Basal forebrain cholinergic projections to the frontal cortex in mice: A combined acetylcholinesterase histochemistry and retrograde tracer study

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The topographical organization of the basal forebrain cholinergic projections to the frontal cortex in mice was assessed through acetylcholinesterase histochemistry and retrograde colloidal gold-wheat germ agglutinin-horseradish peroxidase double labeling. The anterior part of the magnocellular basal nucleus, and the horizontal limb of the diagonal band of Broca project to the mediodorsal anterior frontal cortex, while the posterior parts of the magnocellular basal nucleus project to the mediodorsal posterior and lateral regions of the frontal cortex. The intermediate regions of the magnocellular basal nucleus project diffusely upon all dorsal regions of the frontal cortex.

Key words: *acetylcholinesterase histochemistry, basal forebrain cholinergic system, cholinergic projections, double labeling, frontal cortex afferents, retrograde tracer*

INTRODUCTION

The basal forebrain cholinergic system (BFCS) has been studied in various species of mammals (see 9) including man (25) by means of choline acetyltransferase immunohistochemistry (anti-ChAT) and histoenzymatic assay for acetylcholinesterase (AChE) detection. This system extends from the medial septum to the globus pallidus and includes the septal region and the magnocellular basal nucleus (nBM). Functionally, this system is involved in memory processes (1, 12, 14, 30) and also in Alzheimer's disease (40).

Several studies have shown a specific pattern of projections from the BFCS, the medial septum projecting to the hippocampus (10) and the nBM being the main source of cholinergic projections towards the entire cerebral cortex (3, 6, 24, 26, 27, 33, 34, 39). These studies include retrograde and immunohistochemical or histoenzymatic AChE double labeling (3, 32) using horseradish peroxidase (HRP) or fluorochromes as tracers. However, these cholinergic projections from the nBM to the cerebral cortex appear to be organized topographically. Differential projections from nBM subdivisions to the cerebral cortex (19) or to some cortical areas, such as visual (4) or somatosensory cortices (2), have been described. These studies are particularly relevant because they would enable to determine the precise localization of cholinergic neurons involved in the modulation of high cortical functions. The essential role of the

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mediodorsal frontal cortex in memory processes is well established (16, 30).

The present work was intended to study the differential projections from the BFCS to the dorsal frontal cortex, using the AChE histochemistry and retrograde colloidal gold-wheat germ agglutinin-horseradish peroxidase complex (CG-WGA-HRP) double labeling method.

MATERIAL AND METHODS

The study was performed on twenty-two C56/BalbC mice placed in a conventional stereotaxic apparatus under sodium pentobarbitone anesthesia (50 mg/kg). The CG-WGA-HRP complex was injected in a volume of 45-50 nl at slow pressure through a micropipette (15-20 µm external diameter) coupled to a 1 µl Hamilton syringe, mounted onto a device operated with a step motor. After 24 hours of survival, animals were anesthetized with an overdose of pentobarbitone and were perfused intracardially, first with Tyrode's buffer solution and then with 3% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were extracted, submerged in 20% sucrose in 0.1 M phosphate buffer saline (PBS), pH 7.4, and stored at 4°C overnight. Slices of 10 µm width were cut in a Cryostat (Leica) and mounted on gelatine coated slides.

The CG-WGA-HRP complex was made according to the methods of De Mey (8) and Menetrey and Lee (21), with slight modifications. The colloidal gold particles, 1.52 nm mean diameter, were measured under the electron microscope (x 49,000). The colloidal gold was developed with silver lactate according to Menetrey and Lee (21), and acetylcholinesterase, according to a modification of the Tago *et al* (37) techniques. This modification consisted in replacing the maleic acid with DL-malic acid as buffer, at the same molarity, and doubling the concentrations of other reagents for double labeling. Briefly, the slices were rinsed 3 x 5 min in Tris buffer saline (TBS) 50 mM at pH 7.4 and incubated 30 min for AChE histochemistry, in DL-malic acid (Sigma) buffer 0.1 M, pH 6, containing acetylthiocholine iodide (Sigma) 10 mg, trisodium citrate (Sigma) 14.7 mg, for 500 ml of malic buffer. Sections were rinsed 3 x 10 min in TBS and the reaction product developed with diaminobenzidine (DAB, Sigma) 0.04% as chromogen, and 0.003% hydrogen peroxide. Slices were then rinsed 3 x 10 min in TBS and 1 min in citrate-citric acid buffer 0.1 M, pH 3.8. The physic developer for the colloidal gold was made by mixing 60 ml of gum arabic 50%, 10 ml of citrate-citric acid buffer 1 M, pH 3.8, 15 ml of hydroquinone solution (840 mg, Sigma) and 15 ml of silver lactate solution (100 mg, Sigma) during 40 min. Then, the slices were rinsed 4 x 10 min in TBS and 2.5% sodium thiosulfate (Sigma) in TBS for 5 min and rinsing 3 x 5 min in TBS, dehydrated and coverslipped with Permount.

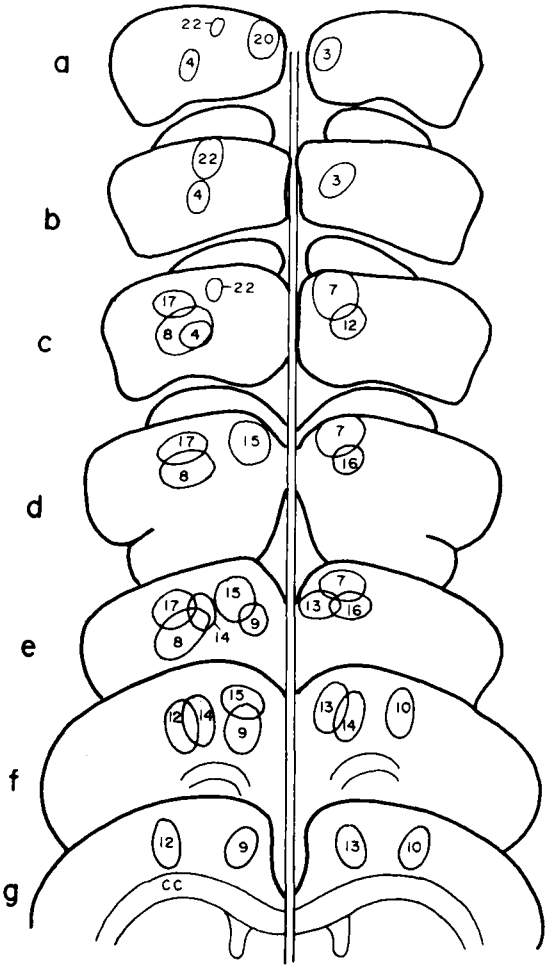

Fig 1. Schematic representation of the injection sites in the frontal cortex. Circles represent the localization of sites, and numbers, the corresponding cases.

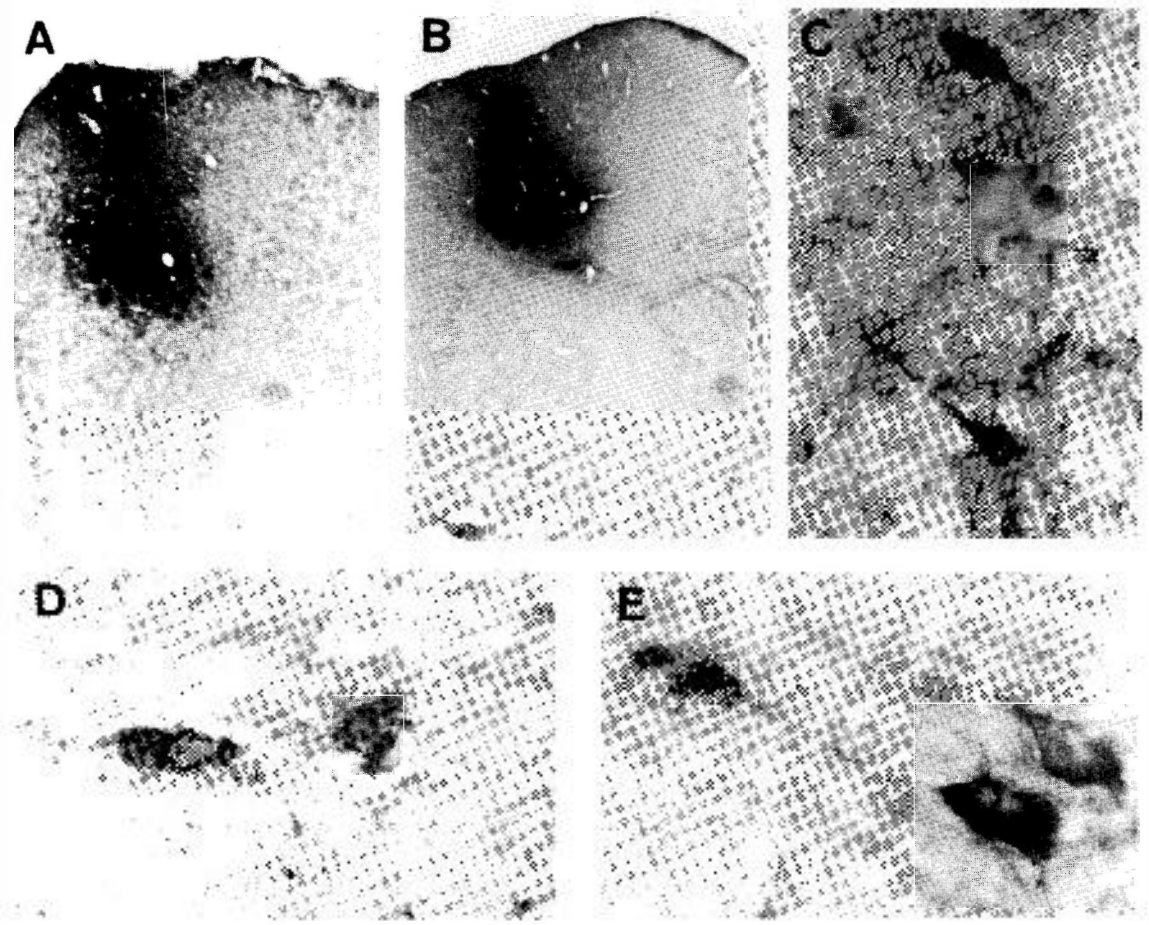

Fig 2. Photomicrography of the injections sites in the medial (A) and lateral (B) anterior frontal cortex. AChE and retrograde double labeled neurons in anterior (C,) intermediate (D) and posterior (E) parts of the nBM.

RESULTS

For the description of the localization of double labeling, retrograde and AChE positive neurons into the nBM, this nucleus was divided into an anterior part (nBMa) from the horizontal limb of the diagonal band of Broca (HLDB) to the anterior commissure caudally, an intermediate part (nBMi) with a ventral region (nBMiv) and a dorsal region (nBMid), and finally a posterior part (nBMp) in the globus pallidus, lateral to the internal capsule, as proposed in a preceding paper (38).

The injection sites were located in the medial and lateral regions of the dorsal frontal cortex at different rostro-caudal levels (Figs 1 and 2). The extent of the injection site varied between 80 and 120 μm. In the cases of injections located in the medial region of the anterior pole of the frontal cortex (cases 3, 16, 7; Fig 1), the double labeled neurons appear in the HLDB and nBMa. Only a few neurons were found in the nBMid, even less in the nBMiv (Fig 2C-D) and none in the nBMp (Fig 3). When the injections were given in the lateral regions of the anterior pole (cases 4, 17, 8; Fig 1), the double labeled neurons were found in the intermediate part, principally in the nBMid and nBMp (Fig 2E). These double labeled neurons appeared without a preferential medio-lateral localization. Very few single labeled neurons, with only CG-WGA-HRP, were found (Fig 3). In cases of injections in posterior regions of the frontal cortex, at medial (cases 9, 13, 15) and lateral (cases 10, 12, 14; Fig 1) levels, we found double labeled neurons in the nBMid, nBMp and less frequently in the nBMiv, with a topo-

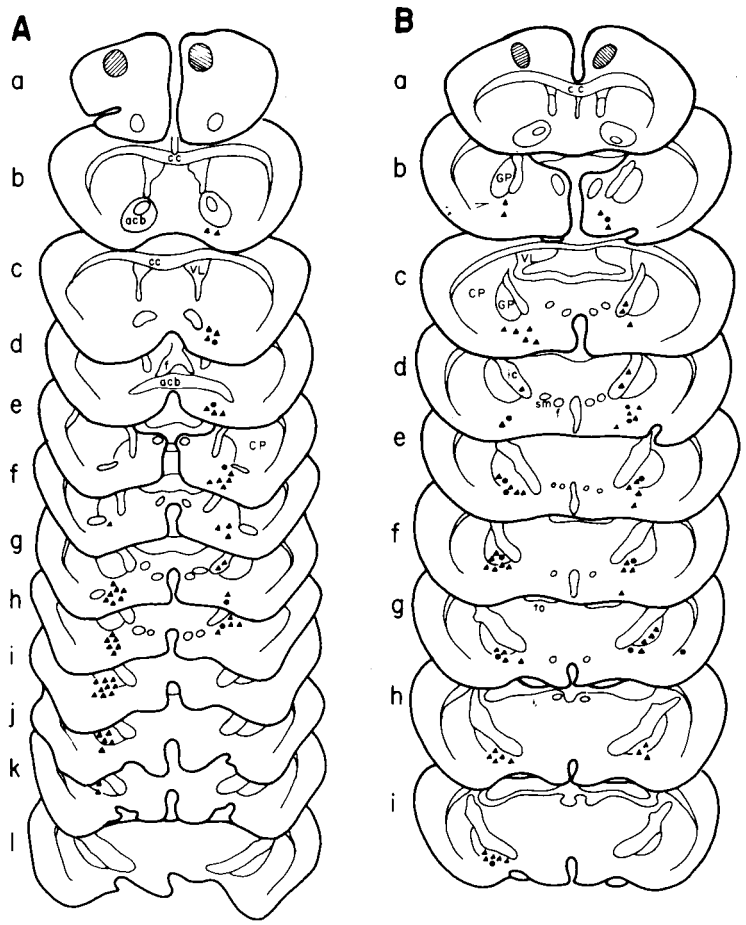

Fig 3. Schematic drawings arranged rostro-caudally, representing the double labeled neurons (black triangles) and single retrogradely labeled neurons. **A**. Injection sites in lateral (left) and medial (right) regions of the anterior frontal cortex. Note double labeled neurons in anterior nBM only, in cases in which the injection sites were located in mediodorsal anterior frontal cortex. **B**. Injection sites in lateral (left) and medial (right) regions of posterior frontal cortex. Double labeled neurons appear in intermediate and posterior parts of nBM.

graphical arrangement. In the cases of medial injections made in the frontal cortex, the double labeled neurons were located preferentially in the medial parts of nBMi and nBMp, while those corresponding to the lateral injections were found in the lateral parts of those regions (Fig 3).

DISCUSSION

Since the description by Shute and Lewis (36) of the AChE enzymatic activity as a good labeling of putative cholinergic activity, several modifications of this method have been used for the study of the cholinergic system neuronal activity (31, 35), corticipetal fibers (15, 28), cholinergic pathways (29) and for the demonstration of the decrease of cortical cholinergic activity in Alzheimer's disease (5, 40). This method has also been used in retrograde and histoenzymatic double labeling methods for the study of cholinergic projections (3, 35). Its sensitivity, when detecting AChE activity, varies according to the methods, fixation conditions, pH and whether or not inhibitors are used (22).

Several authors find a good correspondence between the localization of the ChAT+ and AChE+ neurons (35). Levey *et al* (18) have shown simultaneous localization

of ChAT+ and AChE+ in neurons of the striatum and the nBM. The differences in sensitivity, when detecting neuronal AChE, described by these authors, may be due to inherent factors of the double labeling method using the DAB as a chromogen or the possibility of the presence of various globular forms of AChE (13) as well as the variations of the synthesis of this enzyme (23). Comparative studies in the cerebral cortex show a laminar organization of the AChE+ fibers which coincides with the ChAT+ ones with the exception of the barrels of somatosensory cortex (20). The use of AChE as a good marker of cholinergic activity is very acceptable in studies of regions like the BFCS and the cerebral cortex. The technique proposed by Tago *et al* (37), without inhibitors, appears to be the most sensitive one. In our work, the replacement of maleic acid with DL-malic acid allowed us to decrease the background. Also, when nickel was not used for intensification, a good contrast for double labeling was obtained, without a loss of sensitivity in the staining of neurons and fibers.

The results obtained show a clear and precise topography with regard to the organization of the AChE+ neurons of the BFCS projecting to the frontal cortex. The HLDB and the anterior part of the nBM project to the anterior medial region of the frontal cortex, while the neurons of the most anterior part of the HLDB project to the most medial anterior pole of the frontal cortex. The intermediate part of the nBM projects in a diffuse manner to all the regions of the frontal cortex, according to Luiten *et al* (19), while the posterior part of the nBM projects to the lateral and medial posterior regions with a medial-lateral gradient; the lateral parts of the nBM project to the lateral regions of the frontal cortex and the medial parts of this nucleus to the medial regions of the frontal cortex. These results coincide partially with those described by other authors in some regions of the frontal cortex (3, 25, 34).The advantage of the method used in our work is the easy distinction between the retrograde tracer and the histoenzymatic labeling of the AChE, in contrast to the difficulty in the distinction of double labeling when the HRP (29), or fluorochromes (3) are used as tracers. On the other hand, the small particles of colloidal gold offer us a great sensitivity in retrograde neuroanatomical tract tracing studies.

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