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PRELIMINARY COMMUNICATION

IMMUNOHISTOCHEMICAL EVIDENCE FOR LOCALIZATION OF NMDAR1 RECEPTOR SUBUNIT ON DOPAMINERGIC NEURONS OF THE RAT SUBSTANTIA NIGRA, PARS COMPACTA

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Immunohistochemical evidence for localization of NMDAR1 receptor subunit on dopaminergic neurons of the rat substantia nigra, pars compacta. K. WĘDZONY, K. CZEPIEL, K. FIJAŁ. Pol. J. Pharmacol., 2001, 53, 675–679.

Non-fluorescent, double-labeling techniques were used in order to investigate whether NMDAR1 receptor subunits are localized on dopaminergic (i.e. tyrosine hydroxylase-positive) neurons of the rat substania nigra, pars compacta. It has been found that NMDAR1 receptor subunits are highly abundant in the pars compacta neurons and their dendritic processes. It was also found that vast majority, if not all, of pars compacta neurons which are positive for the presence of NMDAR1 receptor subunits are dopaminergic ones. It is concluded that if NMDAR1 receptor subunits, an indispensable element of functional NMDA receptor ion channel complex, is co-assembled with other subunits of NMDA receptor ion channel complex, NMDA receptors might directly control the activity of dopaminergic neurons.

Key words: *NMDAR1 receptor subunit, tyrosine hydroxylase, substantia nigra, immunohistochemistry, double-labeling, glutamate, Parkinson's disease*

The basal ganglia is a group of interconnected nuclei, localized subcortically and involved in a variety of functions involving control of motor, memory and even cognitive processes [3]. Among important elements of the basal ganglia is the substantia nigra (SN) which consists of two major parts, substantia nigra, pars reticulata (SNpr) and substantia nigra, pars compacta (SNpc). SNpr forms the major output system of the basal ganglia, while dopaminergic neurons of SNpc form the nigrostriatal dopaminergic system which predominantly innervates the striatum [3]. In the course of Parkinson's disease, the degeneration of dopaminergic neurons of pars compacta is observed and this pro-

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cess is not fully understood. The factors which are supposed to initiate above sustained degenerative process include glutamate receptor-mediated neurotoxicity [2]. SN receives glutamatergic inputs from several brain regions including the cerebral cortex, pedunculopontine nucleus, and nucleus subthalamicus [3]. The released glutamate, which is excitatory neurotransmitter in the physiological states, regulates the activity of dopaminergic cells, while its excessive and long-lasting outflow may evoke progressive degeneration of dopaminergic neurons [4, 2]. Two major groups of ionotropic glutamatergic receptors are involved in regulation of the activity of dopaminergic neurons, namely N-methyl-D-aspartate (NMDA) receptors and α-amino-3--hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors [5]. Electrophysiological and pharmacological experiments have provided several arguments suggesting that above receptors are localized on the somata or dendrites of dopaminergic neurons of SNpc [5, 6]. Moreover, the *in situ* hybridization techniques, as well as autoradiographic studies revealed that above receptors are highly abundant in the pars compacta of rodent SN, which has been widely studied in experimental models of Parkinson's disease [5, 8]. Recent progress in the molecular biology has greatly improved our ability to investigate precise receptor localization, e.g. by generation of specific antibodies, which recognize the epitope of various subunits of NMDA receptors and, thus, allowing precisely visualize cellular distribution of subunits of NMDA receptor ion channel complex [1, 10]. Availability of novel goat antibody, recognizing epitope of the carboxyl terminus of R1 subunit of NMDA receptors and the importance of above receptors in the pathology of Parkinson's disease prompted us to investigate the distribution of R1 subunit of NMDA receptor (NMDAR1) in both SNpc and SNpr, and secondly, to investigate with non-fluorescent double-labeling procedure whether above NMDA receptor subunits are present on the dopaminergic cell bodies and their dendrites, i.e. neurons and their processes which are tyrosine hydroxylase (TH)-positive. The experimental protocols were approved by the Committee for Laboratory Animal Welfare and Ethics of Institute of Pharmacology, Polish Academy of Sciences in Kraków, and met guidelines of the International Council for Laboratory Animal Guide for the care and use of Laboratory animals (86/609/EEC).

Immunocytochemical visualization of NMDAR1 subunit has been described previously [10]. Briefly, after perfusion (4% paraformaldehyde in phosphate buffered saline) and 4-hour post-fixation period, 50 μm-thick sections were cut at the level of rat SN, using Leica VT-1000S vibratome. The free--floating brain sections were rinsed, and incubated for 1 h in a blocking buffer containing 5% normal rabbit serum and 0.3% Triton X-100 in 0.01 M PBS (phosphate buffered saline). Subsequently, the sections were incubated for 48 h with affinity-purified polyclonal goat primary antibody recognizing the carboxyl terminus of NMDAR1 receptor (Santa Cruz, Biotechnology, INC., USA) diluted at 1:2500 (0.08 μg of antibody protein in 1 ml of 0.01 M PBS containing 0.3% Triton X-100 and 2% normal rabbit serum). Antigen has been visualized using standard ABC Elite Vectastain kit (Vector Lab. USA). The reaction was visualized using DAB (diaminobenzidine) and hydrogen peroxide [9]. In the experiments investigating the specificity of labeling, we used the primary antibody (diluted as above) which has been pre-adsorbed with the excessive concentration of control peptide (Santa Cruz, Biotechnology, INC., USA) mixed in the proportion of 1:4 with respect to protein content. In the double-labeling experiments, sections were stained first for the presence of TH exactly as it was described previously, however, in order to adjust the procedure for demands of mouse monoclonal antibody recognizing TH (dilution 1:10000, Chemicon, USA), we used normal horse serum (Vector Lab), anti-mouse rat adsorbed (Vector Lab) secondary antibody and standard ABC Elite kit (Vector Lab). The reaction was visualized using a glucose oxidase-DAB method, yielding a light brown staining product [9]. Then, the sections were stained exactly as described above for NMDAR1 receptor subunit, with the exception that the final reaction was visualized using BDHC (benzidine dihydrochloride) substrate prepared exactly as it was reported by Levey et al. [7]. The described protocol of staining leads to the formation of dark blue grainy product which enables good visualization of the second immunogen over the light brown and homogenous TH immunoreactivity. For data presentation and mapping of immuno-positive material, digital images were captured using a SPOT II camera (Diagnostic Instruments, USA) attached to a Nikon Optiphot II microscope. Photo-montages were composed using the Adobe

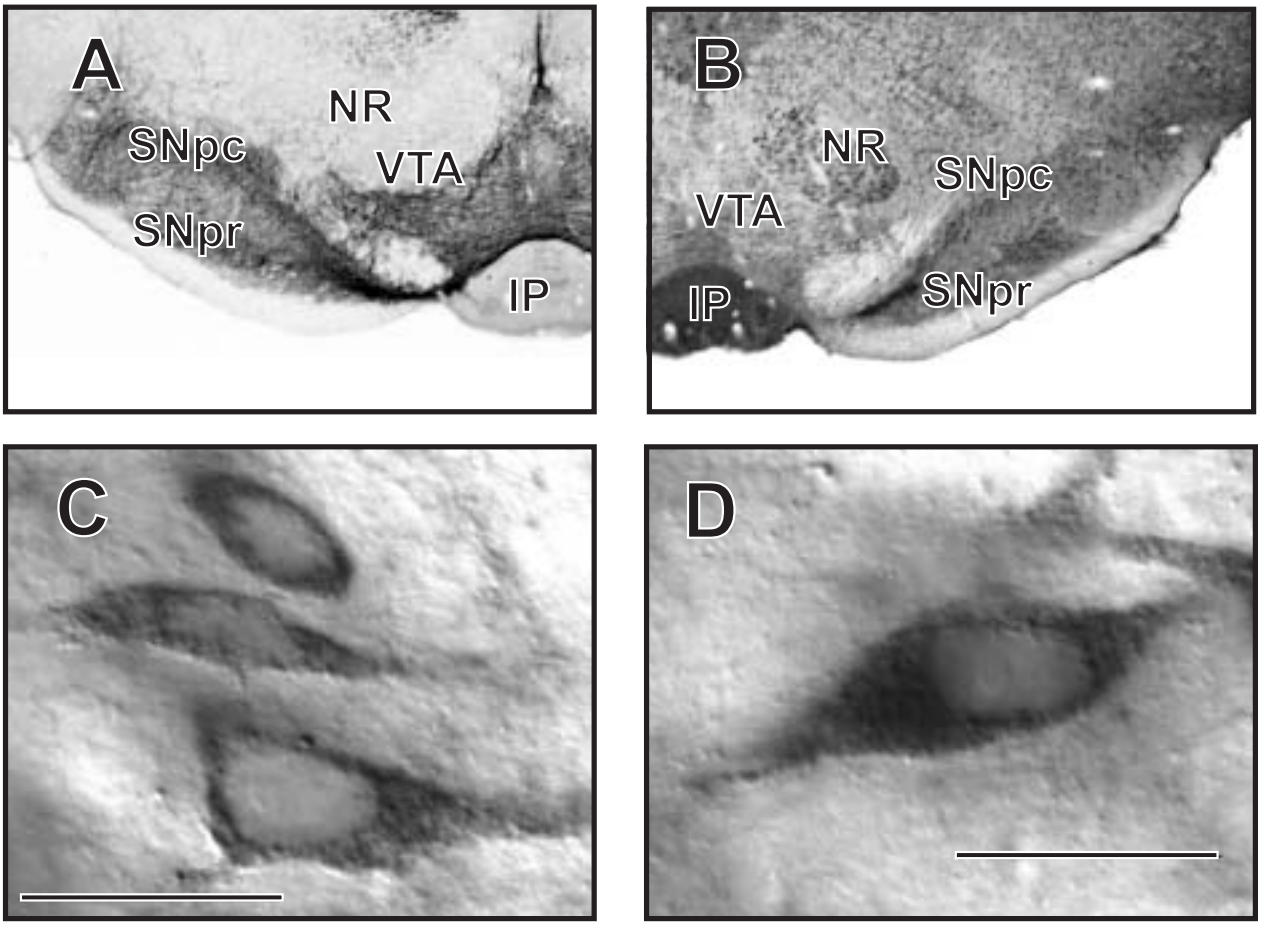

Fig. 1. Photomicrographs of the rat substantia nigra stained for the presence of tyrosine hydroxylase (A) and NMDAR1 receptor subunit (B, C, D). A–B are a low power magnification of two consecutive sections 50 µm apart stained for tyrosine hydroxylase and NMDAR1 subunit, respectively. (C, D) high power (objective 100x, scale bar 3 µm) magnification of neurons positive for the presence of NMDA-R1 receptor subunit from SNpc (C) and SNpr (D). Abbreviations: VTA – ventral tegmental area, IP – interpeduncular nucleus, NR – red nucleus, SNpc – substantia nigra, pars compacta, SNpr – substantia nigra pars reticulata

PhotoShop program and were spatially calibrated using ImagePro Plus (Media Cybernetics, USA).

The NMDAR1 immunoreactivity was heterogeneous but clearly visible in different subregions of the rat SN: it ranged from very intense in the SNpc (Fig. 1B) to moderately weak in SNpr (Fig. 1B), possibly due to the various density of NMDAR1-positive neurons in above regions (Fig. 1B). Sections in which the primary antibody was omitted, or pre-adsorbed with a control peptide did not show evidence of any staining, what speaks for the selectivity of labeling (data not shown). Both, in SNpc and SNpr, intense immunoreactivity was observed in cell bodies (Fig. 1C, D), but there was also a clear staining of dendritic processes emanating from the NMDAR1 positive cell bodies (Fig. 2B). Moreover, some dendritic processes not related to any surrounding neurons were stained (Fig. 2E). At the cellular level, it was observed that NMDAR1 immuno-positive material had relatively uniform cellular distribution in some neurons (Fig. 1B) but in general NMDAR1 immunoreactivity was distributed in the periphery of the cell body (Fig. 1C, D), which is in line with the pattern of distribution of functional receptors [9].

The monoclonal antibody visualising TH showed a typical pattern of distribution of this enzyme in SN (Fig. 1A). Double-labeling experiments revealed that vast majority, if not all of NMDAR1-positive neurons in SNpc were also TH-positive (Fig. 2A, B). Also a sparse TH-positive neurons in SNpr were positive for the presence of NMDAR1 receptor subunit (Fig. 2C, D). However, in SNpr we observed also neurons which were positive for NMDAR1

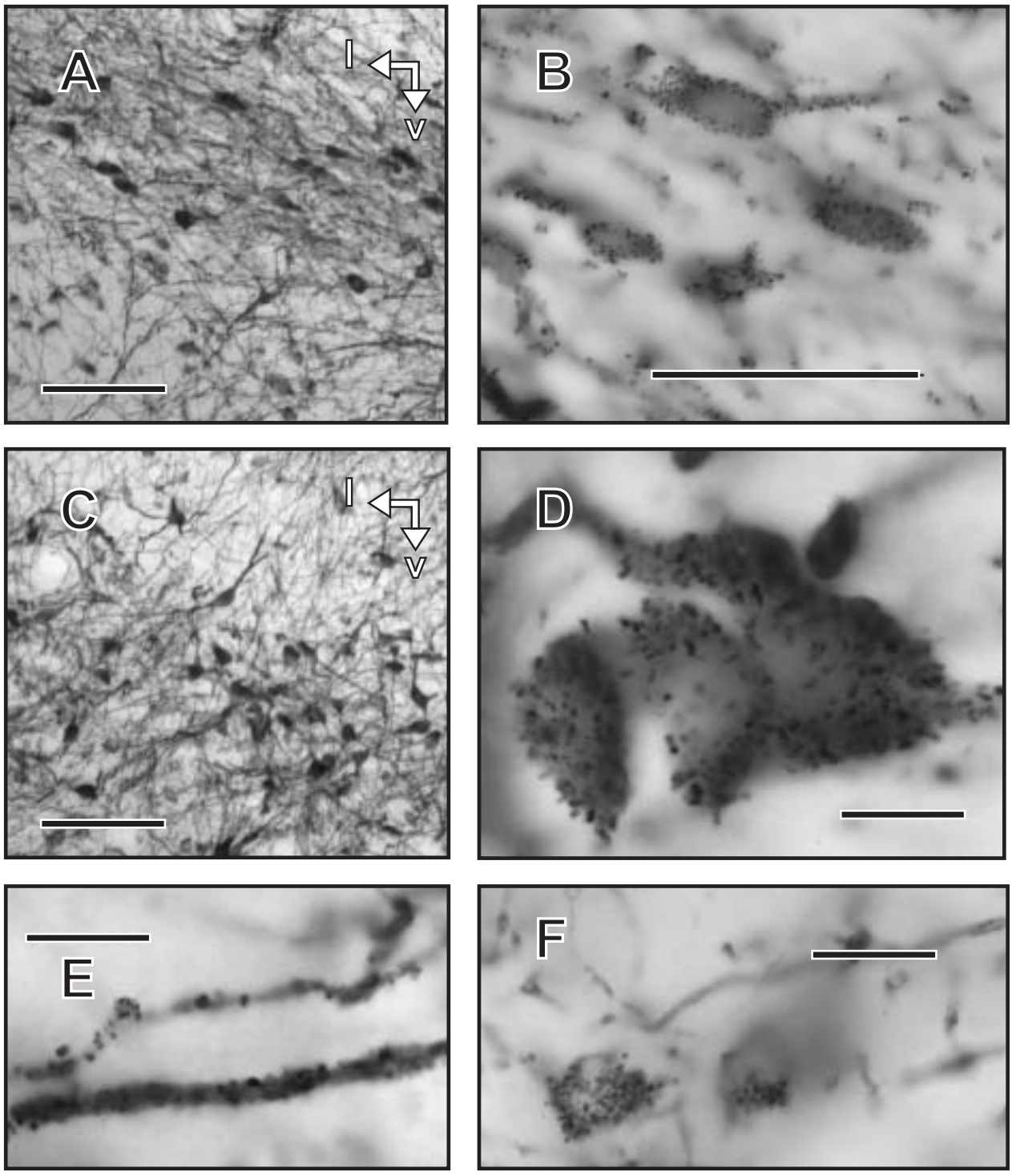

Fig. 2. Photomicrographs illustrating co-localization of NMDAR1 receptor subunit (dark blue deposits) with tyrosine hydroxylase (TH, homogenous brown deposits) in rat SNpc (A, B) and SNpr (C, D). A and C are low power magnifications (objective 10x), while B and D are high power magnification (objective 40x and 100x, respectively). E illustrates the presence of NMDAR1 receptor subunit on TH-positive dendritic processes (objective 100x), while F shows NMDAR1-positive neurons in SNpr, which are devoid of TH immunoreactivity. Arrows in A and C indicate the lateral ventral orientation of photomicrographs

subunit but devoid of TH immunoreactivity indicating the presence of NMDAR1 receptors on non-dopaminergic interneurons or output neurons (Fig. 2A, F). Although BDHC labeling procedure provides a clear evidence of co-localization of NMDAR1 subunit with TH, a full quantification of degree of co-localization is limited by the fact that sensitivity of above labeling is lower than conventional labeling with DAB, leading to the visualization of smaller number of neurons positive for NMDAR1 subunits in comparison to the number of cells stained with DAB.

In conclusion, our present results indicate that novel goat polyclonal antibody recognizing the carboxyl terminus of NMDAR1 receptor epitope visualized the above receptor subunits in the dopaminergic neurons (TH-positive) in the SNpc and SNpr. Moreover, in SNpr, NMDAR1 receptor subunit has been also present on the non-dopaminergic interneurons or output neurons. The observed intracellular distribution of NMDAR1 subunits suggests that it is possible to visualize not only the distribution of elements of active receptors, i.e. receptors attached to the cell membrane, but also certain amount of the receptor protein seen in the cytoplasm which may represent the receptor protein in the process of synthesis and transport to the membrane or receptor protein which is under degradation process [9].

The observed localization of NMDAR1 receptor on the dopaminergic cells may indicate that, if NMDAR1 receptor subunit (indispensable element of functioning of NMDA receptor) is co-assembled with other subunits (2A, 2B, 2C and 2D), which according to the *in situ* hybridization studies [8] are present in the SNpc (for controversy about 2A see [1]), NMDA receptors may directly, without intermediate neurotransmitter, control the activity of dopaminergic cells [4]. This conclusion is supported further by the functional observations that NMDA may control the activity of dopaminergic cells [4, 6]. In awake animals, dopaminergic neurons exhibit both rapid bursting activity, as well as irregular single spike activity [4]. Such bursting activity, which is associated with the neurotransmitter release, has not been observed *in vitro* [6], but can be induced by application of NMDA agonist [6]. Above data are in line with our anatomical observations and provide functional proof supporting the suggestion that glutamatergic neurons and NMDA receptors control the activity of the nigro-striatal dopaminergic system. It will be of interest to investigate in future the co-distribution of other subunits of NMDA receptors like 2A, 2B, 2C and 2D with TH in the rat SN. Further studies may allow to associate vulnerability of certain population of dopaminergic cells to glutamate-mediated neurotoxic insults with NMDA receptors subunit composition [2].

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