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# Cholinergic Systems in the Rat Brain: II. Projections to the Interpeduncular Nucleus

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WOOLF, N. J. AND L. L. BUTCHER. *Cholinergic systems in the rat brain: II. Projections to the interpeduncular nucleus.* BRAIN RES BULL 14(1) 63–83, 1985.—The cholinergic innervation of the interpeduncular nucleus was investigated by use of fluorescent tracer histology in combination with choline-O-acetyltransferase (ChAT) immunohistochemistry and acetylcholinesterase (AChE) pharmacohistochemistry. Following propidium iodide or Evans Blue infusion into the interpeduncular nucleus, brains were processed for co-localization of transported fluorescent label and ChAT and AChE. Control infusions of tracers were made into the ventral tegmental area. In order to delimit the course of putative cholinergic afferents to the interpeduncular nucleus from extra-habenular sources, knife cuts surrounding the habenular nuclei were performed. Somata containing propidium iodide that were highly immunoreactive for ChAT were found primarily in the vertical and horizontal limbs of the diagonal band, the magnocellular preoptic area, and the dorsolateral tegmental nucleus, also referred to as the laterodorsal tegmental nucleus. A few such co-labeled somata were also detected in the medial septal nucleus, substantia innominata, nucleus basalis, and pedunculopontine tegmental nucleus. A good correlation was observed between intensely-staining, AChE-containing and ChAT-positive neurons projecting to the interpeduncular nucleus from the aforementioned structures. Although the medial habenula contained numerous cells demonstrating transported label following interpeduncular infusion of fluorescent tracers, the ChAT-positivity associated with somata in that nucleus was weak compared to ChAT-like immunoreactivity in known cholinergic neurons in the basal forebrain and brainstem. Knife cuts that separated the habenular nuclei from the stria medullaris and neural regions lateral and posterior to those nuclei while leaving the fasciculus retroflexus intact resulted in a reduction of ChAT-like immunoreactivity in the medial habenular nucleus, fasciculus retroflexus, and interpeduncular nucleus. These data suggest (1) that the cholinergic innervation of the interpeduncular nucleus derives primarily from ChAT-positive cells in the basal forebrain and dorsolateral tegmental nucleus and (2) that putative cholinergic fibers having their origin in the medial habenula, if they exist, constitute a minor portion of the cholinergic input to the interpeduncular nucleus.

Choline acetyltransferase immunohistochemistry Basal forebrain Dorsolateral tegmental nucleus
Interpeduncular nucleus Medial habenula Fluorescent tracers

THE interpeduncular nucleus (IPN) is an unpaired structure that lies at the base of the mesencephalon. It is interconnected with various limbic system structures and possesses the highest concentrations of acetylcholine [79] and the greatest activities of choline-O-acetyltransferase (ChAT, EC 2.3.1.6) [60, 62, 72, 79], acetylcholinesterase (AChE, EC 3.1.1.7) [60], and high affinity choline uptake [72] of any region in the mammalian brain. Despite the probable importance of cholinergic mechanisms in the IPN, its cholinergic neuroanatomy is incompletely understood. Immunohistochemical studies have revealed that ChAT, thought by many investigators to be the most specific marker of cholinergic neurons currently available, is associated with fibers and terminals and not cell bodies in the IPN [38], suggesting that the cholinergic innervation of the structure derives from extra-interpeduncular sources. Among the neural regions implicated prominently in this regard have been the habenular complex and basal forebrain, particularly the former structure. Indeed, numerous studies have demonstrated projections from the habenula to the IPN via the fasciculus retroflexus [1, 15, 17, 33, 36, 52, 58, 61, 70, 84], with experiments designed to differentiate medial from lateral habenular projections showing that interpeduncular afferents have their origin primarily in the medial nucleus. In one study, IPN afferents were reported to derive exclusively from the medial habenula [52], but most investigators have observed a sparse projection from the lateral habenula as well [1, 15, 70].

Although the existence of an habenulo-interpeduncular pathway seems well documented, the cholinergic nature of that fiber bundle has not been correspondingly evinced. First, results from recent histochemical experiments have not established unequivocally the existence of cholinergic somata in the habenular complex. Using a monoclonal antibody against ChAT and intensification of the reaction product by multiple bridge labeling and osium treatment, Houser and her associates [38] apparently replicated the earlier observations of Hattori *et al.* [32] by demonstrating

dense ChAT-like immunoreactivity associated, in part, with cell bodies in the medial habenula but not the lateral nucleus. Scattered ChAT-positive puncta were found in the lateral habenula, however [38]. In partial contradistinction, Kimura and his collaborators [44], using a polyclonal preparation, observed no ChAT-immunoreactive perikarya in either the medial or lateral habenula, although they did indicate the presence of presumed cholinergic terminals in both nuclei. The absence of cell bodies displaying ChAT-like immunoreactivity in both divisions of the habenular complex has also been reported with the monoclonal preparation of Satoh *et al.* [68]. Finally, perikarya in the medial and lateral habenula have not been found to demonstrate intense AChE staining after irreversible inhibition of that enzyme by systemically administered *bis*(l-methylethyl) phosphorofluoridate (DFP) [9, 10, 24, 68]. Strong cellular enzyme activity following application of the pharmacohistochemical regimen for AChE signals cholinergic neurons in most regions of the nervous system [9, 10, 24, 68].

Second, although the medial habenula appears to be the major origin of the habenulo-interpeduncular tract (*vide supra*) and although extensive lesions of the habenular complex produce almost complete loss of ChAT activity in the IPN [18, 42, 47, 48, 53], knife cuts separating the medial from the lateral habenula result in a 77% reduction of ChAT in the former nucleus [18], suggesting that most of the cholinergic synthetic enzyme in the medial habenula derives from extrinsic sources. Indeed, Cuello *et al.* [18] have concluded that the ". . . lateral habenular nucleus . . . is the source of cholinergic projection to the interpeduncular nucleus and to the medial habenular nucleus" (p. 413). Similarly, Flumerfelt and Contestible [26] have reported that infusions of kainic acid, a cytotoxin thought to preferentially destroy neuronal somata, into the lateral habenula deplete AChE activity in the fasciculus retroflexus on the injected side, again implicating the lateral habenula as a primary source of cholinergic fibers to the IPN. In another study, however, kainic acid pathology restricted primarily to the lateral habenula but involving some cell loss in dorsal aspects of the medial nucleus produced a reduction of substance P in both the habenular complex and the IPN but left ChAT activities in the two regions unchanged [78], suggesting that cholinergic fibers afferent to the IPN do not arise from somata in either the lateral or dorsomedial habenula. The reduction of substance P in the IPN can be explained on the basis of damage to perikarya in the medial habenula because substance P-containing cell bodies in that nucleus have been suggested to project to the IPN via the fasciculus retroflexus [18,59].

Although the existence of a cholinergic habenulo-interpeduncular pathway eschews certainty on the basis of currently available experimental evidence, acetylcholine-containing projections to the IPN from the medial septal nucleus and diagonal band appear better established. First, these two regions of the rostral basal forebrain are known to contain numerous neurons demonstrating ChAT-like immunoreactivity [4, 38, 56, 57, 68, 83] and staining intensely for AChE after a DFP challenge [5, 9, 10, 68, 81, 83]. And second, electrolytic lesions encompassing the medial septal nucleus and diagonal band, as well as ablations in the stria medullaris, result in an approximately 50% reduction of ChAT activity in both the medial habenula and IPN of the rat [16,27], with lesser enzyme decrements being observed in the rabbit IPN [43]. Somewhat expectedly, however, retrograde tract-tracing studies have both confirmed and denied the existence of basalo-interpeduncular projections: whereas Contestible and Flumerfelt [15] and Hayakawa and Zyo [33] found labeling of diagonal band cells following horseradish peroxidase injections into the IPN, Marchand *et al.* [52] could not replicate that observation unless the tracer also invaded the adjacent ventral tegmental area. Similarly, some investigators have found labeled terminals in the IPN following infusions of radioactive amino acids into the diagonal band region [20,46], but, in other studies, comparable injections have resulted in anterograde labeling of fibers terminating only in the ventral tegmental area and not in the IPN [14,55].

Despite the probable existence of cholinergic basalo-interpeduncular pathways, presumed acetylcholine-containing neurons in the medial septal nucleus and diagonal band apparently account only for 50% of the ChAT activity in the IPN [16,27]. From what neural region or regions, then, does the remaining 50% derive? Although the medial habenula must be considered as a potential candidate in this regard, currently available experimental evidence does not support such a conjecture compellingly (*vide supra*), and alternative hypotheses should be entertained. Among the other possible sources of cholinergic afferents to the IPN are the ChAT-positive and intensely AChE-reactive somata associated with the brainstem cholinergic complex comprised prominently of the pedunculopontine and dorsolateral tegmental nuclei [9, 10, 24, 68, 81, 83]. Indeed,the dorsolateral tegmental nucleus, also referred to as the laterodorsal tegmental nucleus, has been shown by horseradish peroxidase hodology to project to the IPN [15,52], but the possibility that this pathway, or a subset of it, is cholinergic has not been tested.

In the present study, a preliminary report of which has been published [12], we attempt to elucidate the cholinergic innervation of the IPN by infusing fluorescent tracers into the IPN and processing the same tissue section both for retrogradely transported label and ChAT demonstrated immunohistochemically or AChE visualized by the pharmacohistochemical regimen [7]. To the best of our knowledge, these methodologies have not been applied previously to analyses of the cholinergic projections to the IPN. The contribution of possible habenular and non-habenular cholinergic neurons to the innervation of the IPN was assessed by making knife cuts circumscribing the habenula and processing the tissue for ChAT-like and, as a control, substance P-like immunoreactivity.

## METHOD

### *Animals*

Eighteen female rats of the Sprague-Dawley strain (Simonsen Laboratories; Gilroy, CA) were used. The animals weighed 250–320 g at the time of surgery and were housed under conditions of constant temperature (22°C) and relative humidity (50%). They were maintained on a 12 hr light-dark cycle; surgical procedures and animal euthanasia were performed during the light phase of the cycle (6:00–18:00 hr).

### *Stereotaxic Surgical Procedures*

For all surgical procedures, the rats were anesthetized with 350 mg/kg chloral hydrate injected intraperitoneally. Their heads were shaved and then mounted by use of ear plugs within a Kopf stereotaxic instrument (David Kopf Instruments; Tujunga, CA).

*Intracerebral infusion of fluorescent tracers.* Two different neuronally transported fluorescent labels, propidium iodide and Evans Blue, were used. Although Evans Blue produced morphologically superior results compared to propidium iodide [83], it was also less compatible with immunohistochemical procedures (see also [76]). For this reason, the former tracer was used primarily in combination with AChE histochemistry, with which it is compatible [5, 80, 81], and propidium iodide was used in conjunction with ChAT and substance P immunohistochemistry (*vide infra*). For further discussion of polyhistochemical methods, see Butcher [7].

A 30% solution of Evans Blue (Matheson, Coleman, and Bell Co.; Norwood, OH) or a 20% solution of propidium iodide (Sigma Chemical Co.; St. Louis, MO), dissolved in distilled deionized water, was prepared. One-$\mu$l syringes (Hamilton Co.; Reno, NV) with permanently attached stainless steel cannulas (outer diameter: 0.48 mm; inner diameter: 0.15 mm) were used to inject total volumes of 0.3, 0.5, or 1.0 $\mu$l of propidium iodide or 0.05 $\mu$l of Evans Blue. Infusion rates ranged from 0.01–0.2 $\mu$l/min. The cannula was allowed to remain in place for 10 min following the termination of the injection period before being withdrawn slowly.

The interpeduncular nucleus was approached from the dorsal surface of the rat cranium 6.3 mm posterior to bregma, at the midline, and 9.3 mm ventral to the surface of the skull. The rats were positioned so that the surface of their skulls was flat according to the parameters outlined in Paxinos and Watson [64]. Control infusions of propidium iodide or Evans Blue were made unilaterally into the ventral tegmental area at the following stereotaxic coordinates [64]: posterior to Bregma, 5.8 mm; lateral, 0.6 mm; and vertical from the skull surface, 8.0 mm.

Following intracranial introduction of propidium iodide or Evans Blue, the animals were placed on a heating pad maintained at 30°C until they recovered from the anesthetic. They were then housed individually in stainless steel cages until they were sacrificed 48 hr later and their brains subsequently processed histochemically and immunohistochemically (*vide infra*). This time interval has been found to produce optimal labeling of neurons in all of the regions examined by us in the present experiments.

*Knife cuts circumscribing the habenula.* In an attempt to assess the contribution of fibers deriving from extrahabenular sources to the cholinergic innervation of the interpeduncular nucleus, knife cuts were made bilaterally that separated the habenular complex from the neural tissue lying immediately anterior, lateral, and posterior to it (Fig. 1) prior to immunohistochemical procedures. Four rats were used. They were anesthetized and placed in a stereotaxic instrument as described previously in this report. A small animal knife (David Kopf Instruments; Tujunga, CA) was then used to make cuts defining the transverse and sagittal faces of a parallelepiped encompassing the habenular complex (Fig. 1). Attempts were made to avoid encroaching upon the fasciculus retroflexus. Four holes were drilled in the skull, two at the anterior intersections of the sides of the horizontal rectangular plane and two at the posterior corners. The two rostral holes were 2.3 mm posterior to Bregma and 2.0 mm on either side of the midline. Following careful incision of the dura mater, the knife was lowered with the blade retracted to a depth of 6.0 mm below the surface of the skull. Two cuts at each hole were made on either side of the brain. In one set of transections the blade was extended toward the midline, and the knife was lifted to 3.5 mm below the skull

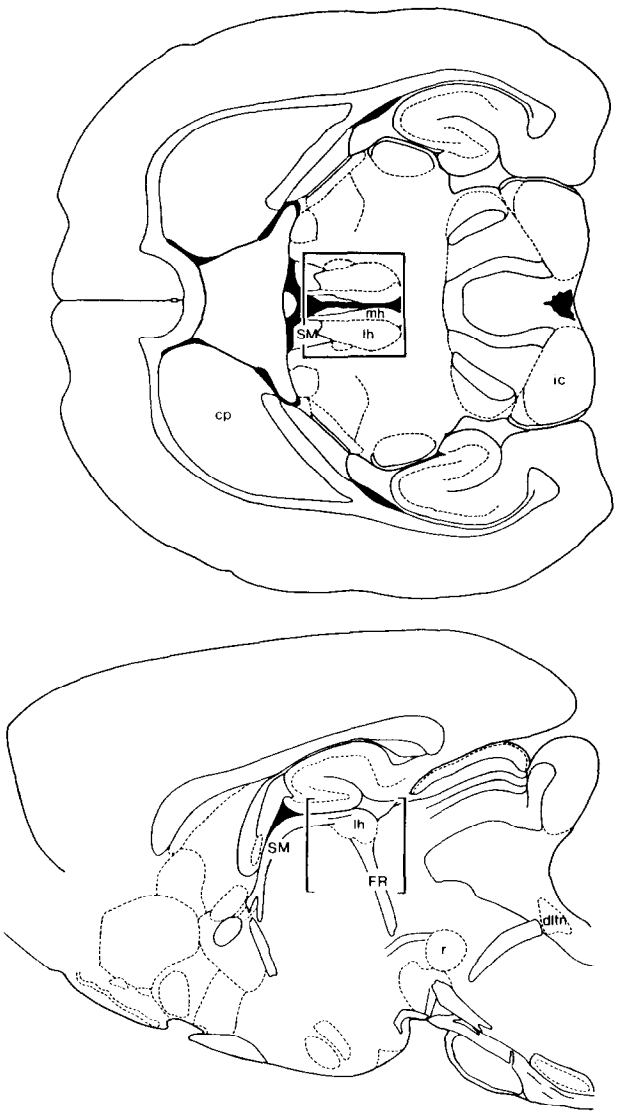


FIG. 1. Representation of the anterior-posterior and lateral (black rectangle, top diagram) and the dorsal-ventral (bracketed black lines, bottom diagram) extents of the knife cuts circumscribing the habenular complex. Both the horizontal section depicted at the top and the saggital section indicated below it are redrawn from Paxinos and Watson [64]. cp, caudate-putamen complex; dltn, dorsolateral tegmental nucleus; FR, fasciculus retroflexus; ic, inferior colliculus; lh, lateral habenula; mh, medial habenula; r, red nucleus; SM, stria medullaris.

surface and then lowered back to 6.0 mm a total of 3 times. The blade was then retracted, the knife rotated 90° posteriorly, the blade extended again, and the entire procedure repeated. Following a "mirror-image" surgical sequence on the opposite side of the brain at the same rostral level, similar set of cuts were made in the two posterior corners at 4.8 mm caudal to bregma and 2.0 mm lateral to the midline on both sides of the brain. Again, the first transection on either side was made with the blade extended toward the midline; the second cut was performed with the blade pointing anteriorly.

Following surgery, the animals were placed on a heating

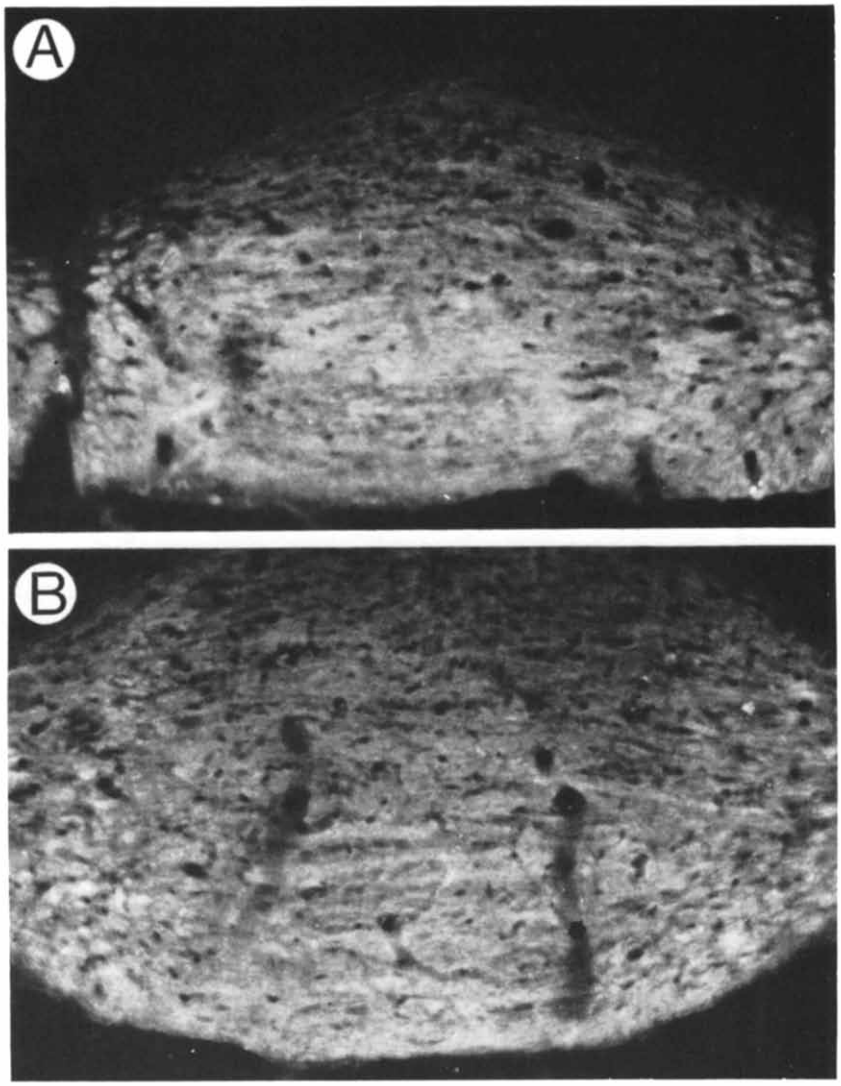


FIG. 2. Distribution of ChAT-like immunoreactivity in the interpeduncular nucleus at four rostrocaudal levels. Arrow in frame C points to a horizontally directed ChAT-positive fiber. Tissue section thickness, 40 $\mu$m. Scale, 100 $\mu$m.

pad maintained at 30°C until they recovered from the anesthetic. They were then housed individually in stainless steel cages until they were euthanized three days later.

*Histochemical Methods*

*ChAT and substance P immunohistochemistry.* Normal rats, rats intracerebrally infused with propidium iodide, or rats given knife cuts were anesthetized with 350 mg/kg chloral hydrate given intraperitoneally and then sacrificed by cardiac perfusion with 50 ml cold (4°C) phosphate buffered saline (PBS; pH, 7.2) followed by 500 ml cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH, 7.2). Brains were post-fixed at 4°C for 90 min in the same fixative and then transferred to cold 30% sucrose in 0.1 M phosphate buffer (pH, 7.2) for an additional 2–3 days. The tissue was then blocked at a 90° angle to the flat surface of the top of the brain, frozen onto a brass specimen holder, and cut at 10 or 40 $\mu$m intervals in a cryostat maintained at −20° or −5°C. The resulting sections were melted onto glass slides precoated with 0.5% gelatin in 0.05% chromium potassium sulfate and were maintained at 4°C until further processing.

After all the tissue was cut, the slides were brought to room temperature (27°C) and tissue sections were covered with PBS, added dropwise. Forty-five min later, the PBS was removed with a Pasteur pipette and replaced with a solution of a monoclonal antibody against ChAT (code 11/255; for characterization, see Eckenstein and Thoenen [22]), diluted 1:50 in PBS containing 0.3% Triton X-100, and, in the case of animals having knife transections, some sections were also incubated with commercially available polyclonal antibodies against substance P (Immuno Nuclear Corp., Stillwater, MN), also diluted 1:50 in the same vehicle. The substance P immunohistochemistry served as a control for possible damage to habenulo-interpeduncular pathways. The brain sections were incubated with the primary antibody

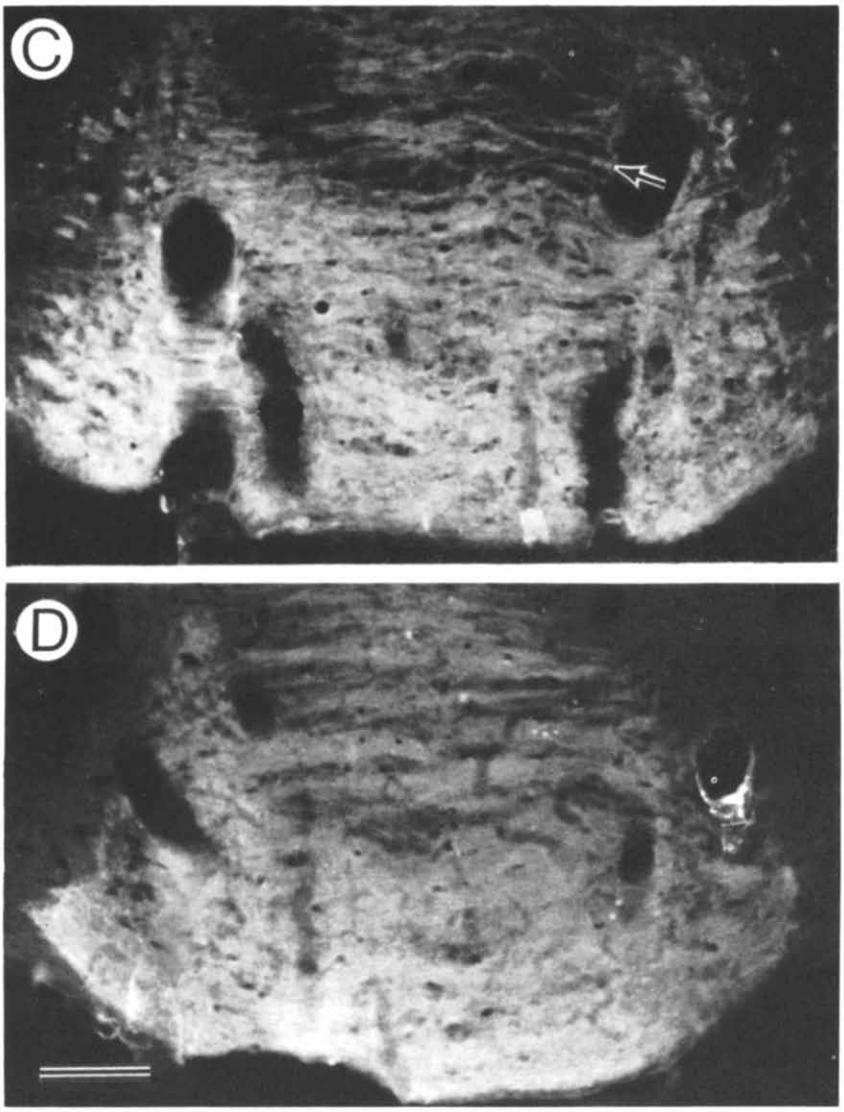


FIG. 2. CONTINUED FACING PAGE

at 4°C for 1–3 days. At the end of the incubation period, the slides were rinsed twice in PBS for 10 min each before being reacted with a solution of affinity purified anti-rat (ChAT) or anti-rabbit (substance P) IgG conjugated with fluorescein isothiocyanate (FITC; Sigma Chemical Co.; St. Louis, MO), diluted 1:50 in PBS, for 30 min at 37°C. The slides were then rinsed with several changes of PBS and coverslipped under a medium consisting of glycerine and PBS (3:1; v/v). Control brain sections were treated in the same manner except that incubation with the primary antibody was omitted. This latter procedure abolished staining for ChAT and substance P.

Orange-red 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. The yellow-green FITC label was observed with epi-illumination and standard Zeiss filters. Double-labeled cells were identified sequentially by changing the filter combinations. Additional commentary concerning these histochemical procedures is contained in Woolf *et al.* [83].

*AChE histochemistry.* Corroboration of the results obtained by use of propidium iodide and ChAT was attempted in separate experiments with AChE and Evans Blue. A detailed treatment of this latter methodology is contained in Butcher [7]. In brief, rats intracerebrally infused with Evans Blue were injected intramuscularly with 2.5 mg/kg DFP (Calbiochem, Inc.; La Jolla, CA) 4 or 6 hr prior to euthanasia. In order to avoid respiratory difficulty during the survival period after DFP administration, all animals were given 10 mg/kg atropine methyl bromide (Sigma Chemical Co.; St. Louis, MO) intraperitoneally immediately prior to DFP injection. In preliminary experiments it was found that DFP or atropine methyl bromide did not influence the transport of Evans Blue.

The animals were anesthetized with 350 mg/kg chloral hydrate intraperitoneally and, subsequently, were sacrificed

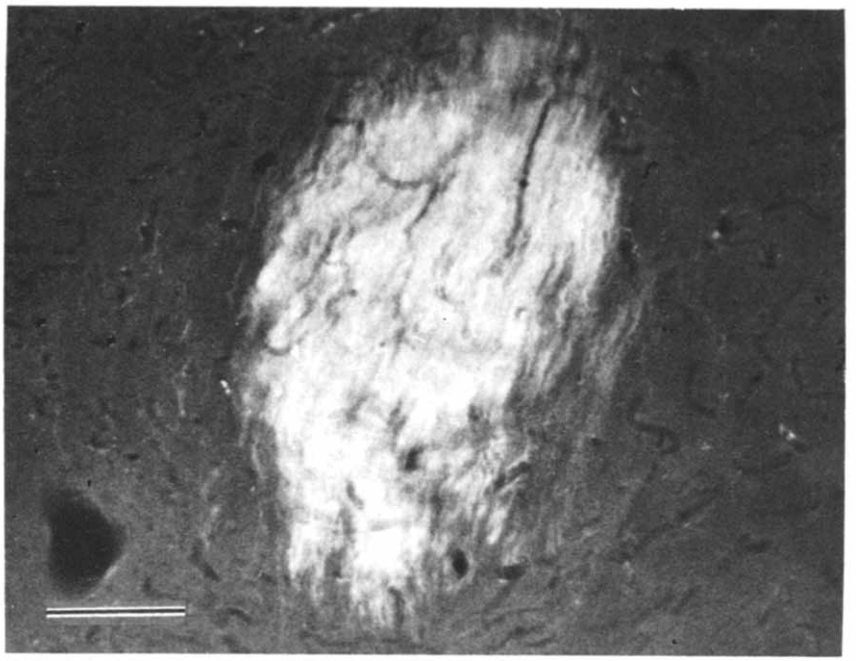

FIG. 3. Fibers demonstrating ChAT-like immunoreactivity in the fasciculus retroflexus. Tissue section thickness, 40 $\mu$m. Scale, 100 $\mu$m.

by cardiac perfusion with 120 ml cold (4°C) 0.9% saline followed by 120 ml cold 10% buffered formalin (pH, 7.2). The brains were removed from the cranial cavity and placed into cold neutral buffered formalin for 48 hr before being transferred to a cold 30% sucrose solution for an additional 48 hr. The brains were blocked and cut on a freezing microtome at 40 $\mu$m intervals. The resulting tissue sections were collected in cold 0.9% saline and immediately thereafter were mounted on glass slides coated with pig gelatin. After the brain sections dried at room temperature (22°C), they were rinsed in distilled deionized water, allowed to dry again, and coverslipped under mineral oil (Nujol®; Plough, Inc.; Memphis, TN).

The slides thus prepared were examined microscopically as described in Bigl *et al.* [5]. Brain regions containing red fluorescent cells labeled with Evans Blue were photographed, and following analyses of the locations of labeled somata, the coverslips of the slides were removed manually. The slides were then blotted on absorbent paper, immersed in xylene for one min to remove the mineral oil, blotted again to remove excess xylene, and allowed to dry at room temperature. The mounted brain sections were then rinsed in 0.9% saline for two min and subsequently processed for AChE according to the procedure of Karnovsky and Roots [41] as modified slightly by Butcher *et al.* [11]. Glass slides were placed for 30 min into Coplin jars containing 30 $\mu$M N,N'-*bis*(l-methylethyl)pyrophosphorodiamidic anhydride (iso-OMPA; K and K Laboratories; Plainview, NY) to inhibit butyrylcholinesterase. The iso-OMPA solution was then poured out and replaced with the AChE reaction mixture containing 50 mg acetylthiocholine iodide, 65 ml of 0.2 M Tris-maleate buffer (pH, 5.7), 5 ml 0.1 M sodium citrate, 10 ml of 0.03 M cupric sulfate, 10 ml of 0.005 M potassium ferricyanide, and 10 ml distilled deionized water per 100 ml of solution. Slides were incubated with mild agitation at 22°C for 2–4 hr. Control experiments for the specificity of the AChE reaction were performed as described in Butcher and Hodge [8].

After the histochemical reaction for AChE was complete, the slides were removed from the Coplin jars, rinsed in distilled deionized water, air dried, immersed in xylene, and coverslipped under Permount® (Fisher Scientific Co.; Fairlawn, NJ). The same regions of the brain examined for cells labeled with Evans Blue were then analyzed with bright-field or dark-field illumination for the presence and intensities of AChE-containing neurons [5, 9, 10, 81]. Some brain sections were additionally counterstained with cresyl violet as detailed in Woolf and Butcher [80].

## RESULTS

### *Cholinergic Neuronal Elements in the IPN, Fasciculus Retroflexus, Habenular Complex, Basal Forebrain, and Dorsolateral Tegmental Nucleus*

The distribution of ChAT-like immunoreactivity at selected rostrocaudal levels of the IPN is shown in Fig. 2. Although a detailed qualitative analysis of the intensity and organization of the enzyme in the different subnuclei of the IPN (e.g., see [34]) was not performed, all of the ChAT appeared associated with fibers, many oriented horizontally, and not with somata (Fig. 2). Indeed, no ChAT-positive perikarya could be detected in our material at any level of the IPN. Similarly, although some interpeduncular cells stained for AChE following a DFP challenge (cf. [10]), enzyme activity in those neurons was weak and, therefore, presumably more characteristic of cholinoceptive than cholinergic entities [9, 10, 24].

Consistent with the conjecture that many, if not all, cholinergic fibers traverse the fasciculus retroflexus before terminating in the IPN (e.g., [50]), numerous vertically oriented filaments demonstrating ChAT-like immunoreactivity were observed in that tract (Fig. 3). The organization

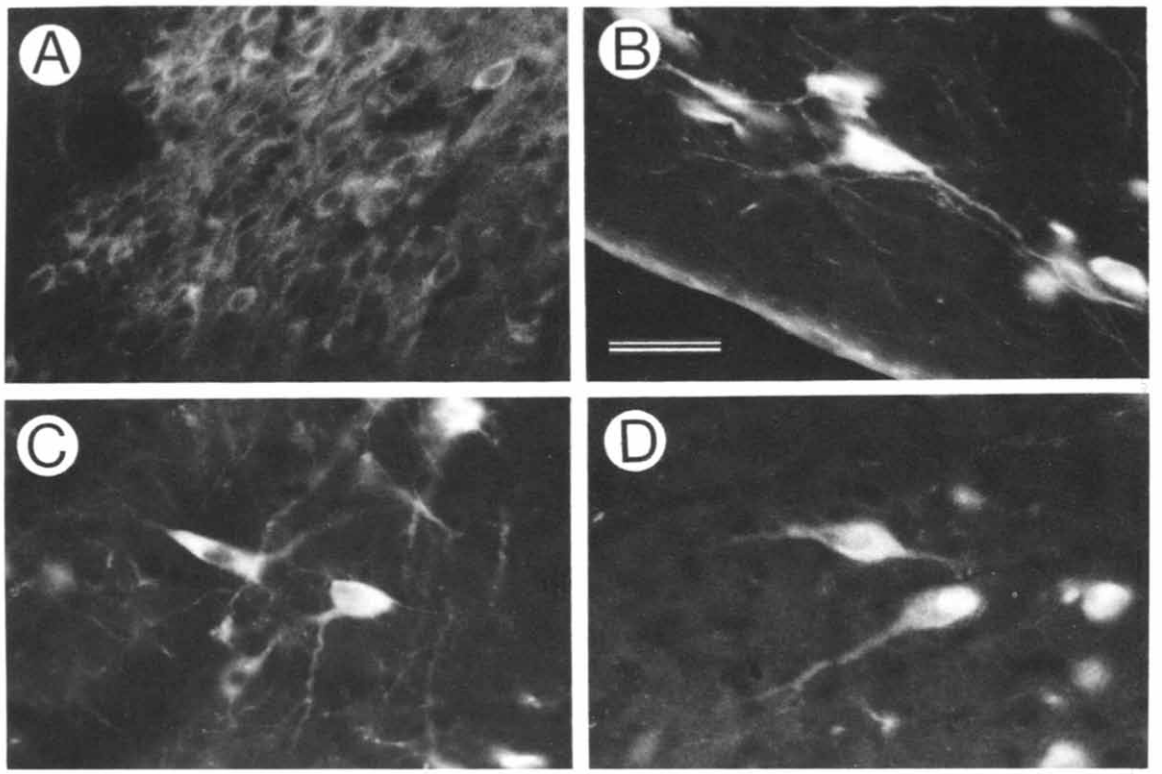


FIG. 4. Neuronal elements demonstrating ChAT-like immunoreactivity in the medial habenular nucleus (A), the horizontal limb of the diagonal band (B), the magnocellular preoptic area (C), and the dorsolateral tegmental nucleus (D). Tissue section thickness, 40 $\mu$m. Scale, 40 $\mu$m.

of the cholinergic synthetic enzyme was similar, if not identical, to that of AChE in the fasciculus retroflexus in non-DFP treated rats (cf. [10,64]).

The immunohistochemical localization of ChAT in the three regions of the brain—the habenular complex, the basal forebrain, and the dorsal tegmental area—for which currently available experimental evidence is most persuasive that they are the origins of cholinergic afferents to the IPN (see Introduction) are exampled in Fig. 4. In the habenular complex, staining for ChAT was found primarily in the medial nucleus, with only scattered puncta being observed in the lateral habenula. The ChAT-like immunoreactivity in the medial habenula was associated both with the neuropil and with cell bodies (Figs. 4A and 10A), where it appeared to be concentrated at the perikaryal margins in many neurons (Fig. 4A). The intensity of ChAT staining in medial habenula somata (Fig. 4A) was clearly less than that associated with known cholinergic neurons in the horizontal limb of the diagonal band (Fig. 4B), the magnocellular preoptic area (Fig. 4C), and the dorsolateral tegmental nucleus (Fig. 4D). Interestingly, the patterns and intensities of somatal ChAT immunoreactivity in these four regions of the brain paralleled somewhat those observed for AChE in the same areas (cf. [10]). That is, an appreciable number of cell bodies in the basal forebrain and dorsolateral tegmental nucleus stain intensely for AChE, whereas somata in the medial habenula are weakly reactive for the cholinergic degradative enzyme (cf. [10]). Whether or not the varying amounts of enzyme reflect dissimilarities in cell volume or in the importance of cholinergic mechanisms in the functioning of the different neuronal populations remains to be established. Neurons in the medial habenula are smaller than those in the basal forebrain and dorsolateral tegmental nucleus (Fig. 4A; cf. Fig. 4B–D), and, therefore, might be expected to contain lower concentrations of subcellular constituents, including enzymes.

### *Retrograde Tracing Experiments*

*Injection sites and spread of fluorescent tracers.* A typical example of the three-dimensional diffusion of propidium iodide infused into the IPN is illustrated in Fig. 5. A volume of 0.5 $\mu$l or less of propidium iodide and, in some cases, 0.05 $\mu$l Evans Blue resulted in such restricted spread. Four of the 9 rats demonstrated diffusions of tracer that were virtually limited to the IPN while filling a major portion of the nucleus as exemplified in Fig. 5. In two other animals, the fluorescent tracer was also restricted to the IPN, but the spread of label was less than that indicated in Fig. 5, and, in 3 of the 9 rats, the dye, although encompassing the IPN, also invaded the ventral tegmental area. An additional three rats served as controls, and, in these, the retrogradely transported label was restricted to the ventral tegmental area (Fig. 5).

*Cholinergic afferents to the IPN.* Regardless of neurochemical signature, neurons labeled with fluorescent tracer following infusion of propidium iodide or Evans Blue into the IPN were found in the basal forebrain, premammillary nucleus, the habenular complex, the dorsal and median raphe, the pedunculopontine tegmental nucleus, the dorsal and dorsolateral tegmental nuclei, the ventral tegmental nucleus, and locus ceruleus (Table 1). Injection of fluorescent dye

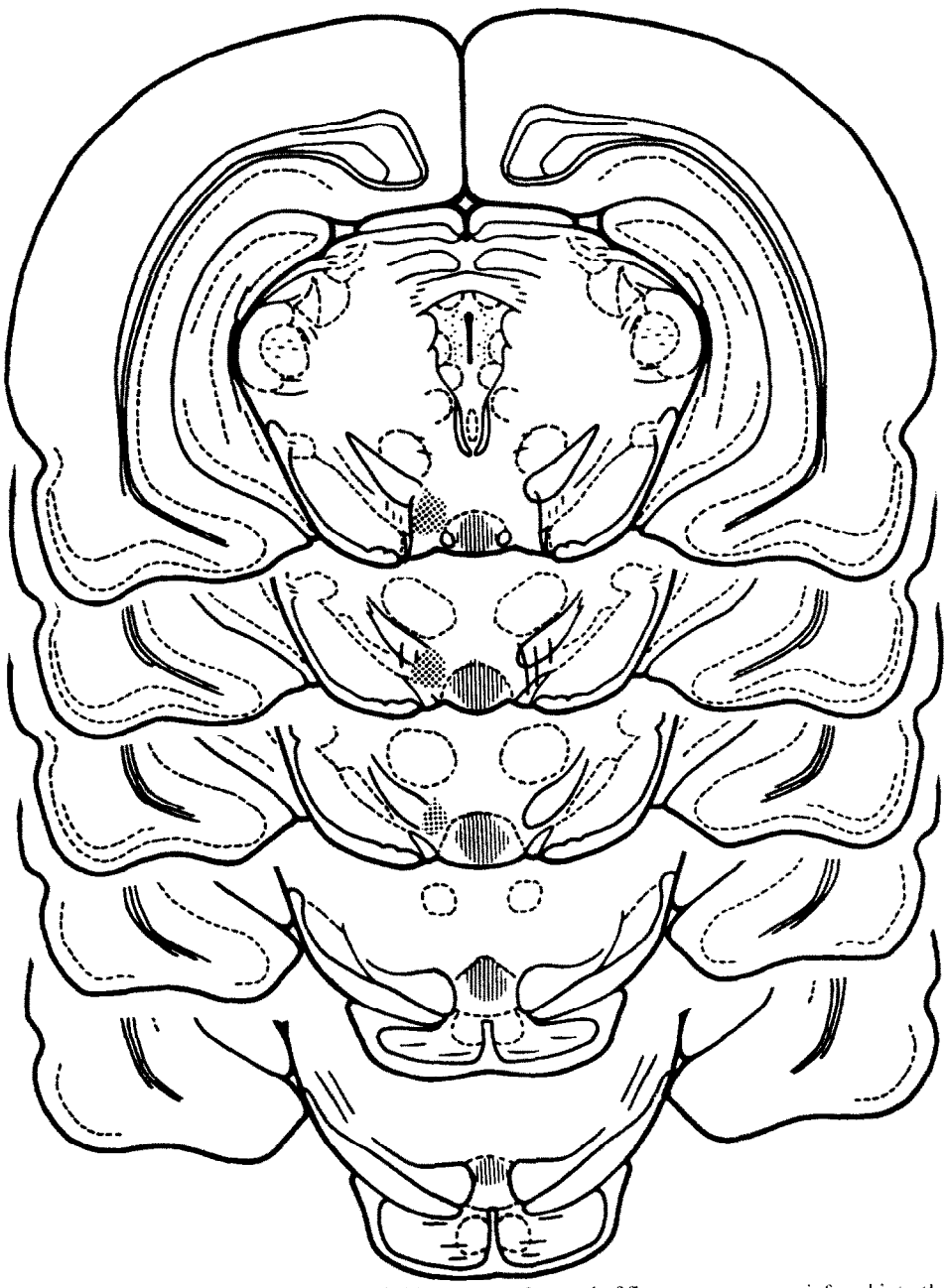

FIG. 5. Representative examples of the visible extents of spread of fluorescent tracers infused into the interpeduncular nucleus (vertical lines in all transverse sections, rat IPN-2) or into the ventral tegmental area (stippling in first three sections, rat VTA-1). Templates are redrawn from König and Klippel [45] and correspond to levels A1950, A1760, A1610, A1270, and A1020 μm.

into the ventral tegmental area also resulted in retrograde labeling of cell bodies in many of these same regions of the brain, as well as in the cingular and frontal cortices, the lateral hypothalamic area, parabrachial nuclei, and the deep cerebellar nuclei (Table 1). Whether or not the labeling following tracer infusion into the ventral tegmental area was due to uptake of dye by terminal fields of projection neurons or fibers of passage was not germane to the goals of the present experiments and, hence, was not investigated. Nonetheless, the patterns of labeling were clearly dissimilar as a function of tracer infusion into either the IPN or ventral tegmental area (Table 1), suggesting, in part at least, the existence of different projections from the same regions to the two adjacent but discrete targets (see Introduction).

Neuronal somata evincing both propidium iodide and ChAT were observed in various regions of the basal forebrain, the pedunculo-pontine tegmental nucleus, and the dorsolateral tegmental nucleus (Figs. 6–9). Although numerous neurons labeled with fluorescent tracer were found in the medial habenula following propidium iodide infusion into the

TABLE 1

NEURAL REGIONS CONTAINING RETROGRADELY LABELED CELLS FOLLOWING INFUSIONS OF FLUORESCENT TRACERS INTO THE VENTRAL TEGMENTAL AREA OR INTERPEDUNCULAR NUCLEUS

| Region containing Retrogradely Labeled Somata | Relative number of labeled somata following tracer infusion into structure indicated* | |
|---|---|---|
| | ventral tegmental area (n=3) | interpeduncular nucleus (n=9) |
| Telencephalon | | |
| cingulate cortex, anterior part | ++++ | |
| frontal cortex | +++ | |
| nucleus accumbens | + | |
| medial septal nucleus | + | ++ |
| vertical limb of diagonal band | ++ | +++ |
| horizontal limb of diagonal band | +++ | ++++ |
| bed nucleus of stria terminalis | +++ | |
| lateral preoptic area | +++ | |
| medial preoptic area | ++++ | |
| magnocellular preoptic area | +++ | ++++ |
| substantia innominata | ++++ | + |
| nucleus basalis | ++ | + |
| Diencephalon | | |
| lateral hypothalamus | ++++ | |
| posterolateral hypothalamus | ++ | |
| premammillary nucleus | | ++++ |
| lateral habenula | ++++ | 0/+ |
| medial habenula | | ++++ |
| Brainstem | | |
| dorsal raphe nucleus | ++++ | ++++ |
| median raphe nucleus | ++++ | ++++ |
| pedunculopontine tegmental nucleus | ++ | ++ |
| dorsolateral tegmental nucleus | ++++ | ++++ |
| dorsal tegmental nucleus | +++ | + |
| ventral tegmental nucleus | +++ | + |
| locus ceruleus | + | + |
| dorsal parabrachial nucleus | ++++ | |
| ventral parabrachial nucleus | + | |
| deep cerebellar nuclei | +++ | |

*Symbols represent the mean number of cells containing propidium iodide or Evans Blue in a 40 $\mu$m thick section (or the mean number multiplied by 4 in a 10 $\mu$m thick section) of the structure indicated. The brain section analyzed contained the greatest number of labeled cell bodies for the particular region shown. +, 1–10 somata; ++, 10–25 somata; +++, 25–50 somata; ++++, >50 somata.

IPN (Table 1), all of these cells stained only weakly for ChAT (Fig. 4A). No other brain regions with neurons projecting to the IPN (Table 1) contained somata in which propidium iodide and ChAT were co-localized.

The basal forebrain neurons demonstrating both propidium iodide label and ChAT-like immunoreactivity were found in the medial septal nucleus, nuclei of the vertical and horizontal limbs of the diagonal band, the magnocellular preoptic area, subpallidal substantia innominata, and nucleus basalis (Fig. 8). Most of these cells were observed in association with the horizontal limb of the diagonal band and the magnocellular preoptic area (Fig. 8), also referred to as the nucleus of the horizontal limb of the diagonal band (e.g., [66]). At most levels of the basal forebrain cholinergic system (for discussion of terminology, see [5, 10, 83]), ChAT-positive projection neurons were intermingled with non-

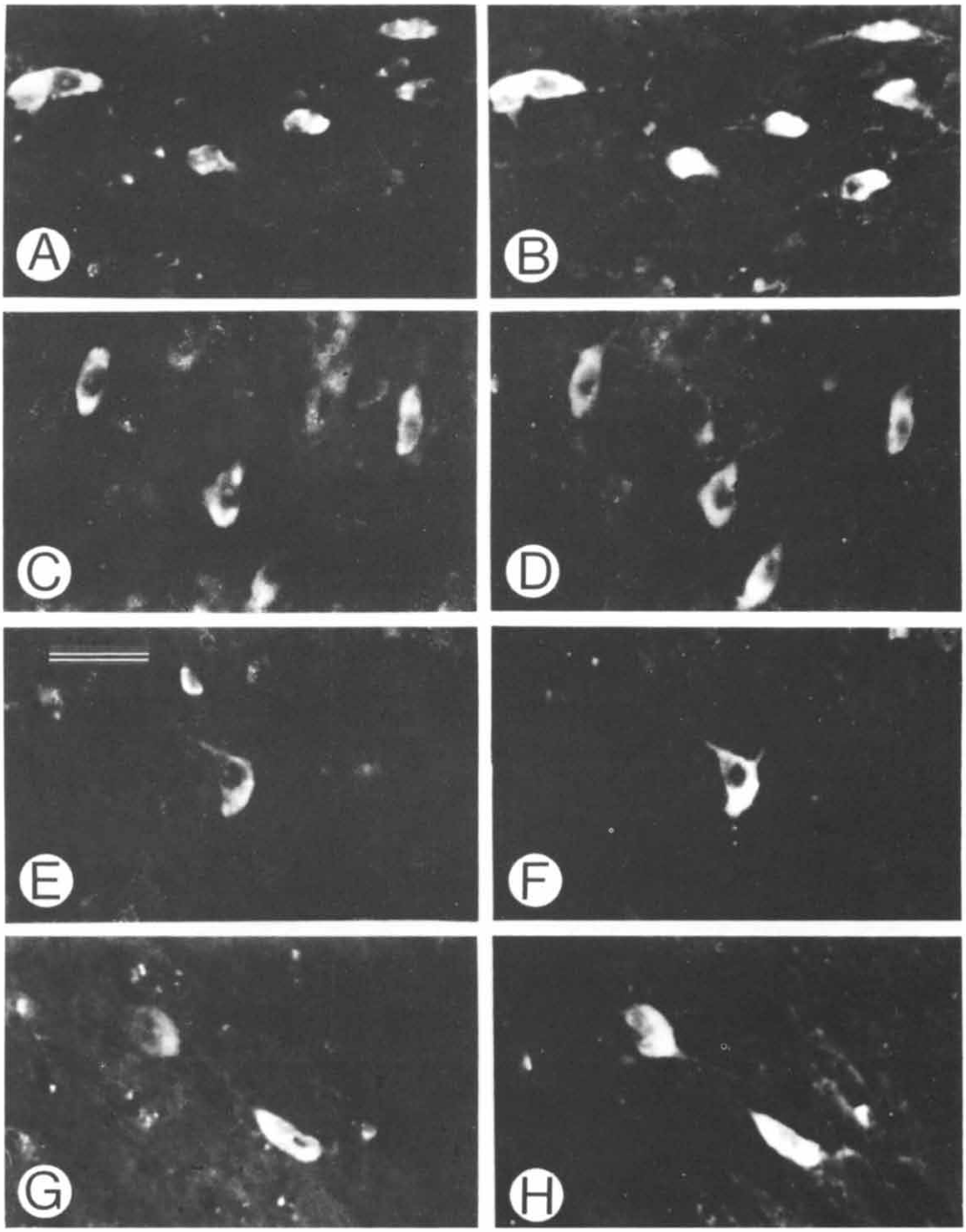


FIG. 6. Neurons in the horizontal limb of the diagonal band (A, B), magnocellular preoptic area (C, D), substantia inominata (E, F), and nucleus basalis (G, H) containing propidium iodide (A, C, E, G) and demonstrating ChAT-like immunoreactivity (B, D, F, H) following infusion of the retrograde tracer into the interpeduncular nucleus. The same tissue section is shown in frames A and B, and this same data-display schema applies to frames C and D, E and F, and G and H. Tissue section thickness, 10 $\mu$m. Scale, 40 $\mu$m.

cholinergic cells providing afferent fibers to the IPN (Fig. 8). Rostrally, these latter neurons were found primarily at caudal levels of the vertical limb of the diagonal band in a region containing few cholinergic somata (Fig. 8C), and, more caudally, they were dorsal to ChAT-containing cells in the horizontal limb of the diagonal band (Fig. 8D). No particular organizational schema was evident at other levels of the basal forebrain with respect to the patterning of presumed cholinergic and non-cholinergic neurons projecting to the IPN (Fig. 8). Many of the ChAT-positive neurons

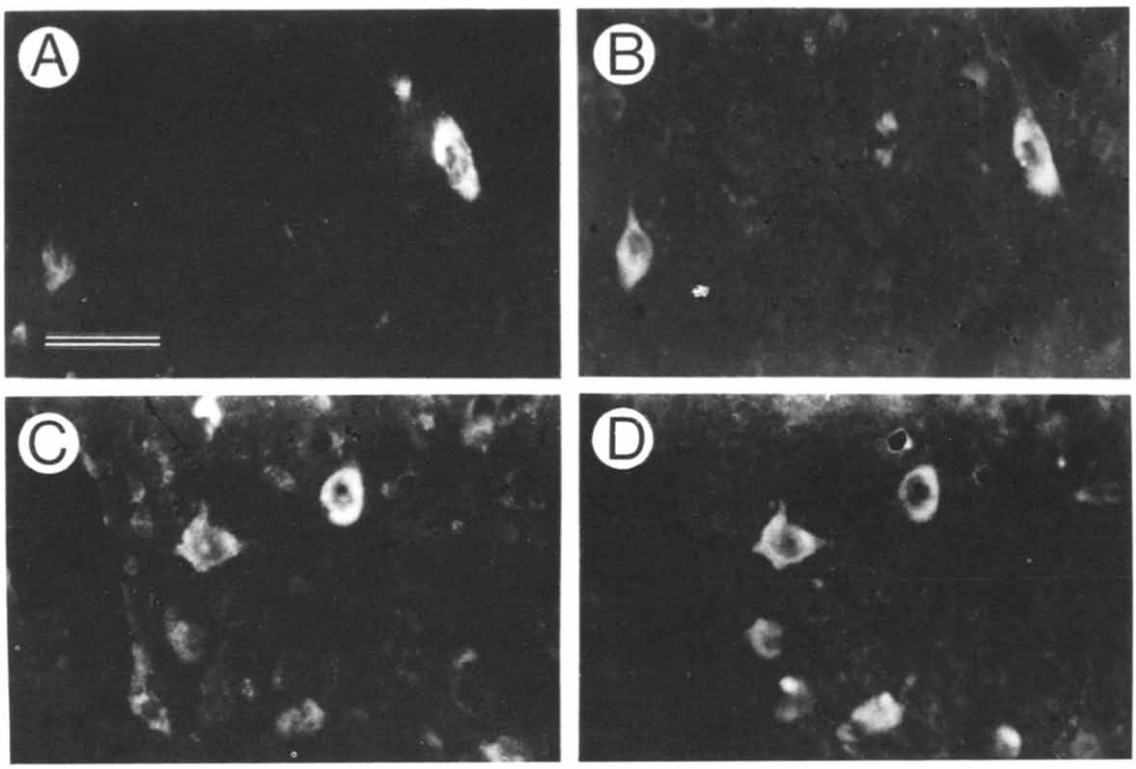


FIG. 7. Neurons in the pedunculopontine tegmental nucleus (A, B) and the dorsolateral tegmental nucleus (C, D) containing propidium iodide (A, C) and demonstrating ChAT-like immunoreactivity (B, D) following infusion of the fluorescent tracer into the interpeduncular nucleus. The same tissue section is shown in frames A and B and in frames C and D. Tissue section thickness, 10 $\mu$m. Scale. 40 $\mu$m.

innervating the IPN from the basal forebrain stained intensely for AChE following a DFP challenge.

In the brainstem, most ChAT-positive cells containing propidium iodide following injection of that fluorescent tracer into the IPN were localized in association with the dorsolateral tegmental nucleus, although a few such neurons were observed in the pedunculopontine tegmental nucleus (Fig. 9). Like the situation in the basal forebrain, these cells were interspersed among non-cholinergic neurons projecting to the IPN (Fig. 9), as well as ChAT-positive cells not giving rise to interpeduncular afferent fibers (Fig. 9), and, although a systematic analysis was not performed, many demonstrated intense AChE activity.

In separate experiments, it was found that presumed cholinergic neurons staining intensely for AChE (pharmacohistochemical regimen) in the basal forebrain and in the pedunculopontine tegmental and dorsolateral tegmental nuclei also contained Evans Blue following infusion of that tracer into the IPN. In those same regions of the brain and in the premammillary nucleus, Evans Blue labeled cells were also present after IPN infusions that stained moderately, lightly, or not at all for the cholinergic degradative enzyme (cf. [10]). The medial habenula contained neurons innervating the IPN that were weakly reactive for AChE (cf. [10]). Interpeduncular projection cells in the dorsal and median raphe stained lightly to moderately for AChE, and those in locus ceruleus demonstrated moderate to intense enzyme activity (cf. [10]).

*Knife Cut Experiments*

In all animals subjected to lesion procedures, the knife cuts resulted in the separation of the habenular complex on both sides of the brain from tissue lying anterior, lateral, and posterior to the medial and lateral nuclei as schematically represented in Fig. 1. The stria medullaris was severed, but there was little or no histologically detectable damage to the fasciculus retroflexus.

The knife cuts produced marked loss of immunohistochemically assessed ChAT in the medial habenula (Fig. 10), the fasciculus retroflexus, and the IPN (Fig. 11C) but no observable depletion of interpeduncular substance P (Fig. 11B, cf. Fig. 11A). This latter finding suggests that neither the medial habenula nor the fasciculus retroflexus was affected appreciably by the surgical procedures (see Introduction), a conjecture further strengthened by the observation that the number of cells and patterns of Nissl staining in the medial nucleus were unaltered in any of the animals as a consequence of the lesions.

## DISCUSSION

*Cholinergic Anatomy of the IPN, Fasciculus Retroflexus, Habenular Complex, Basal Forebrain, and Pontine Tegmentum*

The localization of ChAT in neurons of the basal forebrain and pontine tegmentum found in the present experiments, as well as the organization of those cells in the two

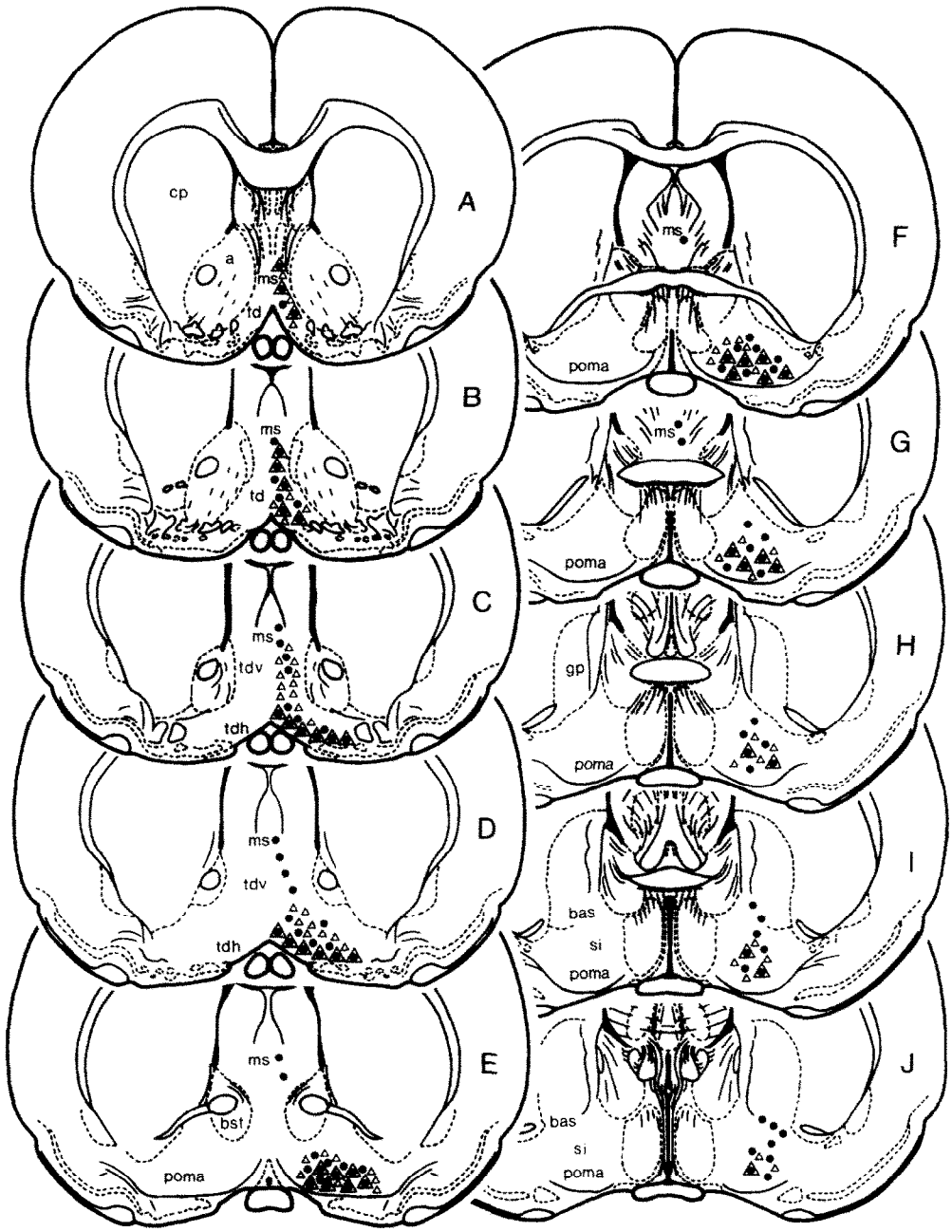


FIG. 8. Representative example of the rostrocaudal distribution of ChAT-positive cells in the basal forebrain projecting to the interpeduncular nucleus (solid black circles within open triangles). At brain levels containing these putative cholinergic projection neurons, somata demonstrating ChAT-like immunoreactivity but not labeled with propidium iodide are depicted as solid black circles. Projection neurons containing propidium iodide but not reactive for ChAT are indicated by open triangles. Each symbol represents approximately five cell bodies. Rat IPN-1. Templates are redrawn from König and Klippel [45] and sequentially depict levels A8920, A8620, A8380, A7890, A7470, A7190, A7020, A6860, A6790, A6670, A6570, A6360, A6280, and A6060 $\mu$m from A to N. a, nucleus accumbens; bas, nucleus basalis; bst, bed nucleus of the stria terminalis; gp, globus pallidus; ms, medial septal nucleus; poma, magnocellular preoptic area; si, substantia innominata; td, diagonal band; tdh, horizontal limb of the diagonal band; tdv, vertical limb of the diagonal band. For other abbreviations, see legend of Fig. 1.

regions, agrees well with previous reports in the literature [4, 21, 38, 44, 56, 57, 68, 82, 83]. The dense plexus of ChAT-positive fibers found in both the IPN and fasciculus retroflexus also is similar to that observed by Houser *et al.* [38]: "The interpeduncular nucleus stained intensely for ChAT that was located in small fibers and in punctate structures suggestive of axon terminals" (pp. 109–110). Unreported by Houser *et al.* [38], however, is the apparent association of some of the interpeduncular ChAT immunoreactivity with horizontally directed fibrils much like the arrangement of

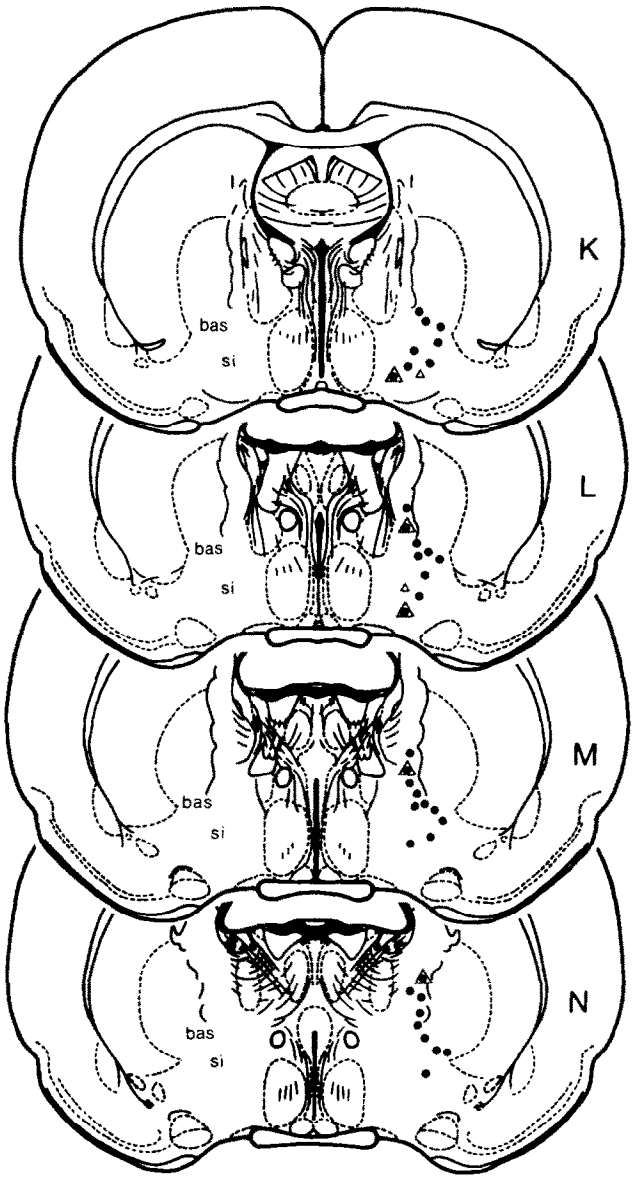


FIG. 8. CONTINUED

IPN fibers noted by Cajal [13] and reproduced in an abbreviated form in Fig. 12. According to Cajal [13], many of the neuronal filaments in the IPN criss-cross the nucleus in "S-shaped" patterns (Fig. 12), and it is possible that some of the interpeduncular ChAT-positive afferents are similarly organized.

The pattern of ChAT-like immunoreactivity found in the habenular complex in the current study differs significantly from the profile reported by Satoh *et al.* [68], who observed an absence of ChAT in both the medial and lateral habenula, and somewhat from the distribution noted by Houser *et al.* [38], who described the majority, if not all, of medial habenula somata as darkly stained. Although virtually all of the cell bodies in the medial habenula demonstrated ChAT-like immunoreactivity in our material as well, the intensity of this staining was less than that associated with known cholinergic neurons in other regions of the brain such as the basal forebrain.

The discrepancy between the present results and those of Satoh *et al.* [68] cannot be explained readily, but one possibility, albeit remote, is that different forms of ChAT exist and that the monoclonal antibody of Satoh *et al.* [68] did not recognize the cholinergic synthetic enzyme associated with the habenular complex. With the observations of Houser *et al.* [38], however, methodologic differences probably account for the dissimilar findings. In the latter investigation, the linking antiserum and peroxidase-antiperoxidase steps were repeated to ". . . intensify staining, particularly of small ChAT-containing structures" (p. 99, [38]). In addition, osmium tetroxide treatment was performed [38], presumably also to augment the optical magnitude of the reaction product. It is conceivable, therefore, that these enhancement procedures, possibly as a consequence of mechanisms involving steric hinderance, obscured the normal intensity relationships among the different populations of neurons demonstrating ChAT-like immunoreactivity. In any case, with the exception of the work of Kimura *et al.* [44], relatively little research has been directed toward assessing the significance of different strengths of ChAT immunoreactivity.

### *Projections to the IPN*

*General observations.* Afferents to the IPN, irrespective of their neurochemical specificities, have been shown consistently to derive from the medial habenula, dorsal tegmental nucleus, dorsolateral tegmental nucleus, locus ceruleus, and the dorsal and median raphe nuclei [15, 33, 52]. Although all of these previous tract-tracing studies employed horseradish peroxidase as the neuronally transported marker, we have corroborated those findings by use of fluorescent tracers. Our observation of labeling in the ventral tegmental and premammillary nuclei following interpeduncular infusions of propidium iodide or Evans Blue is also corroborative (cf. [15]). In the present experiments, however, many more cells in the basal forebrain demonstrated projections to the IPN than have been reported previously. Numerous such neurons were seen in association with the vertical and horizontal limbs of the diagonal band, for example, whereas only a few cells projecting to the IPN from those forebrain loci have been reported by other investigators [15,33]. We also found several retrogradely labeled somata in the magnocellular preoptic area, and lesions involving that region have been reported to produce terminal degeneration near the IPN [17]. Other researchers have not described anatomically the sparse projections observed in the current study from the substantia innominata and nucleus basalis to the IPN.

Notwithstanding that our data suggest more extensive interpeduncular projections from the basal forebrain than previously suspected, it seems unlikely that the greater number of labeled somata we observed could be attributed to tracer spread to and transport from neural regions adjacent to the IPN. First, no detectable spread of fluorescent label could be observed into the ventral tegmental area, the region of the brain lying immediately dorsal and lateral to the IPN, following interpeduncular injections of propidium iodide or Evans Blue. Second, although the lateral habenula is believed to project to the ventral tegmental area [65], only a few or no cells in the lateral nucleus apparently innervate the IPN [15,

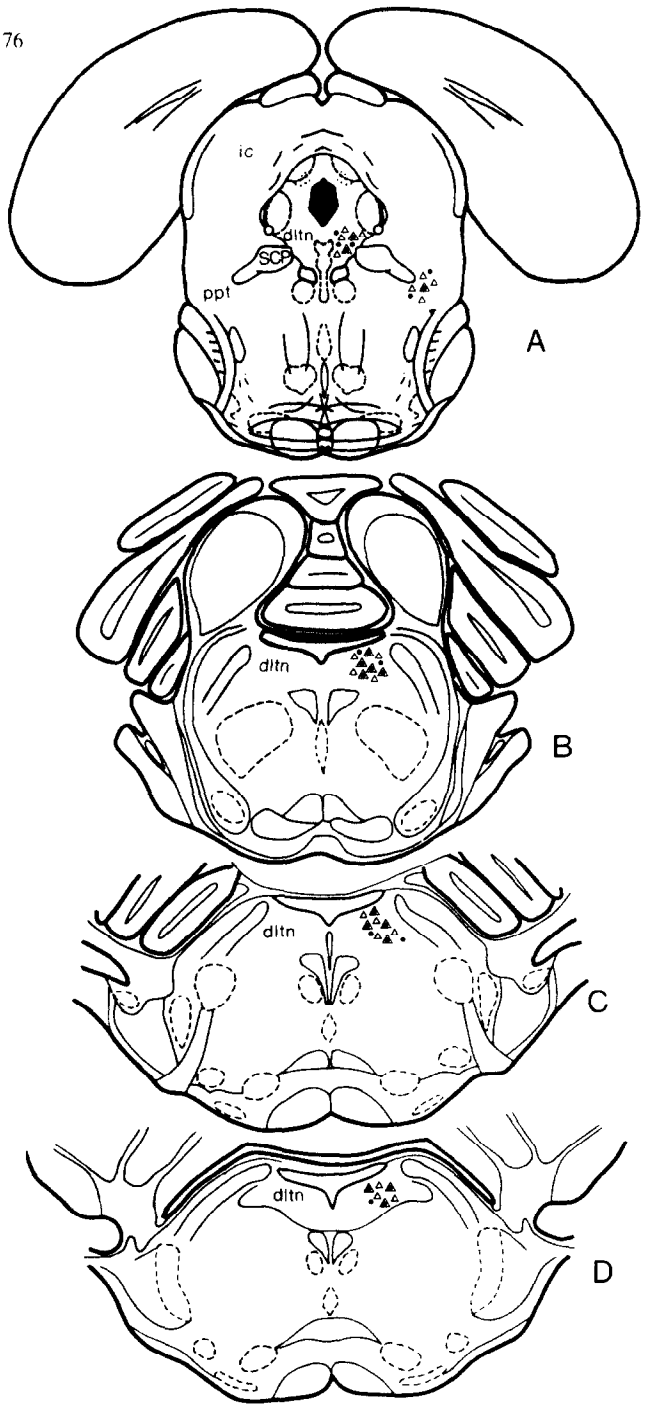


FIG. 9. Representative example of the rostrocaudal distribution of ChAT-positive cells in the pedunculopontine tegmental nucleus (ppt) and dorsolateral tegmental nucleus (dltn) projecting to the interpeduncular nucleus (solid black circles within open triangles). At brain levels containing these putative cholinergic projection neurons, somata in the same two regions demonstrating ChAT-like immunoreactivity but not labeled with propidium iodide are depicted as solid black circles. Projection neurons containing propidium iodide but not reactive for ChAT in the ppt and dltn are indicated by open triangles. Each symbol represents approximately five cell bodies. Rat IPN-3. For the purposes of illustration, some of the symbols unavoidably extend into schematically portrayed regions not containing those cells in actuality. It must be emphasized that all of the symbols are meant to indicate neurons in association with the ppt or dltn and not adjacent structures. Template A is redrawn from König and Klippel [45] and depicts level P480 $\mu$m. Templates B–D are from Butcher and Woolf [10] and correspond to levels P1500, P2000, and P2300 $\mu$m. SCP, superior cerebellar peduncle. For other abbreviations, see legend of Fig. 1.

33, 52]. Consistent with this datum, only a few neurons in the lateral habenula transported label when tracer infusions were confined to the IPN in the present experiments. And third, patterns of labeling in the basal forebrain differed as a function of dye injections into the IPN or ventral tegmental area. Indeed, some forebrain regions (e.g., bed nucleus of the stria terminalis, lateral preoptic area) innervating the ventral tegmental area did not project to the IPN.

*Origins of cholinergic tracts.* That the IPN is cholinergically innervated is suggested by data indicating that acetylcholine release can be measured from that nucleus [67]. The existence of ChAT-containing neurons in the ventral telencephalon and pontine tegmentum is largely undisputed, and many authors have suggested that they use acetylcholine as a neurotransmitter and, hence, are cholinergic [4, 21, 38, 44, 49, 56, 57, 68, 71, 82, 83]. It seems reasonable to suggest, therefore, that the ChAT-positive cells in the basal forebrain and pedunculopontine and dorsolateral tegmental nuclei labeled with propidium iodide following interpeduncular injections of the tracer in the present experiments are sources, perhaps not the only ones, of the cholinergic innervation of the IPN.

The contribution of the medial habenula to the cholinergic innervation of the IPN is less clear. In the present study, ChAT-positive fibrils and puncta were demonstrated in the medial habenula, and some ChAT-like immunoreactivity appeared associated with most of the somata, particularly at perikaryal margins. Knife cuts separating the habenular complex from neural tissue surrounding it, however, resulted in a marked, if not complete, loss of ChAT in the entire medial nucleus and in the IPN, at least as assessed immunohistochemically. One explanation for the former finding is that most of the ChAT positivity in the medial habenula derives from sources external to the habenular complex, a conjecture also intimated by the data of Cuello *et al.* [18] who reported a 77% loss of ChAT activity in the medial habenula following a unilateral transection separating the medial from the lateral nucleus and presumably severing some afferent stria medullaris fibers (see Fig. 12). This surgical procedure also produced a virtually complete loss of histochemically assessed AChE in the medial habenula [18].

The results of Cuello *et al.* [18] suggest that only approximately 23% of the ChAT activity in the medial habenula can be attributed to neurons in that structure. If so, then this relatively small amount of ChAT might not have detected in the present immunohistochemical experiments following circumsection of the habenular complex, and it would be erroneous for us to conclude that none of the ChAT in the medial nucleus has an intrinsic origin. Alternatively, however, it is conceivable that the residual ChAT activity reported by Cuello *et al.* [18] derived from cholinergic fibers that traversed the contralateral, non-transected habenula and entered the ipsilateral habenula through the habenular commissure. In any case, it seems reasonable to propose on the basis of currently available experimental evidence that, although the medial habenula itself receives a cholinergic input, little, if any, of the cholinergic innervation of the IPN derives from that nucleus, as suggested previously by Fibiger [24] in his cogent review of forebrain cholinergic systems (cf. [10, 26, 78]).

*Trajectories and fine structure of cholinergic pathways.* The results of the present experiments indicate that the cholinergic innervation of the IPN derives primarily from cells in the diagonal band, magnocellular preoptic area, and dorsolateral tegmental nucleus, with minor contributions from the medial septal nucleus, substantia innominata, nu-

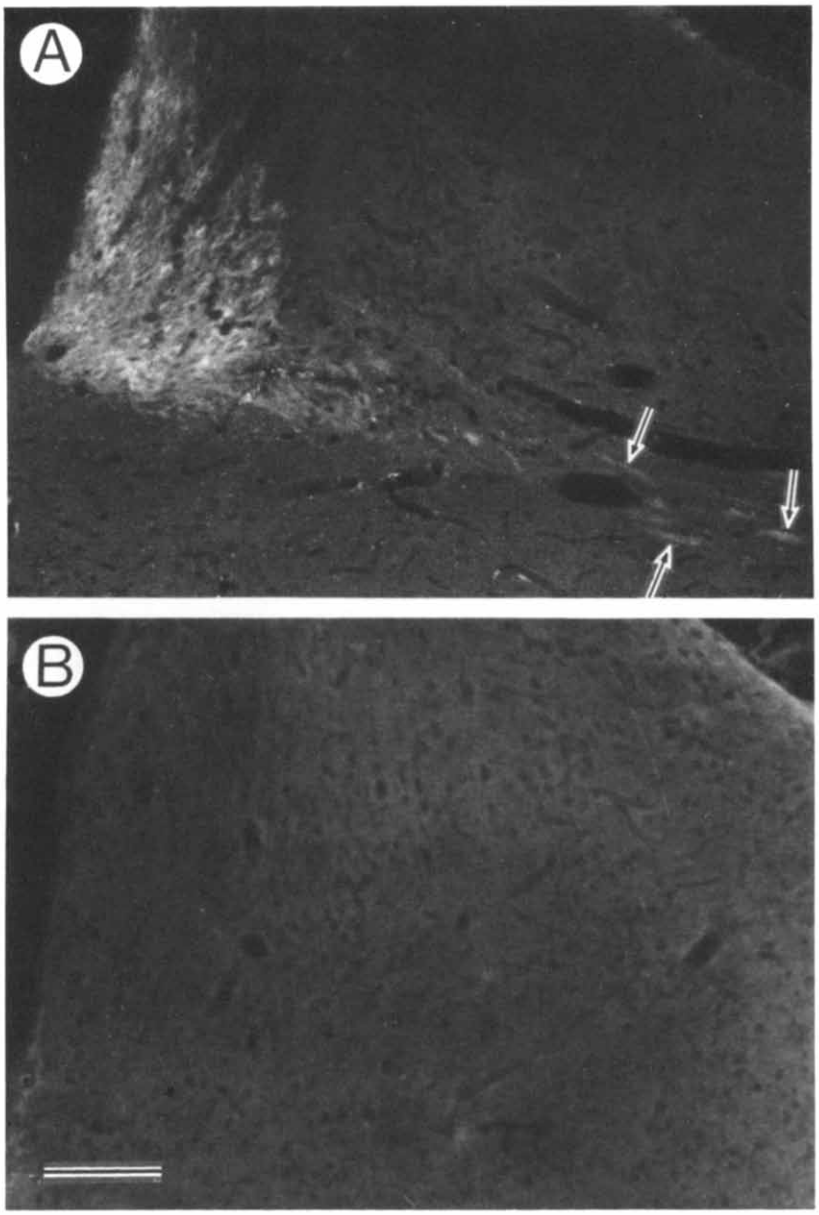


FIG. 10. ChAT-immunoreactivity in the medial habenula and adjacent regions in a surgically unmanipulated preparation (A) and following knife-cut circumscription of the habenular complex (B) as indicated in Fig. 1. Observe that the ChAT-positivity associated with the cell bodies and neuropil of the medial habenula, as well as the fasciculus retroflexus (arrows in A), cannot be detected in B. Tissue section thickness, 40 $\mu$m. Scale, 120 $\mu$m.

cleus basalis, and pedunculopontine tegmental nucleus. Because knife cuts bilaterally circumscribing the habenular complex also depleted ChAT-containing fibers in the IPN, it would appear that cholinergic fibers innervating the interpeduncular area course through or near the habenular nuclei before entering the fasciculus retroflexus. Indeed, bilateral ablations in the region of the habenular nuclei produce a 91–95% decrease of ChAT activity in the IPN-ventral tegmental area ([18,27], cf. [42, 47, 48, 53]).

Cholinergic fibers from the septum and diagonal band region probably course through the stria medullaris, habenular area, and fasciculus retroflexus on their way to the IPN [16, 27, 43]. Indeed, decreases of interpeduncular ChAT by 38–52% and 45–46% have been reported following bilateral ablations of the septum-diagonal band region and stria medullaris, respectively ([16,27], cf. [18,43]). In addition, Cuello *et al.* [18] found a 58% loss of ChAT activity in the IPN-ventral tegmental area after unilateral transection of the fasciculus retroflexus.

The ChAT-positive neurons found in the present study projecting from the magnocellular preoptic area, substantia innominata, and nucleus basalis to the IPN might first traverse the stria medullaris, or the inferior thalamic peduncle, to the habenular complex before entering the fasciculus retroflexus. Fibers from the preoptic area have been traced to the lateral habenula via the stria medullaris [74], as well as

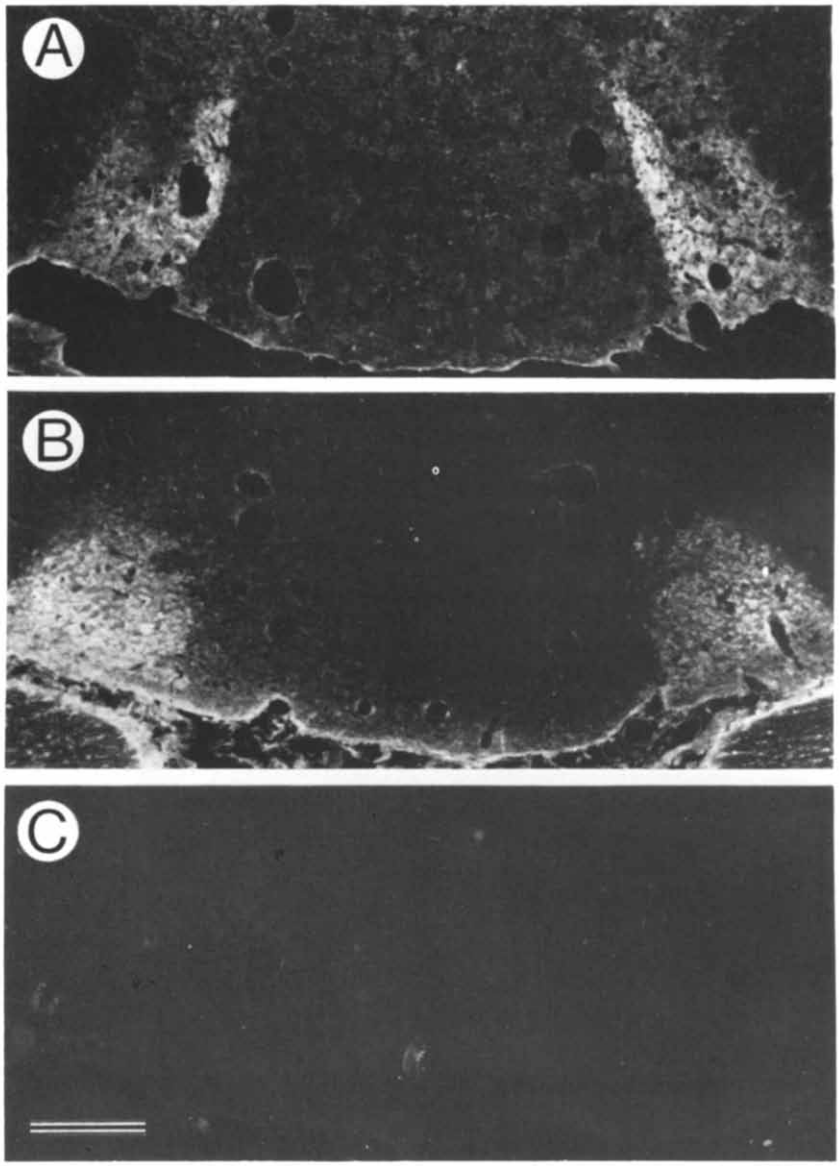


FIG. 11. Effects of knife-cut circumscription of the habenular complex on immunoreactivity for substance P (B) and ChAT (C) in the interpeduncular nucleus. Normal substance P positivity is shown in A. Normal ChAT-like immunoreactivity is displayed in Fig. 2. Tissue section thickness, 40 $\mu$m. Scale, 100 $\mu$m.

through the inferior thalamic peduncle [54]. The substantia innominata also has been shown to project to the lateral habenula [35], and AChE-containing fibrils have been traced through the lateral habenula to the fasciculus retroflexus [26]. In addition, fibers from the basal forebrain to the IPN have been demonstrated to traverse the medial forebrain bundle [20,46], but it is likely that they are primarily noncholinergic because most ChAT-positive interpeduncular afferents appear to course through or near the habenula (*vide supra*).

Some fibers traversing the stria medullaris have been reported to give rise to collaterals ending in dense, net-like terminations in the medial habenula (Fig. 12) before traveling toward the fasciculus retroflexus [40]. If this morphologic profile obtains for some of the afferent cholinergic fibers deriving from the basal forebrain, then such an anatomic arrangement could account, in part, for the ChAT-positivity seen in the neuropil of the medial habenula and IPN, as well as possibly that associated with somata in the former nucleus. Furthermore, it would be compatible with currently available lesion and biochemical data [16, 18, 26, 27, 28, 78]. For example, ablations in the basal forebrain, specifically the septum-diagonal band region, or in the stria medullaris produce reductions of ChAT activity ranging from 26–52% in both the habenular complex and the IPN [27,28]. Putative cholinergic neurons that collateralized in the medial habenula before innervating the IPN would provide an appropriate morphologic substrate for these biochemical findings (cf. [16]). It must be emphasized, however, that it is also possible that separate but confluent cholinergic neurons

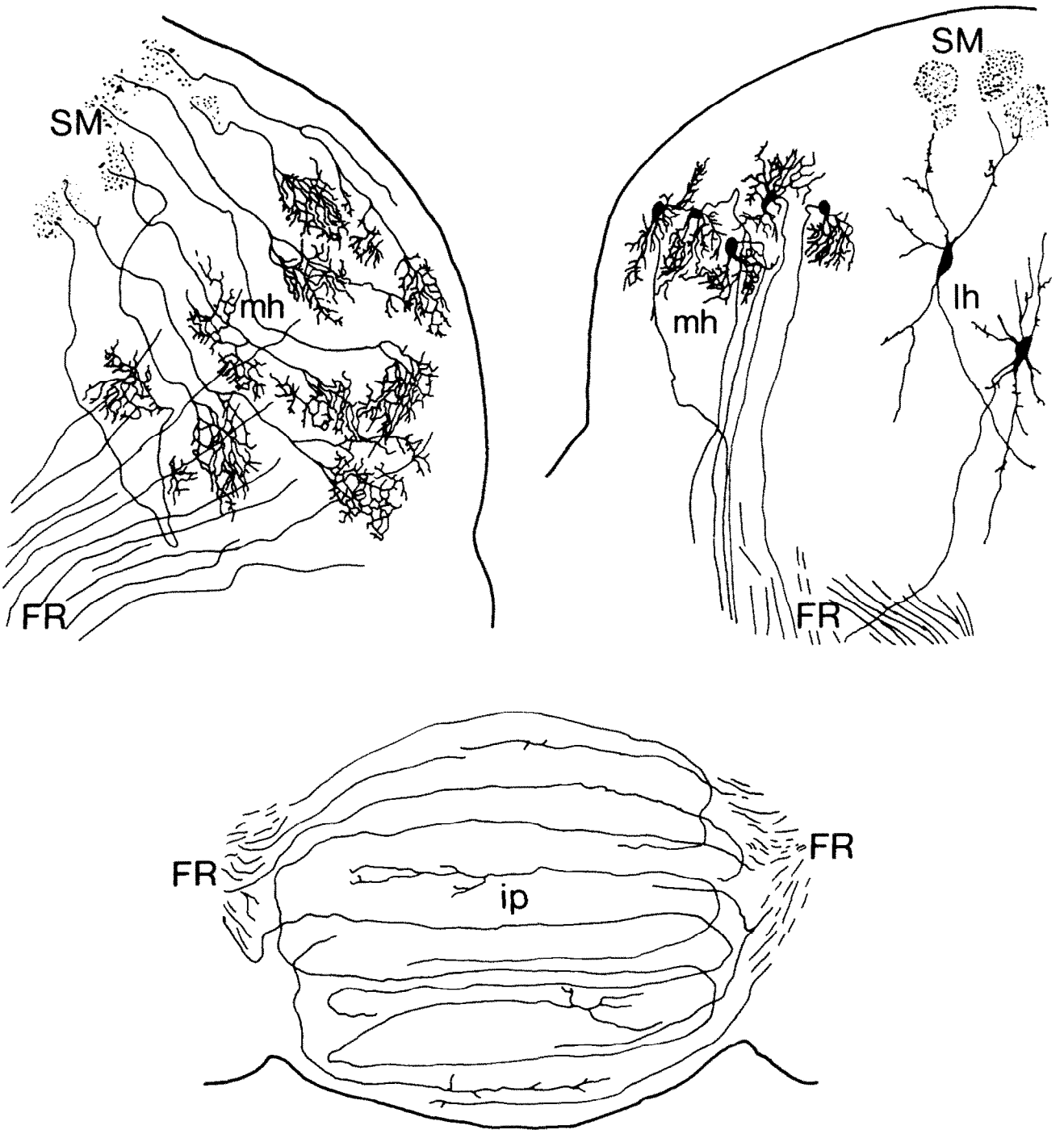


FIG. 12. Golgi demonstration of neurons or their subcellular components in the medial (mh) and lateral (lh) habenula, stria medullaris (SM), fasciculus retroflexus (FR), and interpeduncular nucleus (ip). Individual illustrations are redrawn in an abbreviated form from Figs. 276 (top left), 272 (top right), and 173 (bottom) of Cajal [13].

traversing the stria medullaris project independently to either the habenular complex or IPN from the basal forebrain (cf. [40]).

The net-like terminations of certain fibers innervating the medial habenula (Fig. 12) suggest a possible morphologic basis for the ChAT-like immunoreactivity seen in association with the periphery of many somata in that nucleus. It is conceivable that the cholinergic synthetic enzyme is contained within neuron terminals that, in addition to traversing the neuropil, enmesh the cell bodies of the medial nucleus, thereby giving the visual impression that ChAT-positivity is localized at perikaryal margins. Such an anatomic arrangement also would account for the virtually complete loss of ChAT we observed in the neuropil and somata of the medial

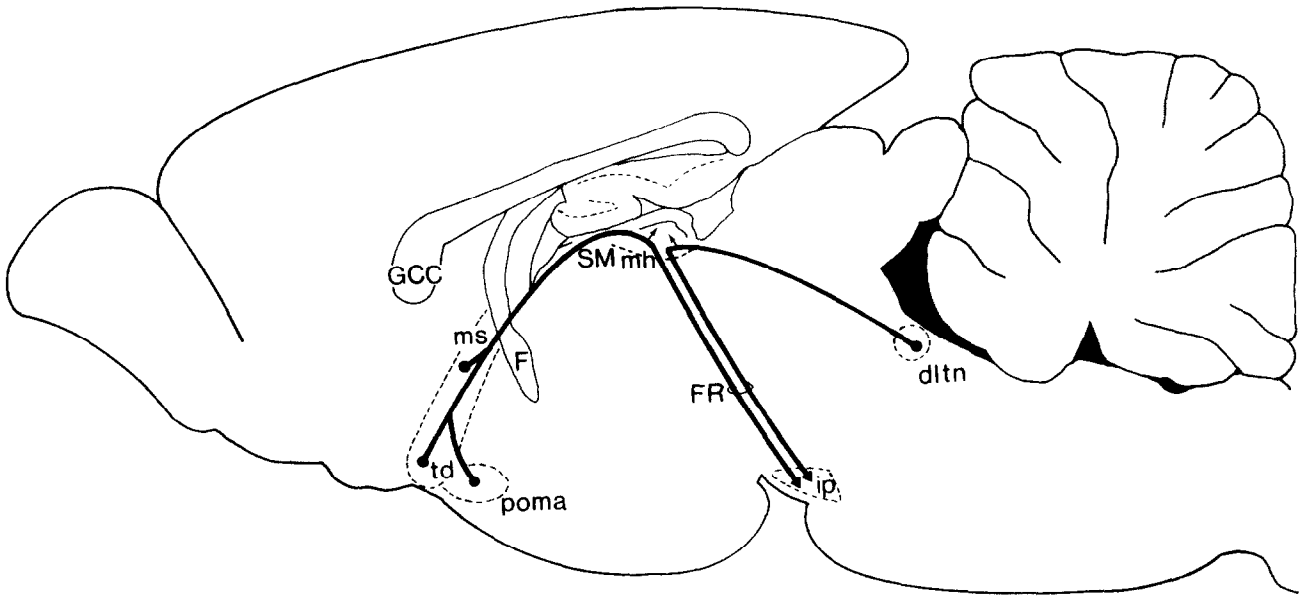


FIG. 13. Schematic representation of the major sources of the cholinergic innervation of the interpeduncular nucleus deriving from the basal forebrain and pontine tegmentum. For further explanation, see text. Template of horizontal section is modified and redrawn from Paxinos and Watson [64]. GCC, genu of corpus callosum; F, fornix. For other abbreviations, see legends of Figs. 1, 8, and 12.

habenula as a consequence of circumscribing knife cuts, as well as possibly the decrease of habenular AChE in the ablation experiments of Cuello *et al.* [18].

The hypothesis that some cholinergic fibers collateralize in a complex terminal network in the medial habenula before proceeding to the IPN might also explain certain observations persuant to the interpeduncular injection and retrograde transport of tritiated choline. According to Villani *et al.* [77], interpeduncularly infused $^{3}$H-choline, which they contend is taken up selectively by cholinergic neurons, could be traced from the IPN to the medial habenula but no further. Interestingly, however, the choline in the medial nucleus could not be identified in association with cell bodies, owing presumably to dense labeling [77]. It is conceivable, therefore, that the choline might have been transported retrogradely to the point or points of collateralization and then down the collaterals rather than toward the somata of origin of the afferent cholinergic fibers.

Although currently available experimental evidence suggests that cholinergic pathways deriving from the basal forebrain traverse the stria medullaris, at least in major part, before terminating in the habenular complex and IPN, the trajectories of putative cholinergic pathways deriving from the pedunculopontine and dorsolateral tegmental nuclei and innervating the interpeduncular area have not been established. Nonetheless, combined lesion and biochemical and histochemical data suggest, as outlined elsewhere in this paper, that such fibers should course dorsally through or near the habenular complex before descending in the fasciculus retroflexus. Possibly at variance with this latter conjecture, however, are the findings that efferents from the dorsolateral tegmental nucleus travel ventrally through the median raphe to the IPN [6,30]. A projection from the dorsolateral tegmental nucleus to the lateral habenula has been described [30,35], but this pathway presumably courses ventrally through the median raphe before turning dorsally near the IPN to travel alongside the fasciculus retroflexus before ending in the lateral habenula [30,35]. Although it seems unlikely that such ventrally projecting pathways are cholinergic, it is conceivable that they are sources of non-cholinergic input to the IPN and habenular complex.

A sparse amount of experimental evidence, albeit somewhat indirect, does indicate the possible existence of dorsally coursing connections between the dorsolateral tegmental nucleus and the habenula. First, a descending pathway from the habenular complex to tegmental nuclei through the central gray has been described as the rostral part of the dorsal longitudinal fasciculus [39,75]. Second, Hamilton [31] has reported that ablations in the dorsal periaqueductal gray produce terminal and fiber degeneration in and near the lateral habenula, an observation prompting Herkenham and Nauta [35] to suggest that ". . . it is possible that Hamilton's lesions interrupted fibers originating in the nucleus tegmenti dorsalis lateralis, the cell group . . . which contained most of the . . . labeled cells found in the central gray in the present study . . . " (p. 141) following infusions of horseradish peroxidase into the habenular complex. Indeed, some fibers deriving from the dorsolateral tegmental nucleus have been traced into the central gray but not far [30]. And third, injection of tritiated leucine into the nucleus cuneiformis, called the pedunculopontine tegmental nucleus in our terminology, but encroaching upon the central gray area has been reported to result in labeling in the lateral habenula [23].

Taking into account all of the experimental findings known to us and cognizant that future research may render some of our conjectures invalid, we propose that the majority of the cholinergic innervation of the IPN derives from two major sources, the rostral basal forebrain cholinergic system and the dorsolateral tegmental nucleus. Cholinergic fibers from these two regions course in or near the habenula, where they may or may not collateralize, before descending in association with the fasciculus retroflexus and terminating in the IPN. This schema is depicted in Fig. 13.

*Non-cholinergic afferents.* Fibers immunoreactive for

ChAT were found in the present study throughout the entire extent of the IPN, whereas substance P was associated primarily with lateral aspects of the nucleus (cf. [34,51]). Similarly, the distribution of interpeduncular AChE has been described as being associated prominently with the lateral, as well as dorsal, subnuclei [34], but the ChAT-containing fibers we observed did not parallel that distribution precisely, probably because the cholinergic degradative enzyme can be localized both pre- and post-synaptically in non-cholinergic, as well as cholinergic, neurons [2,10].

In the current experiments, many cells in the medial habenula retrogradely transported fluorescent tracers from the IPN. The dorsal part of the medial habenula has been shown to project preferentially to lateral aspects of the IPN [36], and this pathway, which courses through the fasciculus retroflexus, is probably substance P-containing [18,59]. Our results are consistent with such a conjecture, because knife cuts circumscribing the habenular complex did not produce a decrement in interpeduncular substance P.

Retrogradely labeled cells were found in the locus ceruleus and the dorsal and median raphe following infusions of propidium iodide or Evans Blue into the IPN. Many of these interpeduncular afferents are probably monoaminergic. Somata in the locus ceruleus contain norepinephrine [19], and noradrenergic fibers have been demonstrated in the dorsal, ventral, lateral, and intermediate interpeduncular nuclei [34]. Similarly, cells in the dorsal and median raphe have been shown to contain 5-hydroxytryptamine [63,73], and serotonergic fibers have been reported to be scattered throughout the IPN [34].

Non-cholinergic afferents to the IPN were also found in the basal forebrain, the premammillary nucleus, and the dorsal and ventral tegmental nuclei. Fibers containing GABA and originating in the basal forebrain may course through the stria medullaris to terminate in the habenula [3, 16, 29] but presumably not in the IPN [16]. A pathway utilizing luteinizing hormone releasing hormone as a possible neurotransmitter has been described, however, from the medial septal nucleus through the stria medullaris, habenula, and fasciculus retroflexus to the IPN [69]. Finally, enkephalin has been localized in cells in the dorsal and ventral tegmental nuclei [25] and in the premammillary nucleus [25,37], and it is conceivable that projections from these nuclei to the IPN are, in part, enkephalinergic.

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## REFERENCES

1. Akagi, K. and E. W. Powell. Differential projections of habenular nuclei. *J Comp Neurol* **132:** 263–274, 1968.
2. Albanese, A. and L. L. Butcher. Locus ceruleus somata contain both acetylcholinesterase and norepinephrine: direct histochemical demonstration on the same tissue section. *Neurosci Lett* **14:** 101–104, 1979.
3. Araki, M., P. L. McGeer and E. G. McGeer. Retrograde HRP tracing combined with a pharmacohistochemical method for GABA transaminase for the identification of presumptive GABAergic projections to the habenula. *Brain Res* **304:** 271–277, 1984.
4. Armstrong, D. M., C. B. Saper, A. I. Levey, B. H. Wainer and R. D. Terry. Distribution of cholinergic neurons in rat brain: demonstrated by the immunocytochemical localization of choline acetyltransferase. *J Comp Neurol* **216:** 53–68, 1983.
5. Bigl, V., N. J. Woolf and L. L. Butcher. Cholinergic projections from the basal forebrain to frontal, parietal, temporal, occipital, and cingulate cortices: A combined fluorescent tracer and acetylcholinesterase analysis. *Brain Res Bull* **8:** 727–749, 1982.
6. Briggs, T. L. and W. W. Kaelber. Efferent fiber connections of the dorsal and deep tegmental nuclei of Gudden. An experimental study in the cat. *Brain Res* **29:** 17–29, 1971.
7. Butcher, L. L. Acetylcholinesterase histochemistry. In: *Handbook of Chemical Neuroanatomy, vol 1: Methods in Chemical Neuroanatomy,* edited by A. Björklund and T. Hökfelt. Amsterdam: Elsevier, 1983, pp. 1–49.
8. Butcher, L. L. and G. K. Hodge. Postnatal development of acetylcholinesterase in the caudate-putamen nucleus and substantia nigra of rats. *Brain Res* **106:** 223–246, 1976.
9. Butcher, L. L. and N. J. Woolf. Cholinergic and serotonergic systems in the brain and spinal cord: anatomic organization, role in intercellular communication processes, and interactive mechanisms. In: *Chemical Transmission in the Brain: The Role of Amines, Amino Acids, and Peptides,* edited by R. M. Buijs, P. Pévet and D. F. Swaab. *Progress in Brain Res,* vol 55. Amsterdam: Elsevier, 1982, pp. 3–40.
10. Butcher, L. L. and N. J. Woolf. Histochemical distribution of acetylcholinesterase in the central nervous system: clues to the localization of cholinergic neurons. In: *Handbook of Chemical Neuroanatomy, Vol. 3: Classical Transmitters and Transmitter Receptors in the CNS,* Part 2, edited by A. Björklund, T. Hökfelt and M. J. Kuhar. Amsterdam: Elsevier, 1984, pp. 1–50.
11. Butcher, L. L., S. M. Eastgate and G. K. Hodge. Evidence that punctate intracerebral administration of 6-hydroxydopamine fails to produce selective neuronal degeneration: comparison with copper sulfate and factors governing the deportment of fluids injected into the brain. *Naunyn Schmiedebergs Arch Pharmacol* **285:** 31–70, 1974.
12. Butcher, L. L., N. J. Woolf and F. Eckenstein. Cholinergic pathways: II. Basal forebrain and pontine tegmentum innervate the interpeduncular nucleus in the rat. *Soc Neurosci Abstr* **10:** 1182, 1984.
13. Cajal, S. Ramon y. *Histologie du Systeme Nerveaux de l'Homme et des Vertebres,* vol 2. Paris: Maloine, 1911.
14. Conrad, L. C. A. and D. W. Pfaff. Efferents from the medial basal forebrain and hypothalamus in the rat. I. An autoradiographic study of the medial preoptic area. *J Comp Neurol* **169:** 185–220, 1976.
15. Contestabile, A. and B. A. Flumerfelt. Afferent connections of the interpeduncular nucleus and the topographic organization of the habenulo-interpeduncular pathway: An HRP study in the rat. *J Comp Neurol* **196:** 253–270, 1981.
16. Contestabile, A. and F. Fonnum. Cholinergic and GABAergic forebrain projections to the habenula and nucleus interpeduncularis: Surgical and kainic acid lesions. *Brain Res* **275:** 287–297, 1983.
17. Cragg, B. G. The connections of the habenula in the rabbit. *Exp Neurol* **3:** 388–409, 1961.
18. Cuello, A. C., P. C. Emson, G. Paxinos and T. Jessell. Substance P containing and cholinergic projections from the habenula. *Brain Res* **149:** 413–429, 1978.

19. Dahlström, A. and K. Fuxe. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol Scand (Suppl)* **232:** 3–55, 1964.
20. Domesick, V. B. Projections of the nucleus of the diagonal band of Broca in the rat. *Anat Rec* **184:** 391–392, 1976.
21. Eckenstein, F. and M. V. Sofroniew. Identification of central cholinergic neurons containing both choline acetyltransferase and acetylcholinesterase and of central neurons containing only acetylcholinesterase. *J Neurosci* **3:** 2286–2291, 1983.
22. Eckenstein, F. and H. Thoenen. Production of specific antisera and monoclonal antibodies to choline acetyltransferase: characterization and use for identification of cholinergic neurons. *EMBO J* **1:** 363–368, 1982.
23. Edwards, S. B. and J. S. DeOlmos. Autoradiographic studies of the projections of the midbrain reticular formation: ascending projections of nucleus cuneiformis. *J Comp Neurol* **165:** 417–432, 1976.
24. Fibiger, H. C. The organization and some projections of cholinergic neurons of the mammalian forebrain. *Brain Res Rev* **4:** 327–388, 1982.
25. Finley, J. C. W., J. L. Maderdrut and P. Petrusz. The immunohistochemical localization of enkephalin in the central nervous system of the rat. *J Comp Neurol* **198:** 541–565, 1981.
26. Flumerfelt, B. A. and A. Contestabile. Acetylcholinesterase histochemistry of the habenulo-interpeduncular pathway in the rat and the effects of electrolytic and kainic acid lesions. *Anat Embryol* **163:** 435–446, 1982.
27. Gottesfeld, Z. and D. M. Jacobowitz. Cholinergic projection of the diagonal band to the interpeduncular nucleus of the rat brain. *Brain Res* **156:** 329–332, 1978.
28. Gottesfeld, Z. and D. M. Jacobowitz. Cholinergic projections from the septal-diagonal band area to the habenular nuclei. *Brain Res* **176:** 391–394, 1979.
29. Gottesfeld, Z., V. J. Massari, E. A. Muth and D. M. Jacobowitz. Stria medullaris: a possible pathway containing GABAergic afferents to the lateral habenula. *Brain Res* **130:** 184–189, 1977.
30. Groenewegen, H. J. and C. A. Van Dijk. Efferent connections of the dorsal tegmental region in the rat, studied by means of anterograde transport of the lectin *Phaseolus vulgaris*-leucoagglutinin (PHA-L). *Brain Res* **304:** 367–371, 1984.
31. Hamilton, B. L. Projections of the nuclei of the periaqueductal gray matter in the cat. *J Comp Neurol* **152:** 45–58, 1973.
32. Hattori, T., E. G. McGeer, V. K. Singh and P. L. McGeer. Cholinergic synapse of the interpeduncular nucleus. *Exp Neurol* **55:** 666–679, 1977.
33. Hayakawa, T. and K. Zyo. Organization of the habenulo-interpeduncular connections in cats: a horseradish peroxidase study. *Brain Res* **240:** 3–11, 1982.
34. Hemmendinger, L. M. and R. Y. Moore. Interpeduncular nucleus organization in the rat: Cytoarchitecture and histochemical analysis. *Brain Res Bull* **13:** 163–179, 1984.
35. Herkenham, M. and W. J. H. Nauta. Afferent connections of the habenular nuclei in the rat. A horseradish peroxidase study, with a note on the fiber-of-passage problem. *J Comp Neurol* **173:** 123–146, 1977.
36. Herkenham, M. and W. J. H. Nauta. Efferent connections of the habenular nuclei in the rat. *J Comp Neurol* **187:** 19–48, 1979.
37. Hökfelt, T., R. Elde, O. Johansson, L. Terenius and L. Stein. The distribution of enkephalin-immunoreactive cell bodies in the rat central nervous system. *Neurosci Lett* **5:** 25–31, 1977.
38. Houser, C. R., G. D. Crawford, R. P. Barber, P. M. Salveterra and J. E. Vaughn. Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Res* **266:** 97–119, 1983.
39. Huber, G. C. and E. C. Crosby. Somatic and visceral connections of the diencephalon. *Arch Neurol Psychiatry* **22:** 187–229, 1929.
40. Iwahori, N. A Golgi study on the habenular nucleus of the cat. *J Comp Neurol* **171:** 319–344, 1977.
41. Karnovsky, M. J. and L. Roots. A "direct-coloring" thiocholine method for cholinesterases. *J Histochem Cytochem* **12:** 219–221, 1964.
42. Kataoka, K., Y. Nakamura and R. Hassler. Habenulo-interpeduncular tract: a possible cholinergic neuron in rat brain. *Brain Res* **62:** 264–267, 1973.
43. Kataoka, K., M. Sorimachi, S. Okuno and N. Mizuno. Cholinergic and Gabaergic fibers in the stria medullaris of the rabbit. *Brain Res Bull* **2:** 461–464, 1977.
44. Kimura, H., P. L. McGeer, J. H. Peng and E. G. McGeer. The central cholinergic system studied by choline acetyltransferase immunohistochemistry in the cat. *J Comp Neurol* **200:** 151–201, 1981.
45. König, J. F. R. and R. A. Klippel. *The Rat Brain: A Sterotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem.* Baltimore: Williams and Wilkins, 1963.
46. Krayniak, P. F., S. Weiner and A. Siegel. An analysis of the efferent connections of the septal area in the cat. *Brain Res* **189:** 15–29, 1980.
47. Kuhar, M. J., R. N. DeHaven, H. I. Yamamura, H. Rommelspacher and J. R. Simon. Further evidence for cholinergic habenulo-interpeduncular neurons: Pharmacologic and functional characteristics. *Brain Res* **97:** 265–275, 1975.
48. Leranth, C. S., M. Brownstein, L. Zaborszky, Z. S. Jaranyi and M. Palkovits. Morphological and biochemical changes in the rat interpeduncular nucleus following the transection of the habenulo-interpeduncular tract. *Brain Res* **99:** 124–128, 1975.
49. Levey, A. I., B. H. Wainer, E. J. Mufson and M.-M. Mesulam. Co-localization of acetylcholinesterase and choline acetyltransferase in the rat cerebrum. *Neuroscience* **9:** 9–22, 1983.
50. Lewis, P. R. and C. C. D. Shute. The cholinergic limbic system: projections to hippocampal formation, medial cortex, nuclei of the ascending cholinergic reticular system, and the subfornical organ and supra-optic crest. *Brain* **90:** 521–542, 1967.
51. Ljungdahl, A., T. Hökfelt and G. Nilsson. Distribution of substance P-like immunoreactivity in the central nervous system of the rat-I. Cell bodies and nerve terminals. *Neuroscience* **3:** 861–943, 1978.
52. Marchand, E. R., J. N. Riley and R. Y. Moore. Interpeduncular nucleus afferents in the rat. *Brain Res* **193:** 339–352, 1980.
53. Mata, M. M., B. K. Schrier and R. Y. Moore. Interpeduncular nucleus: differential effects of habenula lesions on choline acetyltransferase and glutamic acid decarboxylase. *Exp Neurol* **57:** 913–921, 1977.
54. McBride, R. L. Organization of afferent connections of the feline lateral habenular nucleus. *J Comp Neurol* **198:** 89–99, 1981.
55. Meibach, R. C. and A. Siegel. Efferent connections of the septal area in the rat: an analysis utilizing retrograde and anterograde transport methods. *Brain Res* **119:** 1–20, 1977.
56. Mesulam, M.-M., E. J. Mufson, A. I. Levey and B. H. Wainer. Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata), and hypothalamus in the rhesus monkey. *J Comp Neurol* **214:** 170–197, 1983.
57. Mesulam, M.-M., E. J. Mufson, B. H. Wainer and A. I. Levey. Central cholinergic pathways in the rat: An overview based on an alternative nomenclature (Ch1–Ch6). *Neuroscience* **10:** 1185–1201, 1983.
58. Mitchell, R. Connections of the habenula and of the interpeduncular nucleus in the cat. *J Comp Neurol* **121:** 441–453, 1963.
59. Mroz, E. A., M. J. Brownstein and S. E. Leeman. Evidence for substance P in the habenulo-interpeduncular tract. *Brain Res* **113:** 597–599, 1976.
60. Nakamura, Y., R. Hassler, K. Kataoka, I. J. Bak and J. S. Kim. Regional distribution of choline acetyltransferase and acetylcholinesterase activity in baboon brain. *Folia Psychiatr Neurol* **30:** 185–194, 1976.
61. Nauta, W. J. H. Hippocampal projections and related neural pathways to the mid-brain in the cat. *Brain* **81:** 319–340, 1958.

62. Palkovits, M., J. M. Saavedra, R. M. Kobayashi and M. Brownstein. Choline acetyltransferase content of limbic nuclei of the rat. *Brain Res* **79:** 443–450, 1974.
63. Parent, A., L. Descarries and A. Beaudet. Organization of ascending serotonin systems in the adult rat brain. A radioautographic study after intraventricular administration of [$^3$H]-5-hydroxytryptamine. *Neuroscience* **6:** 115–138, 1981.
64. Paxinos, G. and C. Watson. *The Rat Brain in Stereotaxic Coordinates*. Sydney: Academic Press, 1982.
65. Phillipson, O. T. Afferent projections to the ventral tegmental area of Tsai and interfascicular nucleus: a horseradish peroxidase study in the rat. *J Comp Neurol* **187:** 117–144, 1979.
66. Price, J. L. and T. P. S. Powell. Certain observations on the olfactory pathway. *J Anat* **110:** 105–126, 1971.
67. Sastry, B. R., S. E. Zialkowski, L. M. Hansen, J. P. Kavanagh and E. M. Evoy. Acetylcholine release in interpeduncular nucleus following the stimulation of habenula. *Brain Res* **164:** 334–337, 1979.
68. Satoh, K., D. M. Armstrong and H. C. Fibiger. A comparison of the distribution of central cholinergic neurons as demonstrated by acetylcholinesterase pharmacohistochemistry and choline acetyltransferase immunohistochemistry. *Brain Res Bull* **11:** 693–720, 1983.
69. Silverman, A. J. and L. C. Krey. The luteinizing hormone-releasing hormone (LHRH) neuronal networks of the guinea pig brain. I. Intra- and extra-hypothalamic projections. *Brain Res* **157:** 233–246, 1978.
70. Smaha, L. A. and W. W. Kaelber. Efferent fiber projections of the habenula and the interpeduncular nucleus. An experimental study in the opossum and cat. *Exp Brain Res* **16:** 291–308, 1973.
71. Sofroniew, M. V., F. Eckenstein, H. Thoenen and A. C. Cuello. Topography of choline acetyltransferase-containing neurons in the forebrain of the rat. *Neurosci Lett* **33:** 7–12, 1982.
72. Sorimachi, M. and K. Kataoka. Choline uptake by nerve terminals: a sensitive and a specific marker of cholinergic innervation. *Brain Res* **72:** 350–353, 1974.
73. Steinbusch, H. W. M. Distribution of serotonin-immunoreactivity in the central nervous system of the rat—cell bodies and terminals. *Neuroscience* **6:** 557–618, 1981.
74. Swanson, L. W. An autoradiographic study of the efferent connections of the preoptic region in the rat. *J Comp Neurol* **167:** 227–256, 1976.
75. Thompson, E. L. The dorsal longitudinal fasciculus in Didelphis virginiana. *J Comp Neurol* **76:** 239–282, 1942.
76. Van der Kooy, D. and P. E. Sawchenko. Characterization of serotonergic neurons using concurrent fluorescent retrograde axonal tracing and immunohistochemistry. *J Histochem Cytochem* **30:** 794–798, 1982.
77. Villani, L., A. Contestabile and F. Fonnum. Autoradiographic labeling of the cholinergic habenulo-interpeduncular projection. *Neurosci Lett* **42:** 261–266, 1983.
78. Vincent, S. R., W. A. Staines, E. G. McGeer and H. C. Fibiger. Transmitters contained in the efferents of the habenula. *Brain Res* **195:** 479–484, 1980.
79. Vizi, S. E. and M. Palkovits. Acetylcholine content in different regions of the rat brain. *Brain Res Bull* **3:** 93–96, 1978.
80. Woolf, N. J. and L. L. Butcher. Cholinergic neurons in the caudate-putamen complex proper are intrinsically organized: A combined Evans Blue and acetylcholinesterase analysis. *Brain Res Bull* **7:** 487–507, 1981.
81. Woolf, N. J. and L. L. Butcher. Cholinergic projections to the basolateral amygdala: A combined Evans Blue and acetylcholinesterase analysis. *Brain Res Bull* **8:** 751–763, 1982.
82. Woolf, N. J., F. Eckenstein and L. L. Butcher. Cholinergic projections from the basal forebrain to the frontal cortex: a combined fluorescent tracer and immunohistochemical analysis in the rat. *Neurosci Lett* **40:** 93–98, 1983.
83. Woolf, N. J., F. Eckenstein and L. L. Butcher. Cholinergic systems in the rat brain: I. Projections to the limbic telencephalon. *Brain Res Bull* **13:** 751–784, 1984.
84. Yamadori, T. Efferent fibers of the habenula and stria medullaris thalami in rats. *Exp Neurol* **25:** 541–558, 1969.