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Cholinergic and non-cholinergic forebrain projections to the interpeduncular nucleus

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A combined fluorescent retrograde tracing and acetylcholinesterase (AChE) histochemical technique was used for the study of some forebrain projections to the interpeduncular nucleus (IPN). After injections of a fluorescent tracer into the IPN, the distribution of AChE-containing and of fluorescent retrogradely labeled neurons was simultaneously studied in the habenular nuclei, medial septum and diagonal band of Broca. In all these regions, the majority of retrogradely labeled neurons also contained AChE: neurons located in the habenular nuclei stained lightly or moderately for the enzyme, while neurons located in the diagonal band and medial septum displayed intense AChE staining and were classified as putatively cholinergic perikarya. In all regions, a minority of labeled neurons did not stain for AChE, and were identified as non-cholinergic neurons projecting to the IPN. The present study shows the existence of a biochemical heterogeneity in the habenulo-interpeduncular and telencephalo-interpeduncular pathways, and indicates that the latter contains putatively cholinergic as well as non-cholinergic fibers.

Mainly on the basis of lesion experiments, two putative sources of cholinergic afferent projections to the interpeduncular nucleus (IPN) have been classically identified: the habenular nuclei[7,18,23,25] and the nucleus of the diagonal band of Broca[10,19]. Morphological studies have shown, in fact, that these two forebrain regions contain neural somata staining for choline acetyltransferase and for acetylcholinesterase (AChE)[3,9,17,20,27,30,33,36]. Owing to the intensity of the AChE histochemical reaction and to the presence of positive immunochemical staining for choline acetyltransferase, it is commonly believed that neurons located in the diagonal band and medial septum are cholinergic[8,9]. Conversely, uncertainty still exists on the chemical nature of habenular neurons, which stain weakly or moderately for AChE and do not constantly react to antibodies raised against choline acetyltransferase[8,9,20,33,35]. Due to the peculiarly close anatomical relationships of the telencephalo-interpeduncular and habenulo-interpeduncular pathways, which raise important technical and interpretative problems in lesion and anterograde tracing studies[12], their hodological organization has not yet been conclusively described[5,9]. After the introduction of a combined fluorescent retrograde tracing and AChE histochemical technique, which has been recently developed in our laboratory[2], a new tool has become available for effectively studying the efferent organization of identified AChE-containing neurons. This technique has been therefore used in the present study to reinvestigate the occurrence and the chemical identity of forebrain neurons projecting to the IPN.

The fluorescent retrograde tracer true blue (TB) was used in this study. Small injections (0.1–0.3 μl) or a 2% aqueous suspension of TB were placed in the IPN or in the adjacent ventral tegmental area in a total of 32 male Wistar rats, weighing 250–300 g. All the injections were placed stereotaxically, according to the atlas by Pellegrino and Cushman[31], by means of a Hamilton microdrive microsyringe. The needle was inserted vertically downward in the midline to reach the IPN; more lateral penetrations were required for injections into the ventral tegmental area. Following an injection the syringe was left in place for 10 min before removal from the brain: this procedure great-

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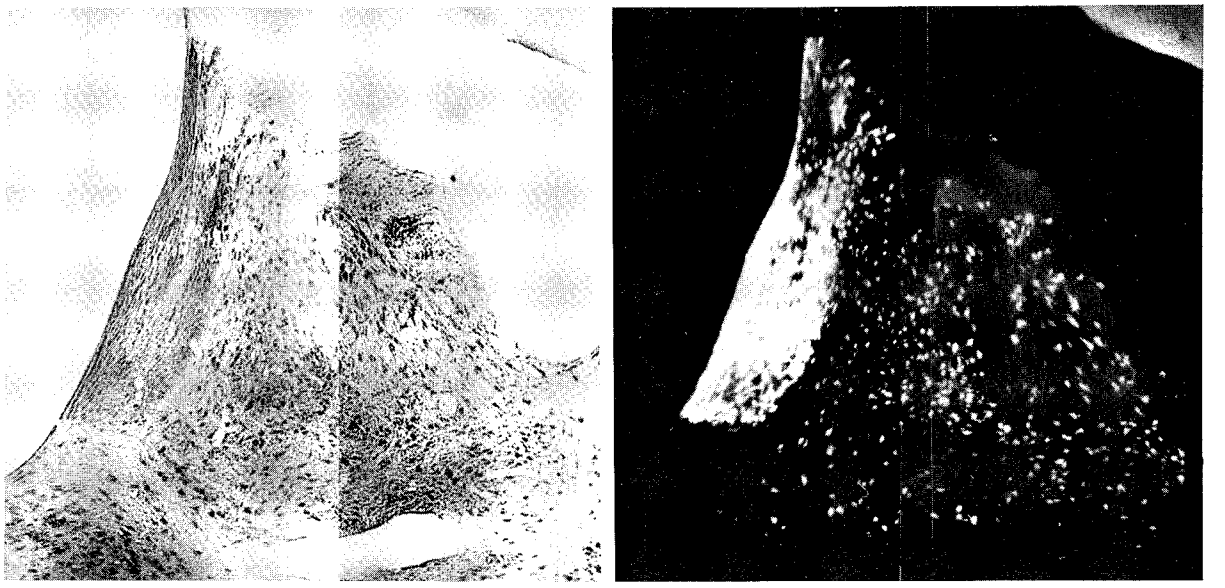

Fig. 1. Fluorescent retrograde labeling and AChE staining in the right side habenular nuclei after an injection involving most of the IPN (case D296). Left: bright-field photomontage showing moderately and lightly stained AChE-containing neurons in the habenular nuclei. Right: dark-field photomontage of the same section showing TB fluorescence in retrogradely labeled perikarya. Scale bar = 0.2 mm.

ly limits the spillage of tracers along the needle tracks. The animals were allowed to survive 10–20 days (usually 14) after surgery. 8 or 4 h prior to their sacrifice, the animals were poisoned with di-isopropylfluorophosphate (DFP). This irreversible AChE inhibitor was injected intramuscularly as 0.15% arachid oil solution in the dose of 1.5 mg/kg body weight. 8 h of recovery from poisoning were allowed to those animals in which the habenular nuclei were studied; 4 h were allowed to those in which the basal

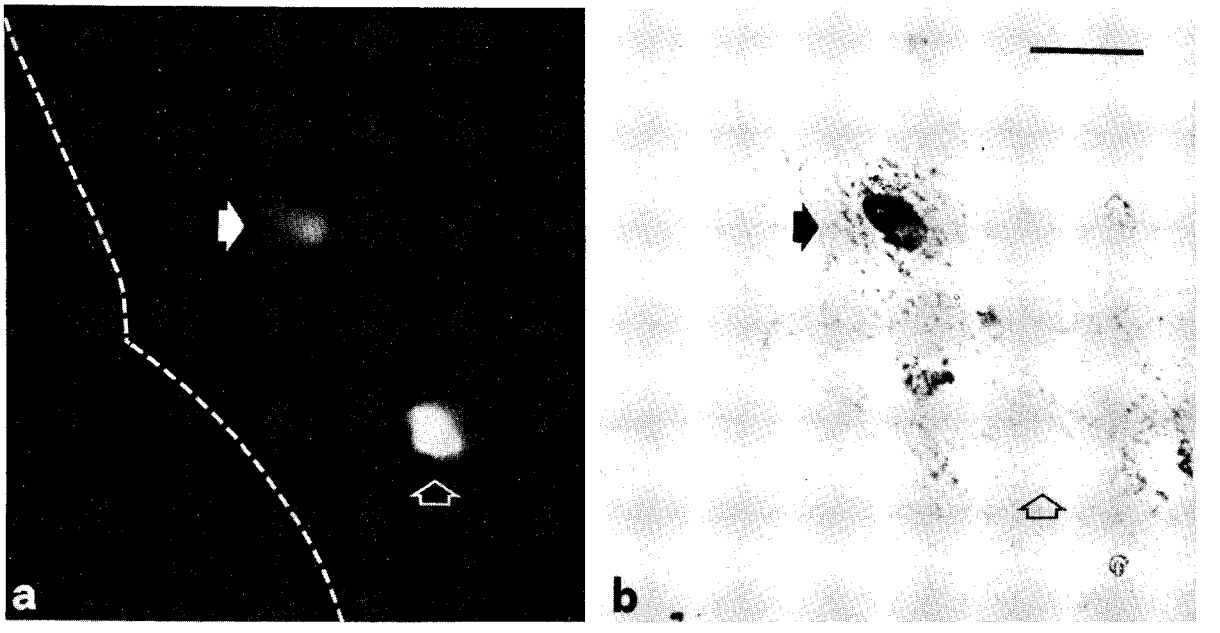

Fig. 2. Fluorescent retrograde labeling and AChE staining in the basal forebrain. a: dark-field microphotograph of two TB fluorescent neurons (arrows) retrogradely labeled from the IPN. b: bright-field microphotograph of the same section, showing AChE staining only in one neuron (solid arrow). Coronal section corresponding to section 1 of Fig. 3. Scale bar = 20 μm.

telencephalon was observed. The animals were perfused intracardially with 0.9% saline solution, followed by 10% phosphate-buffered formalin (pH 7.2). Before surgery and perfusion the animals were deeply anaesthesized with sodium pentobarbital (40 mg/kg, i.p.). Postfixation, histochemistry and microscopy were performed as described by Albanese and Bentivoglio[2]. Serial brain sections through the midbrain were charted for the reconstruction of the size of injection areas; the distribution of retrogradely labeled and of AChE-containing neurons was charted on serial drawings of the forebrain.

In all the injection areas, two concentric zones could be detected around the needle tracks[14]. In the IPN injections the central zone (only from which retrograde transport has been shown to occur[15]) almost always included both pars dorsalis and pars medialis of IPN[16]. In control injections, both zones were contained within the ventral tegmental area.

After injections placed into the IPN, the medial habenular nuclei were particularly rich in retrogradely labeled perikarya, which also displayed a weak AChE staining. In the lateral habenular nuclei several retrogradely labeled neurons, which also stained moderately for AChE, were seen; few retrogradely labeled cells in this nucleus apparently did not stain for AChE. The distribution of retrogradely labeled somata in the habenular nuclei was very reproducible in different experiments: the medial nuclei were bilaterally filled with labeled somata, while the lateral nuclei contained numerous scattered labeled perikarya (Fig. 1). The basal telencephalon also contained a large number of fluorescent, retrogradely labeled, cell bodies: in all cases they were particularly abundant in the nucleus of the diagonal band. Retrogradely labeled neurons were also seen in the medial septal

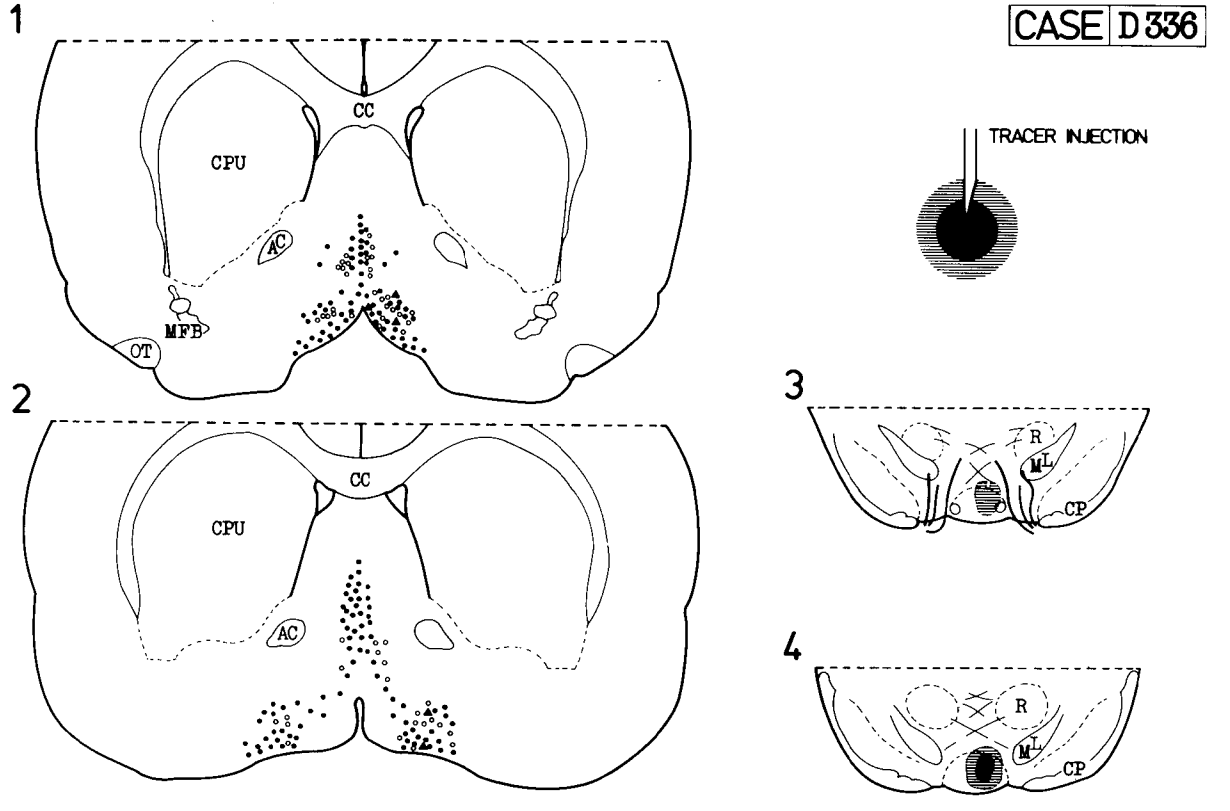

Fig. 3. Schematic representation of the distribution of AChE-containing and retrogradely labeled cells in rostral telencephalon (left column) after a small injection of TB placed into the IPN (right column). Filled circles indicate unlabeled AChE-containing perikarya, open circles indicate retrogradely labeled AChE-containing perikarya, triangles indicate non-AChE-containing retrogradely labeled perikarya. Numbers 1–4 refer to the rostrocaudal coronal sections. Abbreviations: AC, anterior commissure; CC, corpus callosum; CP, cerebral peduncle; CPU, caudato-putamen; MFB, medial forebrain bundle; ML, medial lemniscus; OT, olfactory tubercle; R, red nucleus.

nucleus and in the ventromedial part of nucleus accumbens: their topography varied according to the size and topography of injection sites. Many labeled neurons also stained intensely for AChE, while some others did not (Fig. 2). Non-AChE-containing retrogradely labeled cell bodies were usually multipolar in shape, and they were rather smaller than the AChE-containing cells. Non-AChE-containing somata were mainly located: (1) in the diagonal band, just lateral and medial to the intensely stained somata, which appeared as a fairly packed cell grouping; (2) in the septal region, intermixed with the AChE-containing cell bodies, which are mainly contained within the medial septal nucleus (Fig. 3).

In control cases, in which injections were placed in the ventral tegmental area, retrogradely labeled neurons were seen bilaterally in both habenular nuclei: their distribution was evidently asymmetrical, being largely prevalent on the side ipsilateral to the injection area. Most labeled neurons in the habenular nuclei stained for AChE. In the telencephalon, neurons retrogradely labeled from the ventral tegmental area were almost entirely restricted to the diagonal band. They were distributed bilaterally, and were more represented in the side ipsilateral to the injection area; most of these neurons also stained for AChE.

The distribution of habenular and basal telencephalic neurons projecting to the IPN, as shown in the present study, is in keeping with previous degeneration and tract-tracing studies in mammals. The habenulo-interpeduncular pathway has been the object of several studies, which have identified its origin either from the lateral[6,7,22], or from the medial habenular nuclei[11,13,24,28,37], or from both[1,4,34]. The present study is the only one based on fluorescent retrograde tracing, which is believed to be more sensitive and reliable than other available retrograde tract-tracing techniques[15]; it shows, in fact, that both the medial and the lateral habenular nuclei project to the IPN, and that the projection arising from the lateral nucleus is quantitatively much smaller. In addition, the present study demonstrates that most habenulo-interpeduncular neurons stain either lightly or moderately for AChE. Previous anatomical studies have also analyzed the telencephalo-interpeduncular pathway, and have shown that it takes origin mainly from the nucleus of the diagonal band[10,29]. This nucleus also sends significant efferent projections to the ventral tegmental area[24,32], which directly abuts the IPN. Due to the richness of this latter projection, the existence of an independent telencephalo-interpeduncular pathway has been questioned[24]. By comparing the results obtained after microinjections placed into the ventral tegmental area or into the IPN, the present study demonstrates that the IPN receives innervation from a wider forebrain region than the ventral tegmental area, which is only (but richly) innervated by the nucleus of the diagonal band.

It is commonly accepted that all cholinergic neurons possess AChE, although the contrary is not necessarily true[8,21]. Furthermore, converging evidence indicates that the basal telencephalic neurons, which stain intensely for AChE, also contain choline acetyltransferase[26,33], and are in fact cholinergic[8,9,36]. On the basis of this assumption it can be concluded that: (1) the retrogradely AChE-containing neurons located in the basal telencephalon represent putatively cholinergic telencephalo-interpeduncular cell bodies; (2) the retrogradely labeled non-AChE-containing basal telencephalic neurons, which are shown in the present study, represent non-cholinergic telencephalo-interpeduncular somata. Therefore, the present report indicates that the telencephalo-interpeduncular pathway is, in fact, a chemically heterogeneous, both cholinergic and non-cholinergic tract.

The putative cholinergic nature of neurons located in the habenular nuclei is still a matter of dispute[8,9]. It has been postulated that cholinergic neurons possess high levels of AChE[21,39]; according to this view, the histochemical features of habenular neurons would indicate that they are not cholinergic. However, this statement is contradicted by a recent transmitter-specific retrograde tracing study[37], which shows the existence of a cholinergic projection to the IPN arising from the medial habenular nucleus. This evidence is in agreement with lesion-based experiments[38], indicating that there are no cholinergic perikarya in the lateral habenular nucleus. In addition, immunocytochemical studies on choline acetyltransferase have led to still inconclusive results[20,33,35]. To this regard, the present data do not allow to favour or to exclude a cholinergic nature of the habenulo-interpeduncular tract. They indicate, however, that it is a heterogeneous pathway, originating largely from AChE-containing and partly from non-AChE-con-

taining neurons.

Previous studies have shown that forebrain projections to the IPN possibly contain substance P and GABA in addition to acetylcholine[5,7,38]. Future anatomochemical studies will probably clarify whether these or other substances are the neurotransmitter candidates for non-cholinergic habenulo-interpeduncular and telencephalo-interpeduncular neurons.

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