FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 42, No. 2, 2004
pp. 83-87

The relationships between neurons containing dopamine and nitric oxide synthase in the ventral tegmental area

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Abstract: Ventral tegmental area (VTA) is a heterogeneous group of dopaminergic cells which contains interfascicular (IF), parabrachial (PBP) and rostral linear (RLi) nuclei. Neurons of this area are involved in the regulation of motor and motivational aspects of behavior and reveal high neuronal plasticity. Among many various neurotransmitters and neuromodulators, nitric oxide (NO) is localized in this region. In the present study, we investigated morphology and distribution of nitric oxide synthase (NOS)-positive neurons in VTA and their colocalization with dopaminergic neurons. The study was performed on six adult Wistar rats. After perfusional fixation, the brains were cut, immunostained for tyrosine hydroxylase (TH) and NOS and studied by confocal laser microscopy. In each of the three studied nuclei of VTA we investigated three different neuronal populations. Numerous TH-immunoreactive (TH-ir) and NOS-immunoreactive (NOS-ir) neurons are present in the studied region. Among them, a considerable number showed coexistence of both neurotransmitters. The populations of TH-ir and NOS-ir neurons interact with each other as manifested by the presence of NOS-ir endings on TH-ir neurons and *vice versa*. Taking the above into account, it may be suspected that NO is involved in the modulation of dopaminergic transmission.

Key words: Nitric oxide synthase - Tyrosine hydroxylase - Ventral tegmental area - Rat

Introduction

The dopaminergic (DA) neurons in the ventral tegmental area (VTA) are involved in the regulation of motor and motivational aspects of behavior as well as implicated in the emotional and cognitive disturbances associated with neuropsychiatric diseases such as schizophrenia and certain affective and stress-related disorders. VTA is located within A10 dopaminergic cell group. Catecholamine-synthesizing enzyme - tyrosine hydroxylase (TH) is regarded as a marker of catecholaminergic neurons. It is well known fact that catecholaminergic neurons in VTA are dopaminergic ones. So, localization of TH in this structure corresponds to the distribution of dopamine containing cells. A10 region consists of heterogeneous nuclei, including the interfascicular (IF), parabrachial (PBP) and rostral linear (RLi) nuclei [6].

Functional heterogeneity of dopaminergic neurons may derive from both extrinsic and intrinsic factors. The former concern different sources of their afferents, the latter - variability of their neurochemical properties [10, 16]. According to many authors, neurons of this area reveal high neuronal plasticity [9, 12, 25, 26].

Among many various neurotransmitters and neuromodulators of VTA, nitric oxide (NO) found in this region by Rodrigo *et al.* [20] is the subject of interest of many authors. Nitric oxide has been identified as a unique molecule, which acts as an intercellular messenger in various tissues. NO is synthesized from arginine by nitric oxide synthase (NOS), a Ca^{2+}/calmodulin-dependent enzyme [24]. Three histochemical methods are now available to identify NO-containing neurons: immunohistochemistry, neuronal nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry and *in situ* hybridization for NOS mRNA [1, 20, 21]. Some physiological aspects of NO are still unknown, however it is certain that NO plays an important role during the brain development by influencing processes such as synaptic remodeling and neuronal death. NO is also one of the most important factors modulating the synaptic plasticity [5, 8, 11, 27]. The last studies of Gholami *et al.* [7] suggest that NO may be relevant to rewarding effects of drug abuse.

The aim of our study was to describe the topography of NOS-ir neurons in VTA as well as to evaluate the degree of NOS/TH colocalization in VTA nuclei.

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Materials and methods

The material consisted of 6 adult Wistar rats. Care and treatment of animals were in accordance with guidelines for laboratory animals established by the National Institute of Health as well as by the Local Ethical Committee. Animals were deeply anesthetized with lethal doses of Nembutal (80 mg/kg of body weight), then perfused transcardially with 0.9% NaCl containing heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were postfixed in 4% paraformaldehyde for 3-4 h, and then kept in 0.1 M phosphate buffer containing 10% sucrose (overnight at 4˚C) and 30% sucrose (until sunk). Coronal 40-µm-thick, sections of the brain were cut using Jung 1800 cryostat (Leica, Germany). The free floating sections were blocked with 3% normal goat serum (NGS) containing 0.3% Triton X-100 for 1 h and then incubated for 48 h at 4˚C with the mixture of polyclonal rabbit anti-tyrosine hydroxylase antibody (Chemicon, AB151; diluted 1:2000) and monoclonal mouse anti-nitric oxide synthase-brain antibody (Sigma, N7155; diluted 1:1500) diluted in 3% NGS. After multiple rinses in PBS, sections were incubated for 2-3 h at room temperature with the mixture of the appropriate secondary antibodies: Alexa Fluor 488 goat anti-mouse (Symbios, A1101; diluted 1:150) and Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch, 111-165-144; diluted 1:600).

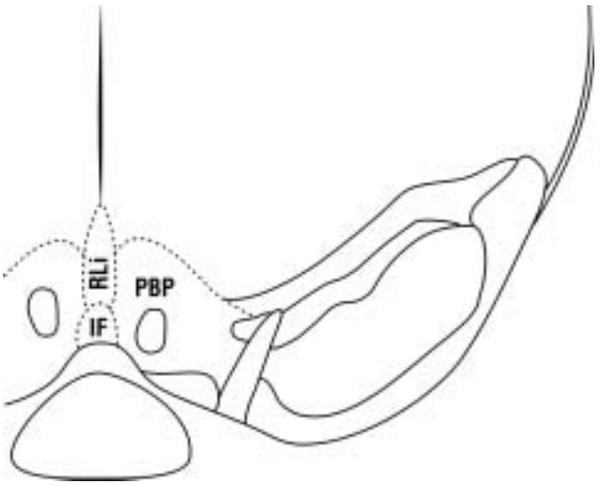

Fig. 1. The schematic drawing of the rat mesencephalon (bregma -5.2). Dotted lines separate the studied nuclei: IF - interfascicular nucleus; PBP - parabrachial nucleus; RLi - rostral linear nucleus.

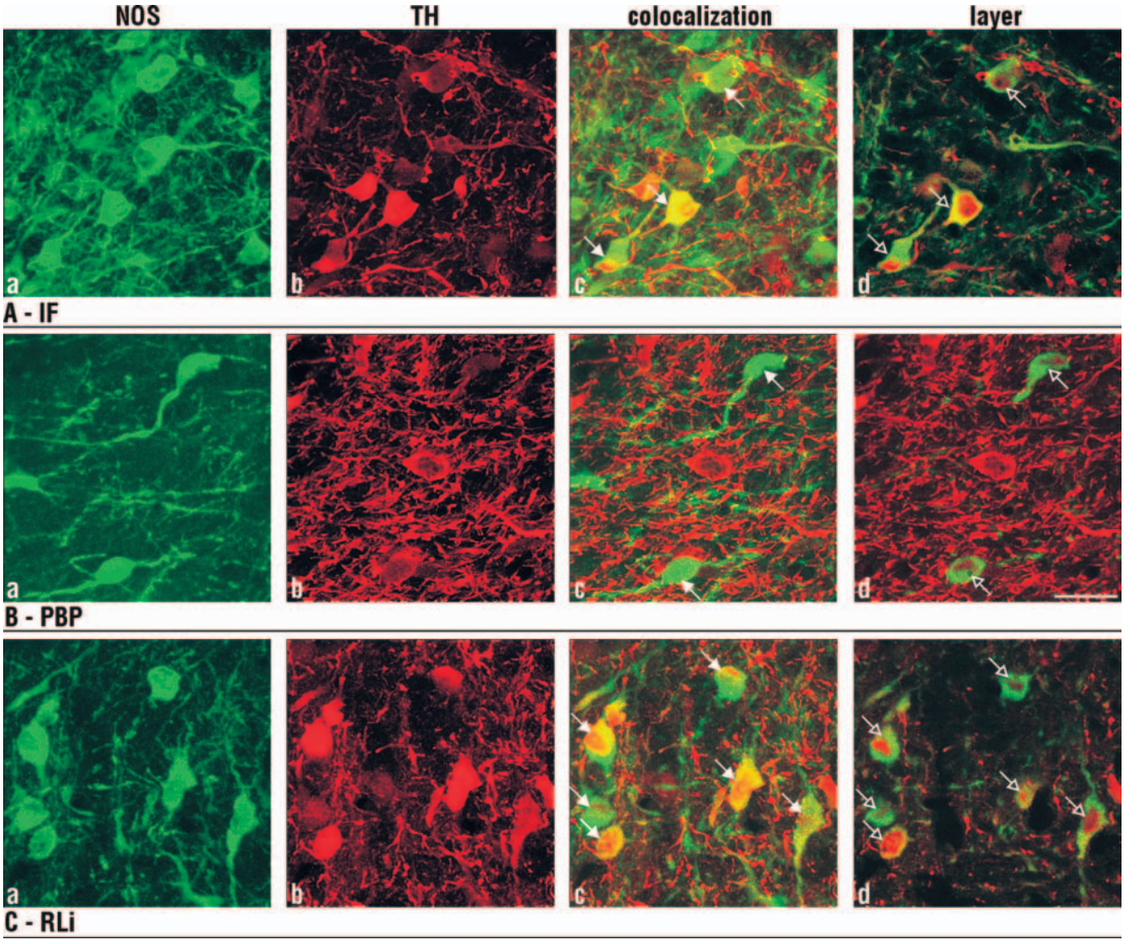

Fig. 2. Micrographs of neurons in the studied nuclei of VTA: **A** - IF, **B** - PBP and **C** - RLi. Green NOS-immunoreactive cells (**a**), red TH-immunoreactive cells (**b**), colocalization NOS-ir/TH-ir (**c**) and (**d**). 3D reconstruction through the whole thickness of the specimen (**a,b,c**). White arrows indicate NOS-ir/TH-ir colocalization neurons. **d** - the selected optical section (thickness 0.6 µm). Empty arrows indicate NOS-ir/TH-ir neurons. Scale bar: 200 µm. Double immunolabeled section, confocal microscopy system.

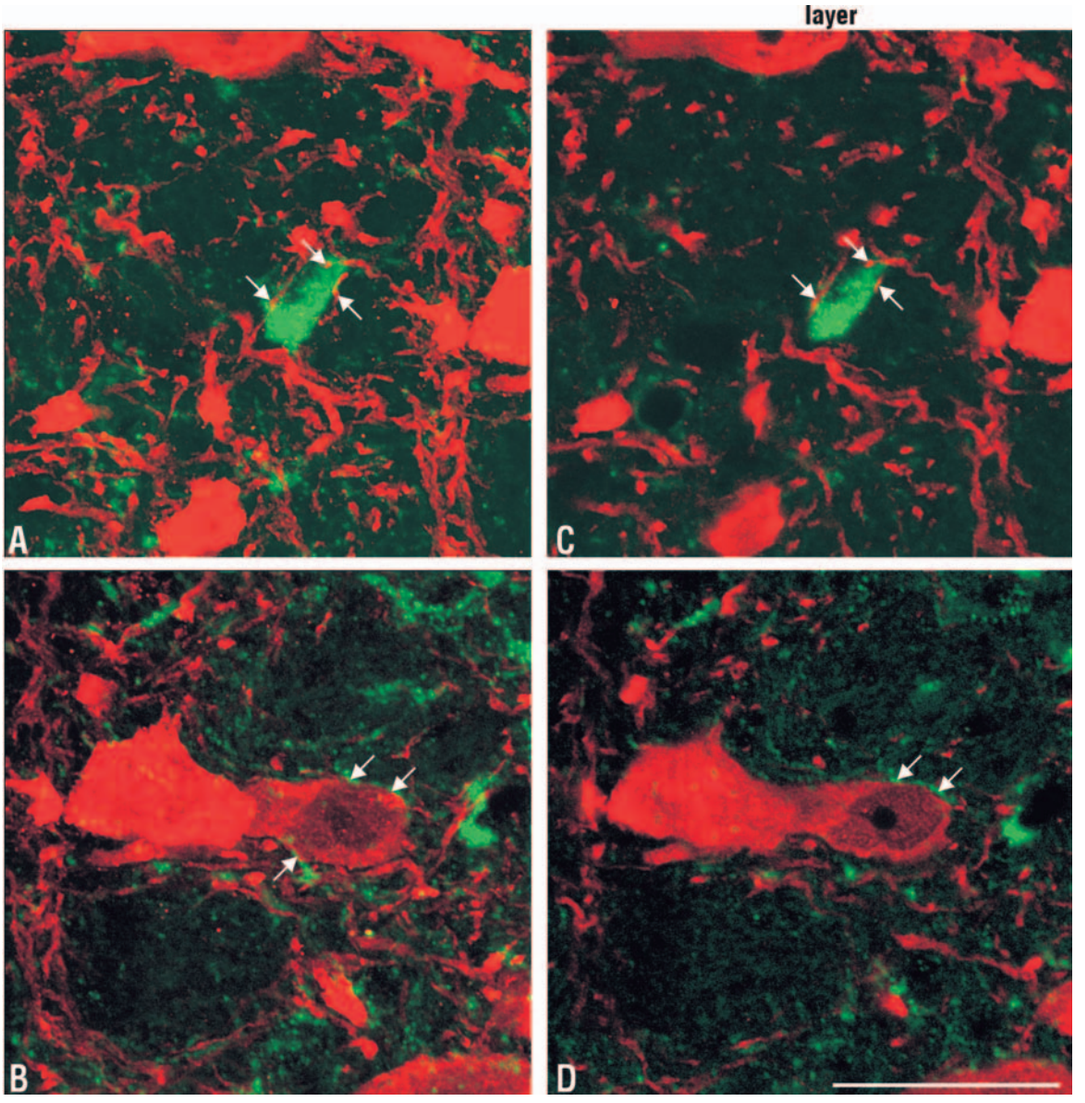

Fig. 3. Micrographs of neurons in VTA. **A** - white arrows indicate TH-ir endings on NOS-ir cell; **B** - white arrows indicate NOS-ir endings on TH-ir cell; **C,D** - the selected optical section (thickness 0.6 μm). Scale bar: 25 μm.

Immunostained slides were examined with a BX-51 (Olympus, Japan) fluorescence microscope and a MicroRadiance (Bio-Rad, UK) confocal system, equipped with an crypton/argon ion laser mounted on the Eclipse 600 (Nikon, Japan) light microscope, using the LaserSharp 2000 v.2.0 (Bio-Rad, UK) software. The confocal laser system microscopy (CLSM) images were obtained using × 40 and × 60 oil immersion objective lenses of N.A.=1.3 and 1.4, respectively.

Criteria used by Paxinos and Watson [18] were employed for subdivision of ventral tegmental area (Fig. 1).

Results

NOS-ir neurons were found in each of the three studied nuclei of VTA. In IF, NOS-ir neurons were densely packed. They were oval or triangular and possessed long dendrites. Their perikarya were stained intensely, and they often showed the presence of intensively fluorescent NOS-ir endings. The neuropil revealed high NOS immunoreactivity. A dense network of NOS-ir fibers with many varicosities was found within the whole IF (Fig. 2Aa).

TH-ir cells observed in IF were mainly oval or triangular in shape with palely stained fibers. The population of TH-ir cells was characterized by differentiated immunoreactivity: intensively stained neurons were accompanied by a population of palely stained cells. The latters often possessed TH-ir endings on their perikarya (Fig. 2Ab).

The population of NOS-positive cells in PBP seemed to be homogeneous. It consisted of fusiform, bipolar, intensely stained cells possessing long intensively

labeled dendrites with varicosities. The neuropil showed lower immunoreactivity than in IF (Fig. 2Ba).

In contrast to the homogeneous population of NOS-ir cells, the population of TH-positive neurons was heterogeneous in shape: cells were oval, triangular or multipolar and they had long dendrites, often with varicosities. Similarly to IF nuclei, these neurons were characterized by differentiated immunoreactivity**.** The long axis of cells and fibers showed a horizontal arrangement (Fig. 2Bb).

NOS-ir neurons of RLi were oval or triangular. They had long dendrites with single varicosities. Immunoreactivity of neuropil was similar to that observed in PBP (Fig. 2Ca). In RLi, the long axis of NOS-ir neurons and fibers revealed a clearly vertical pattern.

TH-positive cells were also oval or triangular with long, intensely stained fibers (Fig. 2Cb).

The colocalization study revealed that in all the studied nuclei NOS-ir cells showed TH-ir endings on their perikarya. Less frequently, NOS-ir endings were present on the perikarya of TH-ir cells.

In the studied area of IF nucleus, among all the tested cells on double stained sections (total number - 221) there were 67.4% TH-positive neurons, 22.2% NOS-ir cells, whereas 10.4% showed colocalization of both enzymes. The latter population constituted 13.3% of all labeled dopaminergic neurons, and 68% of all labeled NOS-ir neurons.

A total of 189 cells were tested in PBP nucleus, out of which 87.3% were TH-positive neurons and 9% NOS-ir cells. The degree of colocalization in this nucleus was only 3.7%. Merely 0.4% of TH-ir cells revealed the colocalization with NOS. However, opposite to IF nucleus, 29.1% NOS-ir neurons were stained for TH (Fig. 2Bcd). The above results differ significantly from the data obtained in the last studied nucleus - RLi, in which the degree of NOS/TH colocalization was 66%. As many as 67.3% TH-ir cells colocalized with NOS. Moreover, in this nucleus almost all NOS-ir neurons (97.1%) revealed colocalization with TH (Fig. 2Ccd).

Discussion

As it is known from the literature, VTA is the basic element of the mesolimbic system involved in various forms of appetitive motivating reactions [12,26]. Moreover, through the dopaminergic system this area is engaged in the development of drug addiction [12]. In this context, the role of NO may be important, because some studies suggest its participation in certain brain functions such as feeding, drinking and regulation of release and uptake of some neurotransmitters, *e.g.* dopamine [3,14]. Recent investigations suggest that NO may play a role in the development of physical dependence on some substances of abuse, for instance cocaine and opioids [2]. According to the literature, NO is diversely involved in regulation of dopamine transmission in the rat striatum [15]. Furthermore, Cox and Johnson [4] observed that NO facilitates NMDA-induced burst firing in DA neurons in VTA. These observations strongly support the hypothesis that enhanced production of NO is the critical factor in dopaminergic activity [23].

Since NOS-positive cells were described in VTA and the role of NO in VTA is associated with its influence on dopaminergic transmission, the aim of our study was to investigate the morphological correlation between the populations of TH-ir and NOS-ir neurons. In the studied nuclei of VTA we described three neuronal populations. Except for cells with NOS/TH colocalization, only TH-ir and NOS-ir neurons were present.

The morphology of dopaminergic neurons in VTA corresponds with results of Phillipson's studies [19] and as it is known, they are projection neurons. Our investigations indicate an interaction between populations of TH-ir and NOS-ir neurons. This is proven by the presence of NOS-ir endings on TH-ir neurons and TH-ir endings on NOS-ir neurons (Fig. 3A-D). Moreover, the influence may vary since both NOS-ir and TH-ir endings were observed both on perikarya and dendrites. According to the literature, perikaryal contact may exert a more rapid and potent effect on the cell than dendritic inputs [13,22]. Somatic synapses regulate action potential firing, whereas dendritic synapses influence efficacy of specific types of afferent innervation [17].

Some authors regard RLi nucleus as one of raphe nuclei (the main source of serotonin). The distinction of this nucleus was proven by our study (the highest percentage of NOS-ir/TH-ir cells). Thus it is probable that its function is slightly different from other VTA nuclei.

Taking the above into consideration, it may be suspected that NO is involved in the modulation of dopaminergic transmission.

Acknowledgements: The authors wish to thank Ms Sylwia Scisłowska for preparation of the illustrations. This study was supported by funds from the State Committee of Scientific Research (KBN), grant no. 4 P05A 068 22.

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Accepted December 5, 2003