CHOLINERGIC PROJECTIONS TO THE SUBSTANTIA NIGRA FROM THE PEDUNCULOPONTINE AND LATERODORSAL TEGMENTAL NUCLEI

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Abstract—The cholinergic innervation of the compact and reticular parts of the substantia nigra in the rat was investigated by use of highly sensitive retrograde and anterograde tract-tracing methods in combination with choline acetyltransferase immunohistochemistry. The fluorescent tracers True Blue, propidium iodide, or fluorogold were infused preferentially into either nigral subnucleus. Cells positive for choline acetyltransferase and retrograde tracer were found in both the pedunculopontine and laterodorsal tegmental nuclei, although considerably more double-labeled somata were observed in the former than in the latter component of the pontomesencephalotegmental cholinergic complex. Approximately 2–3 times more cholinergic cells were labeled in the pedunculopontine and laterodorsal tegmental nuclei when tracer injections were centered in the compact nigral subdivision than when infusions of about the same size were confined totally to the reticular part.

Infusions of the anterogradely transported tracer Phaseolus vulgaris leucoagglutinin into the pontomesencephalotegmental cholinergic complex resulted in uptake and transport of that label to both nigral subnuclei, and some of the Phaseolus vulgaris leucoagglutinin-accumulating somata and proximal processes also demonstrated choline acetyltransferase-like immunoreactivity. The Phaseolus vulgaris agglutinin-labeled entities in the substantia nigra exhibited terminal-like profiles that were reminiscent of the pattern of nigral choline acetyltransferase-positive puncta demonstrated immunohistochemically by use of nickel ammonium sulfate enhancement of the final reaction product in the avidin–biotin procedure.

These observations strongly support the contention that the pontomesencephalotegmental cholinergic complex is the major source of cholinergic projections to both the compact and reticular portions of the rat substantia nigra.

Innervation of the rat substantia nigra by cholinergic neurons (i.e., those that synthesize and use acetylcholine as an intercellular chemical messenger) is suggested by a considerable body of experimental evidence derived from the application of diverse methods.

First, activities of choline acetyltransferase (ChAT, EC 2.3.1.6) and levels of acetylcholine in the substantia nigra, although somewhat low in relation to other regions of the rat brain, appear significant. Activities of nigral ChAT have been calculated to range from 2.5 to 17.7% of the activity of this enzyme in the mammalian central nervous system. Similarly, the level of acetylcholine in the substantia nigra, calculated from values obtained from pooled nigral subdivisions, has been reported to be 18.3% of that in the caudate-putamen complex. Because the concentration of acetylcholine in the rat striatum is between 81 and 98 nmoles/g tissue, it is likely, therefore, that the substantia nigra contains a physiologically important amount of this cholinester (vide infra), a conjecture also compatible (1) with the observations of Jacobowitz and Goldberg that acetylcholine levels in the compact and reticular nigral subdivisions are 49 and 68%, respectively, of that in the caudate-putamen nucleus and (2) with the autoradiographic findings of Clarke et al. showing substantial acetylcholine binding in the substantia nigra, particularly in the compact portion. That these cholinergic indices are associated primarily with efferent fibers rather than with nigral somata and proximal processes (for caveats, see Gould and Butcher), is suggested by the results of Nagy et al. demonstrating that ChAT activity in the substantia nigra is unaltered following infusions into that region of kainic acid, a cytotoxin thought to produce selective degeneration of neuronal somata, and hence all of the processes of those cells, while leaving fibers deriving from loci extrinsic to the injected structure intact.

Second, both presumed muscarinic and nicotinic receptors have been found in association with the substantia nigra. Rotter et al. reported moderate binding densities of propylbenzilycholine, a potent muscarinic antagonist, in both nigral subdivisions, and Clarke et al. observed appreciable nicotine labeling in the compact part of the substantia nigra.

Abbreviations: ChAT, choline acetyltransferase; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PHAL, Phaseolus vulgaris leucoagglutinin; PMT, Pontomesencephalotegmental; WGA–HRP, wheat-germ agglutinin–horseradish peroxidase.
In contrast, the substantia nigra has been reported to demonstrate low binding levels of α-bungarotoxin, a toxin that, although believed by some investigators to demonstrate nicotinic receptors selectively, recently has been questioned in that regard (e.g., see commentary in Clarke et al.12 and Wonnacott et al.).46

Third, systemically or iontophoretically applied acetylcholine and nicotine excite neurons in the substantia nigra, presumably dopaminergic cells preferentially located in the compact part,17,30,31 that can be blocked by the centrally-acting nicotinic cholinergic antagonist mecamylamine (see Clarke et al.).12 Dose-dependent increases in the firing rates of nigral dopamine neurons produced by kainic acid injections into or near the ipsilateral pedunculopontine tegmental nucleus can also be antagonized by mecamylamine administered intravenously.15 Furthermore, intranigral infusion of carbachol inhibits, whereas atropine facilitates, striatal dopamine synthesis.47

And fourth, intranigral applied physostigmine produces stereotyped gnawing, thought by many investigators to be a dopaminergically mediated behavior, that can be antagonized by intraperitoneal administration of atropine49 (see also Ref. 16). Punctate application of physostigmine itself to the caudate-putamen complex or globus pallidus does not elicit such gnawing.40 On the basis of these observations, Smelik and Ernst4* suggested that "...cholinergic nerve fibers end synaptically on the dopaminergic nigral cells" (p. 1487), a conclusion rendered likely in view of recent electron microscopic studies.40,45 The association of acetylcholinesterase, the degradative enzyme of acetylcholine, with dopamine neurons in the substantia nigra8 is also compatible with such a conjecture, as are the observations of Clarke et al.13 indicated in the foregoing discourse.

Despite the collective strength of the preceding data in supporting the existence of a cholinergic projection to the substantia nigra, demonstration of such a pathway in the rat by use of neuroanatomic tract-tracing methods in combination with immunohistochemical procedures for ChAT, currently believed to be the most valid cellular indicator of cholinergic neurons, remains controversial. Woolf and Butcher41 suggested that a cholinergic projection to the substantia nigra derived from the pontomesencephalic tegmentum on the basis of combined fluorescent tracer and ChAT-immunohistochemical studies (see also Clarke et al.).13 The vast majority of neurons in other brain regions projecting to the substantia nigra did not demonstrate ChAT-like immunoreactivity,57 although in an infrequent number of rats a few (i.e., 1–3) such cells were observed in nucleus basalis (unpublished observations of this laboratory). These results were subsequently corroborated by Beninato and Spencer5 using the tract-tracing agent horseradish peroxidase in conjunction with ChAT immunohistochemistry and are compatible with the observations of Henderson and Greenfield21 that puncta demonstrating ChAT-like immunoreactivity are found in the ferret in both the compact and reticular portions of the substantia nigra but particularly in the former nigral subdivision. Nonetheless, Rye et al.37 on the basis of transport of intranigrally infused horseradish peroxidase conjugated to wheat-germ agglutinin (WGA–HRP) and ChAT immunohistochemistry, concluded recently that, in the rat at least, the pedunculopontine tegmental nucleus, defined by the authors as composed entirely of neurons displaying ChAT-like immunoreactivity, "...does not project to the substantia nigra..." (pp. 522, 523, italics theirs). Furthermore, no cartographers of central cholinergic systems2,22,38 have reported the existence of ChAT-positive fibers and puncta in any subdivision of the rat substantia nigra.

In this report, we extend previous observations relevant to the issue of a pedunculopontine tegmental–nigral cholinergic projection in the rat by using both anterograde and retrograde tract-tracing methods in combination with ChAT immunohistochemistry and a protocol for the enhanced demonstration of ChAT-like immunoreactivity. Furthermore, an attempt is made to determine the relative distribution of cholinergic fiber elements in both the compact and reticular parts of the substantia nigra.

**EXPERIMENTAL PROCEDURES**

**Experimental animals**

Forty-six female albino rats of the Sprague–Dawley strain (Simonsen Laboratories, Gilroy, CA, U.S.A.) were used. They were housed in stainless steel cages under conditions of constant temperature (22 °C) and relative humidity (50%). Food and water were available ad libitum. At the time of surgical procedures and killing, the rats weighed between 250 and 350 g.

**Sterotaxic infusion of retrogradely and anterogradely transported tract-tracers**

The rats were anesthetized with 350 mg/kg chloral hydrate administered intraperitoneally. Their heads were then shaved and mounted in a small-animal stereotaxic instrument (David Kopf Instruments, Tujunga, CA, U.S.A.). All intracerebral injections of tract-tracing agents were made unilaterally.

Three different retrogradely transported tracers, each detectable by fluorescence microscopy, were used in separate groups of experimental animals and were dissolved in distilled, deionized water in the following concentrations: True Blue, 10% (Sigma Chemical Co., St. Louis, MO, U.S.A.; n = 8 rats); propidium iodide, 20% (Sigma Chemical Co., St. Louis, MO, U.S.A.; n = 10 rats), and fluorogold, 2.5% w/v (n = 8 rats). All solutions were prepared immediately prior to use. In order to decrease solution time, propidium iodide solutions were sonicated for 10 min; the other solutions were prepared without sonication. Fluorescent tracers were chosen in the present experiments because of the advantages they offer when used in combination with the histochemical and immunohistochemical procedures employed by us (vide infra), as discussed extensively in prior publications from this laboratory. 6,46.
True Blue and propidium iodide were pressure injected as described previously by use of 1-μl syringes (Hamilton Co., Reno, NV, U.S.A.) with permanently attached stainless cannulas (outer diameter: 0.48 mm; inner diameter: 0.15 mm). The following volumes were used for each injection because they attenuated diffusion into adjacent nigral subdivisions and the ventral tegmental area while allowing adequate retrograde transport: True Blue, 0.1, 0.2 or 0.3 μl and propidium iodide, 0.1 or 0.3 μl. Rates of infusion ranged from 0.01 to 0.1 μl/min. In order to diminish flow of tracer along the cannula tract by capillary and suction action, the cannula was allowed to remain in place for 5 min following the termination of the intranigral infusion period before being withdrawn slowly.

Fluorogold was applied iontophotically in an attempt to minimize the size of the injection site even further. Glass micropipettes (tip diameter: 10–50 μm) were vacuum filled with solutions of fluorogold and intranigraly injected by delivering 5–15 μA pulses for 1 s with 1 s interpulse intervals for times ranging from 5 to 10 min as indicated essentially in Woolf et al.22

Infusions of retrogradely transported fluorescent tracers into the compact part of the substantia nigra were made in a plane perpendicular to the surface of the skull or at a 45° angle, whereas injections into the reticular nigral subdivision were performed only at an angle 45° from the horizontal stereotaxic plane. The latter angle of cannula insertion was selected in an attempt to minimize both tissue damage and transport of fluorescent tracer to the contralateral portion of the substantia nigra. The stereotaxic atlas of Paxinos and Watson23 was used for the initial selection of infusion sites. The cannulas were aimed at points within the substantia nigra 5.3 mm posterior to bregma and 2.5 mm lateral to the midline. The vertical distances from the cortical surface were 7.0 and 7.8 mm, respectively, for the compact and reticular nigral parcellations.

The retrogradely transported tracers, Phaseolus vulgaris leucoagglutinin (PHAL), was iontophotically infused into the pontomesencephalic tegmentum at various rostrocaudal levels where retrogradely labeled cells were found following intranigral injections of True Blue, propidium iodide, and fluorogold. Ten rats were used. Glass micropipettes (tip diameter: 10–30 μm) were vacuum filled with solutions of PHAL (Vector Laboratories, Burlingame, CA, U.S.A.) dissolved in 0.1 M phosphate-buffered saline (PBS) as described in Woolf et al.21 These tracer-containing pipettes were then introduced stereotaxically into the pontomesencephalic tegmentum perpendicular to the surface of the skull in the plane of Paxinos and Watson.21 PHAL was iontophotically infused according to the parameters indicated previously for fluorogold, but with total times of current application ranging from 15 to 25 min.

Following surgery, the animals were placed on a heating pad maintained at 30°C until they recovered from the anesthesia. They were then housed in stainless steel cages until they were killed, 48 h after intranigral introduction of retrogradely transported fluorescent tracers or 4 days following intracranial infusions of PHAL. These post-surgical times of killing have been found to produce optimal labeling of cells in the brain regions examined by us.

**Immunohistochemistry and fluorescent tracer histology**

**General procedures.** All brains injected with retrogradely or anterogradely transported labels were processed both for the tract-tracing agent and for ChAT. The rats were anesthetized first with intraperitoneally administered chloral hydrate (350 mg/kg) before being placed on a bed of ice and perfused through the heart with 50 ml cold (4°C) PBS (pH = 7.2) followed by 500 ml cold 4% paraformaldehyde dissolved in 0.1 M phosphate buffer. The brains were removed from the cranial cavities and placed into the paraformaldehyde–phosphate buffer fixative for an additional 90 min, at which time they were transferred to a cold 30% sucrose solution in 0.1 M phosphate buffer (pH = 7.2) where they remained for 2–5 days. The tissue was then cut in the transverse plane of Paxinos and Watson24 at 40 μm intervals on a freezing microtome. The resulting brain sections were then dewaxed and dehydrated in a series of PBS containing 0.3% Triton X-100 and were maintained in this solution at room temperature (22°C) for 24 h.

**Retrograde tracer histology and choline acetyltransferase immunohistochemistry.** Sections from brains into which retrograde tracers were infused were subsequently processed for ChAT as described in detail in Woolf and Butcher.25 In brief, the PBS–Triton X-100 solution was decanted, and a solution containing a monoclonal antibody against ChAT (code: 11/255; Eckenstein and Thoenen),26 diluted 1:50 in PBS containing 0.01% sodium azide, was added to the tissue. The brain sections were incubated with agitation in the primary antibody solution for 24–48 h at room temperature. At the end of this incubation period, the tissue was rinsed twice in PBS and then immersed in the secondary antibody solution which consisted of affinity purified anti-rat IgG conjugated to fluorescein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, MO, U.S.A.) diluted 1:100 in PBS–0.3% Triton X-100. Following 2 h incubation at room temperature in the secondary antibody solution, the brain sections were rinsed twice in PBS, mounted onto gelatinized glass slides, and coveredslipped under a medium of glycerine and PBS (3:1; v/v) containing 0.1 M n-propyl gallate to retard fading of fluorescence.

Orange-red neuronal somata labeled with propidium iodide were visualized with transmitted illumination in a Zeiss fluorescence microscope equipped with a combination LP 520 and KP 560 excitation filter and an LP 590 barrier filter. Both True Blue and fluorogold fluorescence was observed with a BG 365 excitation filter and an LP 435 barrier filter. The yellow–green FITC label signaling the presence of ChAT was visualized with epi-illumination and standard Zeiss filters. Neurons demonstrating fluorescent retrograde tracer and ChAT, both alone and within the same cell, were identified sequentially on the same tissue section by changing the filter combinations. For each experimental animal, the sites and extents of diffusion of the fluorescent labels within the substantia nigra were projected on paper and traced from individual brain sections by use of a Zeiss drawing tube (camera lucida) and fluorescent drawing pens illuminated by an ultraviolet black light.

**Anterogradely transported tracers and choline acetyltransferase immunohistochemistry.** The double immunohistochemical method demonstrating PHAL and CHAT on the same tissue section, used to confirm the uptake of PHAL by cholinergic cell bodies, has been described extensively in Woolf et al.21 In brief, brain sections were transferred from PBS–Triton X-100 to a solution containing goat polyclonal antibodies against PHAL (Vector Laboratories, Burlingame, CA, U.S.A.) dissolved in PBS containing 0.1% sodium azide and rabbit monoclonal antibodies against ChAT (code: 11/255; Eckenstein and Thoenen),26 diluted 1:50 in the same solvent. The tissue was incubated in this medium with gentle agitation for 48 h. This and all subsequent incubations were performed at room temperature. The brain sections were then rinsed twice in PBS and reacted first with FITC-conjugated anti-goat IgG (Sigma Chemical Co., St. Louis, MO, U.S.A.), diluted 1:1000 in PBS containing 0.1% sodium azide, and rat monoclonal antibodies against ChAT (code: 11/255; Eckenstein and Thoenen),26 diluted 1:50 in the same solvent. The tissue was incubated in this medium with gentle agitation for 48 h. This and all subsequent incubations were performed at room temperature. The brain sections were then rinsed twice in PBS and reacted first with FITC-conjugated anti-goat IgG (Sigma Chemical Co., St. Louis, MO, U.S.A.), diluted 1:1000 in PBS containing 0.1% sodium azide, and rat monoclonal antibodies against ChAT (code: 11/255; Eckenstein and Thoenen),26 diluted 1:50 in PBS–0.3% Triton X-100, for 60 min and then, following two additional PBS rinses, with rhodamine-conjugated anti-rat IgG (Cooper Biomedical Co., Malvern, PA, U.S.A.), diluted 1:10 in 0.3% Triton X-100 for an additional hour. Following two more rinses in PBS, sections were mounted on glass slides, coverslipped as described previously in this manuscript, and viewed with darkfield or fluorescent microscopy. With this protocol, sites of CHAT-like immunoreactivity fluoresced red, and loci containing PHAL fluoresced yellow–green. Both ChAT and PHAL on the...
same brain section were photographed on the same or different frames of film following a simple change of
epi-illumination conditions involving sliding in and out of
a standard Zeiss filter cassette.

In order to further demonstrate specific staining, some brain sections were processed immunohistochemically for PHAL only according to the avidin–biotin procedure described essentially in
Gould and Butcher.26 In brief, tissue was washed in three
rinses of PBS followed by incubation in a solution of polyclonal antibodies against PHAL (vide supra). The brain sections were rinsed thereafter in several changes of PBS and placed into diluted normal rabbit serum (1:80 in PBS) at 22°C for 1 h in order to minimize non-specific binding
of the secondary antibody. Following subsequent rinses in
PBS, the tissue was incubated at room temperature for 2 h
in biotinylated rabbit anti-goat IgG (Vector Laboratories,
Burlingame, CA, U.S.A.) diluted 1:200 in PBS, rinsed
again in PBS and placed into a diluted solution (1:50 in
0.3% Triton X-100) of avidin–horseradish peroxidase
(Vector Laboratories, Burlingame, CA, U.S.A.) for 2 h.
They were then rinsed in PBS and reacted for 15 min in a
solution containing equal quantities of diaminobenzidine in
0.1 M Tris buffer (pH = 7.4) and 0.02% H2O2 in distilled,
deionized water. Finally, the sections were rinsed in PBS,
mounted onto glass slides coated with pig gelatin, dehy-
drated in 100% ethanol, cleared in xylene, and coverslipped
under Permount (Fisher Scientific Co., Fairlawn, NJ,
U.S.A.).

Enhanced visualization of choline acetyltransferase. A slightly different immunohistochemical protocol (vide infra)
and a different monoclonal antibody to those described
previously were used to demonstrate ChAT-positive fibers
and puncta optimally. In all other respects, this latter
method was equivalent to that outlined for the avidin–
biotin procedure described in the preceding section of this
manuscript. Because the ChAT antibody was generated in
mouse, however, the second antibody employed by us was
directed against that species.

Rats (n = 10) were anesthetized with 350 mg/kg chloral hydrate administered intraperitoneally and were then
placed on ice. They were perfused through the heart with
50 ml of cold PBS followed by 500 ml of cold 4% para-
formaldehyde containing 7.5% saturated picric acid dis-
solved in 0.1 M phosphate buffer (pH = 7.6). The brains
were removed from the skull and additionally postfixed in
a solution of the same composition as the perfusate for 2 h
before being cut submerged in a PBS bath on a Vibratome
(Lancer Co., St. Louis, MO, U.S.A.) at 50 pm intervals.
Sections were collected into PBS, stored in that solution at
4°C for 24 h, and then incubated with anti-ChAT mono-
clonal antibody 1F6 (for characterization, see Crawford
et al.).15 Sites of ChAT positivity were demonstrated by use
of the avidin–biotin procedure described previously with
the addition of a 2.5% solution of the enhancing agent,
nickel ammonium sulfate, to the final reaction (see German
et al.).19

Immunohistochemical controls. A number of different
control protocols were used, some common to all immunohistochemical methods used in the current experiments and
others applicable to specific procedures. First, in an attempt
to determine whether or not secondary antibody immuno-
globulins produced spurious staining, some brain sections
in experiments in which either PHAL or ChAT were
processed with the fluorescein or peroxidase methods
were processed precisely as indicated in those previously
described protocols except that the corresponding primary
antibodies were omitted from the incubation medium.
Second, in order to insure that the primary antibodies
employed by us yielded specific staining, several tissue
sections in all immunohistochemical experiments were incu-
bated exactly as detailed in the appropriate section else-
where in this manuscript but with the substitution of
normal serum mouse, rat, or goat IgGs for primary
antibodies. Third, some brain sections in the avidin–biotin
procedure were incubated with hydrogen peroxide and
methanol following incubation in biotinylated antibody
in order to destroy endogenous peroxidase activity.24

Finally, in an effort to further eliminate the possibility
of non-specific staining contributing to peroxidase labeling
in the substantia nigra with the use of the avidin–biotin
method, several brain sections were incubated, as described
previously for mouse monocolonal antibody 1E6, with goat
anti-mouse IgGs that had been adsorbed to rat immuno-
globulins (i.e., species-specific second antibodies: American
Qualex Co., La Mirada, CA, U.S.A.), as indicated in
Houser et al.24

RESULTS

Controls

Omission of the primary antibodies in all immuno-
histochemical experiments abolished staining, as did
incubation with normal serum immunoglobulins in place of primary antibodies. Brain sections incubated
with hydrogen peroxide and methanol in the avidin–biotin procedure exhibited shrinkage, but no alterations in the patterns or intensities of ChAT-like
or PHAL immunoreactivites were observed in the
substantia nigra or other structures in the central nervous system examined by us. Although species-
specific second antibodies (for example of use, see
Fig. 1) reduced background staining somewhat in the
substantia nigra, the nature and distribution of ChAT-like immunoreactivity remained unchanged
to non-rat IgG adsorbed mouse second antibodies.

Distribution of choline acetyltransferase-positive
puncta in the substantia nigra

Although structural elements suggestive of termi-
nals and demonstrating ChAT-like immunoreactivity
could not be visualized consistently in the substantia
nigra by use of the avidin–biotin or fluorescent
second antibody procedures alone, such puncta
were observed reliably in nigral material processed
according to the avidin–biotin method with nickel
ammonium sulfate intensification (Fig. 1). These
ChAT-positive puncta were more numerous in the
compact part of the substantia nigra than in the
reticular subdivision (Fig. 1A; compare with Fig. 1B)
and, on the basis of unpublished observations of this
laboratory, were similar in morphologic organization
and size, 1–2 μm in diameter, not only to terminal-
like entities immunostaining for ChAT in known
projection regions of the pontomesencephalo-
tegmental (PMT) cholinergic complex (e.g., certain
nuclei of the thalamus),23 but also to presumed
cholinergic terminals in target structures of the basal
forebrain cholinergic system (e.g., the cerebral
corpus).23 The density of ChAT-positive puncta in the
compact part of the substantia nigra was comparable
to that seen in the reticular thalamic nucleus, whereas
the distribution of such terminal-like elements in the
reticular nigral subdivision resembled that observed
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Fig. 1. Distribution of ChAT-positive puncta in the compact (SNC, frame A) and reticular (SNR, frame B) portions of the substantia nigra demonstrated by use of nickel ammonium sulfate enhancement of the reaction product in the avidin-biotin procedure and species-specific second antibodies. Examples of such puncta are indicated by arrows. Transverse brain sections. Scale bar: 20 μm (applies to both frames).

in the posterior nuclear group of the thalamus (unpublished observations of this laboratory).

Cholinergic projections to the substantia nigra revealed by retrograde tract tracing

All injections of True Blue, propidium iodide, and fluorogold were confined to the substantia nigra with the exception of the largest volume, 0.3 μl, of True Blue and propidium iodide. With this latter amount there was slight diffusion in some cases into the underlying cerebral peduncle. Although some animals displayed tracer in both the compact and reticular parts of the substantia nigra, the majority of rats receiving 0.1 or 0.2 μl True Blue, 0.1 μl propidium iodide, or iontophoretic application of fluorogold had deposits of label that were confined either wholly to the reticular nigral subdivision or importantly to the compact part (e.g., Fig. 2) depending on which subnucleus was targeted; it is these cases that were analysed in detail in the present study. The narrowness of the latter nigral component, however, made it impossible, at least in our hands, to restrict injections entirely to that region. Little, if any, diffusion of label was noted along the cannula tract, particularly following iontophoretic application.

True Blue, propidium iodide, and fluorogold injected into the substantia nigra all yielded comparable patterns of retrograde cell labeling (for detailed discussion and analysis of the totality of brain regions so labeled, see Woolf and Butcher). Consonant with our previous observations, neuronal somata containing retrograde tracer and demonstrating ChAT-like immunoreactivity were found in the PMT cholinergic complex (Figs 3–6), which is composed of cholinergic cells associated with the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus (Fig. 4; for further analysis and discussion of topography and terminology, see Woolf and Butcher). Such double-labelled cells, all medium (>15 μm but <20 μm) to large (>20 μm) in maximum soma extent (cf., Rye et al.), were associated to a greater extent with the pedunculopontine tegmental nucleus than with the laterodorsal tegmental nucleus (Figs 5 and 6) and were
found predominantly ipsilateral to the side of tracer injection, although a few were observed contralaterally (Figs 5 and 6). Consonant with the comparative distribution of presumed cholinergic puncta in the substantia nigra (Fig. 1), approximately 2–3 times as many ChAT-positive neurons in the PMT cholinergic complex were labeled following tracer infusions centered in the compact as opposed to the reticular subnucleus (Fig. 6; compare with Fig. 5). Smaller injection volumes of tracer (e.g., iontophoretically applied fluorogold, 0.1 μl of True Blue and propidium iodide) resulted in the labeling of fewer ChAT-positive PMT cells than did larger injections. For example, injections of 0.2 μl True Blue, which also were confined wholly to the substantia nigra, resulted in the retrograde labeling of significantly more ChAT-containing PMT cells (not illustrated) than did smaller volume injections such as those illustrated in Figs 5 and 6. Still larger injection volumes (i.e., 0.3 μl of True Blue and propidium iodide) resulted in even greater numbers of ChAT-positive PMT cells being retrogradely labeled, but these larger volumes sometimes resulted in a small amount of dye diffusion into neural regions adjacent to the substantia nigra.

**Cholinergic projections to the substantia nigra revealed by anterograde tract tracing**

An example of the extent of diffusion of iontophoretically applied PHAL in the pedunculopontine region is shown in Fig. 7. Such infusions (Fig. 8A) resulted in the local accumulation of the anterograde tracer by several neurons also demonstrating ChAT-like immunoreactivity (Fig. 8C, D), and PHAL-labeled fibers could be traced into both the compact and reticular nigral subnuclei (Figs 8B and 9). Some of these fibers demonstrated bouton-like protuberances reminiscent of ChAT-positive puncta in the substantia nigra (Fig. 9; compare with Fig. 1) which, according to the electron microscopic observations of Wouterlood and Groenewegen, represent regions of synaptic contact.

**DISCUSSION**

The results of the current neuroanatomic experiments are highly compatible with the suggestion,
Fig. 3. Examples of True Blue-labeled somata in the pedunculopontine (arrow, frame A) and laterodorsal (arrows, frame C) tegmental nuclei that also demonstrate ChAT-like immunoreactivity (arrows, frames B and D; FITC second antibody label) following infusions of that retrograde tracer confined to the reticular (corresponding frames: A, B) or centered in the compact (corresponding frames: C, D) portions of the substantia nigra. Frames (A) and (B) depict the same transversely cut brain section under different conditions of illumination and visualization, and the identical situation applies to (C) and (D), although in the latter two frames the tissue section is different from that in (A) and (B). Arrowhead in frame (A) points to an example of a True Blue-labeled cell body that is not positive for ChAT (B). Scale bar in (A) is 50 μm and applies to all frames.

Fig. 4. Transverse section of the pontomesencephalotegmental cholinergic complex at a level depicting the two major nuclear components of the system, the pedunculopontine tegmental (PPT) and the laterodorsal tegmental (LDT) nuclei, as well as the dorsal (PPTd) and the ventral (PPTv) subdivisions of the PPT nucleus formed by the lateral encroachment of the superior cerebellar peduncle (scp). Transverse brain section. Avidin–biotin method for ChAT without nickel ammonium sulfate enhancement. For a complete rostrocaudal portrayal of the pontomesencephalotegmental cholinergic complex, see Figs 2–7 in Woolf and Butcher. Scale bar: 300 μm. Aq, cerebral aqueduct.
derived from and mutually reinforced by an amalgam of diverse neurobiologic data (see Introduction), that cholinergic neurons in the pedunculopontine and laterodorsal tegmental nuclei project to the substantia nigra and that the PMT cholinergic complex is the major source of such nigral cholinergic innervation, as demonstrated first in a previous publication from this laboratory. A cholinergic projection from the PMT region to both sectors of the substantia nigra has been more completely and precisely documented in the present study by the use of immunohistochemical visualization of ChAT combined with an anterograde tracer, as well as with three different retrograde tracers, all of which produced retrograde...
Fig. 6. Distribution of True Blue-labeled neuronal somata simultaneously demonstrating ChAT-like immunoreactivity in the pontomesencephalotegmental cholinergic complex following infusions of that retrograde fluorescent tracer aimed for the compact part of the substantia nigra. Each dot represents one co-labeled cell body. The extent of diffusion of the injected tracer in the substantia nigra for this pattern of labeled somata is shown in Fig. 2D–F. For further details and abbreviations, see legend of Fig. 5.

Fig. 7. Example of extent of diffusion of *Phaseolus vulgaris*-leucagglutinin infused into the region of the pedunculopontine tegmental nucleus (PPT). Transverse brain section (*cf.* Fig. 4). Avidin–biotin method without nickel ammonium sulfate enhancement. Aq, cerebral aqueduct. Scale bar: 500 μm.
Fig. 8. Sagittal brain sections through the pons and mesencephalon depicting the pedunculopontine segmental nucleus (PPT) and substantia nigra. The rostral direction is to the right side of each frame. Frame (A) shows the distribution of ChAT-positive somata demonstrated according to the avidin–biotin method without nickel ammonium sulfate enhancement. The box in (A) delineates the field represented in higher power magnification in frames (B)–(D), which are taken from a brain section adjacent to that shown in (A). Frame (B) shows fluorescent neuronal somata and other subcellular entities that have accumulated Phaseolus vulgaris-leucoagglutinin (PHAL) following infusion of that anterograde tracer into the PPT (FITC second antibody label), and black-on-white arrows point to fluorescent fibers in the reticular part of the substantia nigra (SNR). Larger white arrow indicates a cell body containing PHAL, portrayed in higher power magnification in frame (C) (top arrow), that also demonstrates ChAT (rhodamine second antibody label; top arrow, D). Remaining black-on-white arrows in (C) and (D) depict on the same brain section other neuronal somata demonstrating PHAL (C) and ChAT (D). Scale bars: frame (A) 500 μm; frame (B) 100 μm; frames (C) and (D) 25 μm.
Cholinergic projections to substantia nigra

Fig. 9. Examples of *Phaseolus vulgaris*-leucoagglutinin-containing fibers and puncta (arrows) in the compact (A) and reticular (B) parts of the substantia nigra following injections of that anterograde tracer into the region of the pedunculopontine tegmental nucleus. Avidin–biotin method without nickel ammonium sulfate enhancement.

Scale bar in (B) is 20 μm and applies also to (A).

labeling of cholinergic PMT cells, even after injections of small volumes of dye that were confined wholly to the substantia nigra. Although predominantly ipsilateral, these projections also enforced a small contralateral component, a result in agreement with our earlier findings and with those of Beninato and Spencer and Clarke et al.

Fewer ChAT-positive cells were found to provide cholinergic afferents to the substantia nigra in the present study than in the experiments of Woolf and Butcher, probably due to the generally smaller infusion volumes used in the current investigation. In the present study, the number of retrogradely labeled ChAT-immunoreactive PMT cells increased as the volume, and hence the amount, of tracer infused was increased. It is possible, therefore, that the full extent of cholinergic PMT neurons projecting to that nucleus could be greater than suggested by the current results, since only a portion of the substantia nigra was injected in any one case.

The present experimental series has also shown, in extension of previous observations, that terminal-like ChAT-positive puncta in the rat are more numerous in the compact than in the reticular nigral subnucleus, a finding similar to that in the pigmented ferret. Although a few somata (i.e., 1–5) demonstrating ChAT-like immunoreactivity have been detected within the traditional boundaries of the rat substantia nigra at caudal-most levels, it is likely that these are ectopically located cell bodies of the pedunculopontine tegmental nucleus rather than components of nigral cholinergic neurons.

The present findings fail to support the conjecture of Rye et al. that the cholinergic pedunculopontine tegmental nucleus does not project, however dimmitively, to the substantia nigra (see Introduction), and several methodologic and interpretative differences may account for this discrepancy. First, although Rye et al. employed the peroxidase–antiperoxidase staining procedure for their ChAT immunohistochemical experiments, they did not use the more sensitive double bridge variation of that technique. As pointed out by Henderson and Greenfield, who used the same monoclonal antibody against ChAT as Rye et al., the "... adoption of a less sensitive procedure for ChAT immunohistochemistry (for example, by omission of the double bridge step in the peroxidase–antiperoxidase method) gave rise to fragmentary or very faint staining for ChAT in axons, dendrites, and small cell bodies..." (p. 110, words in parentheses theirs).

Second, Rye et al. used WGA–HRP as the tract-tracing agent, and it is known that this conjugate can be transported trans-synaptically in both retrograde and anterograde directions in a variety of neuronal systems. Such trans-synaptic transport could result in loss of WGA–HRP from some cells originally containing the label and/or accumulation of WGA–HRP by other neurons originally devoid of the tracer (i.e. both false-negative and false-positive results). Rye et al. do not address this potential limitation of their method, however. Finally and somewhat surprisingly, Rye et al. do, in fact, find ChAT-positive neurons in the pedunculopontine tegmental nucleus that project to the substantia nigra following intranigral infusions of WGA–HRP claimed to be restricted entirely to that structure, although not specifically to either the compact or reticular subdivision (case R21; Fig. 28 in Rye et al.).

Notwithstanding the methodologic caveats indicated previously, it is difficult to reconcile these last-mentioned observations with the pronouncement of the authors in the same article that no cholinergic neurons in the pedunculopontine tegmental nucleus project to the substantia nigra (pp. 522–523 in Rye et al.).

Careful consideration of the preceding data and arguments supports strongly the contention that cholinergic projections exist to both the compact and reticular nigral subdivisions. Such a neuroanatomic configuration provides the chemomorphologic substrate necessary (1) to interpret the diversity of biochemical, pharmacologic, physiologic, and behavioral findings relevant to cholinergic influences on the substantia nigra (see Introduction) and (2) to account for possible direct and indirect effects of the PMT cholinergic complex on extrapyramidal motor function, an area of investigation still in a nascent state.
Acknowledgements—This research was supported by USPHS grant NS 10928 to L.L.B. Drs Paul Salvaterra and Felix Eckenstein are thanked for generous supplies of monoclonal antibodies against choline acetyltransferase, and Larry Schmued is thanked for his gift of fluorogold.

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(Accepted 26 July 1988)