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INPUT FROM THE FRONTAL CORTEX AND THE PARAFASCICULAR NUCLEUS TO CHOLINERGIC INTERNEURONS IN THE DORSAL STRIATUM OF THE RAT

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Abstract—Evidence derived from many experimental approaches indicates that cholinergic neurons in the dorsal striatum (caudate–putamen) are responsive to excitatory amino acids. Furthermore, evidence from physiological experiments indicate that the excitatory input is derived from the cortex and/or the thalamus. The object of the present experiment was to anatomically test whether cholinergic neurons receive cortical and/or thalamic input in the dorsal striatum using a combined anterograde tracing and immunocytochemical approach at both the light- and electron-microscopic levels.

Rats received injections of the anterograde tracers *Phaseolus vulgaris*-leucoagglutinin or biocytin at multiple sites in the frontal cortex or parafascicular nucleus of the thalamus. Sections of the striatum were stained to reveal the anterogradely transported markers and then immunostained to reveal choline acetyltransferase immunoreactivity. The striata of these animals contained dense networks of anterogradely labelled fibres that were dispersed throughout the neuropil and interspersed with the choline acetyltransferase-immunoreactive (i.e. cholinergic) perikarya and dendrites. The anterogradely labelled fibres were often closely apposed to the choline acetyltransferase-immunoreactive neurons.

Examination of electron-microscopic sections failed to demonstrate cortical terminals in synaptic contact with the cholinergic neurons even when choline acetyltransferase-immunoreactive structures were examined that had first been identified in the light microscope as having cortical terminals closely apposed to them. In these cases it was often observed that the cortical terminal, although apposed to the membrane of the labelled neurone, made synaptic contact with an unlabelled spine that was in the vicinity.

In contrast to the cortical input, analysis of material that was double-stained to reveal thalamostriatal terminals and choline acetyltransferase-immunoreactive structures, revealed that the thalamostriatal terminals were often in asymmetrical synaptic contact with the perikarya and dendrites of cholinergic neurons.

It is concluded that the cholinergic neurons of the dorsal striatum, like those of the ventral striatum or nucleus accumbens [Meredith and Wouterlood (1990) *J. comp. Neurol.* **296,** 204–221] receive very little or no input from the cortex but are under a prominent synaptic control by the thalamostriatal system. Those pharmacological effects of excitatory amino acids on the cholinergic systems of the striatum are therefore presumably related to the thalamostriatal and not the corticostriatal system.

Morphological studies of the striatum have revealed the presence of a population of large neurons that display immunoreactivity for choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine. These cells, which represent a class of interneurons, have been detected in the striatum of many species including the rat,[2,5,42] cat,[26] monkey[8,37,50] and human.[38] They comprise 1–2% of the neuronal population and possess a large oval or multipolar cell body (diameter of soma 30–40 μm) from which several, infrequently branching dendrites emerge and extend for up to 1 mm from the cell body.

The ChAT-immunoreactive neurons receive afferent input from terminals forming both symmetrical and asymmetrical synaptic contacts. Some of the symmetrical synapses are made by terminals immunoreactive for substance P[4] and GABA,[3] both of which are probably derived from local neurons. They also receive symmetrical synaptic input from terminals that are immunoreactive for tyrosine hydroxylase, the synthetic enzyme for dopamine[30] that are derived from the dopaminergic neurons of the substantia nigra. In addition to these inputs, cholinergic neurons in both the primate and the rat have been shown to receive input from at least two classes of terminals (based on vesicle morphology or type of specilization) that form asymmetrical synaptic contacts.[5,7,42] The origin of these asymmetrical synaptic contacts is unknown.

When examining the striatum as a whole, it is clear that at least some of the terminals making asymmetrical synapses orginate from sources extrinsic to the striatum,[55] two of the major ones being the cerebral

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Abbreviations: ABC, avidin–biotin–peroxidase complex; BDHC, benzidine dihydrochloride; ChAT, choline acetyltransferase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; PBS, phosphate-buffered saline; PHA-L, *Phaseolus vulgaris*-leucoagglutinin; Tris buffer, 0.05 M Tris–HCl buffer (pH 7.4).

cortex[9,11,16,21,22,56,60,65] and the centremedian–parafascicular complex of the thalamus.[6,9,47,66] The main synaptic targets of the corticostriatal terminals are the spines of medium-sized densely spiny neurons[9,11,22,23,56] but they also contact GABAergic interneurons[31] and neurons that display immunoreactivity for neuropeptide Y.[59] The main targets of the thalamic terminals, in contrast, are dendritic shafts, some of which have been characterized as sparsely spiny neurons.[9] Both of these areas, the cortex and the thalamus, therefore represent possible sites of origin of the asymmetrical terminals in contact with striatal cholinergic neurons.

Several other lines of evidence suggest that the corticostriatal and/or thalamostriatal terminals make synaptic contact with striatal cholinergic neurons: (i) both the corticostriatal and thalamostriatal pathways have been suggested to use the excitatory amino acid, glutamate, as a neurotransmitter[8,10,17,24,25,29,35,39,43,44,46,57,58,61] and excitatory amino acids stimulate the turnover and release of acetylcholine in the striatum probably via *N*-methyl-D-aspartate receptors;[1,32,51,52] (ii) destruction of the cortex[54,64] or the thalamus[40,48,49,53] affects the levels and turnover of acetylcholine in the striatum; (iii) following stimulation of the cortex and the thalamus, monosynaptic excitatory responses have been recorded in giant aspiny neurons in the striatum that have all the morphological features of cholinergic neurons;[62] and (iv) in the ventral striatum, cholinergic interneurons, identified by ChAT-immunoreactivity, have been shown to receive a direct asymmetrical synaptic input from thalamostriatal terminals[36] and, albeit to a lesser extent, from the hippocampus.

The object of the present experiments were therefore to determine, by morphological means, whether cholinergic interneurons in the dorsal striatum, receive a direct synaptic input from corticostriatal and/or thalamostriatal terminals. The experiments were carried out using a combination of tracing studies to label the corticostriatal and thalamostriatal pathways and immunocytochemistry to reveal the cholinergic neurons at both the light- and electron-microscopic levels.

EXPERIMENTAL PROCEDURES

Animals and preparation of the tissue

All experiments were carried out on female albino Wistar rats (150–200 g, Banton and Kingman). To examine the corticostriatal projection, bilateral, multiple (3–5) injections of the anterograde tracers, biocytin[19,27] or *Phaseolus vulgaris*-leucoagglutinin (PHA-L),[13] were made into the frontal cortices of 19 anaesthetized rats. The biocytin was administered as a 5% solution in 0.05 M Tris–HCl buffer (pH 7.4; Tris buffer) using a pressure system; approximately 200 nl were deposited at each site. The PHA-L was administered as a 2.5% solution in Tris buffer by iontophoresis (using a positive cathodal current of 6.2 μA, delivered in 7-s pulses every 14 s over a period of 10 min). To examine the thalamostriatal projection, pressure injections of approximately 300 nl of the biocytin solution were made bilaterally into the parafascicular nucleus of the thalamus of 14 anaesthetized rats.

Following a survival time of 18 h for biocytin and 10–14 days for PHA-L, the rats were deeply anaesthetized using chloral hydrate (3.5 mg/kg; i.p.) and then transcardically perfused, initially with Ca^{2+}-free Tyrode's solution (gassed with 95% O_2 and 5% CO_2) and then with fixative that consisted of either 3% paraformaldehyde and 0.5% glutaraldehyde for biocytin experiments, or 3% paraformaldehyde and 0.05% glutaraldehyde for the PHA-L experiments. In both cases 200 ml were administered over 10–12 min and were followed by 100 ml of 3% paraformaldehyde over a period of 5–7 min. All the fixatives were dissolved in 0.1 M phosphate buffer (pH 7.4).

Following perfusion, the brain was removed from the skull and sections including the tracer injection sites in the frontal cortex and the thalamus, as well as the transport sites in the striatum, were cut on a vibrating microtome at 70 μm. The sections were washed with phosphate-buffered saline (PBS; 0.01 M phosphate) and then treated with 1% sodium borohydride in PBS for 5–10 min. Some sections were freeze-thawed in isopentane cooled in liquid nitrogen after equilibration in a cryoprotectant consisting of 25% (w/v) sucrose and 10% (v/v) glycerol in 0.05 M phosphate buffer (pH 7.4). The sections were then processed to reveal the injected and transported biocytin or PHA-L using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen in the peroxidase reaction. The sections that included the striatum were then processed to reveal ChAT immunoreactivity, the marker enzyme for acetylcholine, using benzidine dihydrochloride (BDHC)[34] or DAB as the chromogen for the immunoperoxidase reaction.

Localization of the anterograde tracers

To reveal the injected and transported biocytin, sections were washed in PBS and then incubated overnight in an avidin–biotin–peroxidase complex (ABC; 1:100 in PBS; Vector Labs.), washed and then incubated in a solution containing DAB (25 mg/100 ml Tris buffer) and 0.006% hydrogen peroxide for 10–20 min. To reveal the injected and transported PHA-L, the sections were incubated overnight in a biotinylated antibody against PHA-L (1:200 in PBS; Vector Labs.), washed in PBS and then incubated for 2 h in ABC (1:100). The bound peroxidase was then revealed by incubation in the same DAB and hydrogen peroxide solution as above.

Immunocytochemistry for choline acetyltransferase

Following several washes in PBS, the sections of the striatum were incubated with a monoclonal antibody directed against ChAT[33] diluted at 1:200, for 24–48 h at 4°C. They were then incubated in a 1:50 dilution of rabbit anti-rat IgG (Dako) for 2 h at room temperature followed by a 1:100 dilution of rat peroxidase–antiperoxidase complex (Sternberger–Meyer) for 2 h at room temperature. The sections were then reacted to reveal the ChAT-immunoreactive sites by incubation in a solution of either BDHC (5 mg/50 ml 0.01 M phosphate buffer pH 6.8) and 0.0024% hydrogen peroxide or DAB and hydrogen peroxide as above.

Preparation for electron microscopy and analysis of material

Sections containing the injection sites were mounted on gelatine-coated slides and prepared for light microscopy. All other sections including controls were post-fixed in 1% osmium tetroxide (in phosphate buffer) for 20 min, dehydrated and embedded in resin on microscope slides. The sections were examined in the light microscope and areas of interest were re-embedded and ultrathin sections cut using a Reichart Ultracut E ultramicrotome. The sections were counterstained with lead citrate[45] and then examined in a

Philips 410 electron microscope. A total of 14 ChAT-immunoreactive neurons identified in the light microscope were examined in the electron microscope in serial sections in relation to the corticostriatal pathway and 20 ChAT-immunoreactive neurons were examined in relation to the thalamostriatal pathway. All synaptic inputs to these neurons were noted. In addition, ultrathin sections were scanned in the electron microscope to identify small-diameter ChAT-immunoreactive dendrites and corticostriatal or thalamostriatal terminals. The inputs to these small-diameter dendrites and the targets of the labelled terminals were noted.

The specificity of the antibody against ChAT[33] and the problems of cross-reactivity in the double-immunocytochemical method[34] have been discussed previously. In order to test the specificity of the reactions performed, some sections were treated to reveal only the ChAT-immunoreactive neurons or the tracer by the omission of either the primary antibody or ABC.

RESULTS

Injection sites

In the light microscope, the DAB-labelled neurons and fibres at the injection sites, i.e. those structures that had taken up the injected biocytin or PHA-L, were brown in colour and displayed an amorphous appearance. At the core of the injections most neurons were labelled but the proportion of labelled neurons decreased with distance from the core. The labelling at the injection site with either tracer was characterized by extensive filling of neurons giving them a Golgi-like appearance. The injections were localized either within the frontal cortex or in the dorsorostral region of the parafascicular nucleus of the thalamus with some involvement of the lateral nucleus and the lateral nucleus pars posterior.

Anterograde labelling of corticostriatal terminals

Light microscopy. Following injections of biocytin or PHA-L into the frontal cortex, an extensive terminal field of corticostriatal fibres was seen throughout the dorsal striatum, with the densest labelling of fibres occurring in the more medial regions. The labelling produced by both tracers was similar in appearance and distribution. At higher magnification the labelling was seen to be composed of a dense plexus of fibres and punctate structures distributed throughout the neuropil (Fig. 1A).

Electron microscopy. In the electron microsope the anterogradely labelled structures containing the DAB peroxidase reaction product seen in the light microscope were identified as vesicle-containing axons and boutons (Fig. 2). The labelling and the peroxidase reaction product formed with either of the tracers were indistinguishable, the two groups will therefore be dealt with together. The peroxidase reaction product appeared as an electron-dense, amorphous material that was associated with the surface of all cell organelles as well as the inner surface of the plasma membranes of the labelled structures. The peroxidase-labelled structures were readily distinguishable from non-immunoreactive structures (Fig. 2B).

The corticostriatal terminals were of variable size (diameter of terminal 0.2–0.7 μm) and contained densely packed, round vesicles. The terminals often contained a single mitochondrion. All corticostriatal terminals that were seen to form synaptic contacts had asymmetrical membrane specializations, mainly with unlabelled spines ($n = 68$; 87%) and occasionally with unlabelled dendrites ($n = 10$; 13%). On some occasions corticostriatal terminals were seen to make asymmetrical synaptic contact with two unlabelled spines.

Anterograde labelling of thalamostriatal terminals

Light microscopy. Following injections of biocytin into the parafascicular nucleus of the thalamus, an extensive terminal field of anterogradely labelled thalamostriatal fibres was seen throughout the striatum. At higher magnification, the DAB reaction product was seen to be present in fibres and punctate structures distributed throughout the neuropil (Fig. 1B, C). In addition to small fibres and punctate structures, labelling was also seen in fibres which coursed for long distances through the striatal neuropil. Some of these fibres were smooth in appearance whereas others were highly varicose. The varicose fibres gave rise to collaterals within the striatum.

Electron microscopy. In the electron microscope, the DAB-labelled thalamostriatal fibres and punctate structures were identified as vesicle-containing axons and boutons (Fig. 3). The peroxidase reaction product appeared as that described for corticostriatal fibres. The thalamostriatal boutons were of variable size (diameter 0.2–0.9 μm), possessed round or oval vesicles which filled the entire terminal and contained at least one mitochondrion. All thalamostriatal terminals that were seen to form synaptic contacts made asymmetrical membrane specializations with unlabelled dendrites ($n = 282$, 79%), unlabelled spines ($n = 71$, 20%) and occasionally perikarya ($n = 5$, 1%). The thalamostriatal terminals were sometimes associated with post-junctional dense bodies (Fig. 3B).

Choline acetyltransferase-immunoreactive neurons

Light microscopy. In the light microscope, neurons displaying ChAT immunoreactivity were brown in colour and displayed an amorphous appearance when DAB was used as the chromogen (Fig. 1A, B) and blue–black in colour and granular in appearance when BDHC was used as the chromogen in the immunoperoxidase reaction (Fig. 1C). The more extensive and reliable staining of ChAT-immunoreactive neurons occurred when DAB was

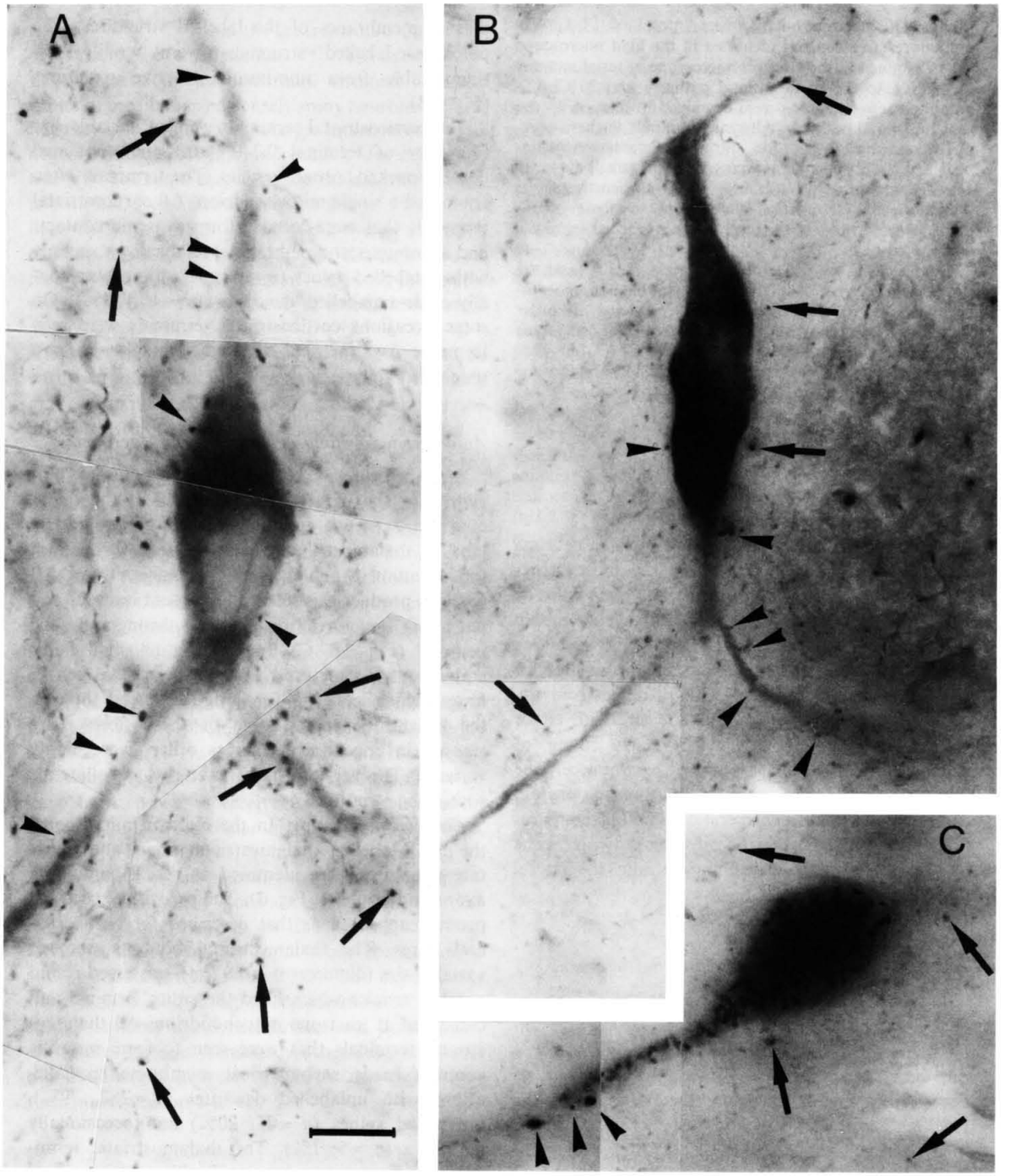

Fig. 1. Partial light-microscopic photomontages of ChAT-immunoreactive neurons in the striatum of rats that were injected with the anterograde tracer biocytin, in the frontal cortex (A) or parafascicular nucleus of the thalamus (B,C). In A the chromogen for the immunoperoxidase reaction that was used to reveal the ChAT was DAB; the immunoreactive neuron shows the typical features of cholinergic neurons in the striatum (see text). The same field contains many corticostriatal axons and terminals (some labelled by arrows) distributed throughout the neuropil. Several of these corticostriatal boutons are in close apposition to the ChAT-immunoreactive neuron (some labelled by arrowheads). In B the same procedure was used as in A, both the ChAT-immunoreactive neurons and the terminals anterogradely labelled from the parafascicular nucleus of the thalamus were localized using DAB as the chromogen for the peroxidase reactions. As with the corticostriatal projection the striatum contained many anterogradely labelled thalamostriatal axons and terminals within the neuropil (some indicated by arrows) which were often closely apposed to the immunoreactive neurons (some indicated by arrowheads). In C the ChAT-immunoreactive neuron was revealed using BDHC as the chromogen for the immunoperoxidase and the anterogradely labelled thalamostriatal using DAB. Note the granular appearance of the BDHC reaction product, the thalamostriatal fibres and boutons in the neuropil (some indicated by arrows) and thalamostriatal boutons closely apposed to the immunoreactive neuron (arrowheads). Scale bar = 25 μm for A–C.

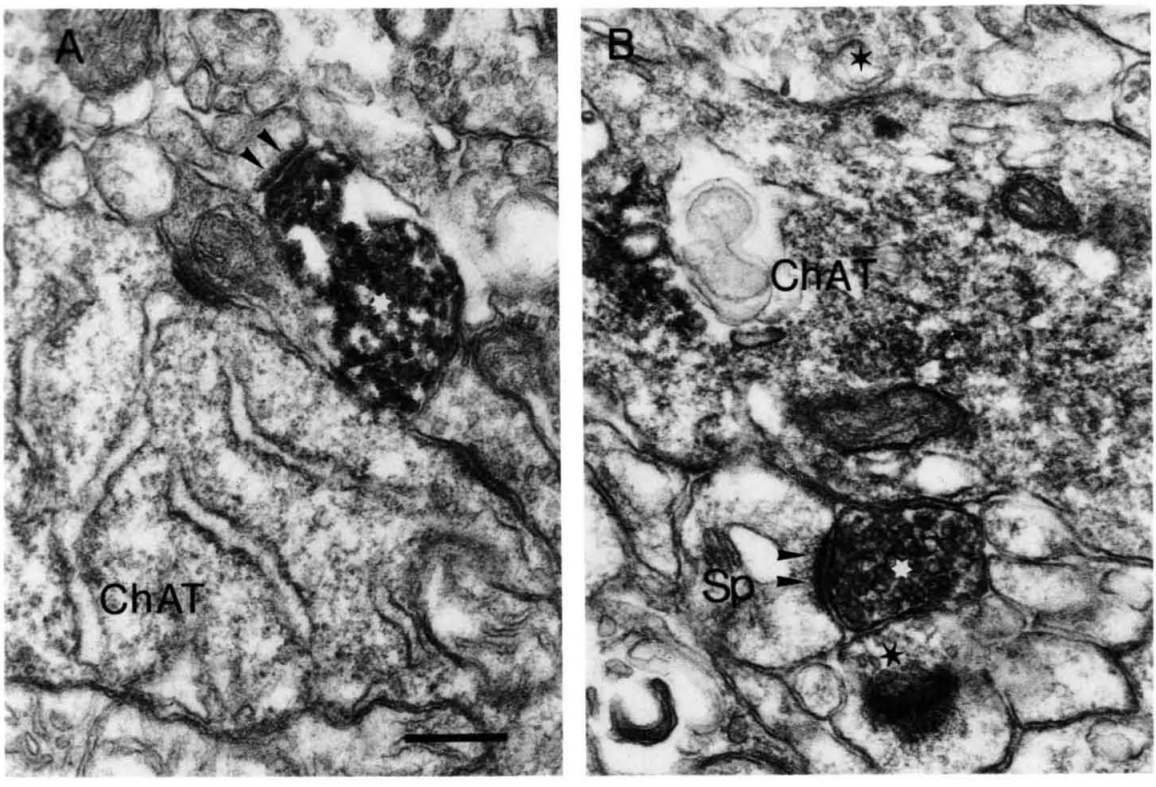

Fig. 2. High power electron micrographs of terminals in the striatum anterogradely labelled with biocytin from the cortex (white stars). Both of the corticostriatal boutons were closely apposed to ChAT-immunoreactive structures (ChAT); a perikaryon in A and a small-diameter, presumably distal dendrite in B. These boutons were followed in serial sections in the electron microscope but neither of them made synaptic contact with the ChAT-immunoreactive neurons. They did, however, make asymmetrical synaptic contact (arrowheads) with adjacent unlabelled dendritic spines (Sp). The black stars in B indicate unlabelled boutons for comparison with the labelled terminals. Scale bar = 0.25 μm for A and B.

used as the chromogen. For this reason most of the electron-microscopic data were obtained using DAB.

A sparse population of ChAT-immunoreactive neurons were distributed throughout the dorsal striatum in an apparently homogeneous manner. The cells possessed a large soma (diameter of cell body 30–40 μm) with an eccentrically placed, invaginated nucleus. The dendrites branched infrequently and were devoid of spines. The characteristics of the immunoreactive neurons were consistent with those described on other occasions.[2,5,7,26,42] In some sections and in parts of others where the staining of cholinergic structures was particularly good, the striatal neuropil contained a dense network of very fine ChAT-positive fibres with small swellings. The fibres generally followed a tortuous path and, due to the light staining and fine nature, the path of individual fibres could not be followed.

Electron microscopy. In the electron microscope, the DAB reaction product was distributed evenly throughout the cell body and the dendrites (Fig 2, 3B, C, D). In confirmation of the light-microscopic findings, the ChAT-immunoreactive neurons had a large cell body containing an eccentrically placed, deeply invaginated nucleus. The perikarya possessed a large volume of cytoplasm that was rich in organelles including endoplasmic reticula. The dendrites were devoid of spines and received numerous synaptic inputs of both the symmetrical and asymmetrical variety, the density of which increased in the more distal regions. At least two classes of terminals making asymmetrical synaptic contact with the ChAT-immunoreactive structures were seen. Both classes of terminals were variable in size and possessed densely packed vesicles; one of them was associated with post-junctional dense bodies.

The ChAT-positive terminals, derived from the local axon collaterals of the cholinergic neurons, were also observed in the electron microscope. In agreement with previous observations,[5,7,20,42] these terminals, when observed to form synapses, always had symmetrical specializations.

Double-labelling of corticostriatal terminals and choline acetyltransferase-immunoreactive neurons

At the light-microscopic level, corticostriatal fibres and ChAT-immunoreactive neurons were seen distributed throughout the dorsal striatum and areas of overlap were seen in all regions. However, the labelling of ChAT-immunoreactive structures appeared

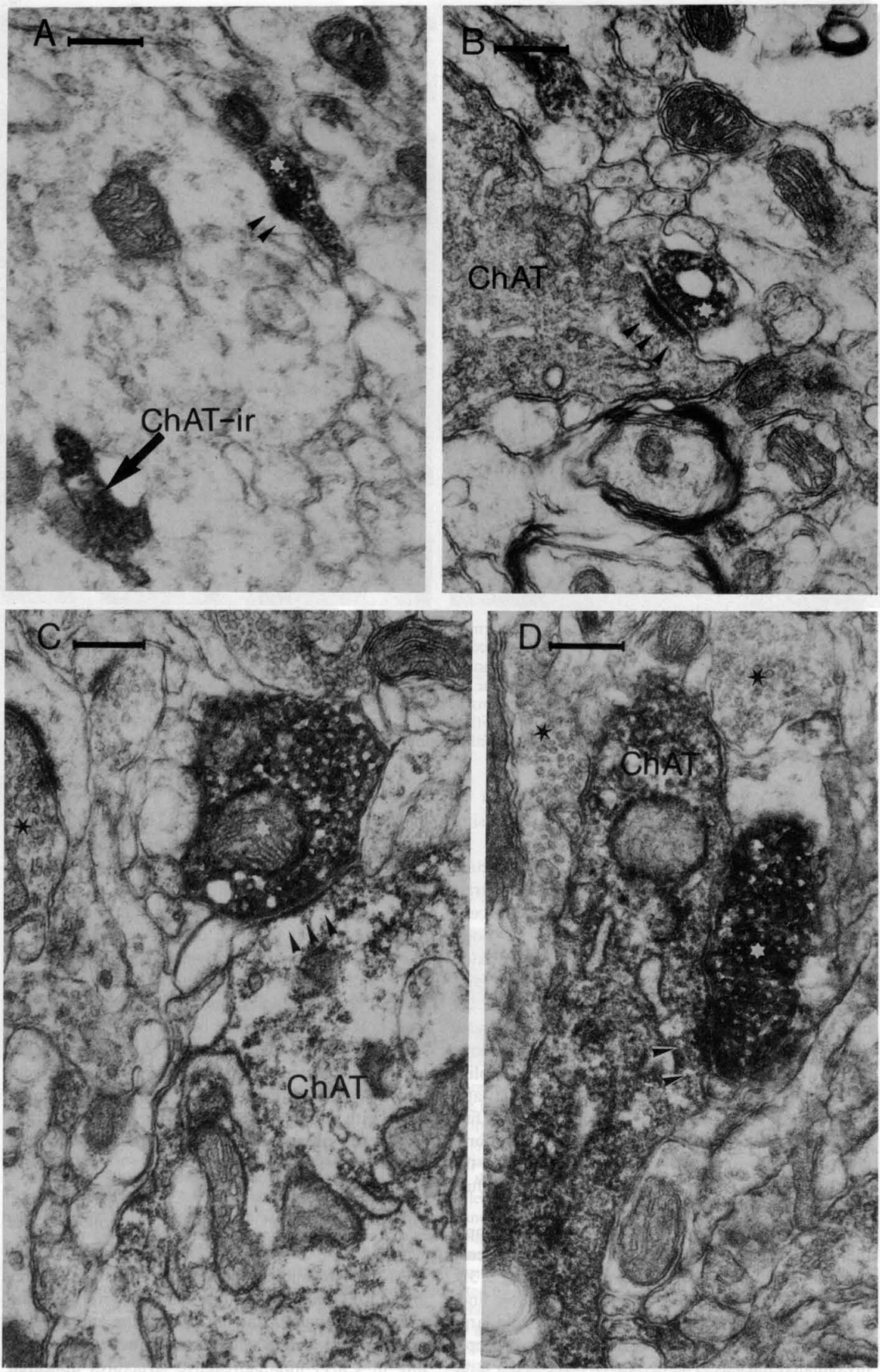

Fig. 3

to be reduced in regions of the densest anterograde labelling. Many of the punctate corticostriatal boutons were in close apposition to the cell bodies and proximal and distal dendrites of ChAT-immunoreactive neurons (Fig. 1A). The anterogradely labelled structures were distinguishable from the collaterals of the cholinergic neurons (when present) on the basis of size and pattern of aborization, the corticostriatal fibres were of a larger diameter, and followed less tortuous paths than did the cholinergic fibres. The areas that were chosen for electron-microscopic analysis had only a few or no ChAT-positive fibres.

In the electron microscope, the corticostriatal terminals and ChAT-immunoreactive structures were identified by the DAB peroxidase reaction product. Corticostriatal terminals were often seen in close apposition to ChAT-immunoreactive cell bodies (Fig. 2A), large-diameter dendrites and small-diameter, presumably distal, dendrites (Fig. 2B). A total of 60 corticostriatal boutons in close apposition to the ChAT-immunoreactive neurons were examined in serial sections, but in no case was a synaptic contact observed between the two structures. The corticostriatal boutons were often directly apposed to the ChAT-immunoreactive neurons over a number of serial sections or were separated from the dendrites by small glial elements. In a number of cases (15) boutons apposed to the ChAT-immunoreactive neurons made asymmetrical membrane specializations, not with the immunoreactive neuron, but with a neighbouring unlabelled spine (Fig. 2). The ChAT-immunoreactive neurons received synaptic input from many unlabelled terminals which formed both symmetrical and asymmetrical synaptic specializations.

Double-labelling of thalamostriatal terminals and choline acetyltransferase-immunoreactive neurons

In the light microscope the thalamostriatal terminals were seen to be distributed throughout the neuropil and often appeared in close apposition to ChAT-immunoreactive cell bodies and dendrites (Fig. 1B, C). Close apposition of terminals was especially prominent when the more distal dendrites were immunostained. As with the corticostriatal system the anterogradely labelled fibres were easily distinguishable from the collaterals of the cholinergic neurons on the basis of diameter of the individual structures and the pattern of arborization. Furthermore, areas for electron microscopy were chosen that were poor in, or free from the cholinergic axons.

In the electron microscope, thalamostriatal terminals identified by the DAB reaction product were interspersed amongst ChAT-immunoreactive structures identified by DAB or BDHC reaction products (Fig. 3). The thalamostriatal terminals were often in close apposition to ChAT-immunoreactive dendrites and perikarya. A total of 33 thalamostriatal boutons that were seen to be apposed to ChAT-immunoreactive neurons and were examined in serial sections, were seen to make direct asymmetrical synaptic contact with 20 ChAT-immunoreactive neurons (Fig. 3). The majority made synaptic contact with small-diameter, presumably distal dendrites ($n = 18$, 55%) (Fig. 3D), whereas smaller numbers were in contact with large-diameter proximal dendrites ($n = 8$, 24%) (Fig. 3B) and perikarya ($n = 7$, 21%) (Fig. 3A, C). In some cases the thalamostriatal terminals making asymmetrical synaptic contact with the ChAT-immunoreactive dendrites were associated with post-junctional dense bodies (Fig. 3B).

Control experiments

Following omission of the primary antibody directed against ChAT, only DAB-positive corticostriatal or thalamostriatal fibres and terminals were observed in the striatal neuropil. On the other hand, in cases where ABC was omitted, only the BDHC- or DAB-positive ChAT-immunoreactive neurons were visualized.

DISCUSSION

The results of the present experiments: (i) confirm that both the frontal cortex and the parafascicular nucleus of the thalamus project heavily onto the striatum and form asymmetrical synapses; (ii) confirm that the major target of corticostriatal terminals are spines whereas the major target of thalamostriatal terminals are dendritic shafts; and (iii) demonstrate that in the dorsal striatum, as in the case in the ventral striatum,[36] cholinergic neurons receive a prominent direct synaptic input from thalamus but not from the cortex.

Fig. 3. High power electron micrographs of terminals in the striatum anterogradely labelled with biocytin from the parafascicular nucleus of the thalamus (white stars). Each of the terminals is in asymmetrical synaptic contact (arrowheads) with ChAT-immunoreactive (ChAT) perikarya (A,C) or dendrites (B,D). Note that in B the asymmetrical synaptic contact is associated with sub-junctional dense bodies. In A the ChAT-immunoreactive neuron was revealed using BDHC as the chromogen for the peroxidase reaction, which gives a crystalline or granular immunoreaction product (one granule is indicated by ChAT-ir). In the other micrographs both the ChAT-immunoreactive structures and the anterogradely labelled terminals were localized using DAB. The black stars in C and D indicated unlabelled terminals for comparison with the labelled ones. Scale bars = 0.25 μm.

Technical considerations

In order to address the question of whether cholinergic neurons receive synaptic input from corticostriatal and/or thalamostriatal neurons it was necessary to combine anterograde labelling with immunocytochemistry for ChAT. The immunocytochemical technique that was used to localize the ChAT and the technique used to localize the anterograde tracer both required peroxidase reactions; it was therefore necessary to carry out double-peroxidase staining at the electron-microscopic level to establish the presence of synaptic contacts. To this end, we used the double-peroxidase method established by Levey *et al.*[34] that has proved successful in the past in the elucidation of the synaptology of striatal cholinergic neurons.[4] In this procedure two different chromogens (DAB and BDHC) for the peroxidase reactions are utilized that give distinguishable reaction products at both the light- and electron-microscopic levels. However, in the present experiments, the use of BDHC as the chromogen to localize the ChAT-immunoreactive structures gave inconsistent results and it was only possible to carry out part of the electron-microscopic analysis with this material (see Fig. 3A). In order to overcome this problem, some of the material was stained using DAB to reveal both the anterograde marker and the ChAT-containing structures. This approach increased the yield of tissue that contained both sets of labelled structures but gave rise to an additional problem of interpretation, i.e. that the tissue may contain two sets of terminals labelled with DAB reaction product, the anterogradely labelled terminals and the terminals of the axon collaterals of the cholinergic neurons. The origin of a DAB-positive terminal would therefore be questionable. This problem, however, was only of minimal importance in the present experiments, as in the light-microscope ChAT-positive terminals were distinguishable from corticostriatal or thalamostriatal fibres on the basis of their morphology and arborization patterns, and areas were selected for re-embedding and electron microscopy that were dense in the anterogradely labelled fibres and terminals and, for technical reasons or because of uneven labelling, were poor in ChAT-positive terminals. Furthermore, at the electron-microscopic level, it was possible to distinguish the two sets of terminals on the basis of their morphological appearance and by the fact that corticostriatal[9,11,16,22,56,60,65] and thalamostriatal terminals[6,9,22,66] make asymmetrical synaptic contracts, whereas ChAT-immunoreactive terminals in the striatum make symmetrical synaptic contacts.[5,7,20,42]

Corticostriatal pathway

The morphology of the corticostriatal terminals was similar to that described in other studies[9,11,22,56,60,65] in that the terminals were of a similar size-range, contained densely packed, round synaptic vesicles and formed asymmetric synapses. In the present study the majority (87%) of the corticostriatal terminals that were observed making synaptic contacts in the striatum, formed asymmetrical specializations with spines, whereas a smaller proportion (13%) were in contact with dendritic shafts. This distribution of postsynaptic targets of the corticostriatal terminals is somewhat different from other studies of the corticostriatal system in the rat[9,60,65] where a much higher proportion were identified in contact with spines. The reason for this difference is uncertain but it may be related to the method of anterograde labelling (Dubé *et al.*[9] used anterograde degeneration) or the experimental situation in which the pathway was being examined since Wictorin *et al.*[60] and Xu *et al.*[65] were examining "spared striatum" following a neurotoxic lesion. However, the distribution of postsynaptic targets in the present study is very similar to that found in the primate striatum (85% spines, 15% dendritic shafts) in which the same system of anterograde labelling was used and there was no neurotoxic insult to the striatum.[31]

As pointed out in the Introduction, the spines that are postsynaptic to the corticostriatal terminals are derived from spiny projection neurons.[11,56] However, since spiny neurons rarely receive input on their dendritic shafts from terminals that form asymmetrical synaptic specializations,[55] the 13% of terminals in contact with dendritic shafts implies that other classes of striatal neurons receive input from the cortex. One class has been shown to be the GABAergic interneuron,[31] identified on the basis of parvalbumin immunoreactivity, and one of the objects of the present study was to test whether cholinergic interneurons also receive this input (see below).

Cortical input to choline acetyltransferase-immunoreactive neurons

Data from a variety of experimental approaches have suggested that cholinergic neurons are under direct control of the corticostriatal system (see Introduction). The observations that we made at the light-microscopic level appeared to support this hypothesis, in that the ChAT-immunoreactive neurons were surrounded by many corticostriatal terminals that were in close apposition to the perikarya, proximal and distal dendrites. However, examination of many of these apposed boutons, that had first been identified at the light-microscopic level, in serial sections in the electron microscope, failed to reveal synaptic specializations between them and the ChAT-immunoreactive structures. Indeed, the corticostriatal terminals, although directly apposed to the membrane of cholinergic cells, often made asymmetrical synaptic contact, not with the cholinergic neuron, but with an adjacent unlabelled spine. These observations imply that the cortical terminals do not make contact with, or make very little contact with, the proximal parts, i.e. perikaryon and first 100–200 μm of the dendritic tree of cholinergic neurons, since it

was only to this extent that the dendritic tree was consistently immunolabelled. It is possible, however, that a cortical input occurs in the more distal dendrites of the cholinergic interneurons, and these parts of the neurons may not have been observed in the present experiments because the immunoreaction product did not extend into the distal dendritic tree. This question was addressed, to some extent, by examination of small-diameter, and therefore presumably distal, ChAT-immunoreactive dendrites that were found when scanning through the electron-microscopic sections. As with the correlated light and electron microscopy, the corticostriatal terminals, although occasionally seen apposed to the ChAT-immunoreactive dendrites, were never seen in direct synaptic contact with them. From a technical point of view, this negative finding is made stronger by the fact that in the same series of experiments, a positive result with the thalamostriatal pathway was found using the same approach; the failure to detect a cortical input to the cholinergic neuron was therefore presumably not due to technical reasons. Another possible explanation for the failure to detect a cortical input is that it is different regions of the cortex that make synaptic contact with the cholinergic neurons; this possibility is under investigation.

Our findings are in agreement with those of Meredith and Wouterlood,[36] who examined the input to cholinergic neurons in the ventral part of the striatal complex, i.e. the nucleus accumbens. These authors demonstrated that terminals anterogradely labelled from the hippocampus (the input that is equivalent to the neocortical input to the dorsal striatum) were often seen apposed to the cholinergic neurons in the nucleus accumbens but, in serial electron-microscopic sections, were never seen to make synaptic contact with the proximal parts of the neurons. A small proportion (2%) of those terminals apposed to distal, small-diameter dendrites did make synaptic contact with the cholinergic neurons. Our observations, therefore, together with those of Meredith and Wouterlood,[36] would suggest that it is a general rule that cholinergic neurons in the striatal complex do not receive input from cortical neurons in their proximal regions and little, if any at all, in their distal regions.

If the corticostriatal pathway is not in direct synaptic contact with the cholinergic neurons, then the changes in cholinergic markers in the striatum that occur following manipulation of excitatory amino acid receptors[1,32,51,52,54] or following destruction of the cortex[54,64] may have been due to two reasons. First, the changes that occur following cortical lesions may be due to an indirect mechanism via other striatal neurons. Corticostriatal terminals make direct synaptic contact with other classes of neurons in the striatum[9,11,22,23,31,56,59] which may in turn contact the cholinergic interneurons.[3,4] Secondly, the effects of manipulation of excitatory amino acid receptors may be due, not to the corticostriatal system, but to effects on receptors associated with an excitatory input from some other region, the most likely candidate being the thalamus (see below). It should be pointed out, however, that on the basis of electrophysiological observations, Wilson *et al.*[62] proposed that striatal cholinergic neurons receive only few inputs from the cortex (and thalamus) but that they are particularly sensitive to these afferents; it may therefore be, that the small number of afferent terminals from the cortex can also account for the pharmacological changes in response to cortical lesions or excitatory amino acid receptor manipulation.

Thalamostriatal pathway

The present results confirm that the parafascicular nucleus of the thalamus projects extensively to the striatum. The morphology of the thalamostriatal terminals is similar to that described in other studies[6,9,22,47,66] in relation to their size, their content of round or oval vesicles and the fact that they form asymmetrical synaptic specializations. In the present study it was observed that 79% of the thalamostriatal terminals that were identified as making synaptic specializations, were in contact with dendritic shafts, 20% with spines and 1% with perikarya. These figures are broadly in agreement with those of other studies of the projection from the parafascicular nucleus in the rat and primate[9,47,66] in that dendritic shafts are the major postsynaptic targets, although in the study of Dubé *et al.*[9] dendritic spines were not identified as a postsynaptic target. It should be noted, however, that the projections from different sub-divisions of the thalamus give rise to markedly different distributions of postsynaptic targets.[47,66]

The fact that a large proportion of the terminals from the parafascicular nucleus make asymmetrical contacts with dendritic shafts, implies, as with the cortical projection, that the postsynaptic neurons are not the typical densely spiny neurons, but one or more of the classes of aspiny interneurons. Indeed, at least one target of parafascicular terminals has been identified as class of sparsely spiny neurons;[9] it leaves open the possibility that cholinergic neurons are also a target of thalamostriatal terminals.

Thalamic input to choline acetyltransferase-immunoreactive neurons

As was observed at the light-microscopic level in material that contained corticostriatal terminals, the cholinergic neurons had many thalamostriatal terminals closely apposed to them. In contrast to the observations on the corticostriatal projection, electron-microscopic examination of thalamostriatal boutons in close apposition to the ChAT-immunoreactive neurons, revealed that many of them made synaptic contact with the cholinergic neurons. The synapses were of the asymmetrical type and were often associated with sub-junctional dense bodies. A

total of 33 thalamostriatal boutons were seen to make direct synaptic contact with 20 ChAT-immunoreactive cells. They made contact with all regions of the cholinergic neurons that were examined; 55% were in contact with distal dendrites, 24% with proximal dendrites and 21% with perikarya. These figures do not give a true indication of the number of boutons of thalamic origin in contact with each cholinergic neuron nor a true indication of their distribution on individual neurons, as the ChAT-immunoreaction product did not extend to the full extent of the dendritic tree and it is unlikely that *all* the terminals derived from the parafascicular nucleus were anterogradely labelled. Furthermore, the variable penetration of the reagents into the tissue and the problems involved in serial ultrathin sectioning will have resulted in a falsely low number of terminals in contact with the immunoreactive neurons. Our results therefore must be considered in a qualitative sense only and the number of terminals in contact with an individual cholinergic neuron is likely to be considerably higher than we observed.

The present findings in relation to the thalamic input, like those in relation to the cortical input, are in agreement with the findings of Meredith and Wouterlood,[36] who demonstrated that in the ventral striatum (nucleus accumbens), thalamic terminals derived from midline/intralaminar nucleus make asymmetrical synaptic contact with cholinergic interneurons.

Previous studies of the thalamostriatal system have shown that at least one of the targets of thalamostriatal fibres are spiny neurons.[6,22,47,66] These neurons have been assumed to be the typical densely spiny neurons that are the most common type of neurons in the striatum but have been shown in the rat at least, to be a class of spiny neurons with a lower density of spines.[9] The present study shows that an additional target of the thalamostriatal fibres are the cholinergic interneurons of the striatum.

In view of the observations that there is very little cortical input to the cholinergic neurons and the likelihood that the thalamic input to the striatum is excitatory[29,43,62] then the effects of manipulation of excitatory amino acid systems on cholinergic function in the striatum[1,32,51,52,54] may be related to the thalamostriatal system. The present findings are also consistent with the observations that lesions of the thalamus result in a reduction of cholinergic markers in the striatum.[40,48,49,53]

CONCLUSIONS

The results of the present and previous studies[9,36] raise some interesting implications concerning the way in which thalamic information is handled in the basal ganglia. It is apparent that the parafascicular nucleus may influence the output of the striatum in at least two ways. First, by a direct route; fibres from the parafascicular nucleus make direct synaptic contact with the dendritic shafts of spiny neurons which are presumably projection neurons.[55] The thalamostriatal fibres therefore directly influence the output neurons of the striatum. The second route is indirect, via the cholinergic interneurons. As shown in the present study the terminals derived from neurons in the parafascicular nucleus make direct synaptic contact with the cholinergic neurons. These neurons, in turn, make direct synaptic contact with the spines, dendritic shafts and perikarya of spiny neurons that project to the substantia nigra.[20] Thus by making contact with the cholinergic interneurons the parafascicular nucleus can influence the output of the striatum. Although the consequence of stimulation of the direct route will be inhibition in the output stations of the basal ganglia (the substantia nigra pars reticulata and the entopeduncular nucleus or internal segment of the globus pallidus) the likely responses to activation of the indirect route through cholinergic interneurons remain to be elucidated.

Whatever the functional consequences of activation of this route it is important to note that it will be confined almost exclusively to the so called matrix division of the histochemical compartments of the striatum (for reviews, see Refs 12, 14). The reasons for this are as follows: (i) although not observed in the present study as all our material was prepared for electron microscopy, the parafascicular nucleus innervates the matrix division of the striatum;[18,47] (ii) the striosome or patch/matrix system of the striatum was originally described on the basis of acetylcholinesterase staining,[14] the matrix is characterized by intense acetylcholinesterase staining whereas the patches or striosomes are weak. Staining for ChAT-immunoreactive structures is consistent with this finding; although the cholinergic neurons are distributed evenly with respect to the histochemical compartments, cholinergic neuropil, i.e. the axons of the cholinergic neurons are almost exclusively present in the matrix.[15] The terminals of parafasicular neurons therefore make synaptic contact with cholinergic neurons in the matrix, which in turn make synaptic contact with striatal output neurons also located within the matrix; these will then project to the substantia nigra pars reticulata and the entopeduncular nucleus or internal segment of the globus pallidus.

Although from the present experiments it is unlikely that the flow of cortical information through the striatum will occur through the *same* routes, it is clear from other studies that there also exists direct and indirect routes for cortical information. Thus cortical terminals make direct synaptic contact with the spiny output neurons of the striatum[56] which represents a direct route. The corticostriatal system also makes synaptic contact with GABAergic interneurons[31] which in turn contact the output neurons of the striatum.[28] This latter pathway represents an indirect route by which the cortex may influence the

output of the striatum and is probably mediating a feedforward inhibition.[28,41,63] The relationship of this pathway to the patch/striosome–matrix system remains to be established.

The present findings demonstrate that although striatal spiny neurons appear to be synaptic targets of most classes of terminals, based on transmitter characteristics or origin,[55] there is selectivity in the innervation of striatal interneurons. The functional significance of this selectivity remains to be established.

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