine tegmental nuclei which contain NOS and acetylcholine (Wotherspoon *et al.*, 1994; Johnson and Ma, 1993).

Dorsal raphe nuclei have a differential sensitivity to neurotoxins and NO has been reported to be both a neuroprotective or neurotoxic agent depending on its oxidative state (Dawson and Dawson, 1994). However, NO contaning neurons are resistant to neurotoxic effects therefore the importance of knowing more about colocalization and about connectivities between nitrergic terminals and other neurons. Besides the knowledge of neurotoxic-resistant neuronal populations containing specific neurotransmitters is also important to understand clinical manifestations of neurotoxic insults.

The ultrastructural localization of the reaction shows reactivity on endoplasmic membranes, Golgi apparatus and nuclear envelope in agreement with previous reports in cerebral cortex, cerebellum, hipocampus and retina (Darius *et al*., 1995; Faber-Zuschratter and Wolf, 1994; Rothe *et al*., 1997; Wolf, 1997; Wolf *et al.*, 1992; Würdig and Wolf, 1994). Previous authors also reported that nNOS is attached to cell membranes while inducible NOS is observed as a ¨sand¨ like deposit in the cytoplasm of microglial cells using the BSPT/NADPH-d histochemical method at EM (Wolf, 1997). The meaning of such membrane attached deposit was suggested to be the consequence of endocytosis and membrane recycling in terminals followed by retrograde transport (Faber-Zuschratter and Wolf, 1994).

An alternative explanation for the localization of reactive deposits observed in endoplasmic reticulum, which is known to be a Ca^{2+} store, could be that NOS is involved in the regulation of store operated calcium channels (SOCC) or second messenger operated channels (SMOC), as is the case in other systems where NO participates in cross-talks with Ca^{2+} (Clementi and Meldolesi, 1997).

Reactive deposits were also observed in outer mitochondrial membrane in cell somas and postsynaptic terminals as was previously observed in other CNS areas (Faber-Zuschratter and Wolf, 1994).

Surprisingly, presynaptic terminals were more frequently stained than postsynaptic terminals. Although this is contradictory, it is also in agreement with previous results in other CNS areas where the labelling of presynaptic membranous elements was suggested to be the consequence of endocytosis processes ocurring after neurotransmitter release (Faber-Zuschratter and Wolf, 1994).

The presence of reactivity on postsynaptic membranes may be the consequence of the localization of the enzyme attached to postsynaptic membrane proteins. In other CNS regions, it was reported that NOS which contains a PDZ domain in the carboxyl terminal of the molecule, interacts with the postsynaptic protein PSD-95 anchoring the enzyme close to NMDA receptor which is a Ca^{2+} channel responsible of the activation of nNOS (Brenman *et al*., 1996). However this is not the only possible protein-protein interaction of NOS, as its PDZ domain also interacts with inhibitory proteins as the Protein Inhibitor of NOS (PIN) and the Carboxyl And PDZ Organizers for nNOS (CAPON) (Snyder *et al*., 1998).

The present results show that BSPT/NADPH-d is a useful technique to study the distribution and ultrastruc-

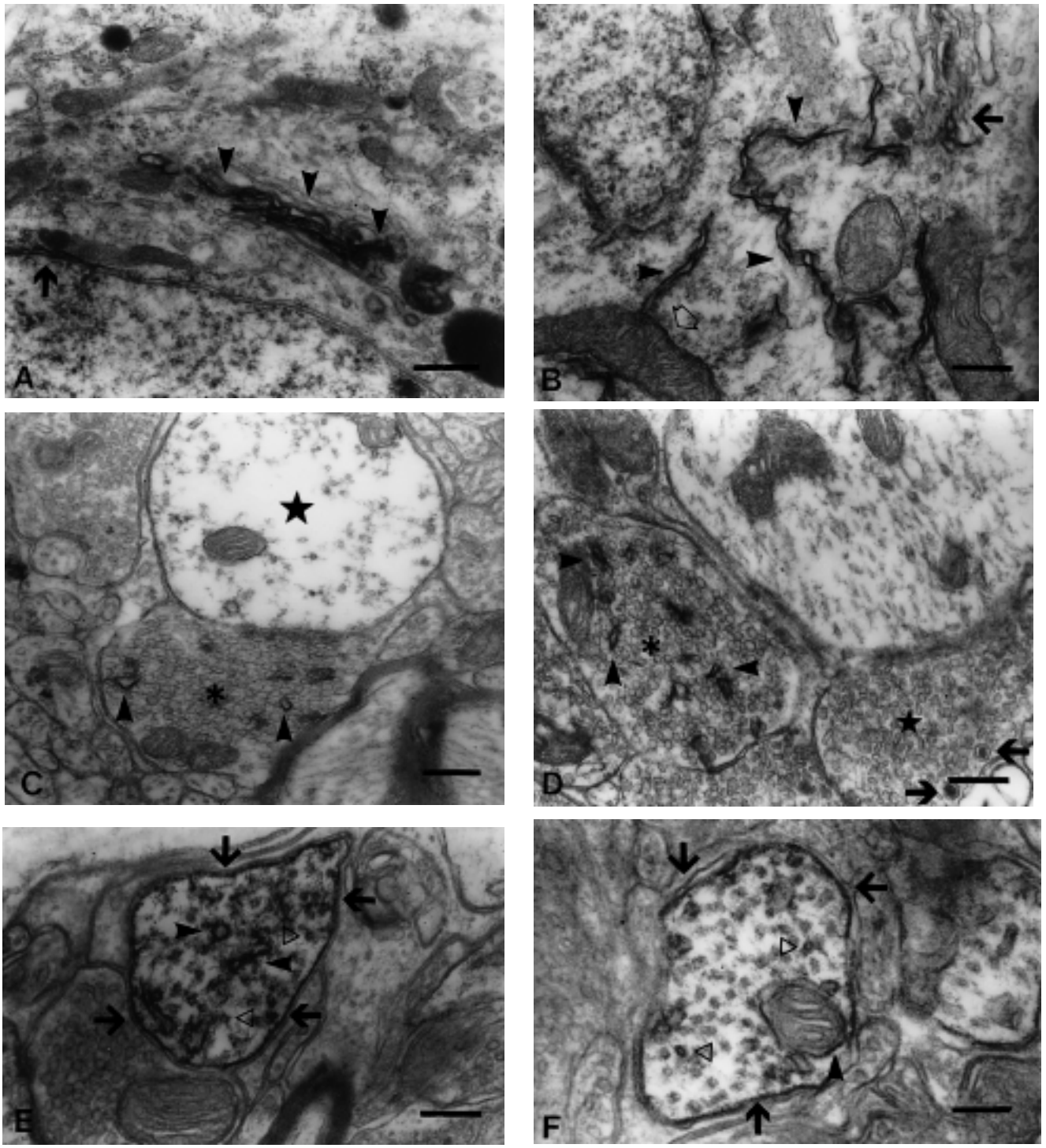

FIGURE 2. A. Electron micrograph of a dorsal raphe neuron. Golgi apparatus is heavily stained with BSPT-formazan deposits on its membranes (head arrows) and on nuclear envelope (arrow). Scale bar: 0.4 μm. **B.** Electron microscopy showing BSPT-formazan deposits on endoplasmic reticulum (head arrows), some Golgi apparatus dictiosomes (arrows) and outer mitochondrial membrane (hollow arrows). Scale bar: 0.28 μm. **C.** Synapsis between a terminal containing clear vesicles (asterisk) and a dendritic spine (star). Observe BSPT/NADPH-d reactive membranous elements in the presynaptic terminal (head arrow). Scale bar: 0.28 μm. **D.** Presynaptic terminal containing clear vesicles (asterisk) in which BSPT-formazan deposits are detected on membranous elements possibly derived from endoplasmic reticulum (head arrows). A non-reactive presynaptic terminal (star) containing clear vesicles and two dense core vesicles (arrows) may be observed in the same field. Scale bar: 0.28 μm. **E and F.** Postsynaptic terminals devoid of synaptic vesicles showing BSPT-formazan deposits attached to their membranes (arrows). In E some microtubules (hollow triangles) and membranous elements (head arrows) are reactive. In F, microtubules (hollow triangles) and outer mitochondrial membrane (head arrows) are stained. Scale bar: 0.17 μm.

ture of NOS mesencephalic neurons and that labeling is found on membranes of the endoplasmic reticulum, Golgi apparatus, nuclear envelope and pre- and postsynaptic terminals of dorsal raphe neurons. However, further work is needed to understand the physiological meaning of the ultrastructural results such as the interactions of NOS with postsynaptic proteins, receptors or diverse membranous channels.

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