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Cholinergic Neurons in the Basal Forebrain of the Cat Have Direct Projections to the Sensorimotor Cortex

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Previous studies showed that projections from the cells in the substantia innominata to the sensorimotor cortex exist in the monkey and rat and that this group of cortical afferent fibers utilizes acetylcholine as a neurotransmitter. Because similar studies have not been made in the cat, the following study was undertaken to analyze the extent and distribution of this projection from the basal forebrain. Horseradish peroxidase (HRP) injections were made into the anterior and posterior sigmoid gyri 24 h before, and diisopropyl fluorophosphate was injected intramuscularly 3 h before the anesthetized cats were perfused. Brain sections from these cats were incubated in solutions to simultaneously detect retrogradely transported HRP and acetylcholinesterase (AChE) reaction product within the somata. The cell bodies that contained reaction product for both HRP and AChE were mostly large (25 to 30 μm) and multipolar. These double-labeled cells were located in (i) the nucleus of the diagonal band in rostral sections, (ii) the globus pallidus, entopeduncular nucleus, and substantia innominata at the level of the anterior commissure, and (iii) the lateral hypothalamus in the most caudal sections. Many of these sites corresponded to that for the basal nucleus of Meynert, an aggregation of large multipolar neurons scattered throughout the basal forebrain. Although the presence of AChE within a cell does not define a cholinergic neuron, recent studies indicated that its presence is a requirement for this neurotransmitter. These data together with biochemical and immunocytochemical data indicate that a cholinergic projection to the sensorimotor cortex of cats arises from the basal forebrain. This pathway may play a vital role in memory and cognition.

Abbreviations: AChE—acetylcholinesterase, HRP—horseradish peroxidase, ACh—acetylcholine, OMPA—octamethyl pyrophosphoramidate.

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INTRODUCTION

Previous anatomical studies in the basal forebrain of the monkey and rat demonstrated a reticular network of large multipolar cells that project to sensorimotor cortex (3, 8, 12). Histochemical and immunocytochemical studies that localized acetylcholinesterase (AChE) and choline acetyltransferase, respectively, indicated that this cortical projection arises from the substantia innominata and may utilize acetylcholine as a neurotransmitter (6, 14, 15, 20, 26). Furthermore, biochemical studies that analyzed the cerebral cortex after the placement of lesions in parts of the substantia innominata showed a loss of these acetylcholine markers; these data are consistent with this notion of a cholinergic projection from the basal forebrain (5, 7, 11, 18, 29).

In the cat, previous anatomical studies utilized a histochemical method to localize AChE in cells of the basal forebrain (23) and in fibers that may project to the cerebral cortex (16). However, the continuity of these AChE-labeled neurons from the basal forebrain to the cortex was not demonstrated. In addition, other studies which reported horseradish peroxidase (HRP) injections into the sensorimotor cortex of cats emphasized the resulting labeled cells in the thalamus, but failed to show labeled cells in the basal forebrain (9, 24). The results of a more recent autoradiographic study (28) provided evidence for a projection to the cortex from the substantia innominata in the basal forebrain of cats. However, the distribution of these projection cells throughout the full extent of the basal forebrain has not been explored as it has for rats and monkeys. Therefore, the following study was undertaken to determine this distribution and if the cells of origin of this projection coincide with the population of large AChE-labeled cells in the cat basal forebrain. A double labeling method for AChE and HRP histochemistry was utilized (19). With this method, developed by Mesulam (19), horseradish peroxidase was used to detect cells with cortical projections, and simultaneously on the same sections, AChE histochemistry was utilized as a marker to label possible cholinergic cells (17).

MATERIALS AND METHODS

Three cats weighing 2.7 to 3.1 kg were used. Each was anesthetized by an i.p. injection of Nembutal (35 mg/kg, administered at a concentration of 50 mg/ml). Subsequently, the cats were placed into a stereotaxic holder, and the cranium was opened by chipping away the frontal sinus and then the underlying frontal bone. After excising the dura mater, injections of 30% HRP were made from a glass micropipet into the anterior and posterior sigmoid gyri of one hemisphere. Each gyrus received four injections that included both medial and lateral parts (Fig. 1A). Each injection consisted

of 0.2 to 0.3 μl HRP solution which was administered during a 5-min period.

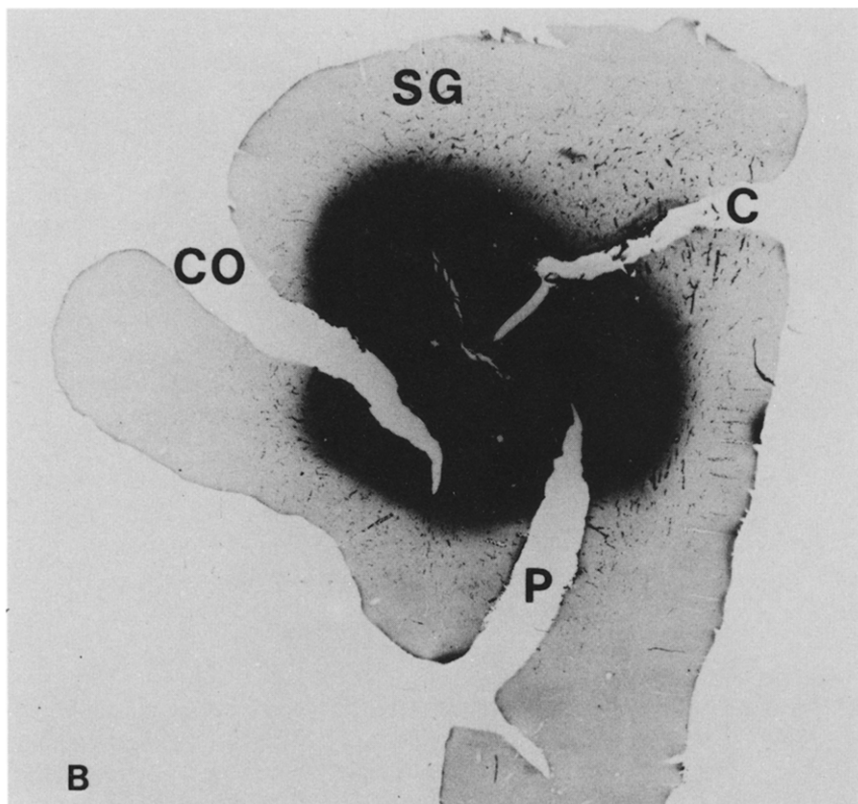
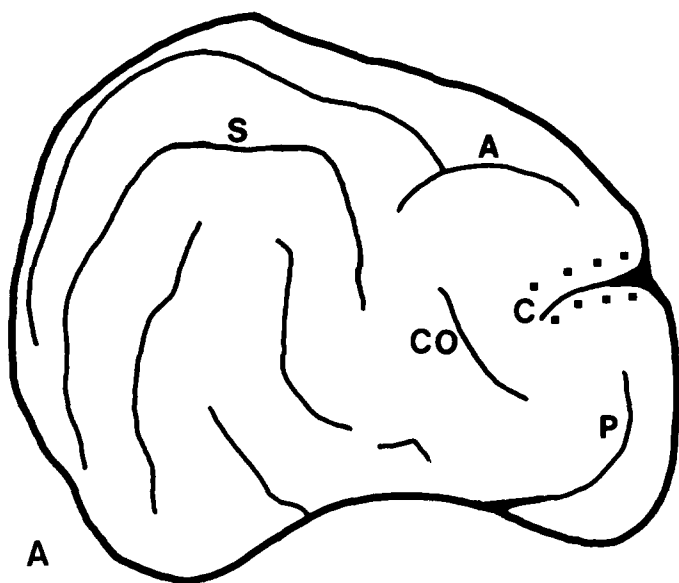
After 22 h, each cat was injected with 1% diisopropyl fluorophosphate (2 mg/kg) into the quadriceps femoris. These injections were made to reduce extracellular AChE activity so that newly synthesized AChE could be detected (2, 22). Approximately 3 h after the drug injection, each anesthetized cat was perfused intracardially with saline, followed by 1.25% paraformaldehyde and 1% glutaraldehyde in a 0.12 M phosphate buffer (pH 7.2). After the 30-min perfusion of fixative, the cat was perfused with a 0.1 M phosphate buffered 10% sucrose solution to rinse out the excess fixative from the brain. The brain was subsequently removed and stored 24 h in this latter solution at 4°C. Frozen sections cut at 50 μm were rinsed with distilled water and then every sixth section was incubated in solutions following a modified procedure described by Mesulam (19).

Briefly, sections were first incubated 1 h in a modified acetylthiocholine solution that contained the pseudocholinesterase inhibitor, iso-OMPA, at a concentration of 4×10^{-5} M instead of ethopropazine as described by Mesulam (19). The sections were rinsed in four changes of distilled water during the next 10 min before they were incubated in solutions of benzidine dihydrochloride according to Mesulam (19). These incubations yielded blue granules at sites containing HRP. The sections were then transferred for 30 min into a stabilizer solution containing nitroferricyanide (19). After a 15-min rinse in distilled water, the sections were developed 15 min for the reddish-brown acetylcholinesterase reaction product in a ferricyanide solution. After another rinsing in distilled water, the sections were placed on gelatin-coated glass slides, and coverslipped without counterstaining.

Each incubated section was drawn at low magnification with a drawing tube mounted on a light microscope. Double-labeled cells were observed at higher magnifications and their loci were marked on these drawings. Triangles were used to mark the double-labeled cells that were heavily stained for HRP, and circles were used for those double-labeled cells with light HRP staining. A composite map for each cat was assembled by taking groups of five consecutive drawings and representing the double-labeled cells for each of the five levels on the middle section in each series.

RESULTS

Injection Sites. The injections of HRP were made into the anterior and posterior sigmoid gyri and the resulting reaction product heavily stained this brain region (Fig. 1A). The apparent spread of HRP was great enough so that these gyri and the banks of the cruciate sulcus were entirely filled in cases where large injections were made (Fig. 1B). In one other cat, the



injected quantities of HRP were smaller and only the crowns of the gyri were labeled. In all cases, the HRP was found to be limited to just these two gyri; other gyri in the hemisphere ipsilateral and contralateral to the injection sites lacked extracellular HRP reaction product. Thus, these injection sites were definitely made into the area of the cerebral cortex known to have a motor function (1).

Criteria for Double-Labeled Cells. Double-labeled cells were defined as containing reaction product for both AChE and HRP. The method used to identify a double-labeled cell was to compare each component of the labeling with well-recognized single labeled cells in other brain regions. For example, large cells in the caudate nucleus and putamen contained flocculent, reddish-brown AChE reaction product within their perikaryal and dendritic cytoplasm (Fig. 2A). These cells served as a guide for recognizing AChE-positive cells in the basal forebrain. Similarly, cells that contained small, blue punctate structures in their perikaryal cytoplasm and were situated in the ventral basal complex in the thalamus (9, 24) were used as examples to determine if a cell had transported HRP from the cortex (Fig. 2B). In determining whether or not a cell was double-labeled, a problem occurred when the HRP reaction product filled the cytoplasm of the cell to such a degree that the AChE reaction product was obscured. In these cases, the cells in question were adjacent to AChE-positive cells, but were not counted as being double labeled. Therefore, the number of double-labeled cells counted in this study is probably a conservative estimation of the actual number.

The cell bodies that contained reaction product for both HRP and AChE were commonly large (25 to 30 μm in diameter) and multipolar (Fig. 2C). Although many of the more lightly stained cells for HRP were medium size (15 to 25 μm in diameter), they were often located in areas mixed with other more heavily stained cells (Fig. 3D). When dendritic staining was present, these medium-size cells appeared as either bipolar or multipolar cells (Fig. 4B).

Distribution of Double-Labeled Cells. The double-labeled cells were found in many different regions throughout the basal forebrain in the cats

FIG. 1. A—schematic drawing of the right hemisphere of the cat brain to show the four injection sites (solid squares) made in each of the sigmoid gyri on either side of the cruciate sulcus (C). Other sulci are drawn on the surface, and they include coronal sulcus, CO; presylvian sulcus, P; ansate sulcus, A; and Sylvian sulcus, S. B—photomicrograph of the posterior sigmoid gyrus (SG) and the fundus deep to the cruciate sulcus (C) heavily stained with HRP reaction product after a series of eight injections in which each consisted of 0.3 μl HRP. The HRP apparently merged from these separate injections because the reaction product appeared as a single mass. In this transverse section, the coronal sulcus (CO) and the presylvian sulcus (P) are also seen. The dorsal aspect is at the top and medial is to the right. $\times 8$.

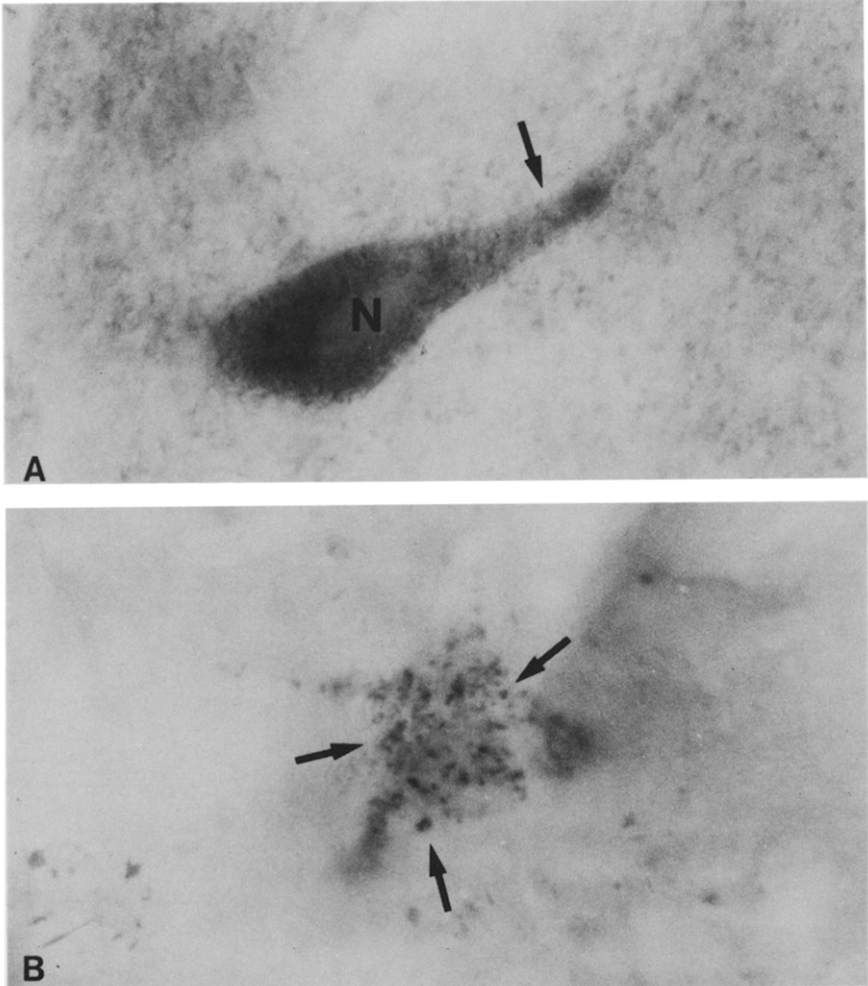


FIG. 2. A—photomicrograph of a cell containing reaction product for AChE. This cell was located in the caudate nucleus, a region dorsal to the basal forebrain, and known to contain cholinergic cells (14, 18). The nucleus (N) was unstained but the cytoplasm contained flocculent reaction product that diminished in density in the dendrite (arrow). $\times 1500$. B—photomicrograph of a multipolar, lightly stained HRP-labeled cell in the ventral basal complex of the thalamus. The HRP reaction product appeared as small distinct punctate structures within the perikaryal cytoplasm. Some of these which were located at the cell's edges are indicated by arrows. $\times 1500$. C—photomicrograph of a large multipolar double-labeled cell (left of center) located in the medial globus pallidus. The HRP reaction product in this cell appeared as punctate structures in the somal cytoplasm and extended into the proximal portion of a dendrite (arrow). The flocculent AChE reaction product extended further (arrowhead) into this dendrite than did the HRP reaction product. The nucleus (N) of this cell was unstained. The other cell in this figure contained AChE reaction product but lacked HRP staining. $\times 1500$.

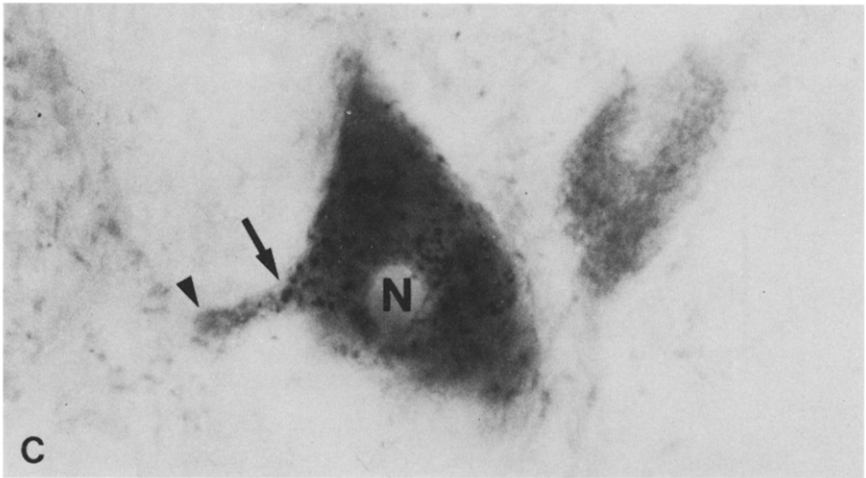
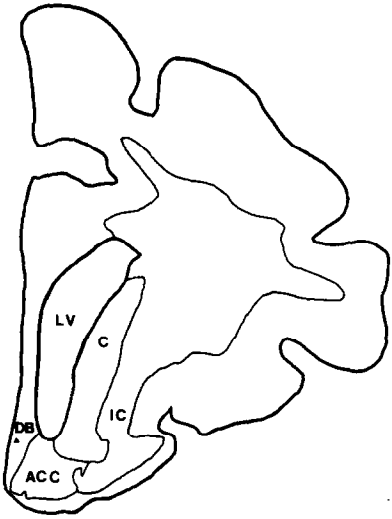


FIG. 2—Continued.

used in this study. All cells were ipsilateral to the injection sites. The rostral-caudal distribution was very similar for each animal even though the number of double-labeled cells varied among animals due to differences in the sizes of the injection sites. The arrangement and number of double-labeled cells is illustrated for cat SI-2, which received large injections of HRP into both anterior and posterior sigmoid gyri (Fig. 3). In rostral sections, most of the double-labeled cells were located in the vertical limb of the nucleus of the diagonal band (Fig. 3A, B). In following the double-labeled cells caudally, the cells were found laterally within the horizontal limb of the nucleus of the diagonal band. At the level of the rostral part of the anterior commissure, the double-labeled cells were found in the globus pallidus, entopeduncular nucleus, and substantia innominata (Figs. 3B, C and 4A, B). At the caudal end of the anterior commissure and beginning at the level of the optic chiasm, many cells were again found in these regions as well as in the ventral putamen bordering the globus pallidus (Fig. 3D). At the most caudal part of this series, the double-labeled cells appeared in the lateral hypothalamus, ventromedial to the internal capsule (Fig. 3F).

DISCUSSION

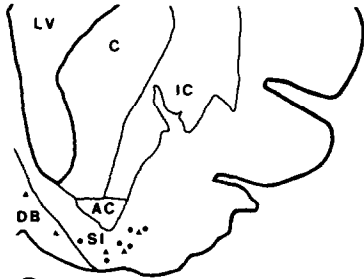
The results of this study indicate that many large, multipolar cells in the basal forebrain of cats project to the sensorimotor cortex and contain AChE. These cells are situated in the nucleus of the diagonal band, globus pallidus, entopeduncular nucleus, substantia innominata, ventral putamen,



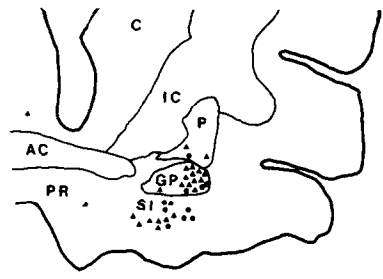
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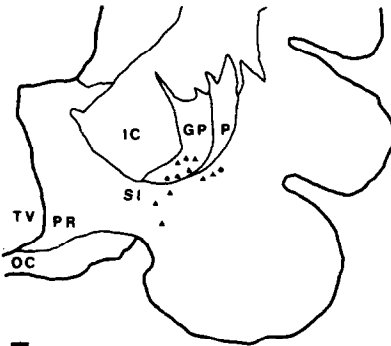
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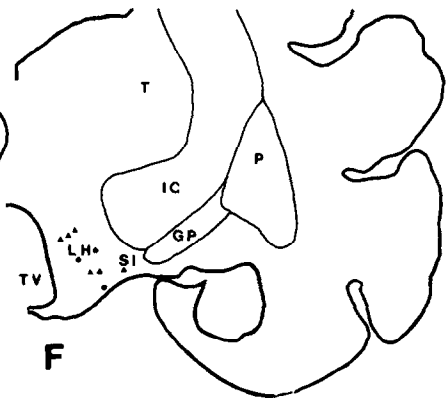
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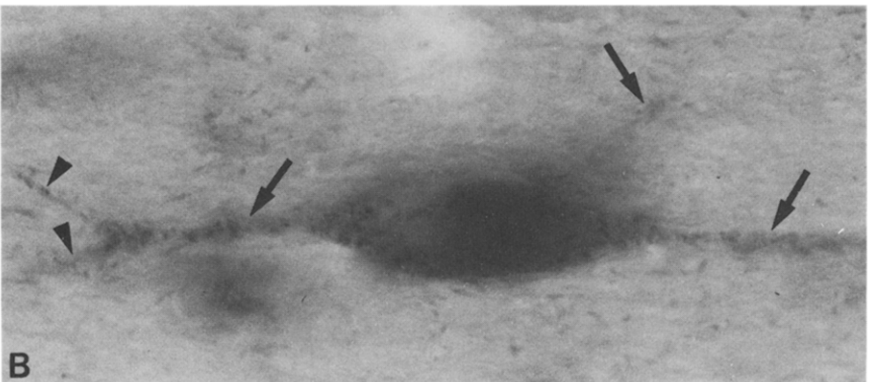


F

and lateral hypothalamus. It is interesting to note that the cells varied in their staining intensity for HRP. Based on previous results, these differences may relate to the density of their axon terminals in the cortical injection sites (10). These results suggest that some cells have concentrated projections to sensorimotor cortex and others have more diffuse projections. The size, location, and histochemical staining of these double-labeled cells for AChE indicate that many of these cells belong to a widely scattered network of large cells in the basal forebrain, the basal nucleus of Meynert (3, 8, 12, 18, 20, 22, 23, 28, 29). Although this nucleus is mainly within the substantia innominata in monkeys, the cells of the basal nucleus in cats occur with high frequency in the globus pallidus (23). This fact can explain the present results which show a large number of double-labeled cells in the globus pallidus. These data as well as other interspecies differences (see below) suggest that caution be used when applying the term basal nucleus to structures outside the substantia innominata.

The distribution of the double-labeled cells found in the cat resembles the distribution of labeled cells in both the monkey and rat that follow numerous HRP injections of the cerebral cortex, including regions other than sensorimotor cortex (3, 8, 12, 18). For example, in the rat, HRP-labeled cells were distributed from a level just caudal to the nucleus of the diagonal band and extended posterior and ventrolaterally into the region of the globus pallidus (3, 18). Labeling was found in the nucleus of the diagonal band only if the frontal cortex was included in the injection site (3). In contrast, the distribution for monkeys with injection sites in the sensorimotor cortex showed HRP-labeled cells from the nucleus of the diagonal band, rostrally, to the lateral hypothalamus, caudally (3, 8, 12). The cells found in this latter region and in the horizontal limb of the diagonal band are considered parts of the basal nucleus of Meynert based on cytologic and connectional data (8). Thus, the rostrocaudal distribution of double-labeled cells in the cat is very similar to that for the monkey (20). However, the presence of large numbers of cells in the cat globus

FIG. 3. Composite drawing of coronal sections taken at various levels of the cat brain ipsilateral to HRP injections in the sensorimotor cortex to show the full extent of double-labeled cells for SI-2 (section A is most rostral, F is most caudal, and the lateral surface is on the right for each drawing). The composite map was assembled by taking five consecutive drawings and representing the cells for each level on the middle section of the series. Triangles mark the double-labeled cells that were heavily stained for HRP and circles mark double-labeled cells with light HRP staining. $\times 4$. List of abbreviations: AC—anterior commissure, ACC—nucleus accumbens, C—caudate nucleus, DB—nucleus of the diagonal band, GP—globus pallidus, IC—internal capsule, LH—lateral hypothalamus, LV—lateral ventricle, OC—optic chiasm, P—putamen, PP—prepyriform cortex, PR—preoptic area, SI—substantia innominata, T—thalamus, TV—third ventricle.



pallidus is more similar to the rat because cells in the monkey appeared ventral to, and within the medullary laminae, of the globus pallidus.

Although there have been no previous studies using HRP to detect cells in the basal forebrain of the cat that project to the sensorimotor cortex, Troiano and Siegel (28) utilized autoradiographic techniques to demonstrate that the substantia innominata projects to all parts of the cerebral cortex in the cat. However, this projection was described after injecting only sites that included the basal nucleus of Meynert and the lateral and caudal parts of the substantia innominata. It was interesting to note that these authors observed this projection whenever the large, angular, multipolar neurons of the basal nucleus were heavily labeled. In addition, results from another autoradiographic study in the cat showed projections from the globus pallidus to undefined regions of the cortex (21). Both these findings are consistent with the results reported in the present study which indicate that these and other basal forebrain regions project to the sensorimotor cortex.

Based on the histochemical data for the localization of AChE, the basal forebrain projections to cat cortex may utilize acetylcholine as a neurotransmitter. Although the presence of AChE does not define a cholinergic neuron, recent studies indicate that its presence is a requirement for this neurotransmitter (17). Furthermore, Kimura *et al.* utilized immunocytochemical methods to localize choline acetyltransferase, the enzyme that synthesizes acetylcholine (ACh), in large multipolar cells in the basal forebrain of rats and cats (14, 15). Their findings in the rostral basal forebrain are consistent with our cytochemical localization of AChE. Recent biochemical data provide further support for these histochemical and immunocytochemical results. After the placement of either electrolytic or kainic acid lesions in the globus pallidus, activity of choline acetyltransferase was significantly reduced in the anterior and middle cerebral cortex of the rat (7, 11, 18, 29). Together these results indicate that the projection from the basal forebrain to the sensorimotor cortex utilizes ACh as a neurotransmitter.

These findings are particularly significant for our intracellular studies of recorded and HRP-labeled cells in the cat sensorimotor cortex (25, 30). Because acetylcholine was shown to change membrane properties of these

FIG. 4. A—low-magnification photomicrograph of the substantia innominata (SI) at a level between Figs. 3C and 3D. Also shown are the globus pallidus (GP) and the ventral medial part of the internal capsule (IC). Numerous AChE positive cells were found in each of those sites. $\times 60$. B—a multipolar double-labeled cell located in the portion of SI indicated by the box in A. Three primary dendrites (arrows) and two secondary dendrites (arrowheads) appear to contain AChE reaction product. $\times 1600$.

cells in the sensorimotor cortex, this cholinergic basal forebrain projection may be important for learning and other cognitive properties (4, 27). This is especially relevant because results from both Lehmann *et al.* (18) and Wenk *et al.* (29) indicate that intrinsic ACh neurons may not exist in the cortex and that the major cholinergic projection to the cortex arises from the basal nucleus of Meynert. These data are relevant to a recent study by Kim and Woody (13) in which changes in membrane potentials of cortical cells similar to those that follow extracellular ACh applications occurred after stimulation of the lateral hypothalamus in cats. Although other fiber pathways may have been stimulated, it is likely that cholinergic cells which project to the cortex were also stimulated and directly gave rise to this ACh effect in cortical cells. The fact that this stimulation caused a decrease in the time needed to acquire a conditioned response suggests that the double-labeled cells demonstrated in our study could give rise to an important cortical, cholinergic projection which is responsible for increases in memory and cognition (7, 29).

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