A LIGHT AND ELECTRON MICROSCOPIC INVESTIGATION OF CHOLINERGIC PROJECTIONS TO THE CM-Pf THALAMIC COMPLEX IN THE DOG

By

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ABSTRACT

A LIGHT AND ELECTRON MICROSCOPIC INVESTIGATION OF CHOLINERGIC PROJECTIONS TO THE CM-Pf THALAMIC COMPLEX IN THE DOG

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The pontomesencephalic-thalamo-striate pathway was examined in the dog with special emphasis on its topography and cholinergic aspects. The thalamostriate projection from the centrum medianum (CM) and parafascicular nucleus (Pf) was studied in detail using lectin-conjugated horseradish peroxidase (WGA-HRP) retrograde tracing techniques. In addition, the organization of cholinergic projections from the nucleus tegmenti pedunculopontinus (PPN) and adjacent areas to the CM-Pf complex was investigated by combining WGA-HRP retrograde tracing with choline acetyltransferase (ChAT) immunocytochemistry. Finally, these two techniques were combined at the electron microscopic level to investigate the cholinergic termination pattern onto identified CM-Pf

thalamostriate projection neurons.

The results of this study indicate that the thalamocaudate projection from the canine CM-Pf complex is topographically organized. Also, the CM-Pf complex receives projections from cholinergic and non-cholinergic neurons within the PPN and central tegmental tract of the pontomesencephalic tegmentum. Finally, some cholinergic terminal boutons contact neurons in the CM-Pf complex which in turn project to the caudate nucleus. It is probable that at least some of these cholinergic boutons belong to neurons which originate in PPN and central tegmental tract.

The determination of a direct contact between cholinergic terminations from PPN and CM-Pf thalamostriate projection neurons provides evidence that PPN may influence basal ganglia activity and possibly have a role in CNS control of movement.

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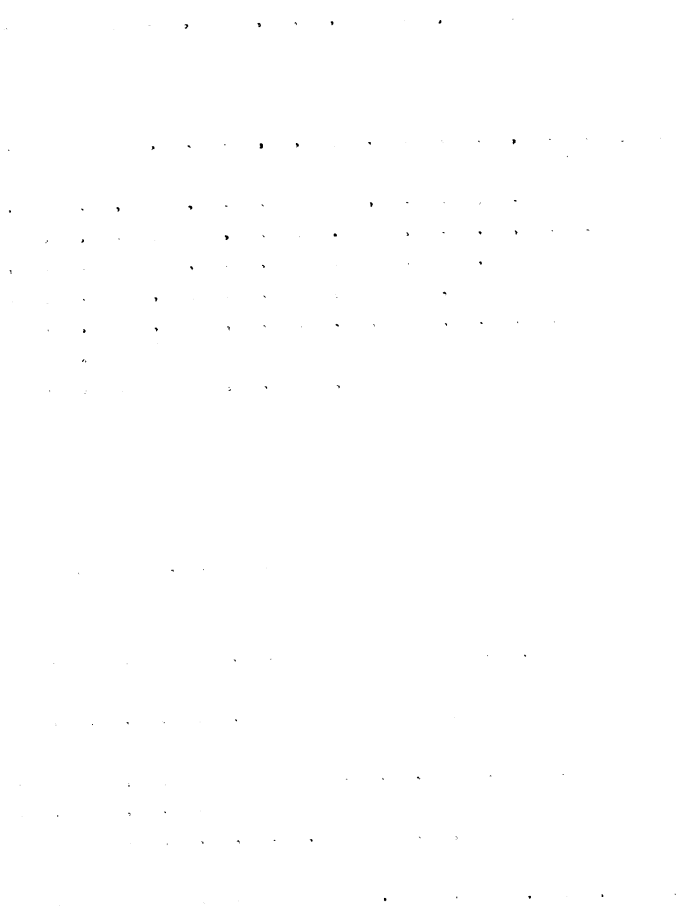

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INTRODUCTION

CYTOARCHITECTURE

The centrum medianum (CM) and parafascicular nucleus (Pf) comprise the caudal intralaminar nuclei of the thalamus. In carnivores, the centrum medianum, located between the mediodorsal nucleus and the ventral posterior nucleus, is formed by a group of homogeneous, closely packed cells enclosed by the splitting of the posterior part of the internal medullary lamina. The neurons are arranged in rows parallel to the internal lamina as they follow its contours. The parafascicular nucleus consists of a densely aggregated mass of large, oval, deeply staining cells located posteroventral to the mediodorsal nucleus. It is split into medial and lateral segments by the habenular-peduncular tract (HPT). It begins rostrally at the level at which the posterior paraventricular nucleus is replaced by the periaqueductal grey and continues caudally to the level of the posterior commissure.

GOLGI STUDIES

Several investigators have briefly described the morphology of CM-Pf neurons during the course of larger studies of the thalamus or brainstem. Leontovich and

Zhukova (1963), in their study of the reticular formation, describe the neurons in the caudal intralaminar region as having poorly ramified dendrites. The Scheibels (1966) found that neurons within the CM-Pf complex of the young cat were multipolar and "reticular" in shape and covered with "fine grained" spines. In the human, Van Buren and Borke (1972) described two types of neurons based on dendritic branching patterns. The first type of neuron has dendrites which branch repeatedly at wide angles and the second type has dendrites which branch infrequently at narrow angles.

Two more recent accounts of Golgi studies describe in more detail the morphological characteristics of neurons within CM and Pf in the lesser bushbaby (Pearson et al. 1984) and the monkey (Hazlett et al. 1976). The majority of cells (up to 90%) within CM are principal neurons which are round to elongate in shape with 3-5 primary dendrites. They possess short somatic and dendritic spines. Their axons arise from either the cell soma or proximal primary dendrites and have few recurrent axon collaterals. In addition to the principal neurons, a smaller Golgi Type II neuron with a round cell soma, 3-4 small diameter spiny dendrites, and somatic spines has been identified. Their axons arise from cell somata and have prominent collaterals arising at right angles to the main axon. In Pf, two types of large radiate principal neurons exist, those with and those without

somatic and dendritic spines. The axons arising from the cell somata and primary dendrites of these two cell types were only partially impregnated and differences in their axonal projection patterns were not detectable. In the monkey , a Golgi Type II neuron was also identified in Pf and appeared to be identical in morphology to that seen in CM (Hazlett et al. 1976). Interestingly, this Golgi type II neuron has not been observed in the lesser bushbaby (Pearson et al. 1984).

ULTRASTRUCTURE

In an ultrastuctural study involving CM, Harding (1973a) described four types of synaptic profiles within this nucleus. The most numerous profile (SR) was small (1.5 um diameter) with spherical vesicles and was observed to be pre-synaptic to dendritic profiles with single asymmetric contacts. The second type also contained spherical vesicles but was larger in size and formed numerous asymmetrical synapses with dendritic and P profiles (described below) within glomeruli. A third, rarer profile (F axon terminal) contained pleomorphic vesicles, formed 1-2 um expansions which have symmetric synaptic contacts with dendritic and P profiles. The F profile also was found to be presynaptic to proximal dendrites and cell somata. The P profile was found within glomeruli, was larger than the F profile and believed to be dendritic in origin. Though the LR bouton

has been found to degenerate following destruction of contralateral deep cerebellar nuclei (Harding 1973b), neither the F nor the P profile were affected by cortical, pallidal, or cerebellar lesions.

Westman and Bowsher (1971) gave only a brief description of boutons within CM. They observed few terminal boutons contacting the cell soma and reported a dense network of non-myelinated fibers surrounding the cell somata located within CM.

EFFERENT PROJECTIONS

The efferent projections of CM-Pf have been the subject of a number of different investigations using degeneration, autoradiographic and retrograde tracing techniques (Powell and Cowan 1956;1967; Mehler 1966; Nauta et al. 1974; Jones and Leavitt 1974; Herkenham and Pert 1981). These investigations conclude that CM-Pf projects heavily to the neostriatum and diffusely to the most superficial part of layer I in widespread areas of the frontal, medial, and dorsolateral cortex. In a more recent study examining cortical projections from CM-Pf in the cat, it was found that, while projections to layer I are most extensive, layer III also contains a substantial number of terminations (Royce and Mourey 1985). This same study has shown a topographical organization of CM-Pf-cortical projections. The rostral CM-Pf projects to the lateral part of the

rostral cortex, including the presylvian and anterior ectosylvian gyrus, as well as the rostral medial cortex, primarily in the cruciate and anterior cingulate gyrus. The caudal and dorsal parts of CM-Pf send projections to more ventral portions of the lateral rostral cortex, including the anterior sylvian gyrus, as well as the rostral medial cortex, including the area surrounding the cruciate sulcus and the area ventral to the cruciate sulcus. Injections into the caudal and ventral parts of CM-Pf resulted in no observed label in any cortical region. In an additional study, Pearson and his colleagues (1984) found in the lesser bushbaby that neurons in CM but not Pf project to the somatosensory cortex. In his studies of collateralization of CM-Pf neurons, Royce (1983a) occasionally encountered thalamocortical neurons in Pf which had collaterals to the neostriatum.

Recent investigations indicate that intralaminar thalamostriate projections are also organized topographically. The CM-Pf complex projects most heavily to the mid- and caudal levels of the head of the caudate while the rostral intralaminar nuclei (paracentral and central lateral nuclei) project primarily to the rostral part of the caudate (Van der Kooy 1979; Jayaraman 1985; Beckstead 1984; Sato et al. 1979). There are also some data to indicate that projections from CM-Pf may be more

discrete than those arising from the more rostral intralaminar nuclei. Retrograde double labeling studies using fluorescent dyes in both the cat and rat reveal that thalamostriate and thalamocortical projections from CM-Pf arise primarily from separate populations of neurons (Royce 1983a; Macchi et al. 1984), while electrophysiological studies suggest that neurons in the central lateral nucleus have axons branching to both the caudate nucleus and cortex (Jinnai and Matsuda 1981). Furthermore, in primates, CM projects only to the putamen and Pf only to the caudate, with no overlap between the two terminal fields, while neurons in the rostral intralaminar nuclei projecting to the caudate nucleus are intermingled with those projecting to the putamen (Parent et al. 1983).

The termination patterns of CM-Pf onto the neostriatum have been shown to be segregated into a complex mosaic of patches and bands in the rat (Herkenham and Pert 1981; Gerfen 1984), cat (Royce 1978b), dog (Isaacson and Tanaka 1984), and monkey (Kalil 1978). In the rat, these projections terminate in enkephalin- and substance P-positive areas and interdigitate with a matrix which is acetylcholinesterase- and somatostatin-positive (Gerfen 1984).

AFFERENT PROJECTIONS

A number of different areas have been shown to project to the CM-Pf complex. These areas include the pars reticulata of the substantia nigra, the superior colliculus, medial and lateral vestibular nuclei, periaqueductal grey, locus coeruleus, cerebellum, and pontomesencephalic tegmentum (McGuiness and Krauthamer 1980; Bowsher 1966; Comans and Snow 1981; Moon Edley and Graybiel 1983; Sugimoto and Hattori 1984). In addition, both the dorsal raphe nuclei and spinal cord project to Pf (Comans and Snow 1981) while the entopeduncular nucleus projects to CM (McGuiness and Krauthamer 1980; DeVito and Anderson 1982).

Certain regions of the neocortex have also been shown to project to CM-Pf. In the cat, Rinvik (1968a;1968b) reported that both CM and lateral Pf receive input from the frontal lobe. Kunzle (1978) found in primates that CM receives afferent input from cortical area 4 while Pf receives input from areas 6 and 9. Recently, in the cat, Royce (1983c) described even more widespread cortical projections to CM-Pf originating from the rostral four-fifths of the neocortex. Using a retrograde double labeling fluorescent technique, he also found cortical neurons with collateral projections to both CM-Pf and the caudate nucleus (Royce 1983b).

FUNCTIONAL DATA

There are some reports in the literature indicating that the CM-Pf complex has some functional association with pain pathways. Stimulation of CM-Pf alleviates pain and motor disorders in patients suffering from painful dyskinesia and dyskinesia alone (Andy 1970). Also, the parafascicular nucleus is responsive to noxious stimuli in the cat (Albe-Fessard and Kruger 1962) and rat (Peshanski et al. 1981) and receives serotonergic projections from the dorsal raphe nuclei (Anderson and Dafny 1983).

Electrophysiological studies have suggested that the CM-Pf complex participates in the cortical recruiting response, a phenomenon believed to be responsible for arousal and awakedness. The diffuse, widespread cortical projections of CM-Pf and other intralaminar nuclei form the anatomical basis for this phenomenon. Royce and Mourey (1985) suggest that their findings lend support for this notion since the distribution of CM-Pf input to the pericruciate area and to the anterior cingulate region is very similar to the map of active recruiting responses as reported by Starzl and Magoun (1951). Their findings that cortical input from CM-Pf terminates primarily in layer I also lends support for this theory since the negative waves associated with the recruiting repsponse are present in the most superficial layers of

the cortex (Sasaki et al. 1970). While CM-Pf appears to be associated with this reponse, other thalamic nuclei, such as the ventromedial nucleus (Herkenham 1979), have also been implicated as being associated with the recruiting response.

Other electrophysiological studies have attempted to characterize the neurons in CM-Pf. These cells have been shown to respond to visual and somatosensory stimuli with a short burst discharge followed by a long post-excitatory inhibition (Dalsass and Krauthamer 1981a). It has been suggested that the substantia nigra "funnels" information from the caudate nucleus to the polysensory neurons located within CM-Pf (Dalsass and Krauthamer 1981b).

CM-PF AND THE PONTOMESENCEPHALIC TEGMENTUM

The CM-Pf complex receives input from the pontomesencephalic tegmentum, primarily from the nucleus tegmenti pedunculopontinus (PPN) (Jackson and Crossman 1983; Moon Edley and Graybiel 1983). This nucleus lies in the caudal mesencephalic tegmentum, beginning at a level just caudal to the red nucleus and extending caudally to the parabrachial nucleus. It has reciprocal connections with the substantia nigra, subthalamus, and pallidal complex (Saper and Loewy 1982) and is frequently regarded as an integral part of the basal ganglia system (Jackson and Crossman 1983). It has been suggested that

PPN provides a "functional link" between the basal ganglia and the lower motor system and that it could influence somatic motor activity either in conjunction with or independent of the known thalamo-corticofugal circuitry (Jackson and Crossman 1983).

Cholinergic neurons have been localized within PPN and adjacent brainstem areas such as the central tegmental tract, nucleus cuneiformis, lateral lemniscus, medial longitudinal fasciculus, and laterodorsal tegmental nucleus (Armstrong et al. 1983; Houser et al.1984). On the basis of their projection patterns and location, these regions containing cholinergic neurons have been included within cholinergic sectors Ch5 and Ch6 in the rat and monkey (Mesulam et al. 1983a; Mesulam et al. 1984). Cholinergic cells in these two groups are considered to give rise to projections to the lateral (Mesulam et al.1983a) and medial (Sofroniew et al. 1985) parts of the thalamus. Cholinergic projections from PPN to the CM-Pf complex have been demonstrated in the rat using retrograde transport of ^{3}H-choline (Sugimoto and Hattori 1984). This finding, suggesting that the PPN--CM-Pf projection may be cholinergic, has yet to be confirmed using the more specific immunocytochemical technique.

RATIONALE AND SPECIFIC AIMS

The PPN-thalamic projection is particularly important in that PPN is regarded as part of the brainstem locomotor region (Garcia-Rill et al. 1983a; 1983b; Skinner et al. 1985). Cholinergic input from PPN to thalamic areas which in turn give rise to neostriatal projections may be of special significance in the mechanisms of movement. In addition, since it is known that PPN receives input from the globus pallidus, a major source of output of the basal ganglia, the investigation of cholinergic terminations onto thalamostriate neurons would provide a link in the circuitry involved with basal ganglia connectivity. Although Sugimoto and Hattori (1984) have demonstrated that ^{3}H-choline is transported retrogradely from CM-Pf to PPN, the uptake and transport of ^{3}H-choline by presumed cholinergic neurons has not been proven to be transmitter-specific. A more reliable and thorough study of cholinergic projections from the brainstem cholinergic cell groups (Ch5-6) to the CM-Pf complex is needed. In addition, none of the studies described above has directly demonstrated whether cholinergic terminals establish synaptic contact with thalamostriate neurons. Finally, some cholinergic systems of the brain have proven clinically relevant. For example, it has been suggested that there exists a selective degeneration of cholinergic neurons in the

nucleus basalis in patients with Alzheimer's disease. In light of the above, I thought it appropriate to initiate a study of the the pontomesencephalic-thalamo-striate pathway in the dog with special emphasis on its topography and cholinergic component. The specific aims of this investigation were:

1) to examine the CM-Pf thalamostriate connectivity in detail to provide information concerning the topographical organization of this projection from CM-Pf to the caudate nucleus.

2) to investigate the organization of the cholinergic projection from the Ch5 (nucleus tegmenti pedunculopontinus) and Ch6 (laterodorsal tegmental nucleus) midbrain groups to CM-Pf at the light microscopic level.

3) to study at the ultrastructural level the cholinergic termination pattern onto identified CM-Pf thalamostriate projection neurons.

Each of the specific aims is dealt with in a separate section. Each section contains its own introduction, materials and methods, results, and discussion directed at that specific aim. Following these sections is a general discussion and a list of appendices.

CHAPTER ONE

ORGANIZATION OF CM-Pf THALAMOSTRIATE PROJECTIONS IN THE DOG

INTRODUCTION

Powell and Cowan (1954; 1967) first demonstrated the thalamostriate projection from the intralaminar nuclei in the monkey using retrograde cell degeneration techniques. Since these early studies, this pathway has been confirmed using modern anterograde and retrograde tracing techniques (Jones and Leavitt 1974; Kuypers et al. 1974; Nauta et al. 1974). These reports have demonstrated a dense neostriatal projection from the intralaminar thalamus which possesses a precise topographical organization in the rat (Veening et al. 1980; Sato et al. 1979), cat (Beckstead 1984; Royce 1978a), monkey (Kalil 1978) and opossum (Hazlett and Bagley 1985). Since previous work in our laboratories has involved cortical afferents to the neostriatum in the dog (Tanaka et al. 1979; Tanaka and Isaacson 1985; Tanaka 1983), we extended our investigation to include thalamic afferents as well. The present study is an assessment of the topographical organization of the thalamostriatal projection from the two caudal intralaminar nuclei, centrum medianum (CM) and parafascicular nucleus (Pf), in the dog. A detailed analysis of the thalamostriatal

projection from the ventral anterior and ventral lateral nuclei in the dog has been reported (Tanaka et al. 1986). Portions of the present study have been presented previously in abstract form (Isaacson and Tanaka 1984).

MATERIALS AND METHODS

Seven mixed breed dogs weighing from 7-10 kg were used in this study. Intravenous injections of sodium pentobarbitol were administered prior to surgery and supplemented during surgery up to a maximal dose of 35-40 mg/kg. Pressure injections of 0.2-0.3 ul of 2.5% lectin-conjugated horseradish peroxidase (WGA-HRP) were stereotaxically placed within various regions of the head and body of the caudate nucleus. One animal received multiple WGA-HRP injections throughout the rostral-caudal extent of the caudate. In the remaining 6 animals, 2 received bilateral WGA-HRP injections, while 4 received unilateral WGA-HRP injections within various regions of the head of the caudate. The injections were administered over a time period of 20-30 minutes. After a two day survival, animals were reanesthetized and perfused transcardially with 1.5% paraformaldehyle/ 1.0% glutaraldehyde solution in 0.1M phosphate buffer followed by a 10% sucrose-buffer solution. The brains were blocked in the coronal plane and placed in sucrose buffer for two days at $4^{o}C$, frozen, and cut at 35um . The sections were then processed for WGA-HRP histochemistry

Figure 1. Low power photomicrographs of coronal sections through the canine thalamus processed for silver-Nissl staining. Note the rostro-caudal change in shape of the CM-Pf complex. In more rostral sections CM is convex in shape. At the level of the HPT, it becomes slightly triangular. Caudal to this level it is somewhat winged in shape (shown in B).

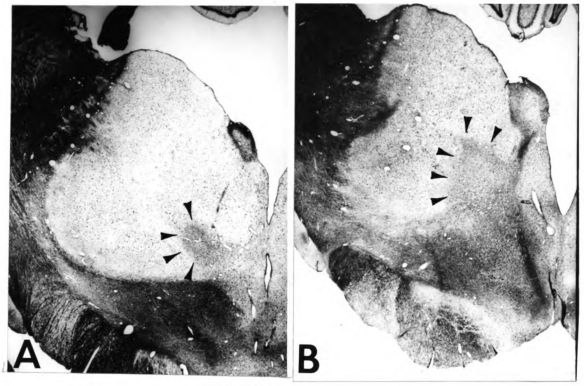

Figure 1

using either the TMB reaction of Mesulam (1978) or glucose oxidase reaction of Itoh et al. (1979). Sections were mounted on gelatinized slides and counterstained with either neutral red or cresyl violet. Labeled cells were mapped using a Zeiss drawing tube. Tracings of thalamic sections were made with the aid of an Aus Jena projection device and mapped cells were transferred to these tracings. A corresponding series of sections was stained using the silver-Nissl staining protocol.

RESULTS

Delineation of CM and Pf

As a guide for defining nuclear boundaries of CM and Pf, the Berman and Jones (1982) atlas of the feline telecephalon was used. As an additional aid for the canine brain, the corresponding series of silver-Nissl stained sections was used in which the internal medullary medulla is differentially stained brown, indicating the location of the intralaminar nuclei.

In sections rostral to the habenular-peduncular tract (HPT) CM is apparent as a convex disc inferior to the mediodorsal nucleus (MD) (Figs.1A, 3, 6). At the level of the HPT, CM becomes almost triangular in shape with its base situated on the tract (Figs. 1B, 3, 6). Just caudal to this level, the nucleus becomes somewhat wing-shaped (Figs. 3, 6). At the level of the posterior

commissure, the cells of CM form a thin wedge which runs in parallel with the fibers of the posterior commissure (Figs. 3, 6).

While it is difficult to separate Pf from CM in silver-Nissl stained sections, it appears that Pf, just medial to CM, is present immediately adjacent to the HPT and is split by this fiber bundle into a medial and lateral segment. At the level of the posterior commissure, CM and Pf appear to merge to form the most caudal aspects of the CM-Pf complex.

HRP labeling

Injections into all levels of the caudate nucleus resulted in at least some retrograde labeling within CM and Pf as well as in the more rostral intralaminar nuclei, the midline nuclei, the substantia nigra, neocortex, and dorsal raphe. Those injections placed more rostrally within the caudate resulted in light label within CM-Pf and heavier in rostal intralaminar nuclei. Conversely, those sites placed in caudal parts of the head of the caudate gave rise to retrogradely labeled neurons primarily in the CM-Pf complex. Results described in this report will concentrate primarily on the organization of retrogradely labeled neurons observed in either CM or Pf.

In case D8401, 3 injections were placed within the rostral, middle, and caudal levels of the medial portion

Figure 2. Line drawings illustrating WGA-HRP injection sites in the caudate nucleus of A) D8406 and B) D8404. Injection site D8406 was centered in the dorsolateral part of the caudate while site D8404 was placed in the ventro-medial part of the caudate.

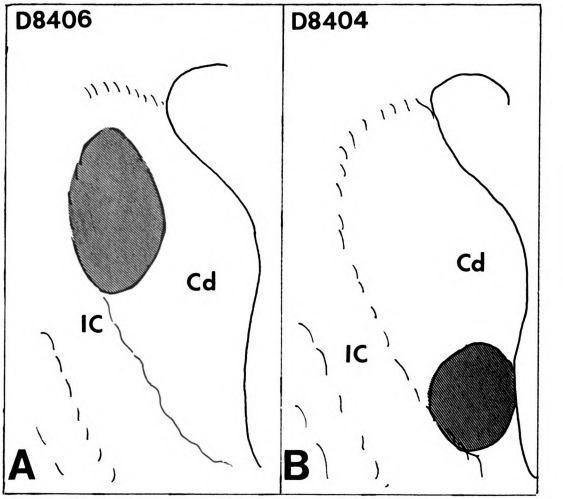

Figure 2

Figure 3. Photomicrographs of a rostro-caudal series of coronal sections through the CM-Pf complex showing retrogradely labeled neurons resulting from the WGA-HRP injection into the dorsolateral caudate (D8406). Note the extensive label in rostro-caudal aspects of CM with little label in Pf.

Abbreviations:
CM, centrum medianum. HPT, habenular-peduncular tract. Pf, parafascicular nucleus.

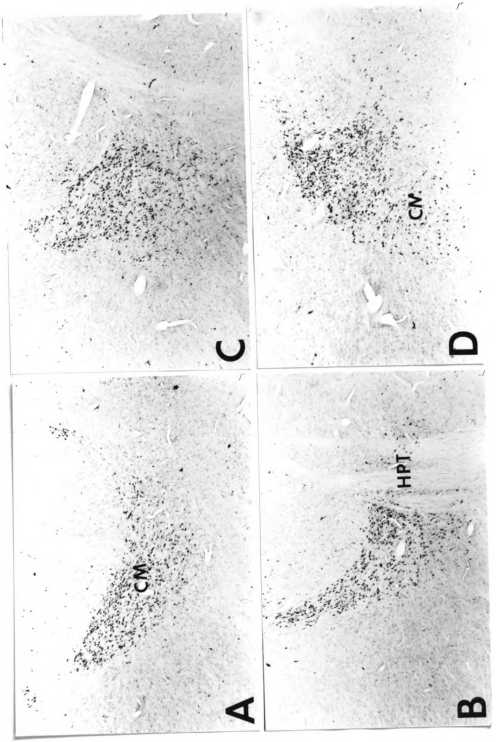

Figure 3

Figure 4. Photomicrographs of a rostro-caudal series of coronal sections through the CM-Pf complex showing retrogradely labeled neurons resulting from the WGA-HRP injection into the ventro-medial caudate (D8404). Note the labeled cells located in Pf and the absence of label in CM. See Fig. 3 for list of abbreviations.

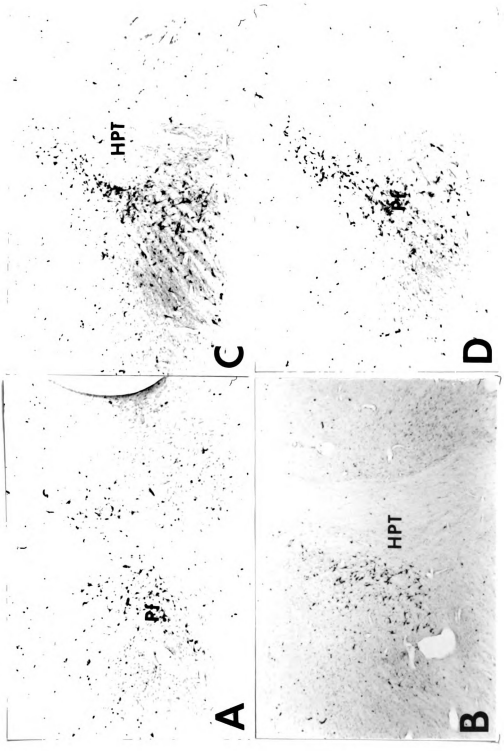

Figure 4

of the head of the caudate nucleus. While these injections filled the head of the caudate nucleus, there was little spread into adjacent structures such as the internal capsule and corpus callosum. These injections resulted in retrograde labeling of the majority of cells located within CM and Pf as well as the rostral intralaminar nuclei. This large injection was helpful in determining the size and extent of the CM-Pf complex in the dog. The boundaries based on thalamostriate connectivity correspond well with the cytoarchitectural determination of nuclear boundaries based on observations of the silver-Nissl stained sections.

In case D8404, the injection site was centered slightly medial and ventral to the dorsolateral corner of the head of the caudate (Fig. 2A). Large numbers of retrogradely labeled neurons were located within CM throughout its rostral-caudal extent (Fig.3). Few labeled cells were observed within Pf.

In case D8406, the injection was confined to the ventral-medial portion of the caudate (Fig. 2B). The retrogradely labeled cells were located primarily in Pf with very few labeled cells within CM (Fig. 4).

In case D8408, bilateral injections were placed in the caudate nucleus at the level of the anterior commissure (Fig. 5). On the right side, a small injection was placed in the dorsolateral corner of the

Figure 5. Diagrams of cross sections through the CM-Pf complex following a bilateral injection of WGA-HRP (D8408) into the caudate nucleus. On the right side, labeled neurons were located in CM and CL with no label in Pf. On the left side, labeled cells were located in Pf as well as in CM.

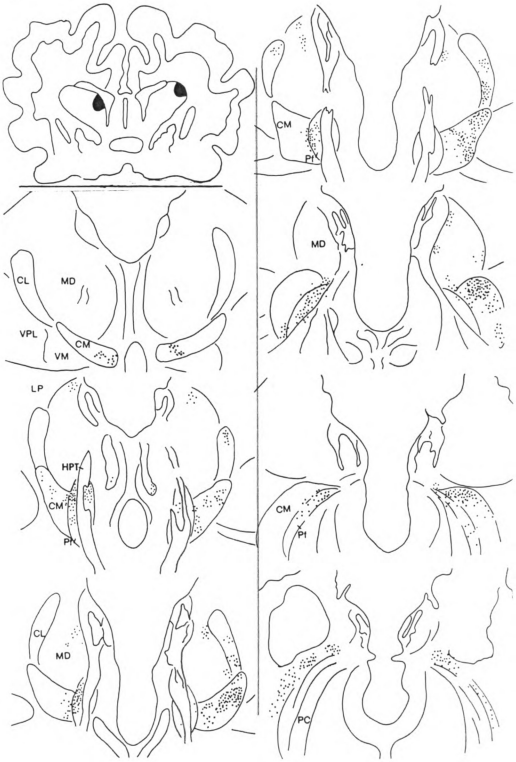

Figure 5.

Figure 6. Diagrams of cross sections through the CM-Pf complex following a WGA-HRP injection (D8504) which filled the dorsomedial part of the caudate. Note the heavy label in both CM and Pf throughout its rostro-caudal extent.

Figure 7. Schematic summary diagram illustrating the convergence of input to the caudate nucleus from A) cortical area 4 (MI) and CM, and B) the orbitofrontal cortex and Pf.

caudate with no spread into any medial or lateral portions of the nucleus. Retrogradely labeled cells were observed within the rostral and mid-portions of CM. Few labeled cells were observed in Pf at any level. On the left side, the injection was confined to the mid-medial part of the caudate. Retrogradely labeled neurons were found primarily in Pf though some labeled cells were identified in the most rostral portions of CM (Fig. 5).

In case D8504, the injection was placed in the dorsomedial portion of the caudate (Fig. 6) with spread into the dorsolateral corner. This injection labeled the entire rostro-caudal extent of CM as well as cells located within Pf.

DISCUSSION

The CM-Pf complex of the dog is similar to that described in the cat (Berman and Jones 1982). However, it appears that the rostral portion of Pf is not as prominent in the dog as in other carnivores. Berman and Jones (1982) as well as Beckstead (1984) describe a group of darkly staining cells lying just ventral to MD before the HPT becomes apparent. Our studies of silver-Nissl stained sections and our connectivity data in the dog do not correspond with this delineation. Also, our observations of the delineations of the canine CM-Pf complex are not in total agreement with some of the

nuclear boundaries depicted by Beckstead (1984) and Royce and Mourey (1985). The CM-Pf complex in the dog does not extend as far dorsally in the caudal aspects of the nucleus as shown in the cat by these authors (See Beckstead 1984, Figs. 12B, 12D; and Royce and Mourey 1985, Fig. 1B). In our own material in the dog, this area comprises the most caudal aspects of the mediodorsal nucleus of the thalamus.

The results of the present study indicate that, in the dog, the CM-Pf complex projects to the caudate nucleus in an organized topographical pattern. The dorsolateral head of the caudate receives input exclusively from CM, though neurons in CM also project to the dorsomedial part of the caudate as well. In contrast, Pf projects to the medial part of the caudate nucleus. A dorsoventral topography exists within Pf in which cells within Pf located within more dorsal portions of Pf project to the dorsomedial caudate, while more ventral located cells project to the ventromedial caudate (not shown).

The topography described here is consistent with previous studies in other mammalian species. A distinct rostro-caudal and medial-lateral topographical organization of thalamostriate neurons has been observed in the rat (Royce 1978a; VanDerKooy 1979), cat (Beckstead 1984; Sato et al. 1979), primate (Kalil 1978) and opossum (Hazlett and Bagley 1985). Our present findings

extend these results to the canine species.

In addition to retrogradely labeled cells in CM, it has been observed that WGA-HRP injections into the dorsolateral head of the caudate nucleus resulted in retrogradely labeled neurons throughout the medial-lateral extent of the primary motor cortex (MI) (Isaacson and Tanaka 1984). Also, following injections into the medial portion of the caudate nucleus, retrogradely labeled neurons were observed as wide bands of labeled neurons located primarily in layer III of the orbitofrontal cortex (OF) (Isaacson and Tanaka 1984). Thus, both MI and CM project to the dorsolateral caudate, while OF and Pf project to the more medial parts of the nucleus. In primates, both the prefrontal cortex and Pf projects primarily to the caudate nucleus while MI and CM project to the putaman (Parent et al. 1983). A similar convergence of input has been described concerning inputs to the caudate nucleus from both the substantia nigra and orbitofrontal cortex (Beckstead 1979). Results from all of these studies suggest that overlapping patterns of afferents from anatomically related cortical, thalamic, and nigral regions may demarcate distinct anatomical and functional regions of the caudate nucleus (See Fig. 7), an idea first introduced by Jones and Leavitt (1974).

In contrast to a recent study (Hazlett and Bagley 1985) reporting several species differences in the

opossum, the canine species appears to possess an organization of thalamostriate projections similar to other non-primate species examined. The findings of the present study reveal that the CM-Pf complex is similar to the cat in morphology except for slight differences in rostral Pf and caudal portions of CM-Pf. Thus, the examination of the thalamostriate projection in the dog has provided a basis for further studies in this species involving the CM-Pf complex and its role in basal ganglia connectivity.

CHAPTER TWO

CHOLINERGIC AND NON-CHOLINERGIC PROJECTIONS FROM THE CANINE PONTOMESENCEPHALIC TEGMENTUM TO THE CM-Pf COMPLEX

INTRODUCTION

On the basis of their projection patterns and locations in the pontomesencephalic tegmentum, several different nuclei containing cholinergic neurons have been included within areas Ch5 and Ch6 in the rat and monkey (Mesulam et al. 1983b;Mesulam et al. 1984). Area Ch5 encompasses the nucleus tegmenti pedunculopontinus (PPN) and the nucleus cuneiformis, as well as neurons located within the central tegmental tract, lateral lemniscus and medial longitudinal fasciculus. Area Ch6 includes only the laterodorsal tegmental nucleus. Several investigators, using autoradiographic and horseradish peroxidase techniques, have shown that some of the nuclei within the Ch5 group, particularly PPN, project to the thalamus (Jackson and Crossman 1983; Moon Edley and Graybiel 1983; Mesulam et al. 1983; Saper and Loewy 1982). Neurons in PPN have also been retrogradely labeled following injections of ^{3}H-choline into the centrum medianum-parafascicular complex (CM-Pf) in the rat (Sugimoto and Hattori 1984). However, this finding,

suggesting that the PPN-thalamic projection may be cholinergic, has yet to be confirmed using the more specific immunocytochemical technique.

Although projections to the thalamus from PPN have been identified autoradiographically in the cat (Moon Edley and Graybiel 1983), there have been no reports identifying the neurotransmitter associated with this projection in any carnivore species. Since our previous work has been centered around an investigation of the neostriatum and its cortical and thalamic afferents in the dog (Tanaka et al. 1979; Tanaka et al. 1981; Tanaka 1983; Isaacson and Tanaka 1984; Tanaka and Isaacson 1985; Tanaka et al. 1986) and since PPN, now believed to be an integral part of the basal ganglia system, has been shown to project to the caudal intralaminar thalamus (Jackson and Crossman 1983; Moon Edley and Graybiel 1983; Sugimoto and Hattori 1984), we thought it appropriate to extend our studies to include PPN and the adjacent nuclei making up the Ch5 group. Using a technique similar to that of Wainer and Rye (1984) in which choline acetyltransferase (ChAT) immunocytochemistry is combined with horseradish peroxidase retrograde labeling, we chose to investigate the organization and morphology of both cholinergic and non-cholinergic neurons projecting to the caudal thalamus from the Ch5 area in the dog. The results of this study have been presented previously in abstract form (Isaacson and Tanaka 1985a).

MATERIALS AND METHODS

Five mongrel dogs weighing from 7-10 kg were used in this study. Intravenous injections of sodium pentobarbital (15-20 mg/kg) were administered prior to surgery and supplemented during surgery up to a maximal total dose of 35-40 mg/kg. Unilateral pressure injections of 0.2-0.3 ul of 2.5% wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) were stereotaxically placed within the CM-Pf complex of the thalamus. After a two day survival, animals were reanesthetized and perfused with 8 l of 2% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) followed by 7 l of 10% sucrose in the same buffer solution. Brains were removed and immediately placed in 30% sucrose in 0.1 M phosphate buffer for 24-36 h at 4°C. Three series of 40 um sections were cut on a freezing microtome. The first series was for WGA-HRP using the glucose oxidase reaction with colbalt chloride pretreatment (Itoh et al. 1979). Subsequent processing for choline acetyltransferase (ChAT) immunocytochemistry using affinity-purified monoclonal rat anti-ChAT (1:200; Immunonuclear, Inc.) was performed at 4°C for 24 hr. After three rinses in phosphate-buffered saline (PBS) with 2% normal rabbit serum, the sections were treated with biotinylated rabbit

anti-rat IgG for 1 hr. Following another series of rinses, tissue sections were treated with the Avidin-Biotin Complex for 1 hr at room temperature. After rinsing in phosphate buffer without saline, sections were processed for localization of ChAT immunoreactivity using the glucose oxidase reaction without cobalt chloride for approximately 30 min at room temperature. As a control, the primary antibody was replaced with normal rat serum in selected adjacent sections. The second series of sections was processed for HRP histochemistry only while the third series was stained for Nissl substance.

Sections were mounted on gelatinized slides and labeled cells mapped at 100X magnification using a Zeiss drawing tube. Cells exhibiting black punctate granules were considered to be projection neurons to the CM-Pf complex (Figs. 11A, 11C, 11D). ChAT-positive neurons exhibiting brown granular precipitate throughout the cytoplasm were assumed to be cholinergic (Fig. 11A). Double-labeled neurons, therefore, were characterized by both a brown granular cytoplasm and black punctate granules (Figs. 11D and 11D). Cell soma areas, calculated using a Bioquant Image Analysis System, were used to divide arbitrarily the single and double labeled neurons into three groups -- small (less than 300 um^2), medium (300-600 um^2), and large (greater than 600 um^2). Only neurons which demonstrated a nucleus and prominent nucleolus were included in cell soma area calculations.

RESULTS

Cholinergic neurons

The Ch5 area in the dog extends rostrocaudally from a level just caudal and dorsolateral to the substantia nigra to the level of the parabrachial nucleus. This area is most extensive between the levels of the trochlear and parabrachial nuclei and includes cholinergic neurons located primarily within the nucleus tegmenti pedunculopontinus (PPN) and the central tegmental tract (ctt). Area Ch5 cholinergic neurons were observed less frequently in the nucleus cuneiformis and among the fibers of the medial longitudinal fasciculus and the lateral lemniscus (Fig. 8).

In the dog, we observed two groups of cholinergic neurons which were associated with the brachium conjunctivum (BC) and which we defined as PPN. The first group was located medially and was composed of diffusely scattered cells intermingling with the fibers of the wing of the BC from the level of its decussation to just rostral to the parabrachial nucleus. A second, more compact, group of cholinergic cells was located dorsolateral to the BC at these same levels (Fig. 8). This compact group was most prominent at its more rostral extent (Figs. 8A, 9A and B, 10A and B). At the level of the trochlear nucleus, it consisted of a small, well-

Figure 8. Low power photomicrographs illustrating the distribution of cholinergic neurons in the Ch5 sector of the dog. Cholinergic neurons were located primarily in the central tegmental tract and in the medial diffuse and dorsolateral portions of PPN.Scale bar = 1 mm.

A. In this section, which is just caudal to the trochlear nucleus, the cholinergic cells in the medial diffuse portion of PPN are intermingled with the brachium conjunctivum (BC) (open arrows). The compact portion of PPN is obvious at this level (arrowheads). Compare this section to Figures 9B and 10B.

B. This section is located at a more caudal level. The diffuse portion of PPN is still prominent while the dorsolateral compact component is not as obvious. Compare this section to Figures 9D and 10D.

Abbreviations:
AQ, cerebral aqueduct. ctt, central tegmental tract. Cu, nucleus cuneiformis. DBC, decussation of the brachium conjunctivum. IC, inferior colliculus. LL, lateral lemniscus. MLF, medial longitudinal fasciculus. PAG, peraqueductal grey. PPNc, nucleus tegmenti pedunculopontinus-compact compact component. PPNd, nucleus tegmenti pedunculopontinus-diffuse component.

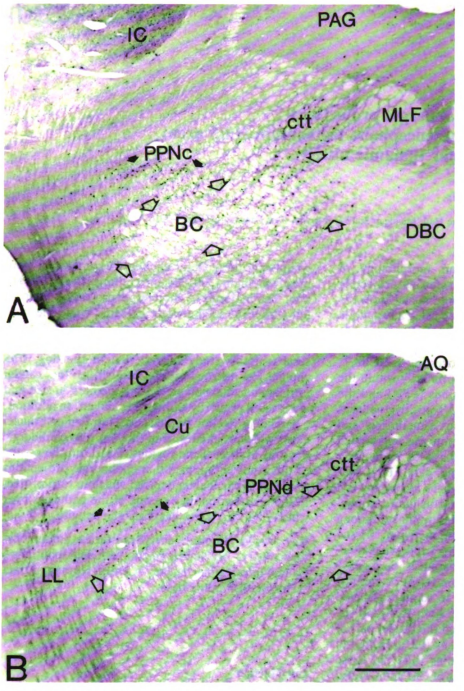

Figure 8

Figure 9. Line drawings of cross sections through the pontomesencephalic tegmentum depicting retrogradely labeled and ChAT-positive neurons following WGA-HRP injections into CM and CL and subsequent processing for ChAT immunoreactivity. Double labeled cells, i.e. cholinergic projection neurons (stars), were numerous in both the medial diffuse and dorsolateral compact components of PPN and in the ctt. Non-cholinergic projection neurons (triangles) were scattered among the cholinergic neurons (dots) comprising the Ch5 sector. In drawing D, note the presence of double labeled cells within the rostral portion of the Ch6 group. Each symbol represents one neuron.

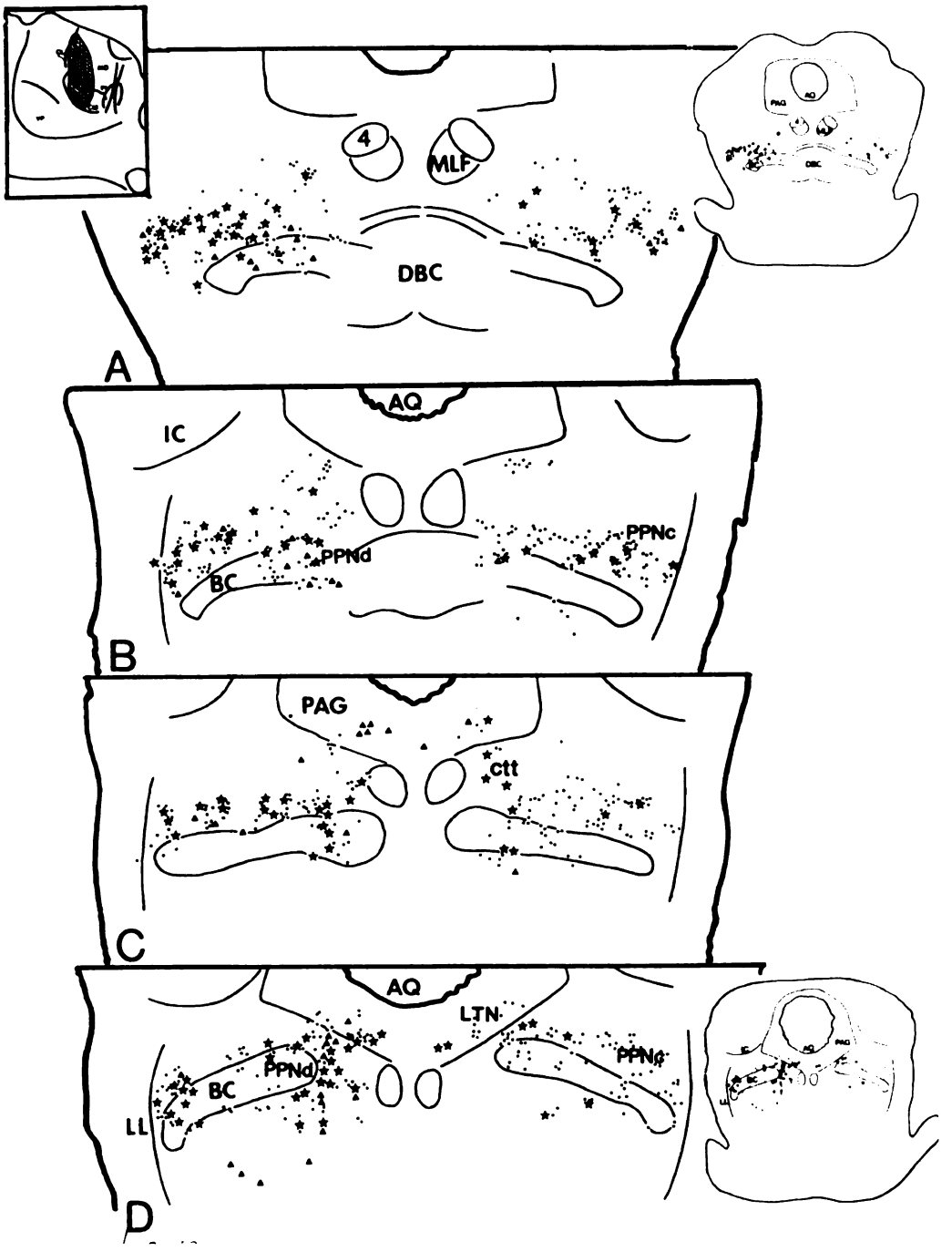

Figure 9.

Figure 10. Line drawings of cross sections through the pontomesencephalic tegmentum depicting retrogradely labeled and ChAT-positive neurons following WGA-HRP injections into Pf and subsequent processing for ChAT immunoreactivity. Double labeled cells, i.e. cholinergic projection neurons (stars), were most numerous in the medial diffuse component of PPN and in the ctt. Non-cholinergic projection neurons (triangles) were scattered among the cholinergic neurons (dots) comprising the Ch5 sector. Note the presence of fewer numbers of double labeled cells within the compact portion of PPN. Each symbol represents one neuron.

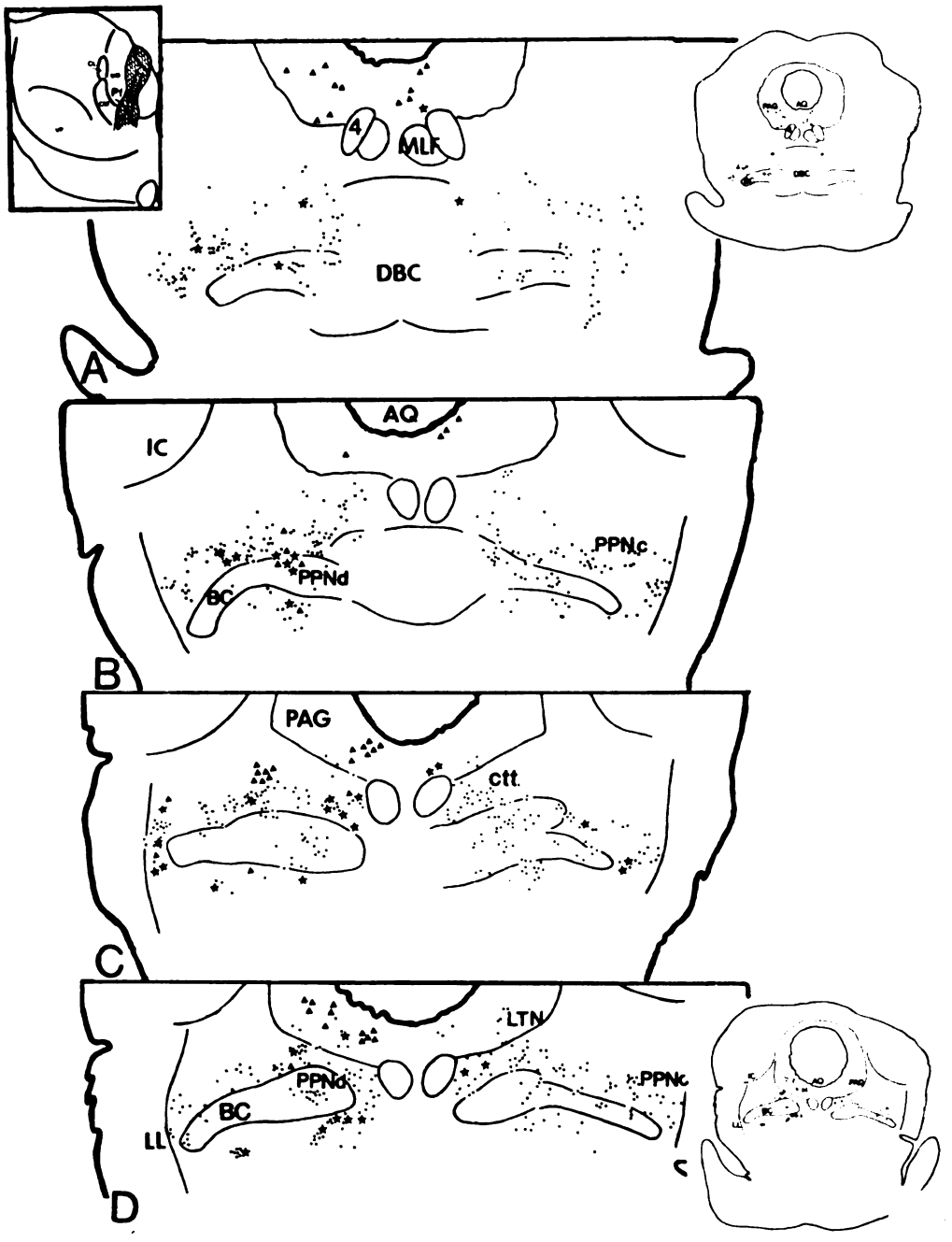

Figure 10.

Figure 11. High power photomicrographs illustrating examples of single and double labeled neurons located in the Ch5 sector.

A. Cholinergic neuron located in the medial diffuse portion of PPN.

B. Non-cholinergic projection neuron (arrowheads) adjacent to a lightly labeled cholinergic projection neuron (open arrows). Note the ventrally projecting primary dendrite of the non-cholinergic projection neuron.

C.and D. Two cholinergic projection neurons. The cell in D is similar in morphology to the non-cholinergic projection neuron pictured in B. Scale bar = 20 um.

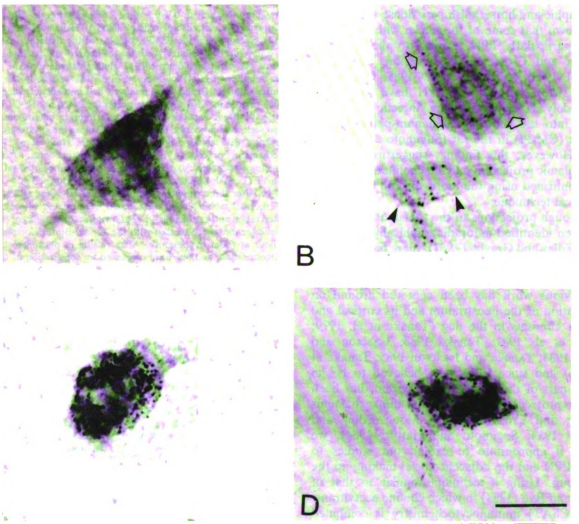

Figure 11

defined cluster of cholinergic neurons directly apposed to the fibers of the lateral lemniscus. At more caudal levels, the cells comprising this compact group were more scattered, and the group appeared less distinct.

At the level of the inferior colliculus, cholinergic cells were observed within the laterodorsal tegmental nucleus (Ch6) which is located in the ventrolateral part of the periaqueductal grey. Although the cell soma areas of these neurons were not measured, they appeared to be smaller than those in Ch5.

Injection sites

In two cases (D8417 and D8428), unilateral injections of WGA-HRP were placed into CM or Pf. In case D8428 the injection site involved primarily CM and the caudal part of the central lateral nucleus (CL) (Fig. 9), while in case D8417, the injection site was located more medially and involved Pf, the fasciculus retroflexus, and the habenular nuclei (Fig. 10). Neither of these injection sites extended beyond the rostro-caudal borders of CM or Pf. However, in both cases there was slight spread of label into the medial (D8417) and lateral (D8428) parts of the mediodorsal nucleus (MD). In a third case (D8415) injections were placed bilaterally with reaction product confined to CM on the left side and Pf on the right side.

In the two remaining cases, injections were centered

in adjacent thalamic nuclei but involved spread into either CM or Pf. The first was centered in MD (D8419), while the second was placed in the lateral border of CM and included a portion of the ventral posterior nucleus (VP) (D8420).

Cholinergic and non-cholinergic projection neurons

Results will be reported in detail from the two cases in which injections were placed unilaterally within CM (D8428) or Pf (D8417) as these cases, along with bilateral case (D8415), yielded the greatest number of double labeled cells in the Ch5 group. Injections which were centered within nuclei adjacent to CM or Pf (MD or VP) resulted in fewer numbers of double labeled cells in the Ch5 group.

Following WGA-HRP injections into either CM or Pf, double labeled cholinergic projection neurons (Fig. 11) were observed in both the medial diffuse and dorsolateral compact cell groups of PPN as well as in the central tegmental tract. Although both PPN cell groups contained retrogradely labeled cholinergic cells, the injections into CM and the caudal part of CL (Fig. 9) resulted in more double labeled cells in the compact PPN cell group than did injections into Pf (Fig. 10). Also, injections into CM and CL resulted in larger numbers of retrogradely labeled neurons when compared to the results seen after Pf injections. A few cholinergic projection

neurons were also seen adjacent to or within the medial longitudinal fasciculus and lateral lemniscus as well as within the rostral part of the Ch6 group (Figs. 9D and 10D). The majority of retrogradely labeled Ch5 neurons were located ipsilateral to the injection site. However, a number of double labeled neurons were also located in the contralateral Ch5 area.

While the majority of retrogradely labeled neurons within PPN and ctt were cholinergic (approximately 70%-80%), a substantial number of projection neurons to the caudal thalamus were not ChAT-positive. In some cases, such as those in which the injection involved CM and CL, these non-cholinergic projection neurons (Fig. 11B) comprised up to 27% of the total population of projection neurons. They did not appear to show any specific topographical organization and were scattered throughout the diffuse and compact components of PPN and ctt (Figs. 9 and 10). Though cholinergic projection neurons were observed both ipsilateral and contralateral to the injection site, non-cholinergic projection neurons were rarely observed on the side contralateral to the injection. Outside of the Ch5 sector, non-cholinergic projection neurons were located in the periaqueductal grey, the raphe nuclei and the nucleus pontis oralis.

Cell soma areas

Measurements of cell soma areas indicated that the cells comprising the general cholinergic population ranged in size from small (less than 300 um) to large (greater than 600)(Fig. 12). The majority of these neurons were medium in size (58%) with a substantial number of large neurons also present (39%). Only a very small percentage of cholinergic neurons (3%) were classified as small. The great majority of cholinergic projection neurons could be classified as medium in size (77%). Relatively few cholinergic projection neurons fell into the small (7%) or large (16%) cell categories. Most of the non-cholinergic projection neurons were small (49%) or medium (49%) in size (Fig. 12).

In terms of cross-sectional areas, non-cholinergic projection neurons were determined to be significantly smaller than cholinergic projection neurons (Mann-Whitney U Test, $p < 3 \times 10^{-5}$). In addition, measurements of cell soma areas of cholinergic projection neurons indicated that these cells tended to fall within the medium size range and were significantly smaller than those neurons which comprised the general cholinergic population (Mann-Whitney U Test, $p < 6 \times 10^{-4}$).

Figure 12. Bar histograms illustrating the frequency distribution of cell soma areas.
A. Cholinergic neurons: n=86, x=563, SD=158.
B. Cholinergic projection neurons: n=42, x=446, SD=114.
C. Non-cholinergic projection neurons: n=17, x=319, SD=92.

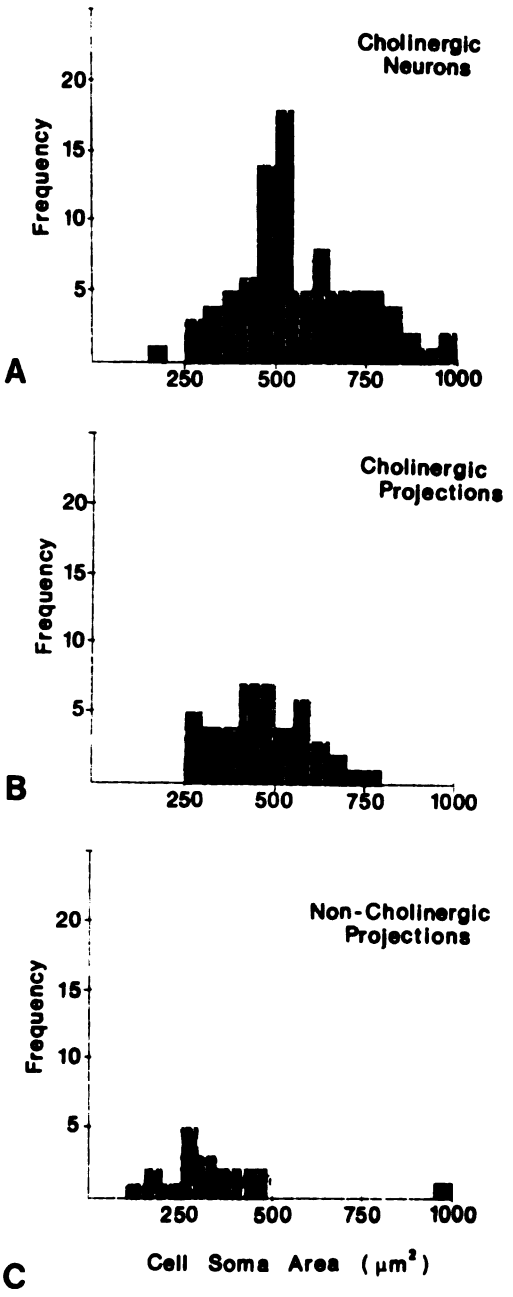
Cholinergic
Neurons
Frequency
20
15
10
5
250
500
750
1000
A
Cholinergic
Projections
Frequency
20
15
10
5
250
500
750
1000
B
Non-Cholinergic
Projections
Frequency
20
15
10
5
250
500
750
1000
Cell Soma Area (μm²)
C

Figure 12

DISCUSSION

Demarcation of PPN

Most of the cholinergic cells in the canine Ch5 group were located within the area defined as PPN. Although some authors do not differentiate PPN from the surrounding reticular formation (Berman 1968; Sofroniew et al. 1985), others have defined it cytoarchitectonically in man (Olszewski and Baxter 1954), monkey (Jackson and Crossman 1981), cat (Taber 1961) and rat (Sugimoto and Hattori 1984). The results of these studies have shown that PPN can be divided into two subnuclei -- a subnucleus compactus, composed of closely aggregated cells located dorsolateral to the brachium conjunctivum, and a subnucleus dissipatus, made up of cells intermingled with the fibers of the brachium conjunctivum. Other investigators have defined PPN on the basis of its connectivity. Thus, the subnucleus compactus has been defined according to the distribution of afferent input from basal ganglia regions in primates (Nauta and Mehler 1966) and cats (Nauta 1979; Moon Edley and Graybiel 1983). However, Jackson and Crossman (1983) found in the rat that projections from the basal ganglia were not confined to the cytoarchitectonically defined subnucleus compactus but extended into adjacent regions as well.

In the present study, we used two criteria to

demarcate the limits of the nucleus tegmenti pedunculopontinus: 1) the cholinergic nature of the cell bodies located in this region and 2) the association of these cholinergic cells with the brachium conjunctivum. Based on these criteria, we divided the cholinergic neurons located in PPN into two major groups -- a diffuse group of cholinergic cells located medially which interdigitates extensively with the BC at levels rostral to the parabrachial nucleus, and a more compact group of cholinergic cells located dorsolateral to the BC. Observations of alternate Nissl-stained sections indicate that these two groups can also be identified and differentiated cytoarchitectonically in the dog. The location and appearance of these two groups in the dog correspond with the cytoarchitectonic division of PPN in man by Olszewski and Baxter (1954).

Cholinergic and non-cholinergic projections

The results of the present study indicate that, in the dog, the caudal intralaminar nuclei receive cholinergic input from medium-sized neurons located in the central tegmental tract and in the medial diffuse and dorsolateral compact components of PPN. In addition, our findings demonstrate a parallel projection to the caudal intralaminar nuclei originating from smaller, non-cholinergic neurons in these same regions.

Sugimoto and Hattori (1984), observing that large

neurons were responsible for ^{3}H-choline uptake and transport following thalamic injections, proposed that cholinergic neurons projecting to the thalamus were mostly large in size. The results of the present study extend their findings in that we observed that both medium and large neurons make up the Ch5 cholinergic population and that, in the dog, cholinergic neurons projecting to the caudal thalamus are primarily medium in size.

The general pattern of retrograde labeling observed here is consistent with results obtained in previous retrograde and anterograde tracing studies. For example, HRP injections into Pf in the rat gave rise to retrogradely labeled neurons associated with the brachium conjunctivum (Jackson and Crossman 1983). In addition, injections of ^{3}H-amino acids placed in the PPN of the cat and rat yielded thalamic anterograde label primarily in CM, CL and Pf (Moon Edley and Graybiel 1983; Jackson and Crossman 1983). These latter results suggest that the sparse retrograde labeling observed in the Ch5 group of the dog after injections centered in nuclei adjacent to CM or Pf (eg. the ventral posterior nucleus or the mediodorsal nucleus) may have been due to spread of injected label into CM or Pf. However, it should also be noted that Moon Edley and Graybiel (1983) reported the presence of light anterograde label in MD after PPN injections. Thus, it may be that some of the retrograde

label observed in the present study resulted from uptake by Ch5 terminals within MD.

Ultrastructural autoradiographic studies using ^{3}H-amino acid injections into PPN in the rat reported labeled terminals in synaptic contact with identified thalamostriate projection neurons located in the CM-Pf complex (Sugimoto and Hattori 1984). Investigations in the dog thalamus have also revealed cholinergic terminals establishing synaptic contact with retrogradely labeled CM-Pf thalamostriate projection neurons (Isaacson and Tanaka 1985b; See Chapter 3). It seems likely that at least some of these cholinergic terminals may arise from cell bodies located in the Ch5 sector.

Many cholinergic neurons in the Ch5 sector of the dog were not retrogradely labeled as a result of injections into the caudal intralaminar nuclei. Other double labeling studies have shown that cholinergic neurons in this area may project to other areas in the central nervous system, including the more rostral thalamic nuclei (Sofroniew et al. 1985; Mesulam et al. 1983b),frontal cortex (Vincent et al. 1983; Mesulam et al. 1983b), caudal pontine and medullary reticular formation (Rye et al. 1984), hippocampus and olfactory bulb (Mesulam et al. 1983b). In addition, Sugimoto and Hattori (1984) observed specific transport of ^{3}H-choline by neurons located in PPN, subnucleus compactus,

following injections into the subthalamus nucleus of the rat. Additional studies using anterograde or retrograde tracing methods alone have demonstrated that neurons in this brainstem region, particularly those located within PPN, also project to the subthalamus (Rinvik et al 1979; Nomura et al. 1980; Moon Edley and Graybiel 1983; Saper and Loewy 1982; Jackson and Crossman 1983; Sugimoto and Hattori 1984), globus pallidus (Saper and Loewy 1982; Jackson and Crossman 1983), substantia nigra, pars compacta (Moon Edley and Graybiel 1983; Saper and Loewy 1982; Jackson and Crossman 1983; Gerfen et al. 1982), the entopeduncular nucleus (Moon Edley and Graybiel 1983; Jackson and Crossman 1983) and spinal cord (Jackson and Crossman 1983; Spann and Grofova 1984). It may be that some of these neurons are also cholinergic and form part of the non-retrogradely labeled cholinergic neuronal population observed in the present study.

The neurotransmitter content of the smaller non-cholinergic projection neurons is still in question. Vincent et al. (1983) provided evidence of co-existence of acetylcholine and the neuropeptide substance P in cells of the pontomesencephalic tegmentum in the rat. Using immunohistochemical methods, they found that cells in this area either were ChAT-positive only or were both ChAT-positive and substance P-positive, but never only substance P-positive. Thus, the question still remains as to the neurotransmitter content of the smaller, non-

cholinergic neurons projecting to the thalamus.

Recent studies indicate that PPN participates widely in extrapyramidal circuitry, establishing reciprocal connections with the neostriatum, subthalamus, substantia nigra, and entopeduncular nucleus (Nomura et al 1980; Jackson and Crossman 1983; Saper and Loewy 1982; Moon Edley and Graybiel 1983). It also appears that this area is part of the mesencephalic locomotor region, stimulation of which can induce movement in a decerebrate animal (Skinner et al. 1985). These anatomical and physiological data have led to the hypothesis that PPN may have a role in the regulation of basal ganglia function. Based on the results of this study, it appears that the cholinergic projections to thalamic nuclei which in turn project to the neostriatum may be one of the pathways over which PPN can affect basal ganglia activity.

CHAPTER THREE

ULTRASTRUCTURAL IDENTIFICATION OF CHOLINERGIC TERMINATIONS ONTO CM-Pf THALAMOSTRIATE PROJECTION NEURONS

INTRODUCTION

As part of our study of the basal ganglia system in the dog, we have been investigating the afferents to the centrum medianum-parafascicular complex (CM-Pf) of the thalamus which originate from the nucleus tegmenti pedunculopontinus (PPN) and from cells near central tegmental tract of the brainstem. In a previous light microscopic double labeling study, we described the overall topographical organization of this projection system in the dog and reported that a significant proportion of the projection neurons from PPN to the CM-Pf complex are cholinergic (Isaacson and Tanaka 1986). In the present study, we have extended these observations to the electron microscopic level.

There is some evidence to suggest that projections from PPN synapse directly onto thalamostriate projection neurons located in CM-Pf. Sugimoto and Hattori (1984) reported that, following injections of tritiated amino acids into PPN, autoradiographically labeled terminals in CM-Pf synapsed directly onto retrogradely labeled

thalamostriate neurons. However, it was not determined whether any of the these terminations were cholinergic. In the present study, we combined choline acetyltransferase (ChAT) immunocytochemistry with WGA-HRP retrograde labeling in order to examine the ultrastructural morphology of cholinergic terminals within the CM-Pf complex and localize cholinergic terminals making synaptic contact with retrogradely labeled CM-Pf thalamostriate neurons. The results of this study have been presented previously in abstract form (Isaacson and Tanaka 1985b).

MATERIALS AND METHODS

Seven adult mixed breed dogs weighing 7-10 kg were used in this study. Four of these animals were used for double labeling procedures. Intravenous injections of sodium pentobarbital (15-20 mg/kg) were administered prior to surgery and supplemented during surgery up to a maximal dose of 35-40 mg/kg. Unilateral injections of 0.2-0.3 ul of 2.5% WGA-HRP were stereotaxically placed within the caudate nucleus. Following a two day survival, the animals were reanesthetized and perfused with 8 liters of 2% paraformaldehyde/ 0.05% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) followed by 7 liters of 0.1M phosphate buffer wash. Brains were removed and 50 um thick sections were cut on a vibratome and processed for WGA-HRP histochemistry using the

glucose oxidase reaction with cobalt chloride pretreatment (Itoh et al. 1979). Subsequent processing for choline acetyltransferase (ChAT) immunocytochemistry using affinity-purified monoclonal rat anti-ChAT (1:200; Immunonuclear, Inc.) was performed at 4°C for 24 hours. After three rinses in phosphate buffered saline with 2% normal rabbit serum, the sections were treated with biotinylated rabbit anti-rat IgG for 1 hour, rinsed, and treated with the Avidin-Biotin Complex for 1 hour at room temperature. After rinsing in phosphate buffer without saline, sections were processed for localization of ChAT immunoreactivity using the glucose oxidase reaction without cobalt chloride pretreatment for approximately 30 minutes at room temperature. As a control, the primary antibody was replaced with normal rat serum in selected adjacent sections.

Three cases served as controls for the double labeling procedure. Two were sacrificed without prior injections of WGA-HRP and brain tissue was processed only for ChAT immunocytochemistry as described above. The remaining animal received a WGA-HRP injection into the caudate nucleus. Vibratome sections were processed for WGA-HRP histochemistry using the cobalt chloride-glucose oxidase reaction with no subsequent immunocytochemical processing. In each case in which ChAT immunocytochemistry was performed, sections through the

caudate nucleus (contralateral caudate if the animal received a previous WGA-HRP injection) were processed for immunocytochemistry as a positive control.

Following treatment for WGA-HRP histochemistry and/or ChAT immunocytochemistry, sections from all seven cases were osmicated in 2% OsO_4 for 1 hour, dehydrated in graded series of alcohols, and flat embedded in Epon plastic between two microscope slides which had been previously treated with Liquid Release Agent (Polysciences). Sections were examined with the light microscope and selected areas through the CM-Pf complex or caudate nucleus were cut out and glued onto blank Epon blocks. Ultrathin sections were cut, collected on Formvar coated slot grids, stained with Reynold's lead citrate and viewed with a JEOL 100 CX II electron microscope.

RESULTS

Light microscopy

In all cases, injections of WGA-HRP were confined to the head of the caudate nucleus. In 3 cases, the injections were located within the dorsolateral part of the head of the caudate nucleus, while in 2 cases they were centered in the more ventral and medial part of the head. Injections placed in the dorsolateral portion of the caudate nucleus resulted in retrogradely labeled

neurons located primarily in CM, while those placed into the medial part of the caudate gave rise to labeled cell bodies confined to Pf (Fig. 13).

Examination of flat-embedded vibratomed sections processed for HRP histochemistry only or for both HRP histochemistry and immunocytochemicial processing revealed black punctate HRP granules located within the cell somata and dendritic processes of retrogradely labeled CM-Pf thalamostriate neurons. These large multipolar and fusiform cells were arranged in rows parallel to the internal medullary lamina (Fig. 13). They contained variable amounts of reaction product. Some cells were heavily labeled while others contained only very light label. Also, in some cells, the reaction product did not spread beyond the proximal dendrites, while in others the reaction product extended into more distal dentritic profiles. It was this latter type of labeled neuron which was most desirable for our study and was chosen for subsequent ultrastructural examination.

Following the immunocytochemical procedure, few punctate structures characteristic of ChAT-postive axonal processes and terminal boutons were distinguishable in CM-Pf at the light microscopic level. However, these thalamic nuclei consistently demonstrated immunoreactivity higher than background, suggesting the presence of ChAT positive structures. In addition, evidence of positive immunoreactivity resulting from the

Figure 13. A and B. Photomicrographs of WGA-HRP injection site through the caudate nucleus and the resultant retrograde labeling in the CM-Pf complex. Cd, caudate nucleus. HPT, habenular-peduncular tract. IC, internal capsule. CM-Pf, centrum medianum-parafascicular complex.

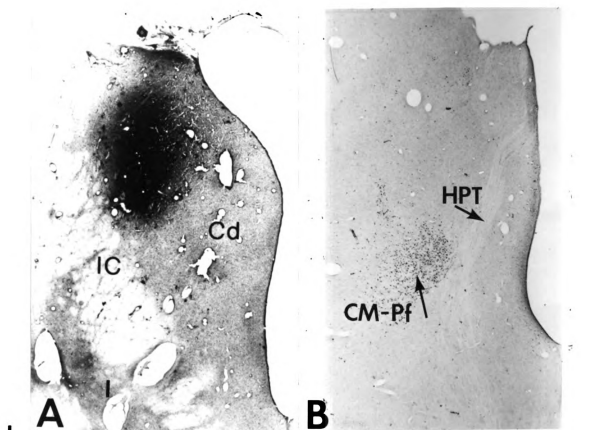

Figure 13.

Figure 14. Low power electron micrograph showing retrogradely labled CM-Pf thalamostriate projection neuron (arrows). These neurons are characterized by deep indentations of the nuclear envelope. N, nucleus. Scale bar = 3 um .

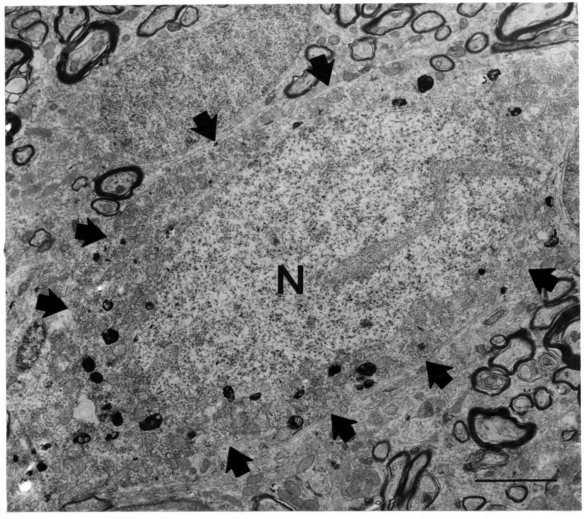

Figure 14

Figure 15. Electron micrograph of retrogradely labeled dendrite and general appearance of WGA-HRP reaction product within dendritic profiles. Note the large round granule (arrowhead) as well as the irregularly shaped reaction product (arrows). Below (open arrow) is a very lightly labeled immunopositive bouton. Scale bar = 0.5 um .

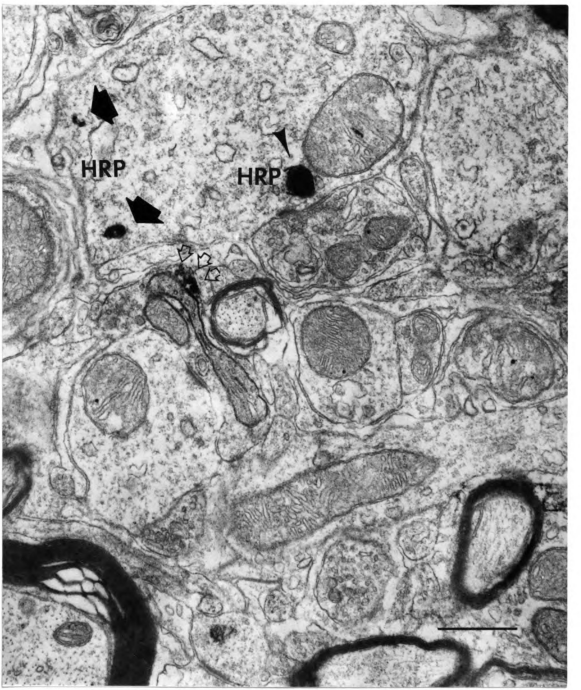

Figure 15

Figure 16. Electron micrographs of ChAT-positive axon terminals synapsing onto dendritic profiles. These terminals were characterized by small pleomorphic synaptic vesicles.

A and B. Small ChAT-positive terminal in series makes slightly asymmetric synaptic contact (arrow) onto dendritic profile. Note the difference in this post-synaptic density in comparison to that of the distinctive assymetric contact in the upper left corner (arrowhead). Scale bar = 0.5 um .

C. ChAT-positive terminal makes symmetrical synaptic contact (arrow) onto small dendrite.

D. ChAT- positive terminal makes probable synaptic contact with dendrite. Scale bar = 0.5 um .

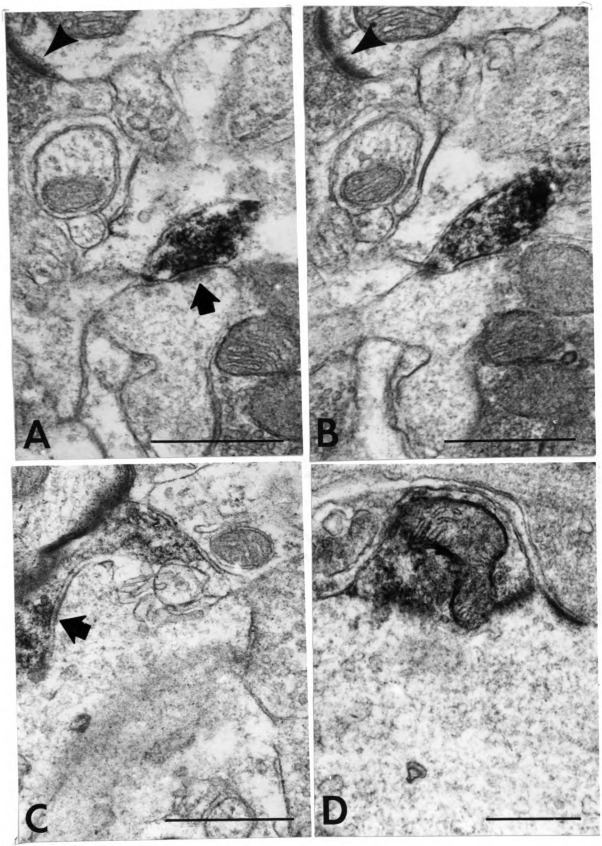

Figure 16

Figure 17.

A. Low power electron micrograph illustrating a ChAT-positive axonal profile (arrowheads) and a ChAT-positive terminal making slightly asymmetrical synaptic contact onto a small dendrite. Note HRP granule (open arrow) in dendrite on left side of micrograph. Scale bar = 0.5 um.

B. At higher magnification, this terminal makes synaptic contact with small dendrite (asterisk). Scale bar = 0.5 um .

C. In a serial section, a puncta adherentes contact (arrow) is made between the two dendritic profiles. See also the summary diagram (Figure 21). Scale bar =0.5 um .

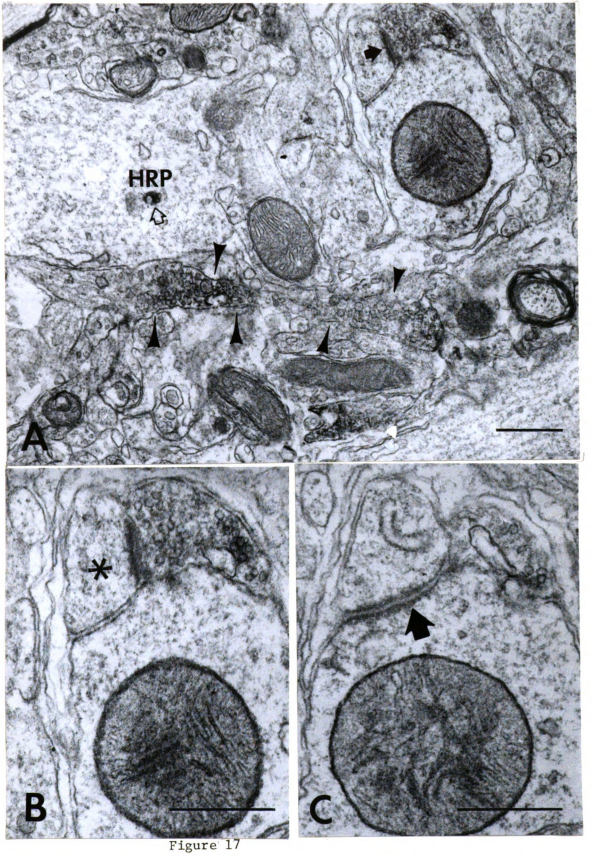

Figure 17

Figure 18.

A. Low power electron micrograph of ChAT-positive axon terminal (arrow) making contact with a small unlabeled dendrite and a larger dendrite containing what may be an WGA-HRP granule (asterisk). Scale bar = 0.5 um .

B and C. In series, the ChAT-positive terminal is seen making a slight asymmetric synaptic contact (arrowhead) with a small dendrite, and a puncta adherentes contact (arrow) with possible retrogradely labeled dendrite. Scale bar = 0.5 um.

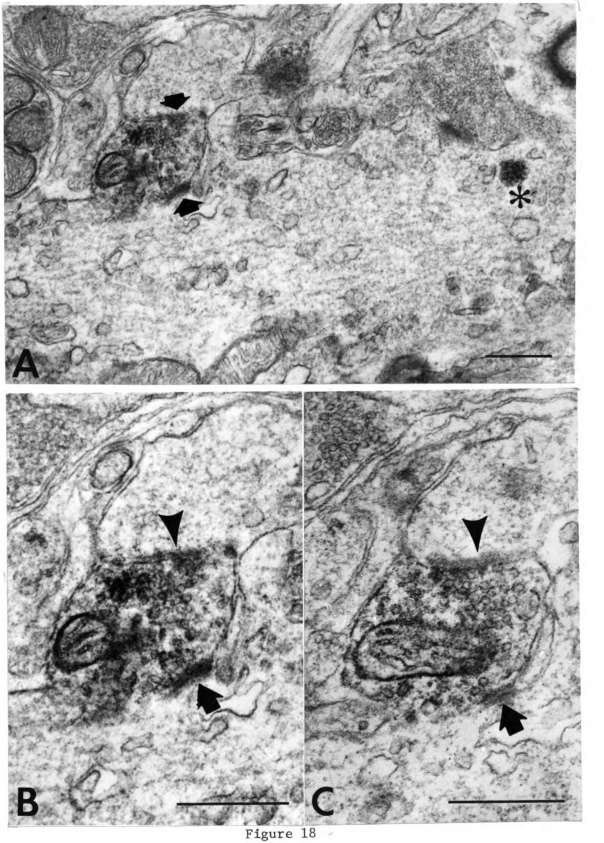

Figure 18

Figure 19. Examples of ChAT-positive axon terminals making contact with dendritic profiles.
A and B. Larger terminal makes symmetrical synaptic contact with HRP labeled dendrite. Note adjacent, unlabeled terminal (asterisk) in A and HRP granules (arrowhead) in B. Bar = 0.5 um.
C. Small ChAT-positive terminal makes synaptic contact (arrow) with dendritic spine of HRP labeled profile. Scale bar = 0.5 um.
D. Small ChAT-positive terminal makes puncta adherentes contact (arrowhead) with HRP labeled dendrite. Scale bar = 0.5 um.

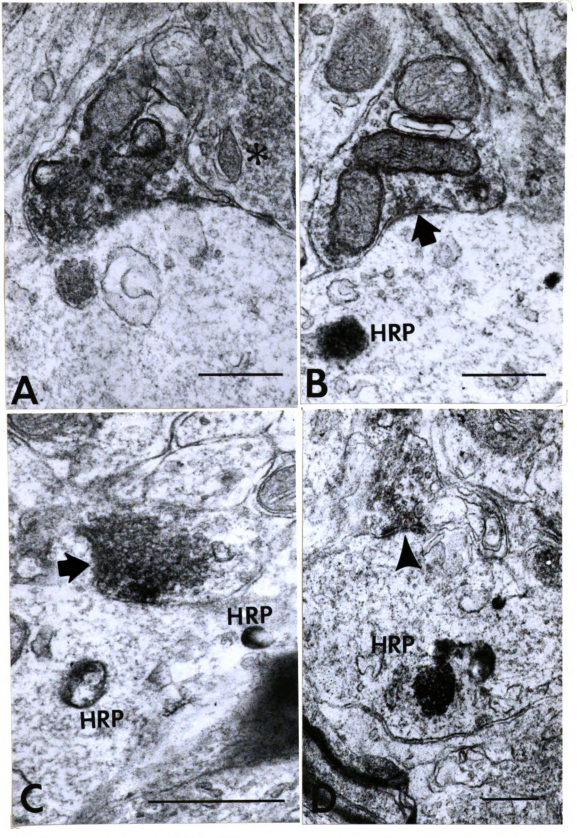

Figure 19

Figure 20. Two large ChAT-positive axon terminals contacting cell somata of striatal projection neurons. These terminals are characterized by multiple symmetrical synaptic contacts (arrows) as well as puncta adherentes contacts (arrowhead).

B. This terminal was observed in series to make double symmetrical contacts with cell soma and puncta adherentes contact onto a spine from the cell soma. See summary diagram (Figure 21G). Scale bar = 0.5 um .

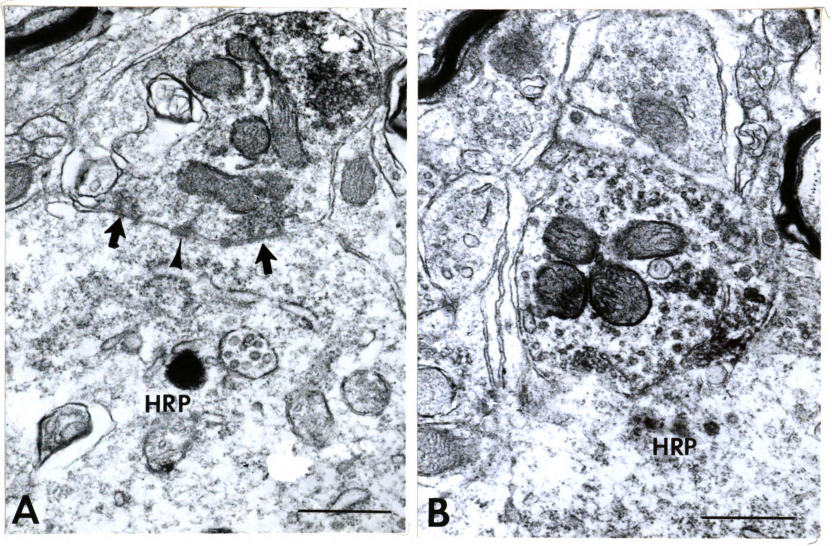

Figure 20

Avidin-Biotin treatment was provided by the identification of ChAT-positive cell bodies and axonal processes in the caudate nuclei.

Electron Microscopy

Retrogradely labeled thalamostriate projection neurons contained membrane bound WGA-HRP reaction product within their cell somata and dendritic processes. In both the cell soma and dendritic processes, the electron dense peroxidase reaction product varied in appearance from large round granules to irregularly shaped pieces of granules (Fig. 14) which appeared to be associated with the smooth endoplasmic reticulum, particularly within dendritic processes (Fig. 15). The retrogradely filled neurons were characterized by indented nuclei and somatic spines. In some cases, serial sections were used to follow and identify more distal dendritic processes arising from WGA-HRP labeled cell somata and proximal dendrites.

At the ultrastructural level, immunoreactive ChAT positive axonal profiles and synaptic boutons in the CM-Pf complex could be identified despite the difficulty in detecting them with the light microscope. The immunoreactive substance within terminal boutons in the CM-Pf complex was associated with the cytoplasmic matrix and synaptic vesicle membrane (Figs. 16-20). Small diameter ChAT-positive non-myelinated axonal profiles

were frequently observed (Fig. 17). In some instances, these processes expanded into vesicle-filled boutons. Heavily myelinated, large diameter axons were also present throughout the region. These myelinated axons were never observed to contain ChAT reaction product.

Within the CM-Pf complex, ChAT-positive synaptic boutons primarily contacted dendritic shafts and dendritic spines, some of which were identified as belonging to WGA-HRP labeled thalamostriate projection neurons. These ChAT positive terminals made either single symmetrical or slightly asymmetrical synaptic contacts, and contained pleomorphic vesicles. They could be classified into two types. The first type was small in size and generally contained one to two small mitochondria (Figs. 16-18; 19C, 19D). This smaller type of ChAT-positive bouton generally was observed in contact with smaller dendritic processes and dendritic spines (Figs. 16 A,B; 17B; 18; 19D). In most cases, the origin of the smaller dendritic profiles could not be identified. For example, in serial sections,one ChAT-positive bouton was found making a double synapse onto two dendritic profiles. Yet neither dendrite could be identified as belonging to a retrogradely labeled neuron (Fig. 17B). However, in one instance, a dendritic spine receiving contact from a small ChAT-positive terminal was cut in the appropriate plane so that it was identified as

part of a WGA-HRP labeled dendrite (Fig. 19C). In another instance, a ChAT-positive bouton made a slightly asymmetrical contact with a small dendritic profile and a puncta adherentes contact with a larger, possibly retrogradely labeled dendritic shaft (Fig. 18). Single puncta adherentes contacts were an occasional occurrance between small and large ChAT-positive boutons and HRP labeled dendritic profiles (Figs. 18, 19D).

A second, larger type of terminal was less frequently observed (Figs.19A, B; 20) and contained more numerous mitochondria and more loosely packed synaptic vesicles. This type generally was found making contact with larger dendritic profiles and, less frequently, with retrogradely labeled cell somata. Those boutons contacting cell somata made several puncta adherentes contacts onto the cell soma (Fig. 20). One bouton was observed in serial sections to contact the cell soma of a projection neuron as well as a somatic spine which was in turn contacted by several other unlabeled terminals (Fig. 21G). ChAT-positive contacts onto proximal dendrites of projection neurons were rarely observed though these dendrites were often covered by numerous unlabeled terminal boutons, most of which made asymmetrical synaptic contacts. Occasionally, these contacts were occasionally present at the branching points of the primary dendrites.

Controls

Sections examined with the electron microscope from material processed only for WGA-HRP histochemistry revealed no difference in appearance or relative numbers of granules in the cell somata and dendritic processes. In addition, no specific immunocytochemical staining was observed in material in which the primary antibody was replaced with normal rat serum. Finally, sections from CM-Pf were examined in which only immunocytochemical processing was performed. No difference in localization of ChAT immunoreactivity was observed.

DISCUSSION

The results of the present study demonstrate that by combining ChAT immunocytochemistry with WGA-HRP retrograde labeling at the ultrastructural level, ChAT-positive terminals can be identified making synaptic contact with retrogradely labeled CM-Pf thalamostriate projection neurons. These findings indicate that at least some striatal projection neurons in the CM-Pf complex are directly innervated by cholinergic terminal boutons.

In a previous report using a polyclonal antibody to ChAT, Kimura et al. (1981) reported intensely stained `terminal dots' in the neuropil of the parafascicular nucleus as well as on neuronal somatic surfaces. These

Figure 21. Summary diagram representing the different relationships observed between ChAT-positive boutons and retrogradely labeled projections in the CM-Pf complex.

A. ChAT-positive bouton makes synaptic contact with two unlabeled dendritic profiles.
B. ChAT-positive bouton makes puncta adherentes contact with unlabeled dendrite.
C. ChAT-positive bouton makes puncta adherentes contact with spine of retrogradely labeled dendrite.
D. ChAT-positive bouton makes synaptic contact with small dendritic profile and puncta adherentes contact with larger,possibly WGA-HRP labeled dendrite.
E. ChAT-positive bouton contacts large unlabeled dendritic process.
F. ChAT-positive bouton contacts small dendritic spine.
G. Large ChAT-positive bouton makes syunaptic contact with cell soma of retrogradely labeled neuron and puncta adherentes contact with somatic spine from the same cell. Note two unlabeled boutons also contacting the somatic spine.

Symbols: b*-ChAT-positive bouton
D*-WGA-HRP labeled dendrite

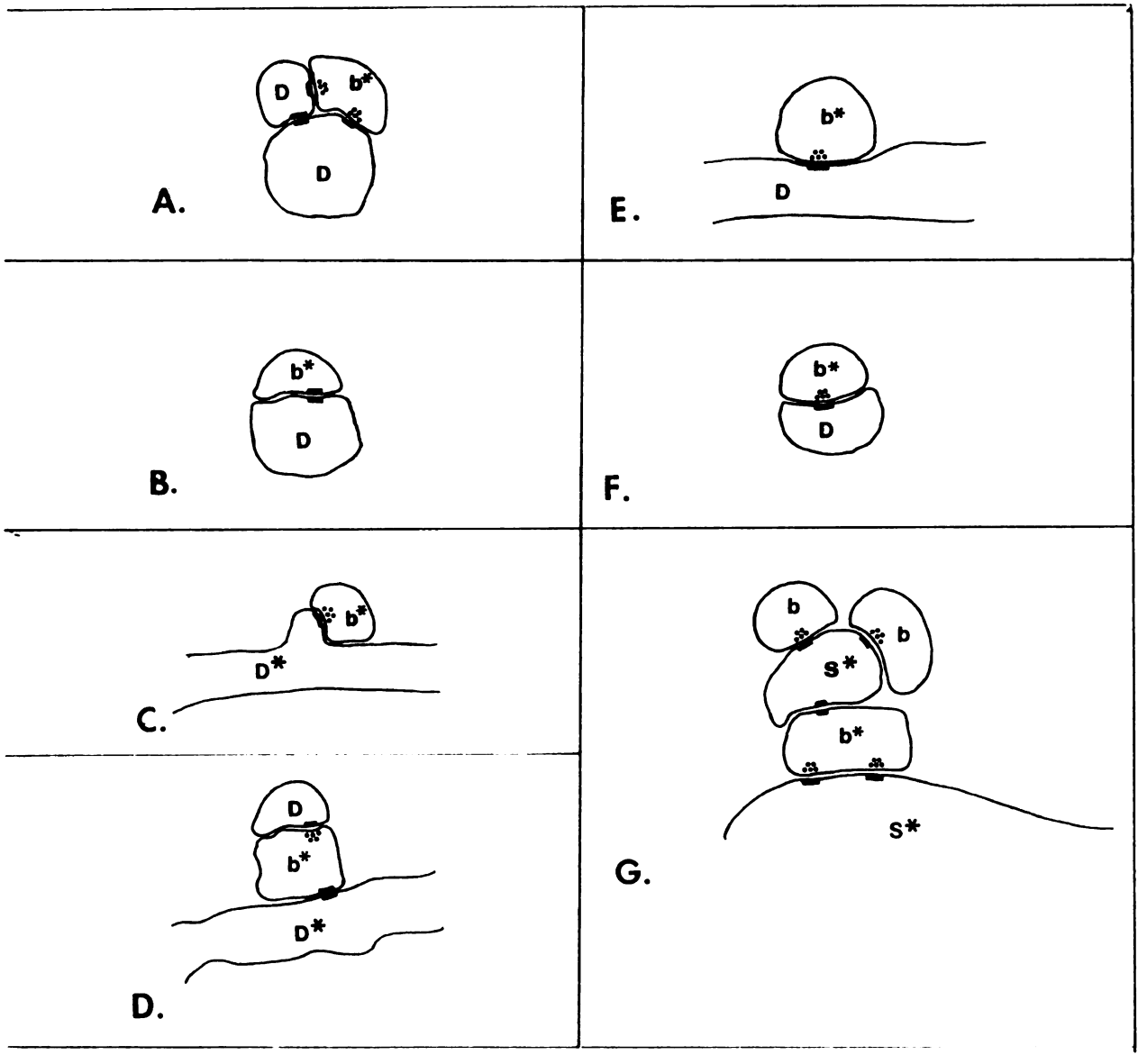

Figure 21

somatic surfaces may belong to the thalamostriate projection neurons which, in the present study, have been shown to receive direct contact from ChAT-positive terminals. In addition, the localization ofChAT-positive terminals in the CM-Pf complex is in direct correspondence to the high activity observed in this area after staining for acetylcholinesterase (Tanaka, unpublished). It was observed that the caudal intralaminar nuclei consistently stained very intensely.

In the present study, the light punctate labeling in the CM-Pf complex at the light microscopic level is difficult to explain. It is possible that a species difference may account for the sparse terminal labeling observed here. The antibody used in this study resulted previously in punctate labeling in the rat spinal cord (unpublished data). ChAT-positive punctate structures have previously been reported in ventral horn of the spinal cord of the rat (Houser et al. 1983). Yet few axonal profiles and only light punctate labeling were also observed in the dog caudate nucleus an area which has been shown to contain heavy punctate labeling in the rat (Phelps et al. 1985). Thus, because the anti-ChAT used in this study was raised against rat enzymes, it is possible that it might not stain terminals as intensely in dog brain tissue as in the rat. Alternatively, the enzyme levels within terminals of cholinergic neurons might be metabolized very quickly in the CM-Pf complex,

resulting in less terminal label than expected. Finally, this problem is not unique to the region of CM-Pf. In a recent report, an absence of intense punctate labeling was observed in the rat interpeduncular nucleus at the light microscopic level (Wainer et al. 1984). When this tissue was subsequently examined with the electron microscope, however, ChAT-positive terminals could be identified.

Recent light microscopic studies using uptake and retrograde transport of ^{3}H-choline (Sugimoto and Hattori 1984) as well as double labeling procedures combining ChAT immunocytochemistry and HRP retrograde labeling (Isaacson and Tanaka 1986) reveal that the CM-Pf complex receives a substantial cholinergic input from neurons located within the pontomesencephalic tegmentum, primarily from PPN and the central tegmental tract. In addition, a double labeling study in the rat using WGA-HRP retrograde labeling in combination with AChE staining demonstrated that, when HRP injections were confined to the thalamus, HRP labeling was observed only in the pontomesencephalic tegmentum (Mesulam et al. 1984). In the same study, it was found that, after larger injections which included areas adjacent to the thalamus, it was found that the vast majority of retrogradely labeled AChE-positive neurons were located in the brainstem. Similarly, following ^{3}H- amino acid

injections into PPN, anterogradely labeled PPN terminals were observed making synaptic contact with thalamostriate neurons (Sugimoto and Hattori 1984). These results lend support to the idea that the cholinergic terminals observed in CM-Pf may arise from cholinergic cell bodies located within PPN and the central tegmental tract.

Because the cholinergic terminals observed here were not labeled with an anterograde marker, their exact origin cannot be identified. Therefore, the possibility does exist that these terminals may originate in regions other than the brainstem. For example, the entopeduncular nucleus, in which cholinergic neurons have been reported (Mesulam et al. 1984; Houser et al. 1983; Armstrong et al. 1983; Kimura et al. 1983 has been shown to project heavily to CM (McGuiness and Krauthamer 1980; DeVito and Anderson 1982; Filion and Harnois 1978). In the dog, however, it appears that the pathway from the entopeduncular nucleus to the centrum medianum is not cholinergic. Double labeled cholinergic projection neurons were never observed in the entopeduncular nucleus following CM-Pf injections of WGA-HRP and subsequent ChAT immunocytochemistry, though separate populations of non-cholinergic thalamic projection neurons as well as cholinergic neurons were observed in this region (unpublished observations). Furthermore, following lesions in the entopeduncular nucleus, Grofova and Rinvik

(1974) observed degenerating boutons in CM which contained pleomorphic vesicles and established symmetrical contacts on cell somata and proximal dendrites. More recent autoradiographic studies at the electron microscopic level extended their findings, demonstrating that pallidal-thalamic terminations in ventral medial and ventral lateral thalamic nuclei establish symmetrical contacts with cell somata and proximal dendrites, contain numerous mitochondria, and consistently have multiple puncta adherentes (Kultas-Ilinsky et al. 1983). Immunocytochemical studies suggest that these terminations are GABAergic (Kultas-Ilinsky et al. 1985). Thus, the boutons described in these studies do not correspond to the ChAT-positive terminals observed in our material which rarely contact cell somata, contain few mitochondria, and establish both symmetric and asymmetric contacts with distal dendrites or dendritic spines. These findings suggest that the ChAT-positive terminals observed in CM-Pf do not arise from ChAT postive cell bodies in the entopeduncular nucleus.

Sugimoto and Hattori (1984) examined autoradiographically labeled PPN terminals at the electron microscopic level and found both asymmetric and symmetric synaptic contacts onto thalamostriate neurons in the CM-Pf complex of the rat, though asymmetric contacts were more prevalent. In addition, they found

that symmetric contacts from PPN were more common in the subthalamus. Though they did not describe the types of vesicles found in the autoradiographically labeled boutons, their photomicrographs indicate that the labeled terminals in CM-Pf contained pleomorphic vesicles, and frequently make puncta adherentes contacts onto their post-synaptic targets. The terminals they describe might correspond to those observed in the present study. These findings suggest that at least two types of terminals originate from PPN . Furthermore, the similarity of the terminals observed in the present study with those described by Sugimoto and Hattori (1984) lends further support for a brainstem (PPN) origin of the ChAT-positive terminals identified within the CM-Pf complex.

In the present study, the type of membrane specializations exhibited by ChAT-positive synaptic contacts were both asymmetric and symmetric. Others have reported a similar mixed population of ChAT-immunoreactive boutons in the hippocampus (Frotchner and Leranth 1985; Wainer et al. 1985), interpeduncular nucleus (Wainer et al. 1985), and amygdala (Wainer et al. 1985; Carlsen and Heimer 1986) Conflicting reports exist, however, concerning ChAT-positive terminals in the rat neostriatum. Phelps and her colleagues (1985) observed that ChAT positive terminals in the neostriatum contained only pleomorphic synaptic vesicles and make only symmetric synaptic contacts primarily on dendritic

shafts. Alternatively, Wainer and his colleagues (1984) reported that, similar to their observations in other parts of the central nervous system, ChAT-immunoreactive terminals in the striatum possess only clear, round vesicles and make both symmetric and asymmetric type contacts.

It appears, then, that ChAT-positive terminals cannot be categorized throughout the CNS according to membrane specializations or synaptic vesicle type. These standard criteria have been used in the past as an indicator of function. It may be that physiological data is the only means by which any functional information concerning ChAT positive terminals in the central nervous system can be ascertained.

It has been suggested that cholinergic interneurons in the neostriatum could modulate the inhibitory output of GABAergic neostriatal projection neurons (Phelps et al. 1985). Also, iontophoretically applied acetylcholine has been shown to have an excitatory effect on neocortical neurons (Lamour et al. 1982) as well as on hippocampal neurons and thalamic neurons in the ventrobasal complex (Kelly et al. 1979) but an inhibitory effect on almost every cell encountered in the nucleus reticularis (Kelly et al. 1979). Electrophysiological data obtained from stimulation of PPN, the suggested source of the observed ChAT terminals, reveal a direct

excitatory effect on the globus pallidus (Gonya-Magee and Anderson 1983). Whether PPN has the same action on the thalamus has yet to be determined.

GENERAL DISCUSSION

The results of this study indicate that:

1) the thalamocaudate projection from the canine CM-Pf complex is topographically organized,

2) the CM-Pf complex receives projections from cholinergic and non-cholinergic neurons within PPN and the central tegmental tract of the pontomesencephalic tegmentum,

3) some cholinergic terminal boutons in the CM-Pf complex contact retrogradely labeled thalamostriate projection neurons.

Functional Implications

The determination of a direct contact between cholinergic terminations from PPN and CM-Pf thalamostriate projection neurons provides evidence that PPN may influence basal ganglia activity, and possibly has a role in central nervous system control of movement. The basal ganglia are believed to be involved with the preparation and initiation of movement (Buchwald et al. 1975), and since PPN is thought to influence basal ganglia activity, the PPN-CM-striatal pathway may be one means by which PPN can affect the processes involved with movement.

It has been suggested that PPN, along with nucleus

cuneiformis and periaqueductal grey may be part of the mesencephalic locomotor region (MLR), an area which induces walking upon stimulation in a decerebrate animal. There is some evidence in favor of this theory. For example, the MLR receives input from the substantia nigra, responds to entopeduncular nucleus stimulation, and projects diffusely to CM (Garcia-Rill 1983; Garcia-Rill et al. 1983a; Garcia-Rill et al. 1983b) ---all characteristics of PPN. Because the nuclear boundaries of PPN are difficult to ascertain, and the location of stimulation electrodes are not easily determined, the question involving the MLR and its exact location has not been determined but is believed to encompass the medial part of PPN, the nucleus cuneiformis and the periaqueductal grey. Nevertheless, others have considered that PPN may be a functional link between the basal gangia system and basal ganglia's effects on lower motor activity (Jackson and Crossman 1983). However, the exact role that this brainstem nucleus plays in basal ganglia function remains unresolved.

It is known that individuals affected with Alzheimer's disease show pathology of cholinergic neurons in the central nervous system (Mesulam et al. 1983a). Correlations exist between the reduction of ChAT activity and the presence of dementia and cortical plaques associated with this disease. It has been demonstrated

that the diagonal band of Broca and nucleus basalis of Meynert provide the major cholinergic innervation of the amygdala, hippocampus, and neocortex (Mesulam 1983a), three areas of the brain which appear to be the most affected in Alzheimer's disease (Brun 1983). Cell bodies of cholinergic cortical projection neurons exhibit characteristics of cell death while the distal axons and nerve terminals form neuritic cortical plaques, degenerate, and consequently result in denervation of the amygdala, hippocampus and neocortex (Bowen 1983).

While most of the cholinergic cortical innervation appears to arise from the nucleus basalis and diagonal band, cholinergic cortical projections to the frontal cortex from PPN also have been demonstrated (Vincent et al. 1983). It is possible that these cortical projections also play a role in the pathology of Alzheimer's disease. Furthermore, brainstem cholinergic projections to the intralaminar nuclei might share some clinical significance in this disease as well since the intralaminar nuclei project to widespread areas of the neocortex. Since the PPN-thalamic pathway has not been studied in individuals affected with Alzeimer's disease, is is not known whether this connection has any role in the pathology associated with the disease. Much more information is needed to determine the role of central cholinergic systems in the pathology of Alzheimer's disease.

Observations from light microscopic studies

While the present study analyzed the distribution of cholinergic PPN projections to CM-Pf, it is unknown whether any of the PPN projections to the subthalamus and substantia nigra, two additional basal ganglia related nuclei, are cholinergic. In addition, it is not known what relationship exists between the populations of cholinergic neurons which project to the hippocampus, olfactory cortex, and rostral thalamus and those which project to the thalamus. It is possible that single neurons in PPN have axon collaterals to two or more of these structures.

There have been no previous reports of cholinergic projection neurons within the central tegmental tract. In addition to containing ascending fibers from the lower brainstem reticular formation, this tract also consists of descending projections from midbrain nuclei to the inferior olivary complex. It was shown in this study that central tegmental tract neurons terminate in the CM-Pf complex of the thalamus. It would be of interest to determine, using autoradiographic techniques, the areas of termination of the remaining ascending and descending projections arising from cholinergic neurons associated with the central tegmental tract.

Additional examination of the smaller non-cholinergic projection neurons in the light microscopic

study is also necessary. Of primary importance is the determination of the neurotransmitter content of these neurons. There does exist the possibility that at least some of these neurons might contain choline acetyltransferase, but had depleted their store of enzyme in the cell soma at the time of sacrifice, thus giving a false negative result following ChAT immunocytochemistry. Also, it is important to use criteria in addition to cell soma area to allow for the catagorization of cholinergic and non-cholinergic projection neurons into separate neuronal populations.

Observations from electron microscopic studies

In order to determine unequivocally the origin of cholinergic terminal boutons identified at the ultrastructural level in CM-Pf, it is necessary to anterogradely label terminals from PPN and, if possible, also label them as ChAT-positive . It may be possible to combine the lectin phaseolus vulgaris leucoagglutinin, PHA-L , an anterograde tracer, labeling with ChAT immunocytochemistry using colloidal gold as one of the markers. Autoradiography at the electron microscopic level combined with ChAT immunocytochemistry also may be a feasible means to study this problem.

Sugimoto and Hattori (1984) anterogradely labeled PPN terminations within CM-Pf, yet their analysis was far from complete. Their photomicrographs depicting terminal

types and synaptic contacts did not adequately substantiate their descriptions in the text. For example, the axodendritic contacts which are described as synaptic contacts in their photomicrographs, are not actually true synapses due to the lack of synaptic vesicles apposed to the presynaptic membrane. Thus, we could not directly compare our results to those found in their study. A complete, thorough ultrastructural examination of PPN terminations in the thalamus is necessary.

In an ultrastructural study concerning CM, Harding (1973a; 1973b) described several terminal types--none of which correspond to the small, cholinergic terminal boutons which make symmetrical and asymmetrical contacts onto dendritic profiles. In our own material, cholinergic terminals were observed much less frequently than other bouton types. It was not uncommon for only one or sometimes no ChAT-positive terminals to be present in an entire ultra thin section. Thus, it is possible that this bouton type was overlooked or not observed frequently enough to be catagorized. In agreement with our ultrastructural study, Westman and Bowsher (1971) observed few terminal boutons in contact with cell somata.

Comments on methods

One limitation of the double labeling technique used in the ultrastructural study involves the penetration of

the primary antibody. The primary antibody cannot penetrate into the depths of the vibratome section beyond 2-3 um. This limited penetration greatly lessens the probability that any immunoreactive terminal boutons are identified - particularly making a synaptic contact. Also, in consideration of the small sampling size involved in each block examined, only a slim possibility exists that any ChAT positive terminal bouton will be observed in contact with a profile containing WGA-HRP granules.

Another limitation of this technique concerns the distal dendritic filling with WGA-HRP reaction product. The WGA-HRP granules normally do not spread into distal dendritic profiles of projection neurons. Therefore, some of the unlabeled distal dendrites and dendritic spines receiving contact from ChAT positive terminals may have belonged to retrogradely labeledthalamostriate projection neurons. In future studies, the origin of the distal dendritic profiles and dendritic spines could be determined by combining Golgi impregnation techniques with immunocytochemistry or HRP labeling. Only this type of analysis could reveal an answer to a question such as whether ChAT-positive terminations contact intrinsic neurons as well as projection neurons in CM-Pf, a phenomenon which is reported to occur with ChAT positive terminals in the lateral geniculate nucleus in the cat (deLima et al. 1985). A more efficient means of

identifying distal processes of projection neurons in double labeling procedures needs to be developed.

Also needed is the development of a primary antibody to choline acetyltransferase which would result in a more accurate comparison of ChAT distribution in carnivores. Using a rat primary antibody to ChAT, we were unable to detect a dense network of axonal labeling in the thalamus of the dog at the light microscopic level. Using two different rat antibodies to ChAT, Henderson (1986) failed to detect labeling of ChAT positive cell bodies in the cortex in the cat and ferret, though he observed the presence of punctate terminal labeling. Perhaps a monoclonal antibody produced against ChAT from a carnivore could provide a more accurate means of comparing cholinergic systems in carnivore species to other mammalian species.

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