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Changes of Dopaminergic Terminals in the Neostriatum Induced by 6-Hydroxydopamine : Immunohistochemistry for Tyrosine Hydroxylase and Electronmicroscopy

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Following a unilateral injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle, changes of the dopaminergic (DA) varicose terminals in the neostriatum were examined by immunohistochemistry for tyrosine hydroxylase (TH) and electronmicroscopy. Using a computer image analyzer, it was clarified that the number of TH immunopositive varicosities averaged 306 in a field of the anterior neostriatum measuring 3500μm^2 on the control side, while the number fell to only 8 on the ipsilateral affected side 3 days after the injection. The reduction of TH immunoreactivity was more pronounced in the anterior part than in the dorsolateral part, suggesting topographical organization of the mesostriatal dopaminergic projections.

Electronmicroscopy showed that TH immunopositive elements in the neostriatum on the control side were composed of small unmyelinated axons (0.1-0.3μm in diameter) and varicose terminals (0.4-1.5μm). Dense reaction products were usually found to precipitate on the axoplasm and microtubules. Some small synaptic vesicles and a few large ones were often labeled with reaction products but none of the mitochondria were labeled. These TH positive terminals seldom displayed synaptic contacts with membrane specialization, although they were closely juxtaposed to non-TH neuronal elements, often forming a "triad" relationship to an axo-spinous synapse.

In the ipsilateral neostriatum, a few degenerating features were first noted on day 1 after injection. After 3 days, the number of degenerating terminals increased, appearing as dense shrunken granular bodies of 0.1-1.2μm in diameter. The content of these granular bodies was just identifiable as an accumulation of a few synaptic vesicles, one or two mitochondria, some dense bodies and membranous materials in the dark axoplasm. The frequency of degenerating terminals was estimated to be 11 in a field of 175μm^2 in the anteromedial neostriatum. Some degenerating terminals still maintained obvious synaptic contacts with dendritic shafts and spines for as long as 7 days after injection, while most of them had lost the postsynaptic element

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and were surrounded by astrocytic processes.

Key words: TH immunohistochemistry, electronmicroscopy, 6-OHDA, neostriatum, dopaminergic terminals, terminal degeneration.

Introduction

It has been demonstrated by various techniques that the mesostriatal pathway is made up of mainly dopaminergic projections derived from the substantia nigra (SN) and the ventral tegmental area (VTA) (Andén et al., '64 ; Björklund & Lindvall, '84 ; Hökfelt et al., '76 ; Nauta et al., '74 ; Ungerstedt '71). The axons from the SN ascend in the H2 field of Forel immediately dorsolateral to the medial forebrain bundle (MFB), in which the DA axons from the VTA also ascend to project to anteromedial and ventral parts of the neostriatum as well as cortical regions (Björklund and Lindvall, '84 ; Oades and Halliday, '87). Therefore, the mesostriatal and mesocortical DA pathways are closely associated. It is well known that the MFB is composed of non-aminergic and aminergic fibers including not only catecholamine but also serotonin (5-HT) fibers connecting the brainstem with the dien- and telencephalic regions (Björklund & Lindvall, '84 ; Moore & Card, '84 ; Steinbusch, '84). Passing through the MFB, DA and 5-HT fibers innervate the neostriatum forming a considerable number of varicosities (Beal and Martin, '85 ; Soghomonian et al., '89). However, an injection of 6-OHDA into the MFB may cause degeneration of mostly the DA terminals rather than 5-HT ones in the neostriatum, because this chemical is useful to induce selective damage to the catecholamine neurons and fibers in the brain (Ungerstedt, '68).

The enzyme TH is well known to be specific for catecholamine synthesis. A demonstration of TH immunoreactivity may indicate the presence of DA as a transmitter and precursors for noradrenaline and adrenaline. However, DA terminals may be distinguished from other catecholamine terminals because of the lack of dopamine-β-hydroxylase (DBH) immunoreactivity. At least it seems to be clear that TH immunopositive varicosities in the neostriatum correspond to DA ones. Since the anti-TH antibodies used in this experiment are very effective for the detection of catecholamine neurons and fibers, we tried to examine the ultrastructure of TH terminals on the control side. Their degeneration features were simultaneously examined on the affected side in order to confirm the reduction in TH immunoreactivity. Although morphological changes of terminals in the neostriatum after electrolytic lesion in the SN have already been reported (Imamoto and Shimizu, '77), it seems necessary to confirm those induced by 6-OHDA and restricted to the DA terminals in the neostriatum. The results were compared with the previous ones (Arluison et al., '84 ; Bouyer et al., '84 a, b ; Pickel et al., '81).

Meterials and methods

Male Sprague-Dawley rats weighing 200-250 g were used in this experiment. Under anesthesia, rats fixed on a stereotaxic apparatus received unilaterally 2$\mu\ell$ of 6-OHDA into the MFB (4mg/mℓ in saline, added 0.02% ascorbic acid). Coordinates were 2.2mm posterior and 1.5mm lateral to the Bregma based on the atlas by Paxinos and Watson ('82). Haminton's syringe was inserted to a depth of 8.5 mm from the surface of the skull and the injection time lasted for 15 minutes. At various survival times ranging from 1 to 7 days after injection the rats were sacrificed. While respiration was maintained artificially, the

chest was opened and 0.1mℓ of heparin and 1 mℓ of 1% sodium nitrate were infused into the blood stream through the heart. Perfusion fixation was performed with 4% paraformaldehyde containing various concentrations of glutaraldehyde (0.1-1 %) in 0.12M PB at pH 7.3 for 15 minutes. Thereafter the whole body was kept in a refrigerator for one hour. Then, the excised brains were put in a fresh fixative of 4% paraformaldehyde for 3 hours and sliced into several pieces using a vibratome. For electron microscopy(EM), 50 μm-vibratome sections were used and for light microscopy (LM), 10 μm-cryostat sections were mounted on gelatin coated glass slides.

In order to increase the penetration of antibodies, 0.2% Triton-X 100 was used for LM and either 0.001% pronase or 0.02% saponine for EM. Immunostaining was carried out by the complete streptavidin-biotin method in a humid box at room temperature. The sequential incubation of tissue sections was as follows : 1) anti-TH antiserum in PBS (1 : 4000) overnight ; 2) donkey anti-rabbit biotinylated Ig (1 : 200) for 2 hours ; 3) the streptavidin peroxidase complex (1 : 400) for 2 hours. Reaction products were visualized by 0.05% of 3, 3'-diaminobenzidine in 0.12M PB, to which 0.025% cobalt chloride, 0.02% nickel ammonium sulfate and 0.005% hydrogen peroxide were added. Immuno-stained and non-stained vibratome sections were postfixed with 2% osmium tetroxide for one hour and dehydrated in graded alcohol and then were flat-embedded in Epon. Areas containing TH positive varicosities were trimmed out from the Epon embedded slices. Ultrathin sections were lightly stained with lead citrate and examined under an H-500 electron microscope.

Calculation of the number of TH positive varicosities in the neostriatum was directly performed under a light microscope using a computer image analyser "Nexus 6400" (Nexus inc., Tokyo). The total number of immunopositive varicosities and the total area occupied by TH positive varicosities in a given area of 3500 μm^2 were calculated at three spots in the anterior part of the neostriatum in each rat based on the difference in depth of colour of the reaction products and finally averaged.

Results

Quantitative analysis of TH immunopositive varicosities in the neostriatum

The neostriatum generally exhibited a strong TH immunoreactivity (Fig. 1, 2). TH immunopositive structures in the neostriatum appeared as numerous varicose fiber networks composed of black dots and fine intervaricose axons. Setting up the limits in the density of the reaction products during computer analysis, we calculated the total number of TH positive varicosities and the total area occupied by such varicosities in a field of 3500μm^2. The quantitative analysis was done in the three different fields of the subcallosal areas from the medial to lateral parts of the anterior neostriatum in a coronal section at the level of the genu of corpus callosum. The results were averaged in each case and are summarized in Table I.

The number of TH immunopositive varicosities in the anterior neostriatum on the control side averaged 306 in a field of 3500μm^2 and 5.5% of the field on an average was occupied by these TH immunopositive elements. However, it seemed there was a slight gradient distribution of TH positive terminals decreasing from anterior to caudal and from dorsolateral to medial. Almost all the TH immunopositive varicosities in the neostriatum were preferentially regarded as dopaminergic terminals, because there are few DBH positive terminals in the rostral part of neostriatum except for in the ventromedial part.

The effect of 6-OHDA caused an extensive reduction of TH immunoreactivity, although a few positive ones often remained scattering in the dorsolateral and the most medial parts of the

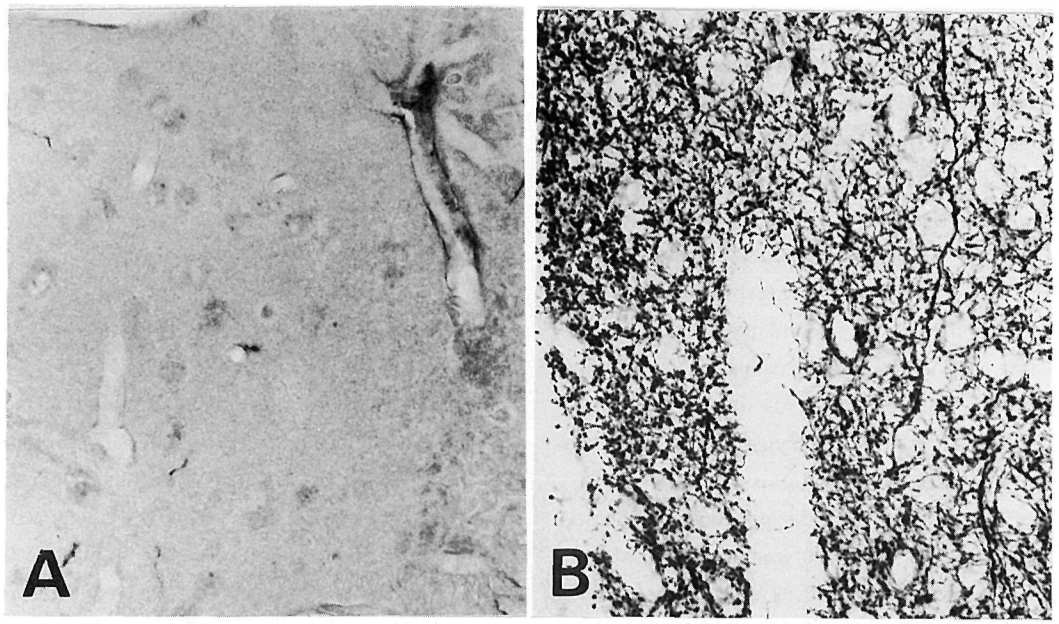

Fig. 1. TH immunopositive fibers in the anterior neostriatum 5 days after 6-OHDA injection into the MFB. TH positive varicosities and intersegmental axons densely distributed in the neostriatum on the conorol side (B), while an almost complete reduction of TH immunoreactivity is conspicuous on the affected side (A). ×450

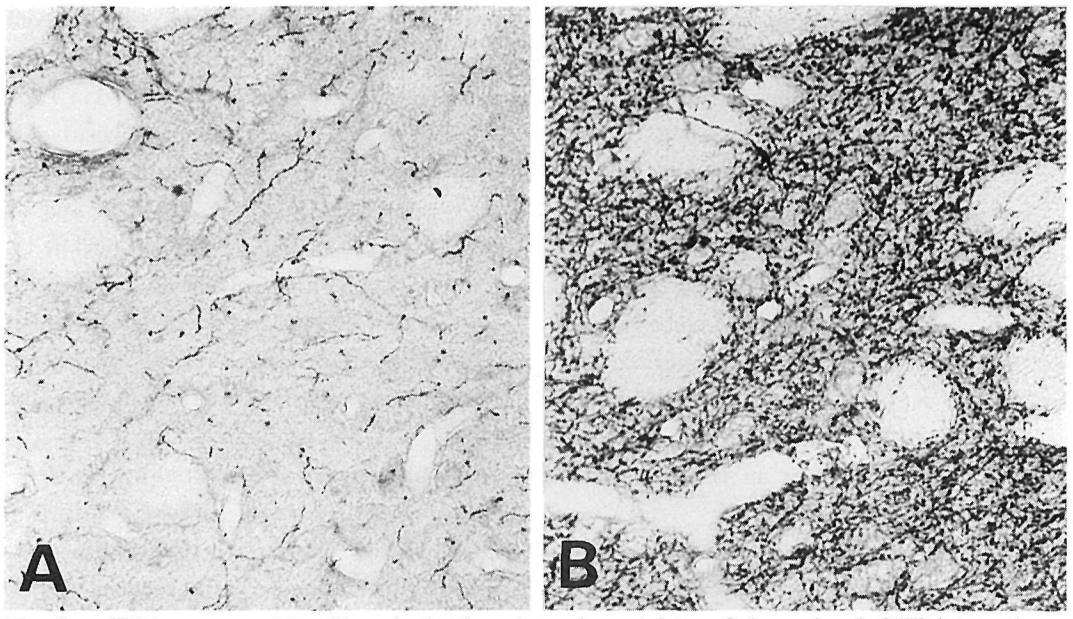

Fig. 2. TH immunopositive fibers in the dorsolateral neostriatum 3 days after 6-OHDA injection into the MFB. A small number of TH positive varicosities remain in the dorsolateral neostriatum on the affected side (A) in comparison with the control side (B). ×400

Table 1. The number of TH immunopositive varicosities in a field of 3500μm^2 in the anterior neostriatum, counted by a computer image analysis system using Nexus 6400. The total area occupied by TH immunopositive varicosities were indicated as a percentage to the field of 3500μm^2 in a parenthesis.

survival day(s)	rats	control side		injection side	
1	2	317	(4.9%)	140	(2.4%) *
2	3	298	(5.1%)	81	(1.3%) *
3	5	329	(5.8%)	8	(0.3%)
4	4	293	(5.5%)	11	(0.3%)
5	2	308	(5.7%)	6	(0.2%)
7	2	291	(5.7%)	5	(0.2%)
	average	306	(5.5%)	8	(0.3%) except *

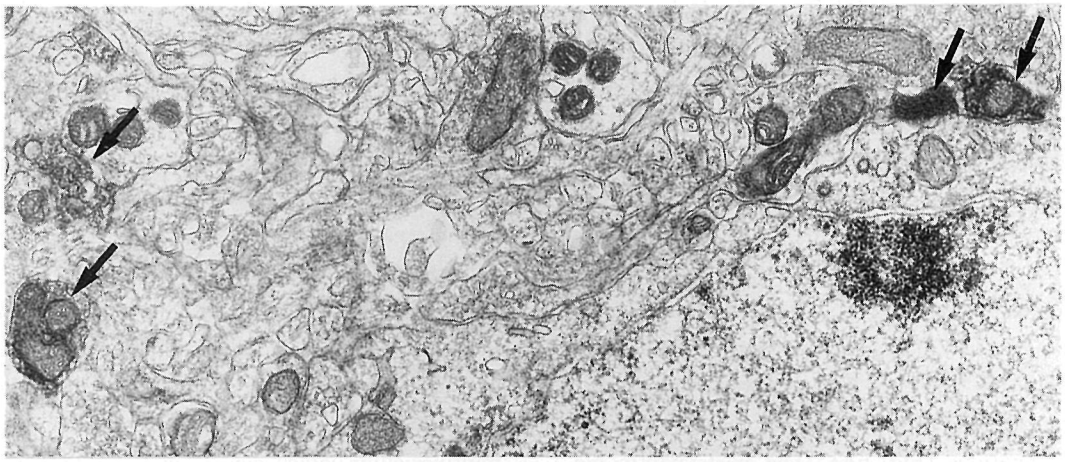

Fig. 3. Four TH positive terminals (arrows) in the dorsolateral neostriatum on the control side. Two are located close to a soma but synaptic specialization is obscure. ×24,000

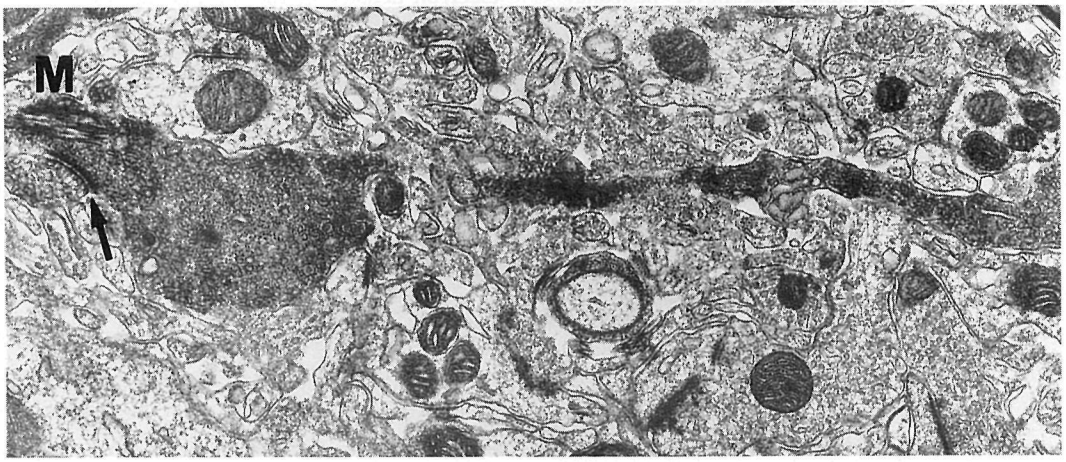

Fig. 4. TH positive varicose fibers observed in the neostriatum on the control side. An axo-axonic synapse showing distinct synaptic membrane thickening on the TH positive side (arrow). TH reaction products deposited on the axoplasm and microtubules (M) and some of synaptic vesicles. ×27,000

neostriatum (Fig.2A). On day 1 after injection, the number of TH positive varicosities fell to half the number of those in the control. On day 3 after injection, however, the number decreased markedly to 8 in a field of 3500μm^2 and only 0.3% of the field was occupied by TH immunopositive varicosities.

Ultrastructure of TH immunopositive terminals

TH immunopositive structures were found to be composed of small unmyelinated intervaricose segments (0.1-0.3μm in diameter) and vesicle-filled varicosities (0.4-1.5μm : Fig. 4). In general, dense reaction products precipitated on the axoplasm and microtubules. Some of the small synaptic vesicles (40-60nm) and a few large ones (80-120nm) were frequently labeled. The mitochondria never displayed a deposit of reaction products except for on the outer limiting membrane (Fig. 3-6). Around such TH positive boutons, synaptic contacts showing a conspicuous postsynaptic thickening or density were rarely found. The incidence of asymmetric synapses of Gray type II was only 5-8% in single section examinations (Fig. 6A). The postsynaptic elements of Gray type I synapses were composed of dendritic shafts and spines but never neuronal somas. Most of the TH positive terminals came in contact with non-TH neuronal elements, but it was very difficult to determine if they formed synaptic junctions of either asymmetric or symmetric types or they had no junctional complex (Fig. 3, 5, 6) in a single section examination. As shown in Fig. 4, the existence of axo-axonic synapses was confirmed between TH positive and negative terminals. These synapses displayed a distinct membrane thickening on the TH positive side. Furthermore, it was noted that some TH positive terminals formed a "triad" relationship with a non-TH axo-spinous synapse (Fig. 5A). There was no membrane specialization around these TH positive terminals, but such a close relationship might act for the modulation or control of the synaptic transmission.

Degenerating dopaminergic terminals after 6-OHDA injection

By electronmicroscopy, terminal degeneration was pursued in the neostriatum on the ispilateral side. Degenerating terminals appeared as dense shrunken granular bodies similar to those observed after electrolytic lesion made in the SN. A marked glycogen accumulation in the swollen astrocytic processes around the degenerating terminals and perivascular areas was not so prominent as reported in the case of electrolytic lesion (Imamoto and Shimizu, '77). These degenerating granular bodies were rarely found at all in the neostriatum on the control side.

During the sequential progression of terminal degeneration, an increase in the electron density of the axoplasmic matrix was noted as the first sign. Such an alteration probably occurred at random during the first few days after 6-OHDA injection, because we noted similar dense terminals even on the 7th day. Another type of terminal degeneration displayed an accumulation of multivesicular bodies, dense bodies and mitochondria in the terminals rather than an increase in the electron density of the axoplasmic matrix (Fig. 7). With time, the degenerating terminals turned into dense shrunken granular bodies of 0.1-1.2 μm in diameter (Fig. 8, 9). In most cases they were surrounded by astrocytic processes, in which there were only a few glycogen particles. Some degenerating terminals still maintained synaptic contacts with the postsynaptic elements, that is to say, with dendritic spines (Fig. 8A), which were probably derived from medium-sized neurons located in the neostriatum (Kemp, '68). However, in no instances did we note that the degenerating terminals maintained a synaptic contact with a neuronal soma. Around the surface of neuronal somas, they were always interrupted by astrocytic endfeet (Fig. 8B). The number of the degenerating terminals was greatest 3-4 days

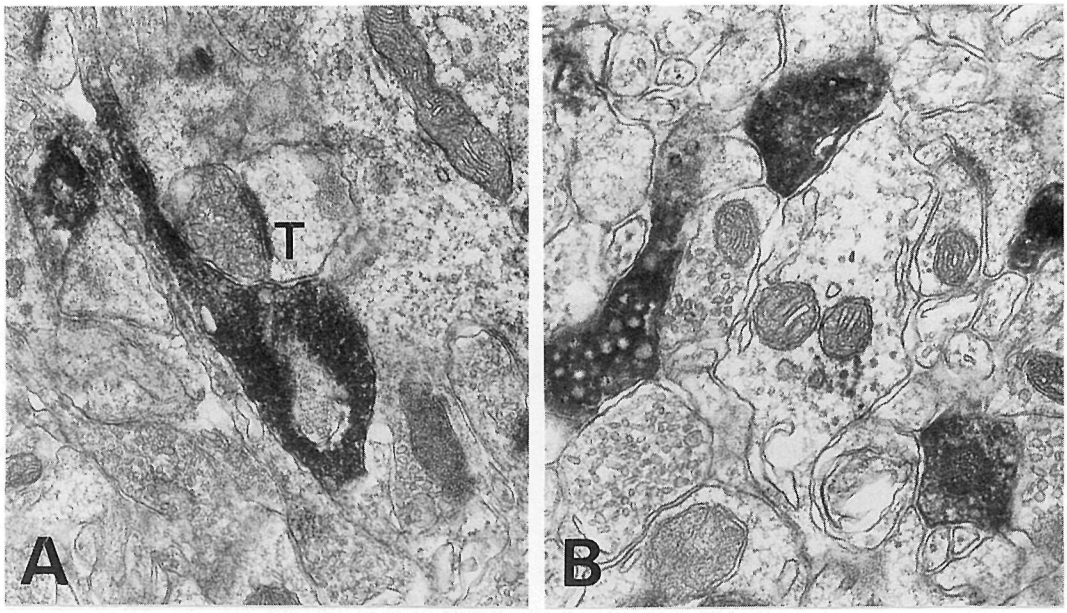

Fig. 5. TH positive varicose fibers in the neostriatum on the control side. A "triad" relationship (T) is seen between a TH positive terminal and a non-TH axo-spinous synapse (A). Mitochondria are always free of the reaction products. In most cases it is hard to confirm synaptic specialization around TH positive terminals (B). A : ×27,000, B : ×38,000

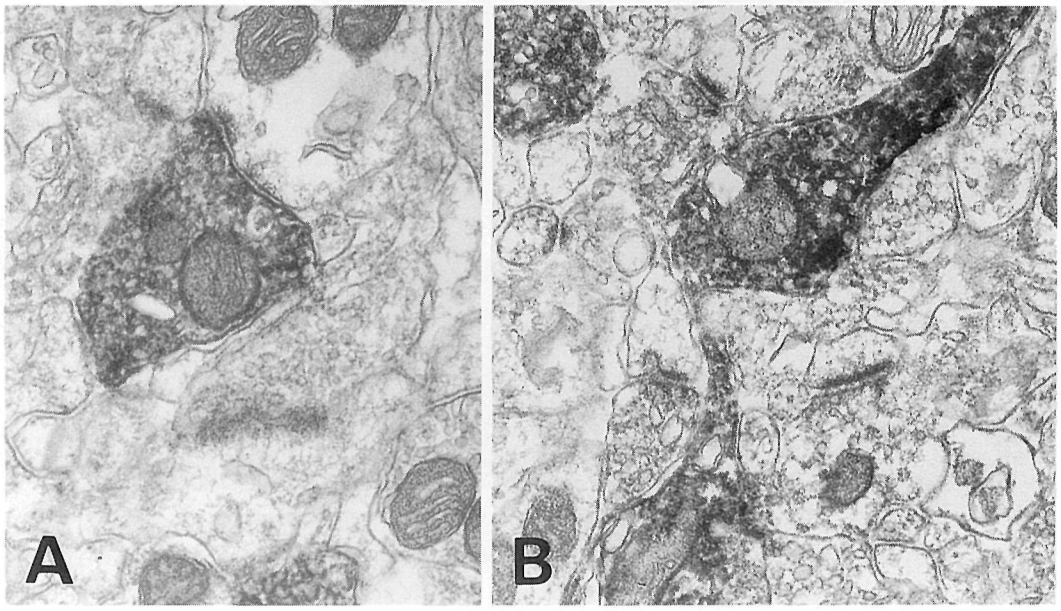

Fig. 6. TH immunopositive terminals observed in the neostriatum on the control side. An axo-dendritic synapse of Gray type I showing a prominent membrane specialization is seen in A, while TH positive terminals in B are merely adjacent to the other neuronal elements without synaptic specialization. A and B : ×38,000

after injection. In such cases about 11 dense shrunken bodies appeared as degenerating terminals in a field of 175μm^2 by electronmicroscopy, which suggests a frequency of 220 per 3500μm^2 of the affected neostriatum. This figure seems to be rather low in comparison with the 306 TH immunopositive varicosities estimated by computer analysis. However, the difference may well be accounted for by the thickness of the sections examined by LM and EM.

Discussion

Concerning the mesostriatal DA projections, the topographic relationships between the origin of DA neurons in the mesencephalon (VTA and SN) and their projection areas in the neostriatum have been a subject of study for a long time. Up to date the topographical principles, that is, the medial to medial and lateral to lateral projections and the rostral to anterior and caudal to posterior projections, are well established (Björklund & Lindvall, '84 ; Fallon & Moore, '78). Therefore, our results obtained after 6-OHDA injection into the MFB may be explained by the topographic relationships of the mesostriatal pathways without any contradiction. The needle track inserted into the brain at the coordinates might be sufficient to reach the MFB, although some cases the infused 6-OHDA solution seemed to spread horizontally along the H2 field of Forel at a level slightly higher than the needle tip. The almost complete reduction of TH immunoreactivity in the anterior neostriatum may suggest that catecholamine fibers derived from the MFB took up

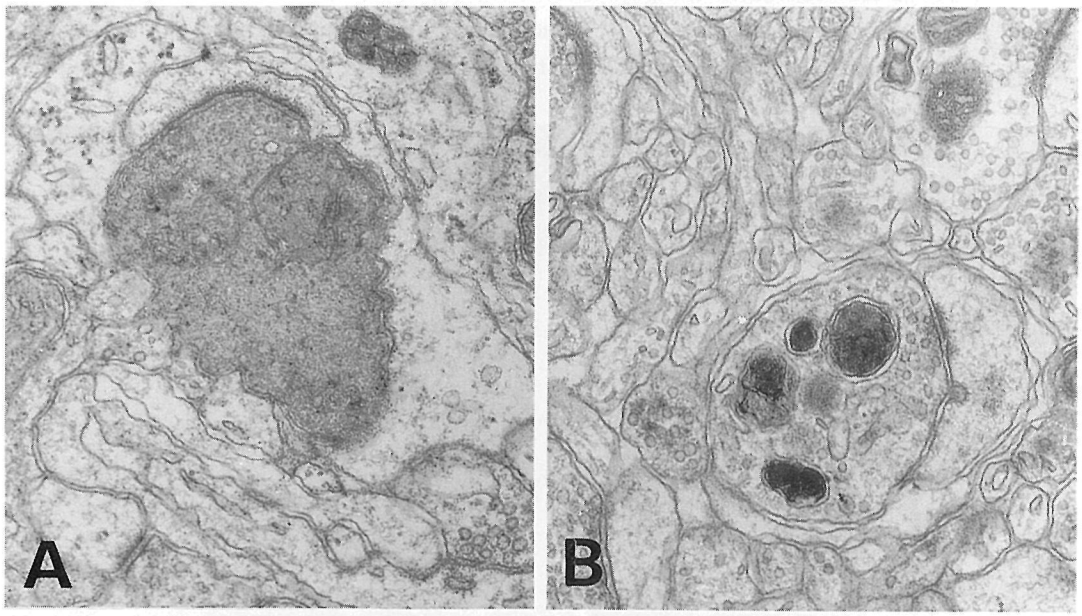

Fig. 7. Two types of degenerating terminals observed in the neostriatum on the affected side 3 days after 6-OHDA injection. One is a dense degenerating terminal showing a synaptic contact with a dendritic spine (A). Synaptic vesicles and mitochondria are barely identifiable. The other is a terminal accumulating dense bodies and vacuoles among synaptic vesicles exhibiting a prominent synaptic junction with a dendritic spine (B). Both terminals are regarded as early features of DA terminals. A and B : × 40,000

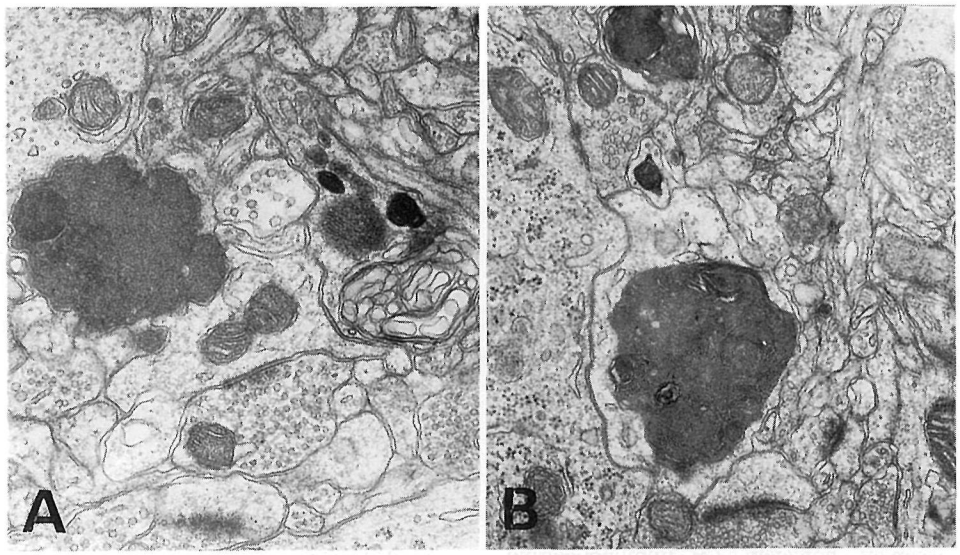

Fig. 8. Degenerating terminals observed in the neostriatum on the affected side 7 days (A) and 3 days (B) after 6-OHDA injection into the MFB. Two types of degenerating terminals are seen in A; one is a dense shrunken granular body and the other is a terminal accumulating dense bodies and vesicular materials. In B, there are a few degenerating elements, one of which is surrounded by astrocytic endfeet next to a neuronal perikaryon. A and B : × 29,000

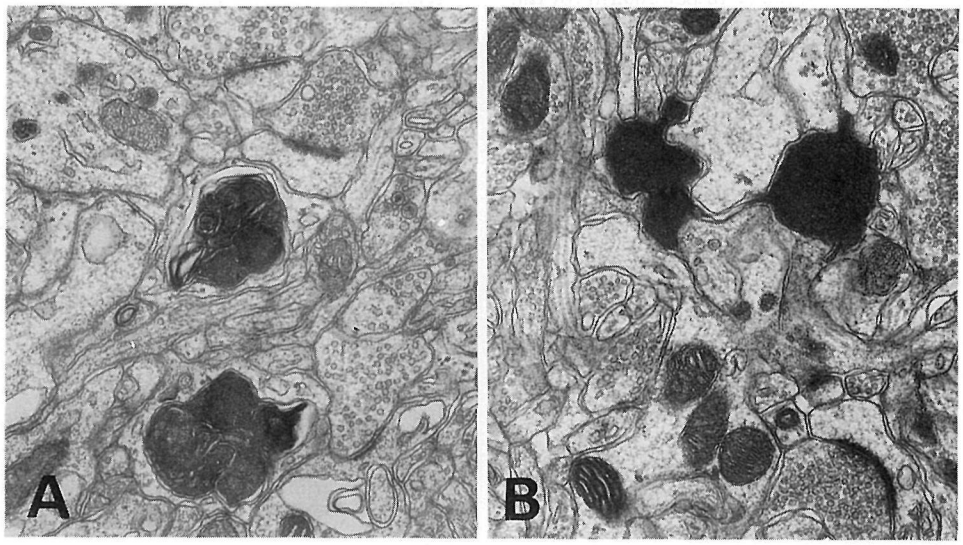

Fig. 9. Degenerating dense irregular terminals in the neostriatum 2 days (A) and 3 days (B) after 6-OHDA injection into the MFB. On day 2 after injection, terminal degene-ration is clearly observable throughout the neuropil of the neostriatum. In most cases, mitochondria and synaptic vesicles are barely identifiable in the electron dense axoplasm. A and B : × 30,000

enough 6-OHDA to cause terminal degeneration. An incomplete reduction of TH immunoreactivity in dorsolateral part suggest perhaps that DA fibers projecting to the dorsolateral part are derived from the lateral part of the SN. Normally, they ascend in the field H2 of Forel and bend sharply laterally at the level of the subthalamic nucleus caudal to the injection site and then ascend into the internal capsule towards the neostriatum (Björklund & Lindvall, '84). Thus, they might remain unaffected by 6-OHDA.

Recently, Tashiro et al. ('89) reported that there were some TH immunopositive neurons in the striatum of control rats and the number of TH immunopositive neurons increased in the ipsilateral neostriatum after 6-OHDA lesions in the SN. In our experiment, however, TH immunopositive neurons were only accidentally observed both on the control and affected sides. We never found an increase in number of TH immunopositive neurons in the anterior neostriatum after 6-OHDA injection.

The quantification of DA innervation has been reported to be about 5-15 per 100μm^2 of the neostriatum by Doucet et al. ('86). The number of DA varicosities estimated by a computer image analysis system in the present experiment averaged 306 per 3500μm^2. This result suggests a frequency of 9 per 100 μm^2, a figure close to the previous result.

Concerning the synaptology of DA terminals, several investigators have described in the striatum by TH immunoelectronmicroscopy (Arluison et al., '84 ; Bouyer et al., '84a ; Freund et al., '84 ; Pickel et al., '81) and those in the cerebral cortex (Séguéla et al., '88 ; Van Eden et al., '87) and in the septum by DA immunoelectronmicroscopy (Onteniente et al., '84). In particular, the synaptic profiles of DA terminals were mentioned with reference to synaptic membrane specialization, that is, pre- and postsynaptic membrane thickenings, width of synaptic cleft and postsynaptic neuronal elements, because there was no characteristic about synaptic vesicles in the DA terminals. In the present experiment we reported that only 5-8% of the total TH positive terminals formed asymmetric synapses of Gray type I while the rest indicated no synaptic specialization, although they came in contact with the dendrites, spines, axon terminals and even neuronal somas. They might belong to symmetric synapses of Gray type II. Because of the poor membrane preservation after TH immunostaining, it may be less meaningful to discuss various synaptic types of TH positive terminals. Even though there is some disagreement concerning the features of DA terminals and synaptic types in the previous reports, it is fairly reliable that a small number of TH positive terminals form asymmetric synapses of Gray type I.

According to Bouyer et al. ('84b), an axo-axonic synapse in the neostriatum provides support for a possible direct interrelationship between inputs from the cerebral cortex and the SN. They have suggested that the motor cortex may possibly stimulate DA release in the neostriatum by axo-axonic synapses. Arluison et al. ('84) mentioned that TH positive terminals making putative axo-axonic synapses were in apposition but usually failed to exhibit pre- or postsynaptic specialization. We noted a few axo-axonic synapses between TH positive and TH negative terminals. The membrane thickening was clearly observable on the TH positive side. This might suggest that the TH positive terminal receives the stimulation of a non-TH one probably projecting from the cerebral cortex. We were also interested in the "triad" relationship between TH positive terminals and non-TH axo-spinous synapses, although there was no membrane specialization around the TH positive terminal. These TH positive terminals might be able to modulate the transmission between the non-TH axon terminal and the dendritic spine. As suggested by Divac ('72) and Groves ('83), it seems that the function of a neostriatal region is determined

by many inputs from the cerebral cortex and the thalamus.

Electronmicroscopy elucidated that some of the degenerating terminals preserved a synaptic contact with a dendritic spine, although most of them lost the postsynaptic element and were surrounded by astrocytic endfeet. The structure of such a synapse was usually composed of Gray type I. Since 5-HT fibers also costitute an element of the MFB, and their terminals form exclusively asymmetric synapses in the neostriatum (Soghomonian et al., '89), we need to be careful in determining the degenerating features to be DA terminals. However, the destructive effect of 6-OHDA seems to be specific to catecholaminergic elements rather than to indolaminergic ones(Descarries et al., '75). Therefore, we consider that most of the degenerating terminals are derived from DA ones, although we can not totally exclude the possibility of them being 5-HT ones. The junctional structure around the degenerating DA terminal might merely remain without any synaptic function.

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6-OHDAによる新線条体ドーパミン線維終末の変化

—TH免疫組織化学的，電顕的研究—

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新線条体におけるドーパミン線維終末を，内側前脳束に6-OHDA注入後，免疫組織化学と電顕で観察した．

画像解析でTH陽性の瘤状終末の数を調べると，健常側では3500μm^2当り306個であるが，注入側では3日目に僅かに8個と低下した．TH陽性線維が占める面積の割合は，3500μm^2の5.6%であったものが0.3%まで低下した．低下は，背外側部よりも前方部に著明であったが，このことは，中脳線条体ドーパミン系の投射が，明確な局在性を構成することを示唆した．

健常側のTH陽性終末を電顕的に観察すると，径0.1～0.3μmの小型の無髄軸索と，0.4-1.5μmの瘤状終末からなり，反応産物は軸索原形質や神経細管上に沈澱していた．大小のシナプス小胞もしばしば陽性となり，ミトコンドリアは常に陰性であった．TH陽性の終末は，他の神経成分と隣接してはいても，膜の肥厚を持ってシナプス接着構造を示すことは希であったが，軸索-樹状突起棘シナプスと接し"三つ組"の関係を構成するのがしばしば見られた．

注入側の新線条体では，変性像は，1日目に小数出現し，3日目に175μm^2に11個の頻度まで増加した．径0.1-1.2μmの電子密度の高い萎縮した顆粒状の変性終末内には，シナプス小胞，ミトコンドリア，緻密な顆粒状または膜状の物質が集積しているのがかろうじて判別された．変性終末の一部は，7日目でも樹状突起や樹状突起棘とシナプス接着構造を保つものもあったが，多くは，シナプス後部から離れ星状膠細胞の突起で包まれていた．