CONNECTIONS OF THE NUCLEUS TEGMENTI PEDUNCULOPONTINUS. A COMBINED LIGHT AND ELECTRON MICROSCOPIC STUDY IN THE RAT.

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ABSTRACT

CONNECTIONS OF THE NUCLEUS TEGMENTI PEDUNCULOPONTINUS.
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The present study was focused on furthering our understanding of the connections of the PPN in the rat. Previous studies have indicated that ascending projections to the basal ganglia and descending projections to the spinal cord originate from the same region of the nucleus tegmenti pedunculopontinus (PPN). Therefore some PPN neurons may have axons which divide into ascending and descending collaterals. Single and double-labeling experiments were conducted to determine the distribution of the cells of origin of these projections and to test for the presence of collateralization. The results indicated that the majority of both ascending and descending fibers arise from separate populations of PPN neurons which are intermingled throughout the nucleus.

Numerous studies have indicated that the substantia nigra pars reticulata (SNR) projects to the PPN. Therefore the distribution and mode of termination of the nigropedunculopontine projection were studied using the

anterograde tracer Phaseolus vulgaris-leucoagglutinin. The results demonstrated that while the nigral fibers terminated in both subdivisions of the PPN, the subnucleus compactus (PPNc) and dissipatus (PPNd), the bulk of the fibers terminated in the PPNd. The nigral fibers appeared directed towards specific neurons and neuropil regions. The majority of the synaptic terminals were seen in contact with medium size dendrites. In light of the previous observations, the nigral input may be preferentially distributed to particular subpopulation(s) of PPN projection neurons.

Using ChAT immunocytochemistry, the final study examined the distribution of cholinergic neurons within the PPN, particularly within the PPNd which receives the bulk of the nigral input, and analyzed the synaptic arrangement of both cholinergic and non-cholinergic PPNd neurons. The results indicated that numerous cholinergic neurons are present in both subnuclei. Both cholinergic and non-cholinergic PPNd neurons are contacted by two morphologically different types of boutons, some of which may be of nigral origin. Thus the present observations suggest, indirectly, that nigral afferents to the PPN may terminate on both cholinergic and non-cholinergic PPN neurons.

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ABBREVIATIONS

c	caudal
CG	central grey
CNF	cuneiform nucleus
cp	cerebral peduncle
d	dorsal
DPB	dorsal parabrachial nucleus
EP	entopeduncular nucleus
f	fornix
fr	fasciculus retroflexus
GP	globus pallidus
HYP	hypothalamus
IC	inferior colliculus
ic	internal capsule
LC	locus coeruleus
LG	lateral geniculate nucleus
LH	lateral hypothalamic area
ll	lateral lemniscus
LPB	lateral parabrachial nucleus
ME5	nucleus of the mesencephalic tract of the trigeminal nerve
me5	mesencephalic trigeminal tract
MHb	medial habenular nucleus
ml	medial lemniscus

MM	medial mammillary nucleus
Mo5	motor trigeminal nucleus
MPB	medial parabrachial nucleus
MRF	mesencephalic reticular formation
MT	medial terminal nucleus of the accessory optic tract
ot	optic tract
Pf	parafascicular thalamic nucleus
PL	paralemniscal nucleus
PN	pontine nucleus
PNO	pontine reticular nucleus, oral portion
PPN	pedunculopontine nucleus
PPNc	pedunculopontine nucleus, subnucleus compactus
PPNd	pedunculopontine nucleus, subnucleus dissipatus
r	rostral
Rmes	mesencephalic reticular formation
RN	red nucleus
RPo	pontine reticular nucleus, oral portion
RR	retrorubral nucleus
RRF	retrorubral field
SC	superior colliculus
scp	superior cerebellar peduncle
sm	stria medullaris of the thalamus
SN	substantia nigra
SNC	substantia nigra, pars compacta
SNR	substantia nigra, pars reticulata
SO	superior olive

SPTg	subpeduncular tegmental nucleus
STH	subthalamic nucleus
TH	thalamus
v	ventral
VLL	ventral nucleus of the lateral lemniscus
VMH	ventromedial hypothalamic nucleus
VPB	ventral parabrachial nucleus
VTA	ventral tegmental area
ZI	zona incerta
3N	oculomotor nerve

INTRODUCTION

The nucleus tegmenti pedunculopontinus (PPN) has been defined by Olszewski and Baxter ('54) in the human as a nucleus which occupies the lateral pontomesencephalic tegmentum and extends from the caudal border of the red nucleus to the parabrachial nuclei. On the basis of cellular density, the authors distinguished two subdivisions of the PPN, the subnucleus compactus (PPNc) and the subnucleus dissipatus (PPNd). While the nucleus and its subdivisions are clearly discernible in other primates, they are far less distinct in non-primates. However, in all species the PPN contains a prominent component of cholinergic neurons which comprise the Cholinergic sector (Ch) 5 of Mesulam and co-workers ('83, '84, '89). It was not until the advent of modern neuroanatomical tracing techniques that the extensive interaction between the PPN and the forebrain as well as brainstem structures and the spinal cord was established. While a large portion of the ascending projections is directed towards the thalamus (Jackson and Crossman, '81, '83; Moon-Edley and Graybiel, '83; Sugimoto and Hattori, '84; Sofroniew et al., '85; Isaacson and Tanaka, '86; Woolf and Butcher, '86; Hallanger et al., '87; Jones et al., '87; Pare et al., '88; Steriade et al., '88; Fitzpatrick et al., '90;

Semba et al., '90), the PPN is also reciprocally connected with the hypothalamus and the basal forebrain (Jackson and Crossman, '81; Moon-Edley and Graybiel, '83; Parent and DeBellefeuille, '83; Swanson et al., '84; Mogenson et al., '85; Woolf and Butcher, '86; Jones et al., '87; Hallanger and Wainer, '88; Jones and Cuello, '89; Sakai et al., '90). Furthermore, both cholinergic and non-cholinergic neurons appear to project to these forebrain targets of the PPN (Sofroniew et al., '85; Isaacson and Tanaka, '86; Woolf and Butcher, '86; Pare et al., '88; Steriade et al., '88; Jones and Cuello, '89; Fitzpatrick et al., '90; Sakai et al., '90; Semba et al., '90).

The ascending projections of the PPN appear to have important roles in sleep and wakefulness. The cholinergic input to the thalamus is postulated to be an important component of the ascending reticular activating system or ARAS (Mesulam et al., '83, '89; Woolf and Butcher, '86; Hallanger et al., '87; Pare et al., '88; Steriade and Llinas, '88; Steriade et al., '88). The ARAS is involved in the regulation of thalamocortical transmission which controls EEG desynchronization. In addition, both the hypothalamus and the basal forebrain also take part in the regulation of EEG desynchronization and thus may be influenced by PPN efferents (Woolf and Butcher, '86; Hallanger and Wainer, '88; Jones and Cuello, '89; Sakai et al., '90). Furthermore, cholinergic projections from the PPN to the lateral geniculate nucleus of thalamus have been implicated in the regulation of

paradoxical or rapid eye movement (REM) sleep (De Lima and Singer, '87; Hallanger et al., '87; Pare et al., '88; Steriade et al., '88; Mesulam et al., '89; Fitzpatrick et al., '90).

In addition to their roles in EEG desynchronization and REM sleep, the PPN neurons appear to participate in various aspects of motor control. Numerous anatomical studies have documented projections from the PPN to the basal ganglia (De Vito et al., '80; Nomura et al, '80; Saper and Lowey, '82; Parent et al., '83; Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Sugimoto and Hattori, '84; Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87; Rye et al., '87; Scarnati et al., '87a; Lee et al., '88; Gould et al., '89;). Several investigators have reported that these projections are partially cholinergic (Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87; Gould et al., '89) and supply an excitatory input.to the basal ganglia (Gonya-Magee and Anderson, '83; Hammond et al., '83; Scarnati et al., '84, '87b). Furthermore, the PPN receives converging input from several basal ganglia nuclei (globus pallidus, entopeduncular nucleus, subthalamic nucleus and substantia nigra pars reticulata) (Nauta and Mehler, '66; Kim et al., '76; Carter and Fibiger, '78; Nauta and Cole, '78; Nauta, '79; Rinvik et al., '79 Beckstead et al., '79; Larsen and McBride, '79; McBride and Larsen, '80; Carpenter et al., '81; Jackson and Crossman, '81, '83; van der Kooy and Carter, '81; Arbuthnott and Wright, '82; De Vito and Anderson, '82;

Beckstead and Frankfurter, '82; Gerfen et al., '82; Moon-Edley and Graybiel, '83; Parent, '86; Schneider, '86; Rye et al., '87; Kita and Kitai, '87; Moriizumi et al., '88; Nakamura et al., '89; Smith et al., '90) and these afferents may have a modulating influence on the excitatory feedback loops.

Recent studies have demonstrated that the PPN gives rise to descending projections to the pontomedullary reticular formation (Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Garcia-Rill, '86; Mitani, et al., '90; Rye et al., '88; Woolf and Butcher, '89; Jones, '90; Semba et al., '90; Yasui et al., '90; 'Grofova et al., '91) and to the spinal cord (Jackson and Crossman, '83; Swanson et al., '84; Jones and Yang, '85; Jones et al, '86; Goldsmith and van der Kooy, '88; Rye et al., '88; Woolf and Butcher, '89). In the rat and cat, the PPN represents the central component of the mesencephalic locomotor region or MLR (Garcia-Rill, '86; Garcia-Rill et al., '87). These locomotor effects may be mediated either indirectly through reticulospinal pathways (Garcia-Rill and Skinner, '87a, '87b) or directly via spinal connections (Goldsmith and van der Kooy, '88; Rye et al., '88). While both cholinergic and non-cholinergic neurons take part in the PPN projection to the reticular formation, only non-cholinergic neurons project to the spinal cord (Goldsmith and van der Kooy, '88; Rye et al., '88; Woolf and Butcher, '89). In addition, the cholinergic projections to the pontomedullary reticular formation have been implicated

as inducing motor atonia during REM sleep (Woolf and Butcher, '89; Jones, '90; Semba et al., '90). Lesions of the PPN in the rat have been associated with impaired motor functions (Kilpatrick and Starr, '81), and clinical studies have shown an association between PPN cell loss in humans and movement disorders related to progressive supranuclear palsy (Zweig et al., '87; Hirsch et al., '87) and Parkinson's disease (Hirsch et al., '87; Jellinger, '88; Zweig et al., '89).

Although significant advances have been made in our understanding of the PPN since the first experimental study 25 years ago (Nauta and Mehler, '66), new knowledge from a variety of disciplines continues to open avenues for further research. At present it is clear that the PPN contains a heterogeneous population of projection neurons with widespread ascending and descending connections which are involved in a variety of motor as well as non-motor functions. The general sources of afferent input to the PPN have also been identified. However, it is still largely unknown whether the different afferent inputs are evenly distributed to all PPN neurons irrespective of their transmitter content or projection site(s). Furthermore, it is still unclear whether there exist intrinsic interactions among the chemically different subpopulation of the PPN neurons and among the PPN neurons projecting to different targets. Such questions have guided our research efforts which focused mainly on the relationship of the basal ganglia to the PPN. The experiments have been carried out in the rat

which has been the most commonly used experimental animal in the previous investigations on the structure and functions of the PPN.

The major objective of the first study was to determine whether the PPN neurons projecting to the basal ganglia and those projecting to the spinal cord were contained in the same portion of the nucleus, and whether they represent two different populations or a single population of neurons with collateralized axons. In the second study, the distribution and mode of termination of the nigro-pedunculopontine fibers has been analyzed both light and electron microscopically. The third study explored the synaptic organization and the presence of cholinergic elements in the region of the PPN receiving nigral input.

CHAPTER I

The Origin of Ascending and Spinal Pathways from the Nucleus Tegmenti Pedunculopontinus in the Rat

INTRODUCTION

The nucleus tegmenti pedunculopontinus (PPN) has been defined in the human brainstem by Olszewski and Baxter ('54) as a nucleus of "unknown connections" which occupies the ventrolateral part of the caudal mesencephalic tegmentum lateral to the superior cerebellar peduncle. Furthermore, on the basis of the cell densities, the authors distinguished two subdivisions, the subnucleus compactus (PPNc) and the subnucleus dissipatus (PPNd). The nucleus remained obscure until it was discovered that it receives converging input from the basal ganglia and associated nuclei (Nauta and Mehler, '66; Kim et al., '76; Carter and Fibiger, '78; Nauta and Cole, '78; Nauta, '79; Rinvik et al., '79; Beckstead et al., '79; Larsen and McBride, '79; McBride and Larsen, '80; Jackson and Crossman, '81, '83; van der Kooy and Carter, '81; De Vito and Anderson, '82; Beckstead and Frankfurter, '82; Gerfen et al., '82; Parent and DeBellefeuille, '82, '83; Moon-Edley and Graybiel, '83; Gonya-Magee and Anderson, '83; Noda and Oka, '84). These findings suggested that the PPN may be involved in relaying information from the basal ganglia to the motor centers of the medulla and spinal cord.

However, further studies have revealed that the majority of the PPN efferent projections stream rostrally toward the basal ganglia and associated nuclei (De Vito et al., '80; Nomura et al., '80; Saper and Loewy, '82; Parent et al., '83a; Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Gonya-Magee and Anderson, '83; Lutze and Rafols, '84; Sugimoto and Hattori, '84; Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87; Rye et al., '87; Scarnati et al., '87a) thereby establishing feedback loops which provide an excitatory, modulating influence on these nuclei (Gonya-Magee and Anderson, '83; Hammond et al., '83; Scarnati et al., '84).

Recent studies on the anatomy and functions of the pontomesencephalic tegmentum have provided convincing evidence that in the rat and cat, the PPN represents the central component of the mesencephalic locomotor region (MLR) (Garcia-Rill, '86). Furthermore, it has been repeatedly demonstrated that the PPN gives rise to descending projections to the medullary reticular formation (Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Garcia-Rill, '86; Rye et al., '88) and to the spinal cord (Jackson and Crossman, '83; Lutze and Rafols, '84; Swanson et al., '84; Jones and Yang, '85; Jones et al., '86; Goldsmith and van der Kooy, '88; Rye et al., '88). These findings substantiate early suggestions that, via connections with the PPN, the basal ganglia may control some aspects of locomotion circumventing the thalamus and cerebral cortex. In addition,

since the PPN is reciprocally connected with various hypothalamic nuclei and limbic regions of the forebrain (Jackson and Crossman, '81; Moon-Edley and Graybiel, '83; Parent and DeBellefeuille, '83; Swanson et al., '84), it has been proposed that the two descending PPN pathways may play an important role in mediating locomotor responses associated with various behaviors subserved by the hypothalamic and limbic regions (Swanson et al., '84).

The interest in the connectivity and functions of the PPN has been further intensified by the results of immunohistochemical studies which have revealed that the pontomesencephalic region including the PPN contains a group of cholinergic neurons designated as the Ch5 sector by Mesulam et al. ('83). These cholinergic neurons appear to contribute to the descending PPN projections to the medullary reticular formation (Garcia-Rill and Skinner, '87; Goldsmith and van der Kooy, '88; Rye et al., '88) and to the ascending projections to the basal ganglia, hypothalamus and limbic regions (Sugimoto and Hattori, '84; Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87). However, they also project heavily to the thalamus (Sugimoto and Hattori, '84; Isaacson and Tanaka, '86; Woolf and Butcher, '86; Rye et al., '87) and provide widespread cholinergic innervation of functionally different groups of thalamic nuclei (Hallanger et al., '87). Thus it seems that the PPN functions are more diversified in that in addition to the locomotor function, the PPN may also be implicated in the

control of thalamocortical transmission and cortical activity (Hallanger et al., '87; Rye et al., '87).

The present study has focused on the relationships of the PPN to the basal ganglia and to the spinal cord. In particular, it analyzes the distribution patterns and morphological features of the two populations of PPN projection neurons, and examines the possibility that some PPN axons may collateralize into long ascending and descending branches which would innervate both the basal ganglia and spinal cord. A preliminary account of this work has been published (Spann and Grofova, '84).

MATERIAL AND METHODS

A total of 25 male Sprague-Dawley albino rats (275-350 g) were utilized for this study. Of these, 12 received injections of horseradish peroxidase (HRP) or wheat germ agglutinin-conjugated horseradish peroxidase (HRP/WGA) into the basal ganglia or cervical cord, while 13 received injections of fluorescent dyes Granular Blue (GB) and Diamidino Yellow Dihydrochloride (DYD) into the same targets. Animals were anesthetized with sodium pentobarbital (50-100 mg/kg, i.p.) and atropine sulfate solution (0.7 mg/kg) was administered intramuscularly in order to prevent brain edema. Pressure injections of tracers were made using 5 µl Hamilton syringes fitted with 26 gauge bevelled needles and mounted in a holder of a Kopf stereotaxic instrument. Stereotaxic coordinates were derived from the atlas of Paxinos and Watson ('82). Injections into the spinal cord were made under direct visual control following laminectomy.

HRP experiments

In the first series of experiments (n=5) multiple bilateral injections of 30% HRP (Sigma VI) in 2% DMSO solution (n=3) or of 2.5% HRP/WGA solution (n=2) were made in the cervical cord (C3-C6 segments). The second group of animals (n=6) received single injections of 2.5% HRP/WGA in 1% DMSO and 1% Poly-L-Ornithine solution in one of the

following basal ganglia nuclei: entopeduncular nucleus, globus pallidus, striatum, substantia nigra and subthalamic nucleus. In one rat, large multiple HRP injections were made bilaterally in the region containing the entopeduncular and subthalamic nuclei and the substantia nigra in order to achieve maximal labeling of PPN neurons projecting to the forebrain. Volumes of HRP/WGA and HRP solutions injected at each injection site ranged from 0.1 to 0.4 µl. Following 2-4 days survival, the animals were deeply anesthetized with sodium pentobarbital and perfused transcardially with a physiological saline solution followed by a fixative consisting of 1% paraformaldehyde and 2% glutaraldehyde in 0.15M phosphate buffer, pH 7.2. Sodium heparin was administered prior to the perfusion. Brains and spinal cords were promptly removed and stored overnight in cold (4°C) phosphate buffer. Blocks of tissue containing the injection sites and the brainstems were cut at 50 µm on a vibratome either in sagittal or coronal planes. Serial sections were collected in cold phosphate buffer and reacted immediately with tetramethyl benzidine according to the protocol of Mesulam ('82). Sections were then mounted on gel coated slides, air-dried, counterstained with neutral red and examined by brightfield and, in some instances, polarized darkfield microscopy. The distribution of labeled cells in selected sections was charted on projection drawings. In representative cases the somatic areas and maximum diameters of labeled cells which exhibited distinct nucleus were

measured using a Leitz Orthoplan microscope equipped with a drawing tube and a Nikon image analysis system. Sizes of neurons were described with reference to their somatic areas as small (125 μm^2), medium (≥125 μm^2, but 350 μm^2) and large (≥350 μm^2).

Fluorescent dyes experiments

The fluorescent dyes were dissolved in physiological saline as a 2.5% solution (GB) or as a 1% solution (DYD) and injected bilaterally into the basal ganglia nuclei and cervical cord in different combinations. Five rats received injections of GB into the spinal cord (C4-C5) and DYD into the entopeduncular - subthalamic nuclei region while eight rats received DYD injections into the spinal cord and GB into the entopeduncular - subthalamic nuclei region. A total of approximately 1.6-4.0 µl of dye solution was injected into each target.

After postoperative survival of 4-8 days, the deeply anesthetized animals were perfused with a fixative consisting of 10% formalin in physiological saline. The brains and cervical cords were immediately dissected out and placed for 36-48 hrs. in graded series of cold (4°C) sucrose solution in 0.2M cacodylate buffer (pH 7.3). The brains and portions of the spinal cord containing the injections were cut in 40 µm thick sagittal or coronal sections on a freezing microtome. Serial sections were mounted on gel-coated slides, air-dried and coverslipped using Entellan mounting medium. The slides

were stored in the dark at 4°C and examined with a Leitz Orthoplan microscope equipped with Ploemopack fluorescence filter system A to provide an excitation light of 360 nm. At this wavelength, GB and DYD emit a blue and yellow fluorescence, respectively. The GB- and DYD- labeled cells are readily distinguishable from one another since, in addition to the differences in color, the GB is selectively labeling the cytoplasm while the DYD is preferentially concentrated in the nucleus. If single cells exhibited a distinctly yellow nucleus as well as silver-gold granules in a deep blue cytoplasm they were considered double-labeled.

In representative cases, the distribution of single- and double-labeled cells in the pontomesencephalic region was recorded on standard projection drawings of sagittal Nissl stained sections through the PPN using major vessels, fiber systems and surface outlines as landmarks. In addition, the distribution of single- and double-labeled cells in the PPN was analyzed on montages of low-power photomicrographs of fluorescent sections. Finally, double labeled neurons in the PPN were counted in every section.

Complete series of Nissl stained sagittal and coronal sections through the rat brain were utilized to study the cytoarchitecture and delineation of the PPN.

RESULTS

Previous studies in the cat (Nomura et al., '80; Gonya-Magee and Anderson, '83; Moon-Edley and Graybiel, '83) and rat (Jackson and Crossman, '81, '83; Hammond et al., '83; Swanson et al., '84; Rye et al., '87, '88; Beninato and Spencer, '87; Clarke et al., '87) have explored connections of the PPN using coronal and horizontal sections. It appears that there is virtually no general consensus as to the delineation and subdivisions of the PPN in the carnivore and rodent brains. Therefore, it was deemed necessary to define the boundaries of the rat PPN which were adopted in the present study. The following description is based on the cytoarchitectural differences distinguishing the nucleus from the surrounding structures in Nissl-stained sagittal sections (Figs. 1A-D).

As in other species, the rat PPN is incorporated in a continuous cell column surrounding the ascending limb of the superior cerebellar peduncle and consists of two subdivisions, the larger subnucleus dissipatus (PPNd) and smaller subnucleus compactus (PPNc). The PPN is medial to the lateral lemniscus and associated nuclei and lateral to the decussation of the superior cerebellar peduncle. The lateral-most part of the PPN (Figure 1A) consists of a rather loose collection of medium-sized and small neurons identified as the PPNd and positioned caudal to the retrorubral nucleus. The PPNc is composed of a small group of larger size neurons

abutting the caudal border of the PPNd and the rostrolateral border of the dorsal parabrachial nucleus. Approximately 300 μm more medially (Figure 1B) the nucleus expands in a rostrocaudal direction and both subdivisions are now fully recognizable. At this level, the PPNc reaches its maximal dimensions and contains a prominent number of larger, darkly stained cells which represents the caudal one-third of the nucleus. The PPNc can be easily delineated from the adjacent cuneiform and dorsal parabrachial nuclei at this level since the latter two nuclei are composed of primarily small densely-packed neurons. On the other hand, the boundaries of the rostroventrally situated PPNd are less distinct. This portion of the PPN consists of loosely arranged predominantly medium-sized and small neurons with fusiform or polygonal cell bodies with the long axes of the elongated neurons often directed parallel to the fibers of the superior cerebellar peduncle. The PPNd represents the principal area of the nucleus at all levels examined (Figs. 1A-D). It borders dorsally on the mesencephalic reticular formation, rostrally on the retrorubral nucleus and the retrorubral field, ventrally on the nucleus reticularis pontis oralis, and caudally on the dorsal and ventral parabrachial nuclei and to a small extent, the subpeduncular tegmental nucleus (Figure 1D). The nucleus reticularis pontis oralis and to a lesser degree the mesencephalic reticular formation harbor a contingent of large multipolar neurons with intensely stained Nissl granules. The presence of these large cells guided the

delineation of the ventral and rostrodorsal boundaries of the PPNd. In addition, at the medialmost level (Figure 1D), the mesencephalic reticular formation adjacent to the PPNd is traversed by fibers coursing toward the superior colliculus and the majority of reticular cells are oriented parallel to the course of these fibers. Rostrally, the PPNd is separated from the substantia nigra by the retrorubral nucleus in the lateralmost section and the retrorubral field more medially. The retrorubral nucleus consists of compact groups of small to medium-sized cells and can be rather easily distinguished from the PPNd. The retrorubral field has a similar cell composition to that of the PPNd. However, the cells are more densely arranged and exhibit a prominent rostrocaudal orientation parallel to the fibers of the superior cerebellar peduncle (Figure 1B).

HRP injections in the spinal cord

Following injections of either HRP or HRP/WGA into the cervical segments of the spinal cord, retrogradely-labeled neurons were observed in various brainstem nuclei known to give rise to spinal projections. Although the general pattern of labeling was similar in all experiments, the number of labeled cells and the intensity of labeling varied. The following description is therefore based mainly on the observations from two animals with bilateral HRP/WGA injections in the C5 and C6 segments which yielded the most prominent labeling. In these animals, the reaction product

filled the entire segment of the spinal cord and involved both gray and white matter.

Large numbers of intensely labeled neurons were observed in the lateral vestibular nucleus and in the nucleus pontis oralis and caudalis of the reticular formation. While a compact group of prominently-labeled cells was present in the medial portion of the red nucleus, only an occasional labeled cell was noted within either the retrorubral nucleus or field. The deep layers of the superior colliculus contained a small number of lightly labeled cells and occasionally labeled neurons were also seen in the mesencephalic reticular formation. In the parabrachial nuclei, the majority of labeled neurons were found within the ventral parabrachial nucleus. The PPN contained a scattered group of lightly labeled cells.

Although the HRP-labeled neurons were seen in both subnuclei of the PPN (Figure 2A-D), the majority were located within the PPNd and often in close proximity to the fibers of the superior cerebellar peduncle. A few labeled neurons within the PPNc were distributed within the ventral border region of the subnucleus (Figure 2B). The number of labeled PPNd neurons slightly increased medially. The overall number of retrogradely labeled PPN neurons was significantly lower when compared to the labeled cells within the nucleus pontis oralis and caudalis of the reticular formation. However, the number was substantially greater when compared to the labeled

neurons located within either the mesencephalic reticular formation or parabrachial nuclei.

The labeled PPN neurons had either polygonal (Figure 5A) or fusiform (Figure 5B) cell bodies ranging in size from small to large. In one of the cases morphometric analyses revealed that the measured neurons ranged between 65 μm^2 and 390 μm^2 (mean area: 188 ± 80 μm^2). In addition, maximum diameters were taken of all neurons with measured somatic areas and these measurements ranged from 15.2 to 41.0 μm.

Retrograde labeling of PPN cells following HRP or HRP/WGA injections in the basal ganglia

Following single unilateral injections of HRP/WGA in the striatum, globus pallidus, entopeduncular nucleus, subthalamic region or the substantia nigra, the PPN on the ipsilateral side invariably contained retrogradely-labeled neurons. In agreement with a previous report (Jackson and Crossman, '83), labeled cells were most numerous following injections in the substantia nigra, subthalamic nucleus and entopeduncular nucleus and least numerous following injections in the striatum and the globus pallidus. The HRP labeled cells were present in both subdivisions of the PPN, but the majority were localized in the PPNd. A moderate number of labeled cells showing a similar distribution was also observed in the contralateral PPN.

In order to compare the distribution, morphology and numbers of PPN neurons projecting to the basal ganglia and to

the cervical cord, multiple bilateral injections of HRP were made in the major targets of the ascending PPN efferents. The localization of the injection sites is illustrated in Figure 4, and the distribution of the labeled cells in the pontomesencephalic tegmentum is shown in Figure 3A-D. Caudal injections were centered in the substantia nigra and the rostral injections were placed in the entopeduncular nucleus or in the subthalamic region. On both sides, the HRP reaction product was observed to encompass the entire subthalamic region and posterolateral thalamus as well as the adjacent internal capsule and cerebral peduncle. These injections resulted in prominent bilateral retrograde and anterograde labeling in the pontomesencephalic region. In particular, numerous labeled cells were present in the retrorubral nucleus and field, the PPN, and the parabrachial nuclei. However, the quantity of labeled neurons within the parabrachial nuclei was less than observed in either the PPN or retrorubral nucleus and field. Since the aforementioned regions are closely associated with the superior cerebellar peduncle, the distribution of labeled cells within these regions gave the appearance of one continuous system extending from the caudal border of the substantia nigra to the rostral half of the parabrachial nuclei. In addition, labeled cells were seen in the cuneiform nucleus, the mesencephalic reticular formation, and the pontine reticular nuclei.

The distribution of labeled cells in the PPNd was similar to that observed after single HRP/WGA injections in the basal ganglia and related nuclei (Figs. 3A-D). However, both lateral and medial portions of PPNc also contained a substantial number of labeled cells (Figs. 3A and B). The HRP-labeled PPN cells had either polygonal or fusiform cell bodies and ranged in size from small to large (Figs. 5A and B). Overall, the fusiform cells were less numerous than the polygonal cells and their long axes were usually aligned parallel to the fibers of the superior cerebellar peduncle. In general, cells within the PPNc tended to be larger than the cells in the PPNd. Histograms displaying the results of the morphometric analyses of the labeled cells are shown in Figure 6. Briefly, the cross-sectional somatic areas ranged between 114 μm^2 and 472 μm^2 (mean area: 229 ± 75 μm^2). Maximum diameters were also taken of all neurons with measured soma areas, and these measurements ranged from 13.5 to 55.2 μm. A quantitative comparison of labeled cells in this experiment and in the representative experiment involving injections of the retrograde tracer in the cervical cord revealed that the PPN cells projecting to the forebrain outnumber 5.4 times those projecting to the spinal cord.

Double-labeling experiments

In these experiments, the retrograde fluorescent tracers GB and DYD were employed for simultaneous labeling of the spinal cord and forebrain-projecting PPN neurons. The GB was

injected bilaterally into the lower segments of the cervical cord while bilateral injections of DYD were made into the entopeduncular nucleus and subthalamic region, or vice versa. Around all injection tracks the dyes appeared as brilliantly fluorescent, structureless masses which completely obscured the morphological features of both the gray and white matter. Adjacent to these regions, the injection spread of the dyes exhibited an intense fluorescence and contained numerous labeled neurons and glial cells. The border region of the injection spread contained only labeled glial cells and faded abruptly into the non-fluorescent tissue. Injections of the GB were often associated with a central necrotic zone and tended to spread more than the DYD injections.

In seven out of thirteen animals, both spinal cord and forebrain injections of the dyes were comparable in size and location to the previously described representative HRP/WGA and HRP injections. In the spinal cord the dyes spread throughout at least one segment and in the forebrain the GB injections involved the entire region of the rostral HRP injection while the DYD was confined primarily within the inner zone of the HRP injection (see Figure 4A). No appreciable difference was observed in the distribution or quantity of labeled neurons within the PPN. The following description of the distribution of labeled neurons in the PPN is based on observations obtained from an experiment in which GB was injected into the forebrain and DYD into C5 and C6 spinal segments (Figure 7). As expected from the HRP

experiments, the GB-labeled cells greatly outnumbered (82% of all labeled PPN neurons) the DYD labeled PPN neurons (14%), and were found throughout the entire anteroposterior and mediolateral extent of the nucleus in both PPNd and PPNc. The DYD-labeled neurons projecting to the spinal cord were present mainly throughout the medial-half (Figure 7B) of the PPNd, and were randomly distributed among the GB-labeled cells. The PPNc contained only an occasional DYD-labeled cell in its ventral-most portion (Figure 7A). The dorsolateral portion of PPNc consistently contained only forebrain projecting neurons. In addition to single-labeled cells, a few double-labeled cells were observed in the intermediate and medial portions of the PPNd. These double-labeled cells were predominantly polygonal in shape and medium to large in size (Figure 5C) and composed less than 5% of the total population of labeled neurons.

DISCUSSION

The present study has focused on the distribution and morphological features of PPN neurons projecting to the forebrain and to the spinal cord. Particular attention has been paid to the relative strength of these connections and to the possibility that they at least partially originate from the same neurons. Since the results were gathered from different experiments and involved quantifying the data, it was essential to define the boundaries and subdivisions of the rat PPN which can be reliably and consistently seen in our preparations.

Cytoarchitecture of the PPN

Since Olzewski and Baxter in '54 defined the nucleus tegmenti pedunculopontinus in human brainstem, numerous studies have explored the connectivity, chemistry and functions of this nucleus. In primates, the PPN stands out as a prominent group of medium-sized and large cells surrounding the decussation of the superior cerebellar peduncle in the pontomesencephalic junction, and both subdivisions, the PPNc and PPNd, are readily identifiable. Unfortunately, this nucleus is far less distinct in non-primate species, particularly in the rat and cat which have been utilized in the majority of experimental studies. Although most authors apply the term PPN to a region of the pontomesencephalic tegmentum cupping the ascending limb of

the superior cerebellar peduncle, there is virtually no consensus as to the extent and subdivisions of the nucleus. In fact, in many reports on the connectivity of the PPN the boundaries of the nucleus remain quite elusive (Nomura et al., '80; Jackson and Crossman, '81, '83; Saper and Loewy, '82; Gonya-Magee and Anderson, '83; Hammond et al., '83; Scarnati et al., '84, '87a; Clarke et al., '87). Other investigators extended the boundaries of the PPN to include adjoining portions of the mesencephalic reticular formation (Swanson et al., '84) or the rostroventral region of the retrorubral field dorsal to the substantia nigra (Paxinos and Butcher, '85; Beninato and Spencer, '87; Rye et al., '87).

Attempts to correlate the two distinct primate subdivisions of the PPN with those present in nonprimate species have created further confusion. Experimental observations in the monkey have revealed that the PPNc is the major target of pallidotegmental (Nauta and Mehler, '66; Kim et al., '76), subthalamotegmental (Carpenter and Strominger, '67; Nauta and Cole, '78), and nigrotegmental (Carpenter et al., '81; Beckstead and Franfurter, '82; Beckstead, '83) fibers. Disregarding the actual cytoarchitecture of the nucleus, the term PPNc has then been used to designate the area of termination of the basal ganglia efferents in the cat and rat (Nauta, '79; Beckstead et al., '79; Larsen and McBride, '79; McBride and Larsen, '80; Beckstead, '83; Moon-Edley and Graybiel, '83). Observations from our laboratory (Grofova and Spann, '87; Spann and Grofova, '88) as well as

work of others (Rye et al., '87) indicate that in the rat the majority of PPN afferents from the substantia nigra terminate more medially in the subnucleus dissipatus of the present description. The subnucleus compactus, characterized by higher neuronal density and a prominent contingent of larger neurons, represents only a smaller dorsolateral and caudal part of the PPN. Our delineation of the PPNc in the rat is consistent with previous descriptions in the same species by Sugimoto and Hattori ('84) and Swanson et al. ('84).

The advent of histochemical and immunocytochemical methods has added a new dimension to the characterization of regional boundaries and connections. A large body of evidence now exists which documents that the pontomesencephalic tegmentum contains numerous cholinergic neurons (Armstrong et al., '83; Mesulam et al., '83, '84; Vincent et al., '83; Butcher and Woolf, '84; Sugimoto and Hattori, '84; Paxinos and Butcher, '85; Satoh and Fibiger, '85; Sofroniew et al., '85; Isaacson and Tanaka, '86; Woolf and Butcher, '86; Beninato and Spencer, '87; Hallanger et al., '87; Jones and Beaudet, '87b; Rye et al., '87). This group of cholinergic neurons is consistently present in different species including primates has been labeled the Ch5 sector (Mesulam et al., '83). The Ch5 sector encompasses primarily the retrorubral region, the PPN, and rostralmost portions of the parabrachial nuclei. In a recent study, Rye and co-workers ('87) employed Nissl-stained preparations, ChAT immunohistochemistry, and anterograde and retrograde

tracing techniques to redefine the boundaries of the rat PPN. In their definition, the PPN consists exclusively of large, multipolar neurons all of which stain immunohistochemically for ChAT. Since such cells are not present within the medial PPN region which receives most of the basal ganglia afferents, Rye and co-workers ('87) labeled this region the midbrain extrapyramidal area (MEA) and excluded it from the PPN. The definition of PPN presented by these authors appears controversial and unorthodox. Evidence presented by Rye and co-workers ('87) as well as by other investigators clearly shows that in the rodent (Mesulam et al., '83; Beninato and Spencer, '86), carnivore (Isaacson and Tanaka, '86; Jones and Beaudet, '87b) and primate (Mesulam et al., '84) both subnuclei of the PPN, particularly the PPNd, contain a substantial number of smaller noncholinergic neurons. Both smaller noncholinergic neurons and larger cholinergic cells are intermingled within the same territory. In accordance with traditional classification schemes, we have defined the nucleus as consisting of all cell populations within a given region, irrespective of their sizes, chemistry, and projections.

Forebrain-projecting vs. spinal cord-projecting PPN neurons.

1) Ascending Neurons

There exists a general agreement that the PPN gives rise to prominent and widespread ascending projections. These include several components which are involved in different circuitries and consist of both cholinergic and non-cholinergic fibers. The two most prominent components of the ascending system are PPN projections to the basal ganglia and related nuclei (Nauta and Cole, '79; Rinvik et al., '79; De Vito et al., '80; Nomura et al., '80; Carpenter et al., '81; Gerfen et al., '82; Saper and Loewy, '82; Gonya-Magee and Anderson, '83; Hammond et al., '83; Jackson and Crossman, '83; Lutze and Rafols, '84; Scarnati et al., '84, '87b; Sugimoto and Hattori, '84; Jones and Yang, '85; Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87; Rye et al., '87), and PPN projections to various thalamic nuclei (Nomura et al., '80; Gerfen et al., '82; Saper and Loewy, '82; Jackson and Crossman, '83; Mesulam et al., '83; Moon-Edley and Graybiel, '83; Parent and DeBellefeuille, '83; Sugimoto and Hattori, '84; Jones and Yang, '85; Isaacson and Tanaka, '86; Woolf and Butcher, '86; Hallanger et al., '87). The primary aim of the present experiments was to label PPN cells projecting to the basal ganglia. The distribution of labeled PPN cells following single unilateral injections of HRP/WGA in the striatum, globus pallidus, entopeduncular nucleus, subthalamus or substantia nigra closely resembled

the documentation presented by previous authors (Jackson and Crossman, '83; Woolf and Butcher, '86). Most of the labeled cells were contained within the territory of the subnucleus dissipatus with only a few located within the ventralmost region of the subnucleus compactus. There was no apparent segregation of such cells in a particular region of the PPNd which would substantiate a delineation of the midbrain extrapyramidal area. In addition, recent observations on the presence of cholinergic fibers in the PPN projections to the substantia nigra (Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87) and other basal ganglia nuclei (Sugimoto and Hattori, '84; Woolf and Butcher, '86) strongly argue against the concept of the MEA postulated by Rye and coworkers ('87). In agreement with previous studies (Jackson and Crossman, '83; Moon-Edley and Graybiel, '83), the numbers of labeled PPN cells were highest following substantia nigra and subthalamic injections and lowest following striatal injections. A moderate number of labeled cells was also observed in the contralateral PPN. Based on these findings, large bilateral injections of HRP into the entopeduncular nucleus subthalamus and substantia nigra were performed with the intention to label all PPN neurons projecting to the basal ganglia. However, there was a considerable spread of the HRP solutions within the subthalamic region, posterior thalamus and internal capsule. Therefore, it is likely that the labeled PPN neurons included also some cells projecting to the thalamus (see above for

references), hypothalamus (Jackson and Crossman, '81; Berk and Finkelstein, '81; Moon-Edley and Graybiel, '83; Parent and de DeBellefeuille, '83; Woolf and Butcher, '86) and basal forebrain (Woolf and Butcher, '86). This probably accounts for the presence of numerous labeled cells within the subnucleus compactus which contained only occasional labeled cells following single injections of the tracer in various basal ganglia nuclei. Most of these ascending neurons were medium-sized and multipolar in shape (67%), with only a moderate contribution of small and large neurons. Labeled PPN neurons with a similar distribution and morphology were encountered following injections of the fluorescent dyes in the entopeduncular nucleus and subthalamic region which invariably involved adjacent thalamic regions. Observations from these double-labeling experiments further support our assumption that the morphometric analysis of PPN neurons labeled following multiple HRP injections in the basal ganglia nuclei also included a proportion of PPN neurons projecting to other targets.

2) Descending neurons

Since the PPN has been identified as a major component of the mesencephalic locomotor region (Garcia-Rill, '86), descending projections involved in the mediation of locomotor effects have been thoroughly investigated. Most authors agree that the PPN gives rise to prominent descending connections terminating in the pontomedullary reticular

formation (Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Garcia-Rill, '86; Rye et al., '88). Although there exists no morphological evidence that the PPN efferents actually synapse onto the reticulospinal neurons, physiological data strongly support this view (Garcia-Rill and Skinner, '87a). In addition, the locomotor effects may also be mediated through a direct projection descending from the PPN to the spinal cord. Such a projection has been repeatedly demonstrated in the rat (Jackson and Crossman, '83; Lutze and Rafols, '84; Swanson et al., '84; Jones and Yang, '85; Jones et al., '86; Rye et al., '88; Goldsmith and van der Kooy, '88) and confirmed in the present experiments. Injections of three different retrograde tracers in the cervical cord invariably labeled some PPN neurons. However, the number of labeled cells was always less than that following forebrain injections of the same tracers. In fact, counts from both representative HRP experiments and double-labeling experiments were fairly consistent and indicated that the ascending PPN neurons outnumbered the spinal cord-projecting cells by a ratio greater than 5:1. Furthermore, there was no noticeable difference in the distribution of spinal cord-projecting PPN neurons in the representative cases. The majority of labeled cells was spread throughout the PPNd with a moderate preponderance in the medial half of the nucleus. The relationship between the distribution patterns of the forebrain-projecting and spinal cord-projecting PPN neurons was clearly illustrated in the

double-labeling experiments. It appears that both populations of PPN projection neurons are intermingled throughout the entire subnucleus dissipatus. However, the subnucleus compactus is overwhelmingly composed of ascending neurons. Observations from single HRP injections in the basal ganglia nuclei suggest that most of the ascending PPNc neurons do not distribute to the basal ganglia. Consequently, we agree with the conclusion of Lutze and Rafols ('84) and Rye et al. ('87) that the PPN projections to the basal ganglia and to the spinal cord originate from the same region of the pontomesencephalic tegmentum. This region coincides with the entire PPNd as delineated in the present study, and appears to be more extensive than the midbrain extrapyramidal area (Rye et al., '87). We also agree that there are no striking morphological differences between the two populations of PPN projection neurons. However, the spinal cord-projecting PPN neurons contain a proportion of small fusiform cells which do not appear to project to the forebrain.

Collateralization of PPN neurons

The diversity of PPN projections and their diffuse origin from morphologically similar cell types suggest that there may exist a great deal of collateralization. This may involve axonal branching into long ascending and descending collaterals, branching of ascending and/or descending axons

into different targets, and axonal branching into ipsilateral and contralateral target nuclei.

In view of the similarities in the distribution and morphology of the spinal cord-projecting and basal ganglia-projecting cells, it was considered likely that these connections might be partially composed of long ascending and descending collateral branches of a single population of PPN projection neurons. However, only 4% of the total number of labeled cells within the PPN were double-labeled following injections of fluorescent dyes into the cervical cord and the entopeduncular nucleus and subthalamic region. The double-labeled cells were predominantly larger multipolar neurons which were randomly distributed throughout the medial two-thirds of the PPNd. Injections of the same fluorescent dyes into the cervical cord and parafascicular thalamic nucleus (unpublished observations) resulted in less than 1% of double-labeled PPN cells. The latter observation is in full agreement with the results of similar experiments performed in different laboratories (Jones and Yang, '85; Goldsmith and van der Kooy, '88). In a recent study, Garcia-Rill and coworkers ('86) analyzed collateralization of neurons in the mesencephalic locomotor region, i.e., PPN and cuneiform nucleus, into branches ascending either to the substantia nigra, or to the entopeduncular nucleus/subthalamus, or to the intralaminar thalamic nuclei, and into descending branches to the medullary reticular formation. Their data indicate that less than 2% of labeled

neurons possess branched axons. In conclusion, there exists a general consensus that the ascending and descending PPN projections originate largely from separate cell populations.

Several studies have examined collateralization of fibers ascending from the pontomesencephalic tegmentum to the basal ganglia. It appears that in the monkey (Parent et al., '83a) as well as in the rat (Scarnati et al., '87a) only a very small population of the PPN neurons project to two different basal ganglia nuclei. Accordingly, only 5% of the cholinergic neurons located in both the PPN and dorsolateral tegmental nucleus appear to collateralize to the substantia nigra and caudate-putamen (Woolf and Butcher, '86). On the other hand, 8% to 18% of these cholinergic neuons provide afferents to both the thalamus and one of the following: the basal forebrain, lateral hypothalamus or the subthalamus (Woolf and Butcher, '86). The collateralization pattern of cholinergic axons distributing to different groups of the thalamic nuclei is at present unknown. However, in a pioneer study in the monkey, Parent and DeBellefeuille ('83) failed to demonstrate double-labeled cells in the PPN region following injections of fluorescent tracers in the VA/VL nuclei and CM/PF complex. This observation suggests that regardless of the chemical nature, the PPN projections to functionally different thalamic nuclei originate from separate cell populations. This also seems to be true for the ipsilateral and contralateral PPN projections to the homonymous thalamic nuclei (Spann and Grofova, '85). Thus

the overwhelming majority of the widespread ascending efferent connections of the PPN seem to originate from specific cells which are intermingled within the nucleus. The lack of a significant collateralization in a system which is known to contain a prominent component of cholinergic fibers is quite surprising since it has been stated that the pontomesencephalic cholinergic neurons show a tendency to collateralize extensively (Woolf and Butcher, '86).

The branching patterns of the descending PPN projections have only recently began to be elucidated. Studies focused on the cholinergic nature of these connections have ruled out the possibility of massive collateralization within the reticular and spinal fiber systems since only the reticular, but not the spinal fibers were found to contain acetylcholine (Goldsmith and van der Kooy, '88; Rye et al., '88). The absence of branched reticular and spinal neurons was further confirmed by double labeling experiments utilizing retrograde transport of fluorescent dyes (Goldsmith and van der Kooy, '88). The degree of collateralization within the PPN projections to different reticular nuclei remains to be established. Our recent observations suggest that at least some of the PPN axons descending to the medulla give off collaterals to the pontine reticular nuclei (Grofova and Spann, '88). Thus it seems that there exists at least two chemically and probably also functionally different descending systems: A direct projection to the spinal cord which has not yet been chemically characterized, and an

indirect PPN-reticular projection which contains a significant proportion of cholinergic fibers.

SUMMARY AND CONCLUSIONS

The nucleus tegmenti pedunculopontinus represents a poorly delineated, but morphologically and functionally distinct portion of a continuous cell column wrapped around the superior cerebellar peduncle in the pontomesencephalic junction. In spite of its widespread projections which to a certain degree mimic the connectivity of the surrounding reticular nuclei, it appears unique by virtue of its reciprocal connections with the basal ganglia. It contains a morphologically and chemically heterogenous population of neurons, but the dominant and best known components are relatively large cholinergic cells. The latter are included in Ch5 sector of Mesulam ('83) which extends throughout the rostral pons and caudal midbrain and encompasses some other nuclei. Therefore the presence of cholinergic neurons can hardly be used as a reliable marker for the delineation of PPN. The complex output organization of the PPN together with a remarkable paucity of collateralization between and within the major outflow systems suggest that the nucleus is involved in a variety of functions. These are probably associated with specific subpopulations of PPN neurons which do not exhibit any distinct morphological differences or definite spatial segregation. It is still largely unknown whether the different subpopulations of PPN projection neurons receive the same afferent input. Ultrastructural and neurophysiological studies are now needed to dissect the

circuits linking the identified output neurons with the specific PPN afferents. Such information in conjunction with chemical characterization of these circuits are essential for our understanding of the entire spectrum of PPN functions.

Figure 1: Location and Subdivisions of the PPN.

Low power photomicrographs of Nissl-stained sagittal sections of the lateral (A) through medial (D) levels of the pontomesencephalic region showing the location and subdivisions of the PPN. A distance of 250 to 300 µm separates any two adjacent sections. Scale bar: 250 µm.

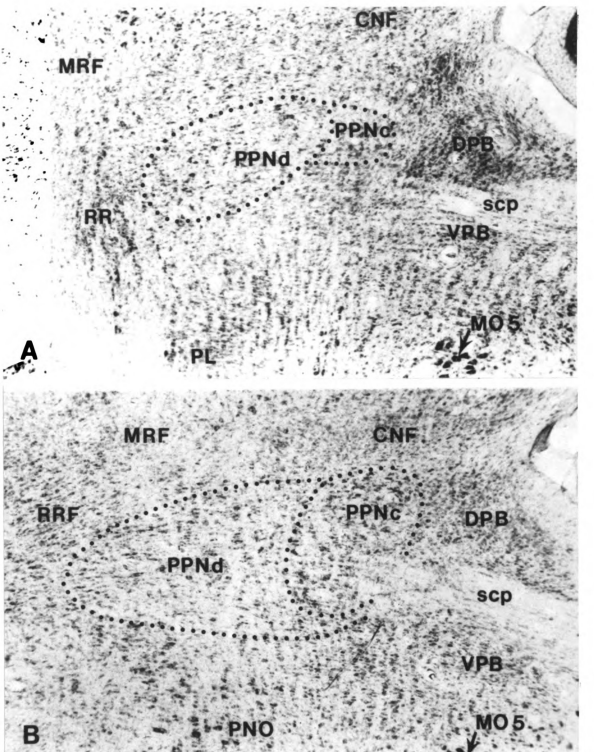
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Figure 1

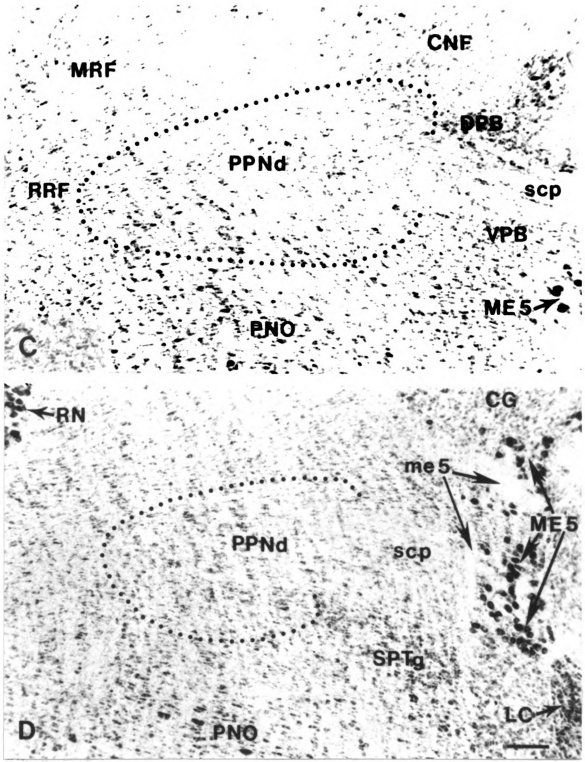

Figure 1 (continued)

Figure 2: Distribution of Descending Projecting Neurons of the Pontomesencephalic Tegmentum.

Standard projection drawings of sagittal sections of the lateral (A) through medial (D) levels of the pontomesencephalic tegmentum showing the distribution of retrogradely-labeled neurons in the PPN and adjacent structures in a representative experiment. In this figure are plotted neurons which were labeled by retrograde transport of HRP/WGA from the cervical cord. Each dot represents four labeled cells.

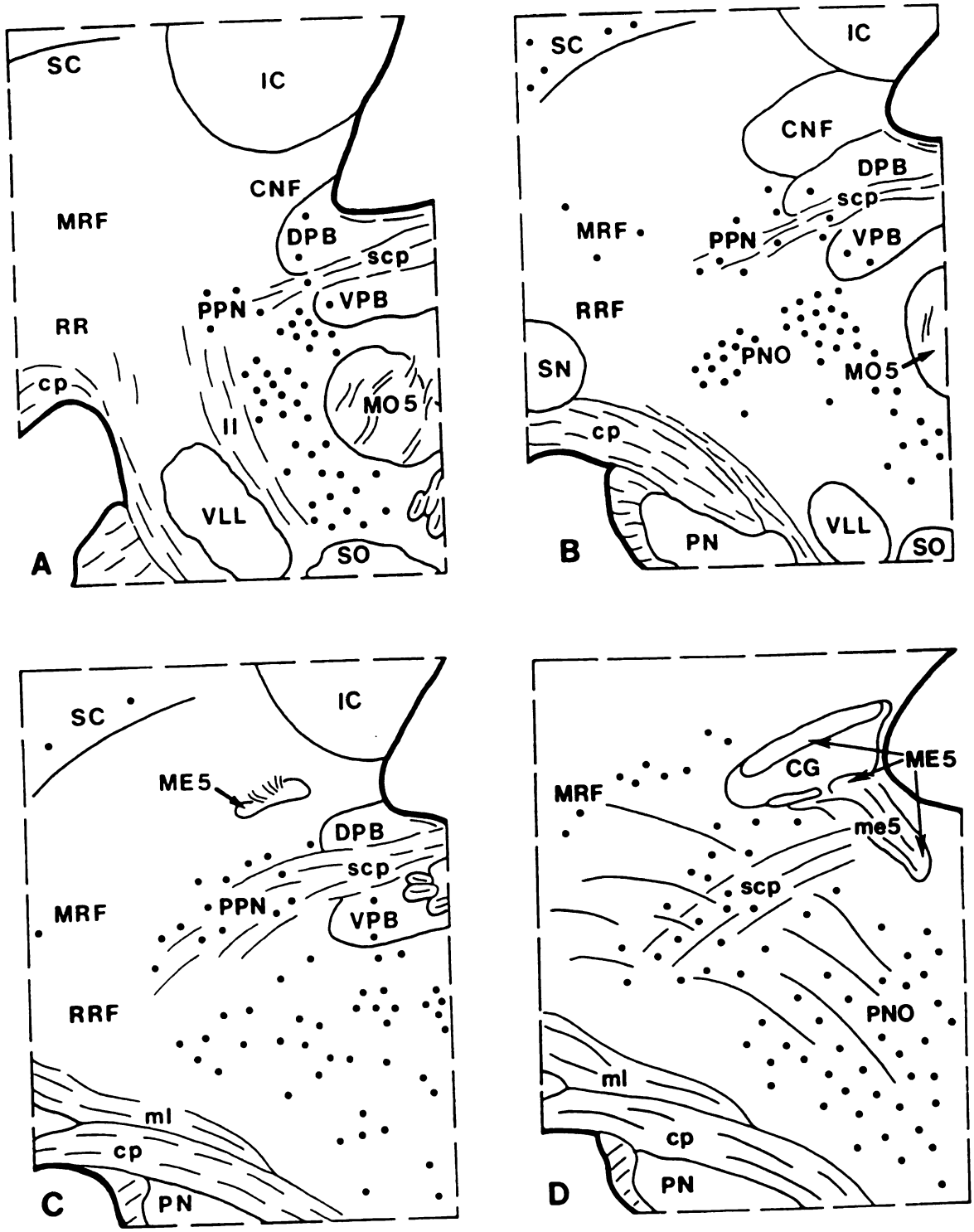
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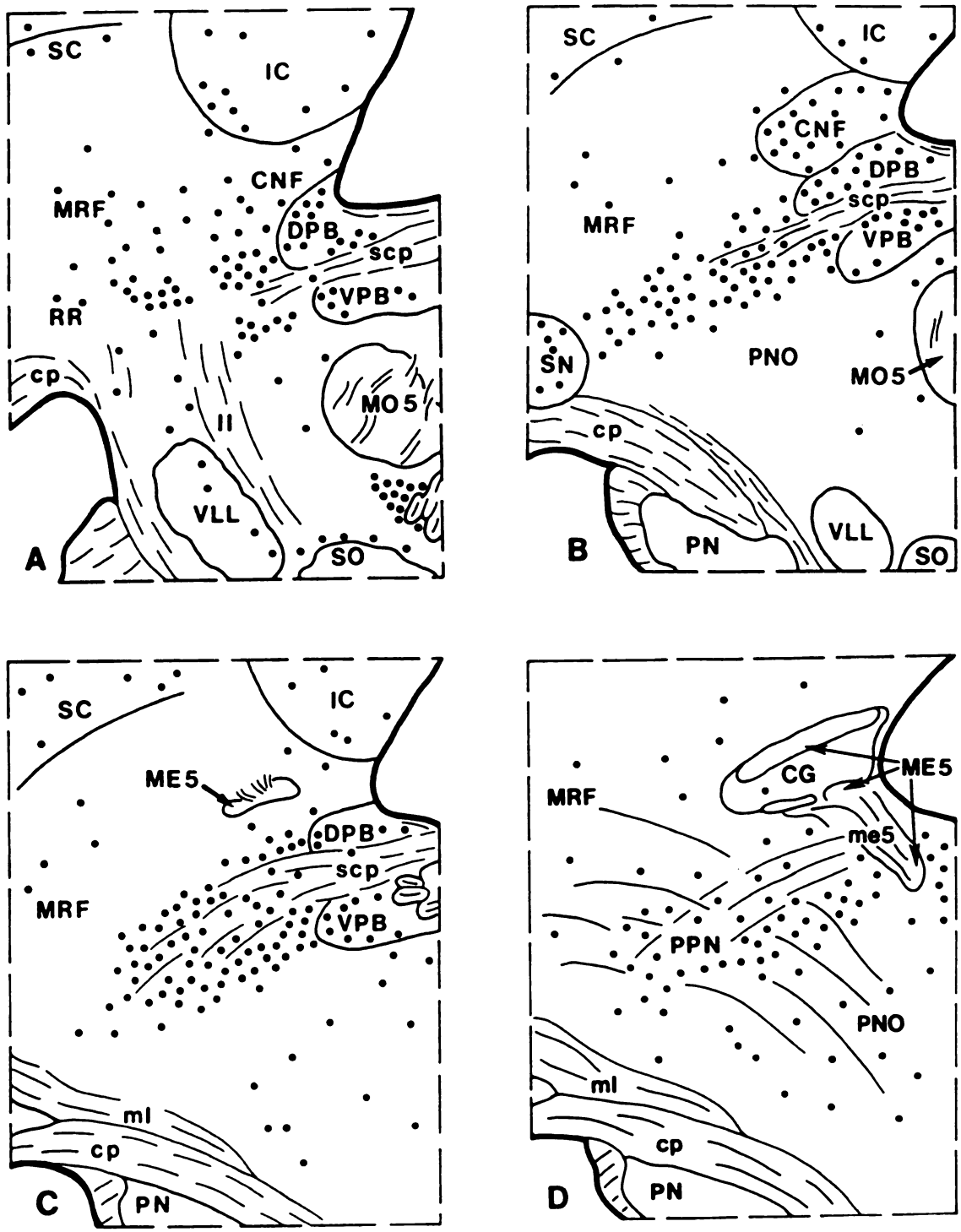
Figure 2

Figure 3: Distribution of Ascending Projecting Neurons of the Pontomesencephalic Tegmentum.

Standard projection drawings of sagittal sections of the lateral (A) through medial (D) levels of the pontomesencephalic tegmentum showing the distribution of retrogradely-labeled neurons in the PPN and adjacent structures in a representative experiment. In this figure are displayed HRP-labeled neurons following forebrain injections illustrated in Figure 4. Each dot represents four labeled cells.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DPB
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Figures 3

Figure 4: Drawings of HRP Deposits in the Forebrain and Midbrain.

Drawings of a sagittal section through the left half of the brain (A) and of coronal sections through the right half of the brain (B, C) showing the extent of HRP injections in the representative experiment described in the text. The distribution of HRP-labeled cells in the left PPN in this particular case is depicted in Figure 3. Needle tracks are drawn in black, the spread and intensity of HRP reaction product are indicated by hatching.

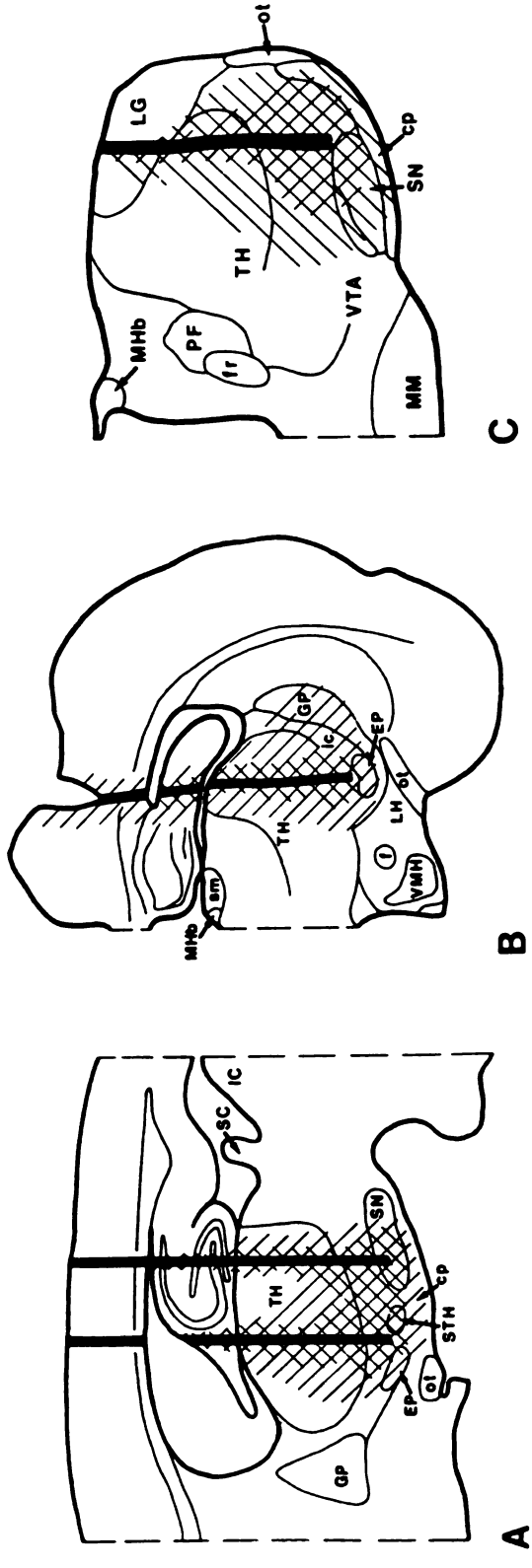
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Figure 4

Figure 5: Photomicrographs of Single- and Double-labeled PPN Cells.

The labeled cells in A are located in the PPNc while all other labeled cells shown are within the PPNd. A and B illustrate the shapes and sizes of HRP-labeled PPN cells projecting to the forebrain. Note the presence of numerous anterogradely-labeled fibers. HRP-labeled cells from spinal cord injections possessed similar features, but exhibited fainter labeling. C and D demonstrate single- and double-labeled cells following DYD injections in the forebrain and GB injections in the cervical cord. The GB-labeled cell (arrow) exhibits numerous fluorescent granules in the cytoplasm while the DYD-labeled cells (arrowheads) show a brightly fluorescent nucleus. One double-labeled cell is illustrated in C. Scale bar: 20 μm.

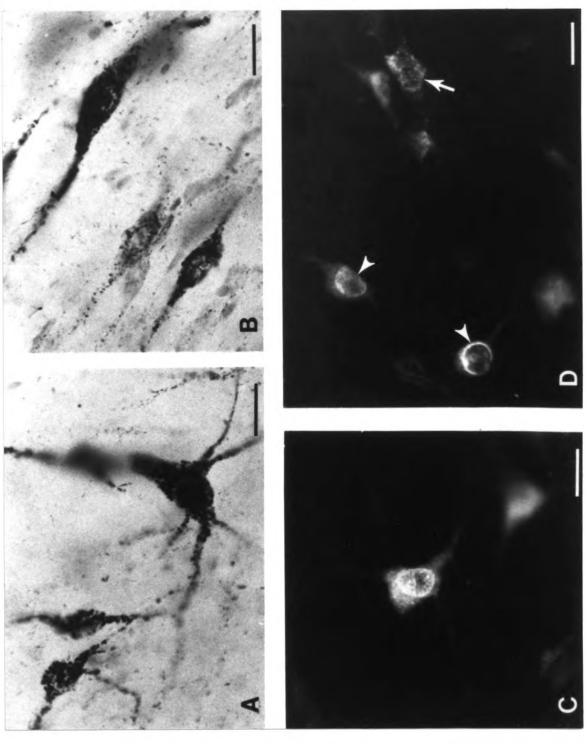

Figure 5

Figure 6: Bar Histograms Comparing the Shapes and Sizes of HRP-labeled PPN Cells Projecting to the Forebrain and/or Spinal Cord.

Although many cells of the two populations of PPN projection neurons have similar shapes and sizes, the spinal cord projecting cells tend to be smaller (mean area: 188 + 80 μm) and more fusiform in shape (50%) than the forebrain projecting neurons (mean area: 299 ± 75 μm ; 33% are fusiform cells).

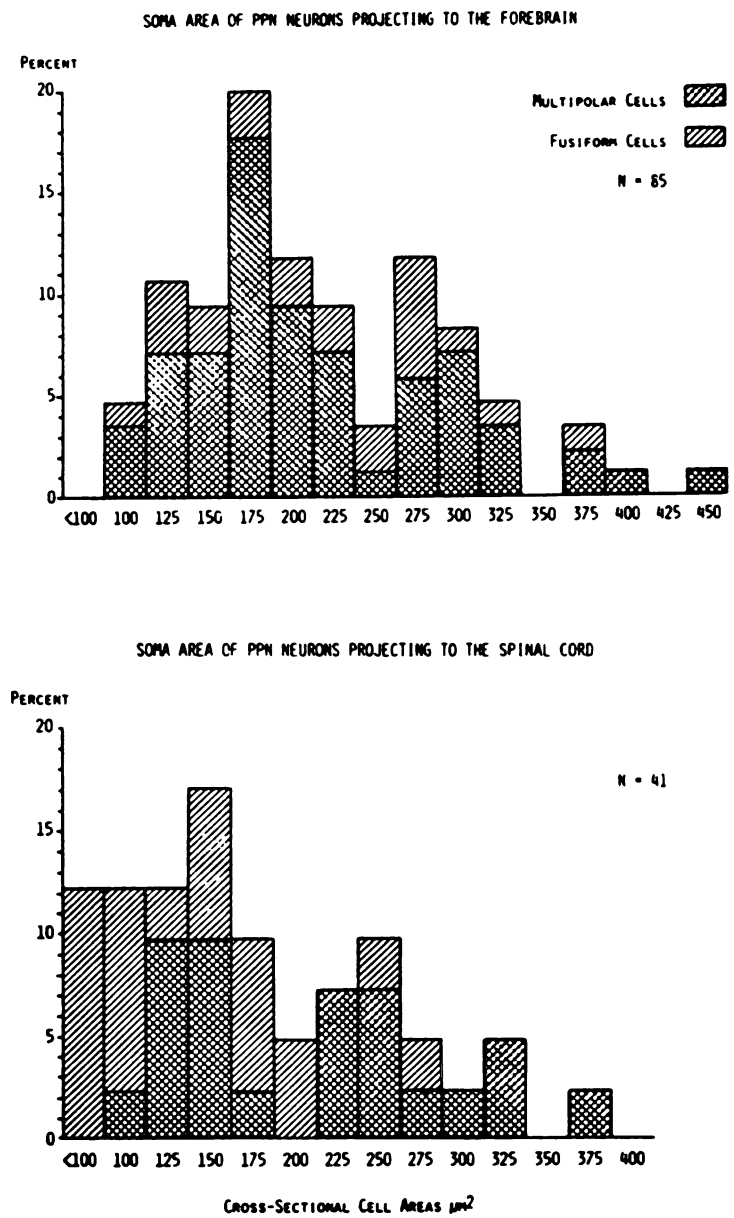
SOMA AREA OF PPN NEURONS PROJECTING TO THE FOREBRAIN
PERCENT
20
15
10
5
0
MULTIPOLAR CELLS
FUSIFORM CELLS
N = 85
<100 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450
SOMA AREA OF PPN NEURONS PROJECTING TO THE SPINAL CORD
PERCENT
20
15
10
5
0
N = 41
<100 100 125 150 175 200 225 250 275 300 325 350 375 400
CROSS-SECTIONAL CELL AREAS μm²

Figure 6

Figure 7: Distribution of Single and Double-labeled Cells in the PPNd.

Projection drawings of sagittal sections through the lateral (A) and medial (B) halves of the pontomesencephalic tegmentum showing the distribution of retrogradely labeled neurons in the PPN and adjacent structures in a representative fluorescent dye experiment. Dots represent cells which were retrogradely labeled following GB injections in the forebrain, and open circles correspond to cells labeled by DYD transported from the cervical cord. Each dot or open circle represents four labeled cells. Stars in B indicate double-labeled neurons. Each star represents one neuron.

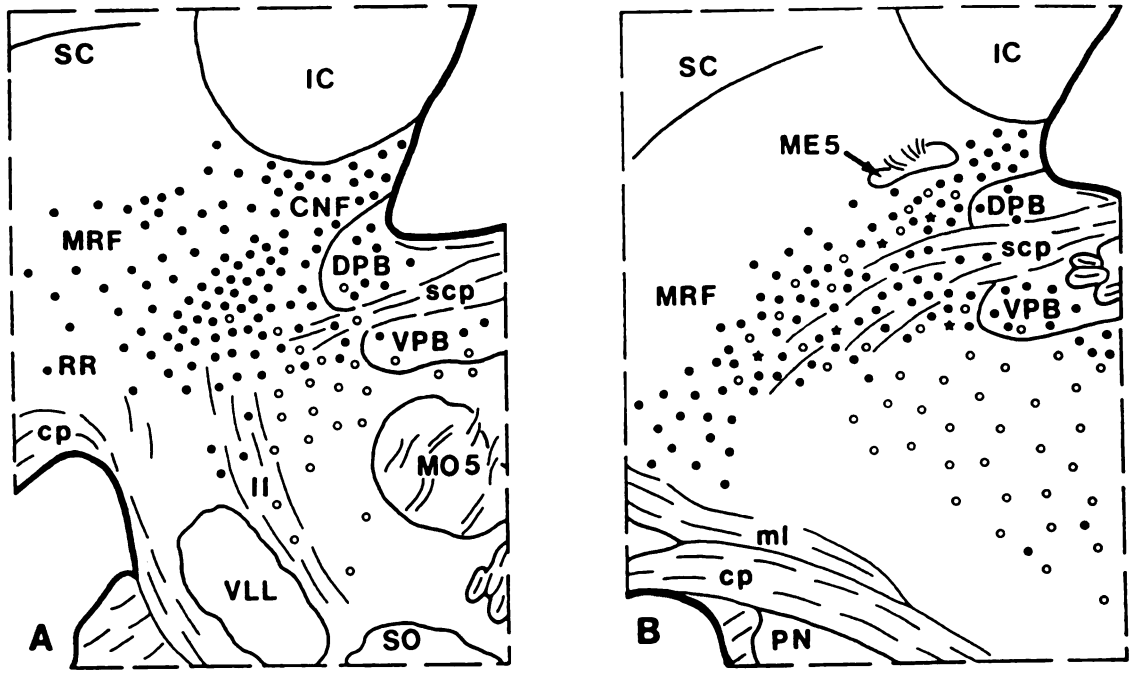
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Figure 7

CHAPTER II

Light and Electron Microscopic Studies on the Nigropedunculopontine projections in the Rat

INTRODUCTION

The nucleus tegmenti pedunculopontinus (PPN) represents a prominent brainstem target of the basal ganglia (BG) outflow (for review, see Parent, '86). It consists of loosely arranged cells that surround the superior cerebellar peduncle (scp) at the pontomesencephalic junction and, in humans, has been subdivided on the basis of cellular density into the pars compacta (PPNc) and pars dissipata (PPNd) (Olzewski and Baxter, '54). The region occupied by the PPN and the adjacent fiber tracts (scp and central tegmental tract) contains numerous cholinergic neurons which peak in density in the PPNc and represent the group Ch5, as defined by Mesulam et al. ('89). A similar arrangement of the PPN is also present in the monkey (Mesulam et al., '84), in which the PPNc has been identified as the major recipient of pallidal fibers (Nauta and Mehler, '66). In carnivores and rodents, the delineation and subdivisions of the PPN are less distinct, but a topographically identical region appears to contain cholinergic cells (Armstrong et al., '83; Mesulam et al., '83; Vincent et al., '83; Butcher and Woolf, '84; Sugimoto and Hattori, '84; Paxinos and Butcher, '85;

Sofroniew et al., '85; Beninato and Spencer, '86; Isaacson and Tanaka, '86; Woolf and Butcher, '86; Hallanger et al., '87; Jones and Beaudet, '87a; Rye et al., '87; Hall et al., '89; Jones, '90) and receives input from the BG (Jackson and Crossman, '83; Moon-Edley and Graybiel, '83). The most prominent BG afferents to the PPN in subprimates are derived from the pars reticulata of the substantia nigra (SNR) (Moon-Edley and Graybiel, '83).

Several lines of research point to the involvement of the nigropedunculopontine (i.e.nigrotegmental or nigroreticular) projections in motor functions. It is well documented that in the cat and rat the PPN is localized within the mesencephalic locomotor region (Garcia-Rill, '86; Garcia-Rill et al., '87) and that the locomotor effects are mediated mainly through the PPN-reticulospinal pathway (Garcia-Rill and Skinner, '87a, b). There exists a definite ultrastructural demonstration that some of the nigral fibers synapse on the PPN-reticular neurons (Nakamura et al., '89), and results of physiological experiments suggest that the nigro-PPN reticulospinal pathway may mediate striatally induced inhibition of muscle activity (Kelland and Asdourian, 89). Furthermore, the BG efferents appear to terminate in the region which gives rise to the ascending projections to the BG (Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Rye et al., '87; Scarnati et al., '87a; Lee et al., '88) thus establishing reciprocal loops which exert excitatory influences on the pallidal (Gonya-Magee and Anderson, '83),

subthalamic (Hammond et al., '83) and nigral cells (Scarnati et al., '84; 87b). However, an increasing amount of evidence has accumulated suggesting that it would be a gross oversimplification to consider the PPN only in terms of motor functions. Recently, Garcia-Rill and Skinner ('88) have concluded that the PPN and adjacent nuclei may be involved in a variety of rhythmic functions including not only REM sleep but also blood pressure regulation, respiration, chewing and other complex cyclic behavioral patterns. In addition, the cholinergic PPN cells projecting to certain thalamic nuclei have been associated with the ascending reticular activating system (Mesulam et al., '89; Steriade et al., 90a, b). It is at present unknown whether the BG input affects these non-motor PPN functions. Furthermore, it is unclear whether the BG input is directed to both cholinergic and noncholinergic PPN cells. While Rye et al. ('87) have concluded that the PPN in the rat consists only of cholinergic neurons and does not coincide with the area that is reciprocally connected with the basal ganglia (i.e.midbrain extrapyramidal area), Hall and co-workers ('89) have demonstrated that an extensive overlap exists between the ChAT immunoreactive cells in the PPN and the terminal field of nigral fibers in the cat.

In view of the increasing interest in the functions of the PPN and in the possible involvement of the BG in these functions, a thorough light and electron microscopic examination of the nigral terminal field in the PPN region is warranted. In order to determine the precise distribution of

the terminal arborizations of nigral fibers, we have used the anterograde tracer *Phaseolus-vulgaris* leucoagglutinin (PHA-L) which provides solid labeling of axons including the terminal arborizations and varicosities at both light and electron microscopic levels. This technique eliminates the limitations inherent to previously used autoradiographic and HRP methods which can not clearly differentiate between fibers passing through and terminating within a given area. The distribution of PHA-L labeled nigral terminal plexus was studied in relation to the subnucleus compactus (PPNc) and subnucleus dissipatus (PPNd) which were defined on the basis of cytoarchitectural criteria in a previous study (Spann and Grofova, '89). A preliminary account of this work has been previously presented in abstract form (Spann and Grofova, '88).

MATERIAL AND METHODS

The brains of four adult, male albino rats were selected for this study from more than 20 brains in which single or multiple unilateral and bilateral stereotaxic injections of PHA-L were made in the substantia nigra pars reticulata (SNR). Animals were anesthetized with sodium pentobarbital (50-100mg/kg, i.p.) and atropine sulfate solution (0.7mg/kg) was administered intramuscularly in order to prevent brain edema. A 2.5% solution of PHA-L (Vector Labs) in 10mM Tris buffer (pH 8.0) was iontophoretically deposited for an interval of 20-60 minutes through a glass micropipette with an inside tip diameter of 10-25 mm using a positive, 7s pulsed 5 mA current (Midgard CS3 power source). The stereotaxic coordinates were derived from the atlas of Paxinos and Watson ('86).

After 10-14 days survival time, the animals were deeply anesthetized with sodium pentobarbital and perfused transcardially with a sodium phosphate buffered saline solution followed by a fixative consisting of 4% paraformaldehyde and 0.2% gluteraldehyde (EM grade) in 0.15 M sodium phosphate buffer at pH 7.35. The brains were immediately removed and stored overnight at 4°C in the fixative. Serial 30 mm thick sections were cut on a vibratome in the sagittal plane, collected in Tris buffered saline (TBS), and divided into two series of alternating sections. The first series was processed for light

microscopic and the second for electron microscopic PHA-L immunohistochemistry using a modified protocol by Gerfen and Sawchenko ('84). The sections were first rinsed in three changes of TBS (10 min. each) and then placed in a blocking solution of 3.0% normal rabbit serum in TBS. Following a 5 min rinse, the sections were transferred to a 1:2000 dilution of primary goat anti-PHA-L (Vector Labs) diluted in TBS with 0.2% Triton X-100 and incubated with gentle agitation for 48 hrs. at 4°C. The sections were then transferred to a 1:200 dilution of biotinylated rabbit anti-goat IgG (Vector Labs) with 0.2% Triton X-100 for 60 mins. at room temperature. After additional rinses, the sections were placed in a 1:1000 dilution of avidin-biotin-peroxidase complex (Vector Labs) in TBS for 60 mins. In order to intensify the reaction, the sections were recycled through the biotinylated secondary antiserum and avidin-biotin-peroxidase solutions an additional time prior to the DAB reaction. The sections were then rinsed and placed in a freshly prepared solution containing 100 mg 3,3' diaminobenzidine or DAB (Sigma), 40 mg NH4Cl, 200 mg beta-D-glucose and 0.4mg glucose oxidase in 100ml of 0.15 M Tris buffer to which 38 mg imidazole has been added. After final rinses in Tris buffer, the sections for light microscopy were mounted onto gelatin-coated slides, air-dried, dehydrated and lightly stained with cresyl violet. The sections were examined in a Leitz Orthoplan microscope using brightfield illumination. The location of the PHA-L deposit in the SNR was charted on a map of sagittal sections

passing through four equally spaced levels of the substantia nigra. The distribution of labeled fibers and plexuses in the PPN was documented on projection drawings of immuno-reacted sections through the latero-medial extent of the nucleus. The borders of the PPN and both subnuclei (PPNc and PPNd) were identified according to the previously established criteria of Spann and Grofova ('89).

The series of sections selected for electron microscopy were processed through all of the aforementioned steps of the PHA-L immunohistochemistry with the following modifications: 1) the concentration of Triton X-100 was reduced to 0.04%; 2) imidazole intensification was omitted from the glucose-oxidase DAB procedure. The reacted sections were thoroughly washed in phosphate buffer and postfixed for 30 mins. in 0.5% osmium tetroxide solution in 0.1 M phosphate buffer. Following several washes in distilled H_2O, the sections were dehydrated in graded acetone solutions and section-embedded in Epon-Araldite between a glass slide and coverslip coated with Liquid Release Agent.

Plastic-embedded sections were first inspected in the light microscope and the distribution of labeled terminal fibers in the PPN was documented on photomicrographs or charted on projection drawings. The coverslip was then removed and a blank block was cemented on the selected region under a stereomicroscope. The region exhibiting the highest density of labeled varicose fibers was then cut out and trimmed for ultrathin sectioning. Serial ultrathin (light-

gold interference color) sections were placed on formvar-coated slotted grids, stained with aqueous uranyl acetate and Reynolds lead citrate and inspected in a JEOL-100CX electron microscope. The synaptic relationship of the labeled nigral terminals were studied and quantified on electron micrographs (final magnification of 35,000) taken from ultrathin sections at two or more mm intervals. The sizes of the labeled fibers, boutons and synaptic vesicles were measured on electron micrographs with a final magnification of 18,000-35,000.

RESULTS

Light Microscopic Observations

1) Large PHA-L deposits in the SNR.

In cases #12 and #15, the tracer was injected through three separate penetrations in order to label most if not all of the SNR efferents. This procedure resulted in large PHA-L deposits that encompassed the ventral portion of the SNR throughout its mediolateral extent. In both animals, the injections were most extensive medially involving the entire thickness of either the rostral two-thirds (#15) or caudal two-thirds (#12) of the SNR (Figs. 1A and 2A,B). At the periphery of the PHA-L deposits, single SNR cells exhibited Golgi-like labeling and their axons could be traced toward the dorsal surface of the SN. No such cells were observed in the SNC or in the tegmentum of the midbrain. Although small loci of PHA-L deposits were found in the cerebral peduncle, no labeled fibers were seen in the medullary pyramids or in the pontine nuclei.

Thick labeled axons of even diameters exited the dorsal surface of the SN and formed two major streams of fibers coursing in a rostromedial and caudolateral direction. The rostromedial fiber system was equally prominent in both cases and distributed mainly to the thalamus. However, a minor component of this fiber system turned caudally and terminated in the rostral portion of the superior colliculus,

mesencephalic reticular formation, periaqueductal gray and the PPN. The caudolateral system of SNR fibers was more prominent in case #15 in which the PHA-L uptake site included the rostralmost portion of the SNR. These fibers supplied the superior colliculus and the PPN. In case #12, in which most of the rostral portion of the SNR was not included in the PHA-L injection, there was only a moderate labeling of the superior colliculus. The caudolateral system of fibers was reduced in size and terminated mainly in the PPN.

The majority of labeled fibers coursing toward the pontomesencephalic tegmentum entered the dorsolateral border of the PPNd and then continued ventromedially and caudally. Although a few fibers were consistently seen to exit the caudal border of the PPN and distribute to the medial and lateral parabrachial nuclei, the bulk of nigral efferents clearly terminated in the PPNd. Within the ipsilateral PPNd, the labeled fibers ramified profusely forming a dense plexus of fine fibers exhibiting numerous varicosities (Figs. 3A-D). The terminal plexus was densest in the medial half of the PPNd with a dorsoventral gradient in a lateromedial direction. In contrast to the PPNd, the PPNc contained only a limited number of fine varicose fibers.

Closer examination of the terminal plexus showed that small regions of the PPNd contained a profuse arborization of labeled varicose fibers surrounded by zones containing only a fine axons of even diameters (Figure 5A). Such dense patches of labeled terminal arborizations were observed both within

the neuropil as well as surrounding clusters of cell bodies or single PPNd neurons (Figure 5B).

A small proportion of PHA-L labeled nigral fibers crossed the midline and distributed to the contralateral PPNd (Figs. 3E,F).

2) Small PHA-L deposits in the SNR.

In animals #13 and #14, single PHA-L injections involved only the ventral (#13) or dorsal (#14) portions of the mid-anteroposterior extent of the SNR (Figs. 1B and 2C, D). The dorsally placed injection was not exclusively limited to the SNR since a number of pars compacta cells also exhibited a Golgi-like labeling.

Only a comparatively small fraction of SNR neurons incorporated the tracer in these cases, yet a prominent plexus of varicose fibers was seen in the ipsilateral PPN. While the general pattern of distribution of PHA-L labeled fibers in the PPN was similar to that seen following large injections of the tracer in the SNR, there was a noticeable difference in the position of the terminal plexus in these two cases (Figure 4). Thus, following the dorsomedially placed injection in experiment #14 the plexus of varicose fibers was most prominent in the caudal PPNd, and following the ventrolateral injection in case #13 in the rostral PPNd. In addition, the density of the terminal plexus was somewhat more prominent dorsally within the PPNd following PHA-L injection in the dorsal SNR (Figure 4-2B), while the

ventrally placed injection in case #13 resulted in higher density of the terminal plexus in the ventral PPNd (Figure 4-1B).

The patchy appearance of the terminal plexus was even more pronounced in the above described cases (Figure 5A) than following large PHA-L deposits in the SNR.

Electron Microscopic Observations

Postfixation with osmium tetroxide greatly enhanced the intensity of DAB reaction product and labeled axons were clearly visible in the microscope even in regions with a heavy contingent of unlabeled myelinated fibers such as the PPNd (Figs. 6A,B). In the electron microscope, electron-dense DAB reaction product was found around microtubuli and mitochondria within both myelinated and unmyelinated axons, as well as in the matrix of nerve terminals (Figs. 6C and 7). In general, most if not all of the varicosities observed in the light microscope contained synaptic vesicles and represented either terminal boutons or boutons en passant (Figs. 7).

The majority of labeled terminals were elongated and of medium size (mean length: 1.5 mm; mean width: 0.9 mm). In most instances, the length of the bouton along the postsynaptic membrane did not exceed 2 mm. Only a few remarkably larger labeled boutons were observed. Labeled boutons commonly contained a prominent centrally placed group

of mitochondria and clusters of pleomorphic synaptic vesicles (Figs. 7). The morphology of the synaptic junctions was frequently obscured by the reaction product or by the plane of sectioning. However, whenever the synapse was distinct, it was invariably of the symmetrical type (Fig 7B). There was often a suggestion of multiple synaptic junctions or puncta adhaerentia with the same postsynaptic element.

The distribution of labeled boutons on the somatodendritic membrane of PPN cells is shown in Figure 8. The data indicate that nigral boutons are most frequently apposed medium size dendrites (1-1.99 mm in width) with thin dendrites (less than 1.0 mm in width) being the next most often encountered target of these terminals. The division of dendrites according to their sizes is purely arbitrary. However, the category of thick dendrites (2 mm or greater in width) includes some primary dendrites which were followed from the somata of PPNd cells. Furthermore, these dendritic profiles always contained numerous polyribosomes, fragments of rough endoplasmic reticulum and an occasional Golgi apparatus (Figure 7B). The presence of all these organelles strongly suggests that this category represents proximal dendrites. On the other hand, it may be assumed that the category of thin dendrites contains distal portions of the dendritic trees, even though some of the thinnest dendritic profiles included in this category may represent spine-like appendages that were occasionally seen on medium-sized and thick dendritic stems.

DISCUSSION

Previous anterograde and retrograde tracing studies in the rat (Beckstead et al., '79; Jackson and Crossman, '81; Arbuthnott and Wright, '82; Gerfen et al., '82; Schneider, '86; Rye et al., '87), cat (Beckstead, '83; Moon-Edley and Graybiel, '83; Moriizumi et al., '88; Hall et al., '89; Nakamura et al., '89), and monkey (Carpenter et al., '81; Beckstead and Frankfurter, '82; Beckstead, '83; Parent et al., '83b; Parent, '86) have firmly established that the PPN receives a substantial input from the SNR. The present results confirm and extend those observations by a demonstration of terminal fields of the nigropedunculopontine fibers in the two subdivisions of the PPN, and by an ultrastructural analysis of the distribution of nigral terminals on the cell bodies and dendrites within the PPN.

Organization of the nigropedunculopontine projection

1) Origin.

There exists a general agreement that the nigrotegmental (i.e.nigropedunculpontine) fibers originate from cells in the SNR. Following HRP injections in the rat PPN, Jackson and Crossman ('81) have observed retrogradely labeled cells in the entire rostrocaudal extent of the SNR except for the dorsal and central regions. These authors pointed out that the distribution of the labeled cells is similar to that of

the cells of origin of the nigrotectal pathway (Faull and Mehler, '78) and have further speculated that the retrogradely labeled cells may give rise to both nigrotectal and nigropedunculopontine projections. In addition, based on anterograde labeling experiments Beckstead et al. ('79) proposed that the nigrotegmental fibers may represent collaterals of the nigrotectal tract. On the other hand, double and triple labeling experiments utilizing retrograde transport of fluorescent dyes in the cat and monkey have clearly shown that the nigrothalamic and nigropedunculopontine cells are widely scattered throughout all parts of the SNR while the nigrotectal neurons are restricted in the rostrolateral SNR (Beckstead, '83). Furthermore, a very low number of the latter cells have been seen to collateralize to the PPN. Our observations suggest that a similar organization exists also in the rat. Thus prominent labeling of the nigrotectal fibers was present only following PHA-L injections involving the rostral SNR, while the nigrothalamic and nigropedunculopontine fibers were consistently labeled irrespective of the SNR region involved in the PHA-L injection.

2) Crossed and uncrossed nigropedunculopontine projections.

Based on the retrograde transport of HRP, Jackson and Crossman ('81) have concluded that the nigropedunculopontine projection in the rat is strictly ipsilateral. The ipsilateral connection from the SNR to the PPN has also been

demonstrated by anterograde transport of tritiated proteins (Beckstead et al., '79; Arbuthnott and Wright, '82) and HRP (Gerfen et al., '82; Schneider, '86). In contrast, our observations indicate that the nigropedunculopontine projection contains a modest contralateral component. This discrepancy can be readily explained by the advantages of PHA-L tracing method which is extremely sensitive and provides a solid Golgi-like labeling of single axons, their ramifications and terminal swellings, thus allowing to detect with certainty even quite sparse connections. It is noteworthy that also in this respect the organization of the nigropedunculopontine projection in the rat is quite similar to that in the cat and monkey since in both these species a small contralateral component has been noted (Beckstead, '83; Parent, '86).

3) Distribution of nigral fibers in the PPN.

In previous studies, the distribution of nigral efferents in the pontomesencephalic tegmentum has been determined in the monkey (Carpenter et al., '81), cat (Moon-Edley and Graybiel, '83) and rat (Beckstead et al., '79; Jackson and Crossman, '81; Arbuthnott and Wright, '82; Gerfen et al., '82; Schneider, '86; Rye et al., '87) using either anterograde transport of tritiated proteins or anterograde transport of HRP. In all species, the illustrations show a remarkably similar distribution of the label in the region surrounding the lateral two-thirds of the superior cerebellar peduncle at the pontomesencephalic junction. In primates,

this region contains a rather well defined group of relatively large cells that has been labeled the pars compacta of the nucleus tegmenti pedunculopontinus by Olzewski and Baxter ('54). However, a corresponding region in the rat and cat is composed of diffusely organized smaller cells, and it is not sharply delineated from the surrounding structures. Therefore, in these species, the denotation of the region of termination of the nigral fibers varies and the terms used reflect particular biases of different investigators. Some authors use the denotation "pedunculopontine region" (Noda and Oka, '86), others "the peribrachial-pedunculopontine area" (Gerfen et al., '82) or simply "the nucleus pedunculopontinus" with no further subdivisions (Jackson and Crossman, '81: Arbuthnott and Wright, '82; Nakamura et al., '89). Furthermore, this region has been referred to as "the nucleus tegmenti pedunculopontinus pars compacta (Moon-Edley and Graybiel, '83; Moriizumi et al., '88), and finally as "the midbrain extrapyramidal area" that evidently does not coincide with the PPN which is located just lateral and dorsolateral to it and consists exclusively of cholinergic cells (Rye et al., '87).

In a previous publication (Spann and Grofova, '89), we have analyzed the cytoarchitecture of the PPN in the rat and described two subdivisions of the nucleus, the pars compacta (PPNc) and pars dissipata (PPNd), that can be reliably recognized in sagittal Nissl-stained sections of the rat

brain. In the present study, we have related the distribution of PHA-L labeled nigral fibers to these subdivisions of the PPN. To avoid confusion, it should be stressed that the PPNd includes the "midbrain extrapyramidal area" as well as portions of the PPTn of Rye et al. ('87), while the PPNc of the present study coincides fairly-well with the PPTn-pc as defined by the aforementioned authors. Furthermore, the PPNd of the present study is likely to include the region of the PPN designated as the pars compacta (TPc) in the cat (Moon-Edley and Graybiel, '83; Moriizumi et al., '88).

The present study, by employing the PHA-L method which is superior to both autoradiographic and anterograde HRP techniques used in previous investigations in demonstrating the terminal arborizations, provides the first accurate description of the termination of the nigropedunculopontine projection. The pattern of PHA-L labeling following large injections of the tracer in the SNR has confirmed that the nigral fibers terminate preferentially but not exclusively in one of the subdivisions of the PPN, i.e.PPNd. The terminal plexus does not fill the entire PPNd but is clearly concentrated in the medial two-thirds of the subnucleus. Furthermore, the location of the terminal plexus with respect to the superior cerebellar peduncle exhibits a dorsoventral shift in a lateromedial direction. At present it is unknown whether inputs from other basal ganglia nuclei converge on

the same territory of the PPN or occupy complementary regions of the nucleus.

4) Topographical organization of nigropedunculopontine fibers.

Based on retrograde transport of HRP/WGA, Moriizumi et al. ('88) have concluded that the nigropedunculopontine projection in the cat exhibits a mediolateral topographical organization. We have not observed any indication of a similar topographical organization in the rat. Admittedly, in our material, the PHA-L injections have always involved a large mediolateral extent of the SNR. However, the smaller injections in cases # 13 and #14 did involve preferentially either the lateral (#13) or medial (#14) portions of the SNR. On the other hand, in these two cases we have noted a slightly different dorsoventral position of the terminal fields of nigral fibers in the PPNd, suggesting that the nigropedunculopontine fibers originating in the dorsal and central region of the SNR terminate more dorsally and caudally in the PPNd than the fibers originating more ventrally in the SNR region adjacent to the cerebral peduncle. However, there exists a considerable overlap and thus a dorsoventral topographical organization of the nigropedunculopontine projection is not distinctly evident.

Termination of nigral fibers in the PPNd

1) Terminal plexus organization.

One of the most interesting observations obtained in this study was the patchy appearance of the terminal plexus of nigral fibers in the PPNd. This was not only due to the scattering of PPN neurons among numerous fiber bundles passing through this region but also to an obvious preference of nigral fibers for some neurons and islands of neuropil. The heterogeneous distribution of nigral terminals was also confirmed in the electron microscope, and strongly suggests that the nigral fibers are preferentially distributed to a particular subpopulation or subpopulations of PPN neurons. It is conceivable that such cells could be characterized by projecting to specific targets or by their neurotransmitter content. The region of PPNd receiving the nigral input is known to contain neurons projecting to the basal ganglia nuclei (Gerfen et al., '82; Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Beninato and Spencer, '87; Rye et al., '87; Lee et al., '88; Spann and Grofova, '89), posterior hypothalamus (Sakai et al., '90), intralaminar thalamic nuclei (Jackson and Crossman, 83; Isaacson and Tanaka, '86), superior colliculus (Beninato and Spencer, '86), pontine and medullary reticular formation (Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Rye et al., '88; Grofova et al., '91) and spinal cord (Jackson and Crossman, 83; Rye et al., 88; Spann and Grofova, '89). At present, there exists

only one study in the cat which provides convincing ultrastructural evidence that some of the nigral fibers terminate on the PPN neurons projecting to the medullary reticular formation (Nakamura, et al., '89). However, our light microscopic observations from double-labeling experiments strongly suggest that the PPN-reticular neurons are not an exclusive target of nigral input since PHA-L labeled nigral fibers were seen to ramify around both PPN neurons that were retrogradely labeled following cholera toxin subunit B injections into the medullary reticular formation as well as around non-labeled cells (Grofova et al., '91). Furthermore, some of the PPN-reticular neurons were located outside of the dense terminal plexus of nigral fibers and no labeled fibers were seen in their proximity. In the light of previous observations that the PPN projection to the medullary reticular formation consists of both cholinergic and non-cholinergic fibers (Rye et al., '88) it is possible that the nigral input is directed to a transmitter-specific population of PPN cells. Further ultrastructural studies utilizing multiple labels are needed in order to understand the entire complexity of the nigropedunculopontine relationship.

Our findings indicate that similar to the previous data in the cat (Nakamura et al., '89), the nigral fibers terminate on both dendrites and cell bodies in the PPN. However, only approximately one third of the nigral terminals were encountered on the somata and thick dendrites, i.e.more

than 2 mm in width, of the PPN neurons. Based on both the observation that some of these dendrites were in continuity with the cell somata, and on the ultrastructural characteristics (the content of organelles within the dendrites was similar to the cell bodies), we have concluded that this category contains primary dendrites which belong to the PPNd neurons. Observations from Golgi material of the rat PPN also indicate that the primary dendrites of both medium and large-size neurons range in sizes between 2 µm and 6 µm (Scarnati et al., '88). The majority of nigral terminals impinge onto medium-sized and small dendrites in the neuropil of the PPNd. The present study does not provide any information on the location of parent cell bodies of these dendrites. Both intracellular HRP and Golgi studies in the rat and cat (Noda and Oka, '86; Granata and Kitai, '89; Newman, '85; Scarnati et al., '88) have demonstrated that the PPN contains isodendritic neurons with extensive dendritic fields, particularly in the mediolateral and rostrocaudal directions. It is therefore possible that at least some of the thin dendritic branches receiving nigral input actually belong to cells located in the PPNc or lateral portions of the PPNd. This would imply that the nigral influence on the PPN may be more extensive than it appears from the light microscopic delineation of the terminal field of nigral fibers.

2) Ultrastructure of the nigral terminals.

The morphology of PHA-L labeled terminals in the PPN is quite similar to the nigral boutons observed in other major targets of the SNR efferents of the rat such as the superior colliculus (Vincent et al., '78; Williams and Faull, '88) and motor thalamus (Grofova, '89). The nigral boutons invariably contained numerous mitochondria and pleomorphic vesicles, and are engaged in symmetrical synaptic junctions. These features are also characteristic for SNR terminals in the cat PPN (Nakamura et al., '89), tectum (Behan et al., '87) and thalamus (Kultas-Ilinsky et al., '83), and in the primate thalamus (Kultas-Ilinsky and Ilinsky, '90). Thus there exists a remarkable inter-species consistency in the morphology of SNR terminals. Furthermore, the morphological similarities of the SNR terminals are accompanied by the same transmitter content. Both nigrothalamic and nigrotectal terminals have been identified as being GABAergic (Vincent et al., '78; Kultas-Ilinsky et al., '85; Kultas-Ilinsky and Ilinsky, '90), and there exists both pharmacological (Childs and Gale, '83) and physiological (Garcia-Rill, '86) evidence that the nigropedunculopontine projection is also GABAergic.

Functional considerations

The involvement of the PPN in motor functions is well supported by anatomical, physiological and neuropathological data. Numerous anatomical studies have documented that the

PPN receives a substantial input from the basal ganglia, in particular from the medial segment of the globus pallidus and SNR, as well as from the limbic areas associated with the basal ganglia such as the substantia innominata (Swanson et al., '84; Mogenson et al., '85) and nucleus accumbens (Groenewegen and Russchen, '84; Haber et al., '90). Furthermore, the PPN is located within the physiologically identified mesencephalic locomotor region (Garcia-Rill, '86;), contributes to exploratory locomotion (Mogenson et al., '89) and may be involved in striatally induced inhibition of muscle activity (Kelland and Asdourian, '89). In addition, lesions of the PPN in the rat have resulted in impaired motor functions (Kilpatrick and Starr, '81). Neuropathological studies in humans have shown an association between PPN cell loss and movement disorders related to progressive supranuclear palsy (Hirsch et al., '87; Zweig et al., '87) and Parkinson's disease (Hirsch et al., '87; Jellinger, '88; Zweig et al., '89). Similarly, significant changes in glucose utilization in the PPN were observed in rhesus monkeys with MPTP-induced hemiparkinsonism (Palombo et al., '90).

There exist multiple anatomical pathways which may be involved in the mediation of PPN influences on motor functions. The PPN gives rise to prominent ascending projections that terminate in the substantia nigra (mostly the pars compacta), the globus pallidus and the subthalamic nucleus (Rinvik et al., '79; De Vito et al., '80; Nomura et

al, '80; Gerfen et al., '82; Saper and Loewy, '82; Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Gonya-Magee and Anderson, '83; Sugimoto and Hattori, '84; Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87; Rye et al., '87; Scarnati et al., '87a; Lee et al., '88; Gould et al., '89; Spann and Grofova, '89). Through these connections, the PPN provides an excitatory influence on the basal ganglia (Gonya-Magee and Anderson, '83; Hammond et al., '83; Scarnati et al., '84; 87b) and may modulate the activity of their outflow to the thalamus and cerebral cortex. This route has been stressed by Moon-Edley and Graybiel ('83) who have suggested that the PPN represents a part of an extrapyramidal loop-circuit which may be involved in the production of simple resting tremors. On the other hand, an increasing evidence has accumulated in favor of Jackson and Crossman's hypothesis ('83) that the PPN may represent a direct link between the basal ganglia and lower motor system. A major support came from the demonstration that the PPN is indeed co-localized with the mesencephalic locomotor region (Garcia-Rill, '86; Garcia-Rill et al., '87), and from anatomical studies showing that the PPN sends numerous descending fibers to the ventromedial portions of the pontomedullary reticular formation which in turn gives rise to the reticulospinal tract (Jackson and Crossman, '83; Garcia-Rill, '86; Garcia-Rill and Skinner, '87a; Mitani et al., '88; Rye et al., '88; Grofova et al., '91; Jones, '90; Robbins et al., '90). Furthermore, a definite

ultrastructural evidence that some of the nigropedunculopontine fibers terminate on the PPN-reticular neurons has been recently provided in the cat (Nakamura et al., '89). At present, similar evidence for the termination of nigral fibers on the PPN neurons giving rise to the ascending projections to the basal ganglia is lacking.

In addition to the motor functions, the PPN, particularly its cholinergic component, has also been implicated as a component in the ascending reticular activating system and rapid eye movement sleep (Mesulam et al., '89; Steriade et al., '90a,b) and in a host of rhythmic activities including not only locomotion but also respiration, mastication, sleep cycle, etc. (Garcia-Rill and Skinner, '88). In view of the present finding that a few nigral fibers terminate in the PPNc which contains the highest density of cholinergic neurons and that the nigral terminals are most frequently encountered on medium size and thin dendrites which may belong to cholinergic cells, it is possible that the nigropedunculopontine fibers may also be implicated in the modulation of some of these functions.

Figure 1: Drawings of PHA-L Deposits in the PPN.

Drawings of sagittal sections through four lateromedial quarters of the SN (1: most lateral; 4: most medial) showing the extent of large (A) and small (B) PHA-L injections. The spread of the PHA-L immunoreactivity is designated by either dashed (C12 and C13) or dotted (C15 and C14) lines.

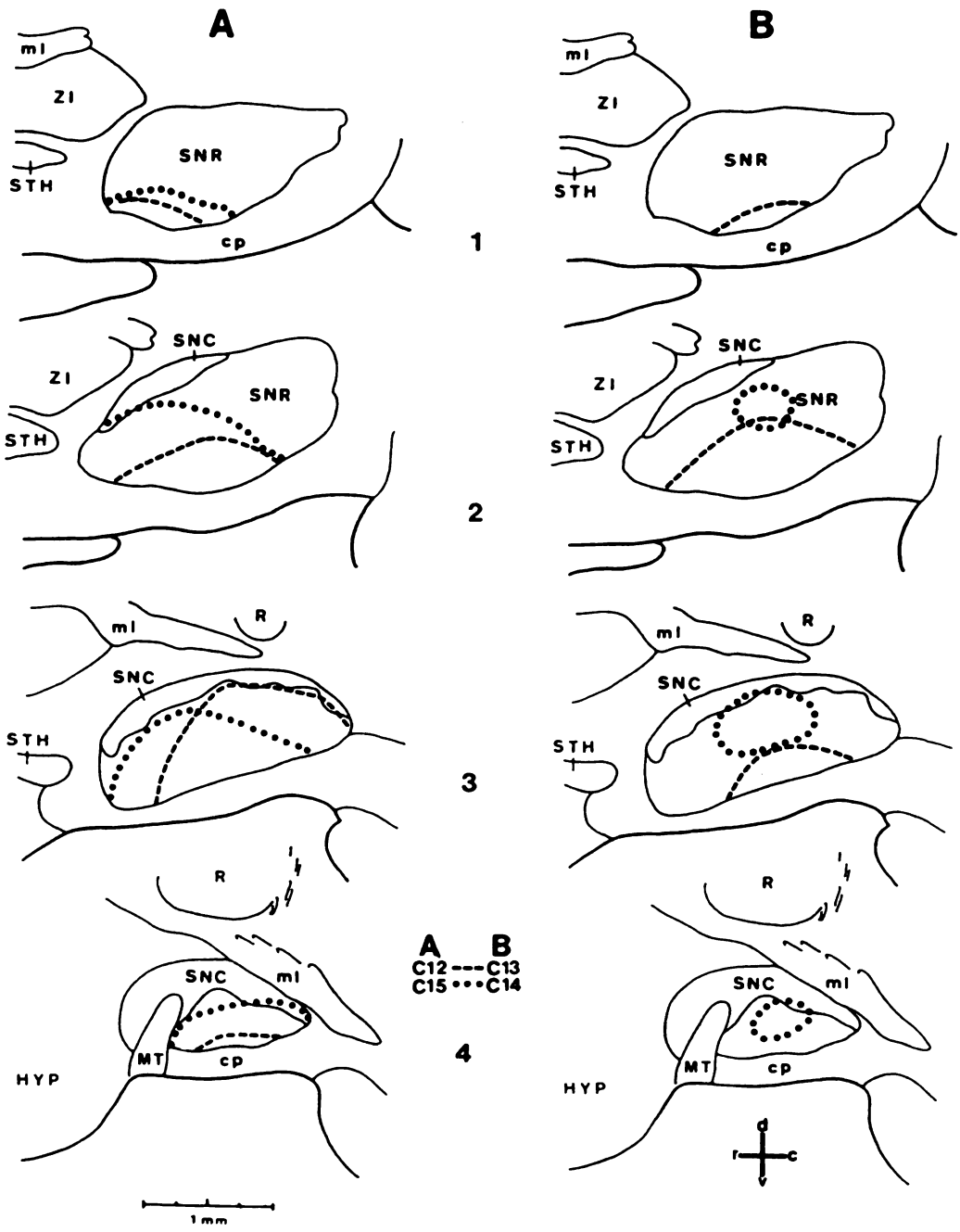


Figure 1

Figure 2: Brightfield Photomicrographs of PHA-L Deposits in the SNR.

Photomicrographs illustrating the central portion of the PHA-L deposits in the SNR in cases #12 (A), #15 (B), #13 (C) and #14 (D). Note that the fibers of the cerebral peduncle do not contain the label. Scale Bar: 0.5 mm.

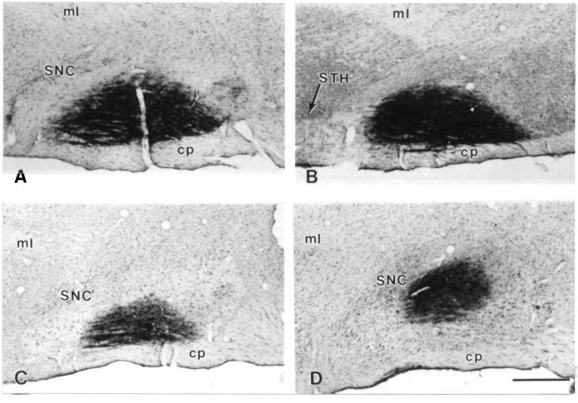


Figure 2

Figure 3: Distribution of Labeled Fibers in the PPN in Case PHA-L #15.

Diagrammatic representation of the distribution of PHA-L labeled fibers in four lateromedial quarters (A: most lateral; D: most medial) of the ipsilateral PPN, and in the lateral (E) and medial (F) halves of the contralateral PPN in case #15. Preterminal and terminal varicosities are indicated by dots.

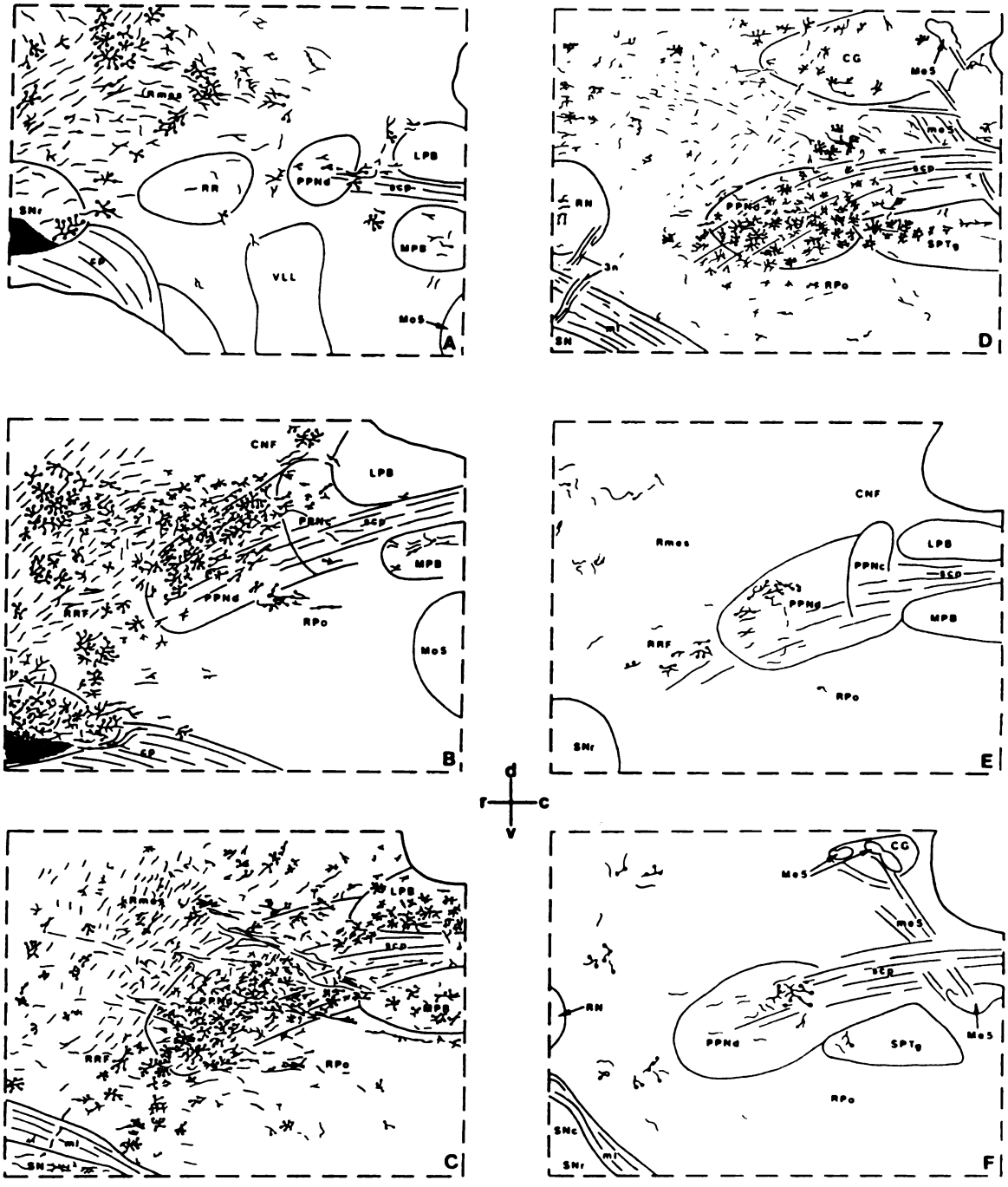

A
LPB
RR
PPNd
SCP
SNr
cp
MPB
VLL
MoS
D
CG
MoS
RN
3n
SCP
PPNd
SPTg
RPo
SN
B
CNF
LPB
PPNc
SCP
MPB
PPNd
RPo
RRF
MoS
cp
E
CNF
Rmos
LPB
PPNc
SCP
PPNg
MPB
RRF
RPo
SNr
d
r
c
v
C
LPB
SCP
RRF
RPo
F
CG
MoS
moS
SCP
RN
PPNd
SPTg
MoS
RPo
SNc
SNr

Figure 3

Figure 4: Distribution of Labeled Fibers in the PPN.

Diagrams comparing the distribution of PHA-L labeled fibers in the PPN following injections in either the ventral (1A-C) or dorsal (2A-C) portion of the SNR. Levels A-C correspond to levels B-D in Figure 3.

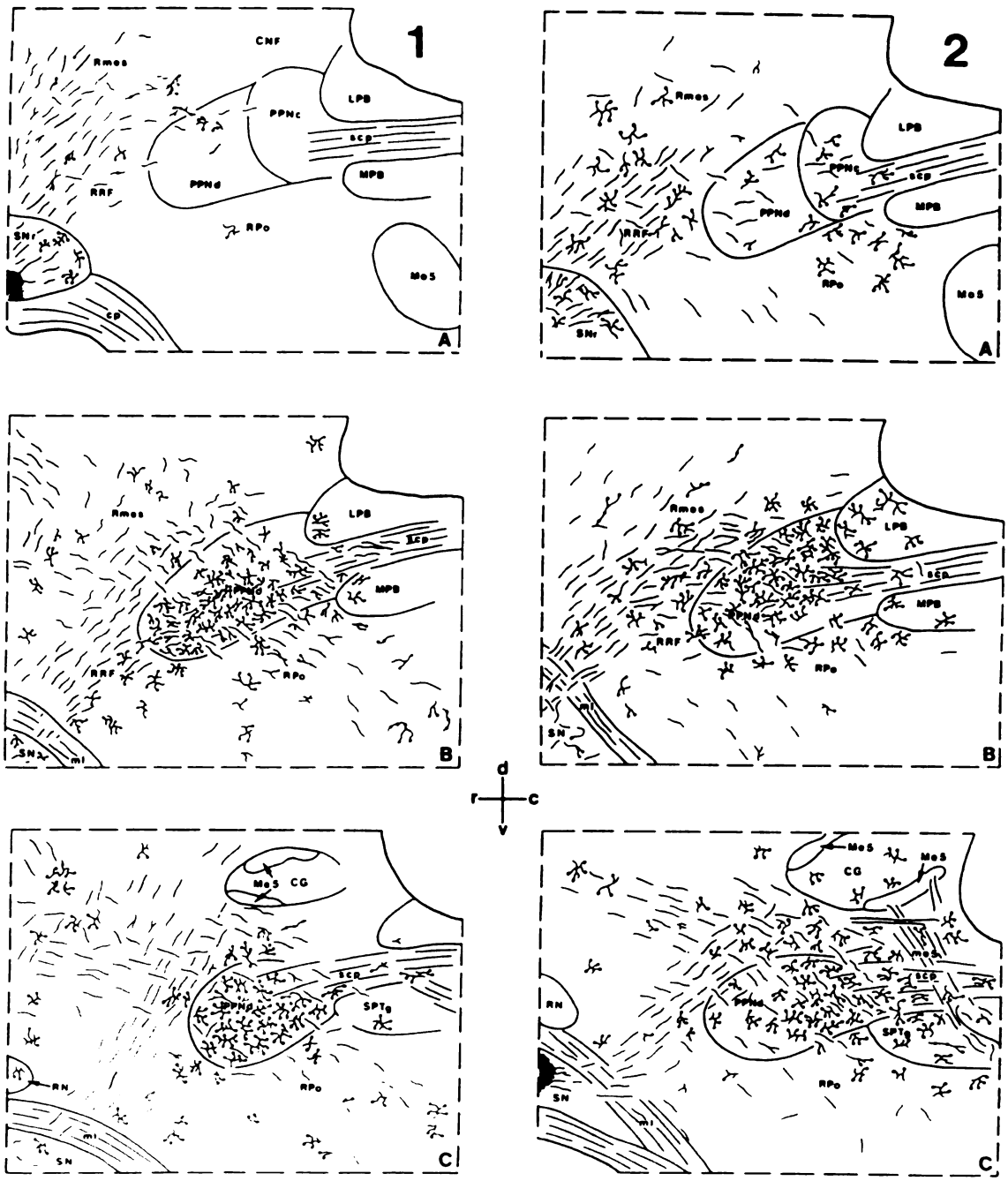

1
CNF
Rmes
LPB
PPNc
scp
MPB
PPNd
RRF
RPo
SNr
MoS
cp
A
2
Rmes
LPB
PPNc
scp
MPB
PPNd
RRF
RPo
SNr
MoS
A
Rmes
LPB
scp
MPB
PPNd
RRF
RPo
SNr
ml
B
Rmes
LPB
scp
MPB
PPNd
RRF
RPo
SN
ml
B
d
r
c
v
MoS
CG
scp
PPNd
SPTg
RN
RPo
SN
ml
C
MoS
CG
MoS
scp
PPNd
SPTg
RN
RPo
SN
C

Figure 4

Figure 5: Brightfield Photomicrographs of PHA-L Immunoreacted Sections Showing the Termination of Nigral Fibers in the PPNd.

A: Arborizations of thin fibers exhibiting numerous varicosities (large open arrows) are concentrated in small areas of the nucleus. Some cells (small open arrows) are located outside of the dense patches of the terminal plexus. Scale bar: 100 µm.

B: Labeled varicose fibers (open arrows) surround the cell body and dendrites of a medium-sized PPNd neuron. Two adjacent cells (arrows) are devoid of apposing nigral fibers.
Scale bar: 20 µm.

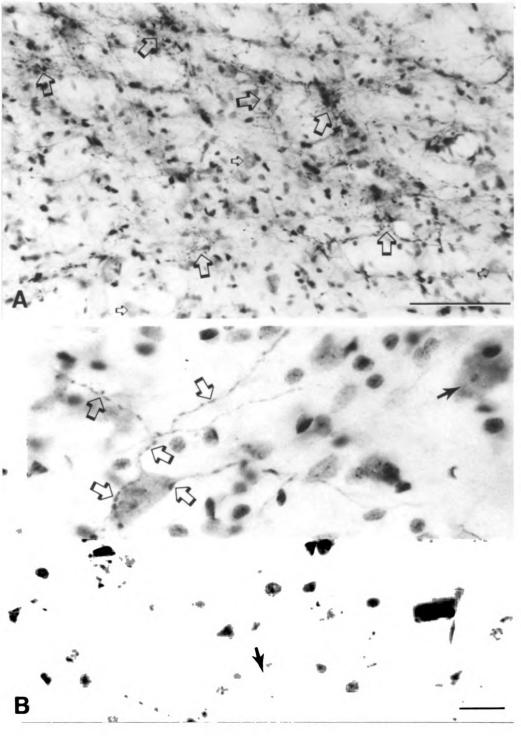


Figure 5

Figure 6: Light and Electron Micrographs of Osmium Postfixed and Plastic Embedded PHA-L Labeled Material.

A: Low-power photomicrograph showing the PPNd region containing a prominent plexus of PHA-L labeled fibers. The borders of the subnucleus are indicated by the discontinuous line while the solid line outlines the area selected for ultrastructural analysis. Note the large diameter fiber dorsal to the PPNd (arrow). Scale bar: 250 mm.

B: Higher magnification of the area outlined by the solid line in A. Arrow indicates PHA-L labeled fiber, arrowhead indicates terminal varicosities. Scale bar: 25 mm.

C: Electron micrograph of a labeled, thinly myelinated axon (arrow) adjacent to two nonlabeled axons (asterisks). Scale bar: 1.0 mm.

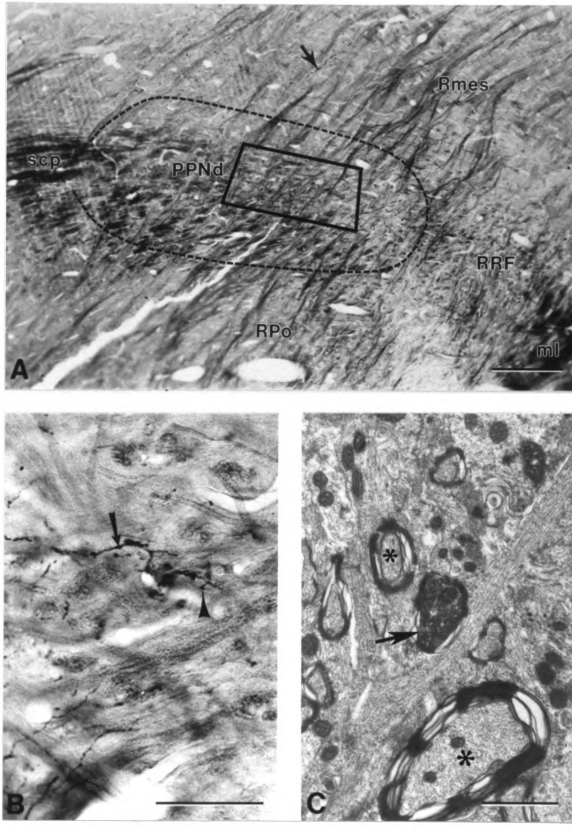


Figure 6

Figure 7: Electron Micrographs Illustrating PHA-L Labeled Nigral Boutons in the PPNd.

A: Two darkly labeled boutons (B1 and B2) containing prominent centrally placed mitochondria contact the cell body (CB) and proximal dendrite (D) of a PPNd neuron.

B: A PHA-L labeled bouton synapsing (open arrow) on a large dendrite (D).

C: A medium-sized dendrite (D) is contacted by a large labeled terminal (B1) and two unlabeled boutons (B2 and B3). Scale bars: 1.0 μm.

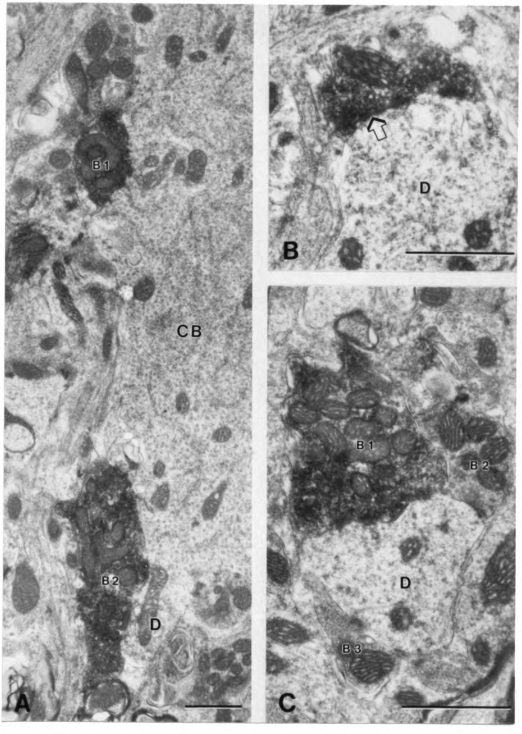


Figure 7

Figure 8: Distribution of Contact Sites of Nigral Terminals.

The distribution of labeled terminals was analyzed in electron micrographs from a region indicated in Figure 6A.

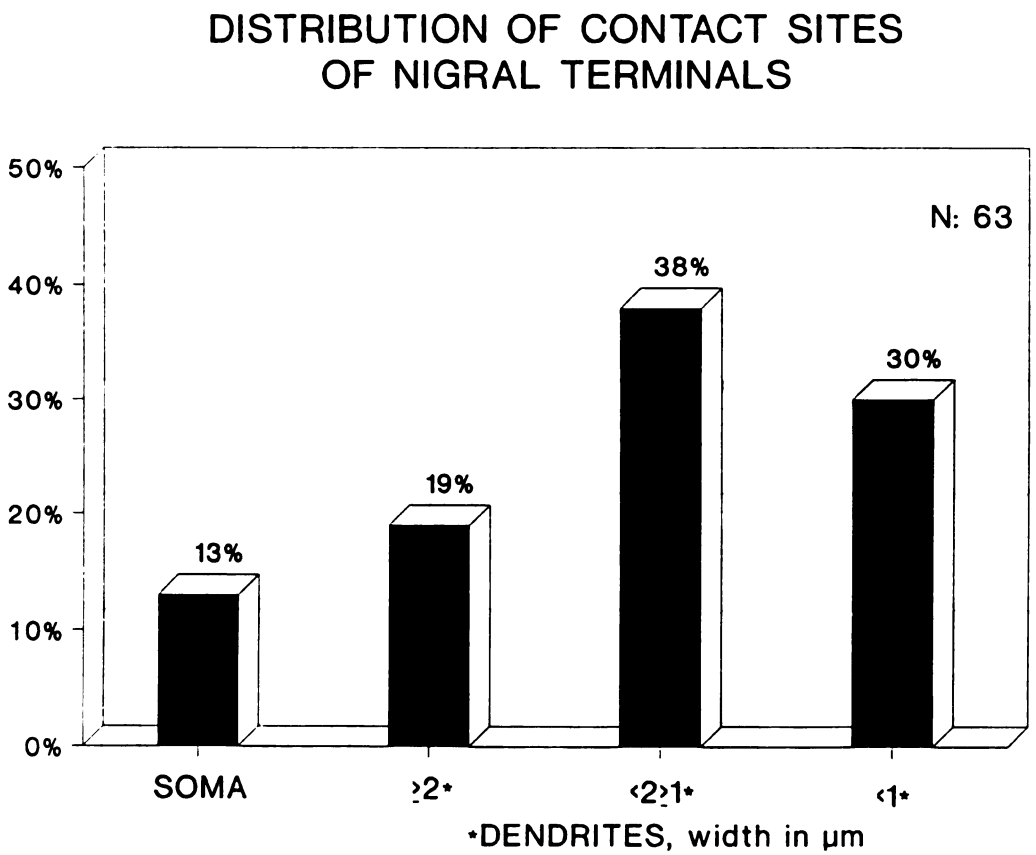

DISTRIBUTION OF CONTACT SITES
OF NIGRAL TERMINALS
50%
40%
30%
20%
10%
0%
N: 63
13%
19%
38%
30%
SOMA
≥2*
<2≥1*
<1*
*DENDRITES, width in µm

Figure 8

CHAPTER III

Ultrastructural study on the cholinergic and non-cholinergic structures of the nucleus tegmenti pedunculopontinus in the rat.

INTRODUCTION

The brainstem of various primate (Mesulam et al., '84, 89; Smith and Parent, '84; Satoh and Fibiger, '85; Mizukawa et al., '86) species contains a prominent group of cholinergic neurons surrounding the brachium conjunctivum and interspersed among its fibers. These cholinergic neurons are largely situated within a region designated as the nucleus tegmenti pedunculopontinus (PPN) by Olszewski and Baxter ('54) in the human brainstem. While cholinergic neurons are found within both divisions of the PPN, the pars compacta (PPNc) and pars dissipata (PPNd), they appear to peak in density in the PPNc (Mesulam et al., '89). Several studies (Mesulam et al., '84, '89) have also indicated that there is a population of non-cholinergic neurons intercalated with the cholinergic neurons throughout the PPN. This terminology has been applied in the non-primate brainstem to the cells similarly located in the tegmentum surrounding the superior cerebellar peduncle and its decussating fibers in front of the parabrachial nuclei (Armstrong et al., '83; Mesulam et

al., '83; Beninato and Spencer, '86, '87; Isaacson and Tanaka, '86; Woolf and Butcher, '86; Clarke et al., '87; Jones and Beaudet, '87a; Gould et al., '89; Hall et al., '89; Jones and Cuello, '89; Fitzpatrick et al., '90; Jones, '90; Semba et al., '90). Double-labeling studies have demonstrated that in both primate and non-primate species the cholinergic and non-cholinergic PPN neurons project to the cortex (Vincent et al., '83), thalamus (Sofriniew et al., '85; Isaacson and Tanaka, '86; Woolf and Butcher, '86; Pare et al., '88; Steriade et al., '88; Fitzpatrick et al., '90; Semba et al., '90), superior colliculus (Beninato and Spencer, '86; Woolf and Butcher, '86; Hall et al., '89), basal ganglia (Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87; Gould et al., '89), basal forebrain (Woolf and Butcher, '86; Jones and Cuello, '89), hypothalamus (Woolf and Butcher, '86; Sakai et al., '90) and pontine (Semba et al., '90) and medullary (Jones, '90) reticular formation.

As a result of these widespread projections, the cells of the PPN have been implicated in a variety of functions. The cholinergic PPN neurons projecting to the thalamus have been considered as important components of the ascending reticular activating system and are also associated with the regulation of paradoxical or REM (rapid eye movement) sleep (Mesulam et al., '83, '89; Woolf and Butcher, '86; De Lima and Singer, '87; Hallanger et al., '87; Pare et al., '88; Steriade et al., '88; Fitzpatrick et al., '90). In addition,

the descending cholinergic projections from the PPN to the pontine and medullary reticular formation may play a role in the induction of motor atonia during REM sleep (Woolf and Butcher, '89; Jones, '90; Semba et al., '90). However, the cholinergic neurons of the PPN may also be involved in other aspects of motor control through ascending projections to the basal ganglia (Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87; Gould et al., '89), intralaminar nuclei which in turn project to the neostriatum (Isaacson and Tanaka, '86), and the motor relay nuclei of the thalamus (Pare et al., '88; Steriade et al., '88). Furthermore, Garcia-Rill and co-workers ('87) have reported that the cholinergic cells of the PPN are co-localized with the mesencephalic locomotor region (MLR).

The sizable population of non-cholinergic neurons intercalated with the cholinergic neuron population throughout the PPN has received very little attention in past experiments. While Steriade and co-workers ('90b) have indicated that both the cholinergic and non-cholinergic neurons of the PPN are responsible for the transfer to the thalamus of brain-stem generated ponto-geniculo-occipital waves, which are closely related to REM, there is little experimental evidence on whether cholinergic and non-cholinergic neurons have similar roles in the other aforementioned functions. Many of the previous studies have focused on examining certain characteristics or functions of only cholinergic neurons and thus de-emphasized the non-

cholinergic neurons. The segregation of cholinergic neurons from non-cholinergic neurons has been taken to its most extreme form by Rye and co-workers ('87). The authors have concluded that, in the rat, the PPN is composed of only cholinergic neurons which are situated outside of the area that is reciprocally connected with the basal ganglia (i.e.midbrain extrapyramidal area or MEA). In addition, they have also suggested that the cholinergic neurons are not involved in either basal ganglia related functions or in the MLR. The segregation of cholinergic neurons and basal ganglia connections has been confirmed by Lee et al., ('88) and a number of authors (Hallanger et al., '87; Hallanger and Wainer, '88; Lee et al., '88; Rye et al., '88) now use the nomenclature of PPN and MEA as defined by Rye and co-workers. Recently, Hall et al., ('89) have indicated there is extensive overlap between the terminal field of nigral fibers and cholinergic neurons of the PPN in the cat. Furthermore, a neurophysiological study on chemically identified neurons in the rat PPN (Kang and Kitai, '90) has suggested that SNR input may be directed to both cholinergic and non-cholinergic cells. Therefore, the definition of the PPN and MEA by Rye and co-workers is becoming increasingly controversial.

In a previous study (Spann and Grofova, submitted), we have demonstrated that in the rat the nigropedunculopontine fibers terminate primarily in the PPNd and suggested that these fibers may be preferentially directed towards a specific subpopulation of PPN neurons. The present study was

performed to gain further insight into the synaptic organization of the PPNd and to establish whether the portion of PPNd receiving nigral input contains cholinergic neurons. Furthermore, particular attention has been paid to the comparison of the fine structure and synaptic organization of cholinergic and non-cholinergic neurons of the PPNd. Preliminary accounts of this work have been previously presented in abstract form (Spann and Grofova, '87, '90).

MATERIAL AND METHODS

ELECTRON MICROSCOPY

The brains of three normal, adult male albino rats were selected from nine brains which were available for the present electron microscopical study. Animals were deeply anesthetized with sodium pentobarbital (80-120 mg/kg, i.p.) and perfused transcardially with 100 mls of sodium phosphate buffered saline solution at 37 °C followed by a fixative consisting of 2% paraformaldehyde and 2% gluteraldehyde (EM grade) in 0.15 M sodium phosphate buffer at pH 7.4 at 4 °C. After perfusion, the brains were immediately removed and kept in the fixative overnight at 4 °C.

The brainstem was blocked and cut sagittally on a vibratome into serial sections of 50 or 100 µm thickness. The sections were thoroughly washed in phosphate buffer and postfixed for 1.0 hrs. in 1.0% osmium tetroxide solution in 0.1 M phosphate buffer. Following several washes in distilled H_2O, the sections were dehydrated in graded acetone solutions and section-embedded in Epon-Araldite between a glass slide and coverslip coated with Liquid Release Agent.

Plastic-embedded sections were first inspected in the light microscope and the borders of the PPN were identified according to previously established criteria of Spann and Grofova ('89). The PPN was divided into four lateromedial quarters or levels and one vibratome section was selected from each of the levels for ultrastructural examination.

With the 50 µm thick sections, the selected region of the PPN for ultrathin sectioning was documented on photomicrographs or line drawings, the coverslip was then removed, and a blank block was cemented on that region under a stereomicroscope. With the 100 µm thick sections, a blank block was first cemented on the PPN and surrounding structures. Semithin sections of 1.0 µm thickness were than taken of this region, stained with P-phenylene-diamine (Holländer and Vaaland, '68) and used to assure correct localization of the PPN region for electron microscopy. Serial ultrathin sections (light-gold interference color) were cut on a Reichart Ultracut E, picked up on Formvar-coated slotted grids and stained with aqueous uranyl acetate and Reynolds lead citrate. The sections were inspected in a JEOL 100 CX electron microscope.

For the analysis of appositional relationships of the cell bodies, 41 cells were randomly selected from various levels of the PPN. Only cells where the plane of section had passed through the nucleolus were used. Montages of electron micrographs having a final magnification of 19,800x were made of each cell. The different profiles contacting the somata were color coded and their total length measured using the Bioquant Image Analysis System. Somatic areas of each cell were measured on electron micrographs with a final magnification of 9,900x using the same system.

Axonal terminals were categorized according to the subsynaptic specialization, vesicle morphology and organelle content from electron micrographs (final magnification of

27,500x) taken at random from the four levels of the PPN. Only terminals with clearly identifiable synaptic contacts were counted. In addition, the length of each terminal (greatest distance between the terminal membranes measured with a line parallel to the synaptic membrane) and postsynaptic target characteristics were recorded. Approximately 3200 μm^2 of neuropil per level was examined.

ChAT IMMUNOCYTOCHEMISTRY

Three normal, adult male albino rats were deeply anesthetized with sodium pentobarbital (80-120mg/kg, i.p.) and perfused transcardially with a sodium phosphate buffered saline solution followed by a fixative consisting of 4% paraformaldehyde and 0.1% gluteraldehyde (EM grade) in 0.15M sodium phosphate buffer at pH 7.2. The brains were immediately removed, stored at 4 °C in the fixative for one hour and then transferred to sodium phosphate buffer (pH 7.2) at 4 °C were they remained overnight. Serial 50 µm thick sections were cut on a vibratome in the sagittal plane, collected in Tris buffered saline (TBS) and divided into two series of alternating sections. The first series was processed for light microscopic and the second for electron microscopic ChAT-immunohistochemistry using a modified protocol of Boehringer-Mannheim Biochemicals (BMB). The sections were first rinsed in four changes of TBS (10 mins. each) and then incubated with gentle agitation in a 4 µg/ml

dilution of primary monoclonal rat anti-ChAT (BMB) diluted in TBS with 2% Bovine Serum Albumin (BSA), 20% Normal Rabbit Serum (NRS) and 0.1% Triton X-100 for either 48 hrs. at 4 °C or overnight at room temperature. After three 10 min. rinses in TBS, the sections were transferred to a 1:250 dilution of rabbit anti-rat antibody (BMB) with 2% BSA, 20% NRS and 0.1 % Triton X-100 for 60 mins. at room temperature, and after additional rinses, placed for 60 mins. in a 1:250 dilution of rat peroxidase-antiperoxidase (BMB) with 2% BSA, 20% NRS and 0.1% Triton X-100. In order to intensify the reaction, the sections were recycled through the secondary antiserum and peroxidase-antiperoxidase solutions an additional time prior to the DAB reaction. Following several rinses, the sections were placed in a freshly prepared solution containing 100 mg 3, 3' diaminobenzidine or DAB (Sigma), 40 mg NH_4Cl, 200 mg beta-D-glucose and 0.4 mg glucose oxidase in 100 ml of 0.15 M Tris buffer for 30-60 mins. After final rinses in Tris buffer, the sections for light microscopy were mounted onto gelatin coated slides, air-dried, dehydrated and either lightly stained with cresyl violet or left unstained.

The sections were examined in a Leitz Ortroplan microscope using brightfield illumination. The distribution of labeled cells and fibers in the PPN was documented on both photomicrographs and projection drawings of immuno-reacted sections through the latero-medial extent of the nucleus.

The series of sections selected for electron microscopy were processed through all of the aforementioned steps of

ChAT immunohistochemistry but the concentration of Triton X-100 was reduced to 0.04%. The reacted sections were thoroughly washed in phosphate buffer and then postfixed for 30 mins. in 0.5% osmium tetroxide solution in 0.1 M phosphate buffer. Following several washes in distilled H_2O, the sections were dehydrated in graded acetone solutions and section-embedded in Epon-Araldite between a glass slide and coverslip coated with Liquid Release Agent.

Plastic-embedded sections were first inspected in the light microscope and the distribution of labeled cells and dendrites in the PPN was documented on photomicrographs. A region of the PPNd exhibiting the highest density of labeled cells or dendritic arborizations and previously shown to contain a dense plexus of nigral fibers (Spann and Grofova, submitted) was then identified. The coverslip was removed, a blank block was cemented on the selected region under a stereomicroscope and then trimmed for ultrathin sectioning as described for the normal material.

For the analysis of the cell somata, 28 cholinergic and 27 non-cholinergic cells cut through the plane of the nucleolus were selected from the PPNd. All 55 cells were selected from regions of the PPNd containing a mixture of both cholinergic and non-cholinergic cells. The largest and smallest diameters of each cell soma were measured from montages of electron micrographs having a final magnification of 9000x. Axonal terminals contacting the somata and primary dendrites of each cell were classified and counted in

ultrathin sections with a microscope magnification of 10,000x through a 10x viewer.

Axonal terminals synapsing on either cholinergic or non-cholinergic dendrites of different sizes in the PPNd were classified and counted from electron micrographs (final magnification of 25,000x) taken at random.

RESULTS

I. NORMAL ULTRASTRUCTURE OF THE PPN

The following description of the fine structure and synaptic organization of the PPN are based on observations from the PPNd and the adjoining portion of the PPNc. Altogether, 41 cells containing the nucleolus and the surrounding neuropil were analyzed.

Nerve Cell Somata

The neurons of the rat PPN exhibited soma shapes which ranged from spindle to oval-round. The cross-sectional somatic areas of these neurons ranged between 48 and 270 μm^2 with a mean of 126.7 μm^2 (S. D.: 49.8 μm^2). The perikaryon of the neurons contained rough endoplasmic reticulum, mitochondria, free ribosomes, Golgi apparatus, lysosomes, multivesicular bodies, microtubules and neurofilaments. The nuclear membrane was indented in nearly 90% of the neurons examined. The depth of the invaginations ranged from shallow to deep and was not restricted to any one cell shape or size. Somatic spines or protrusions from the perikaryon of the neurons were only rarely encountered.

The surface of the neurons was apposed primarily by myelinated and unmyelinated axons which covered on average 52 and 33% of the somatic membrane. The remaining portion of the somatic membrane was apposed by nerve cell bodies and

dendrites, and axon terminals. Particular attention was paid to the number of terminals synapsing on various size somata of PPN neurons (Figure 1). While the overall percentage of the cell membrane covered by axon terminals was relatively small (13%), the range of terminal coverage varied greatly (0-55%). On average, the PPN neurons were contacted by 5 terminals (range: 0-27) per soma. However, the soma of small neurons received fewer terminals than the large neurons. Furthermore, the data suggests that larger neurons (126 μm^2 or greater in area) may be further subdivided into two groups based on the density of synaptic terminals. One group of the larger neurons was covered by very few terminals (0-9%) while the other group received a substantially greater number of terminals (15-55%) establishing contact. In all neurons examined, the terminals appeared to be randomly distributed along the entire length of the somatic membrane.

Of the 41 cells examined, 16 of the neurons had one or more primary dendrites emerging from the cell body. These dendrites did not exhibit any spine-like appendages and nerve terminals seen in contact with these dendrites did not show any particular preference for any specific region of the primary dendrites.

Neuropil

The neuropil of the PPN consisted of myelinated and unmyelinated axons, glial profiles, dendrites, and axon terminals. The myelinated and unmyelinated axons (Figure 2A)

were often organized into bundles. The diameters of myelinated axons (myelin sheath included) ranged between 0.4 and 5.0 µm. Only a few of the myelinated axons had diameters greater than 3.0 µm. The unmyelinated axons had diameters under 0.6 µm and their cytoplasm contained primarily microtubules. Glial profiles mainly belonged to astrocytes.

The majority of dendritic profiles were under 2.0µm in diameters. The cytoplasm of dendrites (Figure 2A) contained microtubules, mitochondria, free ribosomes, small sacs of smooth endoplasmic reticulum and vesicular structures. While dendritic profiles with clusters of synaptic vesicles were occasionally noted (Figure 2A), dendrodendritic synapses were not encountered in the present material. Longitudinally cut dendrites were often irregular in shape but did not exhibit the morphological characteristics of dendritic varicosities (Peters et al., '76). Furthermore, the dendrites were generally smooth with only an occasional spine-like process.

Nerve terminals

The morphology and distribution of 582 nerve terminals were analyzed in detail. Based on the type of synaptic junction and the shape of the synaptic vesicles, two major types of terminals were identified in the PPN.

The Type I terminals (Figure 2A,B) were engaged in asymmetrical synaptic junctions and contained round or slightly ovoid synaptic vesicles. The synaptic cleft measured approximately 25 nm in width. The Type I terminals

contained several mitochondria and could be further subdivided into two Subtypes, IA and IB. The Subtype IB terminal (Figure 2A) could be differentiated from the Subtype IA (Figure 2A, B) by the presence of post-junctional dense bodies beneath the postsynaptic density. The Type I terminals ranged in length from 0.4 µm to 3.0 µm with the Subtype IA being slightly larger than the IB. On random sections these type of terminals often represented the ends of unmyelinated axons.

The Type II terminals (Figure 2A, C) formed symmetrical synaptic junctions with the synaptic cleft being about 20 nm. They contained clusters of pleomorphic vesicles and numerous mitochondria which were often centrally located. Some of these terminals (Figure 2A) were observed to establish multiple synaptic junctions with the same postsynaptic target. In addition to the synaptic junctions, the Type II terminals were also attached to the postsynaptic structures by one or more puncta adhaerentia (Figure 2C). The Type II terminals ranged in length from 0.4 to 3.0 µm and were encountered as either en passant or terminal boutons. Occasionally the type I or type II terminals formed a double synapse on two dendritic profiles of various sizes (Figure 2B). However, complex synaptic arrangement were never observed.

The Type II terminals were found to represent 56% of the bouton population. The Type IA represented 37% and the Type IB accounted for only 7% of the total number of terminals

within the PPN. With the exception of the Type IB terminals which were observed only on dendrites, both Type IA and II were found on somata/primary dendrites as well as on dendritic profiles of various sizes within the neuropil of the PPN. However, there was a significant difference in the distribution of the Type I and Type II terminals. The Type II terminals were concentrated on the somata/primary dendrites which appeared to receive 56% of the total number of the Type II terminals (N=326). Their numbers declined on the medium size dendrites (i.e.1.00-1.99 µm in width), and slightly increased again on small dendritic profiles measuring less than 1 µm in width. On the other hand, 50% of the total number of Type IA terminals (n=215) and 71% of the Type IB terminals (n=41) were found on small dendrites.

II. ChAT IMMUNOCYTOCHEMISTRY

Light Microscopy

The ChAT positive (ChAT+) neurons could be easily identified on the basis of a homogenous dark brown staining of the soma and dendrites. In the lateral pontomesencephalic tegmentum (Figure 3), cholinergic cells were most numerous in the PPN. A smaller number of cholinergic cells were observed in the ill-defined caudal and dorsal border of the SNR, the retrorubral nucleus/field, the rostral poles of the lateral and medial parabrachial nuclei, and the subpeduncular

tegmental nucleus. The cholinergic neurons were seen in both subnuclei of the PPN (Figs. 3, 4A), however a slightly greater number (58%) were located in the PPNd. The majority of the PPNd cholinergic neurons were seen in the middle portion of the subnucleus (Figure 3B,C) and often in close proximity to the fibers of the superior cerebellar peduncle. The ChAT+ PPNd cells had fusiform or polygonal cell bodies and ranged in size from 20 to 60 µm along the longest axis.

The PPNd contained dendrites forming extensive arborizations among a diffusely organized group of cholinergic cell somata (Figure 4A,B). The fusiform cells were seen to give rise to two primary dendrites which were oriented in a rostrocaudal direction. From the polygonal cell somata, several primary dendrites radiated in all directions. The primary dendrites were relatively straight and divided into secondary dendrites approximately within 100µm of the soma. The secondary and tertiary dendrites branched infrequently and exhibited a moderate degree of undulation along their course. While varicosities were occasionally encountered on the secondary or tertiary dendrites, spines or short appendages were not observed arising from any of the dendrites examined. Dendrites could be traced up to 300 µm from the cell somata of the fusiform cells and slightly over 425 µm from the polygonal cell somata. While a moderate number of dendrites were followed beyond the borders of the PPNd, the majority remained confined within the territory of the subnucleus.

The second subdivision of the PPN, the PPNc, contained a densely packed group of ChAT+ neurons. The lateral half of the PPNc consisted of a single compact group of cholinergic neurons (Figs. 3A,B and 4A). At more medial levels (Figure 3C), the PPNc was traversed by the expanding scp coursing rostrally and separated into a dorsal and ventral cluster of cholinergic cells which diminished in number medially. The cholinergic PPNc cells exhibited similar shapes and sizes as the PPNd cells. However, the polygonal cells were more numerous than the fusiform cells and both cell types appeared to be less elongated than the cells in the PPNd.

In addition to the cell bodies, the PPNc contained a dense plexus of ChAT+ dendrites which were interlaced among clusters of cholinergic somata (Figure 4A). In general, these dendrites appeared more undulated and branched sooner than the dendrites of PPNd neurons. They were rarely traced beyond 250 μm from the cell somata but a few could be followed for nearly 400 μm. While a greater number of dendrites appeared to extend beyond the borders of the PPNc when compared to the PPNd, the bulk of the dendrites remained within the PPNc. No varicosities, spines or appendages were encountered on the dendrites of the PPNc.

In addition to the ChAT+ cell bodies and dendrites, we have also observed a small number of ChAT+ thin, frequently varicose fibers. The majority of the varicose fibers were located in the medial PPNd. No such fibers were noted within the PPNc.

Electron Microscopy

At the electron microscopic level, we have observed ChAT+ cell bodies and dendrites, a few myelinated axons and one nerve terminal in the PPNd. All ChAT+ cells (Figure 5) exhibited a dense, homogenous reaction product throughout the cytoplasm of their somata and dendrites. However, the reaction product was excluded from the interior of organelles, vesicles and the nucleus. The ChAT+ reaction product within axons and dendrites (Figs. 7A and 9) of the neuropil appeared to adhere directly to the surface of the microtubules and formed periodic patches along the arrays of microtubules. Cholinergic profiles were studied in tissue from the middle portion of the PPNd and compared to non-cholinergic cells (Figs. 5,6) contained within the same region.

Twenty-eight ChAT+ cells cut through the nucleolus were examined in detail. The size of the somata ranged from 14 x 7 μm to 33 x 16 μm. The endoplasmic reticulum, Golgi apparatus, lysosomes and mitochondria of the perikaryon of ChAT+ neurons could be recognized. The nuclear membrane of both large and small cells often exhibited one or more shallow, randomly-located invaginations (Figure 5). Somatic appendages or spines were only rarely encountered. The somatic membrane of cholinergic neurons (Figure 5) was contacted by only a moderate number of terminals (average: 2/soma; range: 0-7). Five of the twenty-eight neurons

examined had one or more primary dendrites emerging from the cell body.

The 27 ChAT- neurons ranged in size from 12 x 5 to 40 x 14 μm. The nucleus of the ChAT- neurons exhibited one or more rather prominent, deep indentations which were most evident in the smaller size neurons (Figure 5). Terminals contacting the somata and primary dendrites of the non-cholinergic neurons (Figure 6) were observed with greater frequency than for the cholinergic neurons (average: 6/soma; range: 0-26). The somatic and dendritic membranes of all ChAT- neurons were aspinous. Approximately 45% of the neurons examined had one or more primary dendrites emerging from the cell body.

A comparison of cholinergic and non-cholinergic neurons revealed that the sizes of the somata (Figure 8) were roughly similar. However, the non-cholinergic cells tended to be somewhat smaller and more spindle-shaped than the cholinergic ones and received a richer synaptic input. A particularly interesting observation was that nearly 30% of the cholinergic somata were directly apposed to other cholinergic or non-cholinergic cell bodies (Figure 5) or large diameter dendrites. Similar appositions between non-cholinergic profiles were not observed. On the other hand, the ChAT+ and ChAT- dendrites were morphologically quite similar. Both exhibited occasional spine-like processes and were contacted by similar number of terminals.

Only one ChAT+ terminal was encountered in the present study. All of the ChAT+ axons (Figure 7A) were myelinated and had diameters (myelin sheath included) ranging in size from 0.5 to 2.2 μm.

Quantitative analysis of nerve terminals (n=421) found in synaptic contacts or close appositions with ChAT+ profiles (33 somata/proximal dendrites and 84 dendrites in the neuropil), and ChAT- profiles (37 somata/proximal dendrites and 65 dendrites) revealed that the ChAT- PPN cells received almost twice as many terminals than the ChAT+ PPN neurons (Figure 10). Furthermore, there were significant differences in the distribution of the bouton types on the ChAT+ and ChAT- profiles. In particular, over 70% of the Type II terminals were found on ChAT- profiles and represented the majority of terminals on the somata of non-cholinergic PPN cells. The Type I terminals were more evenly distributed between the cholinergic and non-cholinergic neurons. Interestingly, the Type I represented the most frequent type of terminals (60%) encountered on the somata and proximal dendrites of cholinergic neurons. Only rarely a Type I or Type II terminal were seen to synapse onto two cholinergic dendrites or onto a non-cholinergic and cholinergic dendrite (Figure 7A).

DISCUSSION

Apart from a brief description of the ultrastructure of the cholinergic somata in the PPNc (Sugimoto et al., '84), the present study represents the first systematic electron microscopic analysis of the rat PPN. Furthermore, it provides the first data on the fine structure and synaptic relationships of the cholinergic and non-cholinergic PPN neurons and complements previous light microscopic studies on the distribution of cholinergic neurons in the pontomesencephalic tegmentum.

Distribution of cholinergic neurons in the (cytoarchitecturally defined) pontomesencephalic tegmental nuclei

In a previous studies, the distribution of cholinergic neurons in the lateral pontomesencephalic tegmentum has been determined in the rat (Armstrong et al., '83; Mesulam et al., '83; Satoh et al., '83; Vincent et al., '83; Paxinos and Butcher, '85; Sofroniew et al., '85; Beninato and Spencer, '86, '87; Isaacson and Tanaka, '86; Woolf and Butcher, '86, '89; Clarke et al., '87; Hallanger et al., '87; Jones and Beaudet, '87b; Rye et al., '87, '88; Goldsmith and van der Kooy, '88; Hallanger and Wainer, '88; Lee et al., '88; Gould et al., '89; Jones and Cuello, '89; Jones, '90; Semba et al., '90), cat (De Lima and Singer, '87; Jones and Beaudet, '87a;

Mitani et al., '88; Pare et al., '88; Steriade et al., '88; Fitzpatrick et al., '89; Hall et al., '89; Sakai et al., '90), ferret (Henderson, '87), dog (Isaacson and Tanaka, '86), monkey (Mesulam et al., '83; Smith and Parent, '84; Steriade et al., '88), baboon (Satoh and Fibiger, '85), and human (Mizukawa et al., '86; Mesulam et al., '89) using acetylcholinesterase (ACE) histochemistry, nicotinamide adenine dinucleotide phosphate (NADPH) histochemistry or choline acetyl transferase (ChAT) immunohistochemistry techniques. In all species, the illustrations show the cholinergic neurons as surrounding the lateral two-thirds of the superior cerebellar peduncle and extending from the caudal pole of the substantia nigra to the rostral portion of the parabrachial nuclei. The nomenclature used in the description of this region containing the cholinergic neurons has been inconsistent and often confusing. Some authors have referred to this entire region as "the pedunculopontine nucleus" (Paxinos and Butcher, '85; Beninato and Spencer, '86, '87; Woolf and Butcher, '86, '89), "the pedunculopontine nucleus area" (Smith and Parent, '84) or "the parabrachial region" (Pare et al., '88; Steriade et al., '88, 90a, 90b). Other authors have used such denotations as "ascending cholinergic reticular system" (Vincent et al., '83), "the central tegmental field" (De Lima and Singer, "87), "Cholinergic (Ch) 5 sector" (Mesulam et al., '83, '84, '89) and "lateral tegmental reticular formation" (Sofroniew et al., '85). Several investigators have combined the

cholinergic neurons within this region with those located in the laterodorsal tegmental nucleus and refer to this entire cholinergic cell group as the "caudal cholinergic column" (Satoh and Fibiger, '85; Vincent et al., '86) or "the pontomesencephalotegmental complex" (Woolf and Butcher, '86, '89; Gould et al., '89). Rye and co-workers ('87) have developed perhaps the most unusual designation for the cholinergic neurons within the lateral pontomesencephalic tegmentum. In their definition, the pedunculopontine tegmental nucleus (PPTn) of the rat is composed of only large, cholinergic neurons while the adjacent or in some instances apposing non-cholinergic neurons are referred to as "non-PPTn neurons". Numerous studies have focused on a subpopulation of these cholinergic neurons projecting to a specific target and have indicated that they are within the borders of the PPN (Beninato and Spencer, '86, '87; Isaacson and Tanaka, '86; Clarke et al., '87; Jones and Beaudet, '87b; Goldsmith and van der Kooy, '88; Fitzpatrick et al., '89; Hall et al., '89; Jones and Cuello, '89; Jones, '90; Sakai et al., '90; Semba et al., '90). However, the descriptions on the boundaries of the PPN in these studies have ranged from none to fairly detailed delineations.

In a previous study (Spann and Grofova, '89), we have analyzed the cytoarchitecture of the pontomesencephalic tegmental region surrounding the superior cerebellar peduncle in sagittal Nissl-stained sections of the rat brain and described the delineation of the PPN from the more rostral

retrorubral nucleus (RR) and field (RRF) and the more caudal parabrachial nuclei (PB) and subpeduncular tegmental nucleus (SPTg). Furthermore, we have identified two subdivisions of the nucleus, the pars compacta (PPNc) and pars dissipata (PPNd). In the present study, we have related the distribution of cholinergic neurons to this delineation of the PPN.

Our present observations show that the bulk of the cholinergic neurons in the pontomesencephalic tegmentum are indeed located within the PPN with a few neurons also observed in the adjacent portions of the SNr, RR/RRF, PB and SPTg. A similar observation was noted in the rat (Armstrong et al., '83; Mesulam et al., '83; Jones and Cuello, '89; Jones, '90), cat (Jones and Beaudet, 87a; Fitzpatrick et al., '89; Hall et al., '89) and dog (Isaacson and Tanaka, '86). However, several of these investigators (Isaacson and Tanaka, '86; Jones and Beaudet, '87a) may have extended the boundaries of the PPNd to include the ventromedial PPNc and the rostral most portion of the SPTg of the present study. In both of those regions the cholinergic neurons exhibited similar morphological features to those in the PPNd.

Organization of cholinergic somata and dendrites in the two subdivisions of the PPN

Some of the previous investigators have reported that both subnuclei of the PPN contain numerous cholinergic somata

(Armstrong et al., '83; Mesulam, et al., '83; Isaacson and Tanaka, '86; Jones and Beaudet, '87b). The present results confirm and extend those observations by demonstrating that the cholinergic somata within the PPNd slightly outnumber those within the PPNc. On the other hand, the PPNc is characterized by a higher density of cholinergic somata. In addition to the cholinergic cell bodies, both subnuclei of the PPN appear to contain an extensive network of overlapping cholinergic dendrites. Although most of these dendrites remain within the cytoarchitectural boundaries of the PPN, they cross freely from one subnucleus to the other. A particularly significant observation is the occurrence of cholinergic somata and dendrites in the medial three-quarters of the PPNd which appear to receive the bulk of nigral afferents (Spann and Grofova, submitted). This observation together with similar findings by Hall and co-workers ('89) in the cat strongly argue against the concept of midbrain extrapyramidal area (MEA) fostered by Rye et al. ('87). According to Rye et al. ('87), the MEA which is reciprocally related to the basal ganglia does not contain any cholinergic neurons and occupies a region adjacent to the PPTn.

Ultrastructural characteristics of the somata and dendrites of PPN neurons

The neurons of the rat PPN exhibited similar size ranges (small to large) and contained similar organelles as

described in ultrastructural studies of PPN neurons in the cat (Moriizumi et al., '89) and ChAT+ neurons in the rat PPNc (Sugimoto et al., '84). The nuclear membranes of the PPN somata exhibited a broad range in the depth of their invaginations. A similar observation has also been reported by Moriizumi and co-workers. On the other hand, the nuclear membranes of the ChAT+ were generally smooth while the ChAT- somata nuclear membranes exhibited deep indentations. The nuclear membranes of ChAT+ neurons in the PPNc (Sugimoto et al., '84) have been described as being indented.

In both the rat and cat PPN, neuronal appositions were occasionally found between somata or between somata and dendrites. However, a unique observation in the present study was that roughly a third of the cholinergic somata were in direct apposition with either cholinergic or non-cholinergic cell bodies or large diameter dendrites. Similar appositions of non-cholinergic profiles were completely lacking.

Moriizumi and co-workers have described somatic and dendritic spines as being common features of the PPN neurons and thus concluded that a portion of the neurons are spiny in their appearance. In contrast our observations indicated that the PPN neurons somatic and dendritic membranes were generally smooth with only an occasional spine being encountered. Furthermore, both intracellular HRP (Granta and Kitai, '89) and Golgi studies (Newman, '85; Scarnati et al., '88) in the rat did not demonstrate somatic and dendritic

spines as being characteristic of PPN neurons. In a previous intracellular HRP study in the cat, Noda and Oka ('86) noted the PPN and surrounding tegmental neuron dendrites are aspinous or sparsely spinous. Therefore, whether spiny neurons are found within the cat PPN remains controversial.

PPN neurons with dendritic varicosities have been observed ultrastructurally by Moriizumi and co-workers ('89). However, while in the present study we demonstrated that the dendrites were irregular in shape, none exhibited the characteristic morphology of the cat dendritic varicosities. Therefore, this dendritic configuration may represent species difference between the rat and the cat.

Synaptic organization of the PPN.

In the present study, terminals were observed contacting both somatic and dendritic profiles of the rat PPN. The terminal coverage of the somatic membrane of the PPN neurons was measured and in general the soma of larger neurons received more terminals than the smaller neurons. Moriizumi and co-workers ('89) have reported similar results in the analysis of an equivalent number of cat PPN neurons. However, our findings also indicate that there exists a great variation in the density of synaptic terminals on the somata of larger neurons. In one group of large neurons the soma membrane was contacted very infrequently (0-9%) by terminals while in the second group the membrane was covered by a

greater number (15-55%) of terminals. It thus may be an oversimplification to equate somatic size with synaptic density in the rat PPN.

Moriizumi and co-workers have suggested the ChAT+ neurons of the PPN receive a greater synaptic input than non-cholinergic neurons. Our findings indicated that the ChAT+ neurons in the rat PPNd were overall contacted by fewer terminals than the ChAT- neurons. In light of these findings, it would be erroneous to conclude that cholinergic neurons in the PPN receive a greater synaptic input than non-cholinergic neurons.

Terminals in the PPN were classified into two major types (Type I and II) based on the type of synaptic junction and the shape of the synaptic vesicles. Similarly, Moriizumi et al. ('89) classified axon terminals in the cat into asymmetrical and symmetrical types by their synaptic junctions. The Types I and II appear to be morphologically quite similar to the asymmetrical and symmetrical types, respectively. In both species, the Type II terminals were most frequently encountered in the PPN. Furthermore, the distribution of each terminal type on the somata and small size dendrites of PPN neurons was quite similar in both species. On the somata the Type II terminals were the most common while on the small dendrites the Type I terminals were most frequently encountered.

Both cholinergic and non-cholinergic neurons in the PPNd were observed in the present study to be contacted by Type I

and Type II terminals. While the Type I terminals exhibited only a slight preference for non-cholinergic neurons, the Type II terminals were found predominantly on these neurons. In addition, the synaptic input to the non-cholinergic somata was on average three times higher than to the cholinergic somata. A recent neurophysiological study (Kang and Kitai, '90) has indicated that cholinergic neurons exhibit a tonic firing pattern while the non-cholinergic neurons firing pattern is phasic in nature. In light of the differences in frequency and distribution of the terminals on the cholinergic and non-cholinergic neurons, these differences in firing patterns may reflect the variations of the synaptic input to the two populations of neurons.

In the present study, complex synaptic arrangements were limited to a small number of single terminals contacting two dendrites of various diameters. While dendrodendritic synapses were suggested by the presence of a small number of dendritic profiles with clusters of vesicles, none of these profiles exhibited a synaptic junction. Serial synapses involving dendrodendritic synapses are considered one of hallmarks for the presence of interneurons at the ultrastructural level. A recent Golgi study in the rat by Scarnati et al. ('88) has shown that axons of a few PPN neurons ramified near their cell bodies and were judged to be interneurons. Thus the results of the Golgi and present study would appear to complement one another. In the cat, terminals involved in serial synapses and other complex

synaptic arrangements made up less than 3% of the total number of terminals examined. Furthermore, dendrodendritic synapses were only rarely encountered. At present, no light microscopic data is available to confirm that interneurons are present in the cat PPN. The results of these studies would imply that the PPN does not possess a prominent complex synaptic organization or a large network of interneurons. However, further light and electron microscopic studies utilizing various labeling techniques are needed to more fully understand the synaptic organization and the exact degree of its complexity

The morphological characteristics of the Type I terminals allowed us to further differentiate them into two Subtypes, IA and IB. The Subtype IB could be differentiated from the IA by the presence of subjunctional dense bodies beneath the postsynaptic density. The morphology of the Subtype IB is quite similar to some of the asymmetrical terminals described in the cat PPN by Moriizumi et al. ('89). However, the asymmetrical terminals were not further subdivided by these authors even though the major terminal classes were differentiated primarily by their post-synaptic junctions. This variance in classification raises the issue that future studies may identify more than two major types of terminals in the PPN of the rat or cat. It is plausible that variations in tissue preparation or more subtle morphological criteria may lead to a further division of the terminals into different classes. Furthermore, experimental studies

designed to determine the origin of different populations of synaptic terminals may also result in further segregation of the terminal types. Thus these initial studies in the rat and cat should be viewed as providing a basis for future ultrastructural studies on the normal morphology and afferent sources of the PPN.

Afferent sources of the PPN

The predominant type of terminals on PPN neurons were those with pleomorphic vesicles and symmetrical contacts (Type II). Previous studies in the rat (Spann and Grofova, submitted) and in the cat (Nakamura et al., '89) have shown that a portion of these terminals originate from the SNR. In addition, the Type II terminals are morphologically similar to the entopeduncular terminals in the cat motor thalamus (Grofova and Rinvik, '74; Rinvik and Grofova, '74; Kultas-Ilinsky et al., '83) and to the boutons of pallidal origin in the rat substantia nigra (Smith and Bolam, '90). Since numerous light microscopic studies have demonstrated that the PPN receives afferents from both the globus pallidus as well as the entopeduncular nucleus (Nauta and Mehler, '66; Kim et al., '76; Nauta., '79; Larsen and McBribe, '79; McBride and Larsen, '80; DeVito and Anderson, '82; Jackson and Crossman, '81, '83; Moon-Edley and Graybiel, '83; Moriizumi et al., '88), the Type II terminals may also belong to these afferent systems.

The next major type of terminals in the PPN was the Subtype IA boutons containing ovoid synaptic vesicles and forming asymmetric synapses. At present there exists no experimental evidence on the origin of these terminals. They exhibit similar morphology as the boutons of subthalamic origin in the rat substantia nigra and globus pallidus (Kita and Kitai, '87). Since PPN afferents from the subthalamic nucleus are well documented by light microscopic studies (Nauta and Cole, '78; McBride and Larsen, '80; Jackson and Crossman, '83; Moon-Edley and Graybiel, '83; Kita and Kitai, '87; Smith et al., '90), it is likely that a portion of Subtype IA terminals actually originate in the subthalamic nucleus. They may also belong to the fibers of cortical origin (Hartman-von Monakow et al., '79: Jackson and Crossman, '83; Moon-Edley and Graybiel, '83) or they may represent termination of axon collaterals of the PPN neurons (Scarnati et al., '88). The latter suggestion is supported by the similarities in the morphology of these boutons and PPN terminals in the subthalamic nucleus and intralaminar thalamic nuclei (Sugimoto and Hattori, '84; Moriizumi et al., '89).

Finally, some suggestions can be offered as to the origin of the Subtype IB which can be differentiated from the Subtype IA by the presence of post-junctional dense bodies beneath the postsynaptic membrane. Experimental studies in our laboratory (Spann and Grofova, unpublished results) had shown that a portion of the Subtype IB originate from a

region of the mesencephalic reticular formation (MRF). This area of the MRF is located dorsal to the SN and rostral to the level of the RR and RRF of the present study. Other origins of these terminals is at present unknown.

Interactions between ChAT+ and ChAT- neurons

Numerous neurochemical studies in both primate and non-primate species (Vincent et al., '83; Sofroniew et al., '85; Beninato and Spencer, '86, '87; Isaacson and Tanaka, '86; Woolf and Butcher, '86; Clarke et al., '87; Pare et al., '88; Steriade et al., '88; Gould et al., '89; Hall et al., '89; Jones and Cuello, '89; Fitzpatrick et al., '90; Jones, '90; Sakai et al., '90; Semba et al., '90) have firmly established that throughout the PPN there is a mixture of cholinergic and non-cholinergic neurons. Immunocytochemistry studies have suggested that portions of the non-cholinergic population may be catecholaminergic (Jones and Beaudet, '87a), enkephalinergic (Fallon and Leslie, '86; Pollard et al., '89), GABAergic (Nagi et al., '84; Jones, '90) or glutaminergic (Clements and Grant, '90). Thus one of the distinguishing traits of the PPN would appear to be the transmitter-related heterogeneity of the PPN neurons. Observations in the present study had shown that a portion of the cholinergic somata in the PPNd are in direct apposition with either ChAT+ or ChAT- neurons. It is plausible that such appositions are not purely casual but may be of

functional importance. During the refractory period of an action potential, the potassium concentration around the firing cell soma increases. This change in extracellular potassium may have a depolarizing effect on the neuron in apposition to the one firing. It is therefore possible that the firing cell may have an excitatory, modulating effect on the appositional neuron.

In addition to appositional relationships, PPN neurons may interact with one another through local circuit neurons. Observations from a Golgi study in the rat (Scarnati et al., '88) and an electron microscopic study in the cat (Moriizumi et al., '89) have suggested that at least a small portion of the PPN cells are interneurons. The presence of interneurons in the PPN would allow a variety of interactions between PPN projection neurons and would permit various forms of processing of the afferent inputs to the PPN. Interneurons in the PPNd may be cholinergic or non-cholinergic. However, it is more likely that these neurons are non-cholinergic since Mesulam and co-workers ('84, '89) have indicated that the bulk of the neurons in the PPNd are non-cholinergic. Furthermore, in the present study we have observed that the axon terminals within the PPNd are almost exclusively non-cholinergic. In view of these findings it is plausible that the PPNd interneurons are non-cholinergic. The presence of neuronal appositions and interneurons in the PPNd, although small in number, would suggest at least a portion of the

cholinergic and non-cholinergic populations are involved in fairly complex interactions with one another.

There exists a general agreement that the PPN gives rise to prominent and widespread cholinergic projections to numerous regions of the CNS. Previous double labeling studies have described cholinergic projections from the PPN to the cortex (Vincent et al., '83), basal ganglia (Woolf and Butcher, '86; Beninato and Spencer, '87; Clarke et al., '87; Gould et al., '89), basal forebrain (Woolf and Butcher, '86; Jones and Cuello, '89), hypothalamus (Woolf and Butcher, '86; Sakai et al., '90), various thalamic nuclei (Sofroniew et al., '85; Woolf and Butcher, '86; De Lima and Singer, '87; Hallanger et al., '87; Pare et al., '88; Steriade et al., '88; Fitzpatrick et al., '90; Semba et al., '90), superior colliculus (Beninato and Spencer, '86; Woolf and Butcher, '86; Hall et al., '89), and both the pontine (Semba et al., '90) and medullary (Rye et al., '88; Jones, '90) reticular formation. Furthermore, while some of the authors have shown that up to 85% of the PPN projections to any one region are cholinergic (Sofroniew et al., '85), none have demonstrated an all cholinergic projection to any of the aforementioned regions. At present, the only 'homogenous' neurochemical projection of the PPN is the non-cholinergic PPN-spinal cord projection (Goldsmith and van der Kooy, '88; Rye et al., '88). Electrophysiological studies have shown that the PPN provides excitatory input to the lateral geniculate nucleus of the thalamus (Hu et al., '89a; Steriade et al., '90b),

various basal ganglia nuclei (Gonya-Magee and Anderson, '83; Hammond et al., '83; Scarnati et al., '84, '86, '87) and the medullary reticular formation (Garcia-Rill and Skinner, '87b). There has been no report of PPN neurons providing an inhibitory input to any of these nuclei. Thus the question arises if both cholinergic and non-cholinergic neurons projecting to the same nucleus may also be functioning in part together. Numerous neurochemical studies have associated the cholinergic PPN projections to the thalamus with REM sleep (Woolf and Butcher, '86; De Lima and Singer, '87; Hallanger et al., '87; Pare et al., '88; Steriade et al., '88; Webster and Jones, '88; Mesulam et al., '89; Fitzpatrick et al., '90; Jones, '90; Semba et al., '90). Specifically, the cholinergic PPN neurons are believed to convey PGO (ponto-geniculo-occipital) waves, which herald the state of REM sleep and continue throughout it, to the lateral geniculate (LGN) nucleus of the thalamus (Hu et al., '89b; Steriade et al., '90b; Pare et al., '90). The findings of double-labeling studies (Pare et al., '88; Steriade et al. '88) have shown that of the PPN projections to the thalamic nuclei, the PPN-LGN projection contains the one of the highest percentages (87%) of cholinergic fibers. However, based on several lines of neurophysiological evidence, Steriade and co-workers ('90b) have proposed that a portion of the PPN neurons (16%) which convey the PGO waves are non-cholinergic. The aforementioned authors have concluded that the remaining PPN cells are cholinergic. It is therefore

possible that both cholinergic and non-cholinergic neurons projecting to the same nucleus may also be involved in similar function(s).

The cholinergic PPN neurons projecting to the thalamus are probably most frequently associated with the ascending reticular activating system which plays a critical role in EEG activity (Mesulam et al., '83; Sofroniew et al., '85; Woolf and Butcher, '86; De Lima and Singer, '87; Hallanger et al., '87; Pare et al., '88; Steriade et al., '88; Fitzpatrick et al., '90; Semba et al., '90). Furthermore, the cholinergic projections to the reticular nucleus and non-sensorimotor relay nuclei of the thalamus have been implicated as being key components in the initiation and maintenance of the desynchronization of EEG activity (Hallanger et al., '87; Pare et al., '88; Steriade et al., '88). However, the ratio between the number of cholinergic and non-cholinergic neurons projecting to each of these thalamic nuclei varied considerably (Pare et al., '88; Steriade et al., '88). The ratios ranged from 4:1 for the PPN-lateral posterior projection to 1:3 for the PPN-centrum medianum/parafasicular projection. The ratio for the PPN-reticular nucleus projection was approximately 1:1. Steriade and co-workers ('90a) have commented on the failure of various research groups to abolish EEG desynchronization following excitotoxic lesions centered in the PPN. These authors have attributed this lack of abolishment to the existence of multiple systems involved in various components

of EEG desynchronization and the fact that brain-stem reticular territories with still unidentifiable transmitters also have a role in EEG desynchronization. A recent study by Jones and Webster ('88) has indicated that kainic acid injections in the PPN resulted in a greater destruction of cholinergic neurons than non-cholinergic neurons. In light of the previous observations on the PGO wave conduction, it is plausible that the surviving cholinergic neurons in conjunction with non-cholinergic neurons are able to maintain the ability of the ascending reticular activating system to modulate EEG activity.

Authors of two recent neurophysiological studies have hypothesized that the non-cholinergic (Steriade et al., '90) and a portion of the cholinergic (Pare et al., '90) neurons which convey PGO waves are modulated by the SNR since the SNR has been linked to the regulation of PGO wave transmission (Datta et al., '90). We have previously demonstrated that a portion of the terminals in the PPN are of nigral origin (Spann and Grofova, submitted). While both the non-cholinergic and cholinergic PPN neurons are contacted by terminals (Type II) which are morphologically quite similar to the nigral boutons, it is still unresolved whether the nigral input is directed towards one or more transmitter-specific neuronal populations of the PPN. The source and functional significance of the Type I terminals observed in the present study has yet to be determined.

The PPN contains a prominent group of cholinergic neurons intercalated with a large population of non-cholinergic neurons. In nearly every instance, projections from the PPN contain a neurochemically heterogeneous population of fibers. Studies on the PPN-thalamic projection have suggested that both cholinergic and non-cholinergic neurons may take part in similar functions. It is plausible that cholinergic and non-cholinergic PPN neurons projecting to other regions may also be functionally related. Furthermore, both cholinergic and non-cholinergic PPN neurons may receive synaptic input from the same source(s). In addition, the appositional relationships and interneurons within the PPN may allow portions of the two populations to intrinsically influence one another. Neuroanatomical, physiological and pathological data have linked the PPN to a rather diverse number of both motor and non-motor functions. Therefore, relating specific functions to only the cholinergic neurons of the PPN while completely excluding the non-cholinergic neurons may be a gross oversimplification which may hinder our further understanding of the roles of the PPN in the central nervous system.

Figure 1: Synaptic Relationships of PPN Cells.

The slope of the regression line indicates there is a direct relationship between somata area and terminal density.

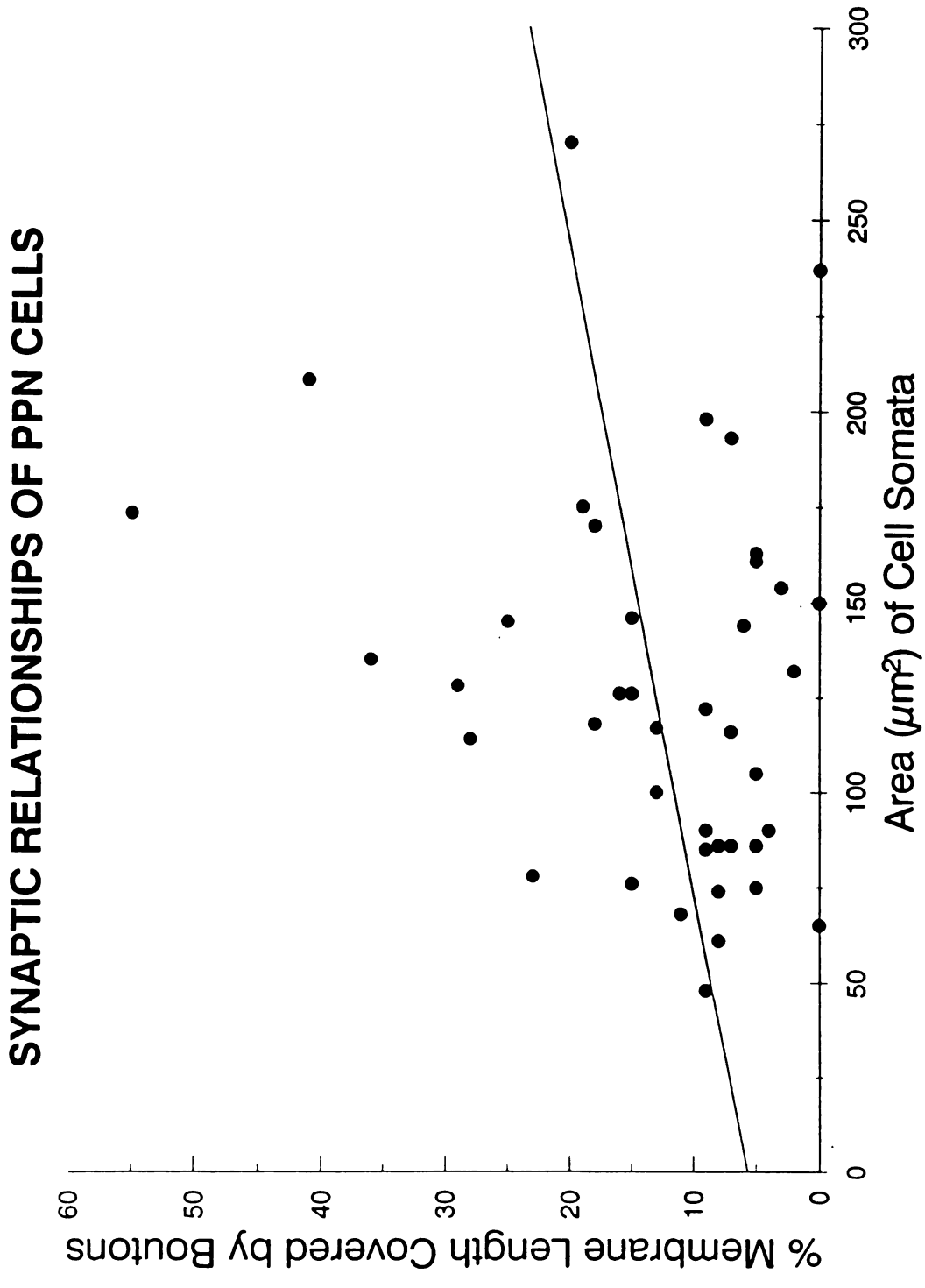

SYNAPTIC RELATIONSHIPS OF PPN CELLS
% Membrane Length Covered by Boutons
0
10
20
30
40
50
60
0
50
100
150
200
250
300
Area (μm²) of Cell Somata

Figure 1

Figure 2: Electron Micrographs Illustrating the Major Terminal Types in the PPN.

A: Type 1 terminals (1A and 1B) containing round synapticvesicle and forming asymmetrical synapses (open arrows) with small-size dendrites. The Subtype 1B is differentiated from the Subtype 1A by its post-junctional dense bodies, which appear to be continuous with a portion of the smooth endoplasmic reticulum in this dendrite (arrowheads). A Type 2 terminal containing pleomorphic vesicles forms symmetrical synapses (open arrows) with a cell somata. Note the small cluster of presumed synaptic vesicles (solid arrows) in a medium-size dendrite.

B: A Subtype 1A terminal synapsing (open arrow) on a small-size dendrite (d_1). Another Subtype 1A terminal is establishing both a definitive synapse (open arrow) on a dendritic spine (solid star) and suggestive synapse on a large-size dendrite (d_2).

C: A Type 2 terminal containing centrally placed mitochondria forming multiple synapses on a cell body (CB). A second Type 2 terminal is attached to the cell body by a puncta adherens (solid arrow).

Scale bars: 1.0 μm

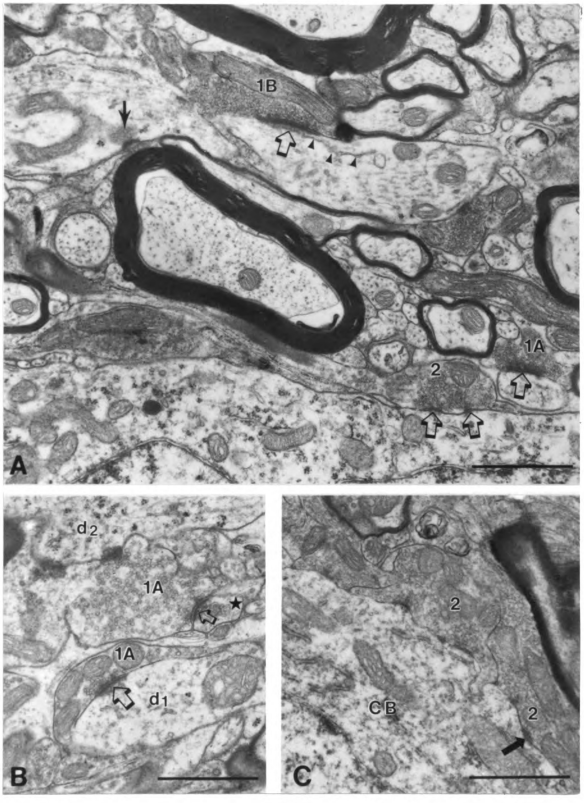


Figure 2

Figure 3: Distribution of Cholinergic Cells in the PPN.

Projection drawings of sagittal sections of the lateral (A) through medial (D) levels of the pontomesencephalic tegmentum showing the distribution of ChAT positive cells in the PPN and adjacent structures. One dot equals one cell.

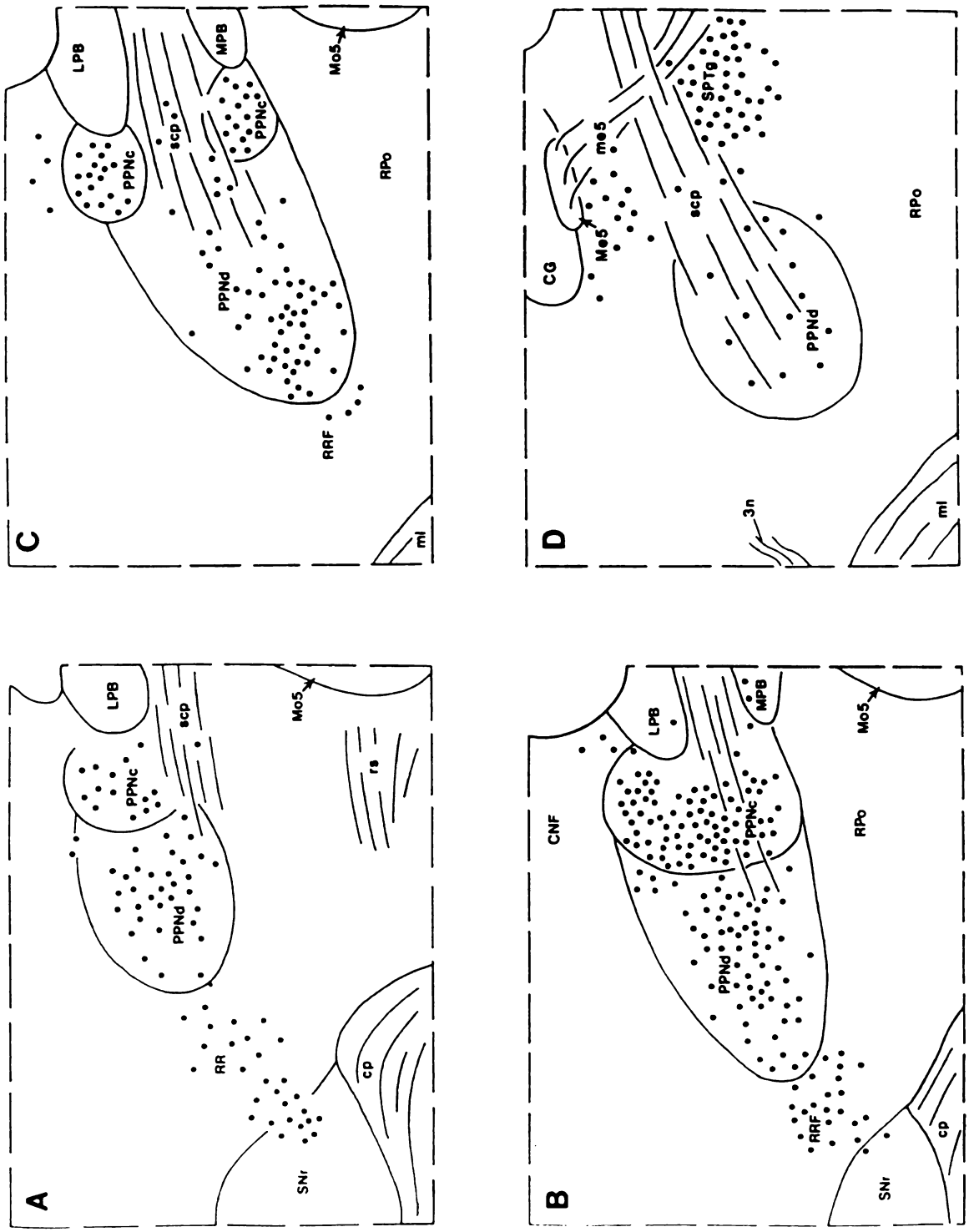
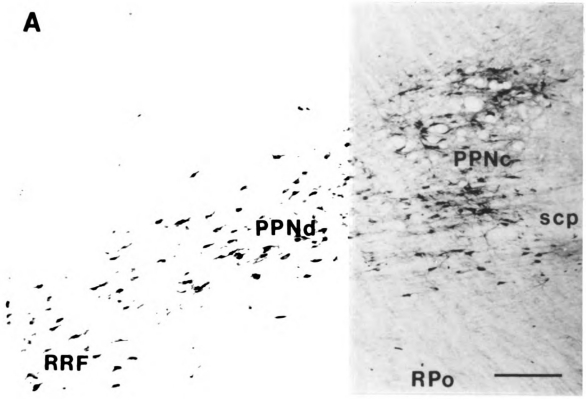

A
LPB
scp
Mo5
PPNc
rs
PPNd
RR
cp
SNr
B
CNF
LPB
MPB
Mo5
PPNc
RPo
PPNd
RRF
cp
SNr
C
LPB
MPB
Mo5
scp
PPNc
PPNc
RPo
PPNd
RRF
ml
D
SPTg
me5
Mo5
scp
CG
RPo
PPNd
3n
ml

Figure 3

Figure 4: Brightfield Photomicrographs of ChAT+ Cells in the PPN.

A: Low-power photomicrograph of the lateral half of the PPN showing numerous ChAT+ cells in both subnuclei. The PPNc has reached its maximum dimensions and contains densely packed ChAT+ cell somata and dendritic plexuses.
Scale bar: 250 µm.

B: Higher magnification of the medial half of the PPNd showing ChAT+ cells interlaced by extensive dendritic arborizations.
Scale bar: 50 µm.



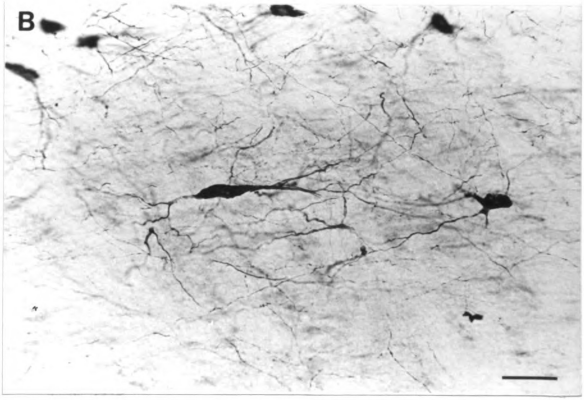


Figure 4

Figure 5: Montage of Electron Micrographs of a Cholinergic PPNd Cell.

Electron microscopic montage from the PPNd of a large ChAT+ cell in direct apposition with a smaller ChAT- cell. Open arrows indicate terminals forming either definitive or suggestive synapses with the somatic membrane of the two neurons. Scale bar: 3.0 μm.

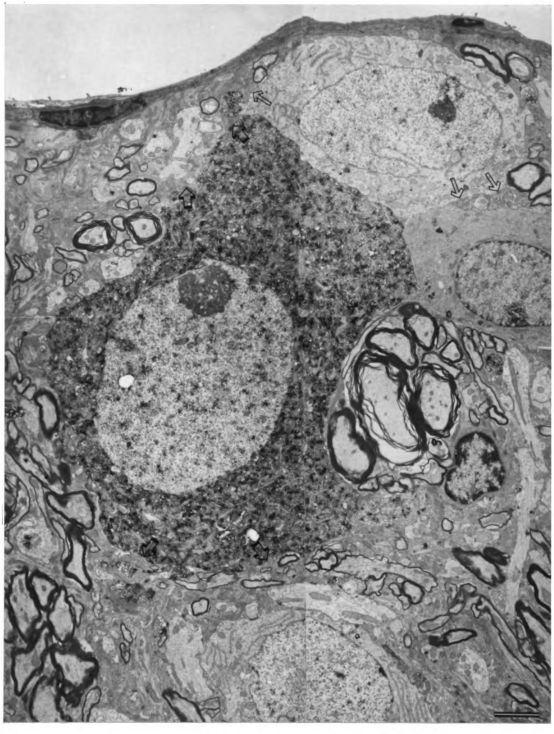


Figure 5

Figure 6: Montage of Electron Micrographs of Non-cholinergic PPNd Cell.

Electron microscopic montage from the PPNd of a large ChAT- cell. Note, the striking increase in the number of terminals (open arrows) contacting the somata in contrast to the ChAT+ cell demonstrated in Figure 5. Scale bar: 3.0μm.

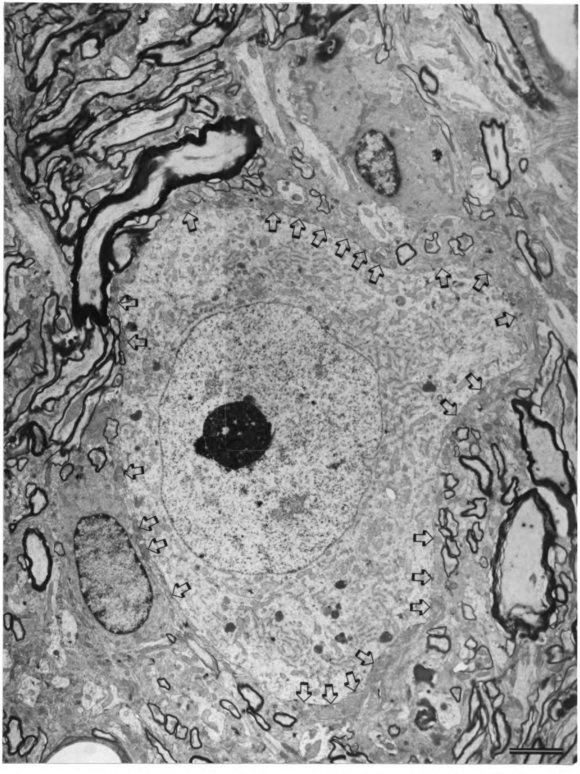


Figure 6

Figure 7: Electron Micrographs of Cholinergic Elements and Their Synaptic Relationships in the PPNd.

A: A Subtype 1B terminal simultaneously synapsing onto ChAT+ (d_1) and ChAT- (d_2) small-size dendrites.

B: Spine-like evaginations (arrows) arising from a ChAT- cell body (CB) are postsynaptic to presumed Subtype 1A and Type 2 terminals.

C: A portion of a ChAT+ cell body (CB) postsynaptic to a Subtype 1A terminal. Scale bars: 1.0 μm.

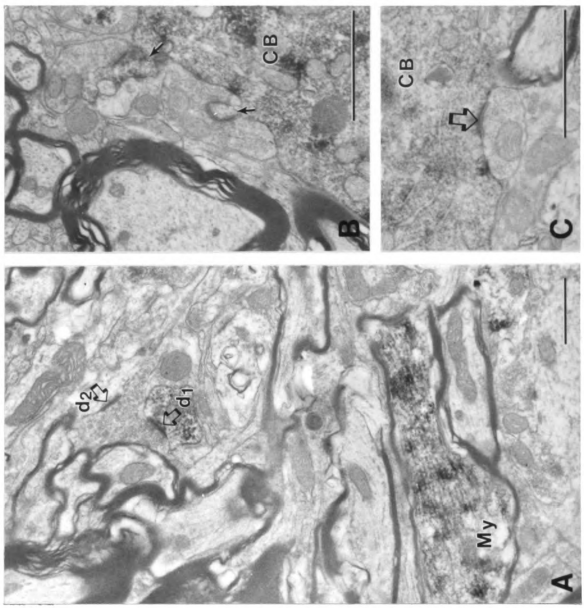


Figure 7

Figure 8: Cholinergic vs. Non-cholinergic Cells. A Size Comparison.

Dot diagram of the sizes of 28 ChAT+ and 27 ChAT- neurons in the central region of the PPNd.

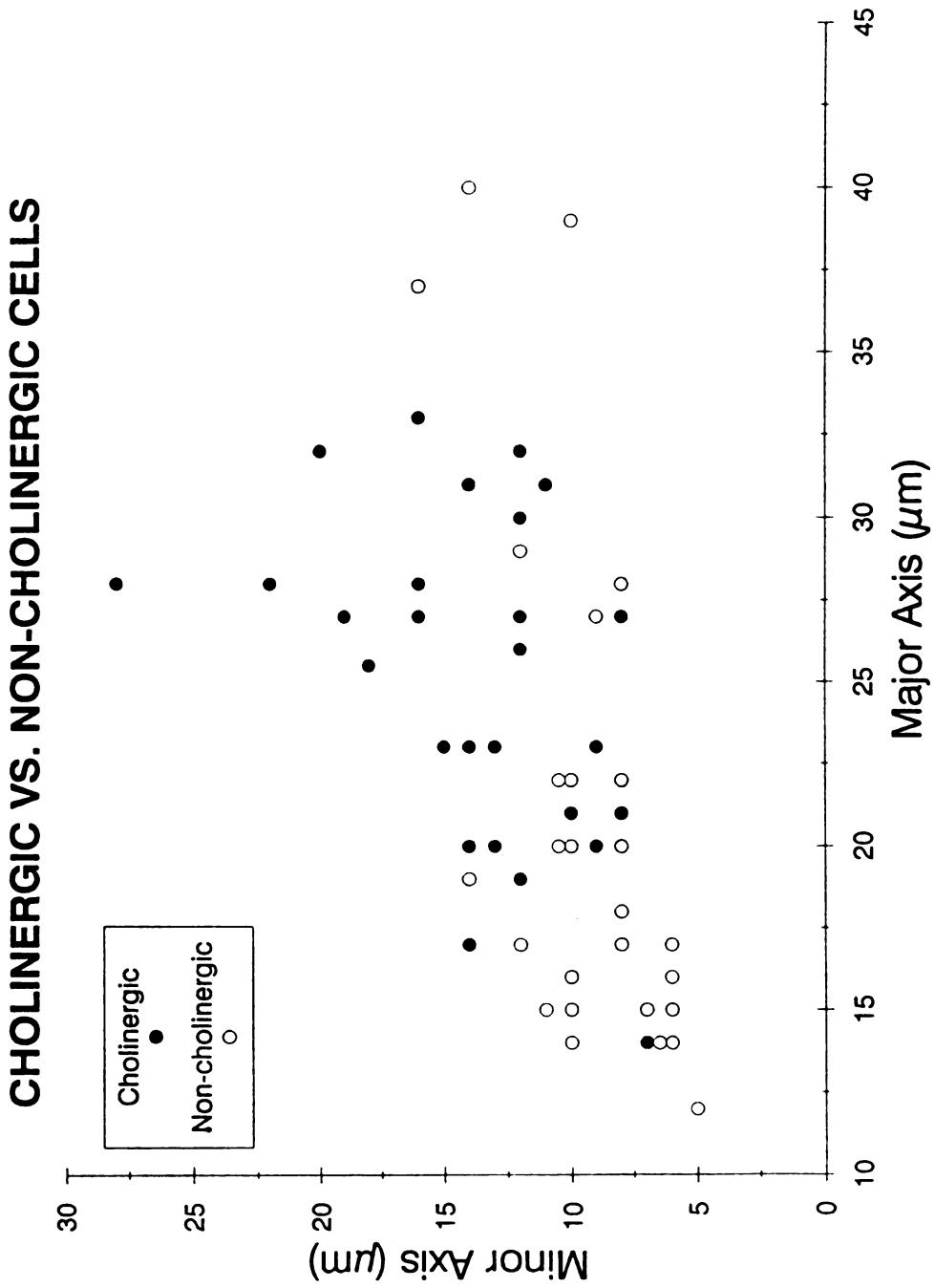

CHOLINERGIC VS. NON-CHOLINERGIC CELLS
Cholinergic
Non-cholinergic
Minor Axis (μm)
Major Axis (μm)
0
5
10
15
20
25
30
10
15
20
25
30
35
40
45

Figure 8

Figure 9: Terminal Types Contacting Cholinergic PPNd Dendrites.

Electron micrographs illustrating the Type 1 (A and B) and Type 2 (C and D) terminals synapsing on various diameter ChAT+ dendrites in the PPNd. Note the subtype 1B (B) post-junctional dense body (arrow) beneath the post-synaptic density is clearly distinguishable from the surrounding reaction product.

Scale bar: 1.0 μm.

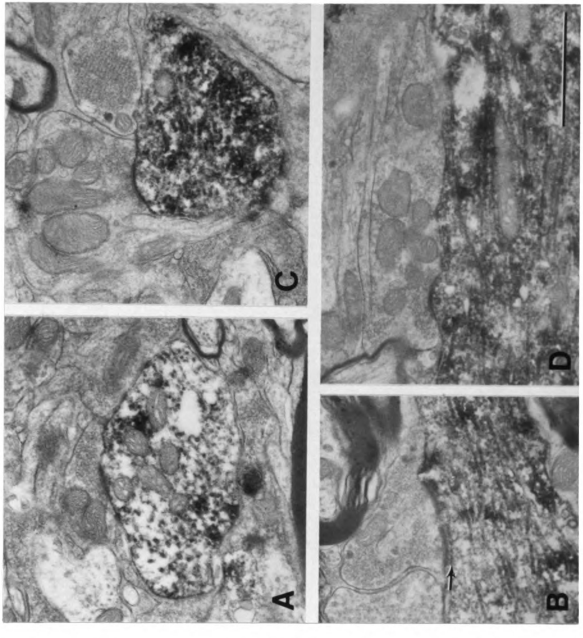


Figure 9

Figure 10: Distribution of Boutons in the PPNd.

The distribution of 421 terminals (Type 1:207; Type 2:214) establishing distinct synapses with 117 ChAT+ and 102 ChAT- profiles were analyzed on electron micrographs containing a mixture of both ChAT+ and ChAT- profiles.

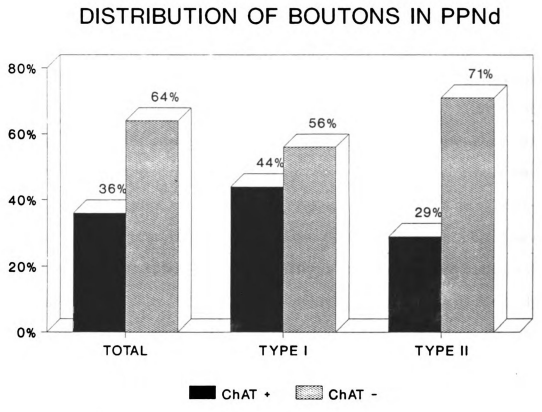

DISTRIBUTION OF BOUTONS IN PPNd
80%
60%
40%
20%
0%
36%
64%
44%
56%
29%
71%
TOTAL
TYPE I
TYPE II
ChAT +
ChAT -

Figure 10

CONCLUDING REMARKS

The present study involved three major projects which were focused on furthering our understanding of the circuits between the basal ganglia and the PPN. The first project examined the distribution of the PPN neurons projecting to the basal ganglia and spinal cord, and established whether some of the cells give rise to collateralized projections. The retrograde transport studies demonstrated that both ascending PPN neurons projecting to the basal ganglia and descending PPN neurons projecting to the spinal cord were distributed throughout the PPNd. An analysis of the labeled ascending and descending projection neurons indicated there was no distinct morphological features to differentiate with certainty one population from the other. The fluorescent dye studies revealed an almost insignificant number of PPN projection neurons had axons branching into an ascending and descending collateral. Therefore, these results indicated that the basal ganglia-projecting cells are neither spatially segregated nor morphologically distinct from the cells projecting to the spinal cord and both represent separate populations of PPN projection neurons.

In the following project, the distribution and mode of termination of the nigropedunculopontine projection was studied light and electron microscopically by using the PHA-L technique. While the SNR was observed to project to both subnuclei, the bulk of the nigral fibers terminated in the

PPNd. Furthermore, the nigral fibers demonstrated a distinct preference for some neurons and islands of neuropil. Electron microscopic examination confirmed that nearly all of the varicosities observed in the light microscope represented either terminal boutons or boutons en passant. The majority of nigral terminals were seen in contact with various-width dendrites. The region containing the nigral plexus has previously been shown to contain both basal ganglia and spinal cord projecting PPNd neurons. However, the patchy distribution of the nigral plexus suggests that the nigral fibers may be related to a specific subpopulation of PPN projection neurons.

The final project focused on the distribution and synaptic organization of both cholinergic and non-cholinergic neurons in the PPNd. The cholinergic neurons were visualized using ChAT immunocytochemistry. This study demonstrated that cholinergic somata and dendrites were present throughout the subnuclei. Furthermore, some of the cholinergic dendrites in the PPNd were observed to arise from cells in the PPNc. Both cholinergic and non-cholinergic somata and dendrites were contacted by terminals which had been classified as Types I and II in the ultrastructural analysis of the PPN. The Type II terminals were morphologically quite similar to the nigral boutons identified in the previous project. Therefore, it is possible nigral afferents to the PPN may terminate on both cholinergic and non-cholinergic neurons of the PPNd. In light of the previous observations that nigral terminals were

also encountered in the PPNc and a portion of the cholinergic dendrites originate from PPNc neurons, it is plausible that the nigral input may modulate cholinergic cells not only in the PPNd, but also in the PPNc. In conclusion, the present results would imply that in the rat the circuit linking the basal ganglia to the PPN (i.e. the nigropedunculopontine projection) is more widespread and complex than previously envisioned.

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