CHOLINERGIC INPUT TO DOPAMINERGIC NEURONS
IN THE SUBSTANTIA NIGRA:
A DOUBLE IMMUNOCYTOCHEMICAL STUDY

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Abstract—In order to determine whether the cholinergic fibres that innervate the substantia nigra make
synaptic contact with dopaminergic neurons of the substantia nigra pars compacta, a double immunocyto-
chemical study was carried out in the rat and ferret. Sections of perfusion-fixed mesencephalon were
incubated first to reveal choline acetyltransferase immunoreactivity to label the dopaminergic terminals and
then tyrosine hydroxylase immunoreactivity to label the dopaminergic neurons. Each antigen was localized
using peroxidase reactions but with different chromogens.

At the light microscopic level, in confirmation of previous observations, choline acetyltransferase-
immunoreactive axons and axonal boutons were found throughout the substantia nigra. The highest
density of these axons was found in the pars compacta where they were often seen in close apposition
to tyrosine hydroxylase-immunoreactive cell bodies and dendrites. In the ferret where the choline
acetyltransferase immunostaining was particularly strong, bundles of immunoreactive fibres were seen to
run through the reticulata perpendicular to the pars compacta. These bundles were associated with
tyrosine hydroxylase-immunoreactive dendrites that descended into the reticulata. The choline acetyltrans-
ferase-immunoreactive fibres made "climbing fibre"-type multiple contacts with the tyrosine hydroxylase
positive dendrites.

At the electron microscopic level the choline acetyltransferase-immunoreactive axons were seen to give
rise to vesicle-filled boutons that formed asymmetrical synaptic specializations with nigral dendrites and
perikarya. The synapses were often associated with sub-junctional dense bodies. On many occasions the
postsynaptic structures contained the tyrosine hydroxylase immunoreaction product, thus identifying
them as dopaminergic.

It is concluded that at least one of the synaptic targets of cholinergic terminals in the substantia nigra
are the dendrites and perikarya of dopaminergic neurons and that in the ferret at least, the dendrites of
dopaminergic neurons that descend into the pars reticulata receive multiple synaptic inputs from
individual cholinergic axons.

The substantia nigra (SN) acts as one of the major
output stations of the basal ganglia transmitting
information received by the striatum, via the pars
reticulata, to the thalamus, superior colliculus or
brainstem. A second function of the SN is to provide
a feedback system that modulates information flow
through the basal ganglia by an action in the stria-
tum. This modulatory feedback is mediated by the
dopaminergic nigrostriatal neurons that are located
in the pars compacta of the SN and that project
heavily and diversely onto the striatum. The func-
tional importance of this feedback system is exem-
plified by the profound motor disturbances that occur
when these neurons degenerate in Parkinson's disease

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Abbreviations: ACh, acetylcholine; AChE, acetylcholin-
esterase; BDHC, benzidine dihydrochloride; Chat,
choline acetyltransferase; DAB, diaminobenzidine; PAP,
peroxidase antiperoxidase complex; PB, phosphate
buffer; PBS, phosphate-buffered saline; SN, substantia
nigra; SNC, substantia nigra pars compacta; SNR,
substantia nigra pars reticulata; TB, tris buffer; TH,
tyrosine hydroxylase.
see below and Discussion). The evidence that ACh acts as a neurotransmitter in the SN is substantial. Thus ACh and the synthetic enzyme for ACh, choline acetyltransferase (ChAT) are present,\textsuperscript{1,12} as are the breakdown enzyme, acetylcholinesterase (AChE)\textsuperscript{8} and ACh receptors,\textsuperscript{13,46} the stimulation of which causes excitation.\textsuperscript{12,33,34} Light microscopic immunocytochemistry has shown that ChAT immunoreactivity is present in axons and terminal-like swellings\textsuperscript{1,2,20,23,39,59} that form asymmetrical synaptic specializations.\textsuperscript{2,3,28} Several observations suggest that at least one of the synaptic targets of the ChAT-positive terminals are the dopaminergic neurons of the pars compacta: (i) ChAT-immunoreactive fibres are most densely distributed in the pars compacta of the SN;\textsuperscript{1,14,20,23} (ii) dopaminergic neurons are rich in AChE;\textsuperscript{8} (iii) dopaminergic neurons possess nicotinic ACh receptors, the stimulation of which causes an increased firing rate;\textsuperscript{12,34} and (iv) stimulation of the region of the pedunculopontine nucleus with kainic acid causes an excitation of dopaminergic neurons that is sensitive to nicotinic receptor blockade.\textsuperscript{14} The object of the present experiment was therefore to test directly whether cholinergic terminals make synaptic contact with dopaminergic neurons in the SN.

**EXPERIMENTAL PROCEDURES**

**Perfusate-fixation**

Experiments were carried out on 11 female albino Wistar rats (160-200 g body weight), and six female ferrets (400-800 g body weight). The rats were deep anaesthetized with chloral hydrate (350 mg/kg, i.p.) and perfused through the aorta initially with Ca\textsuperscript{2+}-free Tyrode's solution followed by 200 ml of fixative consisting of 3% paraformaldehyde and 0.1-0.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) over a period of 10-12 min. This solution was followed by 100 ml of 3% paraformaldehyde in PB over a period of 4-5 min. The brains were removed from the skull and on some occasions postfixed in the second fixative solution for up to 1 h. The ferrets were anaesthetized with a lethal dose of sodium pentobarbitone (80 mg/kg, i.p.) and pre-perfused with NaHCO\textsubscript{3}/HCO\textsubscript{3}\ buffered saline (pH 7.2) and then 500 ml (approx. 10 min) and 300 ml (0.25 min), respectively, of the two fixative solutions described above. On some occasions they were postperfused with 500 ml of PB. Some of the ferret brains were also postfixed in the second fixative solution for up to 20 min. Ferrets that were prepared for light microscopy only, were perfusion-fixed with 2% paraformaldehyde and 15% (v/v) saturated picric acid in 0.1 M PB. The brains from these animals were sectioned on a freezing microtome.

**Preparation of the sections**

After perfusion or postfixation the brains were transferred to PB or phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.4), the meninges were dissected out and then sectioned either immediately or with a few days delay, at 70 \( \mu \)m on a vibrating microtome in PBS. Most sections were then treated with pronase\textsuperscript{17} in order to enhance the immunostaining for ChAT. The sections were washed twice in Tris–HCl buffer (TB; 0.05 M, pH 7.4) and then incubated in a 0.001% solution of pronase (protease Type XXV, Sigma) at room temperature with constant shaking for 10 min. The reaction was stopped by addition of ice-cold TB followed by several washes in the same buffer and then several washes in PBS. On some occasions the sections were left at 4°C overnight at this stage. Most of the sections were then freeze-thawed to increase penetration of the immunoreagents. They were first equilibrated in a cryoprotectant consisting of 25% sucrose and 10% glycerol in 0.05 M PB, placed on nylon mesh nets, initially frozen by immersion in isopentane cooled (but still liquid) in liquid nitrogen and then immersed in liquid nitrogen. They were then thawed in PBS at room temperature and subjected to immunocytochemistry as described below.

**Immunocytochemistry**

All the sections were subjected to either a double immunocytochemical protocol to reveal both ChAT and TH or single staining to reveal each antigen separately. The peroxidase-antiperoxidase staining was carried out according to the method of Levey et al.,\textsuperscript{32} in which both antigens are revealed by peroxidase methods but using different chromogens for the peroxidase reactions. Briefly, the sections were incubated in the following sequence of solutions in PBS: monoclonal antibodies against ChAT, the characteristics of which have been described in detail elsewhere,\textsuperscript{31} diluted 1:200 for 12-48 h; several washes in PBS; either rabbit (Dakopatts) or sheep (Scrothe) anti-rat IgG diluted 1:50 in PBS for 2-18 h; several washes in PBS; rat peroxidase-antiperoxidase (PAP; Sternberger-Meyer Inc) diluted 1:100 for 2-18 h. Some of the ferret sections were incubated in a mixture of primary antibodies against ChAT from two different sources: 1:50 rat anti-ChAT (see Ref. 16) and 1:500 rat anti-CHAT (see Ref. 31) in PBS supplemented with 2% bovine serum albumin. In this case the primary antibodies were localized using a 1:50 dilution of biotinylated rabbit anti-rat and then a 1:25 dilution of an avidin–biotin–peroxidase complex (Vector).

The ChAT-immunoreactive structures were then localized by first washing the sections in TB and then pre-incubation for 10 min in a solution of diaminobenzidine (DAB) in PB (0.05%) followed by 5-10 min in the same solution but containing 0.001% H\textsubscript{2}O\textsubscript{2}. The reaction was terminated by two washes (5 min) in TB and three washes in PBS (5-10 min). Some of the rat sections were incubated to reveal the ChAT-immunoreactive structures using 0.25% DAB and 0.006% H\textsubscript{2}O\textsubscript{2} but including 0.01 M imidazole in the reaction mixture. Occasionally at this stage, there was a delay of a few days before starting the TH immunostaining. In these cases the sections were either stored in PBS at 4°C or at \(-23°C in an anti-freeze solution containing ethylene glycol and glycerol in PB. In the latter case, the procedure enhanced the penetration of the immunoreagents without any detrimental effects on ultrastructure.

The sections were then passed through a series of solutions to reveal TH immunoreactivity. They were incubated for 18-48 h in a 1:1000 dilution of rabbit anti-TH.\textsuperscript{24} The TH antibodies were then localized using either the PAP technique or an avidin–biotin–peroxidase procedure. For the former, after several washes in PBS the sections were incubated for 2 18 h in a 1:50 dilution of goat anti-rabbit IgG (ICN Immunobiologics) followed by several washes in PBS and then 2 18 h in a 1:100 dilution of rabbit PAP (ICN Immunobiologics). For the latter, after the washes in PBS they were incubated for 2 h in a 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector), washed in PBS and then 2 18 h in a 1:100 dilution of an avidin–biotin–peroxidase complex. The bound biotin and the peroxidase in both cases was then localized using benzidine dihydrochloride (BDHC) as the chromogen. Briefly, they were washed (10 min) in 0.01 M PB at pH 6.8 and then incubated in a solution of BDHC (0.01% in the same buffer) containing 0.025% sodium nitroprusside for 10-15 min. Hydrogen peroxide, to a final concentration of approximately 0.005%, was then added and the sections incubated for a further 2-6 min. The reaction was terminated by several washes in the low pH PB (for details see Refs 4, 32).
On some occasions the double immunocytochemical protocol was carried out by incubation of the sections in a cocktail of the two primary antisera. The ChAT-immunoreactive sites were localized first as described above and using DAB as chromagen. The TH-immunoreactive sites were then localized using the avidin-biotin-peroxidase method and BDHC as the chromagen.

Some sections were used for light microscopic analysis only; these were usually incubated to reveal only one of the antigens in which case BDHC was not used as a chromagen. Triton X-100 was included in the antibody solutions and they were mounted on gelatine-coated slides on completion of the immunostaining. These sections were air-dried, dehydrated and mounted in XAM.

Control incubations were carried out to ensure that cross-reactivity was not occurring between the different antibody solutions. The controls were also carried out to ensure that, where there was the possibility of recognition of an antibody from one of the series by those from the other series (e.g. recognition of the rabbit anti-rat by the goat anti-rabbit), the peroxidase reaction product obscured the antigenic sites. Thus the whole double immunocytochemical protocol was carried out with the omission of each of the primary antibodies in turn.

Preparation of sections for electron microscopy

On completion of the immunostaining the sections were washed twice in the low pH PB and immersed in a 1% solution of osmium tetroxide in the same buffer at room temperature for 20-30 min. They were then washed three times in the low pH PB, dehydrated in an ascending series of alcohols and two changes of propylene oxide and transferred to an electron microscopic resin (Durcupan ACM, Fluka). After equilibrating overnight in the resin, they were mounted on microscope slides and cured at 60°C for 48 h. One per cent uranyl acetate was included in the 70% ethanol to improve contrast in the electron microscope. After examination in the light microscope, areas of interest were cut out from the slides, glued to blank cylinders of resin and sectioned on an ultramicrotome. The ultrathin sections were collected on Pioform-coated single slot grids, stained with lead citrate and examined in a Philips 410 or CM10 electron microscope.

Analysis of the material

All sections were carefully examined in the light microscope for the distribution and relationships of ChAT-immunoreactive and TH-immunoreactive structures in the substantia nigra. Areas of interest were cut from the slides and re-sectioned for electron microscopy and examined for ChAT-immunoreactive boutons in synaptic contact with TH-immunoreactive structures. Some of the ChAT-immunoreactive boutons seen in close apposition to the TH-immunoreactive structures in the light microscope were examined in the electron microscope by correlated light and electron microscopy.4

RESULTS

Light microscopic observations

The TH-immunoreactive structures were identified by the granular BDHC reaction product or in light microscopic sections, by the DAB reaction product. Their distribution in the mesencephalon of both the rat and ferret was consistent with that of previous observations in these species.22,24 Of importance to this study was the dense band of immunopositive cells occupying the pars compacta of the substantia nigra (SNC) and the ventral tegmental area. The substantia nigra pars reticulata (SNR) contained only a few TH-positive cells except in the caudal aspects where larger numbers were present. However, throughout the rostrocaudal extent of the pars reticulata there were many TH-positive dendrites that were derived from cells located in the pars compacta (Fig. 1A).

The distribution of ChAT-positive structures, identified by the presence of the amorphous DAB reaction product, was also similar to that described previously.2,19,20,23,37,38,39 Thus, the most caudal aspects of the nigra contained a few scattered, ChAT-positive neurons (considered to be a rostral extension of the pontomesencephalotegmental cholinergic cell groups), the mid-region (rostrocaudal) of the medial aspects of the nigra contained the ventrally projecting oculomotor nerve and the whole extension of the nigra contained ChAT-positive axons and axonal boutons (Fig. 1B). The density and distribution of the ChAT-positive fibres and terminals were often difficult to assess because of the difficulty in staining the terminals. It was apparent, however, that both the SNR and the SNC contained immunoreactive fibres that gave rise to axonal swellings. The density of fibres was greatest in the SNC where their distribution mirrored that of the TH-positive neurons (Fig. 1). In the ferret it was evident that, in addition to the apparently randomly dispersed ChAT-positive axons, the pars reticulata contained bundles of long positive axons that gave rise to many boutons and followed a course perpendicular to the pars compacta. The appearance of these bundles of fibres was reminiscent of the bundles of dendrites of dopaminergic neurons of the compacta that descend into the reticulata.

In the double-stained material the ChAT-positive structures coursed in amongst the TH-positive perikarya and dendrites in both the SNC and SNR and were often seen in close association with each other (Figs 2D; 4A, E). Some of the cases where distinct associations were identified at the light microscopic level were subsequently examined in the electron microscope (Figs 2D-F; 4). In the ferret, sections in which the ChAT immunostaining was strong, it was evident that the bundles of ChAT-positive axons seen in the SNR were closely associated with bundles of TH-immunoreactive dendrites. Individual ChAT-immunoreactive axons were seen to follow the course of individual TH-positive dendrites and give rise to many boutons that were closely apposed to the dendrites (Fig. 4A, E). At the light microscopic level single ChAT-positive axons were seen to give rise to as many as 10 boutons closely apposed to a single dendrite. Occasionally more than one ChAT-positive axon was seen converging onto the same TH-positive dendrite.

Electron microscopic observations

In the electron microscope the ChAT-immunoreactive structures (containing the amorphous DAB reaction product) were identified as axons, pre-terminal boutons and synaptic boutons. No
Fig. 2.
Fig. 1. Light micrographs of adjacent sections of part of the ventral mesencephalon of the ferret incubated to reveal TH immunoreactivity (A) and ChAT immunoreactivity (B). In both cases the chromogen for the peroxidase reaction was DAB. Note the dense band of TH-immunoreactive cell bodies occupying the SNC and the few cells but many dendrites in the SNR. The distribution of ChAT-immunoreactive fibres (B) appears to parallel the distribution of TH-immunoreactive structures. Thus, the SNC contains a dense plexus of ChAT-immunoreactive fibres and boutons whereas the SNR is far less dense. Note the ChAT-immunoreactive oculomotor nerve coursing through the nigra (III) in both micrographs. Scale bar = 200 μm.
differences in the morphology of the boutons were identified between the two species; therefore they will be described together. The boutons were of about 1–1.5 μm in diameter, they contained many vesicles that were tightly packed and were of variable shape and size that is best described as round and oval (Figs 2A–C; F; 3A–D). Mitochondria were also often present within the boutons (Figs 2A; 3A–D). When followed in serial sections the boutons were found to form synaptic specializations with spines (Fig. 2A), proximal (Figs 2E, F; 3C) and distal (small diameter) dendritic shafts (Figs 2B, C; 3A, B, D) and perikarya. Specializations were of the asymmetrical type (Figs 2A–C; 3A–D) although on occasions the postsynaptic density was not extensive (Fig. 3A). The synapses were often associated with sub-junctional dense bodies (Figs 2B, C; 3B–D).

In the double-immunostained material, whether examined by correlated light and electron microscopy or by scanning the ultrathin sections, the ChAT-positive boutons were often seen apposed to TH-immunoreactive dendrites and sometimes perikarya, containing the granular BDHC immunoreaction product (i.e. TH-positive). In serial sections they were generally found to form asymmetrical synapses with the TH-positive structures (Figs 3A–D; 4). The postsynaptic structures were often seen to receive synaptic input from non-immunoreactive boutons (Figs 2B; 3A, D).

Correlated light and electron microscopy of the ChAT-positive climbing fibres along the TH-positive dendrites revealed that individual cholinergic axons made multiple synaptic contacts with the TH-positive dendrites (Fig. 4). The boutons formed asymmetrical synaptic specializations. The postsynaptic dendrites also received inputs from non-immunoreactive boutons forming both symmetrical and asymmetrical synapses.

In the control sections in which the primary antibodies against ChAT were omitted, DAB reaction product was not detected at either the light or electron microscopic levels. Similarly the omission of the antisemum against TH resulted in sections without any specific BDHC reaction product. These controls indicated that the two reaction products are only associated with their appropriate antigens. In no case was the DAB reaction product associated with cell bodies or dendrites nor was the BDHC reaction product present in axon terminals, indicating that there was no detectable cross-reactivity between the two sets of immunoreagents.

DISCUSSION

The results of the present study confirm the presence of a cholinergic input to the SN and confirm that the cholinergic axons form asymmetrical synaptic contacts with nigral neurons. More importantly, the present findings demonstrate that one of the synaptic targets of the ChAT-positive, i.e. cholinergic, boutons in the substantia nigra of both rat and ferret are TH-immunoreactive structures, i.e. the dopaminergic neurons of the SN.

Technical considerations

The object of this study was to determine whether dopaminergic neurons in the SN receive a direct synaptic input from cholinergic axon terminals. To address this type of question it is necessary to apply a double immunocytochemical technique so that the chemical nature of both the pre- and postsynaptic structures can be identified. To this end we applied a double pre-embedding peroxidase procedure that has been used extensively in the study of the synaptic connections of chemically characterized structures in the SN. This procedure enabled us to identify both TH-positive neuronal perikarya and dendrites and ChAT-immunoreactive terminals at the electron microscopic level and so identify synaptic contacts between dopaminergic and cholinergic structures. The problems of the deposition of one peroxidase reaction product on the other and the problems of cross-reactivity of the immunoreagents have been addressed extensively before. In addition, the control experiments in the present study ensured that only ChAT-immunoreactive structures were labelled by the DAB reaction product and that only TH-immunoreactive structures were labelled by the BDHC reaction product. These control experiments indicated that we were labelling structures with each chromogen that were specific to the antibodies used.

Although the staining of TH-immunoreactive structures is generally not a difficult procedure since the enzyme seems particularly robust, this is certainly

Fig. 2. (A–C) Electron micrographs of ChAT-immunoreactive boutons (ChAT) forming asymmetrical synapses (arrowheads) with non-immunoreactive dendritic shafts (B, C) and a spine (A) in the SN of the rat. The immunoreactive structures are recognized by the amorphous electron-dense precipitate associated with membranes. Compare the immunoreactive boutons with the non-immunoreactive boutons labelled by asterisks. Also present in A are two TH-immunoreactive dendrites (TH) identified by the BDHC immunoreaction product. D–F illustrate correlated light and electron microscopy of a ChAT-immunoreactive bouton apposed to a TH-immunoreactive neuron. D is a light micrograph of a TH-immunoreactive neuron in the rat SN. The neuron is closely apposed by a ChAT-immunoreactive bouton (b). The proximal dendrite of the same neuron is shown in E. The dendrite (d) is identified by the electron dense granules of the BDHC reaction product dispersed throughout the cytoplasm. The ChAT-positive bouton (b) is shown at higher power (in a serial section) in F. The bouton is closely apposed to the TH-positive (granules of immunoreaction product indicated by arrows) dendrite and the junction which is probably synaptic is associated with a subjunctional cistern (arrowheads). A, B, C, and F are at the same magnification; scale bar = 0.5 μm; in D = 10 μm; in E = 2 μm.
Fig. 3. Electron micrographs of ChAT-immunoreactive boutons (ChAT) forming synaptic contacts (arrowheads) with TH-immunoreactive dendrites (the TH immunoreaction product is indicated by TH and arrows) in the SN of the rat (A, B) and the ferret (C, D). In B, C and D the synapses are clearly asymmetrical and associated with subjunctional dense bodies whereas in A the synapse appears symmetrical at this level. The asterisks indicate non-immunoreactive boutons. All micrographs are at the same magnification; scale bar in A = 0.5 μm.
not the case for the immunostaining of cholinergic terminals. The staining of cholinergic terminals in most parts of the brain has proved very difficult, particularly when preparing material for electron microscopy; it seems that the enzyme is very sensitive to the method of fixation and the primary antisera very sensitive to the secondary antibodies. To overcome this problem it was necessary to adopt special procedures of fixation and tissue treatment (pronase treatment and freeze-thawing). However, these procedures, as well as the low concentrations of glutaraldehyde that were used, are generally detrimental to the ultrastructural preservation of the tissue. Furthermore, the use of pronase was detrimental to the TH immunostaining. For these reasons compromises had to be made between the quality of preservation and the degree of immunostaining.

Cholinergic terminals in the substantia nigra

The detection of a ChAT-immunoreactive axonal plexus in the SN of both the rat and ferret confirms previous immunocytochemical studies (ferret, rat) that have identified a cholinergic innervation of the SN. Our observations also confirm previous descriptions that it is the pars compacta that receives a more dense innervation than the pars reticulata. In fact, in the ferret there was a remarkable correspondence between the distribution of the dopaminergic neurons of the pars compacta and the densest region of cholinergic axons and terminals (see Fig. 1).

The electron microscopic demonstration that the ChAT-positive axonal terminals form asymmetrical synapses with nigral neurons also confirms previous ultrastructural studies in the rat. The majority of the terminals made synapses with dendritic shafts although a small number were identified in contact with dendritic spines or perikarya. This distribution, however, probably reflects the relative proportions of dendrites, spines and perikarya in the SN and not a selective or preferential innervation of dendrites. The major object of the present experiment, however, was to determine whether any of the cholinergic terminals in the SN made contact with the dopaminergic neurons. This question arose because of the indirect evidence that dopaminergic neurons receive a cholinergic input (see Introduction) and because of the co-distribution of TH- and ChAT-immunoreactive structures in the SN. The analysis of the double-stained material revealed that the dopaminergic neurons do indeed receive a direct synaptic input from cholinergic terminals. This input occurred on all parts of the neurons so far examined, i.e. proximal and distal dendrites and perikarya. The quantitative nature of this input cannot be assessed in the present material because of the difficulty in labelling the cholinergic terminals and because of problems of penetration of the immunoreagents, both of which give rise to false negatives. Nevertheless, it is clear from the present study and from other studies of the synaptology of dopaminergic neurons that most of the afferent boutons to dopaminergic neurons form symmetrical synapses. Thus, in a study of the GABAergic input, in the region of 70% of the afferent terminals to dopaminergic neurons were identified as GABAergic and forming symmetrical synapses. Furthermore, other chemical classes of terminals form symmetrical synapses, e.g. neurotensin-positive terminals. The proportion of asymmetrical terminals in contact with the dopaminergic neurons is thus low and includes several different classes, i.e. 5-hydroxytryptamine-containing terminals from the raphe, enkephalin-immunoreactive terminals, a population of substance P-immunoreactive terminals and possibly terminals derived from the cortex and subthalamic nucleus. It is thus likely that the cholinergic terminals identified in contact with dopaminergic neurons account for only a small proportion of those terminals forming asymmetric synapses which in turn account for only a small proportion of the total input. The functional significance of this input is therefore likely not to depend on a massive innervation of the neurons like that from the striatum nor a heavy and strategically located input like that from the globus pallidus; it is more likely to depend on the topographical relationship of this input to other inputs and the electrophysiological responses of the neurons to released ACh. The situation, however, may be different in the pars reticulata where individual cholinergic fibres make multiple contacts with the dopaminergic dendrites. The cholinergic input to this part of the neuron represents a higher proportion of the total input and one might predict the ACh released from

Fig. 4. Correlated light and electron microscopy of "climbing-fibre-like" ChAT-positive fibres in synaptic contact with TH-positive dendrites in the SNR in the ferret. A and E are light micrographs of TH-immunoreactive dendrites (d) in the pars reticulata with ChAT-positive axons apposed. The axons give rise to many boutons (small arrows). Bouton b, in A is shown in the electron microscope in B and C where the ChAT immunoreaction product is visible (b, ChAT). The bouton forms asymmetrical synaptic contact with the dendrite containing the TH immunoreaction product (TH arrows). Three myelinated axons are indicated by arrowheads for correlation between the light and electron microscopic levels. Similarly the bouton b, in E is shown in D to contain the CHAT immunoreaction product and make asymmetrical synaptic contact with the TH-immunoreactive dendrite (some immunoreaction product indicated by TH and arrows). A TH-positive dendrite (d,) and a myelinated axon (arrowhead in E) are indicated by arrowheads for correlation between the light and electron microscopic levels. The asterisk denotes a non-immunoreactive terminal forming a synapse with the TH-positive dendrite. Scale bars in A and E = 10 μm; in B = 1 μm; in C = 0.2 μm; in D = 0.5 μm.
these terminals may have a more pronounced effect on the neurons.

**Origin of the cholinergic input to dopaminergic neurons**

Several lines of evidence suggest that the cholinergic terminals identified in contact with the dopaminergic neurons are derived from cholinergic neurons of the pedunculopontine tegmental and laterodorsal tegmental nuclei, some of which may be ectopically placed in the nigra itself. They contain dense populations of cholinergic neurons and have been shown by many authors to project to the SN (for Ref. see 30, 45). Lesions of the pedunculopontine tegmental nucleus in the cat lead to the degeneration of terminals that form asymmetrical synapses with retrogradely labelled nigrostriatal neurons. That these projections are likely to be cholinergic, at least in part, has been demonstrated by several groups using the combination of retrograde labelling with ChAT immunocytochemistry. It must be noted, however, that some authors consider that it is not the cholinergic neurons in these cell groups that give rise to the projection to the SN. Additional evidence in favour of the cholinergic projection has come from the work of Clarke et al. who showed that stimulation of neurons in the tegmental nuclei by infusion of kainic acid leads to a dose-related excitation of nigral neurons that were characterized as dopaminergic neurons on the basis of their responsiveness to apomorphine and haloperidol, and that the excitation is blocked by intravenous administration of the nicotinic receptor antagonist, mecamylamine. The weight of evidence, therefore, makes it likely that the cholinergic terminals identified in synaptic contact with the dopaminergic neurons in the present study are derived from cholinergic neurons in the pedunculopontine tegmental and laterodorsal tegmental nuclei.

The fact that the cholinergic terminals form asymmetric synapses that are often associated with sub-junctional dense bodies is of interest since two other classes of terminals form similar types of synapses; i.e. enkephalin-positive terminals and a sub-population of substance P-positive terminals both of which contact dopaminergic neurons. Because of this similarity in morphology, the possibility must be considered that there is a co-existence of these peptides with ACh. This idea is more likely for substance P as a proportion (15–40%) of the rostrally projecting cholinergic neurons in the pedunculopontine tegmental and laterodorsal tegmental nuclei also contain substance P immunoreactivity. Furthermore, the only other type of axon that has been shown to make multiple, climbing-fibre-like contacts with nigral neurons is a population of substance P-positive axons that form asymmetrical synapses often with sub-junctional dense bodies. The remarkable similarity between these and the cholinergic fibres (especially in the reticulata of the ferret) adds weight to the possibility of the co-existence of the two substances. It is likely, therefore, that some of those neurons that project to SN contain both ACh and substance P. To determine whether this is the case or not requires the application of double immunocytochemical staining of terminals or double immunocytochemical staining of the perikarya combined with retrograde labelling.

**CONCLUSIONS**

There seems to be little doubt that the SN receives a substantial cholinergic input that is directed mainly towards the pars compacta and that is probably derived from tegmental cholinergic neurons. The projection, at least in part, is directed towards the dopaminergic neurons, where asymmetrical synapses are formed between the cholinergic terminals and the dopaminergic dendrites and perikarya. These synapses thus represent the anatomical substrate for the excitatory action of cholinergic agonists on dopaminergic neurons, for the excitatory effect on dopaminergic neurons of kainate stimulation of the pedunculopontine nucleus, possibly for the dopamine-dependent behavioural effects of cholinergic agents injected into the nigra and for changes in the turnover of dopamine in response to ACh receptor agonists (see Ref. 51). Although AChE has been proposed to have functions in the SN that are independent of the cholinergic system, the presence of cholinergic synapses on dopaminergic neurons suggests that at least one of the functions of AChE associated with this class of neuron is the hydrolysis of released ACh. Finally, although the classical view that the balance between the levels of activity of ACh and dopamine for “normal” basal ganglia function occurs within the striatum (see Ref. 29), the detection of cholinergic terminals in contact with dopaminergic neurons implies that at least part of this balance resides in the SN.

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