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Morphology

Parkin-Immunoreactivity in the Basal Ganglia and Diencephalon of the Rat. Comparison of Two Antibodies

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Parkin is an intracellular protein that plays a significant role in the etiopathogenesis of autosomal recessive juvenile parkinsonism. By using two polyclonal anti-parkin antibodies the distribution of immunoreactive (IR) nerve cell bodies and their processes in the basal ganglia and diencephalon of the rat was investigated and a comparison of the results between both antibodies (AB I, cat No 5112; and AB II, cat No 5978) is presently described. The parkin-IR material occurred in the perikaryal cytoplasm and in the dendrites, but only rarely in the cell nuclei. The immunoreaction was stronger with the I AB in neostriatum, nucleus entopeduncularis and substantia nigra, but with application of the II AB the immunoreactivity was stronger in the neurons of nucleus basalis gigantocellularis of Meynert, globus pallidus, nucleus paraventricularis thalami, bed nucleus of stria terminalis and in the neurosecretory nuclei of the hypothalamus.

Key words: Neostriatum, nucleus basalis gigantocellularis of Meynert, thalamus, hypothalamus, immunohistochemistry.

Introduction

The Parkinson disease (PD) is a neurodegenerative disorder characterized by akinesia, tremor and disturbances of gait and posture. The main finding in the pathology of PD is neuronal degeneration of the pars compacta of the substantia nigra (SNc), and to a lesser extent – of the locus coeruleus neurons. SNc neurons synthesize the neurotransmitter dopamine (DA) and the consequence of the selective loss of this mesencephalic neurons is a decrease of DA content in the forebrain regions receiving SNc axons, such as caudate nucleus and putamen. These brain areas, along with neighboring neural circuits are an integral part of the basal ganglia, a group of forebrain nuclei facilitating or inhibiting the

motor function. The cause of degeneration of the DAergic neurons in PD is unknown, but a number of factors such as mutations and exposure to environmental toxins have been implicated in the etiology of the disease [reviewed in 18, 23]. K i t a d a et al. [13] found out that by the autosomal recessive juvenile parkinsonism a mutation in a newly identified gene takes place. This gene encodes a protein of 465 amino acids, and K i t a d a et al. [13] nominated this protein product "Parkin". Data on the functions of Parkin and its involvement in the pathology of PD immediately followed [21, 7, 8, 20, 16, 17, 2] to mention only a few. There are several immunohistochemical reports on the expression of the protein Parkin in the brain [22, 24, 10, 11, 15, 12, 19]. The aim of the present study is to investigate the immunohistochemical distribution of the parkin in the basal ganglia and diencephalon in the rat and to compare this finding by using two Parkin antibodies.

Material and Methods

All procedures were carried out according to a standard protocol established by the Ethic Commission of Institute of Neurobiology at the Bulgarian Academy of Sciences. Under deep ether anesthesia seven male Sprague-Dawley rats were perfused transcardially initially with 0.05 M phosphate buffered saline (PBS) pH 7.3 followed by 500 ml fixative solution containing 4.0% paraformaldehyde in 0.1 M phosphate buffer pH 7.3 for 30 min. Brains were removed and postfixed overnight in the same fixative at 4°C and then were transferred in 20% sucrose in 0.1 M PB for further 24 h in refrigerator. Thirty μm thick tissue sections were cut on a freezing microtome "Reichert Jung" and collected in the same solution of sucrose in subset of 5. They were washed overnight in 0.05 M PBS in several changes. Free floating sections were immunostained for Parkin by using an indirect immunohistochemical procedure. All incubations were carried out at room temperature. Sections were treated with 50% ethanol, followed by 0.3% H_2O_2 in 100% methanol in order to block endogenous peroxidase activity for 15 min. Then the sections were incubated in 10% normal donkey serum in PBS for 1 h, rinsed in PBS and then incubated overnight in anti-parkin polyclonal antibodies (Chemicon Int. USA). We used two antibodies. The first one (I AB), cat. No AB 5112 is a 19-amino-acid peptide and corresponds to amino-acid sequence 305-323 of the human Parkin molecule. It was used in working dilution 1:500. The second antibody (II AB) – AB 5978 is an 18-amino-acid peptide and corresponds to amino-acid sequence 295-311 of the human Parkin molecule. It was used in working dilution 1:1000. Sections were incubated overnight in rabbit anti-Parkin polyclonal antibody, followed by donkey anti-rabbit IgG diluted 1:200, and in ExtrAvidin Peroxidase Conjugate, diluted 1:5000. The reaction was visualized with Ni/DAB/glucose oxidase. In control sections the primary anti-parkin serum was omitted, and the results were negative. After the development tissue sections were mounted on chrome alum gelatin coated slides, air dried overnight, dehydrated in 96% and 100% ethanols, cleared in xylene and coverslipped with Entellan. The preparations were investigated on "Jenaval" light microscope. Microphotographs were taken by using "Kodak 200" film.

Results

The present experiments demonstrate that the protein Parkin is broadly distributed in the neuronal populations of the basal ganglia and the diencephalon. The immunostaining is present in the perikaryal and dendritic cytoplasm, and spares the cell nuclei. In most cases the application of the two antibodies provides comparable results, with mild quantitative

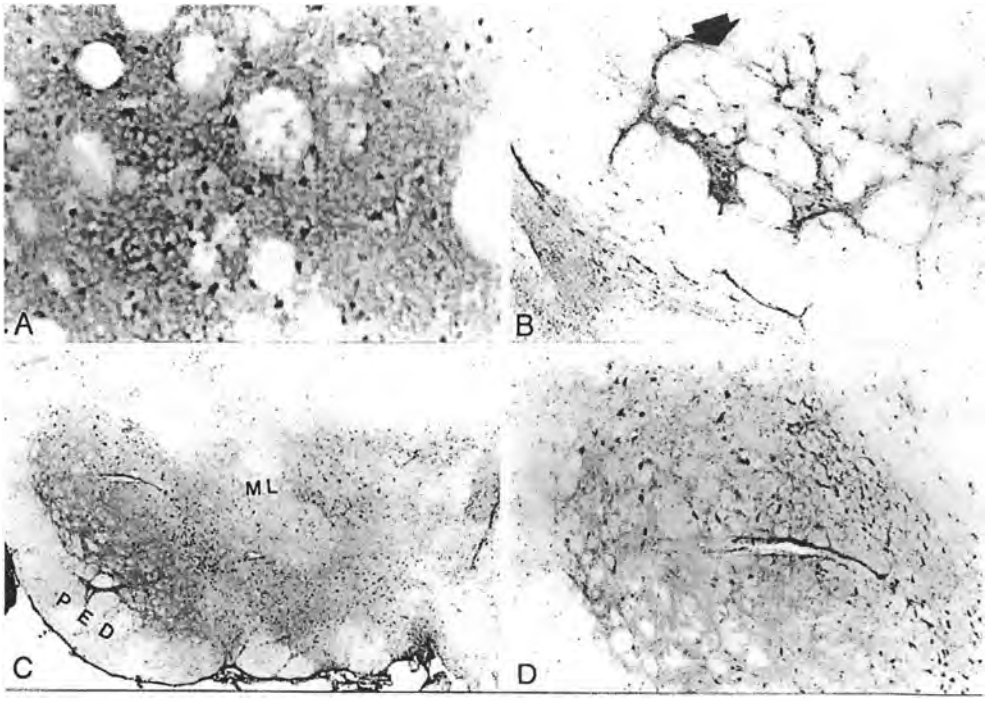

Fig. 1. First antibody
A — Rostral striatum. The immunoreactive neuropil is pearced by numerous immunonegative bundles of cortical fibers. Numerous strongly stained small neurons, that represent "medium spiny" GABAergic projection neurons.×400; **B** — The arrow points to nucleus entopeduncullaris. Within the strongly stained neuropil of the entopeduncullar nucleus, also its GABAergic neuronal perikarya display a strong immunoreaction.×100; **C** — Low power view of the ventral mesencephalic tegmentum. The strongly stained substantia nigra (SN) is recognized between the immunonegative cerebral peduncle (PED) and the medial lemniscus (ML).×40; **D** — Detail from **C**. The right part of the figure is occupied by the densely arranged neurons of the SN pars compacta. To the left — large immunoreactive neurons of the substantia nigra pars reticulata. In the lower left part of the figure — strongly stained neuropil of the substantia nigra pars lateralis is seen.×100

differences. However, in some regions also drastically different results appear. I AB stains the axons only rarely, while II AB labels various fiber systems.

In the neostriatum the application of I AB results in strong immunoreactivity. Parkin-positive are the medium spiny neurons (the main projection cells of the neostriatum), as well as their axons (Fig. 1A). The bundles of corticofugal axons that traverse the neostriatum are immunonegative, although the cells of origin of these fibers (the pyramidal neurons of the frontal cortex) strongly immunoreact for Parkin, giving the neurons in the fifth layer almost a Golgi silver impregnation-like appearance. The entopeduncular nucleus (the equivalent of the internal pallidal segment in subprimate species) is clearly outlined within the pale axons of the internal capsule, due to the significant number of Parkin-positive perikarya and dendrites (Fig. 1B). Also strong immunoreaction is present in the basal magnocellular nucleus of Meynert. Its large, multipolar perikarya, as well as the long, robust dendrites, are intensively labelled. On the other hand, by the use of II AB the neostriatal neurons are only faintly labelled (Fig. 2C, D). The neurons of the globus pallidus and nucleus entopeduncularis display similar immunoreactivity by both antibodies (Fig. 2C, D). In the substantia nigra there is a significant Parkin immunoreactivity (Fig. 1C, D). The three subnuclei of substantia nigra (pars compacta, pars reticulata and pars lateralis) contain strongly immunolabelled neurons. Along the perikarya, also the den-

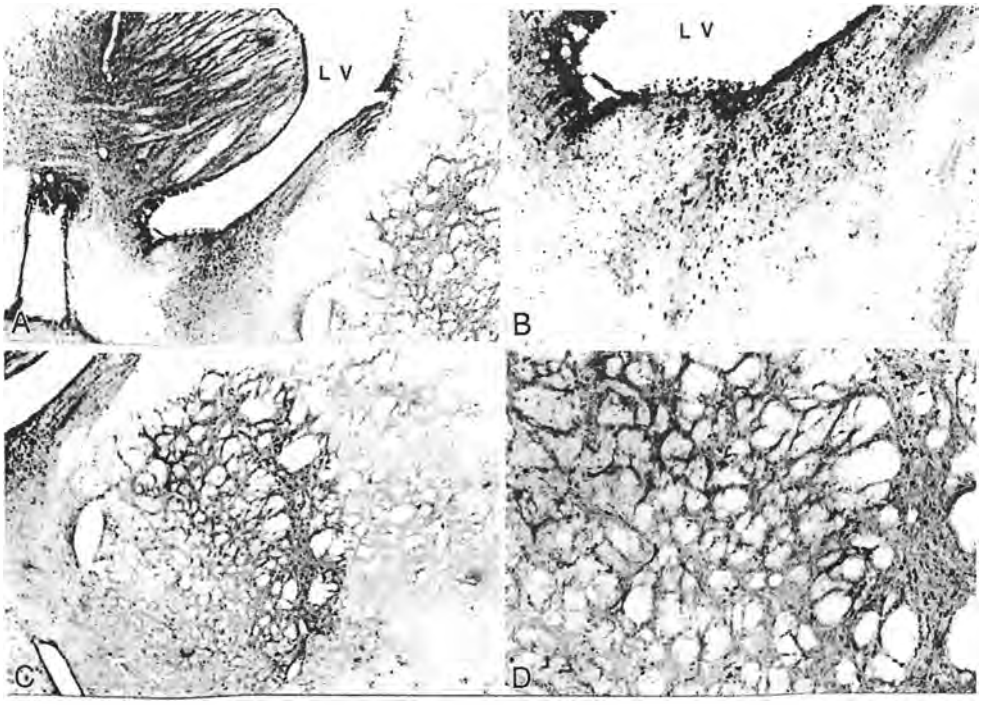

Fig. 2. Second antibody
A — Bed nucleus of the stria terminalis surrounded by nucleus septofimbriatus (upper left), columnae fornicis and commisura anterior (lower left), stria terminalis and globus pallidus (to the right). ×40; **B** — Detail from **A**. Strongly stained neurons of the bed nucleus of stria terminalis. ×100; **C** — Overview of the bed nucleus of stria terminalis (upper left), globus pallidus (center) and neostriatum (to the right). ×40; **D** — Detail from **C**. Scattered strongly immunoreactive neurons of the globus pallidus. At the right part of the figure is the most dorsal portion of the magnocellular basal nucleus of Meynert. LV — lateral ventricle. ×100

drites are labelled but in most cases the dendritic outlines are obscured by the diffuse neuropil labeling. The latter is due to the enormous number of axonal endings of the medium spiny neostriatal neurons that terminate on the nigral dendrites.

Nucleus septofimbriatus (Fig. 2A) and bed nucleus of stria terminalis (Fig. 2A, B) demonstrate intensive immunoreactivity with the II AB.

In the thalamus following the application of the I AB, a moderate intensity of the immunoreaction is present in most of the nuclei, and it is slightly stronger in the reticular thalamic nucleus. By II AB the intensity of the reaction is lower, with the exception of the paraventricular thalamic nucleus (Fig. 3A). In the hypothalamus, the I AB display a rather homogenous reaction in most nuclei, and the neurosecretory supraoptic and paraventricular nuclei are more intensively labelled. By II AB there is a sharp contrast between the neurosecretory and supachiasmatic nuclei and the remaining hypothalamic areas. Nucleus supachiasmaticus (Fig. 3B), nucleus supraopticus (Fig. 3C), and nucleus paraventricularis hypothalami (fig. 3D) are strongly Parkin-IR to the last neuron, while the remaining nuclei display only a faint immunostaining.

Discussion

As described in the Introduction, through the last decade the protein Parkin is immediately associated with autosomal recessive juvenile parkinsonism. Therefore, it is important to

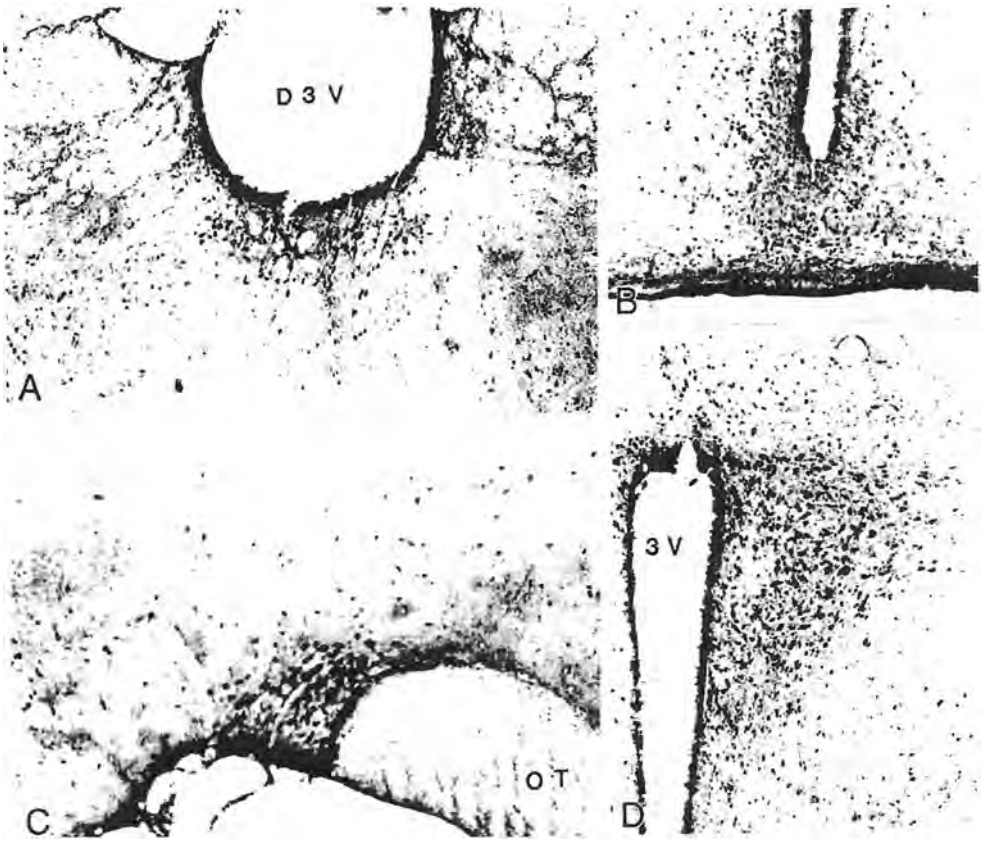

Fig. 3. Second antibody. Strongly immunoreactive neurons in the paraventricular thalamic nucleus (**A**), suprachiasmatic nucleus (**B**), supraoptic nucleus (**C**) and paraventricular hypothalamic nucleus (**D**). D3V — dorsal 3rd ventricle, 3V — 3rd ventricle, OT — optic tract. × 100

point out that several immunohistochemical studies listed in the Introduction, and supported by the present data, indicate that Parkin is to be found in normal CNS, and not only in the basal ganglia but is present throughout the neuraxis, and it is detectable also in the peripheral nerves [6, 1]. Along the human and primate brain [24], Parkin is commonly found in the rodent CNS [9, 3, 4, 5, 12, 19; the present study]. H o r o w i t z et al. [10] declared the presence of Parkin also in the brain of birds, lower vertebrates (frog), and even in the "brain ganglion" of insects (fruit-fly). Parkin appears early in the phylogenesis but is not present in the early ontogenetic stage. H u y n h et al. [11] found out that in mouse fetuses Parkin was expressed only after neuronal differentiation. Further K ü h n et al. [14] specified that in the developing mouse brain Parkin mRNA and protein appear as early as E10/E12 but a marked increase in expression level takes place during midgestational development (E15-18), followed by a steady increase until adulthood.

The present data, as well as previous light microscopic immunohistochemical investigations, indicate that Parkin is absent in the cell nucleus but appears to be diffusely distributed in the perikaryal cytoplasm, in the dendrites, and in some axons. The "patchy" staining observed by us in the pars reticulata of substantia nigra strongly suggests the presence of Parkin also in the synaptic boutons of the strionigral axons, e.g. Parkin is to be found both presynaptically and postsynaptically. Indeed, S h i m u r a et al. [21] reported a broad cytoplasmic distribution – both in the Golgi complex, and in the cytosol.

Kubo et al. [15] investigated the intracellular localization in cultured cells, and detected Parkin in the trans-Golgi network and in secretory vesicles. A detailed ultrastructural localization of Parkin was presented by Mouatt-Prigent et al. [19] in the rat brain. Notably, they found Parkin also in glial cells that raises further questions on the functions of this protein. In the neurons, Parkin was mostly localized on the periphery of large vesicles, some rare mitochondria and endoplasmic reticulum in the cell bodies, and on the periphery of large vesicles in the dendrites and terminals of the neurons. In addition, Parkin immunoreactivity was also found around synaptic vesicles in the presynaptic elements of some axons. The latter observation of Mouatt-Prigent et al. [19] supports our suggestion that Parkin is localized also in the strionigral axon terminals.

Since Parkin is involved in a prominent movement disorder - autosomal recessive juvenile parkinsonism - its presence is to be expected in brain structures responsible for motor function: the neostriatum, both segments of the globus pallidus, and in both DAergic and GABAergic neurons of substantia nigra. We found only few discrepancies with previous reports, for example the data of Horowitz et al. [10] on the paraventricular hypothalamic nucleus do not correspond to the present findings. It should be noted, however, that also in our own hands, the use of antibodies for two Parkin isoforms provided in some cases different results.

In conclusion, the present study provides unequivocal data on the presence of Parkin in the extrapyramidal motor brain centers. The strong immunolabelling of the large cholinergic neurons of the Meynert's nucleus in substantia innominata immediately recalls the fact that these cells are the most deteriorated neuronal elements in another severe neurodegenerative disorder – the presenile dementia of Alzheimer. And the prominent immunostaining in the bed nucleus of stria terminalis raises the question if Parkin is also not involved in the limbic circuitry. That Parkin is prominently involved in autosomal recessive juvenile parkinsonism is firmly established. But since this protein is neither restricted to a single functional system nor associated with a particular transmitter system, also other functions of Parkin remain to be elucidated.

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