

CENTRAL CHOLINERGIC PATHWAYS IN THE RAT: AN OVERVIEW BASED ON AN ALTERNATIVE NOMENCLATURE (Ch1-Ch6)

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Abstract—Monoclonal antibodies to choline acetyltransferase and a histochemical method for the concurrent demonstration of acetylcholinesterase and horseradish peroxidase were used to investigate the organization of ascending cholinergic pathways in the central nervous system of the rat. The cortical mantle, the amygdaloid complex, the hippocampal formation, the olfactory bulb and the thalamic nuclei receive their cholinergic innervation principally, from cholinergic projection neurons of the basal forebrain and upper brainstem. On the basis of connectivity patterns, we subdivided these cholinergic neurons into six major sectors. The Ch1 and Ch2 sectors are contained within the medial septal nucleus and the vertical limb nucleus of the diagonal band, respectively. They provide the major cholinergic projections of the hippocampus. The Ch3 sector is contained mostly within the lateral portion of the horizontal limb nucleus of the diagonal band and provides the major cholinergic innervation to the olfactory bulb. The Ch4 sector includes cholinergic neurons in the nucleus basalis, and also within parts of the diagonal band nuclei. Neurons of the Ch4 sector provide the major cholinergic innervation of the cortical mantle and the amygdala. The Ch5-Ch6 sectors are contained mostly within the pedunculopontine nucleus of the pontomesencephalic reticular formation (Ch5) and within the laterodorsal tegmental gray of the periventricular area (Ch6). These sectors provide the major cholinergic innervation of the thalamus. The Ch5-Ch6 neurons also provide a minor component of the corticopetal cholinergic innervation.

These central cholinergic pathways have been implicated in a variety of behaviors and especially in memory function. It appears that the age-related changes of memory function as well as some of the behavioral disturbances seen in the dementia of Alzheimer's Disease may be related to pathological alterations along central cholinergic pathways.

Neurons in the basal forebrain provide the major source of cholinergic innervation for the entire neocortex, the hippocampus, the amygdala and the olfactory bulb.^{5,20,21,30,35,37,40,41,42,50,52,54,57,58,59,66,68,73,76} Based on observations in the macaque brain, we subdivided these cholinergic projection neurons of the basal forebrain into four major sectors which we designated Ch1-Ch4.⁵⁷ In our initial report, the Ch designation referred to the entire nucleus that contained cholinergic projection neurons. We have since modified our position so that we reserve the Ch designation only for the subset of cholinergic neurons within the relevant nuclei. In the macaque, the Ch1 and Ch2 sectors are contained within the medial septal nucleus and the vertical limb nucleus of the diagonal band, respectively. They collectively provide the major source of cholinergic projections to the hippocampus. The Ch3 sector is contained, at least in part, within the horizontal limb nucleus of the diagonal band and provides the major source of Ch1-Ch4 projections to the olfactory bulb. The Ch4 sector

most closely corresponds to the nucleus basalis of Meynert but also includes cholinergic neurons within the nucleus of the ansa lenticularis, the nucleus of the ansa peduncularis, the medullary laminae of the globus pallidus, and the substantia innominata. The Ch4 neurons provide the principal cholinergic innervation for the amygdala and neocortical areas. This extensive and continuous system of Ch1-Ch4 neurons gives rise to topographically organized cholinergic projections which innervate the entire neocortical mantle as well as many limbic and olfactory structures. In addition to these telencephalic structures, thalamic nuclei also appear to receive substantial cholinergic innervation.^{17,24,69,83} Several lines of investigation strongly suggest that this innervation originates in the region of the pontomesencephalic reticular formation.^{15,32,33,51,76}

The experiments that we report here served two purposes. First, we wanted to see if an analogous Ch1-Ch4 nomenclature, based on the same set of cytochemical and anatomical considerations which we used in the macaque brain, could be proposed for the cholinergic neurons of the rat forebrain. A second purpose was to identify the individual neurons which give rise to the cholinergic innervation of the thal-

Abbreviations: AChE, acetylcholinesterase; ChAT, choline acetyltransferase; HRP, horseradish peroxidase; WGA, wheat germ agglutinin.

amus and to see how these cholinergic neurons relate to the Ch1–Ch4 sectors of the basal forebrain.

EXPERIMENTAL PROCEDURES

Our observations are based on material obtained from 23 Long-Evans male hooded rats weighing between 250 and 350 g. Each animal received a single injection (0.01–0.03 μ l of a 10% tracer solution) of horseradish peroxidase (HRP) conjugated covalently to wheat germ agglutinin (WGA).^{26,56} The injections were placed in the olfactory bulb, the hippocampus, the neocortex or the thalamus. In several of the thalamic injections, the tracer was administered through an implanted cannula in order to decrease spread of tracer to the overlying hippocampus and cortex. Following a survival time of 24–48 h, each animal was perfused with a mixture of either 1.25% glutaraldehyde–1% paraformaldehyde or 0.1% glutaraldehyde–4% paraformaldehyde. The latter combination was used for cases in which immunohistochemical procedures were contemplated. Although the latter fixative combination was compatible with HRP histochemistry, it afforded a lower level of overall sensitivity than the combination of 1.25% glutaraldehyde and 1% paraformaldehyde. Timed fixation was obtained according to procedure II of Rosene and Mesulam.⁷⁴ Each animal was also pretreated 6–18 h before death with the irreversible acetylcholinesterase (AChE) inhibitor diisopropyl-fluorophosphate (DFP) at a dose of 0.5–2 mg/kg, i.m. in peanut oil. The DFP pretreatment allowed the AChE-containing perikarya to be visualized with greater clarity.^{7,48} Following fixation the brain was cut on a freezing microtome into 8–10 series of matching 40 μ m-thick sections. Individual series of sections were then processed according to the following procedures.

Horseradish peroxidase histochemistry was performed with tetramethyl benzidine as the chromogen and neutral red for a counterstain.^{55,56} Choline acetyltransferase (ChAT) immunohistochemistry was done using rat monoclonal antibody AB8 and the peroxidase–antiperoxidase method of Sternberger.⁸¹ Monoclonal antibody AB8 binds active ChAT in a specific double antibody assay, binds specifically to ChAT using the Western blotting system and localizes known cholinergic neurons immunohistochemically.^{44,46} Control material was obtained by using rat IgG instead of AB8. Some of the sections processed for ChAT immunohistochemistry were counterstained with thionin in order to elucidate the relationship between ChAT-positive and ChAT-negative neurons within the same nuclear group. Acetylcholinesterase (AChE) histochemistry was done with acetylthiocholine as the substrate and ethopropazine as the inhibitor of non-acetyl cholinesterases.^{22,56} Control material was obtained by using butyrylthiocholine as the substrate. The AChE reaction-product was black and granular. In these preparations, a wide range of intensity was observed in the staining pattern. Only neurons with intensely positive perikarya and dendrites were identified as AChE-rich. Some sections processed for AChE were also counterstained with thionin in order to observe the relationship between AChE-rich neurons and the other perikarya in the same nucleus.

Choline acetyltransferase and AChE were demonstrated concurrently in one set of sections.⁴⁵ In this procedure, the AChE reaction-product is black and coarsely granular whereas the ChAT reaction-product is brown and diffuse. With this procedure it is possible to differentiate perikarya that contain only AChE or only ChAT from those that

contain both reaction products. Acetylcholinesterase and HRP were demonstrated concurrently in another series of sections.⁵⁶ In this procedure, the AChE reaction-product was reddish brown and diffuse whereas the HRP reaction-product was coarsely granular and blue. This method has a considerable false negative rate for HRP labeling. Therefore, the extent of retrograde labeling is best assessed by examining matching series of sections processed only for HRP histochemistry. However, there are no false positives so that the HRP-labeled AChE-rich neurons that are visualized provide unequivocal information about the projections of AChE-rich neurons. The staining of Nissl substance was done with thionin in order to obtain cytoarchitectonic information.

RESULTS

We focused our observations on non-striatal and non-oculomotor cholinergic projection neurons in the basal forebrain and upper brainstem. In the forebrain, ChAT-like immunoreactivity in non-striatal neurons was seen within the medial septum, the diagonal band nuclei and the nucleus basalis. In the upper brainstem ChAT-positive non-oculomotor cholinergic neurons were found in the pontomesencephalic reticular formation within regions that overlap with the nucleus cuneiformis, the nucleus pedunculopontinus, the parabrachial area and the nucleus tegmentalis laterodorsalis (Figs 1–2). These observations corroborate the reports of Kimura *et al.*³⁸ Sofroniew *et al.*⁷⁸ and Armstrong *et al.*¹ Following DFP pretreatment, we noted that these areas also contained AChE-rich neurons as described by others.^{5,8,21,34a,42,49,65,76} The concurrent demonstration of ChAT-like immunoreactivity and AChE in the same tissue section showed that in each of these areas AChE-rich neurons were almost always (95–99%), also ChAT-positive even though not all ChAT-positive neurons were necessarily AChE-rich. This corroborates our prior observations in the basal forebrain of the rat and monkey.^{45,57} These results indicated that in the area of the upper brainstem and basal forebrain, the AChE-rich staining pattern could be used as a reliable, albeit conservative, marker for cholinergic neurons. This conclusion is especially useful in justifying the use of the concurrent HRP-AChE stain to trace the projections of cholinergic neurons*.

Horseradish peroxidase injection sites

In three cases, the injection of tracer was confined to the olfactory bulb. In two cases, the injection site was centered within the dorsal hippocampus with minor spread of tracer to overlying dorsal neocortex. In five cases, the injection site was confined to dorsolateral frontoparietal neocortex. In nine cases, the center of the injection site (2 mm in diameter) was within the dorsal thalamus and involved mostly the anterior, reticular and lateral nuclei with relatively minor spread of tracer to overlying hippocampus and dorsal neocortex. In four cases, the tracer injection had a maximal diameter of 0.5 mm and was entirely

*The AChE-rich staining pattern does not necessarily indicate the presence of a cholinergic neuron everywhere in the brain. In the hypothalamus there are AChE-rich neurons which are ChAT-negative.^{45,57} Thus, the relationship between these two enzymes needs to be determined individually for each region of the brain.

confined to the thalamus. The center of these smaller injections was within the lateral thalamic area and there was minor spread to the lateral geniculate and parafascicular nuclei.

The Ch1–Ch6 nomenclature

Choline acetyltransferase immunohistochemical preparations counterstained with thionin indicated that the cholinergic projection neurons of the basal forebrain and upper brainstem are constituents of heterogeneous nuclei which contain cholinergic as well as apparently non-cholinergic neurons. These nuclei are also incompletely demarcated either from adjacent non-cholinergic cell groups or from passing fiber bundles. The nomenclature for the nuclei that contain these cholinergic cells has engendered considerable inconsistency and confusion.^{14,27,57,72} These considerations have prompted us to propose the alternative Ch1–Ch6 designation for the cholinergic projection neurons of the basal forebrain and upper brainstem. The prefix Ch focuses the emphasis on the subset of cholinergic neurons in each area. These cholinergic neurons are further subdivided into six individual sectors each of which share common connectivity patterns and cytoarchitectonic characteristics. We have proposed an analogous nomenclature for the cholinergic cell groups in the basal forebrain of the monkey.⁵⁷

The Ch1 sector. Cholinergic neurons analogous to the Ch1 and Ch2 sectors of the monkey brain can also be identified in the rat. The Ch1 sector consists of ChAT-positive neurons contained within the medial septal nucleus (Fig. 1A). In general, these neurons are the smallest in the Ch1–Ch6 cell groups. The cholinergic neurons of Ch1 are intermingled with ChAT-negative perikarya (Fig. 3A). Within the traditional boundaries of the medial septal nucleus, the ChAT-positive perikarya are concentrated along the midline raphe of the septum and also along the outer edge of the nucleus (Fig. 1A). Although the thionin counterstain obtained in these immunohistochemical preparations does not offer optimal staining of Nissl substance, it appeared that only 30–50% of the perikarya within the traditional boundaries of the medial septal nucleus were ChAT-positive. It is conceivable that immunohistochemical methods with better sensitivity may yield a higher proportion of ChAT-positive perikarya in this nucleus. However, one alternative conclusion is that the medial septal nucleus contains a sizeable population of non-cholinergic neurons. A similar observation was made in the Ch1 sector of the monkey.⁵⁷ Substantial HRP labeling of AChE-rich (cholinergic) neurons in this area occurred only after hippocampal injections of tracer (Fig. 4A–D).

The Ch2 sector. There are no definite boundaries between Ch1 and Ch2. The Ch2 sector consists of ChAT-positive neurons within the vertical limb nucleus of the diagonal band (Fig. 1A). The Ch2 neurons are, in general, larger than those in Ch1. The

ChAT-thionin preparations showed that approximately 50–75% of the identifiable neuronal perikarya in this region are ChAT-positive (Fig. 3B). At some levels ChAT-negative neurons were evenly distributed among ChAT-positive ones; at other levels, each type of neuron formed small clusters. Substantial retrograde labeling of Ch2 occurred in cases with HRP–WGA injections into the hippocampus (Fig. 4A–D). In these cases, the vast majority of all the retrogradely-labeled AChE-rich cholinergic neurons were in Ch1–Ch2. Therefore, the Ch1 and Ch2 groups collectively provide the major component of cholinergic projections to the hippocampus. In this respect, the Ch1–Ch2 neurons of the rat are analogous to the Ch1–Ch2 neurons of the monkey.⁵⁷ A sizeable proportion of retrograde labeling in the medial septum and the vertical limb nucleus following hippocampal injections of tracer was seen within AChE-negative perikarya. This raises the possibility of an alternate non-cholinergic septohippocampal projection. A similar observation was made in the macaque brain.⁵⁹

Cholinergic neurons just lateral to the vertical limb nucleus create potential difficulties for the Ch nomenclature (Fig. 1A). This region has been called the ventral part of the vertical limb by DeOlmos *et al.*¹⁴ and includes the pars angularis of the diagonal band of Bigl *et al.*⁵ Cytochemical and topographical criteria suggest that these neurons belong to Ch2. The substantial retrograde labeling in this area in cases with hippocampal tracer injections supports this conclusion. However, the observations of Bigl *et al.*⁵ and Lamour *et al.*⁴¹ show that these neurons may provide the major source of neocortical projections to the occipital lobe. This connectivity pattern suggests that some elements of this region could also be included in Ch4 since one of the major characteristics for Ch4 in the monkey was that it provided the major cholinergic projections to neocortical regions.

The Ch3 sector. A group of neurons analogous to the Ch3 sector of the monkey can be identified within the horizontal limb nucleus of the diagonal band. With respect to nomenclature we have followed the example of DeOlmos *et al.*¹⁴ so that we have not attempted to differentiate the horizontal limb nucleus from the preoptic magnocellular nucleus. Within the boundaries of the region traditionally designated as the horizontal limb nucleus (for example, by Price and Powell,⁷² or DeOlmos *et al.*¹⁴) there are considerable variations in the density of cholinergic perikarya. Sections stained for ChAT and counterstained with thionin showed that the proportion of cholinergic perikarya was 50–75% in the medial parts of the horizontal limb nucleus but only 20–25% in its lateral aspects (Figs 1B, 3C). Heimer²⁹ has also observed that the lateral part of the horizontal limb nucleus contained fewer AChE-rich neurons than its medial part. This is clearly illustrated in Fig. 2 of the paper by Bigl *et al.*⁵

Retrograde labeling in the horizontal limb nucleus

was very intense after olfactory bulb injections of HRP-WGA. The neurons that projected to the olfactory bulb were more concentrated in the lateral part of the horizontal limb nucleus (Figs 5A–B). Many of these HRP-labeled neurons were AChE-negative; others were also AChE-rich. In fact, the horizontal limb nucleus, and especially its lateral part, provided the major source of projections from AChE-rich (cholinergic) Ch1–Ch6 neurons to the olfactory bulb (Figs 4E–H, 5A–C). As in the case of septo-hippocampal projections, the presence of many HRP labeled, AChE-negative neurons raises the possibility of a parallel non-cholinergic pathway from the horizontal limb nucleus to the olfactory bulb.

In the macaque, we defined Ch3 as a nuclear group along the horizontal limb of the diagonal band which provided the bulk of projections from cholinergic basal forebrain neurons to the olfactory bulb.⁵⁷ We also noted that in this region of the macaque brain few cholinergic neurons were embedded among a majority of non-cholinergic perikarya. It appears, therefore, that the closest analogue for Ch3 in the rat brain is provided by the cholinergic perikarya within the lateral half of the horizontal limb nucleus (Fig. 1B). It is not yet entirely clear how the cholinergic neurons in the medial part of the horizontal limb

nucleus in the rat fit our Ch nomenclature. Topographically, these neurons are in continuity with Ch2 anteriorly and with Ch4 dorsally. The observations of Bigl *et al.*⁵ and Lamour *et al.*⁴¹ indicate that these neurons may have substantial neocortical projections so that they may form, perhaps in substantial part, a component of Ch4.

The Ch4 sector. In the monkey, the Ch4 sector consists of neurons which provide the major source of cholinergic projections to the neocortical mantle. In the rat, the most obvious components of an analogous Ch4 are contained within the nucleus basalis as designated by Lehmann *et al.*⁴² and Bigl *et al.*⁵ (Fig. 2A). These same neurons have been considered part of the entopeduncular nucleus and of the globus pallidus by Shute and Lewis⁷⁶ by Jacobowitz and Palkovits^{34a} and by Emson *et al.*²⁰ As indicated above, connectivity studies suggest that neurons lateral to the vertical limb nucleus as well as those on the medial part of the horizontal limb nucleus may also belong to Ch4. In addition, cholinergic neurons which form a band ventral to the anterior commissure, in a region usually called the substantia innominata^{5,42} as well as those which bridge the gap between the horizontal limb nucleus and the nucleus basalis (labeled, at least in part, as components of the

Fig. 1 A–B: These photomicrographs are from sections stained for the immunohistochemical demonstration of choline acetyltransferase-like immunoreactivity. Perikarya which are ChAT-positive are seen in the striatal complex (cp, otb, fs and na) as well as in the basal forebrain. Magnification $\times 23$. (A) The ChAT-containing perikarya within the traditional boundaries of the medial septal nucleus constitute Ch1; those within the boundaries of the vertical limb nucleus of the diagonal band constitute Ch2. (B) ChAT-containing perikarya are seen within the boundaries of the horizontal limb nucleus (nhl) of the diagonal band. Laterally, the ChAT-containing perikarya are less densely packed and constitute Ch3. The ChAT-containing perikarya of the medial nhl are more densely packed and probably constitute more rostral elements of Ch4. Under the anterior commissure, there are ChAT-containing non-striatal neurons in a region that is generally designated as the substantia innominata. These neurons are also likely to belong to Ch4.

Abbreviations used in figures

a	cerebral aqueduct	hy	hypothalamus
ac	anterior commissure	ic	internal capsule
amg	amygdala	LL	lateral lemniscus
cb1	cerebellum	ltm	laterodorsal tegmental nucleus
cc	corpus callosum	mlf	medial longitudinal fasciculus
cg	central grey	ms	medial septum
Ch1	cholinergic cell group 1	na	nucleus accumbens
Ch2	cholinergic cell group 2	nb	nucleus basalis
Ch3	cholinergic cell group 3	nc	neocortex
Ch4	cholinergic cell group 4	nhl	nucleus of the horizontal limb of the diagonal band
Ch5	cholinergic cell group 5	nvl	nucleus of the vertical limb of the diagonal band
Ch6	cholinergic cell group 6	ob	olfactory bulb
ci	inferior colliculus	olt	olfactory tract
cn	cuneiformis nucleus	otb	olfactory tubercle
cp	caudate-putamen complex	pno	oral division of the pontine reticular nucleus
cs	superior colliculus	ppn	pedunclopontine nucleus
dsp	decussation of the superior cerebellar peduncle	si	substantia innominata
f	fornix	sm	stria medullaris
fi	fimbria of the fornix	sp	superior cerebellar peduncle
fs	fundus of the striatum	th	thalamus
gp	globus pallidus	v	ventricle
h	hippocampus		

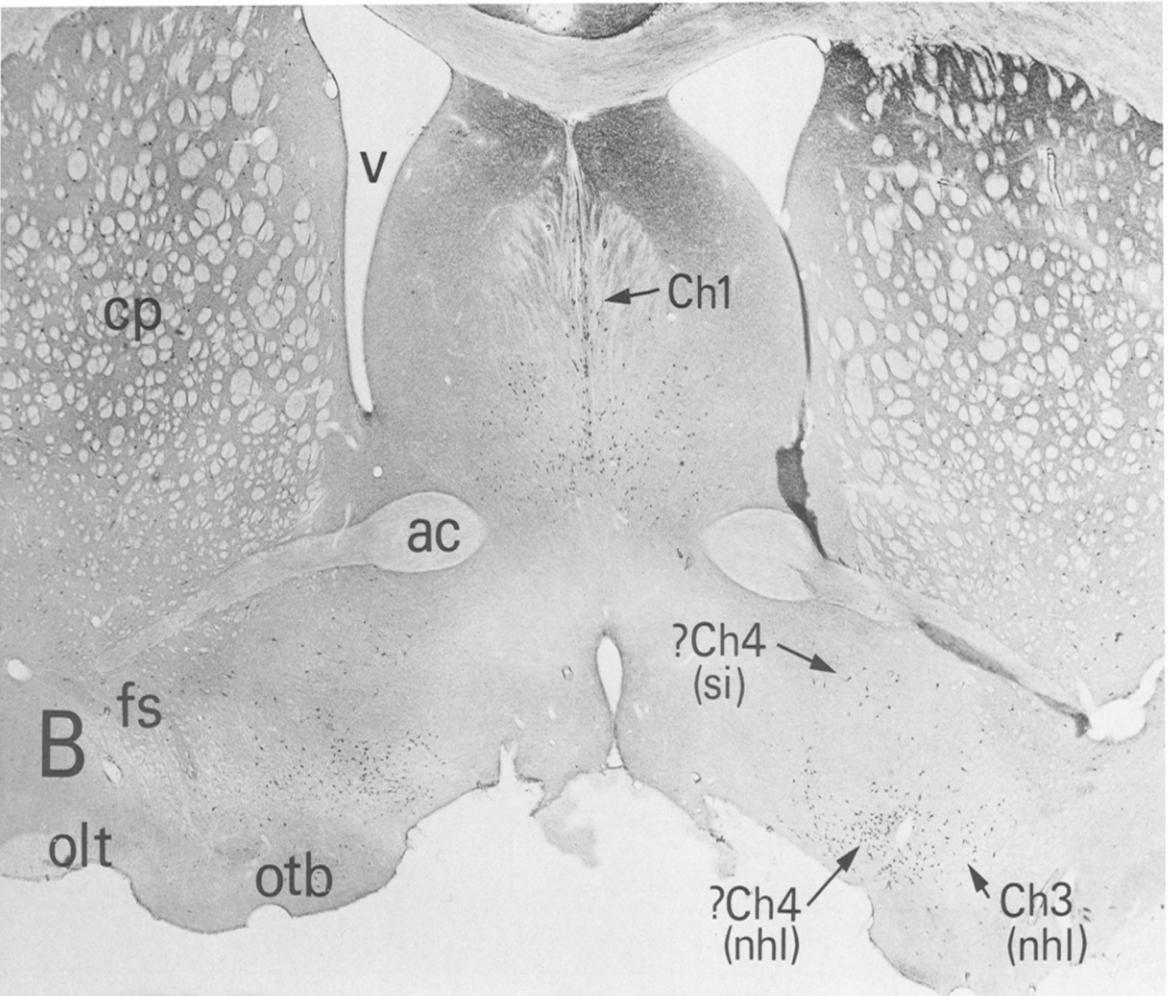
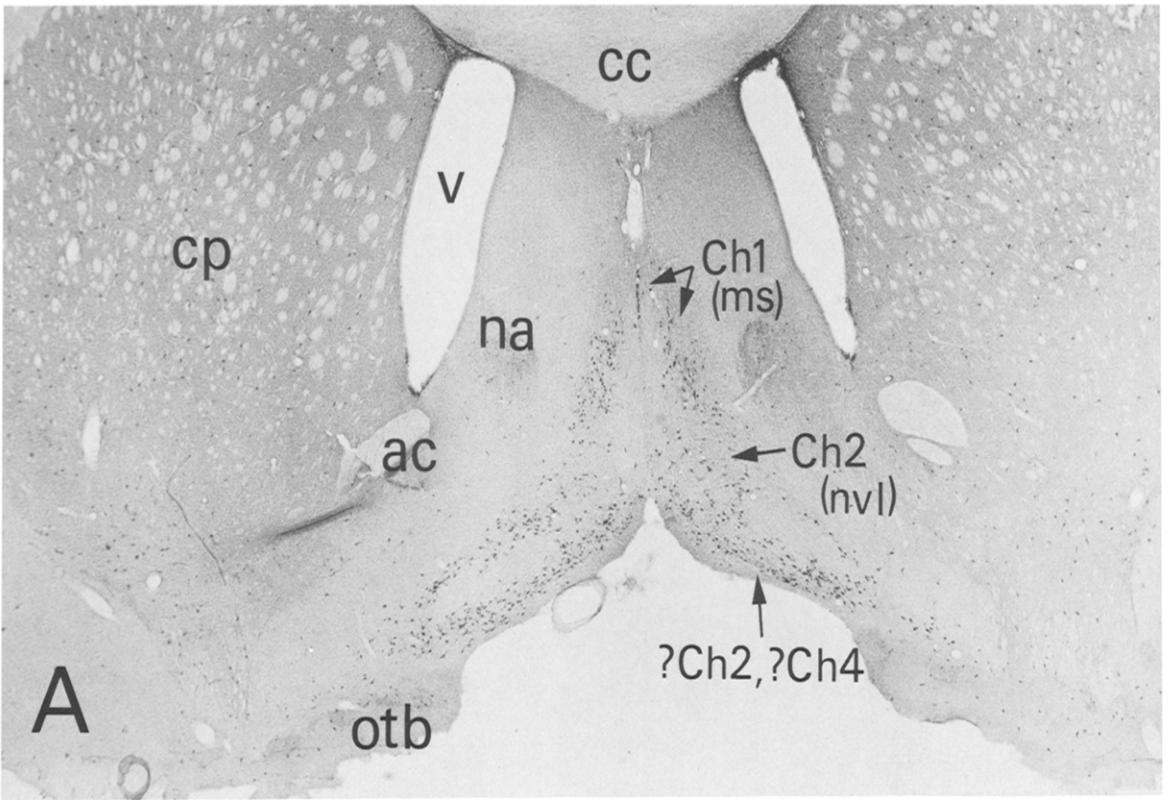


Fig. 1.
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Fig. 2(A)–(E). These photomicrographs are from sections prepared for the immunohistochemical demonstration of choline acetyltransferase-like immunoreactivity. (A) There are non-striatal ChAT-containing neurons located between the internal capsule and the globus pallidus. These neurons are often designated as the nucleus basalis.^{5,42} They belong to Ch4. The neurons of the globus pallidus are ChAT-negative. Magnification $\times 25$. (B)–(C) There are two groups of non-oculomotor ChAT-positive neurons in the pontomesencephalic junction. The Ch5 group is made up of ChAT-positive perikarya which are mostly within the traditional boundaries of the pedunculopontine nucleus. Some Ch5 neurons extend into the nucleus cuneiformis, the parabrachial region, the lateral lemniscus, the superior cerebellar peduncle and the medial longitudinal fasciculus. Ch5 has a compact component which abuts on the lateral lemniscus and a diffuse component which extends more medially. Choline acetyltransferase-containing cell bodies within the boundaries of the laterodorsal tegmental nucleus constitute the Ch6 sector. Magnification $\times 17$. (D) Detail of Ch5 neurons. Magnification $\times 150$. (E) Detail of Ch6 neurons. Magnification $\times 150$.

Fig. 3(A)–(C) Choline acetyltransferase immunohistochemical preparation counterstained with thionin. These photomicrographs show that ChAT-positive perikarya (double arrowhead) are intermixed with ChAT-negative perikarya (single arrowhead). (A) Medial septal nucleus. Ch1 neurons (double arrowhead) are intermixed with apparently non-cholinergic neurons (single arrowhead). Medial is to the left and dorsal towards the top. Magnification $\times 300$. (B) Vertical limb nucleus of the diagonal band. Ch2 neurons (double arrowhead) are intermixed with other apparently non-cholinergic neurons (single arrowhead). Dorsal is towards the top. Magnification $\times 280$. (C) Horizontal limb nucleus of the diagonal band. Medial is to the left and dorsal towards the top. The medial part of the horizontal limb nucleus contains a high density of ChAT-positive perikarya (double arrowhead) intermixed with relatively few ChAT-negative cell bodies (single arrowhead). The lateral part of the horizontal limb nucleus contains fewer ChAT-positive perikarya interspersed among many ChAT-negative cell bodies. The cholinergic neurons on the lateral part of the horizontal limb nucleus belong to the Ch3; those on the medial part probably belong to the Ch4. Magnification $\times 150$.

Fig. 4(A)–(P). Distribution of acetylcholinesterase-rich Ch1–Ch6 perikarya with and without retrograde horseradish peroxidase labeling in four representative cases. Each horizontal row of four sections represents one case. The HRP injection site is written on the left side of the Figure. In two of the cases the injection site is also indicated by the striped area. The AChE-rich, HRP-negative neurons of Ch1–Ch6 are shown as open circles. Acetylcholinesterase-rich neurons that are also labeled with HRP (and which therefore project to the injection site) are shown as black circles. The location of these neurons was determined from sections processed for the concurrent demonstration of AChE and HRP. The distribution of each type of neuron was charted with the help of an X-Y plotter electronically coupled to the movable stage of the examining microscope. The highest concentration of AChE-rich perikarya also labeled with HRP occurs in Ch1–Ch2 when the injection is in the hippocampus (A–D); in Ch3 when the injection is in the olfactory bulb (E–H); in Ch4 when the injection is in neocortex (I–L); and in Ch5–Ch6 when the injection is in the thalamus (M–P). The medial septal nucleus and the vertical limb nucleus also contained many HRP-labeled AChE-negative neurons after hippocampal HRP injections. A similar situation existed in the lateral part of the horizontal limb nucleus after HRP injections in the olfactory bulb. These HRP-positive, AChE-negative neurons are not indicated in this illustration which demonstrates only the AChE-rich neurons in Ch1–Ch6.

Fig. 5(A)–(B). The concurrent demonstration of acetylcholinesterase and horseradish peroxidase following pretreatment with DFP. (A) The horizontal limb nucleus of the diagonal band in a rat injected with HRP into the olfactory bulb. Medial is to the left and dorsal towards the top. The HRP reaction-product is black whereas the AChE reaction-product is a lighter gray. Most of the retrogradely labeled neurons (the black perikaryal profiles) are in the lateral part of the horizontal limb nucleus. Some of the HRP-labeled neurons are AChE-rich (triple arrowhead) but the majority are not (single arrowhead). Acetylcholinesterase-rich neurons (double arrowhead) are more concentrated on the medial part of the horizontal limb nucleus even though some are also found more laterally. These AChE-rich neurons within the lateral part of the horizontal limb nucleus provide the major AChE-rich projections to the olfactory bulb and constitute the Ch3 sector. Magnification $\times 150$. (B) Detail from the same case showing an AChE-rich neuron labeled with HRP (triple arrowhead), a cell body with only the HRP reaction-product (single arrowhead) and cell bodies with only the AChE reaction-product (double arrowhead). Magnification $\times 450$. (C) The concurrent demonstration of AChE and ChAT in Ch1–Ch6. In such preparations, the ChAT reaction product results in a diffuse precipitate in the form of a perikaryal profile. The AChE reaction-product precipitates as coarse granules. The neuron in the center of the field gives an appearance consistent with an AChE-rich ChAT-positive perikaryon. Virtually all AChE-rich perikarya in Ch1–Ch6 give this staining pattern, indicating that the AChE-rich perikarya in these regions are also ChAT-positive. Magnification $\times 450$.

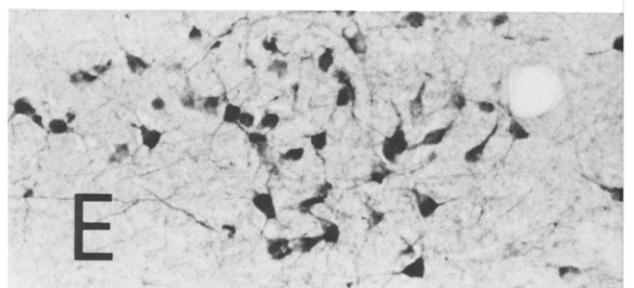
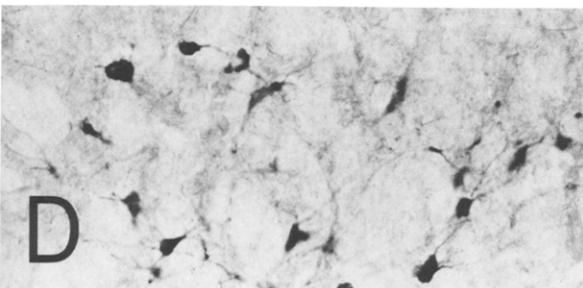
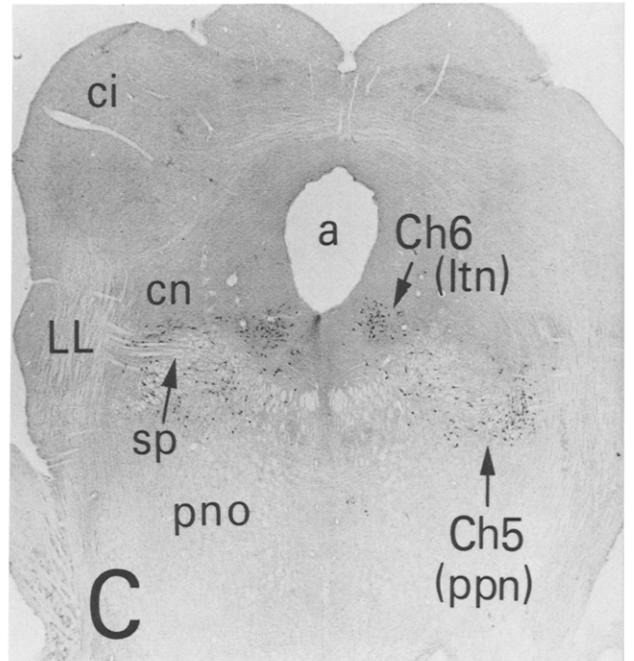
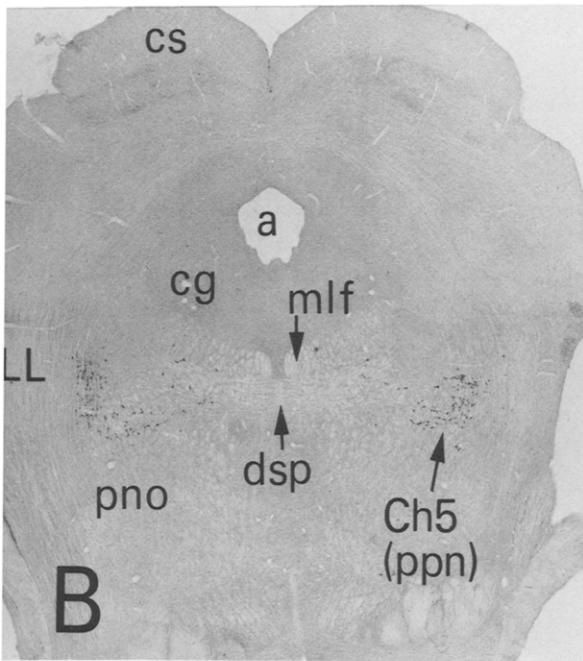
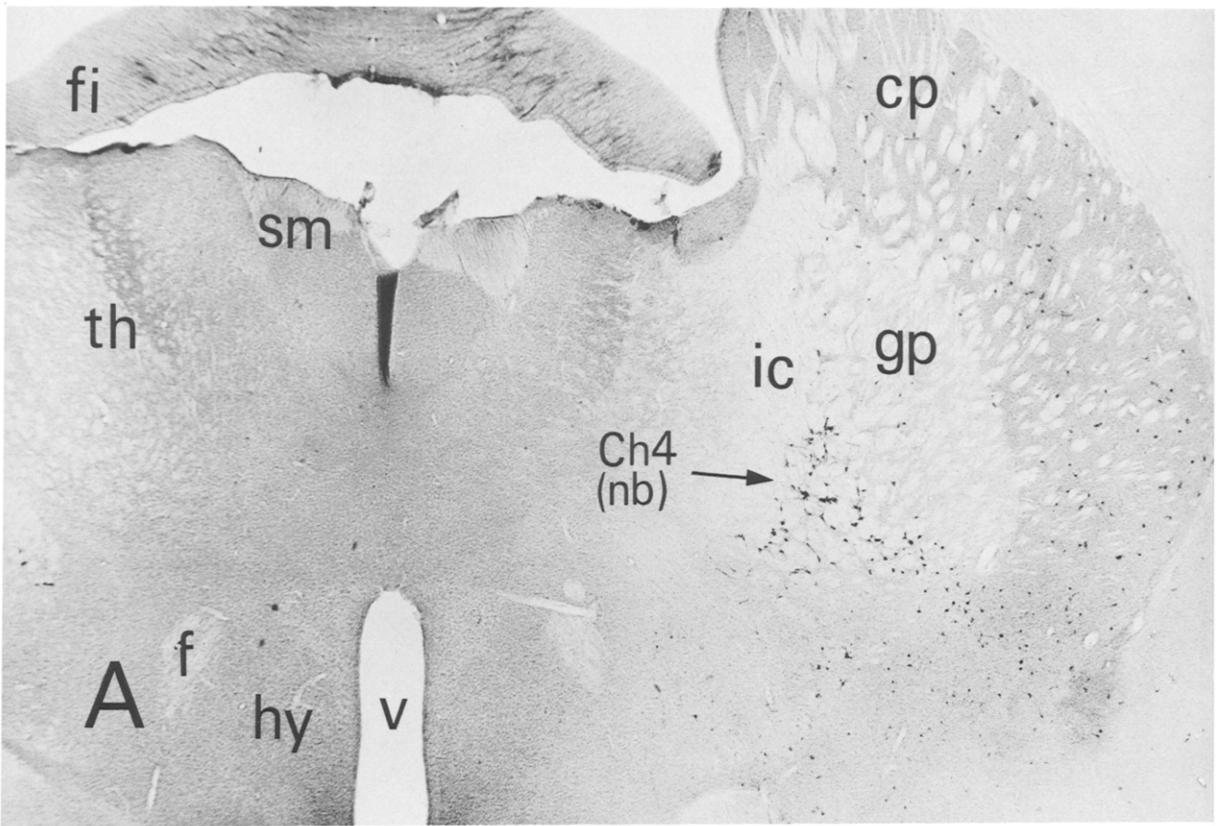


Fig. 2.
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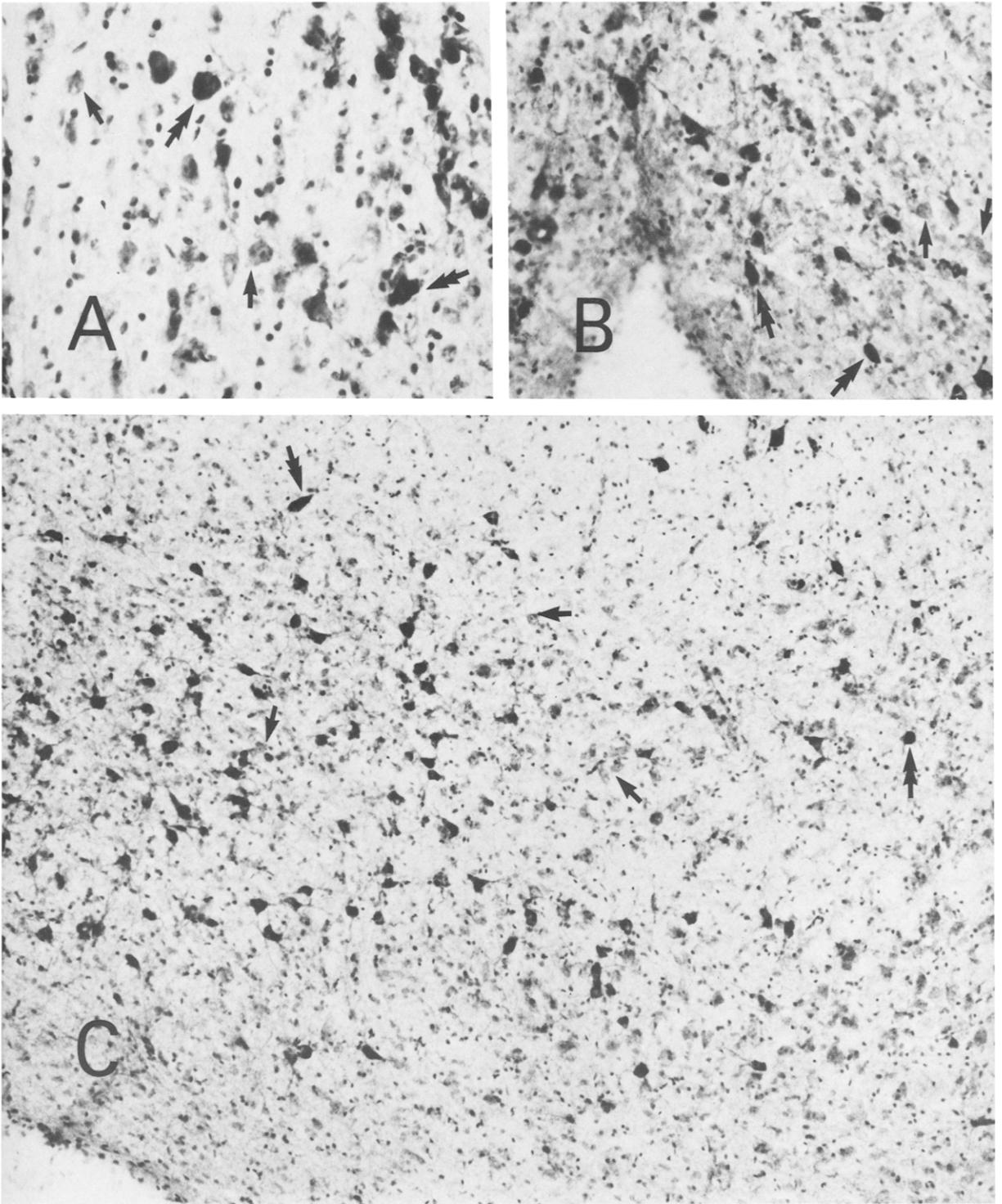
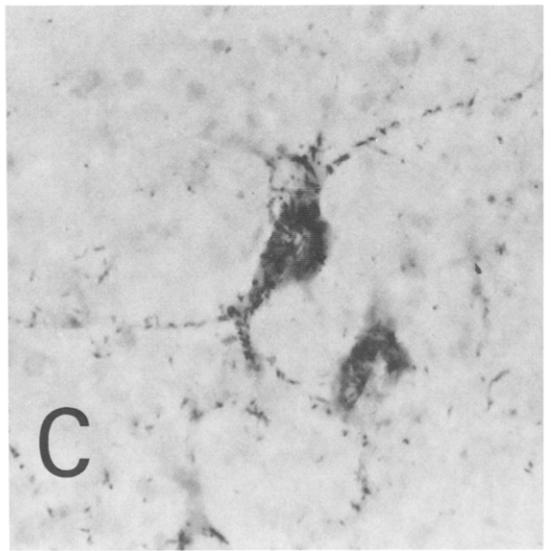
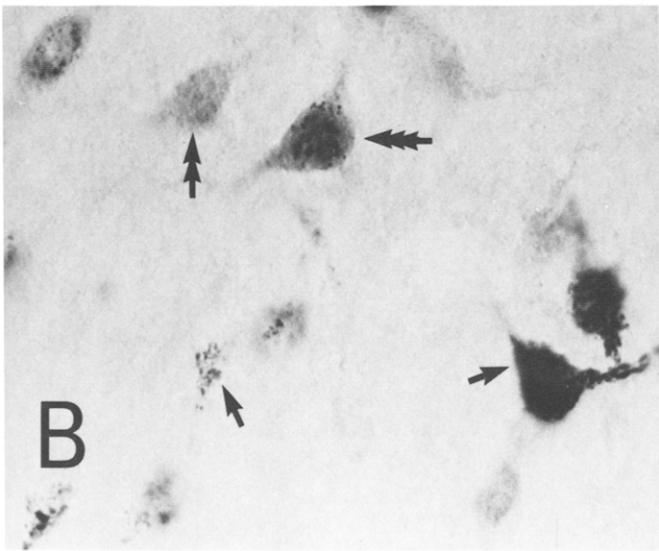
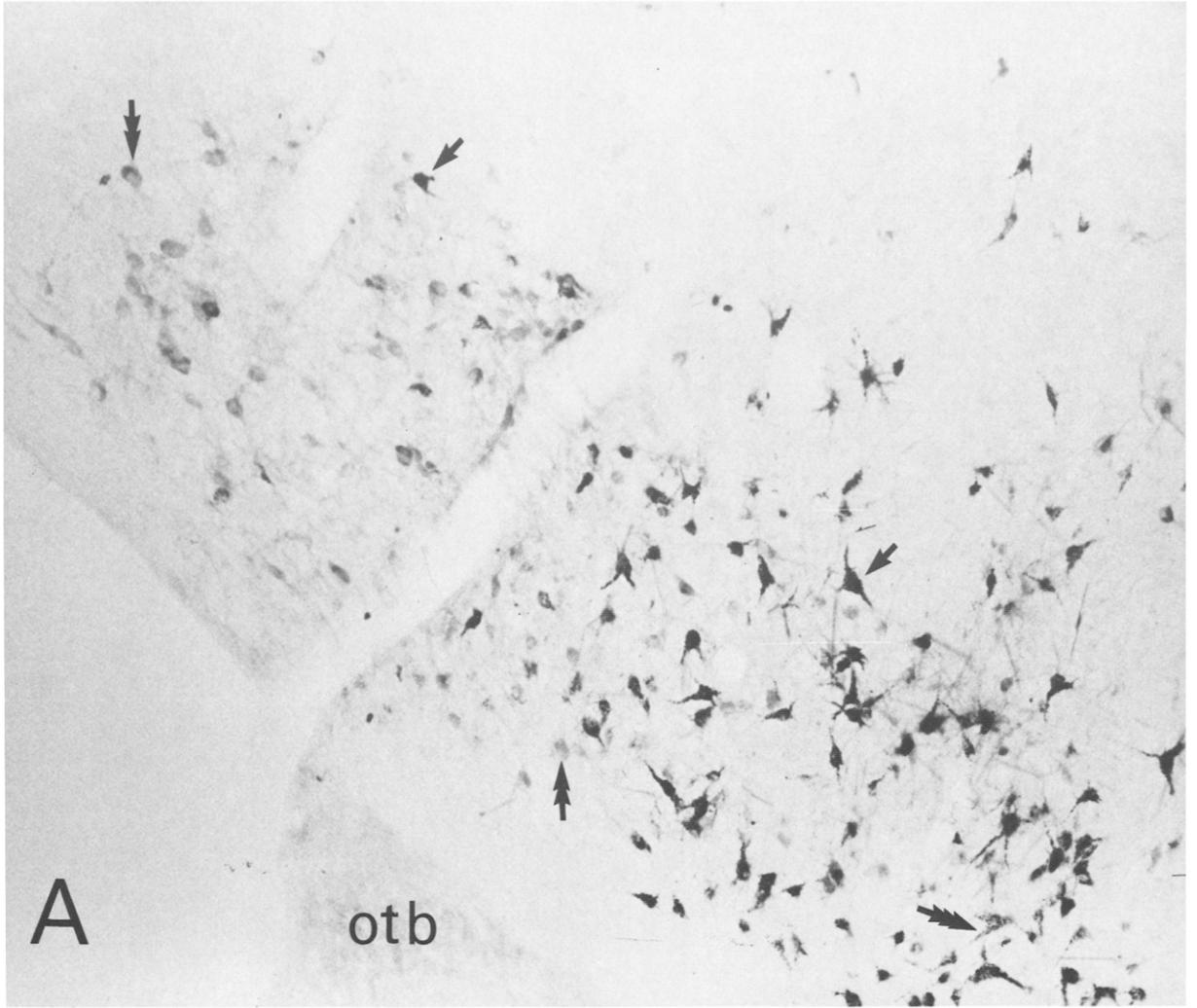


Fig. 3.



preoptic magnocellular nucleus and of the substantia innominata by Bigl *et al.*⁵) should probably also be included in Ch4 on the basis of their neocortical projections.^{5,41} (Figs 1B, 2A).

In general, the Ch4 neurons, especially those in the nucleus basalis, are larger and more hyperchromic than those in Ch1–Ch3. Differentiation from the globus pallidus and from lateral hypothalamic neurons is readily made since these latter two regions do not contain ChAT-positive perikarya. In the monkey, at least 90% of the neurons in the nucleus basalis are ChAT-positive.⁵⁷ A similar relationship appears to exist in the rat. The Ch4 sector contains most of the retrogradely labeled AChE-rich Ch1–Ch6 neurons after neocortical injections of tracer and therefore constitutes the principal source of cholinergic projections to the neocortex (Figs 4I–L). In our cases, almost all of the HRP labeled perikarya in the region of the nucleus basalis were AChE-rich, suggesting that the corticopetal projection from the basal forebrain is overwhelmingly cholinergic.

The Ch5 and Ch6 sectors. The pontomesencephalic reticular formation contains two groups of medium-sized cholinergic neurons (Figs 2B–E). Most of the Ch5 neurons are within the boundaries of the pedunculopontine nucleus. Shute and Lewis⁷⁶ as well as Palkovits and Jacobowitz⁶⁵ appear to have included these neurons within the cuneiform nucleus. We have followed the terminology of Armstrong *et al.*¹ in placing them within the pedunculopontine nucleus. However, individual Ch5 neurons also extend into the more dorsally placed cuneiform nucleus and the more caudally situated parabrachial nuclei. Some Ch5 neurons are embedded within the adjacent lateral lemniscus, superior cerebellar peduncle, central tegmental tract and medial longitudinal fasciculus. The most rostral elements of the Ch5 group appear just lateral to the caudal end of the substantia nigra. More caudally, the Ch5 neurons reach a more dorsal position within the pedunculopontine nucleus (Fig. 2B). At this level, the Ch5 neurons are organized into a compact lateral subgroup which abuts on the lateral lemniscus and a more diffuse medial group (Figs 2B–C). Thionin counterstain of ChAT immunohistochemical preparations show that ChAT-positive Ch5 neurons are intermingled with many non-cholinergic neurons in the cuneiform and pedunculopontine nuclei of the pontomesencephalic reticular formation. Most caudally, the Ch5 neurons surround the superior cerebellar peduncle (Fig. 2C). Even though some Ch5 neurons are found within the confines of the parabrachial nucleus, the cholinergic Ch5 neurons are easily distinguished from the smaller non-cholinergic neurons of the medial and lateral parabrachial nuclei.

The Ch6 group remains within the periventricular gray (Fig. 2C). Most of the Ch6 neurons are within the boundaries of the laterodorsal tegmental nucleus. These cholinergic neurons are intermingled with smaller non-cholinergic perikarya in the same region.

The cholinergic Ch6 neurons are more radially symmetrical than the generally elongated and angular cell bodies of the Ch5 neurons (Figs 2D–E).

In our cases, the Ch5–Ch6 sectors contained substantial numbers of retrogradely labeled AChE-rich neurons only after tracer injections which were centered within the thalamus (Figs 2, 4M–P). Many of these cases also contained some spread of the HRP-WGA tracer into overlying hippocampus and dorsal neocortex. However, experiments in which the tracer was specifically injected into hippocampal and neocortical regions did not contain substantial Ch5–Ch6 labeling (Figs 4A–D, 4I–L). Therefore, it appears that the Ch5–Ch6 labeling in the cases with thalamic injections can be attributed to retrograde transport from the thalamus. Following HRP-WGA injections within the thalamus the vast majority of all retrogradely labeled AChE-rich Ch1–Ch6 neurons was in Ch5–Ch6. It appears, then, that the principal cholinergic projection to the thalamus originates from Ch5–Ch6. Furthermore, almost all of the retrograde labeling in the pedunculopontine and laterodorsal tegmental areas with thalamic injections of tracer occurred within AChE-rich perikarya, suggesting that the thalamopetal projection from these regions is overwhelmingly cholinergic. Most of the thalamic tracer injections involved several nuclei including mostly the anterior, lateral and reticular nuclei. Injections which definitely spared the adjacent habenular nuclei and zona incerta also resulted in substantial Ch5–Ch6 labeling. However, we cannot rule out the possibility that the Ch5–Ch6 sectors provide additional cholinergic projections to the habenula, zona incerta and even to the hypothalamus.

The Ch5–Ch6 neurons may also provide a minor cholinergic projection to telencephalic structures including cortex (Fig. 4). In order to obtain a semi-quantitative estimate of the relative Ch5–Ch6 contribution to the cholinergic innervation of telencephalic structures, we counted all HRP labeled AChE-rich neurons in Ch1–Ch6 in representative cases with HRP injection into the hippocampus, the olfactory bulb, the neocortex or the thalamus (Table 1). The numbers in Table 1 show that as much as 10% of the cholinergic perikarya projecting to the olfactory bulb, hippocampus and neocortex may be located in Ch5–Ch6. This estimate may need to be revised since our counts are based on retrograde labeling of AChE-rich neurons, 1–5% of which may be non-cholinergic and since some ChAT-positive neurons may have lower AChE contents. We have shown that the Ch5–Ch6 sectors in the macaque also provide a minor corticopetal cholinergic projection.^{61a} Table 1 also indicates that up to 30% of cholinergic perikarya projecting to thalamus may be located in Ch1–Ch4. This number almost certainly represents an overestimation because of the spread of HRP-WGA to the hippocampus and cortex along the needle tract in these cases. In four cases, small thalamic injections (maximum diameter 0.5 mm)

Table 1. Relative contribution of Ch1–Ch6 to the cholinergic innervation of telencephalic and diencephalic structures

Site of horseradish peroxidase injection	Numbered of retrogradely labeled telencephalic cholinergic neurons (Ch1–Ch4)	Number of retrogradely labeled pontomesencephalic cholinergic neurons (Ch5–Ch6)
Hippocampus	235	18
Olfactory bulb	106	8
Dorsal neocortex	136	13
Thalamus	34	128

were made through an implanted cannula within the lateral thalamic nuclei with minor extension into the adjacent parafascicular and lateral geniculate nuclei. No spread of the injected tracer into neocortex or hippocampus could be detected. In these cases, the number of HRP-labeled neurons in the pedunculo-pontine and laterodorsal tegmental regions ranged from 9 to 12 whereas no labeling was seen in the basal forebrain regions that contain the Ch1–Ch4 sectors. This suggests that at least some thalamic nuclei may receive their cholinergic innervation exclusively from Ch5–Ch6. These cases could not be included in Table 1 since the small number of neurons and the low sensitivity of the AChE–HRP method for detecting retrograde transport did not result in reliable HRP labeling of AChE-rich neurons.

The essential outlines for the overall organization of ascending cholinergic pathways as shown in Fig. 6 have been confirmed in additional experiments based on the concurrent demonstration of HRP and ChAT.^{74a} Since ChAT is the most specific marker for cholinergic neurons, these experiments established, with even greater certainty, that the projections to the neocortex, the olfactory bulb, the hippocampus and the thalamus did arise, at least in part, from the cholinergic cell bodies of Ch1–Ch6.

DISCUSSION

Organization of ascending cholinergic projections

The forebrain and upper brainstem of the rat contain six groups of cholinergic projection neurons which we have designated Ch1–Ch6 on the basis of cytoarchitectonic criteria and patterns of connectivity (Table 2). The Ch terminology was originally described in the monkey brain⁵⁷ and is now proposed in extended form in the rat. Recently, choline acetyltransferase containing cell bodies have been described in the cortex of the rat brain.^{33a} These neurons probably contribute an intrinsic component to the cortical cholinergic innervation. Nevertheless, it is clear from pharmacological experiments that the majority of the cortical cholinergic innervation is extrinsic^{35,86} and that it originates in the subcortical cholinergic neurons of the basal forebrain and reticular formation. In the rat brain, the Ch1–Ch2 sectors are contained within the medial septal nucleus and the vertical limb nucleus of the diagonal band, respectively. They provide the major cholinergic projection to the hippocampal formation. The Ch3 sector is mostly contained within the lateral part of the horizontal limb nucleus of the diagonal band and provides the primary cholinergic projection to the

Table 2. Nomenclature for cholinergic projection neurons of the basal forebrain and upper brainstem in the rat

Cholinergic cell groups	Traditional nomenclature for the nuclei that contain the cholinergic neurons*	Major source of cholinergic innervation for
Ch1	Medial septal nucleus ^{5,76}	Hippocampus
Ch2	Vertical limb nucleus of the diagonal band ⁵	Hippocampus
Ch3	Lateral part of the horizontal limb nucleus of the diagonal band ¹⁴	Olfactory bulb
Ch4	Nucleus basalis of Meynert, ^{5,42} globus pallidus ^{20,34a,76} Substantia innominata, ^{5,42} Nucleus of the ansa lenticularis, ² neurons lateral to the vertical limb nucleus and those on the medial parts of the horizontal limb nucleus of the diagonal band (including parts of the preoptic magnocellular nucleus ⁵)	Neocortex and amygdala
Ch5	Nucleus pedunculopontinus, ¹ neurons within the cuneiform nucleus ¹ and in the parabrachial area ¹	Thalamus
Ch6	Laterodorsal tegmental nucleus ⁷⁶	Thalamus

*Reference is made to other papers whose usage we have followed for the relevant nomenclature.

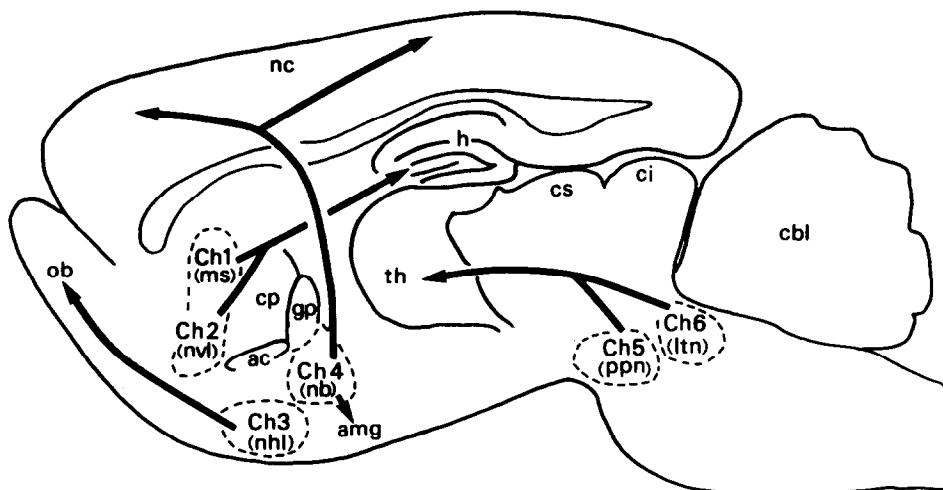


Fig. 6. Schematic representation of some ascending cholinergic pathways. The traditional nuclear groups which most closely correspond to the Ch subdivisions are indicated in parenthesis. However, as indicated in Table 2, the correspondence is not absolute.

olfactory bulb. The Ch4 sector contains the cholinergic neurons of the nucleus basalis, the substantia innominata and probably also laterally situated neurons of the vertical limb nucleus as well as medially situated cells of the horizontal limb nucleus. One unifying feature of Ch4 is that its components provide the major cholinergic projection to neocortical targets. Although we did not prepare cases with tracer injections confined to the amygdala, the observations of Woolf and Butcher⁸⁹ in the rat and our own observations in the monkey⁵⁷ suggest that the major cholinergic projections to the amygdala also arise from the Ch4 region. The Ch5–Ch6 sectors are located in the pontomesencephalic reticular formation and they provide the major cholinergic innervation to the thalamus. There is overlap in connectivity so that individual Ch sectors also provide lesser sources of cholinergic innervation for areas other than their primary targets. The essential outlines of each of these projections and their cholinergic nature have already been demonstrated either directly or indirectly in the rat, hamster, cat and monkey.^{5,9,15,25,30,32,33,34a,35,37,40,41,42,50,51,57,58,59,66,68,69,73,76,83,}

^{86,87} The observations reported in this paper corroborate this large body of evidence and allow an overview of this extensive network of cholinergic projections from the vantage point of an alternative nomenclature which emphasizes common patterns of cytochemistry and connectivity across different species of animals (Fig. 6).

The boundaries among Ch1–Ch4 and that between Ch5 and Ch6 are not always distinct. However, each sector has an individual pattern of salient characteristics with respect to topography, architectonics, cytochemistry and connectivity. Nevertheless, considerable overlap of these patterns also occurs, especially at the boundaries. In the brain of the monkey, individual Ch1–Ch4 sectors, each with a distinct set of projections, could be associated with relatively well

delineated nuclear entities (e.g. medial septum—Ch1, vertical limb nucleus—Ch2, horizontal limb nucleus—Ch3, nucleus basalis—Ch4). In the rat brain, this is only partially possible. The greatest difficulty is encountered in the horizontal portion of the diagonal band. If connectivity patterns of analogous sectors in the monkey were used as the guiding criteria, then it appears that the most medial part of this region belongs to Ch2, the most lateral to Ch3 and the intermediate part to Ch4. It is possible that the ascending cholinergic projections from the basal forebrain are not as differentiated in the rat as they are in the monkey so that this part of the diagonal band nucleus may contain an interdigitation of Ch2, Ch3 and Ch4.

Gorry²⁷ and Parent *et al.*⁶⁷ emphasized the dramatic changes that occur in the organization of the nucleus basalis (Ch4) in the course of evolution. They showed that this cell group increases its size as well as in its differentiation from adjacent structures with advancing phylogenetic development. Our observations in the macaque and human brains showed that the Ch4 sector was by far the most extensive and most differentiated cell group within the Ch1–Ch4 complex.⁵⁷ In the rat, however, the Ch4 sector is not as well differentiated from adjacent neural structures, especially the globus pallidus. Furthermore, its size is comparable to the other components within the Ch1–Ch4 complex. In the monkey brain, we subdivided the Ch4 sector into five distinct components, each with a preferential set of cortical connections. The detailed experiments by Lehmann *et al.*,⁴² Bigl *et al.*⁵ and Lamour *et al.*⁴¹ suggest that an analogous topography may also exist in the brain of the rat.

Reticulothalamic cholinergic pathways

The Ch5–Ch6 neurons of the pontomesencephalic reticular formation provide the major source of cholinergic innervation for thalamic nuclei. This conclu-

sion confirms and extends previous anatomical and physiological investigations of reticulothalamic pathways.^{15,19,28,32,33,51,60,64,69,76,83} The ascending projections from the reticular formation to the thalamus have attracted a great deal of interest ever since Moruzzi and Magoun⁶¹ described an ascending reticular activating system which acted to desynchronize the cortical EEG via a relay in the thalamus. Much subsequent work has provided definitive confirmation for this concept.⁷⁹ The intralaminar and midline thalamic nuclei are thought to provide the principal thalamic relays for the reticular influence upon cortex.^{19,23,28,60,64,79,80} The widespread cholinergic pathway from Ch5-Ch6 to the thalamus undoubtedly provides an essential component of the ascending reticular activating system and may play a pivotal role in regulating cortical activation. Our observations also indicate that the cortical mantle is under dual cholinergic influence. On one hand, there is a monosynaptic cholinergic corticopetal pathway which arises mostly from Ch4. On the other hand, there is also an indirect pathway which has a cholinergic segment from Ch5-Ch6 to the thalamus and then a non-cholinergic segment from the thalamus to cortex.

Possible functional implications

Behavioral correlates of central cholinergic pathways. There has been a longstanding interest in the behavioral correlates of central cholinergic pathways, especially in the area of memory and learning. Cholinergic antagonists cause amnesia in monkeys and humans,^{3,16} whereas cholinergic agonists cause a small but reliable enhancement of memory processes.^{4,12,77} In another line of investigation it has been shown that there are genetic differences in acetylcholine turnover and ChAT levels among inbred strains of mice and that strains with higher levels of cholinergic markers show better learning performance.¹⁸ The locomotion-induced hippocampal theta activity and muricidal behavior in rats have also been correlated with the activity of central cholinergic pathways.^{84,90} It is reasonable to assume that the multiple behavioral correlates of central cholinergic activity reflect the multiple targets of ascending cholinergic projections from the basal forebrain and the reticular formation. For example, the cholinergic pathway to the hippocampus and other medial temporal areas may regulate memory; those to the amygdala may modulate affect; those to neocortical regions may play a role in perception and other cognitive processes; and those to the thalamus may influence states of attention, arousal and sleep.

Neurons in the basal forebrain show a very complex pattern of electrical activity. Whereas units in the globus pallidus of the monkey fire in temporal relationship to push-pull movements, the activity of units in the nucleus basalis (Ch4) vary in relationship to the delivery of a juice reward.¹³ Furthermore, Burton *et al.*⁶ reported that units in the nucleus basalis changed their rate of firing in response to the slight or taste of food and in relationship to the state of hunger. These observations suggest that the activity of Ch4 neurons may be responsive to shifts in motivational states. The Ch4 region in the monkey receives projections predominantly from limbic and paralimbic parts of the brain.^{36,43,56a,65,71,75} The Ch4 complex is therefore in a unique position to act as a cholinergic relay between limbic-paralimbic areas and the entire extent of neocortex in a fashion that may influence all complex behavior according to the prevailing emotional and motivational state. It is

thus conceivable that pathological alterations in these neurons may lead to widespread changes in many aspects of complex behavior.

Central cholinergic pathways in age and disease. There is reason to believe that some of the mental state changes that occur with aging, especially those related to memory, may be the outcome of altered cholinergic transmission. For example, experiments done in the laboratory of R. Bartus⁴ show that young rats and monkeys which are given anticholinergic drugs develop a memory disorder identical to that which appears in aged animals. Similar observations have also been made in humans.¹⁶ If a definite relationship can be demonstrated between age-related memory dysfunction and physiological alterations along central cholinergic pathways, then vast opportunities would arise for treating and perhaps even preventing certain age-related cognitive alterations.

The relationship of the central cholinergic system to human disease has recently started to attract a great deal of attention. Alzheimer's Disease, the single most common cause of dementia and senility, has been associated with a relatively selective loss of presynaptic cortical cholinergic markers.¹⁰ Loss and degeneration of Ch4 (nucleus basalis) neurons have also been described in Alzheimer's Disease.^{34,70,88} Since Ch4 provides the major source of cortical cholinergic innervation, the loss of cortical cholinergic markers and the loss of Ch4 neurons may be very much related to each other in Alzheimer's Disease even though the direction of causality has not yet been determined. It would be of interest to see if thalamic presynaptic cholinergic elements are also depressed in this disease and if this is associated with neuronal loss in Ch5-Ch6. It also remains to be seen whether some disease conditions, perhaps those with salient disturbances of attention, arousal or sleep, may be associated with a predominant involvement of the reticulothalamic cholinergic pathway. In Alzheimer's Disease, the presence of altered cholinergic innervation has stimulated specific therapeutic efforts aimed at enhancing cholinergic function. There are now encouraging reports that some patients with Alzheimer's Disease may show an improvement of memory function when given cholinergic agents such as physostigmine and lecithin.^{11,87}

Pathological alterations in the nucleus basalis and associated cell groups have also been reported in Parkinson's Disease, the Parkinsonism-Dementia Complex of Guam, Pick's Disease, Huntington's Disease and Schizophrenia.^{2,31,47,62,82,85a} The neuropathology of these diseases clearly extends much beyond the nucleus basalis and there are vast differences among their overall clinical profiles. It is nonetheless conceivable that some of the mental state alterations in each of these diseases might be attributed to a disturbance in individual components of central cholinergic pathways.

Considerable advances have occurred in the recent past in unraveling the anatomy of monoaminergic pathways and their clinical relevance to human disease, especially with respect to mood and motivation. The anatomy of central cholinergic pathways, following the pioneering work of Shute and Lewis,⁷⁶ is now enjoying a period of similar growth. As in the case of monoaminergic pathways, it is almost certain that these advances will also have profound clinical implications for understanding and treating neurological and psychiatric disease.

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