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Synaptic Contacts in Schizophrenia: Studies Using Immunocytochemical Identification of Dopaminergic Neurons

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Immunocytochemical identification of dopaminergic neurons was performed using an immunoperoxidase method employing antibodies to tyrosine hydroxylase. The ultrastructure of synaptic contacts on dopaminergic (tyrosine hydroxylase immunopositive (TP) cells) neurons was investigated in the substantia nigra in the brains of four patients with schizophrenia and three mentally healthy subjects (controls). The substantia nigra of schizophrenia patients differed from control material in showing the following changes in the ultrastructure of presynaptic terminals contacting TP neurons: reductions in the size of terminals with dense matrix and poorly distinguished vesicles; swelling of terminals with small numbers of vesicles displaced from the active zone of the synapse; hyperplasia of mitochondria in some presynaptic boutons; appearance of membranous lamellar structures within or adjacent to presynaptic boutons. These changes to terminals were located mostly on the distal (small and intermediate) TP dendrites in the compact zone of the substantia nigra, where nearly all the dendrites detected belonged to dopaminergic neurons and the altered terminals formed asymmetrical contacts with short active zones. In the reticular part of the substantia nigra of schizophrenic patients, changes in the ultrastructure of presynaptic terminals were relatively rare; altered terminals contacted both tyrosine hydroxylase immunopositive as well as with the tyrosine hydroxylase immunonegative dendrites located in this structure.

Recent years have seen the publication of new data on morphological rearrangements in the brains of patients with schizophrenia, which are important both for understanding a number of the typical clinical manifestations of this disease and for furthering our knowledge of its pathogenesis [2, 3, 6, 7, 11, 14, 15, 20]. In particular, studies of the brain dopaminergic system (the structures relating to the most widespread—dopaminergic—hypothesis of the pathogenesis of schizophrenia) have demonstrated changes in the numbers and ultrastructure of synapses in the prefrontal cortex and substantia nigra [2, 3]. However, apart from dopaminergic neurons, these zones are known to contain neurons with other neuromediator specificities (GABAergic, serotoninergic, peptidergic neurons). Thus, the relationship with previously established changes in structures relevant to neuron transmission is of major importance for assessment of the state of the brain dopaminergic system. Contemporary immunocytochemical methods allow this approach. Dopaminergic neurons in the substantia nigra can be detected by an immunoperoxidase method using antibodies against tyrosine hydroxylase (tyrosine hydroxylase is the key enzyme in catecholamine synthesis).

The aim of the present work was to study the ultrastructure of synaptic contacts in immunocytochemically identified dopaminergic (tyrosine hydroxylase-positive (TP)) neurons in the substantia nigra, where most catecholamine neurons are dopaminergic.

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Studies were performed using autopsy brain specimens from patients with schizophrenia and control material from the brains of mentally healthy subjects.

Brains from patients with schizophrenia were collected at autopsy in the Department of Pathological Anatomy, N. A. Alekseev Moscow City Clinical Psychiatric Hospital No. 1. Brains were obtained from four patients with schizophrenia (MKB-10 No.: F20.0). Two patients were male and two were female, and they were 66-75 years old at death, and had suffered from schizophrenia for 18-47 years. The control group consisted of brain specimens from three mentally healthy subjects (two males, one female) who died at ages of 35-65 years. This material was obtained from the Department of Pathological Anatomy, Moscow City Clinical Hospital No. 55. The causes of death in all seven cases were identical: cardiovascular failure. The time between determination of death and collection of brain specimens was in no case no more than 6 h.

Extracted tissue blocks containing substantia nigra material were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 1 day at 4°C. Vibratome sections of 30-40 μm thickness were washed for 60 min in 0.02 M phosphate buffer pH 7.4 containing 0.9% NaCl and 10% normal rabbit serum. The same buffer, containing 10% normal rabbit serum, was used for diluting antibodies and washing slices. Slices were incubated with monoclonal antibodies against tyrosine hydroxylase (Boehringer Mannheim, Germany) at dilutions of 1:100 to 1:200 for 24-48 h at 4°C and were then washed (for 30 min) and incubated with rabbit anti-mouse IgG conjugated with peroxidase (Serva, Germany) for 3-4 h at room temperature, and were washed again. Peroxidase activity was assessed by incubating slices in solution containing 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 in 0.05 M tris-HCl buffer pH 7.6, for 15-20 min at room temperature. Reactions were stopped by transferring slices into 0.1 M phosphate buffer. Some of the slices were used for light microscopy. The remainder were used for subsequent electron microscopy and were fixed in 2.5% glutaraldehyde for 0.5 h, washed, and prefixed in 1% OsO_4 for 1 h. After fixation, material was dehydrated in ethanol solutions of increasing concentration and embedded in Araldite by fixing slices to prepared blocks. Areas of slices predominantly containing material from the compact or reticular zones of the substantia nigra were identified by microscopy. Ultrathin sections were cut using an Ultracut E ultramicrotome (Reichert, Austria), contrasted with uranyl acetate and lead citrate, and examined in an EM-420 electron microscope (Phillips, Holland).

Control slices were incubated at the first stage in the absence of antibodies to tyrosine hydroxylase in phosphate-containing buffer with 10% normal rabbit serum for 24-48 h at 4°C. Subsequent processing of control slices was as described above. Immunoperoxidase staining, detected by DAB precipitation, was absent from control slices.

Light Microscopy

TP neurons were detected by light microscopy from the presence of intense brown staining by DAB. Their distributions in the substantia nigra of the brains from schizophrenic patients and mentally healthy patients were identical (Fig. 1, *a*). Most neurons in the compact zone of the substantia nigra were immunopositive, as were separated groups of cells in the reticular zone. The sizes and shapes of TP neuron perikarya varied. There were also numerous immunopositive dendrites of various diameters; most dendrites in the reticular zone were oriented mainly in the ventrocaudal direction. Additionally, the compact zone of the substantia nigra contained point accumulations immunopositive structures located mainly in its dorsal part.

Electron Microscopy

TP structures were identified in electron microscopic studies from the presence of electron-dense peroxidase reaction product formed from DAB (Fig. 1, *b*). Reaction product was associated with the inner surface of cytoplasmic membranes of perikarya and dendrites and with the membranes of internal organelles. TP perikarya with preserved ultrastructure were filled uniformly with DAB precipitates, while the distribution of peroxidase reaction product in dendrites was less uniform and depended on the level of ultrastructural preservation of the organelles.

The vast majority of neuron perikarya in the compact zone of the substantia nigra of brains from schizophrenic patients and mentally healthy subjects were TP. Among these were large melanin-containing neurons and intermediate-sized neurons with cytoplasm containing occasional pigment or lipofuchsin granules. Most dendrites, which were of various diameters, were also immunopositive. The dorsal part of the compact zone contained TP dendrites of small cross section; these corresponded to the point accumulations immunopositive structures seen at the light

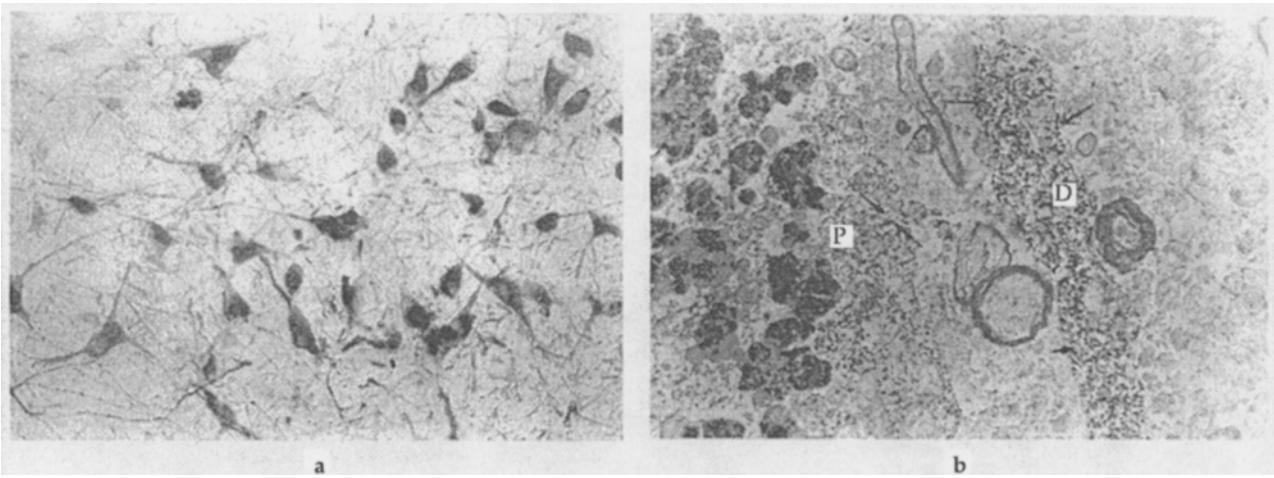

Fig. 1. TP neurons in the compact zone of the substantia nigra of the brain of a mentally healthy subject at the light (*a*) and electron (*b*) microscope levels. *a*) A group of dopaminergic neurons with processes. Magnification 16 × 25; *b*) perikaryon (P) and dendrite (D) containing microdisperse electron-dense DAB precipitate (arrows). Magnification ×12,000.

microscopy level. The diameters of these TP dendrites were no more than 1.5-2 times greater than the sizes of the axon terminals in contact with them. Larger proximal dendrites were frequently surrounded by axon terminals forming structures resembling sleeves around them.

In the reticular zone of the substantia nigra in the brains of schizophrenic patients and mentally healthy subjects, large and intermediate-sized dendrites were mostly immunopositive. These were in contact with numerous presynaptic boutons, often forming rosette-like structures on cross sections around dendrites.

Axon terminals making contacts with TP dendrites in the substantia nigra in controls were generally characterized by size variability and quite uniform ultrastructure (Fig. 2, *a*). The vast majority of these terminals had a multitude of synaptic vesicles, completely filling the terminals. The vesicles were pleomorphic in shape, though some were indistinguishable because of their high density. Additionally, large vesicles with dense centers could often be seen (isolated or as groups of vesicles delimited by an additional membrane), with 1-3 mitochondria at the terminal periphery. In general, the number of mitochondria depended on the size of the terminal. Synaptic contacts formed on small and intermediate-sized cross-sections of TP dendrites in the compact zones of the substantia nigra were in most cases of the asymmetrical type, while terminals forming sleeves around larger dendrites had mostly symmetrical contacts. Some of the axon terminals in the brains of patients with schizophrenia and mentally healthy subjects showed various extents of swelling. In terminals with these size increases, the numerous densely packed vesicles and mitochondria were surrounded by electron-transparent edematous zones.

The compact zone of the substantia nigra from the brains of patients with schizophrenia showed a series of changes in the ultrastructure of axon terminals making contact with TP dendrites. Altered terminals mostly contacted small TP dendrites. Both normal terminals, with the ultrastructure described above, and several terminals with a variety of ultrastructural changes (in different directions), could form contacts with any particular dendrite (Fig. 2, *b*). There was a reduction in the size of terminals having a dark matrix and densely packed vesicles, whose membranes were poorly distinguished on the background of the dense matrix; round terminals with an electron-transparent matrix were swollen, and the internal organelles consisted only of a few vesicles, which were aggregated and lay outside the synaptic contact zone. In the former case, the severity of changes varied; terminals with extreme changes showed signs of dark degeneration. Additionally, there were quite large terminals with occasional vesicles and hyperplasia of mitochondria, which occupied nearly all the terminal. Another characteristic feature of presynaptic terminals in contact with TP dendrites in the compact zone of the substantia nigra of the brains of schizophrenic patients was the presence of membranous lamellar structures within or adjacent to the presynaptic bouton. These structures were horseshoe-shaped formations consisting of several layers of membranes, generally in contact with the presynaptic bouton membrane. Regardless of the type of changes in the presynaptic bouton ultrastruc-

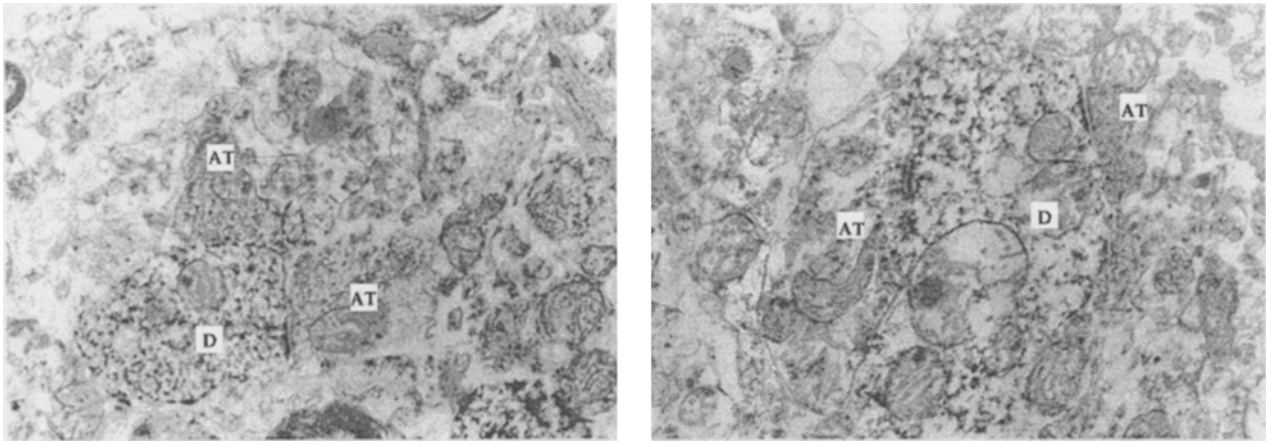

Fig. 2. Axodendritic synapse on dendrites of TP neurons in the compact zone of the substantia nigra in normal (*a*) and schizophrenic (*b*) patients. AT = axon terminal; D = dendrite with electron-dense DAB precipitate. Magnification: *a*) ×25,000; *b*) ×35,000.

ture, altered synapses were characterized by the asymmetrical type of contact with very short active contact zones.

Pathological changes to presynaptic terminals were also found in the reticular zone of the substantia nigra of the brains of schizophrenic patients; these were similar to those described for the compact zone, including the appearance of membranous lamellar structures, though these were relatively rare in this part of the substantia nigra and were found only in immunonegative dendrites. The main type of change seen in the reticular zone were reductions in the sizes of terminals with a dense matrix and filled with numerous synaptic vesicles.

The use here of a two-stage immunoperoxidase method resulted in the detection of numerous TP neurons in the substantia nigra of the human brain, along with a dense network of TP dendrites. The structure and distribution of TP neurons in the substantia nigra of brains from schizophrenic and mentally healthy patients were virtually identical at the light microscopy level, and were comparable with data on the substantia nigra in animals obtained using antibodies to tyrosine hydroxylase [9, 17].

The virtually complete absence of staining due to DAB precipitation in control sections incubated in the absence of anti-tyrosine hydroxylase antibodies during the first incubation supports the specificity with which tyrosine hydroxylase-reactive structures were detected in the present investigations. Another important methodological aspect of immunocytochemical studies of autopsy material is the possibility of autolytic ultrastructural changes. Material was selected for the current study such that the period between death and initial fixation was no more than 6 h; published data show that during this time the ultrastructure of brain tissue is quite well preserved [1]. Nonetheless, the peripheral chromatolysis which was seen in some small neurons, along with the swelling of a proportion of presynaptic boutons, was probably due to chromatolysis. These changes were encountered with the same frequency in brain tissues from patients with schizophrenia and from controls. However, in the cases selected for analysis, these changes were not widespread and the structure of most synaptic contacts remained intact. The ultrastructure of most presynaptic boutons forming contacts with dopaminergic dendrites in the substantia nigra of brains from mentally healthy subjects was very similar to that of unchanged terminals in the substantia nigra from the brains of schizophrenic patients, and corresponded to the ultrastructure described for presynaptic axon terminals contacting TP dendrites in the substantia nigra of animal brains [8, 17, 22]. Existing data show that most of these terminals (about 70%) in the rat brain substantia nigra are GABAergic; these and the ultrastructurally similar substance P-ergic terminals come from neurons in the striatum [8]. However, most investigators believe that virtually all animal brain substantia nigra synapses are contacts of the symmetrical type, while in the present study, a significant proportion of synaptic contacts formed on small dopaminergic dendrites in the compact zone of the substantia nigra of the human brain were asymmetrical.

The use of an immunocytochemical method resulted in the demonstration that axon terminals making contact with dopaminergic neurons in the

substantia nigra of brains of schizophrenia patients were characterized by a series of structural changes not encountered in the substantia nigra of brains from mentally healthy subject: small terminals with dense matrix and numerous vesicles; swollen terminals with small quantities of aggregated vesicles; terminals with mitochondrial hyperplasia; and presynaptic boutons containing lamellar membranous structures. Pathologically changed presynaptic terminals, most containing lamellar membranous bodies, were predominantly located in the distal branches of immunopositive dendrites (small and intermediate-sized) in the compact zone of the substantia nigra. Changes in the ultrastructure of synaptic contacts formed on dopaminergic dendrites of the reticular zone of the substantia nigra similar to those described above were found relatively rarely in the brains of patients with schizophrenia. The predominant type of change for this zone of the substantia nigra, small terminals with dark matrix, sometimes also occurred on immunonegative cells.

The simultaneous detection of terminals with preserved ultrastructure and with various differently directed changes making contacts with the same TP dendrite might be explained on the basis that dendrites of dopaminergic neurons in the substantia nigra often make contact with several presynaptic boutons of different neurotransmitter specificity [8]. These features, along with the predominance of the asymmetric type of contacts formed on the distal branches of dendrites in the compact zone of the substantia nigra, are evidently due to the characteristics of the afferent sources feeding the compact and reticular zones of the substantia nigra. However, as mentioned above, animal studies have demonstrated that most of the synaptic contacts on neurons of the substantia nigra are formed by terminals of GABAergic and substance P-ergic neurons of the striatum; several authors believe that innervation of dopaminergic neurons in the compact zone of the substantia nigra is characterized by a lower density on striatal input and a greater variety of sources [8]. Symmetrical contacts in the compact zone of the substantia nigra have been shown to form terminals belonging to neurons in the midbrain tegmental nuclei [16, 21], to serotoninergic neurons in the cervical nuclei [18] and to cortical neurons [19]. This latter point, considering existing data on atrophy of the gray matter and "loss" of neurons in the frontal parts of the cortex in schizophrenia [4, 5, 10, 23], is of particular interest from the point of view of putative lesions in the cortical regulation of subcortical dopaminergic formations [9, 12, 13]. In addition, the predominant location of axon terminals with lamellar membranous structures in the compact zone of the substantia nigra detected here and the high frequency of these structures in the neuropil of the caudate nucleus, previously demonstrated by Uranova [3], may represent evidence of the common (cortical) origin of these types of changes to terminals.

Thus, these studies have demonstrated that the ultrastructural changes to presynaptic terminals mostly affect synapses formed on the distal dendrites of dopaminergic neurons in the compact zone of the substantia nigra of patients with schizophrenia. These lesions, including polymorphic changes to presynaptic axon terminals and the presence of short active zones in the synaptic contacts, support the existence of pathology affecting synaptic transmission in the dopaminergic system of the schizophrenic brain.

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