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CHOLINERGIC PROJECTIONS FROM THE BASAL FOREBRAIN OF RAT TO THE AMYGDALA[1]

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Abstract

Cholinergic afferents to the amygdala from the basal forebrain were studied using di-isopropyl fluorophosphate-AChE histochemistry in combination with retrograde tracing using various fluorescent dyes. Cells sending their axons to the amygdala and staining intensely for AChE were located mainly in the nucleus of the substantia innominata. They also were found in the ventral part of the globus pallidus, the horizontal limb of the nucleus tractus diagonalis Broca, and the nucleus interstitialis ansae lenticularis. A correspondence was established between these cells and cells staining for choline acetyltransferase by immunohistochemistry in both distribution and morphology. Non-cholinergic neurons which send their axons to the amygdala also were found in the substantia innominata complex.

It has been well established that there is a high concentration of "marker" enzymes for cholinergic neurons, i.e., choline acetyltransferase (ChAT) and AChE, in the amygdaloid complex (Palkovits et al., 1974; Ben Ari et al., 1977). Recently, immunohistochemical staining using high titer Fab fragments of anti-ChAT antibodies on feline brain (Kimura et al., 1981) has shown that the bulk of cholinergic cells in the basal forebrain exists in the substantia innominata complex and that no cholinergic cells can be found in the amygdala. The basal and lateral subnuclei of the amygdala contain, however, a high concentration of cholinoceptive cells (Kimura et al., 1981). These data indicate that the cholinergic innervation of the amygdala must originate outside of this nucleus. In agreement with this supposition, knife cuts of the so-called ventral amygdalofugal pathway produced a large depletion of ChAT in the amygdala (Emson et al., 1979). Some early information on possible afferent cholinergic pathways to the amygdala using AChE histochemistry was obtained by Schute and Lewis (1963), but it has since proved difficult to confirm their precise origin.

In the present study, cholinergic afferents to the amygdala from the basal forebrain were studied using a retrograde fluorescent tracing technique (Bjorklund and Skagerberg, 1979; Kuypers et al., 1977; Nagai et al., 1981) in combination with a pharmacohistochemical method for AChE. In this latter procedure, which involves injections of the irreversible cholinesterase inhibitor di-isopropyl fluorophosphate (DFP) some hours before sacrifice (Butcher, 1977), the only intense AChE staining is in cells which rapidly synthesize new enzyme. Lehmann and Fibiger (1979) have provided evidence that "intense" AChE staining after DFP treatment is a simple and convenient method for identifying neuronal perikarya that are possibly cholinergic. Confirmation is possible using ChAT immunohistochemistry, and this technique was applied to adjacent sections in this study.

Materials and Methods

Forty albino rats, weighing 150 to 200 gm, were used. In 30 rats, 0.1 to 0.2 μl of a fluorescent tracer was injected into the amygdala. Four tracers, i.e., Evan's blue (10% in saline), true blue (5% in distilled water), 4',6-diamidino-2-phenylindole hydrochloride (DAPI; 2.5% in distilled water), and Primuline yellow (PR, 10% in saline), were tried in different rats in combination with AChE histochemistry. Each rat was anesthetized with Nembutal (50 mg/kg) and placed on its back in a stereotaxic apparatus. In order to obviate the involvement of structures located dorsal to the amygdala (such as the cerebral cortex and caudate-putamen), most injections of tracers were made through a ventral approach. To accomplish this, the foramen ovale was located and a small burr hole was made in the bone just laterally. The amygdala was

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reached 2.5 to 4.0 mm beyond the surface of the bone. The tracer was injected through this hole by a glass micropipette system equipped with an oil pressure pump.

The rats were processed for AChE histochemistry and ChAT immunohistochemistry as follows. After 2 or 3 days survival, DFP (Butcher, 1977) was injected into the thigh muscle at a dose of 1.5 mg/kg of body weight (Lehmann et al., 1980). Six hours later, the rats were anesthetized deeply and perfused through the heart with 100 ml of fixative containing 4% paraformaldehyde (PFA) and 0.35% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4°C. Brains were removed and immersed for 6 hr in postfixative containing 4% PFA and 15% sucrose in 0.1 M PB. Then they were washed in 15% sucrose solution dissolved in 0.1 M PB. They were cut on a cryostat into 20-μm-thick sections and collected into the same solution used for washing. Sections were divided serially into three sets. The first set was used only for analysis of the retrogradely labeled neurons without any further histochemistry. They were put on glass slides, dried, and observed under a fluorescent microscope (Olympus, BH-RFL), using the appropriate excitation system for each tracer. The second set was processed for AChE by the Karnovsky and Roots (1964) method which was partly modified as follows (Imai et al., 1980): the amount of acetylthiocholine iodide was increased up to 10 mg/10 ml of incubation medium. Specimens were immersed for 60 min at 4°C for the enzymatic reaction and washed in 15% sucrose solution. Then they were mounted similarly to the first set. Using this modification, both tracer fluorescence and the AChE reaction product could be seen on the same specimen under the fluorescent microscope and light microscope, respectively. The third set was used for immunohistochemical staining with anti-human ChAT rabbit Fab serum (Peng et al., 1981) by the peroxidase-antiperoxidase technique (Kimura et al., 1980, 1981; Sternberger et al., 1970). Specimens were pretreated with 0.3% Triton X-100 for 6 days and then were treated in turn with 0.1% normal goat serum for 2 hr, anti-ChAT rabbit Fab serum (× 1/20) overnight, anti-rabbit goat IgG (× 1/400) for 2 hr, and peroxidase-rabbit antiperoxidase complex (× 1/800) for 1 hr. Each antiserum was diluted with phosphate-buffered saline (PBS) containing 0.01% Triton X-100. Specimens were washed in PBS containing 0.05% Triton X-100 before they were transferred from one serum to another. Finally, ChAT could be visualized as a brown precipitate after the diaminobenzidine reaction (Graham and Karnovsky, 1966). ChAT immunohistochemistry also was done on normal rat brains by the same procedure.

Results

AChE histochemistry combined with axonal retrograde labeling using a fluorescent tracer. Primuline proved to be the most satisfactory of the four fluorescent tracers tested. The conditions are such that the yellow fluorescence of Primuline (excited at 365 nm) can be easily distinguished from the dark brown reaction product of the AChE reaction even in the same perikarya. By contrast, DAPI and true blue fluoresce as fine pale granules in the perikarya and are sometimes invisible against the light blue background fluorescence seen after the AChE reaction. Evan's blue is also unsuitable because of strong background fluorescence after the AChE reaction as seen under the required excitation light of 546 nm. Primuline was used therefore for the experiments described below.

When Primuline was injected in a volume of 0.2 μl, centered in the basolateral nucleus of the amygdala, examination indicated that the injection did not spread into the caudate-putamen situated just above the central nucleus of the amygdala or into the hippocampus. Figure 1 shows adjacent sections of the basal forebrain demonstrating the correspondence between AChE and ChAT neurons. Figures 2, 3, and 4 are representative pictures of neurons staining for AChE alone admixed with others staining for AChE plus Primuline which has been retrogradely transported from the amygdala. The distribution in the basal forebrain of cells found to be intensely positive for AChE, with or without labeling by Primuline, is indicated in Figure 5 by *solid* and *open circles*, respectively. The distribution of cells in the forebrain intensely reactive for AChE after DFP treatment was identical to that reported previously (Lehmann et al., 1980). They were found in the septum, the area of the tractus diagonalis, the substantia innominata, the medial or ventral part of the globus pallidus (GP), and the ansa lenticularis. They also were scattered throughout the caudate-putamen. The positive cells outside of the caudate-putamen are classified here into five cholinergic cell groups following our previously developed map in the feline: the medial septal nucleus, the nucleus tractus diagonalis Broca (NDB), the nucleus of the substantia innominata (SI), the medial part of the GP, and the nucleus interstitialis ansae lenticularis. At the most rostral level, a small number of intensely AChE-reactive cells, labeled by Primuline, appeared in the horizontal limb of the NDB.

Caudolaterally, the number of such cells increased. The largest intensely AChE-reactive cell cluster labeled by Primuline was located in the SI at the level of the commissura anterior. Medium to large size (20- to 30-μm), round or ovoid shaped, intensely AChE-reactive cells labeled by Primuline were densely packed in the ventral part of this area. Dorsally, large (25- to 35-μm) and irregularly shaped, doubly labeled cells were scattered and spread into the ventral margin of GP. Rarely, a few intensely AChE-reactive cells labeled by Primuline were found in the ventral part of the GP. They were of medium size (20 to 25 μm), oval in shape, and located in the most ventral margin of this nucleus. A small number of non-cholinergic neurons also were labeled in the ventral part of the SI. They were medium in size and round or ovoid in shape. Almost all of the large neurons labeled by Primuline in the SI showed an intense AChE reaction.

Caudally, AChE-positive neurons, labeled by Primuline, extended into the nucleus interstitialis ansae lenticularis. Some intensely AChE-reactive neurons of that nucleus also showed labeling by Primuline. They were of large size (25 to 30 μm) and spindle shaped and were embedded in the fiber bundle of the ansa lenticularis. Neurons labeled by Primuline but not stained for AChE were found in the thalamus, the ventromedial nucleus of the hypothalamus, the peripeduncular nucleus, the nucleus parabrachialis dorsalis, and the basal nucleus of the amygdala (contralateral side). Neurons labeled by Primuline and stained for AChE also were found in the

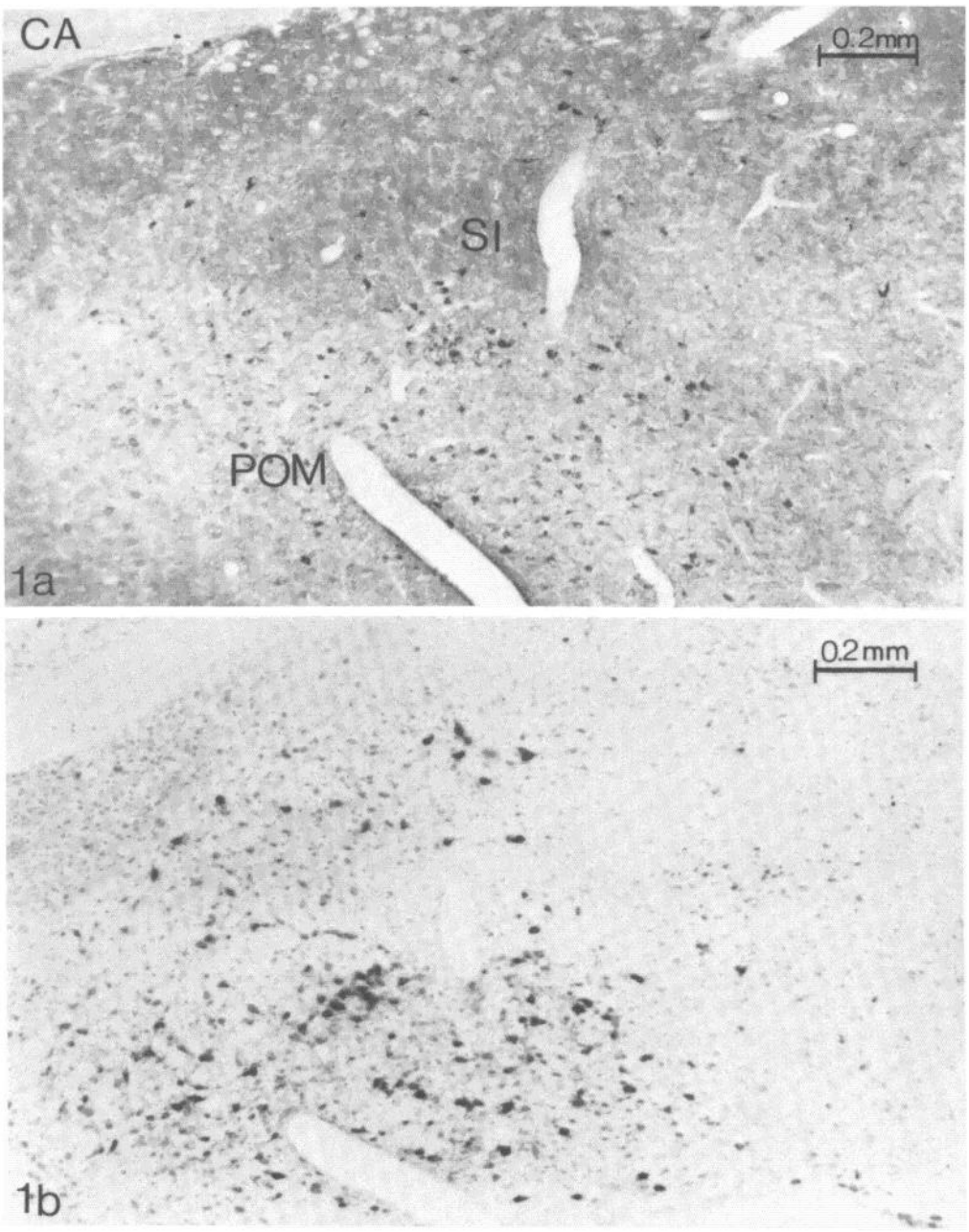

Figure 1.[3] Adjacent sections of rat brain showing neurons of the nucleus substantia innominata stained (*a*) for ChAT immunohistochemistry and (*b*) intensely reactive for AChE following DFP treatment. Neurons, visualized by two different histochemical techniques, show similar distribution.

aminergic groups of the ventral tegmental area of the midbrain, the pars compacta of the substantia nigra, the nucleus raphe dorsalis, and the locus coeruleus. Of all of these areas containing such Primuline-labeled cells, only the nucleus parabrachialis contains cholinergic neurons (Kimura et al., 1981).

Correspondence between ChAT-containing cells and intensely reactive cells for AChE in the basal forebrain. The distribution of somata positively stained by ChAT immunohistochemistry in the forebrain was the same as the distribution of intensely AChE-reactive cells in the rats used in this study. As previously described (Kimura et al., 1981), no ChAT-containing cells were found in the thalamus, hypothalamus, or amygdala.

ChAT-containing cells in the forebrain were generally moderate (20 μm) to large (40 μm) in size. On the frontal sections at the level of the commissura anterior, dense

[3] The abbreviations used on the figures are: bl, nucleus amygdaloideum basalis, pars lateralis; bm, nucleus amygdaloideum basalis, pars medialis; c, nucleus amygdaloideum centralis; CA, commissura anterior; CAI, capsula interna; CC, corpus callosum; co, nucleus amygdaloideum corticalis; CO, chiasma opticum; CP, caudate-putamen; GP, globus pallidus; HDB, horizontal limb of the nucleus tractus diagonalis Broca; HI, hippocampus; IA, nucleus interstitialis ansae lenticularis; lp, nucleus amygdaloideum lateralis, pars posterior; m, nucleus amygdaloideum medialis; POM, nucleus preopticus magnocellularis; SI, substantia innominata; SM, nucleus septi medialis; TO, tractus opticus; VDB, vertical limb of the nucleus tractus diagonalis Broca; ZI, zona incerta.

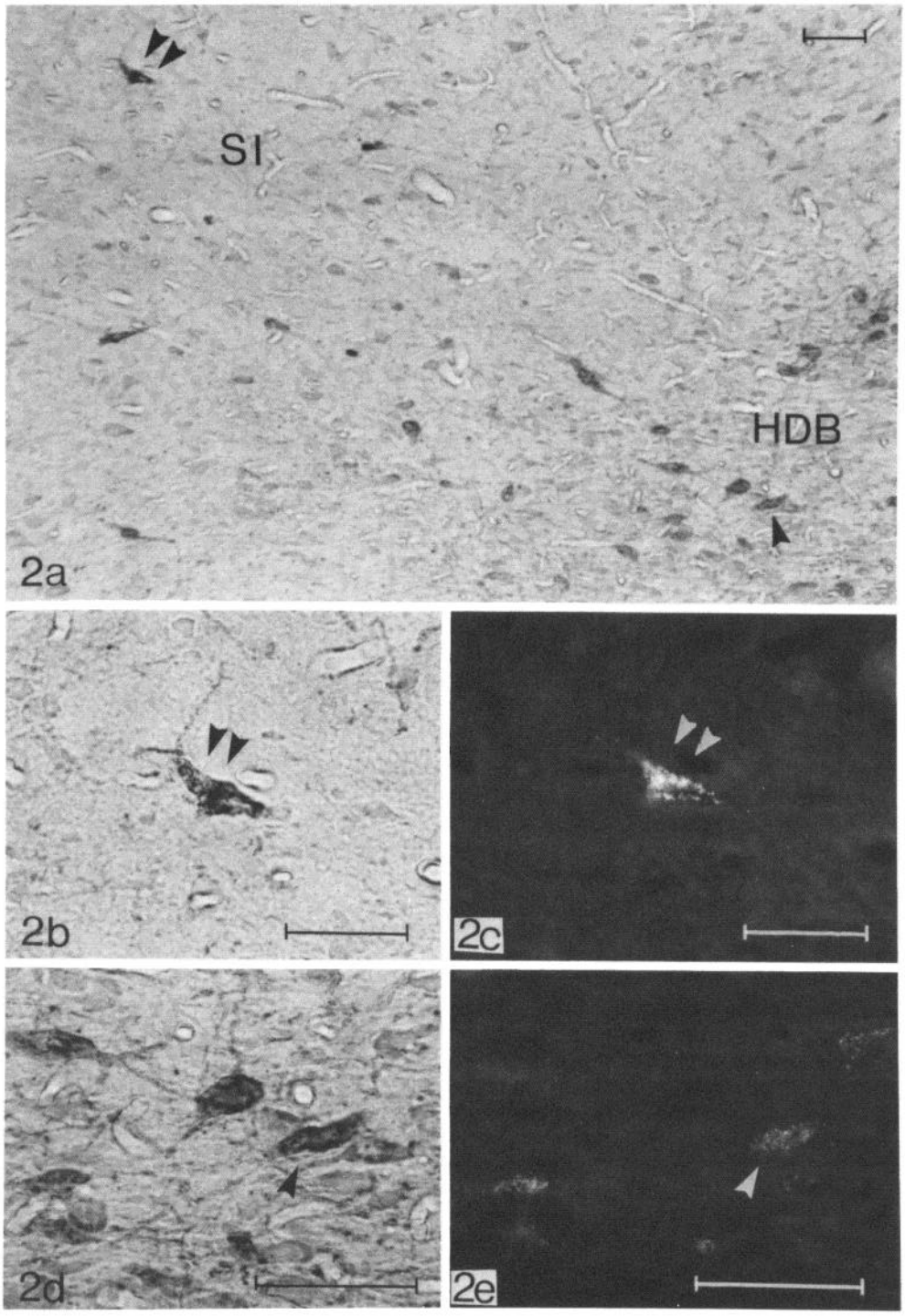

Figure 2. a, Low power photomicrograph of intensely reactive AChE cells of the substantia innominata. *b*, High power photomicrograph of a large intensely reactive AChE neuron, indicated by the *double arrowhead*, seen in the nucleus substantia innominata. *c*, The same neuron photographed under fluorescent light. The neuronal cell body was labeled by retrogradely transported Primuline, visualized here as granular fluorescence. *d* and *e*, Intensely reactive cells found in the horizontal limb of nucleus tractus diagonalis Broca. Some examples (indicated by the *single arrowhead*) in *e* are labeled by the typical granular fluorescence of Primuline. *Calibration bars*, 50 μm.

ChAT-containing cell clusters are seen in the nucleus septi medialis and NDB; these cell groups, which also stain for AChE, consist of round or ovoid cells. Caudolaterally, ChAT-containing cells of the horizontal limb of the NDB extend to the SI which is densely packed with large cholinergic cells. Dorsomedially, cells are less densely packed, are somewhat larger in size, and are more irregular in shape. Further dorsally, the numbers

increase, extending into the ventral part of the caudate-putamen and touching the medial part of the GP. Caudally, large cholinergic perikarya are distributed in the ventral part of the capsula interna. At the more caudal levels, large and spindle-shaped ChAT-containing cells are seen along the fiber bundle of the ansa lenticularis between the capsula interna and the tractus opticus in an area designated here as the nucleus interstitialis ansae lenticularis. Further, many large, aspiny ChAT-containing somata are found throughout the caudate-putamen.

Discussion

The source of cholinergic fibers to the amygdala from the basal forebrain appears to have been identified. The distribution of the cells retrogradely labeled by Primuline injected into the amygdala was similar to previous reports using the horseradish peroxidase method in the rat (Ottersen and Ben-Ari, 1978, 1979; Ottersen, 1980; Veening, 1978) and other species (Norita and Kawamura, 1980; Mehler, 1980; Aggleton et al., 1980). Proceeding caudally, the intensely reactive AChE neurons labeled by Primuline were distributed from the level of the rostral margin of the commissura anterior to a restricted region of the ansa lenticularis between the capsula interna and the tractus opticus. They were located mainly in the SI, the horizontal limb of the NDB, and the nucleus interstitialis ansae lenticularis. A few were found occasionally in the ventral margin of the GP. It can be concluded that the former three nuclei are the main origin of basal forebrain cholinergic afferents to the amygdala. This evidence is consistent with a previous report that kainic acid injections placed in the lateral preoptic area result in substantial depletions of both AChE and ChAT in the amygdala (Emson et al., 1979).

Satisfactory staining for ChAT and Primuline or other dyes on the same tissue section is technically not feasible at the present time. Accordingly, the distributions of intensely positive AChE cells and ChAT-containing cells in the rostral forebrain were compared on consecutive sections. In lower brain areas, such as the hypothalamus,

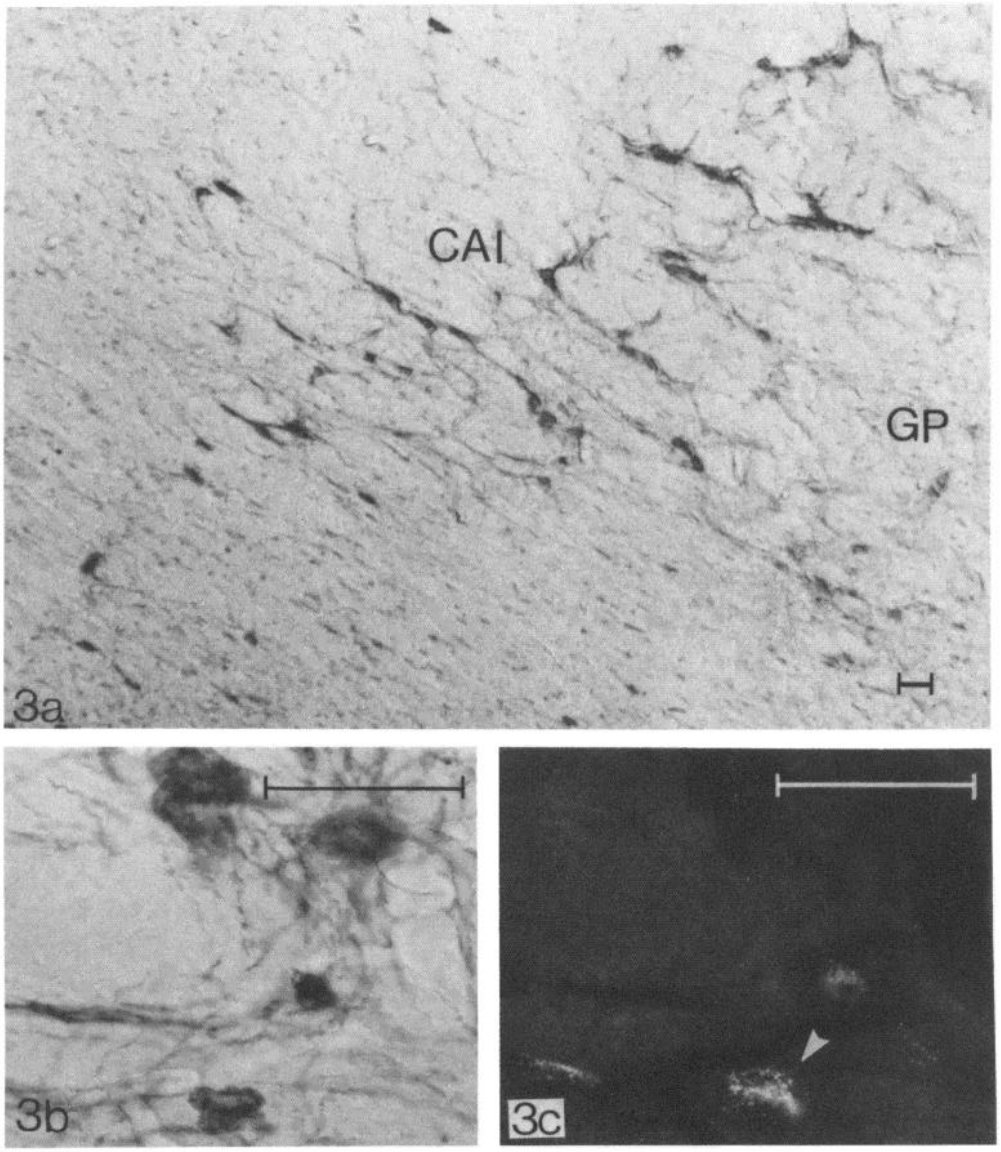

Figure 3. a, Low power magnification photomicrograph shows intensely reactive AChE cells in the medial part of the globus pallidus. *b*, A middle size neuron, at the *bottom*, shows intense AChE reaction. *c*, The same neuron is labeled by Primuline. *Calibration bars*, 50 μm.

the substantia nigra, the nucleus raphe, and the locus coeruleus, discrepancies have been noted between the neuronal distributions of cells intensely reactive for AChE and those staining for ChAT (Kimura et al., 1981). However, as shown in the present study and in confirmation of previous reports (Kimura et al., 1981; Lehmann and Fibiger, 1979), the correspondence is excellent in basal forebrain areas. It is concluded therefore that cells in the basal forebrain which are intensely reactive for AChE can be regarded as cholinergic.

A combination of the horseradish peroxidase tracer technique and AChE histochemistry has been used previously (Mesulam and Van Hoesen, 1976; Lehmann et al., 1980), but this is time consuming and complicated compared with fluorescent tracers. A fluorescent tracer such as Primuline can easily be distinguished from the AChE reaction product when they are in the same somata except when the neuron is faintly labeled by the Primuline and the AChE reaction is extremely strong. In such circumstances, the fluorescence can be covered by the reaction product.

A precise anatomical concept of the substantia innominata has never been advanced. This region has been described as the nucleus basalis (Meynert) as well as by some other terminologies (Mesulam and Van Hoesen, 1976; Kievet and Kuypers, 1975; Heimer and Willson, 1975). The term substantia innominata, as used here, indicates a large area of the ventral forebrain caudal to the nucleus tractus diagonalis Broca and rostral to the medial amygdaloid nucleus. The "substantia innominata" of Swanson (1976) and the nucleus preopticus magnocellularis of Loo (1931) are included within this area. Moreover, the rostral part of the substantia innominata possibly corresponds to the ventral pallidum of Ottersen (1980). We have chosen to designate this area by a single term because virtually all of the giant neurons within the area are cholinergic (Kimura et al., 1981; present results). We have, however, distinguished it from

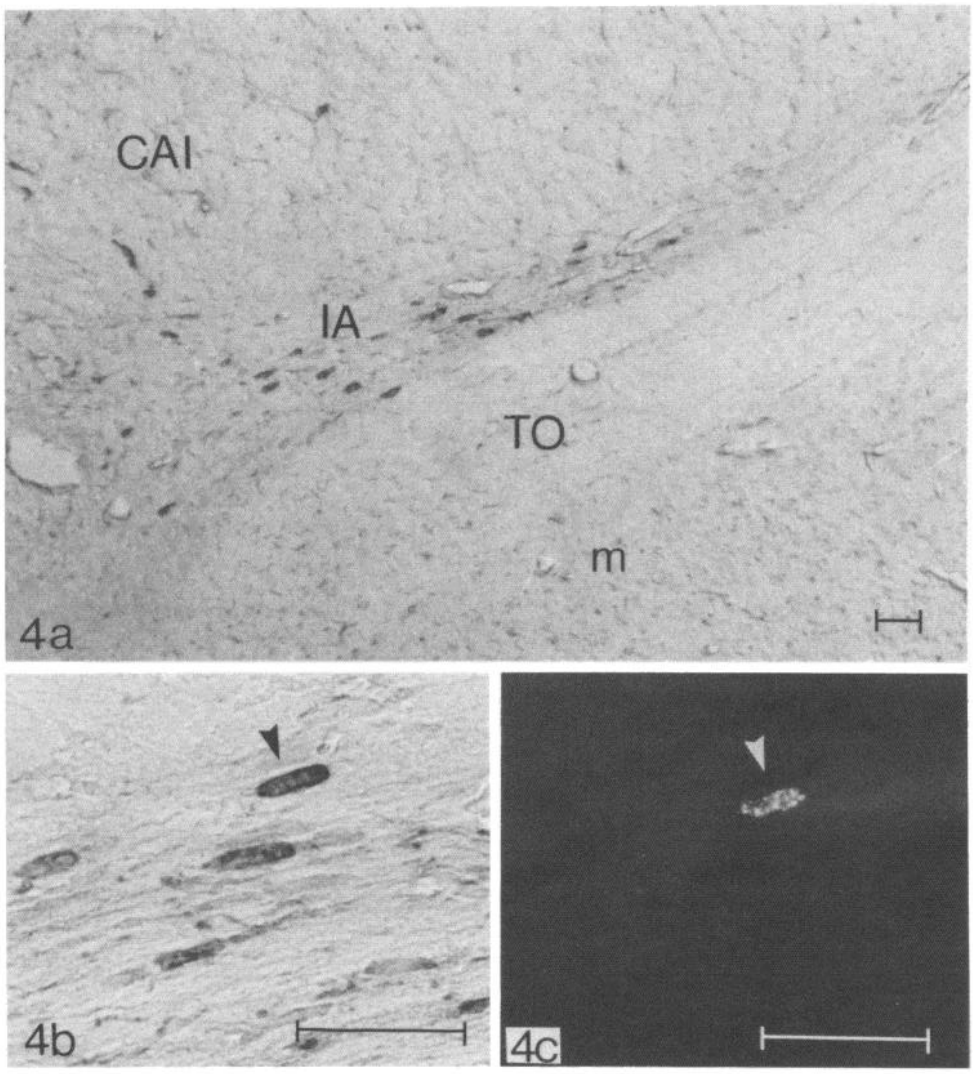

Figure 4. a, Low power magnification of nucleus interstitialis ansae lenticularis AChE reaction. *b*, High power magnification of intensely stained cells indicated by the *arrowhead*. *c*, Fluorescent microscopic photograph of same neurons shows retrograde labeling by Primuline. *Calibration bars*, 50 μm.

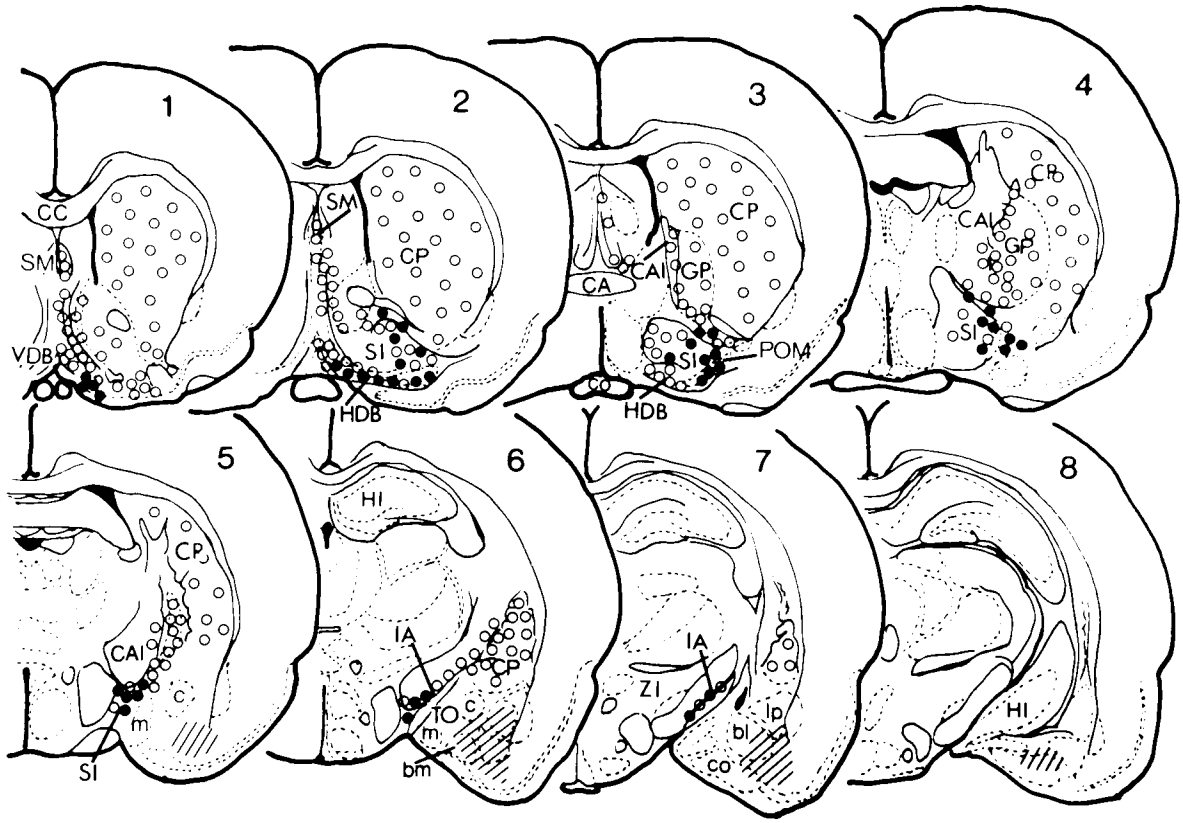

Figure 5. Distribution of cells intensely stained for AChE in the basal forebrain. The *solid circles* indicate cells labeled by Primuline following injection into the amygdala, while the *open circles* indicate those without such Primuline labeling. The *shaded portion* of the figure shows extent of Primuline around the injection site.

the more caudal nucleus interstitialis ansae lenticularis, in which large, spindle-shaped cholinergic cells are intermingled with the fiber bundles of the ansa lenticularis (Kimura et al., 1981).

The afferent fiber pathway to the amygdala from the substantia innominata is presumed to consist of two major pathways: the amygdalopyriform association bundle (Johnston, 1923) that also is referred to as the ventral amygdalofugal pathway and the stria terminalis (Cowan et al., 1965; Heimer and Nauta, 1969; de Olmos and Ingram, 1972; Hamilton, 1976; Swanson, 1976). Knife cuts of the ventral pathway caused large depletions of ChAT in the amygdala, especially in the posterolateral and basolateral nuclei (Emson et al., 1979). It might be supposed, therefore, that the majority of the cholinergic afferent fibers to the amygdala from neurons in the substantia innominata pass through the amygdaloventral pathway.

It is worthy of note that some neurons in the substantia innominata which were labeled by Primuline did not show intense positive staining for AChE. This indicates that there is another, non-cholinergic input from the substantia innominata to the amygdala.

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