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Light and electron microscopic observations of the cat periaqueductal grey

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LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS OF THE CAT PERIAQUEDUCTAL GREY: RELATION TO PAIN MODULATION

by

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DISSERTATION

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DEDICATION

This thesis is dedicated to my husband Willy whose love, continual encouragement, and sense of humor helped me to to accomplish my goal.

LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS OF THE PEPTIDERGIC ORGANIZATION OF THE CAT PERIAQUEDUCTAL GREY: RELATION TO PAIN MODULATION

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ABSTRACT

Despite the significant contribution of the periaqueductal grey (PAG) to endogenous pain suppression systems, little is known about its neurochemical organization. Previous studies have indicated regional variations in the effectiveness with which the midbrain can generate potent analgesia in response to either opiate microinjection or electrical stimulation. In addition to opiates and opioid peptides, the non-opioid peptides substance P (Sub P) and vasoactive intestinal polypeptide (VIP) also produce analgesia when injected into the PAG.

As a first step towards elucidating the neurochemical organization underlying the PAG's contribution to endogenous analgesia systems, we used immunocytochemistry to map the distribution of the endogenous opioid peptide leucine enkephalin (ENK), and the non-opiate peptides substance P (Sub P) and vasoactive intestinal polypeptide (VIP) in the cat PAG.

Throughout the rostral-caudal extent of the PAG, ENK-containing neurons and terminals are consistently clustered in discrete populations. The distribution of Enk neurons and terminals undergoes a ventral to dorsal shift from caudal to rostral PAG. Conceivably this clustered distribution of ENK cells and terminals contributes to the differential effectiveness of various PAG regions in generating analgesia. The ventral-dorsal shift in ENK immunoreactivity may correspond either to a somatotopic organization within the PAG or may mirror the topographic relationship of PAG neurons to other afferent or efferent components of the endogenous analgesia system.

The distribution of Sub P immunoreactivity overlaps considerably with that of ENK. Sub P-containing neurons and terminals are also located in discrete populations which also undergo a ventral to dorsal shift from caudal to rostral levels of the PAG. This overlap in Sub P and ENK may contribute to the naloxone sensitivity of the analgesia produced by Sub P injection into the PAG.

The distribution of VIP immunoreactivity differs completely from that of either ENK or Sub P. Regardless of the rostral-caudal level examined, VIP-containing neurons are located in the subependymal neuropil of the ventromedial PAG. The lack of overlap between the distribution VIP and ENK immunoreactivity, and the fact that VIP-produced analgesia is not reversed by naloxone, provides evidence for a non-opiate, VIP-mediated, analgesia system involving the PAG.

To determine the PAG microcircuitry through which opiates generate analgesia we examined the synaptic interactions of ENK labelled profiles. We concentrated on the caudal, ventral PAG, a region particularly effective in generating analgesia. To relate the ENK picture to other elements of the PAG, we also did a quantitative analysis of the normal ultrastructure of the caudal PAG. ENK-containing neurons and dendrites are found postynaptic to a variety of axonal-terminals. The great majority of ENK terminals are presynaptic to dendrites. The remainder are presynaptic to neuronal perikarya or occassionaly to vesicle-containing profiles. The quantitative analysis of normal PAG revealed few axo-axonic interactions. Thus a primary opiate action is as convergent input onto postsynaptic dendrites.

INTRODUCTION

It has been almost two decades since the midbrain periaqueductal grey (PAG) was first shown to be a potent locus for the production of analgesia by morphine and by electrical brain stimulation (Tsou & Jang, 1962; Reynolds, 1969). Subsequent studies have elucidated the properties of the analgesia elicited by either intracerebral opiate microinjection into or electrical brain stimulation of this region (Mayer et al, 1971; Mayer & Liebeskind, 1974; Oliveras et al 1974, 1979; Mayer & Price 1976; Lewis and Gebhart, 1977; Gebhart and Toleikas, 1978; Yeung et.al., 1977; Bennet and Mayer, 1979). Of particular interest are the studies demonstrating that the PAG is an important component of an endogenous pain control system that involves, at least in part, a descending pathway to nociceptive neurons of the spinal cord dorsal horn (see Mayer & Price, 1976; Basbaum & Fields, 1978 for review). However the mechanisms within the PAG by which opiates or focal brain stimulation initiate their antinociceptive effects are unknown. In effect, the PAG remains a "black box" containing unknown, analgesia relevant, circuits.

I. PAG and Endogenous Pain Control Systems

As stated above, focal electrical stimulation of the PAG produces potent analgesia. This effect is remarkably specific; pain suppression is produced without any general motor or sensory changes (Mayer and Price, 1976). Although some stimulation-produced analgesia (SPA) sites in the PAG are also effective sites for self stimulation, (a behavior which is an index of the reinforcing properties of brain stimulation) the two effects can be dissociated: certain spinal lesions will abolish SPA, while self-

stimulation behavior remain intact (Basbaum et. al., 1977). In humans, stimulation of the periventricular grey also genrates analgesia without adverse sensations and behavioral effects (Adams, 1976; Hosobuchi, et. al., 1977); PAG stimulation is generally avoided because of involvement of the III nerve nuclei.

While previous studies had demonstrated powerful descending control from the brainstem, analgesia production by brain stimulation provided the strongest evidence for the existence of central mechanisms of pain modulation. The discovery of endogenous opiate binding sites (Kuhar et.al., 1973) and endogenous opiate receptor ligands (enkephalins) (Hughes et. al., 1975; Simantov and Snyder, 1976) further implied the existence of an opiate-mediated endogenous analgesia system.

It was proposed (Mayer & Price, 1976) that exogenous opiates and brain stimulation suppress pain through similiar mechanisms with the PAG. The PAG contains both endogenous opiate receptors and enkephalins (Pert et. al., 1976; Simantov et al 1976). The most effective sites for opiate and SPA are in the ventrolateral PAG. More importantly, SPA from the PAG has been shown to be reversed by naloxone, the specific opiate antagonist (Adams, 1976; Hosobuchi et. al., 1977; Akil et. al., 1976). In addition, tolerance can develop to the analgesic actions of brain stimulation-produced analgesia after prolonged morphine administration (Mayer and Price, 1976). Finally, both opiate induced and stimulation produced analgesic mechanisms involved serotonergic neurons. For example, the serotonin synthesis inhibitor PCPA antagonizes both opiate and SPA (Akil et. al., 1972; Tenen, 1968).

Since the PAG receives afferents from the anterolateral quadrant of the spinal cord (Mehler, 1969), it was posible that the pharmacological and electrophysiological manipulations of the midbrain central grey produced analgesia by blocking pain-transmission pathways. Several lines of evidence, however, argued against this proposal. First, excitation of PAG neurons by electrical stimulation (Reynolds, 1969; Mayer et al., 1971; Mayer and Price, 1976; Lewis and Gebhart, 1977; Gebhart an Toleikas, 1978; Oliveras et. al., 1974, 1979; Yeung et. al., 1977) or by an injection of glutamate into the PAG (Behbehani and Fields, 1979) produces potent analgesia. Second, neither lesions of the PAG (Dostrovsky and Deakin, 1977), nor injection of local anesthetic into the PAG (Mayer and Price 1976; Lewis and Gebhart, 1977; Gebhart and Toleikas, 1978) suppresses pain. This indicates that activation of the PAG is necessary to generate analgesia. Thus the mechanisms of PAG produced analgesia requires activation of an antinociception system rather than a blockage of pain transmision.

Stimulation of the PAG produces both behavioral analgesia and inhibition of spinal cord dorsal horn nociceptors (Oliveras et. al., 1974). Moreover, the pain tests used to assess the degree of analgesia (ex. tail flick) involve spinally mediated reflexes. An endogenous pain control system with a major descending component seemed likely. Conclusive proof of such a pathway was provided by studies of the effect of subtotal lesions of the spinal cord. Lesions of the dorsolateral funiculs (DLF) of the cord antagonized both SPA and opiate analgesia (Basbaum et.al. 1976; 1977).

The PAG, however, projects minimally to the spinal cord (see section III[2]). Thus, a brainstem relay must be involved. The importance of serotonin in the analgesia produced by opiates and brain stimulation (see Messing, 1977 for review) was suggestive of a link with midline serotonergic nuclei in the medulla. For example, the analgesia produced by opiate microinjection into the PAG is blocked by the serotonin antagonists methylsergide and cinanserin (Yaksh et. al., 1976).

Retrograde transport studies (Basbaum and Fields, 1979) revealed that the axons of the medullary nucleus raphe magnus (NRM) projected to the cord via the DLF. Anterograde studies of the efferent projections of the NRM (Basbaum et. al., 1978) confirmed the existence of a pathway to the spinal cord dorsal horn, via the DLF, which terminated in laminae I,II, and V. In addition, stimulation of the NRM selectively inhibits spinal cord nociceptors (Fields and Anderson, 1978) and produces potent analgesia (Oliveras et al., 1975; 1979). Finally, in the rat, cat and primate, the NRM receives a major afferent projection from the PAG (Abols and Basbaum, 1981; Mantyh, 1981; Ruda, 1976; Gallaher and Pert, 1978). Thus the abolition of SPA and opiate analgesia by a lesion of the DLF is consistent with an interruption of the NRM- spinal cord pathway.

In summary, the previous discussion demonstrated that the midbrain PAG is involved in both opiate and stimulation produced analgesia. A pain suppression system from this region involves, at least in part, neurons of the nucleus raphe magnus and a pathway descending via the DLF, and terminating on or near spinal dorsal horn nociceptors. In addition to the endogenous analgesia system

described above, there is also evidence of multiple endogenous pain suppression systems involving the PAG, some opiate, others non-opiate in nature (Watkins and Mayer, 1982). For example, while microinjection of the non-opiate peptides substance P and vasoactive intestinal polypeptide (VIP) produce potent analgesia; only the former is naloxone sensitive.

II. Organization of the PAG: Behavioral Studies

Although treated as a homogenous unit extending several millimeters through the midbrain, behavioral an histological studies have indicated regional differences within the PAG. Recordings of somatosensory evoked responses in the rat indicate that there is a crude somatotopic organization to the PAG. At any recording site within the PAG, evoked potentials could be obtained by stimulating anywhere on the body. However, for each individual recording locus, stimulation of a particular body area consistently produces a greater evoked response than stimulation of other peripheral regions. Thus, face and anterior limbs are better represented in the rostral PAG while the hindlimbs are better represented in the caudal PAG (Liebeskind and Mayer, 1971). Behavioral studies provided further evidence for somatotopia. Opiate and opioid peptide microinjection into the rostral PAG is more effective in suppressing pain in the rostral half of the body (Rosenfield and Kereszls-Nagy, 1980; Yaksh et. al., 1976b) while whole body analgesia can be generated when morphine is injected into the caudal PAG (Yaksh et. al., 1976b). Analgesia produced by electrical stimulation of the PAG has also been reputed to be restricted to only half of the body (Mayer and Liebeskind, 1974).

There are differences within the PAG, in the effectiveness of the analgesia produced by either electrical stimulation (Liebeskind et. al., 1973; Oliveras et. al., 1974; 1979; Yeung et. al. 1977; Lewis and Gebhart, 1977; Gebhart and Toleikas, 1978) or opiate microinjection (Yaksh et. al., 1976; Yeung et. al., 1977; Lewis and Gebhart, 1977). In the rat, the caudal ventrolateral PAG is the region most sensitive to both microinjected morphine and electrical stimulation.

The situation is not as well delineated in the cat because adverse behavioral reactions are often elicited from stimulation of more rostral PAG sites (Liebeskind et. al., 1973; Oliveras et.al.,1974). Nevertheless, both caudal ventromedial sites (ie in or near the nucleus raphe dorsalis) (Oliveras et. al., 1979) and caudal ventrolateral sites (Gebhart and Toleikas, 1978) have been reported to be the most effective regions for stimulation produced analgesia.

It must also be stressed that many functions other than pain modulation have been attributed to the PAG. For example, rage-fear reactions (Skultety, 1963) vocal expression (Jurgens and Pratt, 1979) and reproductive behavior such as lordosis, prolactin release, and inhibition of lutenizing hormone release (Sakuma and Pfaff, 1980; Johnson et. al., 1982; Lakoski and Gebhart, 1982) involve the PAG. This is consistent with both the diversity of the somatosensory input to the PAG (Hara et. al., 1961) and it extensive reciprocal connections with the hypothalamus. However no studies have revealed regional differences in the PAG that correspond to these different functions.

III. Organization of the PAG: Anatomical Studies

(1) Cytoarchitecture

Early studies of the cytoarchitecture of the midbrain produced several classification systems within which to subdivide the PAG. Cajal (1955) subdivided the PAG into internal and external layers. Taber (1961) divided the cat PAG into three regions: dorsal, lateral and ventral. All the divisions contained small to medium-sized cells, that are fusiform or oval in shape; however each individual region exhibited differences in this general pattern: the dorsal PAG had a larger amount of glia, the lateral PAG a higher cell density and the ventral PAG contained primarily small cells. Olszweski and Baxter (1954) based their subdivisions of the human PAG on a concentric nuclear arrangement: subnucleus medialis was the innermost ring, subnucleus lateralis, the outermost ring, and subnucleus dorsalis spanning both, in the dorsal PAG.

Other neuroanatomists (Ramon-Moliner and Nauta, 1966) viewed the PAG as more homogeneous. They classified the PAG as part of the "isodendritic core" of the brainstem. Like other "isodendrite core" elements, the PAG was considered to be populated with a neuronal type whose morphology has been preserved throughout phylogeny. In Golgi stained material the dendritic arborization of these neurons is rectilinear, with little branching and a great overlap of dendritic fields.

More recently, Hamilton reexamined the possible subdivisions of the PAG with cytoarchitectural and connectivity studies. Based on cytoarchitectural differences observed in Nissl stained material (1973), she divided the cat PAG into three subdivisions that were

reminiscent of the schema of Olszweski and Baxter. The nucleus medialis and lateralis form concentric rings around the aqueduct. The nucleus medialis is the relatively acellular inner layer. The neuronal type found here is designated as class I: cells are approximately 8-12 um in diameter, spindel-shaped and darkly stained. Nucleus lateralis is the outer layer of the PAG and contains the largest neurons; class II neurons, 12-30um in diameter. Their neuronal perikarya are spherical or triangular in shape and only lightly stained. The nucleus dorsalis contains small, darkly stained, fusiform or spherical neurons designated as class III. This nucleus is a wedged shape cell group localized just dorsal to the aqueduct.

Golgi studies of the PAG indicate that each of the cell classes defined by Nissl staining is, in fact, heterogenous. Lamle, (1979) in a Golgi study of the N. lateralis of the human PAG, described three predominant neuronal types based on cell orientation and configuration: vertical cells, stellate cells, and horizontal cells.

A Golgi study of all three subdivisions of the cat PAG (Liu and Hamilton (1980) classified the different neuronal types on the basis of the shape of their perikarya reconstructed from three different planes of sectioning. Type I neurons are spindle-shaped, type II triangular shaped and type III polygonal-shaped. Additional subclassifications based on size (Ia: small, Ib: medium) and specific geometory (IIIa: rectangular, IIIb; spherical, IIIc: periform, IIId: rhomboid (largest cell type in the PAG) brings to seven the total number of different cell types in the cat PAG. As stated previously, in Golgi stained material each of the subdivisions of the PAG

demonstrated a heterogeneity of cell types not revealed by Nissl preparations. However, each subdivision had a preponderance of several cell types over others. For example, nucleus medialis contained mostly Ia, II and IIIb neurons, while the nucleus dorsalis contained primarily IIIa an IIIb. Nucleus lateralis contained all cell types, especially IIIc and IIId; type Ia was rarely seen. Liu and co-workers feel that these results confirm their original PAG subdivisions based on the Nissl stained material.

Beitz and co-workers (Neuroscience Abstracts, 1980) have divided the PAG of the rat into four subdivisions. Based on an analysis of Golgi material, PAG regions were classified both on the basis of the different patterns of cell types in each area and on the angle of dendrite orientation with respect to the aqueduct. The subdivisions are (1) medial: small, bipolar cells, (2) ventrolateral: small bipolar, large multipolar and fusiform, (3) dorsolateral: small bipolar, small multipolar and triangularly shaped neurons, (4) dorsal: small bipolar an multipolar neurons.

Mantyh (1982) also observed a heterogeneous population of neuronal types in Nissl and Golgi stained material. However, he did not feel the data warranted the subdivision of the PAG of the rat, cat or primate into different nuclear regins, since no one area was clearly defined by any particular cell types. Comparison of Mantyh's results with those of the other investigators indicates more of a difference in interpretation than in observation. Hamilton, Liu and Beitz all stress the great amount of overlap of the different cell types among the PAG subdivisions. In spite of this, they felt that the pattern formed by the different neuronal types (i.e., a

predominance of one class of neurons over another) provided significant cytoarchitectural differences between the medial, lateral and dorsal regions of the PAG.

(2) Connectivity Studies

Further studies attempting to discern the anatomical organization of the PAG examined the region's afferent and efferent connections. The efferent projections of the cat PAG have been studied both by degeneration (Hamilton, 1974, Hamilton and Skultety, 1970; Chi, 1970) and anterograde transport tracing techniques (Ruda, 1976; Mantyh, 1982). Lesion studies of PAG are at a disadvantage because of the small axonal diameter of the neurons and the numerous fibers of passage travelling through this area. Nevertheless, although Hamilton and co-workers (1970; 1974) reported overlapping terminal-fields (that could be ascribed to damaged fibers of passage) they also observed differential projections from the three different PAG divisions. It was felt that these results supported the different subdivisions based on PAG cytoachitecture.

Ruda (1976), using autoradiographic tracing methods also observed differences in the efferent projections of the PAG. For example, the lateral PAG seemed to have the most extensive projection to the hypothalamus. Nevertheless, there was a great degree of overlap in the neuronal terminal-fields; the differences observed were often quantitative rather than qualitative. Although Mantyh (1982) also found extensive efferent projections with overlapping terminal-fields he did not report on significant qualitative differences in the connections of the different PAG regions.

Regardless of the investigator or technique, all of the above

studies demonstrated that the PAG has widespread efferent projections; ascending projections to the periventricular grey, paraventricular, intralaminar and midline thalamic nuclei, all regions of the hypthalamus, Fields of Forel, zona incerta. Descending projections, leaving the PAG and travelling caudally near the pyramidal tract, terminate in the raphe nuclei of the brainstem, nucleus cuneiformis, locus coeruleus, and the reticular formation of the medulla.

Of special interest to descending pain control mechanisms , is whether the PAG projected directly to the spinal cord. Retrograde transport studies found numerous labelled cells in the midbrain, but few, if any, of these cells were found in the PAG of the rat and primate, and none were found in the cat PAG (Watkins et. al., 1981; Castiglioni et. al., 1978). An anterograde transport study (Mantyh, 1982), failed to find a direct PAG projection to the spinal cord in any of the above species.

The PAG does, however, receive a direct projection from the spinal cord. Early lesion studies (Mehler, 1960) described the terminal field of this spinal projection predominantly in dorsolateral PAG. Recent investigations indicate that other regions of the PAG receive spinal input as well. (Willis et. al., 1979., Mantyh, 1982; Beitz, 1982). The projection neurons are located in the spinal cord dorsal horn in laminae I (Willis et. al., 1979) and the deeper laminae V-VII (Mantyh, 1982; Beitz, 1982).

The PAG also receives a wide variety of inputs from other CNS regions including the prefrontal cortex, hypothalamus, nucleus cuneiformis and pretectal nuclei. While Mantyh(1982) sees no

regional variation in the pattern of afferent projections to the PAG, Beitz (1982) reports not only rostral-caudal differences but ventral dorsal PAG differences as well. These are mostly quantitative differences, although some are qualitative. For example the caudal ventrolateral PAG receives a dense projection from the locus coeruleus, while the rostral PAG does not. As stated previously, a different interpretation of the significance of the quantitative differences seen in the terminal fields originating from each PAG region probably accounts for the disagreement between Mantyh and the other investigators.

In conclusion, anatomical studies have shown the PAG to be composed of a heterogeneous population of neuronal types and diverse afferent and efferent connections. There is a great degree of overlap not only in the different morphological neuronal types found from region to region, but also in the afferent/efferent regional connections as well. However, the majority of investigators have subdivided the PAG, based on the patterns formed by the different neuronal types and connections, into different nuclear regions.

IV. Rationale

The midbrain central grey had been classified as a homogeneous integration center, based in its diverse functional involvements and the generalized dendrite morphology of its neurons. Recent anatomical studies, however, have indicated the possible existence of PAG subdivisions based on Nissl and Golgi stained material. Unfortunately, easily distinguishable three-dimensional neuronal groupings, such as laminae or nuclei, are not readily apparent in the concentric subdivisions of the PAG. Although behavioral studies have

also indicated regional variations in the PAG, for example some regions of the PAG are more effective than others in pain modulation, the different anatomical subdivisions have not been found to correspond to any behavioral mappings. These anatomical and behavioral studies do provide important clues that the PAG is not a homogeneous structure. However the circuitry underlying the functional organization of the PAG and specifically its role in pain modulation, is still unknown.

Clearly, to understand the neuroanatomical substrate underlying the PAG's role in pain supression requires a detailed examination of its neurochemical organization at both the light and electron microscopic levels. To this end we mapped the distribution of the opioid peptide leucine enkephalin and the nonopiate peptides substance P and VIP, all of which have been reported to produce analgesia when injected into the PAG. In addition, we examined the synaptic contacts made by and with immunoreactive ENK-containing profiles, in the caudal, ventral PAG, a region particularly effective in generating analgesia in response to electrical stimulation and opiate microinjection. There results were analyzed within the framework of the normal fine structure of the caudal PAG thus providing new information on the synaptic circuits within the PAG through which opiates may exert their analgesic effects.

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CHAPTER 1: ENKEPHALIN IMMUNOREACTIVE PERIKARYA IN THE CAT RAPHE DORSALIS

ABSTRACT

The serotonin-containing nucleus raphe dorsalis (RD) of the cat contains numerous leucine-enkephalin immunoreactive cells, throughout its rostra-caudal extent. The distribution of the enkephalin neurons closely parallels the cytoarchitectural boundaries of the RD, as described in previous Nissl preparations. Enkephalin perikarya are most numerous along the midline of the RD, but also extend ventrally, into the dorsal portion of the nucleus centralis superior, and laterally, into the 'wings' of the rostral RD, at the level of the IV nucleus. The possible contribution of these enkephalin cells to endogenous pain control systems is discussed.

INTRODUCTION

The midbrain raphe dorsalis (RD) is one of several midline serotonergic nuclei implicated in an endogenous pain suppression system which can be activated either by electrical stimulation or by opiates (3, 10, 12). Studies of the RD's role in pain suppression have stressed the serotonergic component of this nucleus. For example, the serotonin syntheis inhibitor p-chorophenylalanine (pCPA) (1)antagonizes stimulation-produced analgesia (SPA). Similarly, LSD counteracts the inhibition of spinal nociceptors (6) and the behavioral analgesia (7) produced by RD stimulation. Other studies indicate that stimulation or lesions of the RD potentiate or diminish morphine analgesia by, respectively, increasing or decreasing serotonin levels of the forebrain (14, 19). Although recent studies have concentrated on the contribution of the periaqueductal gray (PAG) to opiate and stimulation produced analgesia, it has, in fact, been argued that the RD is the more effective locus, particularly in the cat (12) As part of our continuing studies of the anatomical basis of opiate and SPA we have examined the distribution of leucine-enkephalin (Enk) in the cat central gray. In this report we demonstrate that the dorsal raphe of the cat contains numerous Enk-immunoreactive perikarya throughout its rostral caudal extent. A preliminary description of this work has been published (11).

MATERIALS AND METHODS

Four adults cats, pretreated 24-48h before sacrifice with a third ventricle injection of colchicine (Sigma) (5 ul; 20 ug/ul) were perfused with a solution of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer. The midbrain was sectioned

serially at 100 um on a Vibratome or embedded in paraffin and sectioned at 30-50um. Enkephalin was localized using peroxidase-antiperoxidase (PAP) immunocytochemistry (16) in representative sections, at 150-300 um intervals. The distribution of the labeled neurons was plotted on projection drawings of the appropriate sections. Control sections were treated with leucine-enkephalin antisera (Immuno Nuclear Corp.) preabsorbed with excess Enk (100 ug or 1 mg Enk/ml diluted antisera). Both absorption controls were negative (Fig. 1C). Other sections were treated with Enk antisera incubated with either 1 mg substance P (Sigma) or 1 mg serotonin (Sigma)/diluted antisera. These preincubations had no effect on the Enk staining.

RESULTS

As defined by Taber (17) the nucleus raphe dorsalis extends through the midbrain, begining just caudal to the dorsal tegmental nucleus of Gudden and ending just caudal to the oculomotor complex. The RD is a midline structure which extends ventrally between the medial longitudinal fasiculi and expands laterally into the central grey, at the level of the IV nucleus.

In general, the distribution of the Enk perikarya parallels the boundaries of the raphe dorsalis. At the caudal pole of the DTN, the Enk-immunoreactive cells of the RD are located in two separates clusters, dorsomedial to the MLF. At slightly more rostral levels, these two Enk cell populations merge along the midline. At this point there are also scattered Enk perykarya between the MLF. These eventually merge both with Enk cells located dorsally and with a population of labeled cells in the dorsal portion of the nucleus

centralis superior. Although the DTN itself contains neither Enk-positive cells nor terminals, it is capped by a prominent group of small, bipolar Enk-staining perikarya. The dendrites of these cells curve around the perimeter of the DTN. At the rostral pole of the DTN the Enk perikarya in the dorsal portion of the raphe dorsalis increase in number and begin to spread laterally. The number of Enk cells between the MLF also increases. By the level of the IV nucleus fewer Enk cells are found between the MLF. In contrast, the dorsal portion of raphe dorsalis is densely packed with Enk perikarya, especially along the midline (Fig. 1A). Enkephalin cells are also dispersed throughout the bilateral wings of the RD at this level.

Viewed in the coronal plane, most of the Enk cells in the dorsal portion of the raphe dorsalis are round and either bipolar ofr multipolar; the dendrites show no preferential orientation (Fig. 1B). The Enk perikarya between the MLF are predominantly bipolar with dendrites aligned dorsoventrally. Preliminary quantification of our results indicates that at the level of the IV nucleus the midline Enk cell population of the RD comprises at least 30% of the total cell population.

DISCUSSION

The presence of Enk-containing neurons in the RD of the cat raises interesting questions concerning the mechanisms underlying SPA and its reversibility by the opiate antagonist, naloxone. Previous studies have emphasized the contribution of serotonin to the analgesia produced by RD stimuation (1, 6, 7, 14). In light of the present results, it is inevitable that electrical stimulation of the RD in the cat will not only excite serotonin-containing neurons but

also large numbers of Enk-containing cells. Conceivably, the effectiveness of RD stimulation in the cat is a direct reflection of its high concentration of Enk cell bodies. For the same reason , the analgesia generated by electrical stimualtion of RD would probably be particularly susceptible to antagonism by naloxone.

It has in fact, been recently demonstrated that analgesia generated by stimualtion of the dorsal and median raphe (centralis superior) in the rat is antagonized by naloxone. In contrast, SPA from the dorsal PAG was naloxone insensitive (5). Although previous studies reported that Enk perikarya labeling in the rat RD is either absent (9, 15) or is restricted to the dorsolateral aspect of the rostral RD (18), studies in our laboratory have demonstrated Enk cells along the midline of the rat RD, throughout the rostral caudal extent of the nucleus. Enk perikarya were also found in the dorsal portion of the median raphe. Although the distribution of Enk cells is similar in both the rat and the cat, there are considerably fewer cells in the rat, especially along the midline. The functional significance of this difference in the Enk neuron concentration between these two species remains to be determined. Conceivably the discrepancy between our results in the rat and those of other investigators is due to differences in the site of colchicine administration and its subsequent ability to penetrate into the neuropil.

Previous studies hypothesized serotonin-Enk interactions in stimulation produced and opiate analgesia (2, 3). In that case, it was suggested that the descending serotonergic terminals from the medullary nucleus raphe magnus (NRM) activated spinal interneurons

which, in turn, inhibit spinal nociceptors. The serotonin and Enk cells of the RD may also feed into this descending analgesia system. In addition, they may contribute to the modulation of the affective component of pain through the RD projections to limbic and other forebrain structues (4, 13). An understanding of the differential contribution of the RD serotonin and Enk cells to opiate and stimuation-produced analgesia awaits the analyses of the terminal fields of each neuronal type.

The present results also raise the interesting possibility that the analgesia generated by stimulation of the cat dorsal raphe results from the activation of neurons in which serotonin and Enk co-exist. This would be somewhat analogous to the substance P/seortonin-containing neurons of the rat NRM (8). Since considerable information is known about the contributions of NRM and RD to pain suppression sytems, these brain- stem regions are ideal loci in which to study the functional significance of the coexistence of indoleamines and peptides in the central nervous system.

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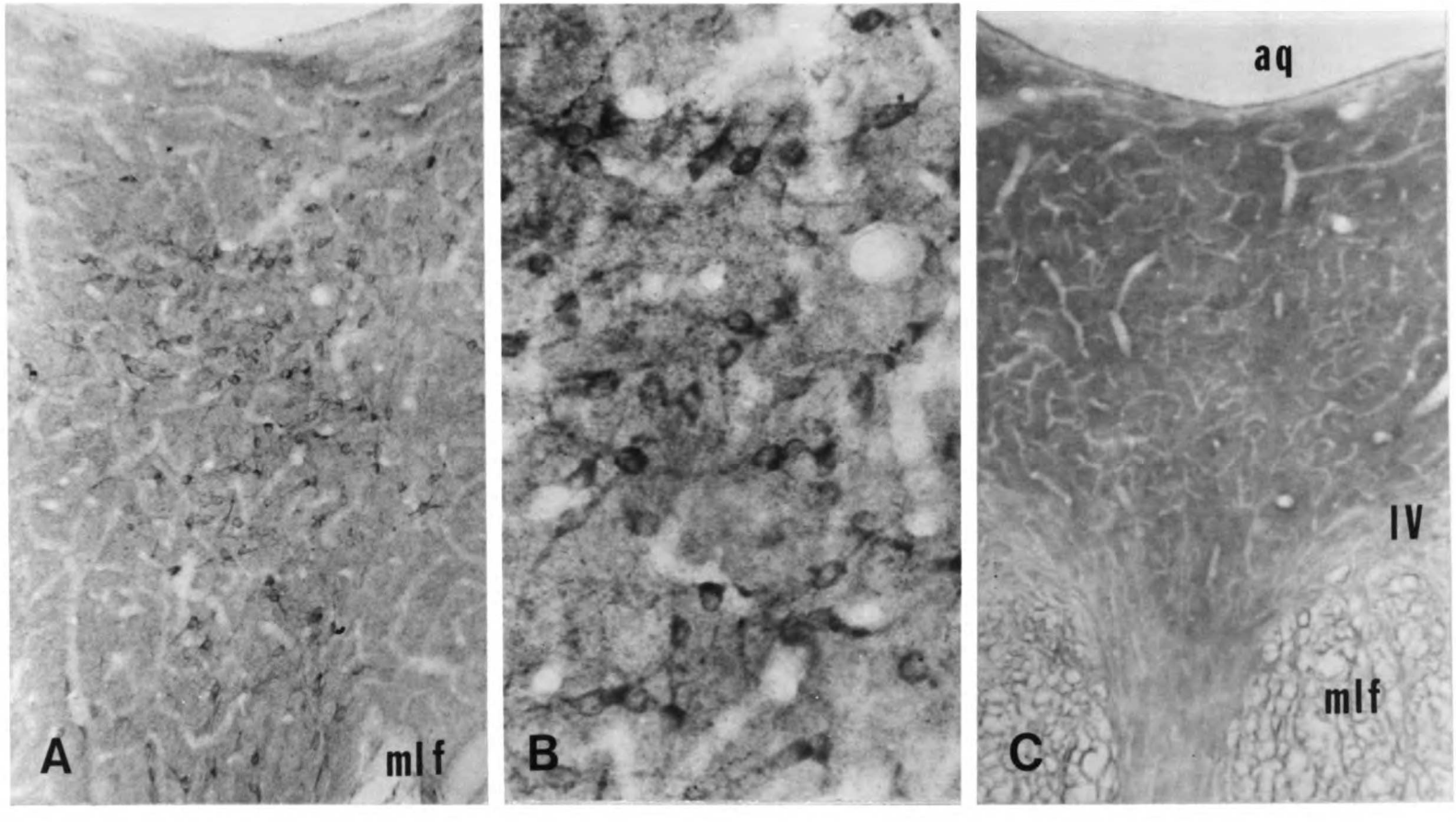
FIGURE LEGENDS

Fig. 1. A: Enkephalin-immunoreactive neurons in the raphe dorsalis at the level of IV nucleus. mlf, medial longitudinal fasciculus; aq, cerebral aqueduct, 30 um paraffin sections. (x 50). B: higher power of dorsomedial portion of A. Note the relatively uniform population of round perikarya (approximately 25 um diameter), with dendrites showing no preferential orientation. (x 132). C: absorption control. This section, 450um caudal to that in A, was reacted with primary antiserum preabsorbed with 1mg Enk/ml diluted antiserum, 30 um paraffin. (x 33).


A
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CHAPTER 2: THE DISTRIBUTION OF IMMUNOREACTIVE ENKEPHALIN-CONTAINING NEURONS AND TERMINAL FIELDS IN THE PERIAQUEDUCTAL GREY

ABSTRACT

Despite the significant contribution of the periaqueductal grey (PAG) to an endogenous pain suppression system, little is known about its neurochemical organization. Previous pharmacological and physiological studies have indicated regional variations in the effectiveness with which the midbrain periaqueductal grey (PAG) can generate potent analgesia in response to either opiate microinjection or electrical stimulation. There is, however, no anatomical correlate of this regional variation. As a first step towards elucidating the neural circuitry underlying the PAG's contribution to endogenous pain suppression systems, we have mapped the distribution of leucine enkephalin (ENK)-like immunoreactivity in the cat PAG. Thoughout the rostral-caudal extent of the PAG, ENK-containing neurons are clustered in discrete populations. ENK terminal-field staining is somewhat more diffuse, however, there are several regions where terminal staining is consistently more intense. The distribution of ENK perikarya and terminals undergoes a ventral to dorsal shift from caudal to rostral PAG. Conceivably, the clustered distribution of ENK cells and terminals contributes to the differential effectiveness of various PAG regions in generating analgesia. The ventral-dorsal shift of ENK immunoreactivity may 1) correspond to a somatotopic organization within the PAG or 2) mirror the topographic relationship of the PAG's interactions with other components of the endogenous analgesia system. In addition, the changing pattern of ENK immunoreactivity may also reflect the involvement of the PAG, and of endogenous opiates, in systems other than those of pain control.

INTRODUCTION

In 1962 Tsou and Jang implicated the central grey region surrounding the third ventricle and midbrain aqueduct in the generation of morphine analgesia. Subsequently, Reynolds (1969) reported that electrical stimulation of the midbrain periaqueductal grey (PAG) produced analgesia profound enough to perform painless abdominal surgery on rats. Further studies elucidated the properties of the analgesia elicited from the PAG by either intracerebral opiate microinjection or electrical brain stimulation (Mayer et. al., 1971; 1973; Mayer and Liebeskind, 1974; Oliveras et. al., 1974; 1979; Mayer and Price, 1976; Lewis and Gebhart, 1977, Yeung et. al., 1977; Gebhart and Toleikas, 1978; Bennet and Mayer, 1979).

The discovery of endogenous opiates (Hughes et. al., 1975; Simantov and Snyder, 1976), the presence of considerable opiate binding sits in the PAG (Kuhar et. al., 1973) and the reversal of stimulation produced analgesia (SPA) by naloxone (Akil. et al., 1976; Adams, 1976; Hosobuchi et al., 1977), further implicated the midbrain central grey, as an important component of an endogenous pain suppression system. In part, this system involves connections with the medullary nucleus raphe magnus (Abols and Basbaum, 1981; Gallager and Pert, 1978; Ruda, 1976), the axons of which descend to the spinal cord via the dorsolateral funiculus and inhibit dorsal horn nociceptors (Basbaum, et. al., 1976; 1977; 1978; Basbaum and Fields, 1979; Fields and Anderson, 1978).

While many details of this descending pain control system have been determined, the mechanism by which opiates or electrical brain stimulation initiate or modulate pain suppression at the level of the

PAG is unknown. In effect, the PAG then remains a "black box". It is not known, for example, whether intracerebrally injected opiates or naloxone act on afferents to the PAG, on PAG projection neurons or on PAG local circuit neurons. Because the site of morphine's action may be inferred from the distibution of endogenous opiates, we examined the distribution of leucine enkephalin (ENK) - like immunoreactivity in the cat periaqueductal grey. We report the presence of discrete populations of ENK-containing neurons and terminal fields thoughout the rostral-caudal extent of the cat periaqueductal grey. Preliminary results of this work have been reported previously (Moss et, al., 1980, Moss et. al., 1981a).

MATERIALS AND METHODS

Ten adults cats, some pretreated 24-48 hours before sacrifice with a third ventricle injection of colchicine (Sigma, 5ul, 20ug/ul), were used in this study. Cats were anesthetized and perfused with 0.1M phosphate buffered saline (pH 7.4, 37^{0}C) containing 0.1% heparin. This was immediately folowed by a 4^{0}C fixative solution consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1M phosphate buffer containing 4% sucrose. After approximately 30 minutes the animal was perfused with fresh cold buffer to wash out excess fixative. The midbrain was then serially sectioned at 100 um using a Vibratome, or at 50um using a freezing microtome or embedded in paraffin and sectioned at 30-50um.

Leucine-enkephalin (ENK) was localized on representative sections taken at 150-400um. intervals, using the peroxidase antiperoxidase (PAP) method (Sternberger, 1974). In vibratome and frozen sections antibody penetration was enhanced by either

preincubation in a sodium ferricyanide methanol solution (Strauss, 1976) or by the addition of 0.3% Triton to all solutions. Sections were incubated in ENK antisera for 48 hours at 4^{0}C. Bridging antisera and PAP incubations were at least 30 minutes in duration at room temperature.The PAP reaction product was visualized by using $3,3^{1}$-diaminobenzidine as the chromagen.

The ENK antibody (Immunonuclear Corp.) used in this study has been shown by radioimmune assay to have a 20-30% cross reactivity with methionine enkephalin and no demonstrable cross reactivity with B-endorphin. However, its cross reactivity with other peptides containing a leucine enkephalin sequence (e.g. dynorphin, alpha neoendorphin) has not been determined. For this reason, although the term ENK-like immunoreactivity is not consistently used throughout this report, it is implied at all times. To establish histochemical specificity adjacent control sections were incubated in ENK antisera preabsorbed with excess ENK (Sigma) (100ug ENK/ml diluted antisera). ENK staining in these control sections was abolished. In addition, several sections were treated with ENK antisera pre-incubated with either 1mg of substance P or serotonin (Sigma) per milliliter of diluted antisera. These pretreatments had no effect on the ENK staining.

The distribution of the ENK-labelled perikarya and terminal fields was plotted on projection drawings of appropriate sections from several animals. The results from these projection drawings were collated and plotted on representative sections of the PAG. In this manner, variability among animals introduced by slight differences in the plane of sectioning and/or colchicine

administration were minimized. The visualization of ENK perikarya was highly dependent on the presence of colchicine. Despite consistent third ventricle injections, the penetration of colchicine into the surrounding neuropil was not consistent, perhaps being influenced by CSF flow and the natural boundaries formed by a axonal tracts. Therefore, the comparison of labelling from several animals was esential.

The schematic drawings and nucler designations used in this study are derived from the atlases of Berman (1968), Mehler (1958) and Taber (1961).

RESULTS

To facilitate the discription of the distribution of ENK-like immunoreactivity the PAG will be divided into three major rostral-caudal regions. Caudal PAG (Fig. 1) includes levels from the dorsal tegmental nucleu of Gudden to a level immediately caudal to the IV nerve nucleus. Mid-PAG (Fig 2) extends from the level of the IV nucleus to the level of the nucleus Edinger-Westphal. Finally, rostral PAG (Fig. 3) includes levels from the posterior commissure to the periventricular grey. In addition, the distibution of ENK immunoreactivity in the midbrain raphe nuclei and the dorsolateral pontine tegmentum is discussed.

Midbrain Raphe Nuclei. (Fig. 1, 2, 4 and 7)

As described previously (Moss et. al., 1981b), ENK containing neurons are found throughout the rostral-caudal extent of the raphe dorsalis (RD); their distribution parallels the cytoarchitectural boundarie of the nucleus (Taber 1961). The densest terminal-field staining is found at the peripheral borders of this nucleus. The

raphe nucleus centralis superior contains both ENK neurons and dense terminal staining along its lateral borders.

Locus Coeruleus (LC)/Parabrachial (PB) Nuclei (Figs. 4,5, and 6).

Large ENK neurons are found in the LC dorsalis, LC pars alpha and the subcoeruleus region (Leger and Hernandez-Nicaise, 1980). These cells are easily distinguished from the smaller ENK cells found in the more rostral, but contiguous ventrolateral PAG. Many ENK cells are also present in the lateral Pb nucleus and in the nucleus Kolliker Fuse. In addition, several ENK cells are also found in the medial Pb nucleus. The ENK neurons in the Kolliker Fuse nucleus are comparable in size to those in the LC. In contrast, ENK cells of the Pb nuclei are smaller, similar in size to those seen in the PAG. Terminal-field labelling in the dorsolateral pontine tegmentum is heaviest at the dorsal tip of the mesencephalic tract. Dense terminal labelling is also found in Pb lateralis. Despite the presence of many immunoreactive ENK perikarya in the LC and subcoeruleus, there is only a moderate amount of terminal-field staining in these nuclei.

Caudal PAG (Fig. 1 and 7)

In the caudal PAG, ENK-containing neurons are clustered predominantly in the ventrolateral PAG. ENK cells are also found arching around the perimeter of the dorsal tegmental nucleus of Gudden (DTN). The DTN itself is devoid of ENK immunoreactivity.

Although ENK terminal-field staining is found throughout the caudal PAG, the staining pattern is marked by areas of denser labelling. The densest terminal staining is found in the ventrolateral PAG and in the dorsal PAG. In the dorsal PAG, the

distribution of ENK labelled terminals forms paired, vertically oriented columns.

Dense ENK staining is also present in the nucleus cuneiformis. In one animal, a small number of ENK cells were seen in the nucleus cuneiformis. This staining pattern may be an example of the incomplete penetration of colchicine into this region.

Mid-PAG (Fig. 2, 8 and 9)

In contrast to the caudal PAG, the ventral region of the mid PAG contains few ENK perikarya. Instead, the distribution of ENK neurons shifts dorsally and appears as two separate populations: one is located in the lateral PAG, the other in the dorsal PAG.

At the levels of the III and IV nerve nuclei, the lateral cluster of ENK cells is located adjacent to the aqueduct. More rostrally, at the level of the Edinger-Westphal nucleus, this population shift outward, away from the aqueduct. The ENK cell population in the dorsal PAG becomes prominent at the level of the III nerve nucleus where it is located adjacent to the dorsal midline. At more rostral levels this dorsal ENK neuronal cluster shifts laterally, away from the midline.

At mid-PAG levels the densest terminal-field staining is located in the dorsal and the lateral PAG, paralleling the dorsal shift of the ENK-cell distribution. At the level of the Edinger-Wesphal nucleus, large beaded immunoreactive ENK fibers are seen coursing ventrally from the PAG, along the midline.

Rostral PAG (Fig. 3 and 10)

In the rostral PAG, at the level of the posterior commissure, the ENK neuron populations remain as two separate clusters. The

dorsal cluster now extends from the dorsal PAG, through the fibers of the posterior commissure and into the overlaying cap of the PAG neuropil. At this level, ENK terminal-field staining is densest in the dorsal PAG, and also extends into the portion of the PAG that caps the posterior commissure.

The rostrally contiguous periventricular grey contains scattered ENK neurons and light terminal field labelling; the terminal labelling is somewhat denser dorsally.

ENK Cell Morphology

In favorably preparations considerable information about ENK cell morphology can be collected. Both the shape of the somata and the primary dendrite branching patterns of the cells are evident. Viewed in coronal section, the ENK cells in the PAG are generally smaller than ENK neurons found in the surrounding brain regions, specifically, the nucleus locus coeruleus. Two cell types predominate in the locus coeruleus (Fig. 4). The larger LC ENK cells (40um) (measured on vibratome sections) are generally round to fusiform, bipolar cells; the majority of smaller LC ENK cells (30um) are multipolar. In contrast, the PAG ENK cell bodies range in diameter from 15u to 30u. The ENK neurons in the PAG are bipolar or multipolar with round, fusiform or triangular-shaped cell bodies. We were unable to recognize either a consistent cell morphology or orientation with which to define all ENK-containing cells in the PAG. There are, however, some regional differences in ENK cell morphology. At the levels of the III and IV nerve nuclei, the ENK-containing perikarya in the lateral ENK cell population (Fig. 9) are generally bipolar fusiform cells, while the ENK cells located

dorsally (Fig. 8A, B) have round or triangular cell bodies which are generally multipolar. ENK cells in the dorsal cell population at the level of the posterior commisure are more variable both in shape and branching pattern (Fig. 10C, D).

DISCUSSION

While several immunohistochemical studies of ENK-like immunoreactivity in the central nervous system have included observations of labelling in the PAG, this region has never been the prime focus of any one study. Our report is the first detailed description of the distribution of ENK immunoreactivity in the PAG of the cat, an animal used extensively in anatomical and electrophysiological studies of endogenous pain suppression systems. Throughout the entire cat PAG, ENK-containing neurons are found clustered in discrete populations. Although ENK terminal-field staining is more diffuse, there are also several regions where terminal staining is consistently more intense.

In both the rat and cat, intraventricular colchicine administration is necessary for the visualization of immunoreative ENK cell bodies in the PAG. Early immunohistochemical studies in the rat, indicated that ENK perikarya are present only in the caudal, ventrolateral PAG (Hokfelt et.al., 1977a, b). Subsequent studies, however, have revealed a wider distribution (Uhl et. al., 1979). There are two major differences between the distribution of ENK-containing neurons in the PAG of the rat and the cat. Although a population of ENK perikarya is present in the lateral PAG of the rat, it is found only towards the perimeter of the PAG, away from the aqueducts at all rostral-caudal levels. This unlike the similiar

cell group in the cat, which is located adjacent to the aqueduct at more caudal levels. A second difference in the pattern of ENK neuronal staining between the rat and the cat is in the dorsal PAG. The distinctive columnar pattern formed by ENK perikarya in the dorsal PAG of the cat, is not seen in the rat.

Differences are also apparent in the terminal-field staining patterns of the two species. ENK terminal-field staining in the rat PAG is more uniform than in the cat (Elde et al., 1976; Sar et. al., 1978; Uhl et. al., 1979). The only concentration of terminal staining in the rat is found in the lateral PAG (Uhl et. al., 1979), however, its distribution is different from the similiar population of ENK terminals in the lateral PAG of the cat. In addition, the dense terminal-field staining found in the caudal ventrolateral PAG and in the dorsal PAG of the cat are not present.

The dorsal tegmental nucleus of Gudden and the parabrachial nuclei are two regions where the distribution of ENK-like immunoreactivity in the rat and cat is similiar (Sar et. al., 1978; Uhl et. al., 1979). In fact, dense concentrations of ENK-containing perikarya and terminals surround the borders of this nucleus not only in the cat and rat but also in fetal human tissue (Miller and Pickel, 1980).

In summary, the distribution of ENK-like immunoreativity in the rat PAG is more uniform than that in the cat, with fewer populations of ENK neurons and terminals. In contrast to the cat, there is much less perikaryal and terminal-field labelling present in the dorsal PAG of the rat. It is possible that some of the differences in the distribution of immunoreactive ENK cell bodies may be due to the

variable penetration of colchicine along the entire extent of PAG neuropil. However, this cannot be true for the differences in the distribution of ENK terminal-field staining, and indicates species differences in the PAG pattern of ENK staining. The functional implication of this remains to be determined, but may indicate species-specific differences in the opiate-mediated circuitry within the PAG.

Cytoarchitectural studies, based on Nissl and Golgi staining, (Hamilton, 1973; Liu and Hamilton, 1980) subdivided the cat PAG into three nuclear regions; nucleus medialis, which surrounds the aqueduct; nucleus lateralis, which is concentric with nucleus medialis; and nucleus dorsalis, a wedge-shaped region in the dorsal PAG. Although ENK-immunoreactive perikarya in the PAG exhibit differences in shape and in dendrite branching patterns, it was not possible to draw analogies between the previous cytoarchitectural studies and either the distribution or morphology of ENK-labelled neurons. The presence of discrete clusters of ENK neurons and terminals is, however, consistent with the conclusions of Hamilton's (1973) and Liu's (1980) studies, namely that the PAG is an anatomically (and one presumes functionally) heterogeneous structure.

The clustered distribution of ENK perikarya and terminals may have important functional consequences. For example, it may underlie the differential effectiveness of various PAG regions in generating analgesia in response to electrical stimulation (Liebeskind et. al., 1973; Oliveras et. al., 1974; 1979; Yeung et. al., 1977; Lewis and Gebhardt, 1977; Gebhardt and Toleikas, 1978). It is also consistent with the nonuniform opiate sensitivity of the PAG. For example in

the rat, where extensive mapping studies have been done, (Yeung et. al., 1977; Yaksh et. al., 1976; Lewis and Gebhart, 1977), the caudal ventrolateral PAG is the region most sensitive to both microinjected morphine and to electrical stimulation. The situation is not as well delineated in the cat, because adverse behavioral reactions are often elicited from stimulation of more rostral PAG sites (Liebeskind et. al., 1973; Oliveras et. al., 1974). Nevertheless, both caudal ventromedial sites (i.e. in or near RD) (Oliveras, et. al., 1979) and caudal ventrolateral sites (Gebhardt and Toleikas, 1978) have been reported to be the most effective regions for stimulation produced analgesia in the cat. Both of these regions contain dense concentration of ENK-containing neuons and terminal-fields.

Although the analgesia produced by intracerebral injection of opiates is readily anatogonized by intracerebral naloxone (Lewis and Gebhardt, 1977; Yaksh et. al., 1976; Yeung et. al., 1977), there are conflicting reports on naloxone's ability to reverse SPA from PAG stimulation (Adams, 1976; Akil et. al., 1976; Hosobuchi et. al., 1977; Yaksh et. al., 1978; Gebhardt and Toleikas, 1978). Conceivably this reflects differences in the location of the stimulation electrode relative to the clusters of ENK-containing neurons or terminals. Electrical stimulation in a region of ENK-containing cells is more likely to generate a naloxone sensitive analgesia. In contrast, electrical stimulation distant to the ENK perikarya, but in ENK-containing terminal regions may, by activating a postsynaptic neuron, bypass the enkephalinegic link in the analgesic pathway. It is also possible that electrical stimulation of the PAG could directly activate a parallel, non-opioid containing

pain suppression system. This would also produce an analgesia that is not naloxone sensitive.

Since SPA can result from the activation of either ENK neurons or of any of the elements in their postsynaptic chain, it is clear that electrical stimulation is less selective than opiate microinjection in elucidating actual endogenous opiate pathways. On the other hand, sites of dense terminal staining would be most sensitive to pharmacological manipulation and presumably offer a better clue to the locus of opiate action. Unfortunately a study of this kind has not yet been done in the cat.

What is the significance of the changing patttern of clustered immunoreactive ENK cells and terminals as one proceeds through the rostral-caudal extent of the midbrain central grey? Recordings of somatosensory evoked responses in the rat indicate that there is a crude rostral-caudal somatotopic organization (Liebeskind and Mayer, 1971) to the PAG. Pharmacological studies provide further evidence for somatotopia. It has been reported that opiate micro-injection into the rostral PAG suppresses pain only in the rostral half of the body; whole body analgesia is generated when morphine is injected into the caudal PAG (Yaksh et. al., 1976). Microinjection of an opiate peptide analogue into the PAG produces an analgesia more potent for facial pain than for tail flick (Rosenfeld and Kereszls-Nagy, 1980). Finally, there are reports that the analgesia produced by stimulation of the PAG is restricted to only half of the body (Mayer and Liebeskind, 1974). Thus one possible functional correlate of the rostral-caudal shift in the distribution of ENK neurons and terminals in the PAG is that it corresponds to an underlying somatotopic organization, i.e. a "body map" for antinociception.

A second possible role for the rostral-caudal change in the distribution of ENK-like immunoreactivity in the PAG is that it mirrors the topographic relationship of the PAG's interaction with other components of the endogenous analgesia system. For example, the ENK cells and terminals in the dorsal region of mid-PAG overlap with the distribution of PAG cells which project to the nucleus raphe magnus in the medulla (NRM) (Abols and Basbaum, 1981). The NRM is recognized as an important link in the analgesia generated by PAG activation (Basbaum, et. al., 1976; Fields and Andersen, 1978). Although there are similarities in the cell shape and dendrite branching pattern between the ENK cells in the dorsal PAG and the dorsal PAG cells which project to the NRM, it is not known whether ENK neurons in the PAG project directly to the NRM. While enkephalin and other opiate agonists generally have inhibitory postsynaptic effects (Nicoll et. al., 1980; Pepper and Henderson, 1980), an excitatory connection between the PAG and NRM appears to mediate the production of analgesia (Bebehani and Fields, 1979). It follows then that activation of the NRM by the PAG ENK neurons would involve disinhibitory circuits. Alternatively, ENK cells and terminals in the dorsal PAG may modulate the activity of the cells projecting to the NRM.

Unfortunately this study cannot determine whether ENK neurons in the PAG are local circuit neurons which act directly on PAG output neurons or on afferents to the PAG involved in the antinociceptive system, or alternately whether some of the ENK-containing PAG cells project to other regions, such as the nucleus locus coeruleus (Sakai et. al., 1977) or the nucleus cuneiformis (Mantyh, 1981) which also

contain ENK terminals. Since the densest clusters of ENK cells and terminals do not always overlap, it is possible that the PAG ENK terminals derive either from local-circuit neurons whose axonal arbors extend somewhat beyond their perikarya or from distant ENK neurons that project into the PAG. We suggest that most of the analgesia-relevant ENK neurons in the PAG are inhibitory local circuit neurons that modulate a tonic inhibitory input to PAG pain suppression projection neurons. Thus the analgesia from opiate injection would result from a disinhibition of the PAG-medullary connection. The origin of the hypothesized tonic inhibition of the PAG output neurons is unknown, however, there is some suggestion that norepinephrine inputs may be involved (Basbaum et, al., in press).

It must also be stressed that the PAG contains other opiate peptides besides enkephalin, such as B-endorphin and dynorphin (Bloom et. al., 1978; Goldstein and Ghazavossian, 1980; Hollt et. al., 1980; Zakarian and Smyth, 1982; Gramsch et. al., 1982). In addition, some neurons have been shown to contain more than one opiate receptor subtype (Egan and North, 1982). Thus opiate injection into the PAG could produce analgesia via the neuronal circuitry through which these other peptides, or some combination of them, act.

We have emphasized the possible functional subdivisions of the PAG as they relate to the topography of the descending endogenous analgesia system. It is conceivable that the PAG contributes to ascending pain control pathways. Reciprocal connections between the PAG and limbic and prefrontal cortical areas (Beitz, 1982; Hardy and Leichnetz, 1981; Mantyh, 1981; Ruda, 1976) indicate a possible involvement in the affective component of pain perception. There is

some evidence (Rosenfeld and Kereszls-Nagy, 1980) however, that opiates, at the level of the PAG, do not activate an ascending pain suppression system.

Finally, many functions other than pain suppression have been attribuited to the PAG (Skultety, 1963; Jurgens and Pratt, 1979; Sakuma and Pfaff, 1980; Johnson et. al., 1982; Lakoski and Gebhart, 1982). The rostral-caudal differences in the distribution of ENK immunoreactivity may thus reflect the involvement of the PAG, and of endogenous opiates, in systems other than those of endogenous pain control. To determine the systems involved, it is essential to establish the identity of the neurons upon which PAG enkephalin neurons act. It is also important to dissect the microcircuitry through which the PAG ENK terminals exert their effects. For example, are pre or post-synaptic inhibitory mechanisms involved? Towards this end, we have initiated electron microscopic studies of imunoreactive ENK varicosities in the PAG (Moss et. al., 1981a). Hopefully these studies will provide insight into the mechanisms by which exogenous opiates, by mimicking the action of PAG endogenous opiates, elicit such powerful behavioral effects.

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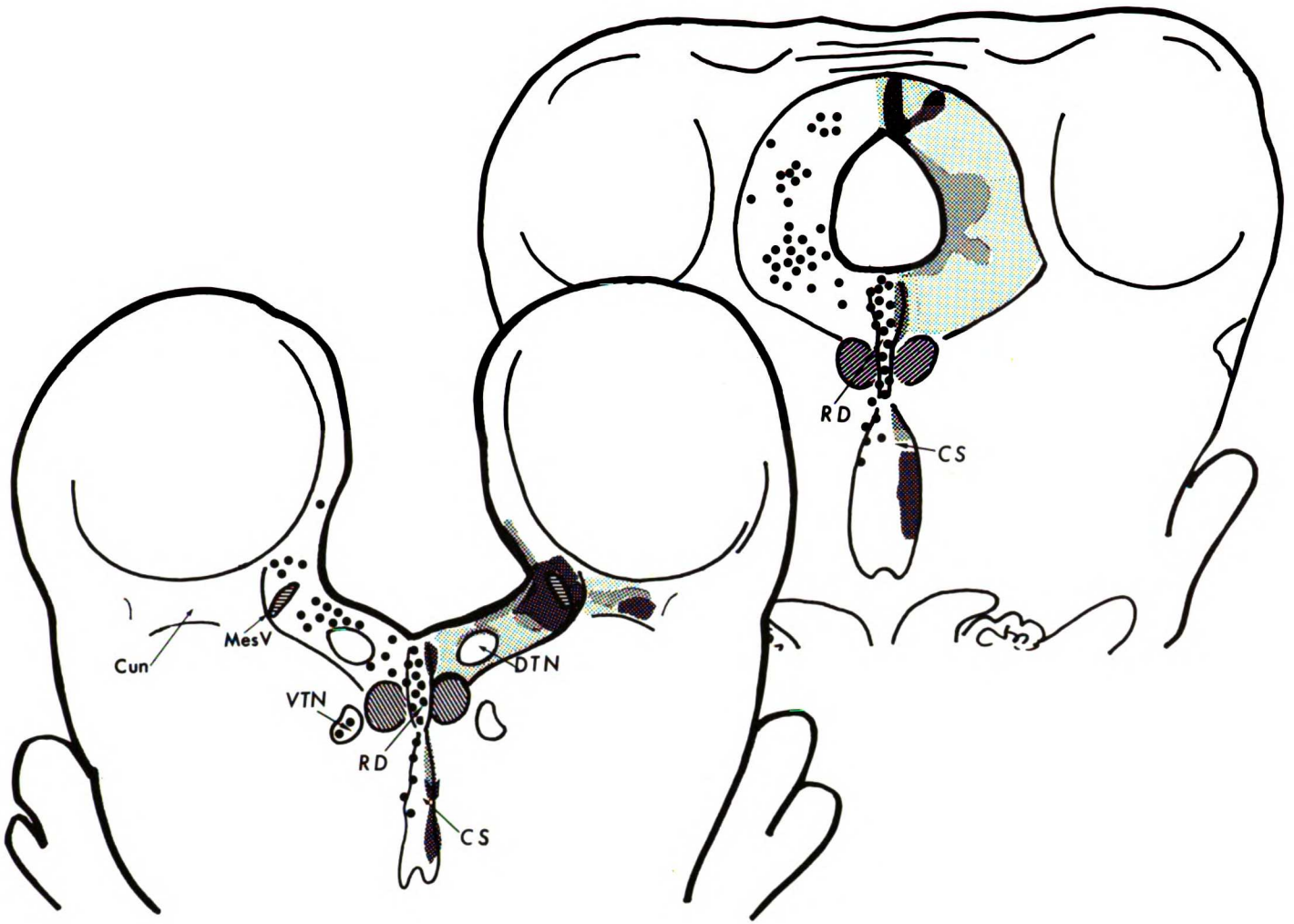
FIGURE LEGENDS

Figs. 1,2,3 Drawings of representative sections through the periaqueductal grey illustrating the distribution of enkephalin-containing perikarya (dots) and terminals (cross-hatching ranging in density from 0-3). CS: centralis superior, CUN: nucleus cuneiformis, DTN: dorsal tegmental nucleus of Gudden, EW: nucleus Edinger-Westphal, Hb: habenulae, LGB: lateral geniculate, LI: linearis intermedius, LR: linearis rostralis, Mes V: mescencephalic tract of V, MLF: medial longitudinal fasiculus, Post C: posterior commissure, Pulv: pulvinar, RD: raphe dorsalis, RN red nucleus, VTN: ventral tegmental nucleus of Gudden, III: third nerve nucleus, IV: fourth nerve nucleus Mes V, MLF and Hb-Interpeduncular tract are indicated by diagonal hatching

Fig. 1. Caudal periaqueductal grey levels

Fig. 2. Mid-periaqueductal grey levels

Fig. 3. Rostral periaqueductal grey levels


RD
CS
Cun
MesV
DTN
VTN
RD
CS


EW
III
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MLF
LR
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LI
CS

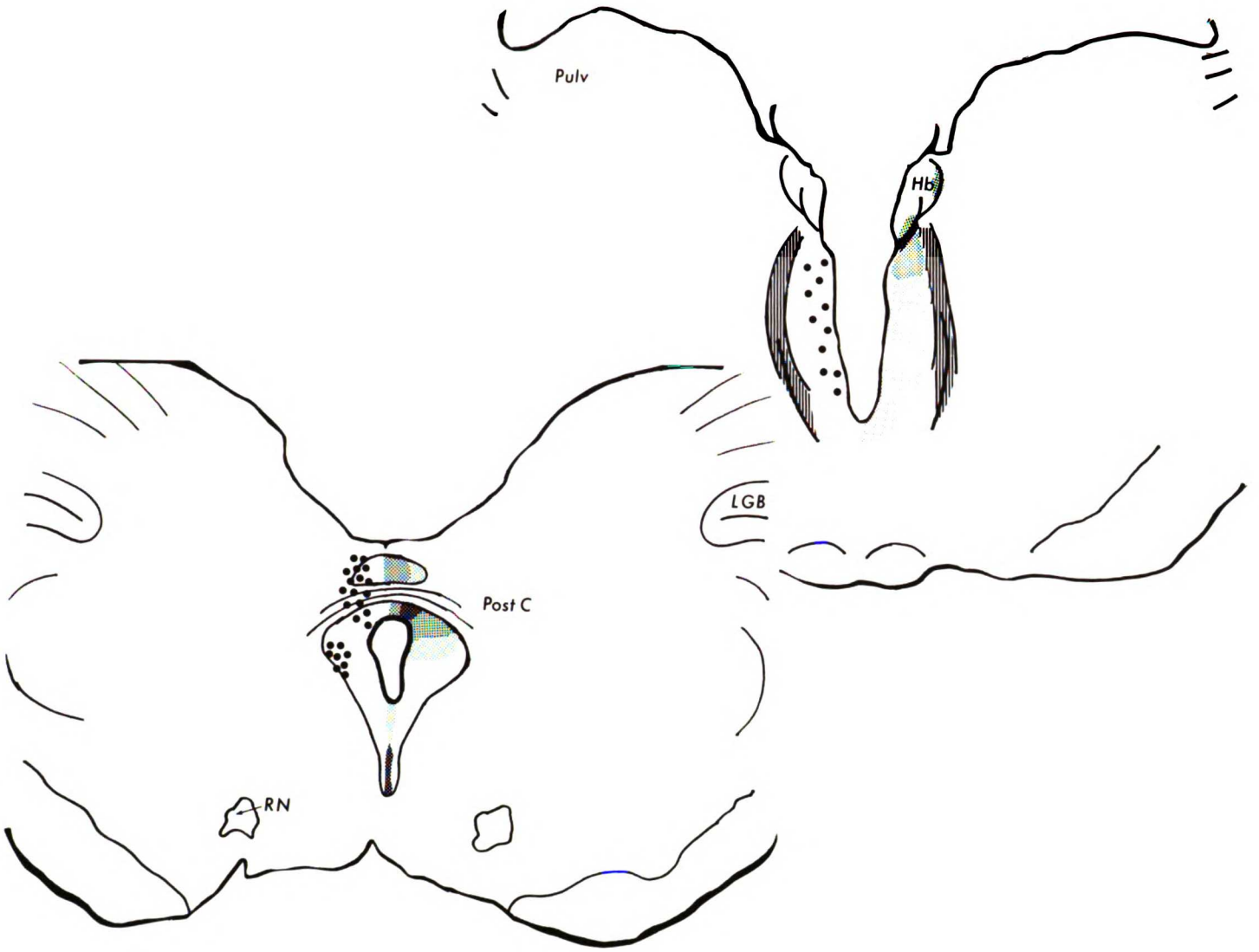

Pulv
Hb
LGB
Post C
RN

Fig. 4. The distribution of enkephalin (ENK) immunoreactivity in the midbrain, just caudal to the periaqueductal grey (PAG). Compare the distribution of ENK neurons in the nucleus raphe dorsalis (RD) at this level with a more rostral level shown in Fig. 7. ENK perikarya and terminals surround the dorsal tegmental nucleus (DTN). The nucleus itself is relatively free of ENK immunoreactivity. This is the same staining pattern seen at caudal PAG levels. Note the different ENK cell types in the nucleus locus coeruleus (LC). (47x) Mes V: mesencephalic tract of V, MLF: medial longitudinal fasciculus, IV: IV ventricle.

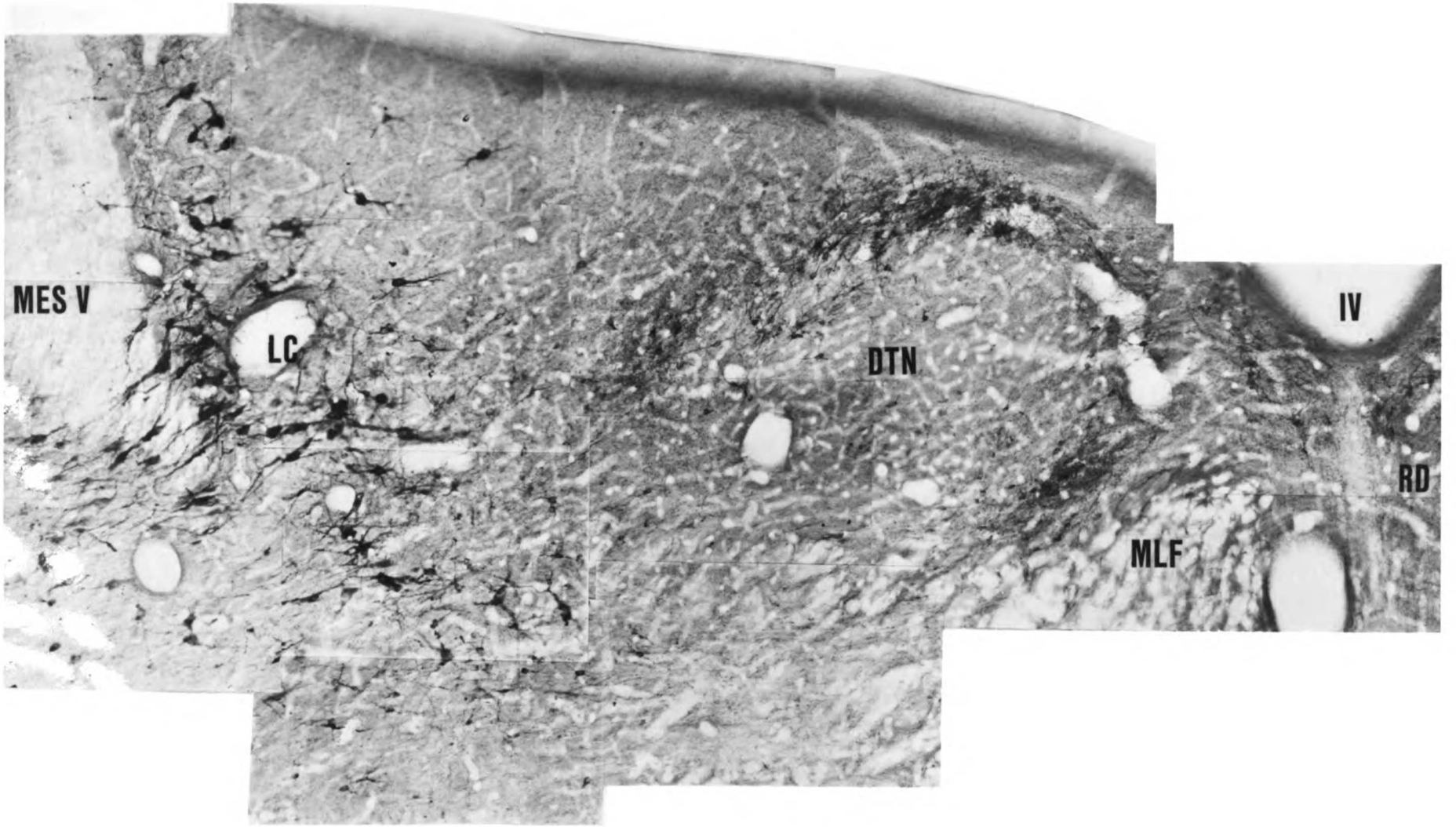

MES V
LC
DTN
IV
RD
MLF

Fig. 5. Enkephalin neurons in the lateral parabrachial (Pbl) and Kolliker Fuse (KF) nuclei at the same level pictured in Fig. 4. Note the difference in cell size between these two enkephalin neuron populations (17X). BC: brachium conjunctivum.


PbI
BC
KF

Fig. 6. Locus coeruleus. (A) Enkephalin neurons in the dorsal portion of the locus coeruleus. (B) Absorption control of a section 360 um from (A) (55X). Mes V: mesencephalic tract of V.

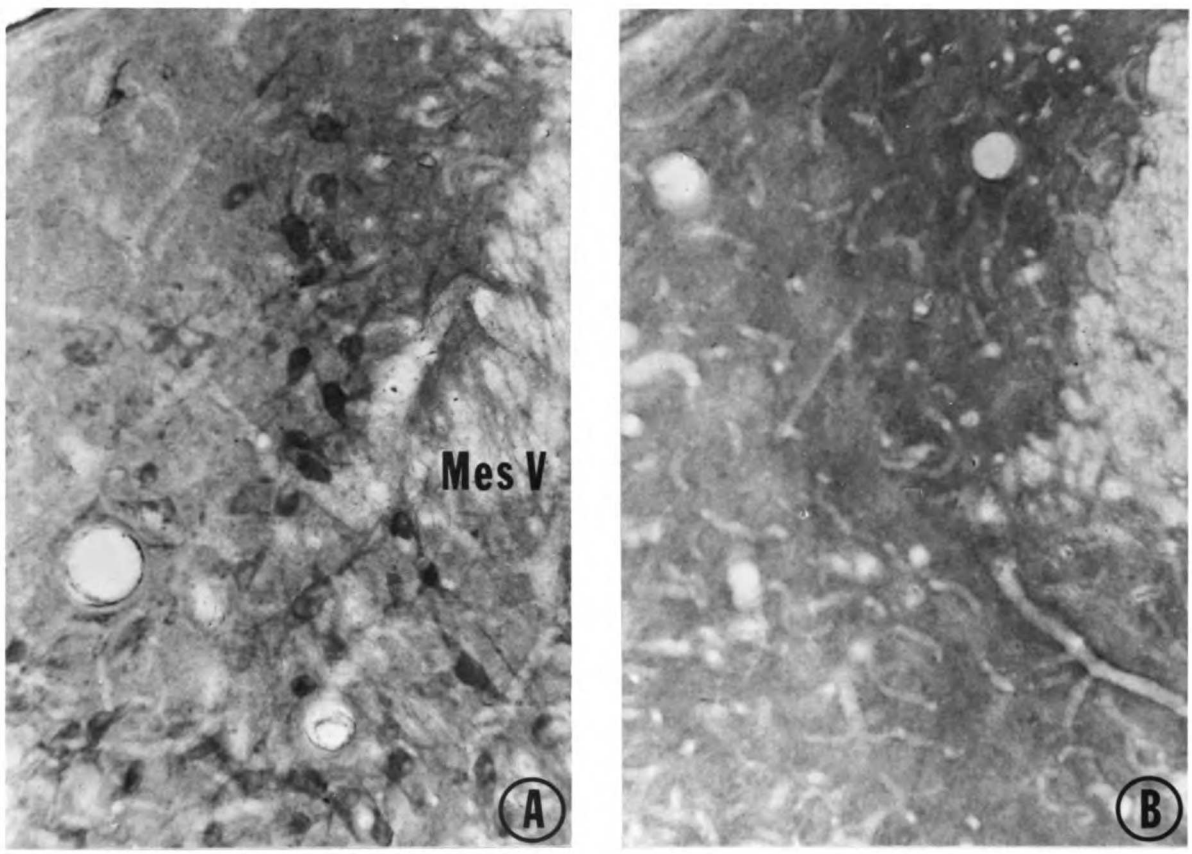
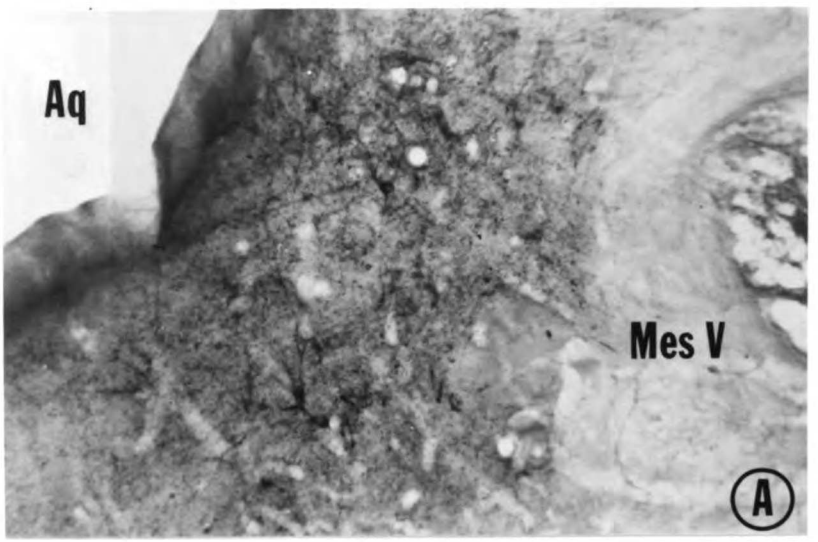

Mes V
A
B

Fig. 7. The distribution of immunoreactive enkephalin (ENK) in the caudal periaqueductal grey (PAG). (A) ENK neurons and terminals in the caudal ventrolateral PAG (55X). (B) ENK-containing perikarya along the midline of the nucleus raphe dorsalis. At the level of the IV nerve nucleus the "wings" of the raphe dorsalis also containing ENK cells (55X). Aq: aqueduct of Sylvius, Mes V: mesencephalic tract of V.


Aq
Mes V
A

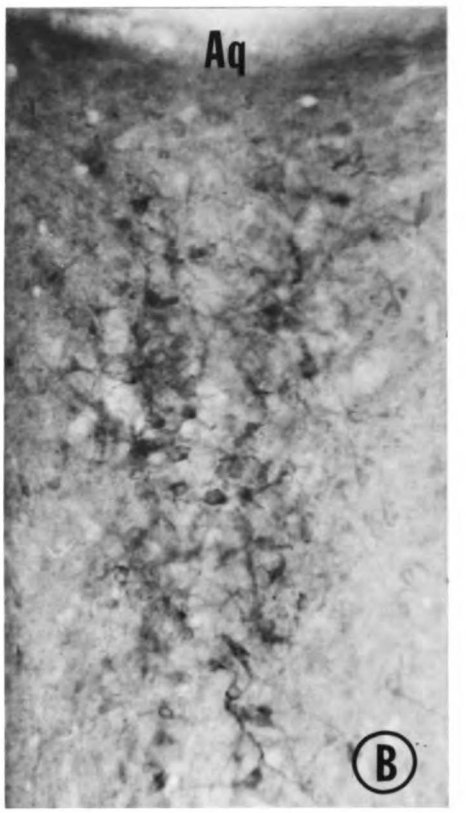

Aq
B

Fig. 8. The distribution of imunoreactive enkephalin (ENK) in the dorsal periaqueductal grey at the level of the IV nerve nucleus. (A) ENK neurons form a dorsal midline column (28X). (B) High power micrograph of a pyramidal ENK cell from this same region (116X). (C) ENK terminals in the PAG form two sets of paired columns on either side of the dorsal midline (23X). Aq: aqueduct of Sylvius.

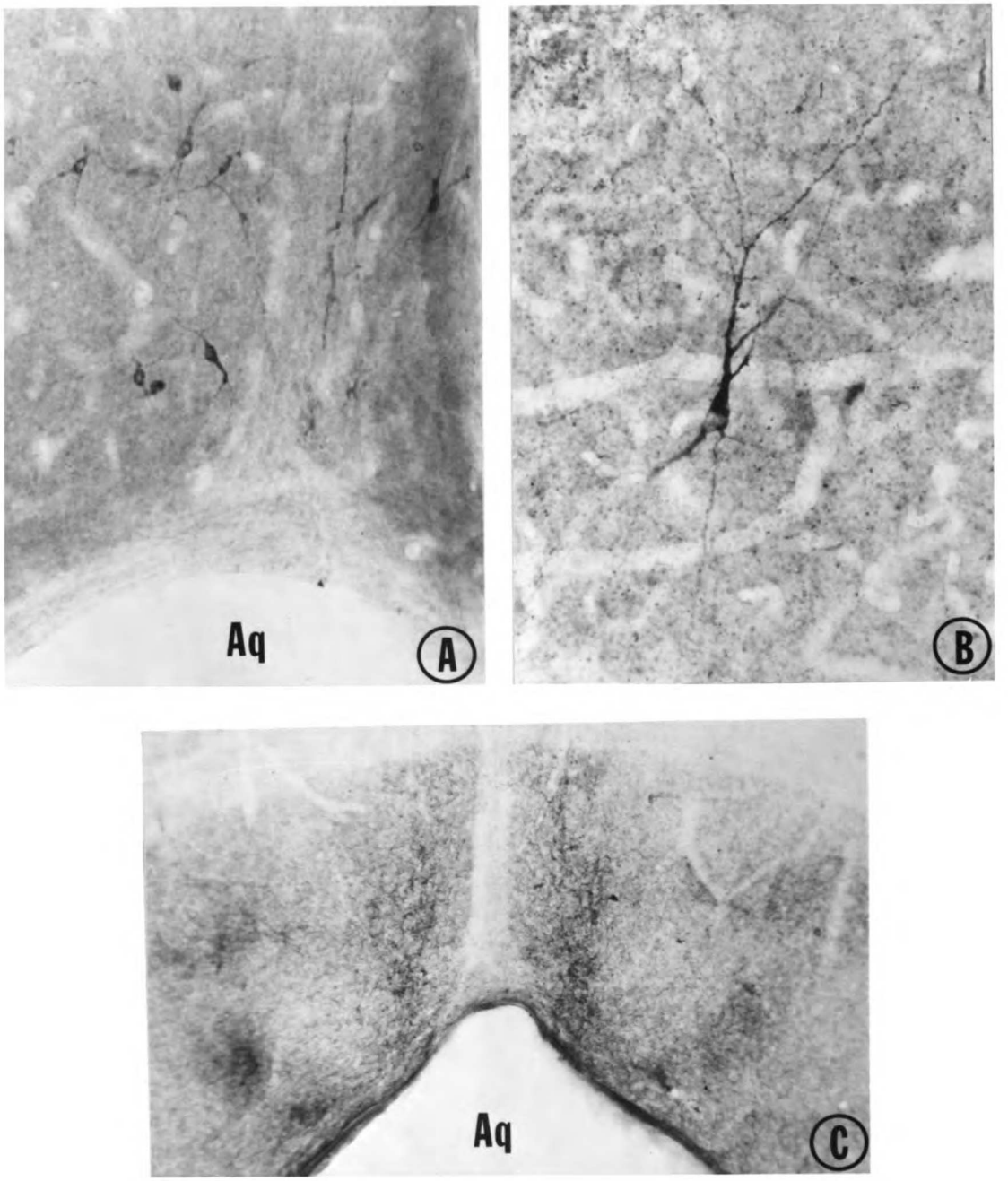

Aq
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Fig. 9. Enkephalin neurons in the lateral periaqueductal grey at the level of the III nerve nucleus (72X). Aq: aqueduct of Sylvius.

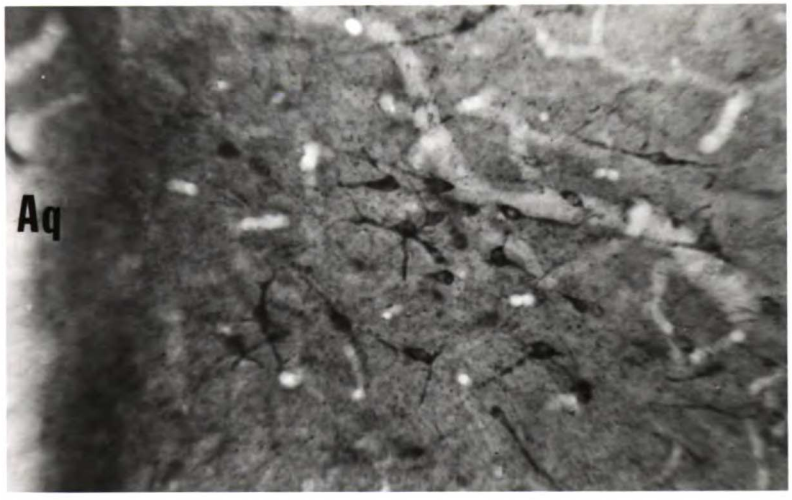
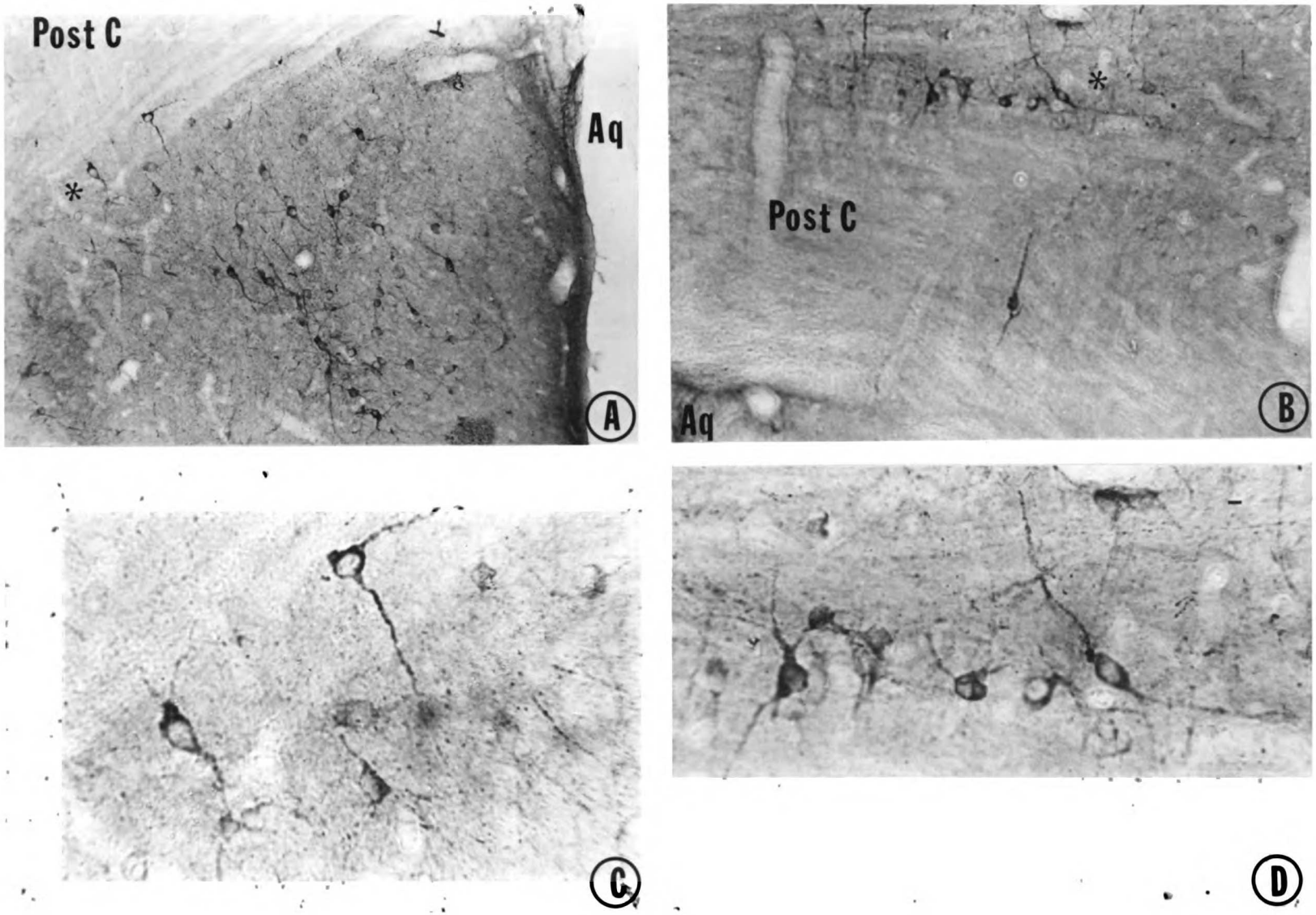

Aq

Fig. 10. The distribution of enkephalin-containing neurons in the dorsal periaqueductal grey at the level of the posterior commissure (Post C). (A) Neurons ventral to (81X) and (B) dorsal to (55X) the commissure. (C) and (D) are a higher magnification (139X) of the cell groups marked with an asterisk in (A) and (B), respectively. Note the variety of neuronal shapes. Aq: aqueduct of Sylvius.


Post C
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CHAPTER 3: THE DISTRIBUTION OF IMMUNOREACTIVE SUBSTANCE P AND VASOACTIVE INTESTINAL POLYPEPTIDE-CONTAINING NEURONS AND TERMINAL FIELDS IN THE PERIAQUEDUCTAL GREY

ABSTRACT

Despite the important contribution of the midbrain periaquectal grey (PAG) to endogenous pain suppression systems, little is known about the neuroanatomical basis of its functional organization. In a previous study of the distribution of the endogenous opiate leucine enkephalin (ENK) in the PAG we found that immunoreactive ENK-containing neurons and terminals are clustered in discrete populations. In this study we we have extended our analysis of the neurochemical organization of the PAG by mapping the distribution of two non-opiate peptides that also produce potent analgesia when administered at central grey levels: substance P (Sub P) and vasoactive intestinal polypeptide (VIP).

Sub P and VIP neurons and terminals, like of those of ENK, are clustered in discrete populations within the PAG. The distribution of immunoreactive Sub P is very similiar to that of immunoreactive ENK; it includes a ventral to dorsal shift in individual Sub P neuronal populations, from caudal to rostral PAG, and a shift away from the aqueduct that characterizes the ENK neuronal population in the lateral PAG. However, at all PAG levels, the individual populations of Sub P neurons and terminals are more extensive than that of ENK.

The staining pattern of immunoreactive VIP is totally different from that of ENK and Sub P. Regardless of the rostral caudal level examined, VIP-containing neurons are found tightly clustered in the subependymal neuropil of the ventromedial PAG.

The striking differences between the distribution of Sub P and VIP immunoreactivity in the PAG indicates that the neural circuitry

underlying pain suppression by Sub P and VIP may also differ. Conceivably, two completely different analgesia systems are activated when Sub P or VIP is injected into the PAG. The naloxone sensitive analgesia produced by Sub P may result from an opiate-Sub P interaction at the level of the PAG, where there is considerable overlap of Sub P and ENK neurons and terminals. The naloxone-insensitive analgesia produced by injection of VIP into the PAG, together with the fact that its distribution in the PAG does not overlap with that of ENK, suggests non-opiate pathways of pain control.

INTRODUCTION

The periaqueductal grey (PAG) is a component of an endogenos pain suppression system that has been implicated in stimulation-produced and opiate analgesia (Mayer and Price, 1976; Oliveras et. al., 1979; Basbaum, et. al., 1976; 1977; 1978; Basbaum and Fields, 1978; 1979; Fields and Anderson, 1978; Lewis and Gebhart, 1977; Yeung et. al., 1977). Despite the important contribution of the PAG to pain suppression systems, studies are only beginning to dissect the complex neuroanatomical basis of its functional organization. In a previous paper (Moss et. al., 1982) we described the PAG distribution of the endogenous opiate peptide, leucine enkephalin (ENK). Immunoreactive ENK-containing neurons and terminal-fields are clustered in discrete populations; the distribution of this clustered pattern changes at different rostral-caudal PAG levels. These results illustrated the heterogeneity of the PAG's neurochemical organization and provided clues to the differential sensitivity of the various regions of the PAG to opiate microinjection.

In addition to the endogenous opiates, the non-opioid peptides substance P (Stewart et. al., 1976; Fredricksen et. al., 1978; Malick and Goldstein, 1978; Mohrland and Gebhart, 1979; Sullivan and Pert, 1981; Naranjo et. al., 1982) and vasoactive intestinal polypeptide (Sullivan and Pert, 1981) also generate a profound analgesia when injected either intraventricularly or directly into the PAG. These two peptides are of particular interest since they generate, respectively, a naloxone sensitive and naloxone insensitive analgesia. Thus a comparison of the degree of overlap of the distribution patterns of these two peptides may provide some

information on the interrelationships of opiate and nonopiate peptides in endogenous pain control mechanisms.

As part of our continuing effort to define the neurochemical organization through which the PAG initiates or modulates pain suppression, we have mapped the distribution of both immunoreactive substance P (Sub P) and vasoactive intestinal polypeptide (VIP) containing neurons and terminal-fields throughout the PAG. We found that the distribution of Sub P and VIP, like that of ENK, is not homogeneous. While there are considerable similarities in the distribution of Sub P and ENK immunoreactivity, the staining pattern for VIP is totally different. Preliminary results of this work have been reported previously (Moss et. al., 1980; Moss and Basbaum, 1982).

MATERIALS AND METHODS

Eleven adult cats were used for this study. To enhance perikaryal labelling, several of the animals received a third ventricle injection of colchicine (Sigma, 5ul, 20 ug/ ul) 24-48 hours before sacrifice. Cats were anesthetized and perfused with a 37^{0}C solution of 0.1M phosphate buffered saline (pH 7.4) containing 0.1% heparin. This was immediately followed by a 4^{0}C solution consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1M phosphate buffer containing 4% sucrose. A postwash of fresh cold phosphate buffer containing 4% sucrose was administered approximately 30 minutes after the perfusion of the fixative. The brain was immediately removed and stereotaxically blocked. The midbrain was serially sectioned at 100um using a Vibratome, or at 50um using a freezing microtome, or embedded in paraffin and sectioned at 30-50um.

Sub P-like and VIP-like immunoreactivity were localized on representative sections at 150-400um intervals using the peroxidase-antiperoxidase (PAP) method (Sternberger, 1974). To enhance antibody penetration, Vibratome and frozen sections were either preincubated in a sodium ferricyanide-methanol solution (Strauss, 1976) or 0.3% Triton was added to all solutions. Sections were incubated in primary antisera for 48 hours at 4^{0}C. Bridging antisera and PAP incubations were at least 30 minutes in duration, at room temperature. The PAP reaction product was visualized using 3,3^{1} diaminobenzidine as the chromagen.

To establish histochemical specificity, adjacent control sections were incubated in either Sub P antisera (Immunonuclear Corp) preabsorbed with an excess of Sub P (Sigma) (100ug/ml diluted antisera) or in VIP antisera (Dr.J. Walsh, UCLA) preabsorbed with excess VIP (Sigma) (200ug/ml diluted antisera). Both Sub P and VIP staining was abolished in the respective control sections. Although the terms Sub P-like and VIP-like immunoreactivity are not consistently used throughout this report, they are at all times implied. Some portion of the immunoreactivity demonstrated with the antisera to Sub P and VIP may be due to other unknown peptides with similar sequences.

Pretreatment with colchicine was necessary to visualize the Sub P perikarya in the PAG. Although colchicine was always administered via third ventricle injections, differences in cell labelling suggest that its penetration into the surrounding neuropil varied from animal to animal. This probably resulted from variations in CSF flow or from diffusion barriers set up by axonal tracts. Therefore it was

essential that we compared the perikaryal labelling from several animals. In contrast, some VIP perikarya in the PAG could be visualized without colchicine pretreatment. Colchicine pretreatment increased the number of immunoreactive VIP neurons at each level but the distribution pattern of the neurons did not change.

The distribution of the labelled perikarya and terminal-fields was plotted on projection drawings of appropriate sections from several animals. The results from the projection drawings were collated and plotted on representative sections through the PAG. This was done to reduce variability among the different animals due to slight differences in the plane of sectioning and/or colchicine administration.

The schematic drawings and nuclear designations used in this study are derived from the atlases of Berman (1968), Mehler (1958) and Taber (1961).

RESULTS

To facilitate the description of the distribution of Sub P and VIP immunoreactivity, the PAG will be divided into three major rostral-caudal regions. Caudal PAG (Fig. 1) includes levels from the dorsal tegmental nucleus to a level just caudal to the IV nerve nucleus. Mid-PAG (Fig. 2) extends from the level of the IV nucleus to the level of the nucleus of Edinger-Westphal. Rostral PAG (Fig. 3) includes levels from the posterior commissure to the periventricular grey. To complete the analysis, the distribution of Sub P and VIP immunoreactivity in the midbrain nucleus raphe dorsalis is also described.

Nucleus raphe dorsalis (Fig. 1, 2):

Several Sub P-containing perikarya are found within the cytoarchitectural boundaries (Taber, 1961) of the nucleus raphe dorsalis. Most are found in the lateral wings of the nucleus, at the level of the IV nerve nucleus. In comparison, numerous ENK neurons are found at all levels of the RD both along the midline and lateral borders of the nucleus. The densest Sub P terminal-field staining is located in more dorsal regions of the nucleus and along its midline (Fig. 4C). This contrasts with ENK terminals which are located along the lateral borders of the nucleus.

Caudal PAG (Fig. 1):

In the caudal central grey, at the level of the dorsal tegmental nucleus of Gudden (DTN), Sub P perikarya are clustered in the ventrolateral PAG. Just caudal to the IV nerve nuclei, this distribution changes. While ENK cells at this level are still located in ventrolateral PAG, Sub P neurons are now predominantly found in the lateral PAG, adjacent to the aqueduct. Scattered Sub P containing neurons are also located adjacent to the nucleus raphe dorsalis and in the dorsal PAG.

Sub P terminal-field staining is densest in a ring around the aqueduct, and in the ventrolateral (Fig. 4A) and dorsal PAG. The nucleus cuneiformis also contains terminal labelling but no Sub P cell bodies are seen. The staining pattern at this level is very similiar to that of ENK immunoreactivity.

Mid-PAG (Fig. 2.):

At all mid PAG levels, the population of Sub P neurons in the lateral PAG is still present. At the levels of the III and IV nerve

nuclei (Fig. 5), this lateral cell population is still located adjacent to the aqueduct. With the appearance of the Edinger-Westphal nucleus, however, this Sub P cell cluster shifts outward, away from the aqueduct, towards the lateral perimeter of the PAG. Another prominent population of Sub P perikarya is located in the dorsal PAG. It extends dorsally, away from the aqueduct, along the midline, and then sweeps outward towards the dorsolateral PAG. Many Sub P neurons are also found in the Edinger-Westphal (EW) nucleus (Fig. 6).

The distribution of Sub P terminal-fields at mid PAG levels is similar to that observed more caudally; dense patches of labelling are still found in the dorsal PAG and in the dorsolateral PAG, however the ventrolateral PAG no longer contains terminal staining. A ring of dense terminal staining around the aqueduct is also present (Fig. 4B) and is quite prominent in the lateral PAG, fanning outward from the aqueduct. Although ENK terminals are also found in the lateral PAG, the heaviest ENK labelling at this level is found in paired columns in the dorsal PAG.

Rostral PAG (Fig. 3):

The distribution of Sub P-containing neurons and terminal-fields at the level of the posterior commissure, is similar to that observed at mid PAG levels. There are two principle Sub P neuronal populations: one is located in the lateral PAG (away from the aqueduct), the second is found in the dorsal-dorsolateral PAG (Fig. 7). Sub P perikarya are also found in the cap of PAG neuropil situated dorsal to the posterior commissure. Terminal-field staining is densest in the lateral PAG, adjacent to the aqueduct, and overlaps the distribution of the cell bodies in the dorsal-dorsolateral PAG.

Sub P neurons and terminals are also found in the rostral continuation of the PAG, i.e. the periventricular grey. In some animals it appeared that the dorsal periventricular grey contained slightly more Sub P immunoreactivity than the ventral periventricular grey. The pattern of Sub P cell and terminal staining in the rostral PAG is quite similiar to that of ENK staining except for the greater amount of immunoreactive Sub P terminals found adjacent to the aqueduct in the lateral PAG.

Vasoactive Intestinal Polypeptide: (Fig. 8):

Regardless of the rostral-caudal level examined, VIP-containing neurons are found tightly clustered in the subependymal neuropil of the ventromedial PAG (Fig. 9). The largest number of subependymal VIP perikarya are located in the mid-PAG, at the levels of the III and IV nerve nuclei (Fig. 9B, C). Scattered VIP neurons are also found in the ventral PAG at these levels and in the raphe dorsalis. No VIP cells were seen in the most caudal region of the PAG, at the level of the dorsal tegmental nucleus of Gudden.

Terminal-field labelling, when present, was limited to the ventral and ventrolateral PAG. Staining was sparse and more often consisted of scattered beaded varacosities than well defined regions of terminal-field staining. Many VIP-labelled processes (possibly axonal or dendritic) arborize within the subependymal neuropil. Several fibers entered the ependymal layer, but could not be followed to their termination.

DISCUSSION

As we found previously for ENK (Moss et. al., 1982), Sub P and VIP cell body and terminal-field staining is concentrated in several

discrete regions throughout the rostral-caudal extent of the PAG. Although ENK and Sub P have similiar distributions, VIP staining is completely different.

Several aspects of the staining pattern of Sub P and ENK perikarya are remarkably similiar. Particularly comparable are the ventral to dorsal shift in these peptide-containing neuronal populations from caudal to rostral PAG, and the shift away from the aqueduct, toward the perimeter of the PAG, which characterizes the lateral neuronal populations. The closest degree of overlap of Sub P and ENK neuronal populations occurs at the more rostral levels of PAG. There are differences, however, in the staining pattern of ENK and Sub P cells. For example, although there are many ENK-containing neurons in the caudal ventrolateral PAG and the nucleus raphe dorsalis (RD), few Sub P cells are present in these areas. In addition, at all PAG levels the individual populations of Sub P neurons are more extensive than those of the ENK cells i.e. Sub P neuronal populations contain more cells and/or are more widely distributed.

Given that Sub P and ENK coexist in some avian PAG neurons (Erichsen et. al., 1982), it is possible that the overlapping distribution of Sub P and ENK neurons in the cat PAG reflects the coexistence of these putative transmitters in individual neurons. It is of interest, however, that the reported area of maximum coexistence in the avian brain is in the EW nucleus. Although the cat EW contains many Sub P cells, it does not contain ENK perikarya.

Just as the distribution patterns of Sub P and ENK cell populations are similar, so the Sub P terminal-field staining in the

PAG follows the pattern seen for ENK terminal-field labelling. This is evident at the level of the III nerve nucleus where the densest terminal-field staining is located in the lateral PAG, adjacent to the aqueduct and in the dorsal and dorsolateral PAG. At all levels, however, Sub P terminal-field staining is consistently more dense around the aqueduct than is the ENK terminal staining. As described above, many ENK-labelled neurons are found in the caudal ventrolateral PAG and the RD while few Sub P cells are seen. Nevertheless, very dense Sub P and ENK terminal labelling is found in both areas.

Unlike the extensive distribution of Sub P and ENK immunoreactivity in the PAG, immunoreactive VIP neurons are tightly clustered in the subependymal neuropil of the ventromedial PAG. Although the cat RD contains numerous ENK perikarya and terminals along its midline (Moss et. al., 1981; Moss et. al., 1982), the labelling is located ventral to the VIP staining. Some VIP neurons are occasionally found in the RD and ventral PAG, but in much fewer numbers than either ENK or Sub P cells.

While this is the first map of the distribution of Sub P and VIP immunoreactivity that focused on the cat PAG, previous generalized mappings of the distribution of VIP and Sub P in the rat central nervous system have included observations in the PAG. The distribution of Sub P neurons in the rat PAG resembles that of the cat (Hokfelt et. al., 1977; Ljungdahl et. al., 1978). In both species Sub P cell populations are located in the lateral and in the ventral PAG (in and adjacent to the nucleus raphe dorsalis). However,except for the level of the posterior commissure, the large

population of Sub P perikarya seen in the dorsal/dorsolateral cat PAG are not seen in the rat PAG. The functional significance of these species differences in the distribution of Sub P immunoreactivity is unknown.

Sub P terminal-field labelling throughout the rat PAG is reported to be uniform (Cuello and Kanazawa, 1978), although a higher density of Sub P in the dorsal PAG has been described (Ljungdahl et. al., 1978). The micrographs of immunoreactive Sub P at more caudal PAG levels, however, clearly shows denser staining in regions adjacent to the aqueduct. Because the action of Sub P presumably reflects the action of the peptide at a receptor, it follows that the terminal, not the cellular, distribution of Sub P is a better indicator of its site of action. Thus, administration of Sub P into areas of the PAG with dense Sub P terminal labelling would have the most potent effects. Given of the dense distribution of Sub P terminals around the aqueduct, it is likely that the peptide acts at this region. It follows that intraventricular injection of Sub P would have relatively easy access to Sub P receptors.

In the rat PAG, VIP-like immunoreactive cells were reported adjacent to the ventral and lateral aspects of the aqueduct; a moderately dense concentration of fibers was seen (Sims et. al., 1980; Loren et. al., 1979). The great majority of VIP cells in the cat PAG are also found adjacent to the aqueduct, but only in its ventral aspect. Scattered cells are also located further ventrally, away from the aqueduct, for example, in the dorsal raphe nucleus. The major difference between the rat and cat in the distribution of immunoreactive VIP is the much reduced terminal-field labelling in

the cat. When present, it is restricted to more caudal, ventral regions of the PAG. Unlike the other peptides, immunoreactive VIP cells can be seen in the cat PAG without colchicine pretreatment, indicating that this peptide may be processed differently from ENK and Sub P in the PAG. Whether this difference contributes to the minimal staining of VIP terminal-fields is unknown.

While the PAG is generally associated with antinociceptive properties, several lines of evidence implicate Sub P in the transmission of noxious messages within the CNS. For example, Sub P-like immunoreactivity has been demonstrated in small diameter primary afferent fibers (Hokfelt et. al., 1976; Cuello et. al., 1978). Iontophoretically applied Sub P excites spinal neurons which respond to noxious input (Henry, 1976) and Sub P is released into the CSF by high intensity peripheral nerve stimulation (Yaksh et. al., 1980). Intrathecal Sub P produces behavioral patterns that are indicative of a pain response (Seybold et. al., 1982) while patients with decreased pain perception (Riley-Day Syndrome) have depleted Sub P levels (Pearsen et. al., 1982).

Sub P has also been implicated in pain control systems. Sub P is located in neurons of the medullary nucleus raphe magnus (NRM) (Hokfelt et. al., 1977; 1978) and, in fact, co-exists with serotonin in many NRM neurons (Hokfelt et. al., 1978) and in their axonal terminals in the dorsal horn (Pelletier et. al., 1981; Gilbert et. al., 1982). Since stimulation of NRM inhibits spinal nociceptors (Fields et. al., 1977; Rivot et. al., 1980) and produces behavioral analgesia (Oliveras et. al., 1975; Zorman et. al., 1982), it appears that spinal nociceptors receive both excitatory primary afferent

input and descending inhibitory input from different populations of Sub P-containing neurons.

This paradox, namely that Sub P contributes both to nociception and antinociception, is also evident if one examines the effects of Sub P at midbrain levels. In the majority of studies, intraventricular or intracerebral (PAG) injection of Sub P in mice or rats generates a naloxone reversible analgesia (Stewart et. al., 1976; Fredrickson et. al., 1978; Malich and Goldstein, 1978; Naranjo et. al., 1982). It has been reported, however, that intraventricular Sub P does not produce analgesia in mice (Hayes and Tyers, 1979) or at best produces a transient analgesia in rats only when administered at high doses (Sullivan and Pert 1981). Apparently, Sub P's analgesic potency depends both on the dose administered (Fredricksen et. al., 1978) and on the initial responsiveness of the animal to a noxious stimulus (Oehme et. al., 1980).

The midbrain opiate link in Sub P generated analgesia is particularly interesting. It has been demonstrated that opiates block the release of Sub P by spinal and trigeminal neurons (Jessell and Iversen, 1977; Mudge et. al. 1979). Furthermore, since enkephalin is located in probable nociceptors in the dorsal horn, it has been suggested (Basbaum and Fields, 1978; Glazer and Basbaum, 1981) that Sub P in the spinal cord activates these opioid neurons, which in turn, inhibit primary afferent fibers. The reversal of Sub P-mediated analgesia by intraventricular methionine-enkephalin antisera (Naranjo et. al., 1982) or by naloxone (Stewart et. al., 1976; Fredrickson et. al., 1978; Malick and Goldstein, 1978; Naranjo et. al., 1982) indicates that in the midbrain, Sub P may also

activate opioid neurons. Thus in both the spinal cord and midbrain Sub P may cause the release of endogenous opiates. However in the cord, the opiate neuron is part of a negative feedback loop, while in the midbrain, it is part of a feedforward circuit, which generates analgesia.

In these midbrain studies, naloxone was administered intraperitoneally or subcutaneously and therefore, could be acting at any level of the central nervous system. Given the extensive overlap of ENK and Sub P-labelled neurons and terminals in the PAG, it is conceivable that the Sub P opiate interaction occurs at the level of the PAG.

Stimulation-produced analgesia (SPA) elicited from the PAG has been reported to be both naloxone sensitive and (Adams, 1976; Akil et. al., 1976; Hosobuchi et. al., 1977) and insensitive (Gebhart and Toleikas, 1978; Yaksh et. al., 1978). Electrical stimulation in regions of the PAG containing ENK cell populations would be more likely to generate a naloxone sensitive analgesia. On the other hand, stimulation of the PAG either in regions distant from ENK-containing neurons or in ENK terminal regions could produce analgesia that is unaffected by opiate antagonists. This would result from activation of neurons postsynaptic to the ENK-containing elements, bypassing the midbrain opiate link. However, the naloxone insensitive analgesia that is sometimes produced by PAG stimulation may also indicate of the existence of multiple endogenous analgesia systems involving the PAG; some opiate, others nonopiate in nature (See Watkins and Mayer, 1982 for review).

The VIP-containing neurons adjacent to the aqueduct in the ventromedial PAG may be part of one such non-opiate pain suppression system. Microinjection of VIP into the PAG produces a naloxone-insensitive analgesia. In addition, Cannon and co-workers (1982) have shown that while analgesia can be produced by stimulation of either the raphe dorsalis or more dorsal sites located in the ventromedial PAG, SPA from the ventromedial PAG sites, unlike the RD sites, is not antagonized by naloxone. Our study has shown that the distribution of VIP cells and processes in the PAG does not overlap the distribution of ENK immunoreactivity. Taken together this evidence indicates a non-opiate, VIP-mediated, analgesia system involving the PAG.

While we have concentrated on descending control systems activated from the PAG, the circuitry underlying VIP's pain suppressive effect may involve ascending connections. At least some of the VIP cells in the PAG are considered to be the origin of the VIP terminals in the hypothalamus, nucleus accumbans, bed nucleus of the stria terminalis and the amygdala (Marley et. al., 1981). Whether these same neurons are involved in the affective component of pain perception through VIP input to limbic structures remains to be determined.

Also of interest is the close association of VIP cells and processes with the subependymal neuropil. Conceivably, this could underlie some type of CSF-VIP interaction in VIP produced analgesic. In addition, although both Sub P and VIP they have completely different patterns of distribution in the PAG, they share a common ability to act as cerebral vasodilators (Edvinsson et. al.,

1981; Larsson et. al., 1976; Duckles and Said, 1982). The possibility exits that their analgesic effects are related to a general physiologic affect on cerebral blood flow. It would be of interest to examine whether the analgesia produced by Sub P and VIP injection into the PAG is antagonized by concurrent administration of vasoconstricting drugs.

Finally, it must be emphasized that the PAG is involved in many other functions besides pain suppression (Skultety, 1963; Jurgens and Pratt, 1979; Sakuma and Pfaff, 1980; Johnson et. al., 1982; Lakoski and Gebhart, 1982). It is almost certain that some of the different populations of Sub P or VIP-containing neurons and terminal fields in the PAG are related to these other PAG activities.

In conclusion we have demonstrated that two non-opiate peptides which generate profound analgesia upon intracerebral microinjection, have totally different distributions within the PAG of the cat. The distribution of immunoreactive Sub P is quite similar, although somewhat more extensive, than that of ENK. In contrast, immunoreactive VIP cells and processes are tightly clustered in the ventromedia PAG, just ventral to the aqueduct. Since Sub P and VIP generate, respectively, a naloxone sensitive and naloxone insensitive analgesia upon microinjection into the PAG, these data provide additional evidence for the existence of separate opiate and non-opiate mediated analgesia systems involving the midbrain central grey.

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FIGURE LEGENDS

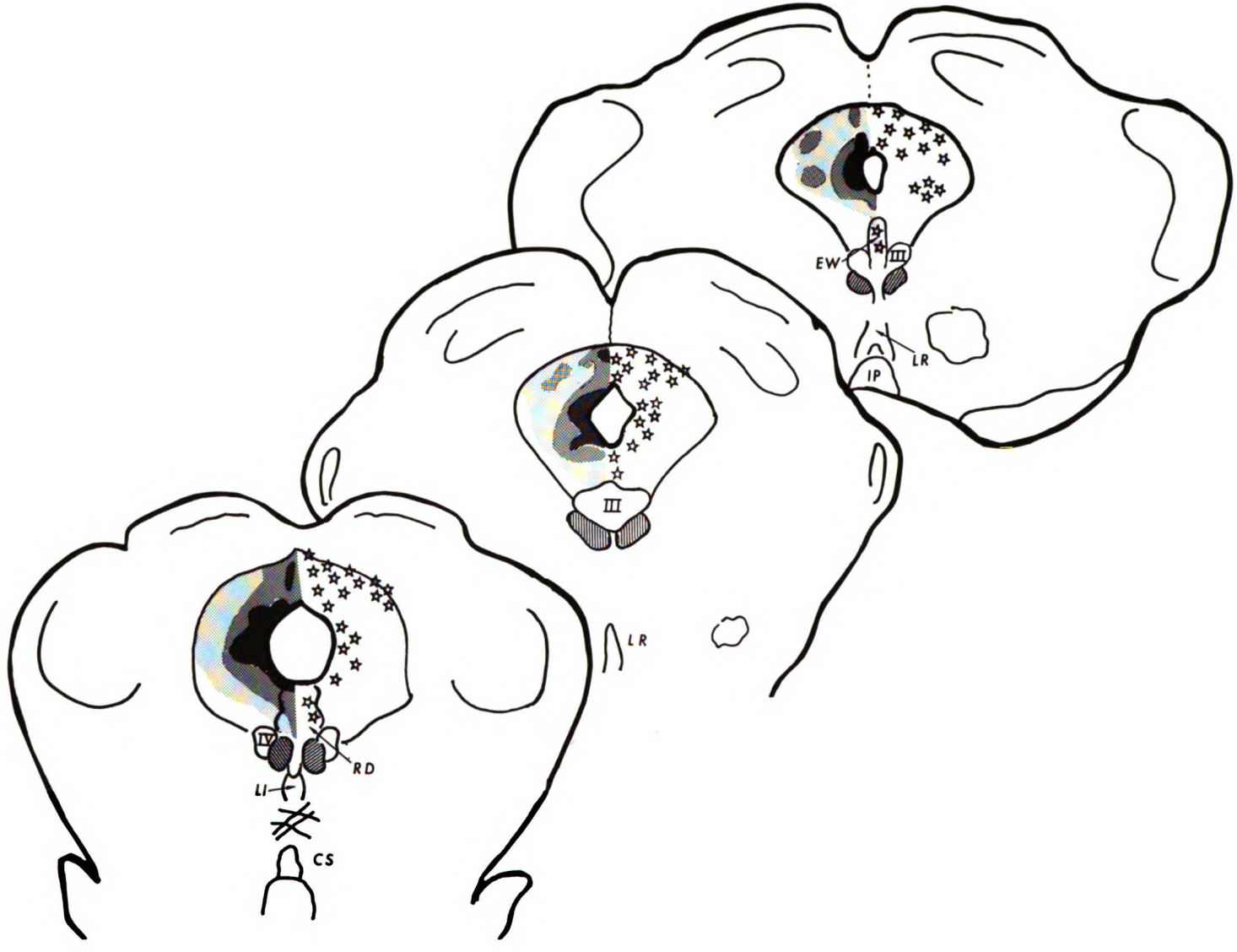
Figs. 1, 2, 3. Drawings of representative sections through the periaqueductal grey illustrating the distribution of Substance P containing perikarya (stars) and terminals (crosshatching),ranging in density from 0-3). CS: centralis superior, CUN: nucleus cuneiformis, DTN: dorsal tegmental nucleus of Gudden, EW: nucleus Edinger-Westphal, HB: habenulae, LGB: lateral geniculate, LI: linearis intermedius, LR: linearis rostralis, Mes V: mescencephalic tract of V, MLF: medial longitudnal fasiculus, Post C: posterior commissure, Pul: pulvinar, RD: raphe dorsalis, RN red nucleus, VTN: ventral tegmental nucleus of Gudden, III: third nerve nucleus, IV: fourth nerve nucleus. Mes V, MLF and the Hb-interpeduncular tract are indicated by diagnal hatching.

Fig. 1. Caudal periaqueductal grey levels

Fig. 2. Mid-periaqueductal grey levels

Fig. 3. Rostral periaqueductal grey levels

Cun
VTN
RD
CS
DTN
Wes V
RD
CS


EW
III
LR
IP
III
LR
IV
RD
LI
CS

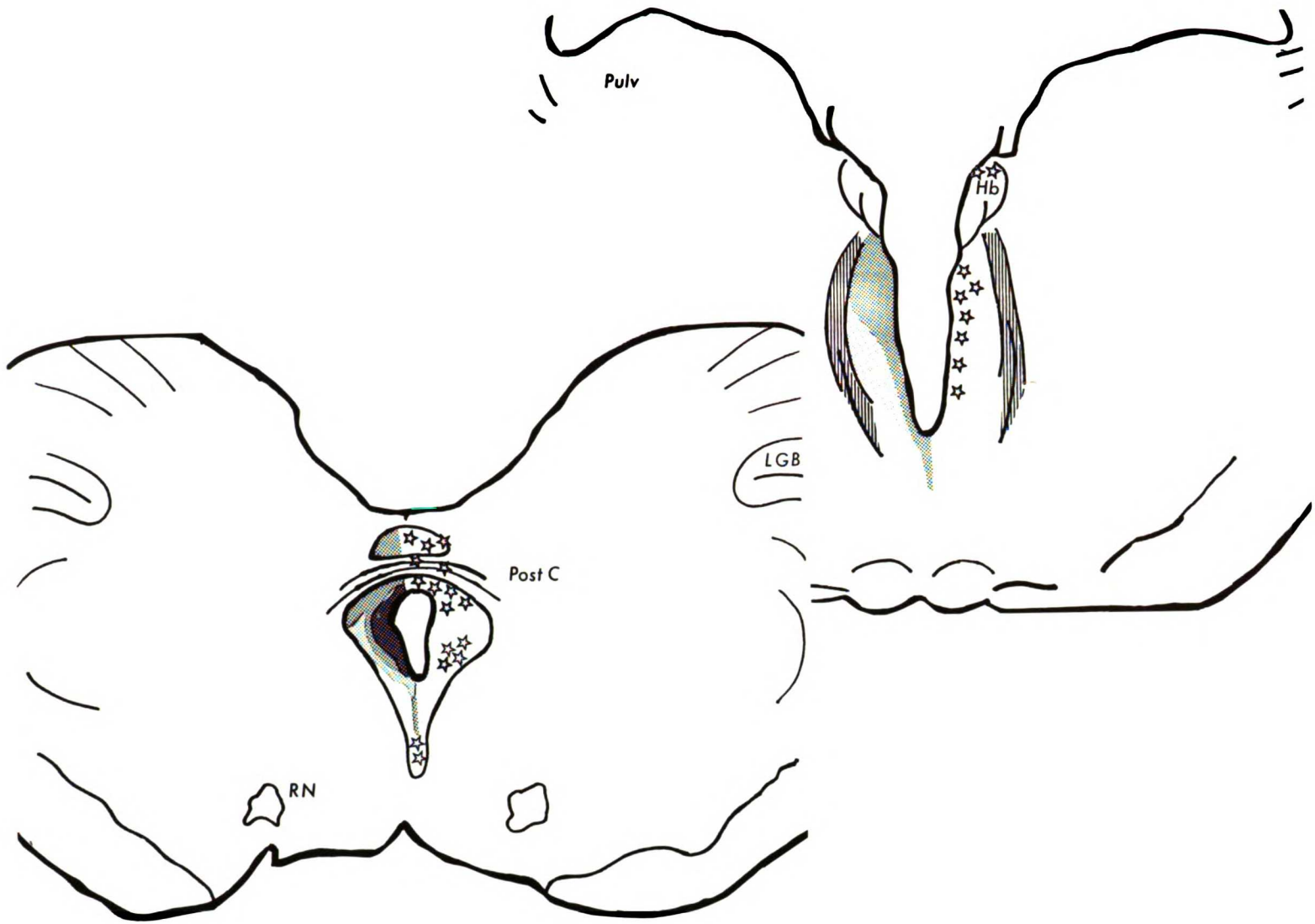

Pulv
Hb
LGB
Post C
RN

Fig. 4. The distribution of Substance P (Sub P) terminal-fields in: (A) ventrolateral periaqueductal grey (PAG) at the level of the dorsal tegmental nucleus of Gudden (88X); (B) lateral PAG at the level of the III nerve nucleus (88X); (C) raphe dorsalis at caudal PAG levels (63X). Aq: aqueduct of Sylvius, Mes V: mesencephalic tract of V, MLF: medial longitudinal fasciculus.

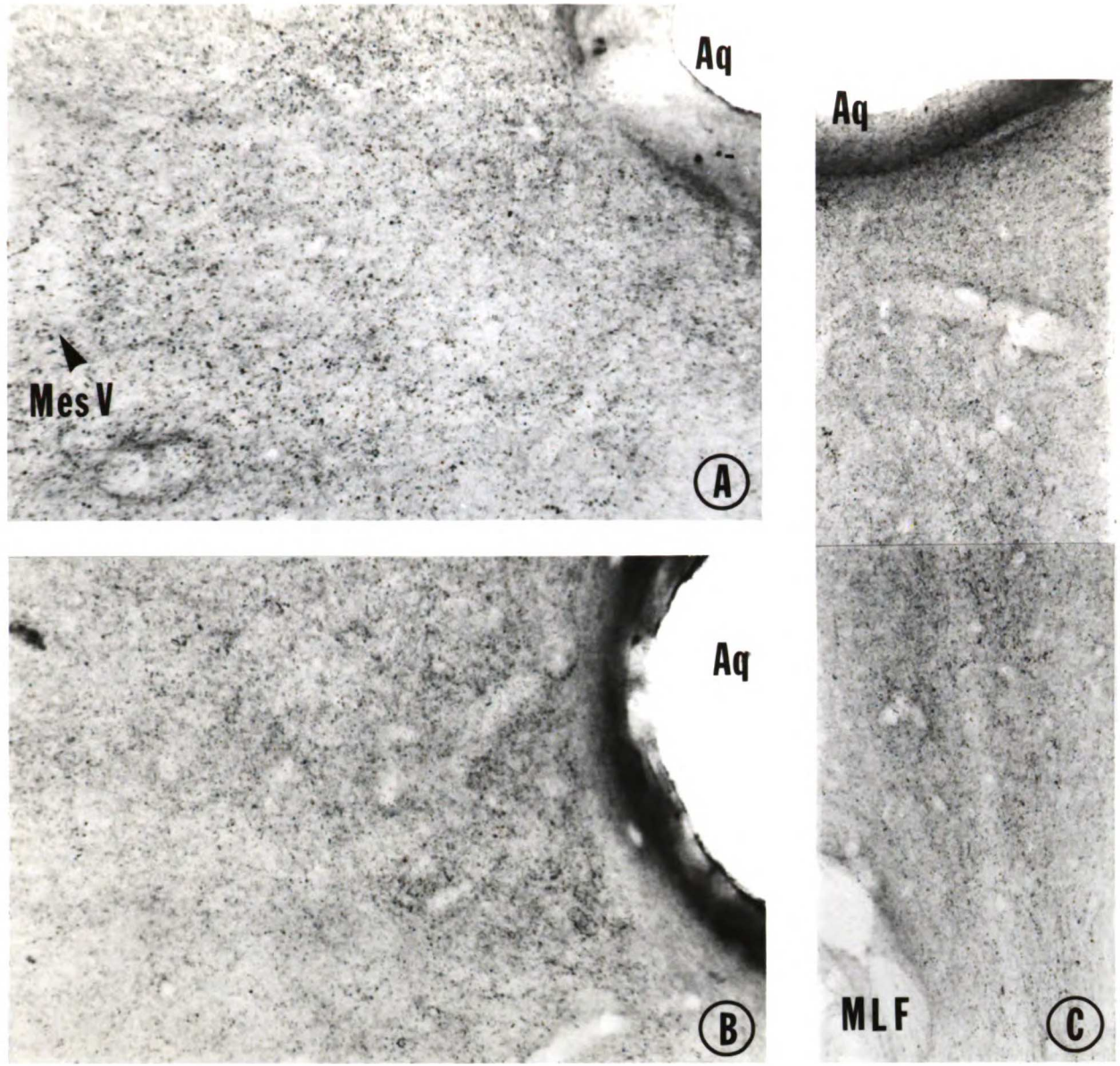

Aq
Mes V
A
Aq
Aq
B
MLF
C

Fig. 5. Substance P neurons and terminals in the lateral periaqueductal grey at the level of the IV nerve nucleus (88X). Aq; aqueduct of Sylvius.

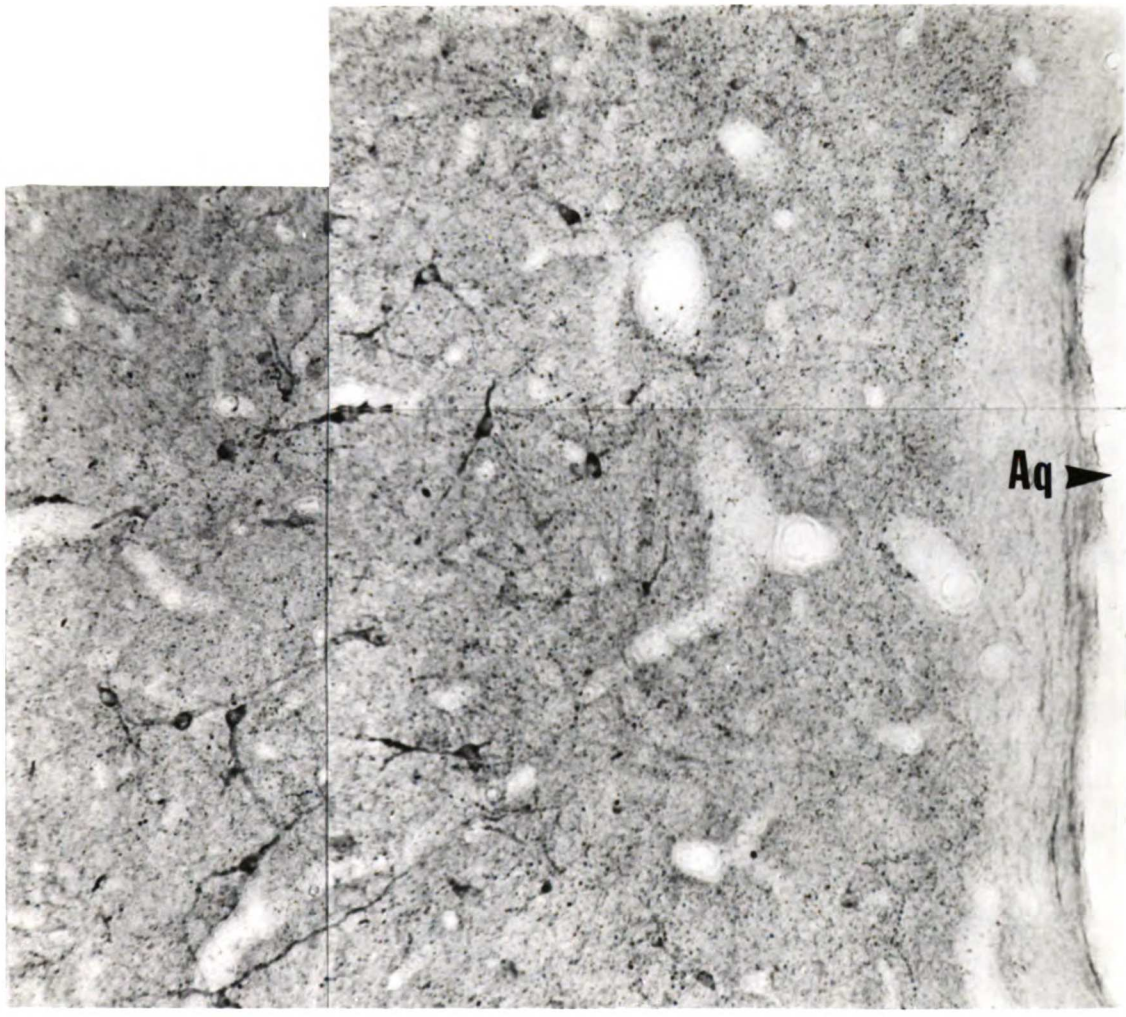

Aq

Fig. 6. (A) Substance P-containing neurons in the nucleus of Edinger-Westphal (88X). (B) Absorbtion control (88X).

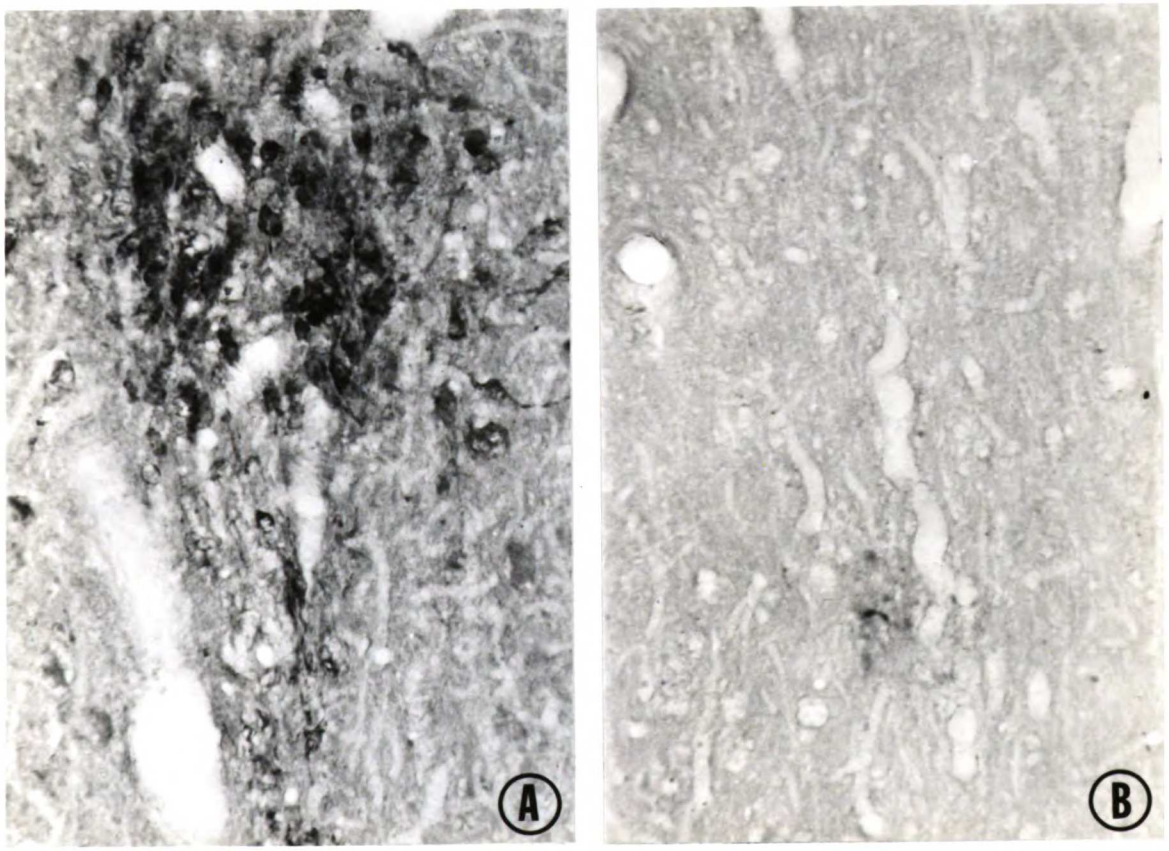

A
B

Fig. 7. The distribution of Substance P (Sub P) immunoreactivity in the dorsal periaqueductal grey at the level of the posterior commissure (88X). Note the difference in size between the cells dorsal and ventral to the commissure. Aq: aqueduct of Sylvius.

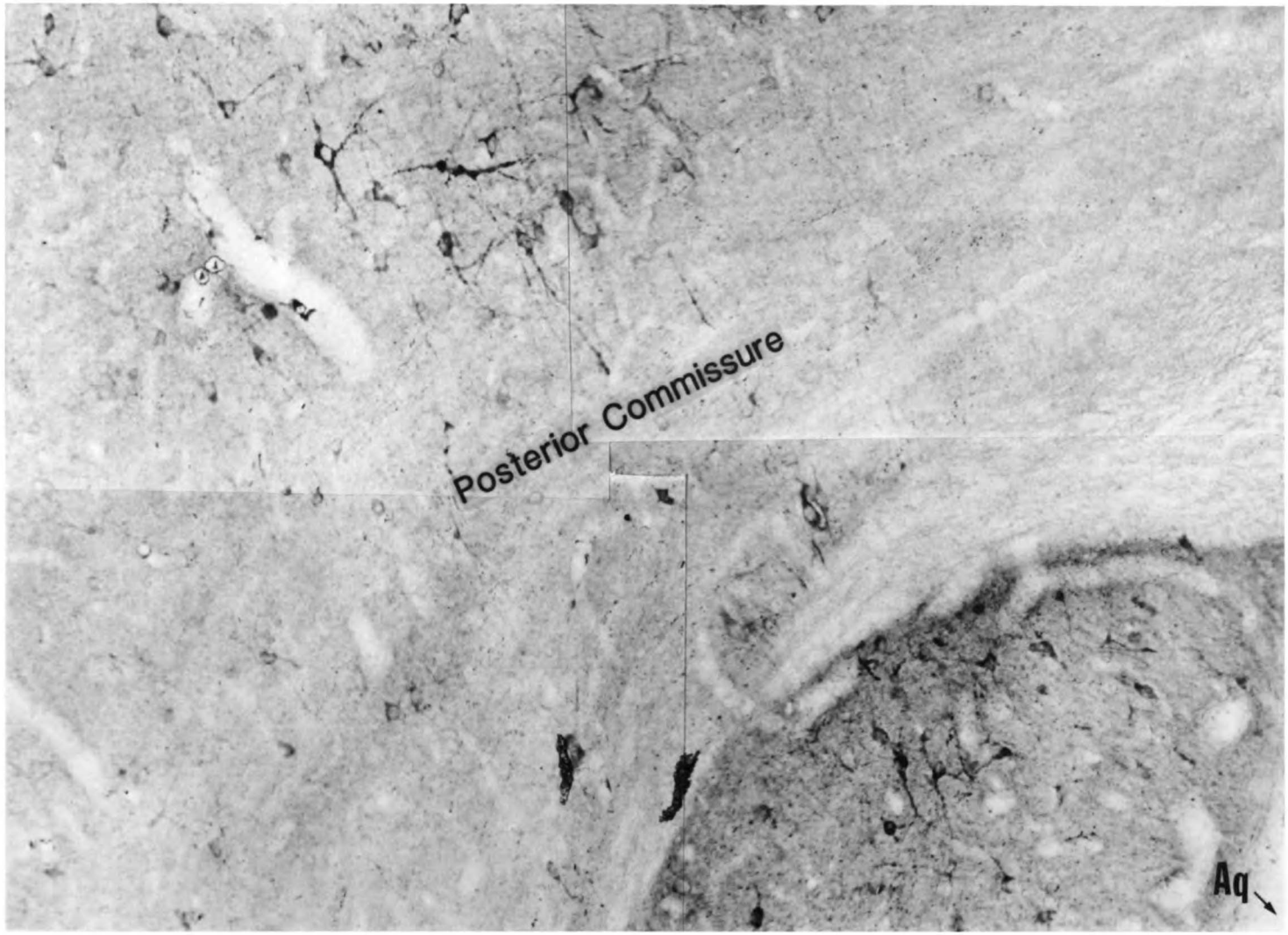

Posterior Commissure
Aq

Fig. 8. Drawings of representative sections through the periaqueductal grey illustrating the distribution of immunoreactive vasoactive intestinal polypeptide-containing neurons (asterisk).

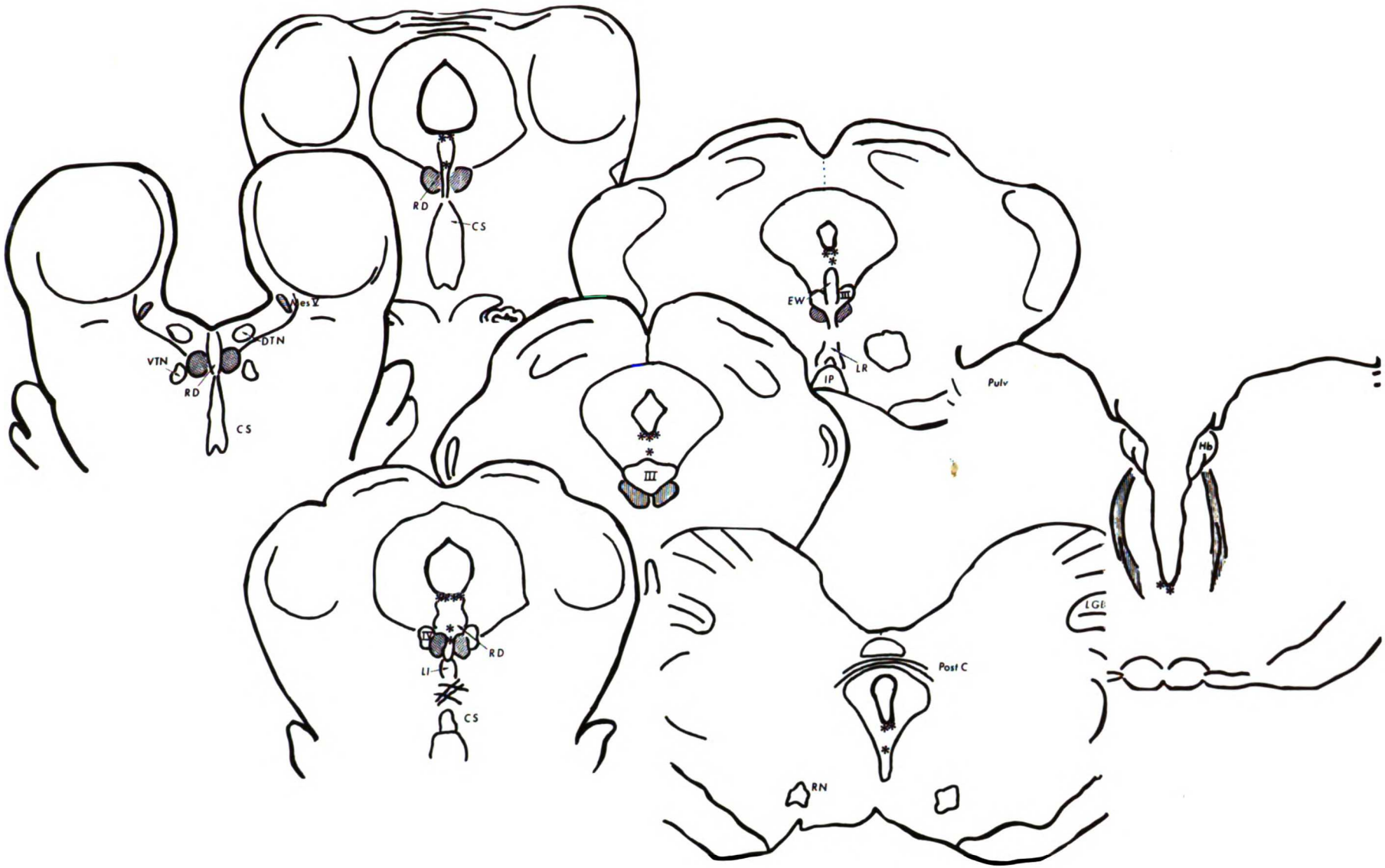
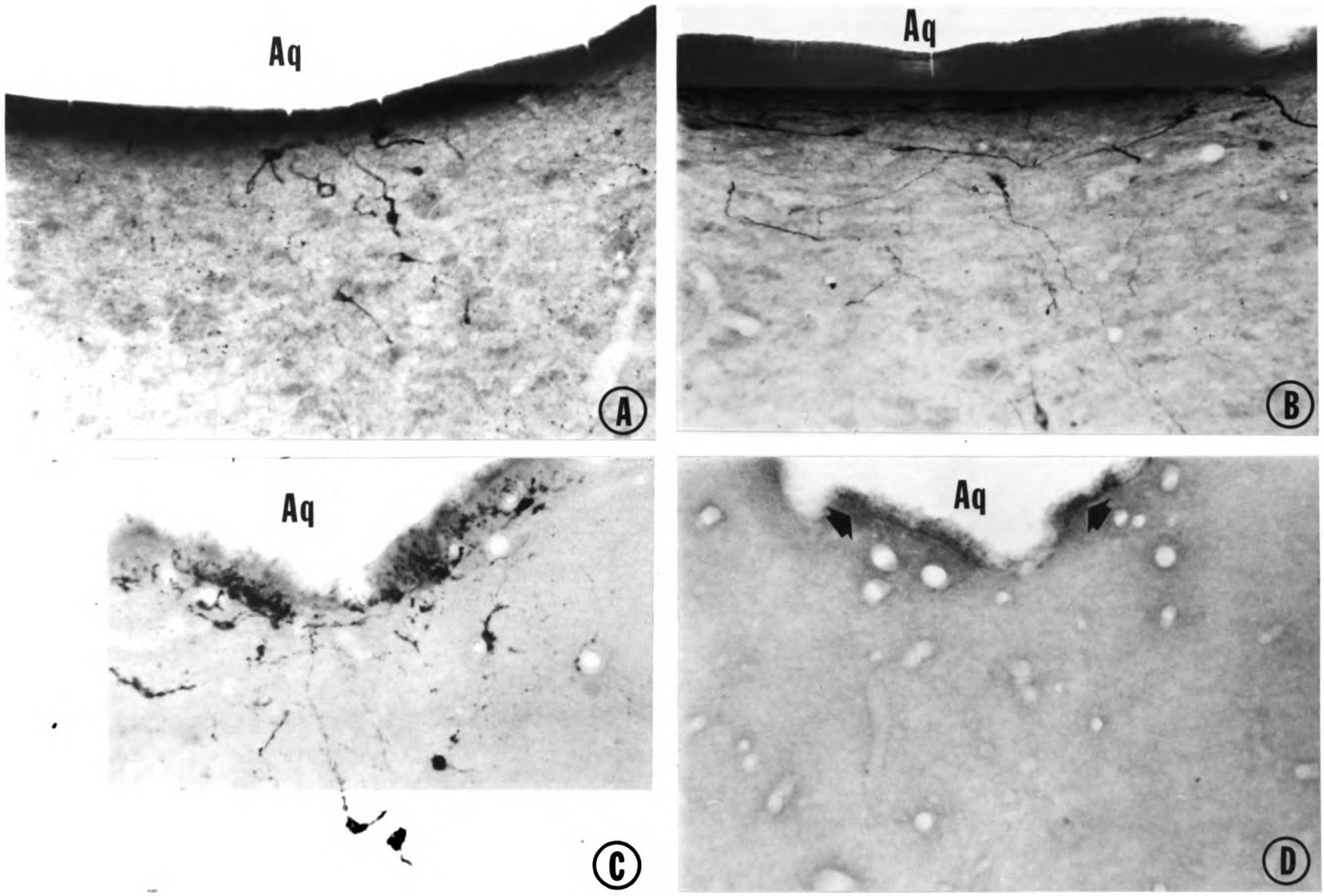

RD
CS
Mes V
DTN
VTN
RD
CS
III
EW
III
LR
IP
Pulv
Hb
LGB
IV
RD
LI
CS
Post C
RN

Fig. 9. VIP neurons in the ventromedial periaqueductal grey: (A) at caudal PAG Levels (153X); (B), at the level of the IV nerve nucleus (153X); (C) at the level of the III nerve nucleus (112X). In (A), (B) and (C) note the arborization of VIP neuronal processes in the subependymal zone. (D) Absorption control of a section adjacent to (C). Arrows indicate nonspecific ependymal staining (112X).


Aq
A
Aq
B
Aq
C
Aq
D

CHAPTER 4: THE SYNAPTIC ORGANIZATION OF THE CAT PERIAQUEDUCTAL GREY: RELATION TO PAIN MODULATION

INTRODUCTION

The midbrain periaqueductal grey (PAG), a component of an endorphin- mediated pain suppression system, plays a major role in the analgesic action of opiates and electrical brain stimulation (Tsou and Jang, 1962; Reynolds, 1969; Mayer et. al., 1971; Mayer and Price, 1976; Basbaum et. al., 1976; 1977, Basbaum and Fields, 1979; Bennett & Mayer, 1979; Fields and Anderson, 1978; Lewis and Gebhart, 1977: Gebhart and Toleikas, 1978; Yeung et. al. 1977; Oliveras et. al., 1974; 1979). Little is known, however, about the neural circuitry of the PAG in general and, more specifically, there is no information about the PAG circuitry through which endogenous or exogenous opiates act.

In a previous paper (Moss et al., 1983b) we described the light microscopic distribution of leucine enkephalin (ENK)-like immunoreactivity throughout the rostral-caudal extent of the PAG. We reported that ENK-containing perikarya and terminal-fields are clustered in discrete populations. One such population is located in the caudal, ventral PAG, a region previously shown to be particularly effective in generating analgesia in response to electrical stimulation or opiate microinjection (Yaksh et. al., 1976; Yeung et. al., 1977; Lewis and Gebhart, 1977; Gebhart and Toleikas, 1978; Oliveras et. al., 1979). These data, however, provide no information about the synaptic mechanisms through which opiates act. Thus, to determine the microcircuitry through which PAG opioid- containing neurons function, we examined the synaptic contacts made by and with ENK-containing profiles in the caudal ventral PAG of the cat.

Since we wished to relate this information about ENK circuitry to the normal PAG cytoarchitecture, we first performed a quantitative analysis of the normal ultrastructure of the caudal PAG. To determine whether there was a characteristic circuitry intrinsic to different regions of the PAG we examined three the caudal PAG areas: ventromedial, ventrolateral and dorsolateral. Preliminary results of this work have been published (Moss et al., 1981, 1983a).

MATERIALS AND METHODS

Fine structure of Caudal Periaqueductal Grey

Preparation of Tissue

For the analysis of the normal PAG, one adult cat was anesthetized and transcardially perfused with a 37^0C solution of 0.1M PO4 buffer containing 4% sucrose and 0.1% heparin. This was followed by a fixative solution consisting of 4% paraformaldehyde, and 2% glutaraldehyde in 0.1M phosphate buffer containing 4% sucrose. The brain was left in situ for approximately 30 minutes, then was removed and blocked in the stereotoxic plane. The caudal PAG (defined as PAG levels caudal to the IV nerve nucleus) was coronally sectioned at 100 um using a Vibratome. Sections were postfixed in 2% buffered O_sO_4 either for 1 hour at 23^0 or overnight at 4^0C and subsequently en bloc stained in a saturated solution of aqueous uranyl for 1 hour. Each PAG section was trimmed and divided into three regions: ventromedial, ventrolateral and dorsolateral (Fig. 1). The trimmed blocks were dehydrated in alcohol and flat-embedded in Epon-Araldite.

Thin sections of silver-gold interference color were collected onto Formvar coated 150 mesh grids with the long axes of the sections

oriented parallel to the grid bars. Grids were stained with lead citrate (Reynolds) and examined with an AEI electron microscope.

Quantitative analysis of synaptic contacts

Our primary interest was in the synaptic interactions in the caudal PAG through which opiates could possibly act. Therefore we counted the types of synapses and the various kinds of profiles involved in synaptic contacts. To insure a random sampling of the caudal PAG neuropil, photomicrographs were taken at one or more corners of the grid squares, across the entire area of the thin section. No other criteria were used to select areas chosen for photography. A total of 386 micrographs, taken at a magnification of 10,000X and printed at a final magnification of 27,500, were examined. Approximately 2000 profiles were categorized and over 600 synapses were counted. Presynaptic profiles were divided into the following categories: axonal-boutons, dendrites, and unclassifiable elements. Synapses were classified according to their vesicle shape and the symmetry or asymmetry of the density of their pre and postsynaptic membranes.

The criterion for identification of presynaptic profiles was that they contain 40-60 nm vesicles. Profiles in this category included (a) axonal boutons: profiles filled with vesicles, (b) dendrites: pale cytoplasm, clumping of vesicles, larger size than axonal boutons and/or presence of ribosomes, (c) unclassified: those profiles that did not clearly fit into the above two categories. Such profiles contained electron lucent cytoplasm and clumped vesicles, but neither their size nor the presence of ribosomes could establish whether they were dendrites or axonal boutons.

Synapses were identified by the presence of vesicles clustered against the presynaptic membrane and discernable membrane specializations. When a synaptic cleft was not visible, the synapse was classified as obliquely cut. Vesicles were clasified as a) round (spherical vesicles of uniform size) or b) flattened (mixture of smaller ovoid, round, and flat vesicles). In addition, if a synaptic cleft was visible, the synaptic contact was described as symmetric or asymmetric by comparing the width of the pre and postsynaptic densities. In asymmetric synapses the postsynaptic density was more marked than that seen presynaptically.

Because axodendritic synapses are the dominant form of synaptic contact in the PAG, they were further categorized according to the amount of convergence onto individual dendrites that was evident in a single plane of section. The categories were defined as follows: (a) dendrites with only one synaptic input, (b) dendrites surrounded by two axonal boutons, at least one making synaptic contact or (c) dendrites surrounded by three or more boutons, at least one making a synaptic contact. In addition, to determine whether there was any preferential input onto either proximal or distal dendritic branches, dendrites cut in cross section were also classified on the basis of size (largest diameter across the profile), as either small ($\frac{1}{4}$ 1.3 um) or medium ($\frac{1}{2}$ 1.3 um).

These arbitrary definitions and categories were arrived at after careful survey of the PAG micrographs. The relevant profiles were traced with difference colored inks and counted.

EM Immunocytochemistry

One adult cat, pretreated 48 hours before sacrifice with an injection of colchicine (Sigma, 5ul, 20ug/ul) into the third ventricle and seven untreated cats were anesthetized and perfused with 0.05 M phosphate buffered saline (pH 7.4, $37^{0}C$) containing 0.01% heparin. This was immediately followed by a $4^{0}C$ fixative solution consisting of 4% paraformaldehyde and 0.2 % glutaraldehyde in 0.1M phosphate buffer containinig 4% sucrose. Approximately 30 minutes later, the animal was perfused with fresh cold buffer to wash out the excess fixative. The brain was removed and the midbrain sectioned on the Vibratome as described above.

Leucine enkephalin-like immunoreactivity was localized on sections from the caudal PAG using the peroxidase-antiperoxidase (PAP) method (Sternberger, 1974). Antibody penetration was enhanced either by preincubating the sections in a graded ascending and descending glycerol series or by the addition of 0.15% Triton to all solutions. Sections were incubated in ENK antisera for 48 hours at $4^{0}C$. Bridging antisera and PAP incubations were at least 30 minutes in duration at room temperature. Following the PAP incubation, the sections were postfixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 5 minutes, washed several times and then reacted with 0.05% $3,3^{1}$-diaminobendizine and .01% H_2O_2.

Reacted sections were postfixed in 2% buffered OsO_4 for 2 hours at $4^{0}C$. They were then washed several times, trimmed into small pieces (See Fig 1) consisting of only the caudal ventral PAG, and rapidly dehydrated in acetone. The tissue was then flat embedded in Epon-Araldite between two plastic slides. Thick sections were cut

and used for the orientation of the tissue blocks, which were then retrimmed, sectioned and stained as described above.

The ENK antiserum (Immunonuclear Corp.) used in this study has a 20-30% cross reactivity with methionine enkephalin and no cross reactivity with B-endorphin. Since cross-reactivity with unknown peptides cannot be assessed, the term leucine enkephalin-like immunoreactivity, while not used throughout this report, is implied at all times. Histochemical specificity was established by incubating adjacent control sections in ENK antisera preabsorbed with ENK (Sigma, 100ug ENK/ml diluted antisera). ENK staining was abolished in these control sections.

RESULTS

Quantitative analysis

The results of the analysis of the synaptic morphology and interactions found within the caudal ventrolateral, ventromedial and dorsolateral PAG are presented in Table I. The totals are given as percentages of each category, rounded off to the nearest whole number.

There is no characteristic of synaptic morphology that distinguishes the individual regions of the caudal PAG from one another. All have approximately equal numbers of symmetric and asymmetric synapses. Not suprisingly, there is a greater tendency for flattened synaptic vesicles to be associated with symmetric synaptic densities. However there is a more equal distribution of flat and round vesicle associated with asymmetric densities. Obliquely cut synapses in the caudal ventral PAG were more often associated with flattened vesicles. In the dorsal PAG, however,

there was a slightly greater number of round vesicle-containing synaptic boutons associated with obliquely cut synapses.

In addition to small clear synaptic vesicles, small (70-80 nm) and large (90-100nm) dense-core vesicles are also present in PAG axonal profiles. Approximately 10% of all boutons in the caudal PAG contained at least one large dense-core vesicle. In the dorsolateral PAG, 8% of all axonal boutons contained at least four small dense-core vesicles. Boutons containing these small dense core vesicles were even less common in the ventral PAG where they made up only 4% of the total population of axonal profiles. Only a few of these profiles contained both large and small dense-core vesicles.

Axodendritic synapses are the dominant form of synaptic interaction in the caudal PAG, comprising 93%, 95% and 97% of the total number of synapses in the dorsolateral, ventromedial and ventrolateral PAG, respectively. In all regions of the PAG the majority of axodendritic synapses are found on dendrites surrounded by one or two axonal boutons. In addition, 16 to 27% of the central dendrites are surrounded by more than two axon terminals. The greatest degree of convergence of inputs onto a single dendrite occurs in the ventrolateral PAG.

The percentage of axodendritic synapses made onto small versus medium sized dendrites varied among the different caudal PAG regions sampled. In the ventromedial PAG, twice as many axodendritic synapses involve small rather than the medium sized dendrites. In the dorsolateral PAG, the majority of synapses is also into small diameter dendrites. In the ventrolateral PAG, however, almost equal numbers of synapses are found on small and medium sized dendrites.

Although axodendritic synapses predominate, other types of synaptic interactions are seen in the caudal PAG including axosomatic, axoaxonic, dendrodentritic, and synapses where it could not be determined whether the presynaptic element was an axonal bouton or a dendrite. These various types of synapses constitute only a small proportion of the total synaptic population in the PAG; the frequency of each particular type varying among the different PAG regions sampled.

Dendrodendritic synapses are seen only in the ventrolateral PAG, where they comprise 1% of the synaptic population sampled. The greatest number of axosomatic synapses are found in the ventromedial and dorsolateral PAG, constituting 3% and 2% of the synapses, respectively. Although perikarya were counted with equal frequency in the ventrolateral and dorsolateral PAG, axosomatic synapses are seen only in the latter. In contrast, the areas sampled in the ventromedial PAG contained approximately twice the number of cells as in the dorsolateral PAG, yet the same number of axosomatic synapses were seen in both regions.

Axoaxonic synapses in the PAG were not as clearly identifiable as the other types of synaptic interactions. The distinct vesicle clustering and membrane specializations present in the other synaptic contacts were absent in axoaxonic contacts. Nevertheless, there did exist examples of adjacent axomal boutons whose membranes and vesicle polarization gave indications of some type of contact specialization. These presumed axoaxonic synapses were found almost exclusively in the dorsolateral PAG where they make up 4% of the total number of synapses. This should be considered an upper limit

on the percentage total of axoaxonic contacts in the caudal PAG.

Immunocytochemistry

ENK-containing perikarya and dendrites

As reported previously (Moss et al., 1983b) there is no consistent cell morphology or orientation with which to define ENK-containing neurons in the PAG. ENK perikarya are bipolar or multipolar and round, fusiform or triangularly shaped.

The reaction product in both ENK-containing perikarya and dendrites appears as dense, membrane bound organelles. Whether this is a normal storage organelle for the ENK antigen or a result of the colchicine pretreatment is not known. Labelling was not seen in dendrites or neuronal cell bodies unless colchicine had been administered.

ENK-containing dendrites are found postsynaptic to round, and flattened vesicle-containing axonal profiles. Occasionally, a profile classified as an unknown type in the normal material or one containing dense core vesicles can also be found presynaptic to an ENK-labelled dendrite. Although ENK-labelled perikarya are surrounded by the same types of profiles, there are fewer synaptic contacts upon cell bodies and primary dendrites.

ENK-containing axonal boutons

ENK-labelled axonal boutons in the ventral caudal PAG contain small, clear, round vesicles. Approximately half of the boutons also contain several large dense-core vesicles. The immunoreactivity is associated with the outer surface of the small vesicles, the inside of the large vesicles and the outer membranes of mitochondria. Often the reaction product was restricted to one or more regions of the

axon profile.

The quality of the PAG tissue processed for immunocytochemistry did not permit a random sampling of any area studied, or a consistent choice of particular regions to examine within a trimmed block. It was also difficult to follow labeled profiles for more than 3 or 4 serial sections. Instead, the criteria that determined the tissue area sampled was that region which exhibited the best antibody labelling and quality of tissue preservation. Thus, the immunocytochemical study is restricted to limited areas, usually very close to the surface of individual blocks. This introduces problems in any attempt at a quantitative analysis of the synaptic interaction of ENK-containing profiles. In the event that there is preferential preservation of certain labelled neuropil elements after the tissue is processed for EM immunocytochemistry, a bias would be introduced in the sampling procedure. Therefore, only qualitative descriptions of the different types of ENK interactions can be made.

Consistent with the predominence of axodendritic synaptic contacts in the caudal PAG, the great majority of ENK-labelled axon profiles which synapsed were found presynaptic to unlabelled dendrites. The remainder are synaptically associated with either unlabelled neuronal perikarya or occasionally with other vesicle containing profiles. ENK boutons form both symmetric and asymmetric synaptic densities with their postsynaptic elements.

Immunoreactive ENK profiles are presynaptic to all sizes of dendrites; axo-dendritic contacts with small dendrites predominate. In a single plane of section, an ENK terminal may be the only source of input to the post-synaptic dendrite. Often a central unlabelled

dendrite receives synaptic contacts from both labelled and unlabelled profiles. The unlabelled axonal boutons can contain either round or flattened synaptic vesicles. On several occasions a central dendrite received input from more than one ENK-containing bouton. Whether these arise from separate neurons or are branches of a single ENK-labelled axon was not determined.

While ENK-labelled profiles are seen adjacent to perikarya, axosomatic synapses, as in the normal material, are infrequent. In addition, although very rare, ENK-containing boutons are found presynaptic to vesicle-containing profiles.

DISCUSSION

Normal Fine Structure

The major aim of this study was to provide information about the circuitry within the caudal PAG through which opiates exert their analgesic action. While our focus was on enkephalin circuitry, the quantitative analysis of the normal fine structure provided a useful framework within which to relate the information about the immunoreactive ENK-labelled elements. It also revealed several important charateristics of PAG synaptology that bears on a possible "pain control" circuits.

All three regions of the caudal PAG examined in this study have a similiar synaptic morphology. Symmetric synapses with flattened vesicles are more common than asymmetric, round vesicle synapses. Although there is a tendency for the presynaptic axonal boutons in symmetric synapses to contain flattened synaptic vesicles, the consistent relationship of flat vesicles/symmetric densities, round vesicles/asymmetric densities found in other CNS regions such as the

ventrobasal thalamus (Ralston and Hermann, 1969), visual cortex (Colonnier, 1968) an spinal cord (Ralston, 1968) is not found in any of the caudal PAG regions.

In all three regions of the caudal PAG axodendritic synapses are the predominent form of synaptic interaction, making up from 93-97% of all synapses sampled. These percentages are comparable to other regions of the CNS such as the spinal cord dorsal horn of the rat, cat and monkey (Zhu et. al., 1981; Duncan and Morales, 1978; Ralston, 1979) the cat ventrobasal thalamus (Ralston & Hermann, 1969) and the nucleus tractus solitarius of the cat (Leslie et al 1982).

The axodendritic synaptic contacts in the PAG consist of central postsynaptic dendrites surrounded by one, two, or more axonal boutons. In all three regions of the caudal PAG the great majority of postsynaptic dendrites are contacted by only one or two inputs in the single plane of section examined.

The majority of axonal profiles in the caudal ventromedial and dorsolateral PAG contact small dendrites. However in the ventrolateral PAG, almost equal numbers of axonal boutons end on small and medium sized dendrites; there is no preferential input onto the smaller, more distal dendrites.

Counted within the category of small dendrites is a subset of very small dendrites, less than 0.7um in diameter. Found with equal frequency in all three regions of the caudal PAG, they are probably derived from dendritic spine processes. Dendritic spines have been noted on Golgi stained PAG neurons from the PAG regions examined in this study (Liu and Hamilton, 1980; Mantyh, 1981; Lamle, 1979). These spine-like dendrites occasionally contain synaptic vesicles, as

well as multivesicular bodies and mitochondria. Their morphology is similar to that of the presynaptic dendritic spines of the islet cells of the spinal cord dorsal horn and nucleus caudalis of the medulla (Duncan and Morales, 1978; Ralston, 1979; Gobel, 1974; Gobel et. al., 1980).

The three caudal PAG regions do exhibit some differences in the relative frequencies with which axosomatic, axoaxonic and dendrodendritic synapses are seen. Axosomatic synapses are not seen in the ventrolateral PAG but make up 2-3% of the total number of synapses in the ventromedial and dorsolateral PAG. Axoaxonic synapses make up 4% of synaptic interactions in the dorsolateral PAG, but are rarely seen, in the ventral PAG. Also rare are presynaptic dendrites, found only in the ventrolateral PAG. The postsynaptic elements is always a dendrite; dendrodendritic synapses making up 1% of the total number of synapses in the ventrolateral PAG. Thus the caudal PAG joins the growing list of CNS regions, including the spinal cord, thalamus, lateral geniculate, medial geniculate, superior colliculus, retina, olfactory bulb etc. (see Ralston, 1971 for review) that have been shown to contain presynaptic dendrites.

There are several problems inherent in the quantitative analysis used to determine the percentage totals of the various synaptic interactions listed in Table I. Making up 1 to 3% of the total population of synapses in the caudal PAG are synapses in which the presynaptic element can not be classified as either a dendrite or an axon in the single plane of section examined. In these synapses, the postsynaptic element was always a dendrite. Correct identification of the presynaptic profile as an axon or a dendrite would thus affect

the total reginal percentages given for axodendritic and/or dendrodentritic synapses. This would have an especially noticeable effect on the percentage totals for the ventromedial PAG where this type of synaptic contact makes up 3% of the total number of synapses.

Besides the problem of the unclassified synaptic interactions, another possible source of inaccuracy in the Table I percentage totals might be introduced by the actual morphology of certain synaptic contacts. For example, if the synaptic junction made by certain types of synaptic associations takes place over only a very small portion of the surface of the pre and postsynaptic elements, a synaptic contact could be missed in analyses done from only a single plane of section (Groves, 1980). Immunoreactive ENK-containing synapses extending less than 0.1 um have been reported in the dorsal horn (Glazer and Basbaum). This problem can be resolved only by serial section analysis.

It is also possible that the percentages of the different types of synaptic morphologies and interactions given in Table I would differ if a greater number of synapses had been counted in each of the three PAG regions. For these reasons, the percentage totals of each of the different categories in Table I should not be regarded as exact numbers. Nevertheless they present a clear indication of the relative frequency of the different synaptic morphologies and interactions in the caudal ventromedial, ventrolateral and dorsolateral PAG.

Immunoreactive ENK-containing circuitry

Immunoreactive-ENK labelled terminals in the PAG have features

in common with ENK-terminals found elsewhere in the CNS including the spinal cord (Hunt et. al., 1980; Glazer & Basbaum), locus coeruleus (Pickel et. al., 1979) and neostriatum (Pickel et. al., 1980). The synaptic densities are symmetric or asymmetric, however small round synaptic vesicles, are seen consistently. Dense-core vesicles are seen in only a small proportion of axonal boutons in the caudal PAG. These vesicles have been associated with peptide-containing and biogenic amine-contaning terminals in other CNS regions (Pickel et al 1977, Bloom, 1973). Approximately half of the immunoreactive ENK-labelled boutons in the caudal ventral PAG contain dense-core vesicles. The presence or absence of dense vesicles does not apear to be associated either with a particular type of synaptic interaction or articular region, such as is indicated in the ENK-labeled terminals in the superficial dorsal horn (Glazer and Basbaum).

ENK boutons in the caudal PAG, like those in the cord (Glazer and Basbaum) and neostriatum (Pickel et. al., 1980), and most commonly form axodendritic synapses; they are much less frequently found presynaptic to cell bodies or other vesicle containing profiles. Not only do ENK-labelled boutons in the PAG resemble immunoreactive ENK terminals described in other CNS regions, but the synaptic morphology and the relative frequencies of the various kinds of ENK synaptic interactions agrees well with the quantitative study of the normal PAG neuropil. This is true in spite of the more inconsistent sampling procedure inherent in the immunocytochemical study of ENK-labelled profiles.

What can be hypothesized about the midbrain microcircuitry

underlying opiate induced analgesia, given the information obtained from the normal fine structure? Previous studies have indicated that activation of a PAG output neuron is necessary to generate analgesia. Excitation of PAG neurons by focal electrical stimulation (Reynolds, 1969; Mayer et. al., 1971; Mayer and Price, 1976; Lewis and Gebhart, 1977; Gebhart and Toleikas, 1978; Oliveras et. al., 1974; 1979; Yeung et. al., 1977) or injections of glutamate (Bebehani and Fields, 1979) produces potent analgesia. However, neither lesions of the PAG (Dostrovsky and Deakin, 1977) nor injection of local anesthetic into the PAG (Mayer and Price, 1976; Lewis and Gebhart, 1977; Gebhart and Toleikas, 1978) suppresses pain. In addition, enkephalin has been shown to have a hyperpolarizing effect on postsynaptic elements; its excitatory affect in various neural systems is produced by blocking inhibitory pathways (Pepper and Henderson, 1980; Nicoll et. al., 1980). Assuming that enkephalin exerts an inhibitory action in the PAG, it follows that enkephalin activates the PAG-endogenous pain suppression system by disinhibitory circuitry within the midbrain central grey.

Evidence from this study indicates that in the caudal ventral PAG, 95-97% of the synapses are axodendritic. In our immunocytochemical studies, the largest population of ENK-containing axonal boutons are presynaptic to dendrites. Given this information, it is highly probable that the analgesia produced by opiate action in the caudal, ventral PAG is caused by the disinhibition of intrinsic PAG inhibitory neurons through axodendritic synapses.

A second, far less common opiate circuit exists. Although very rare, several examples of immunoreactive ENK labelled axonal profiles

have been found presynaptic to vesicle-containing profiles. While over 98% of vesicle-containing profiles in the caudal ventral PAG can be identified as axonal boutons, less than 0.5% of the synapses are axoaxonic. The remainder of the vesicle-containing profiles can either be identified as dendrites or cannot be classified (approximately 2% of the total number of vesicle containing profiles). Therefore, although it is possible that opiates are also involved presynaptically in axoaxonic synapses, it is more probable that the unlabelled postsynaptic vesicle-containing profiles are presynaptic dendrites under opioid control.

The source(s) of the proposed tonic inhibition to analgesia generating PAG output neurons that might be "shut off" by opiates, is not known, however a catecholamine involvement has been postulated (see Basbaum, Moss and Glazer for review). It would be of interest to determine whether opiate receptors are located on norepinephrine containing axons in the PAG as they are, for example, in the cortex (Lorens et. al., 1978). This would implicate an opiate presynaptic control of norepinephrine inputs to the PAG. Given the rarity of axoaxonic synapses in the ventral PAG, this type of interaction is unlikely. However, a low number of axoaxonic synases does not prelude a major functional contributions.

It must be emphasized that the origins of the immunoreactive ENK-labelled boutons could not be determined in this study, and could be the axonal processes from either PAG local circuit ENK neurons or distant ENK projection neurons. The close overlap in the distribution of ENK-containing neurons and terminal fields in the caudal ventral PAG would seem to suggest the former possibility.

In conclusion, this study provided the first information on of the synaptic interactions within the caudal periaqueductal grey of the cat. Our analysis of the morphological types and the relative numbers of the various kinds of synaptic contacts in the normal caudal PAG provided a framework within which to relate the observations about immunoreactive ENK microcircuitry. From these data it is clear that the most common site of opiate action in the caudal PAG is as convergent input onto the postsynaptic dendrites of intrinsic PAG analgesia producing neurons. Most probably, this circuitry reflects the postsynaptic inhibition, by enkephalin, of inhibitory interneurons that act upon the PAG analgesia producing output neurons. Much less frequently, there are indications of presynaptic dendrites, or possibly axonal boutons, under opioid modulation. Whether either or both of these circuits are of equal functional weight, or even whether these synaptic contacts are analgesia relevant interactions cannot be determined from this study. Future studies will focus on determining the presynaptic inputs modulating the PAG opioid neurons and identifying the postsynaptic elements under ENK control.

ACKNOWLEDGEMENTS

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Table 1 Classification of synaptic morphology and interactions in three different regions of the PAG. Results have been rounded off to the nearest percent, consequently total sums may differ from 100%

TABLE I: SYNAPTIC MORPHOLOGY AND INTERACTIONS IN THE CAUDAL PAG

PAG REGIONS	VENTROMEDIAL	VENTROLATERAL	DORSOLATERAL
SYNAPSE MORPHOLOGY:			
1. Symmetric	39	35	37
a) round vesicles	10	10	13
b) flattened vesicles	29	25	24
2. Asymmetric	29	28	29
a) round	16	16	16
b) flattened	13	12	13
3. Oblique	33	27	33
a) round	13	15	19
a) flattened	20	22	14
SYNAPTIC INTERACTIONS:			
1. Presynaptic element unclassified	3	1	1
2. Dendrodendritic	0	1	0
3. Axosomatic	3	0	2
4. Axoaxonic	$<.5$	0	4
5. Axodendritic	95	97	93
a) X-section dendrites	63	55	59
i) 1 input	35	27	34
small dendrite	28	17	24
medium dendrite	7	10	10
ii) 2 inputs	20	17	18
small dendrite	10	7	11
medium dendrite	10	10	7
iii) >2 inputs	8	11	7
small dendrite	4	4	$<.5$
medium dendrite	4	7	7
b) Sagittal dendrites	37	44	40
2 inputs	26	28	31
ii) $>$ 2 inputs	11	16	9

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FIGURE LEGENDS

Fig. 1 Diagram of PAG regions examined in this study: a) ventromedial, b) ventrolateral, c) dorsolateral PAG. Quantitative study of the normal PAG fine structure compared regions a, b and c. Immunocytochemical study of immunoreactive ENK-labelled profiles encompassed the combined area of a and b.

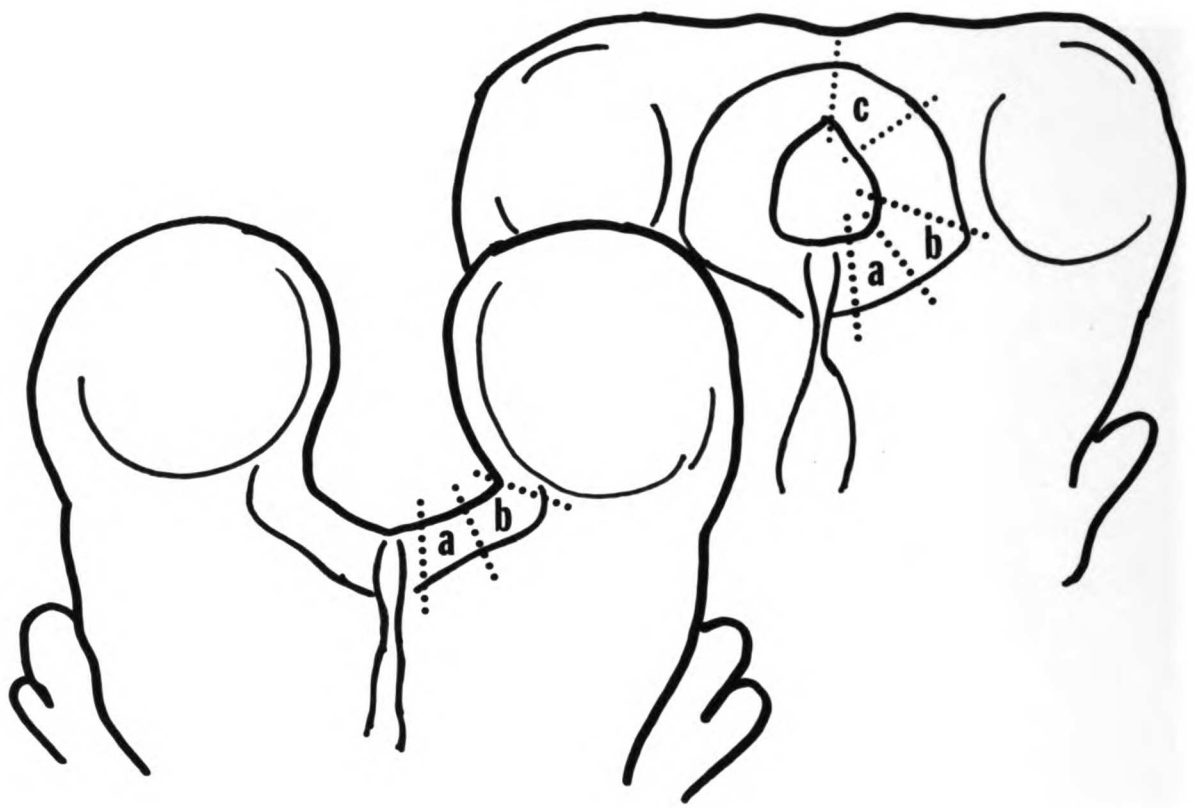
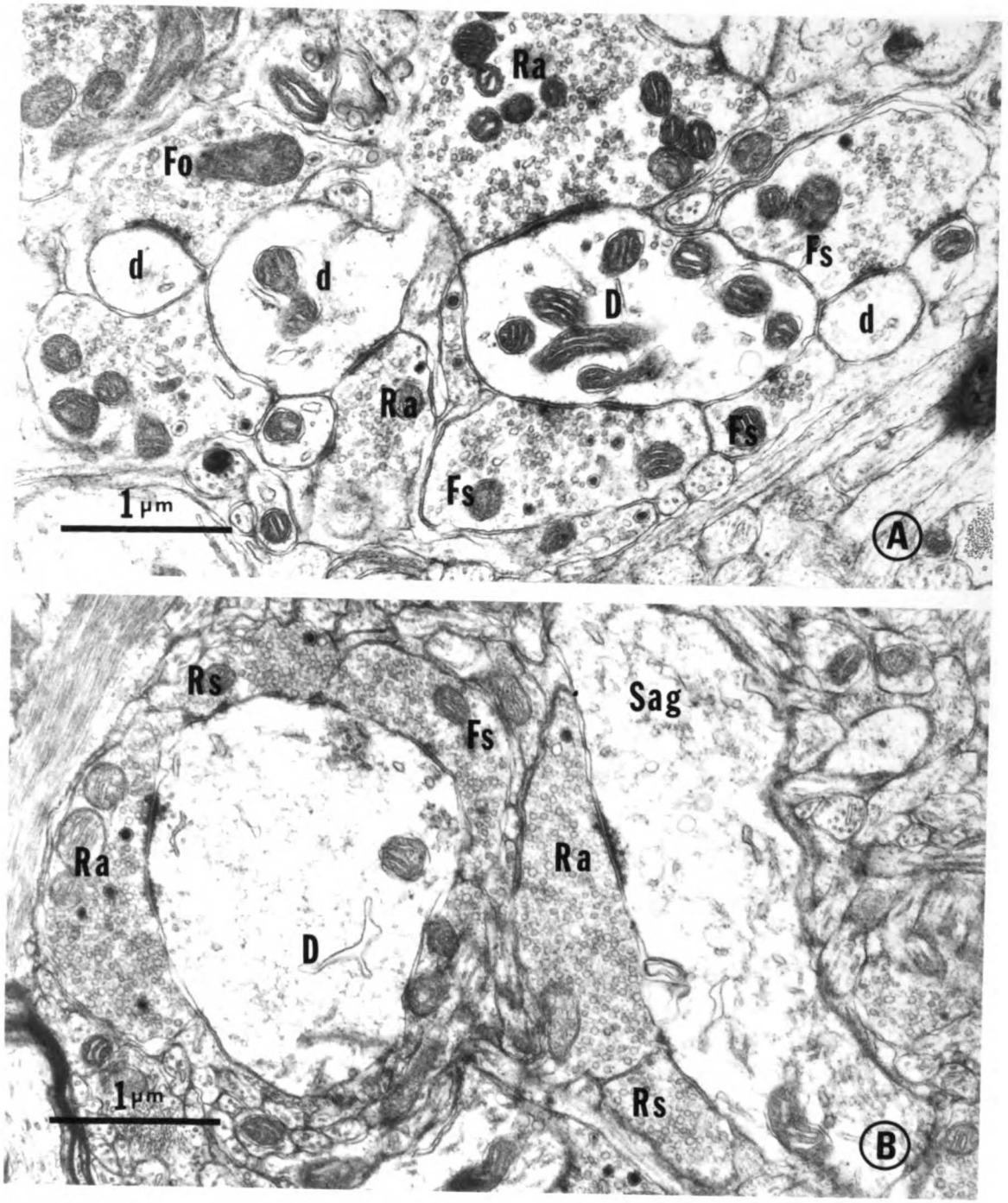

c
b
a
a
b

Fig. 2 Convergent axonal input onto postsynaptic central dendrites. Note the differing synaptic morphologies of the surrounding boutons including the presence of dense-core vesicles. A) ventromedial PAG, B) dorsolateral PAG. R = round vesicles, F = flattened vesicles, s = symmetric density, a = asymmetric density, D = medium-sized dendrite, d = small dendrite, SAG = sagittal dendrite.


Ra
Fo
d
d
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d
Ra
Fs
Fs
1 µm
A
Rs
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Sag
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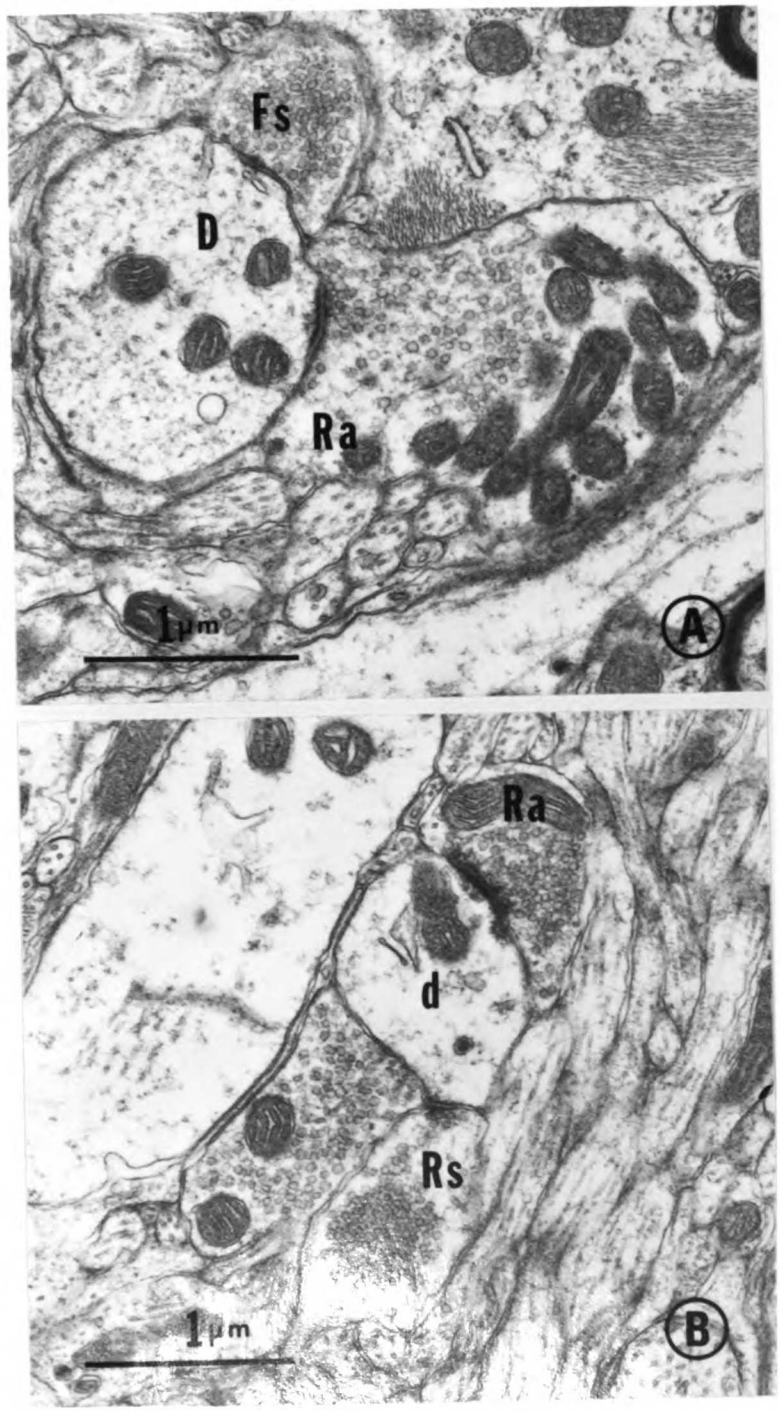
Rs
B

Fig. 3 A) and B)

Convergent axonal input onto postsynaptic central dendrites in the ventrolateral PAG.


Fs
D
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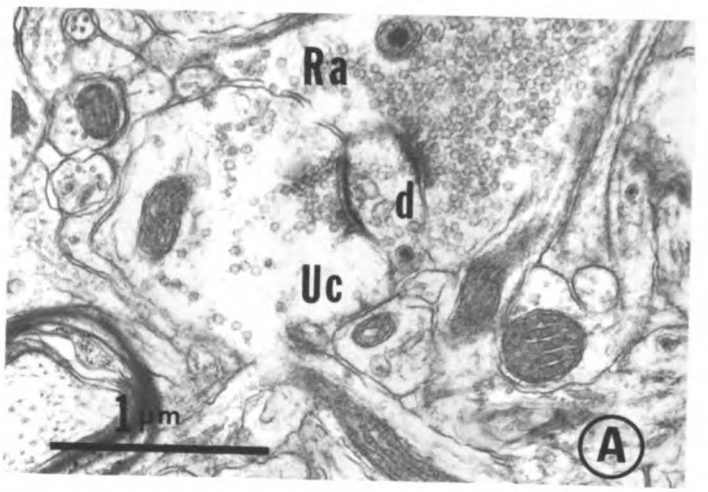
A
Ra
d
Rs
1μm
B

Fig. 4 A) Convergent input onto a spine-like, small dendrite in the ventrolateral PAG. Presynaptic elements include a round vesicle-containing (R) axonal bouton and and unclassified profile (Uc). Both synaptic densities are asymmetric. Note the presence of both clear and dense-core vesicles in the post synaptic dendrite. B) Single axonal input onto postsynaptic dendrite in the ventrolateral PAG and C) ventromedial PAG. o = obliquely cut synaptic density.


Ra
d
Uc
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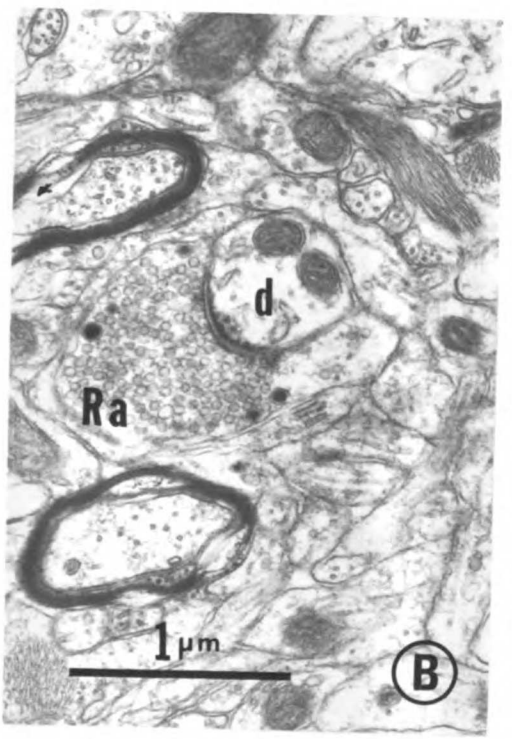
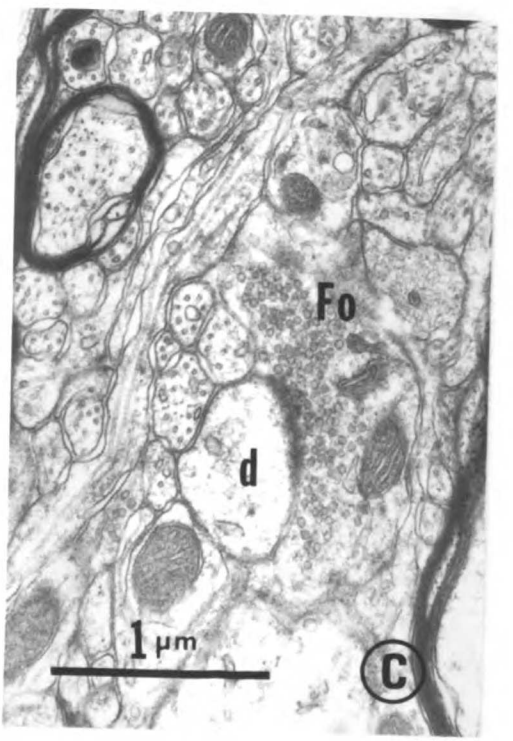
A


d
Ra
1 μm
B


Fo
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C

Fig. 5 Axosomatic synapse in the ventromedial PAG. Ra = round vesicle containing bouton, asymmetric density. Also axodendritic synapses onto small (d) and medium sized (D) dendrites. Note dense-core vesicles. N = nucleus, Fs = flattened vesicles, symmetric density.

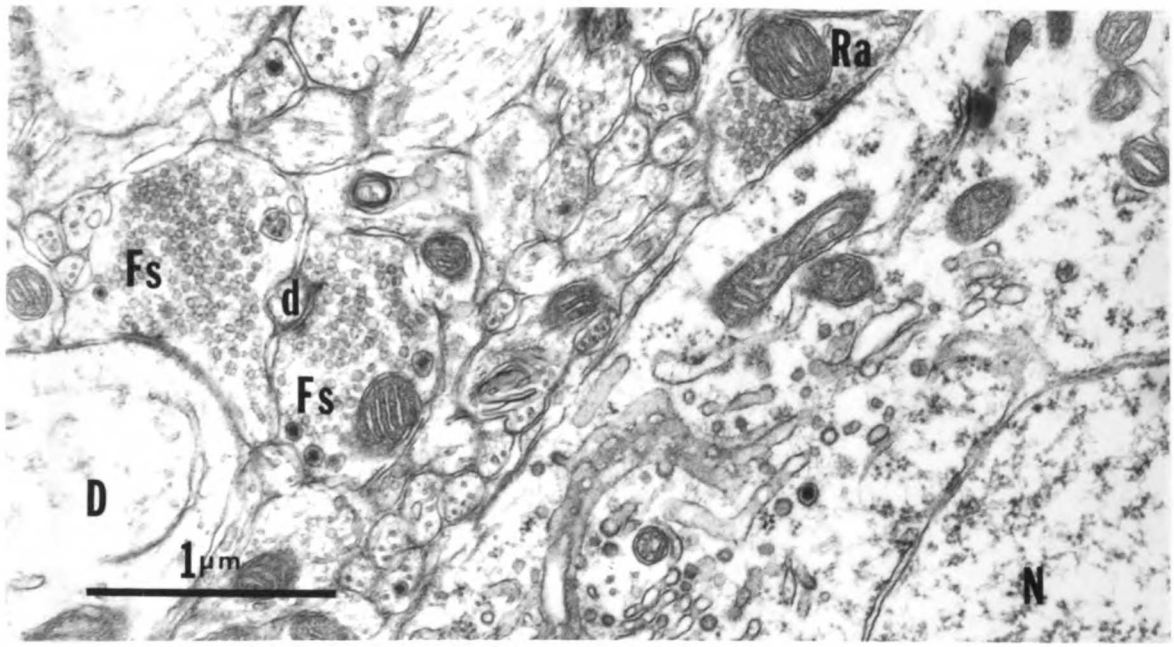
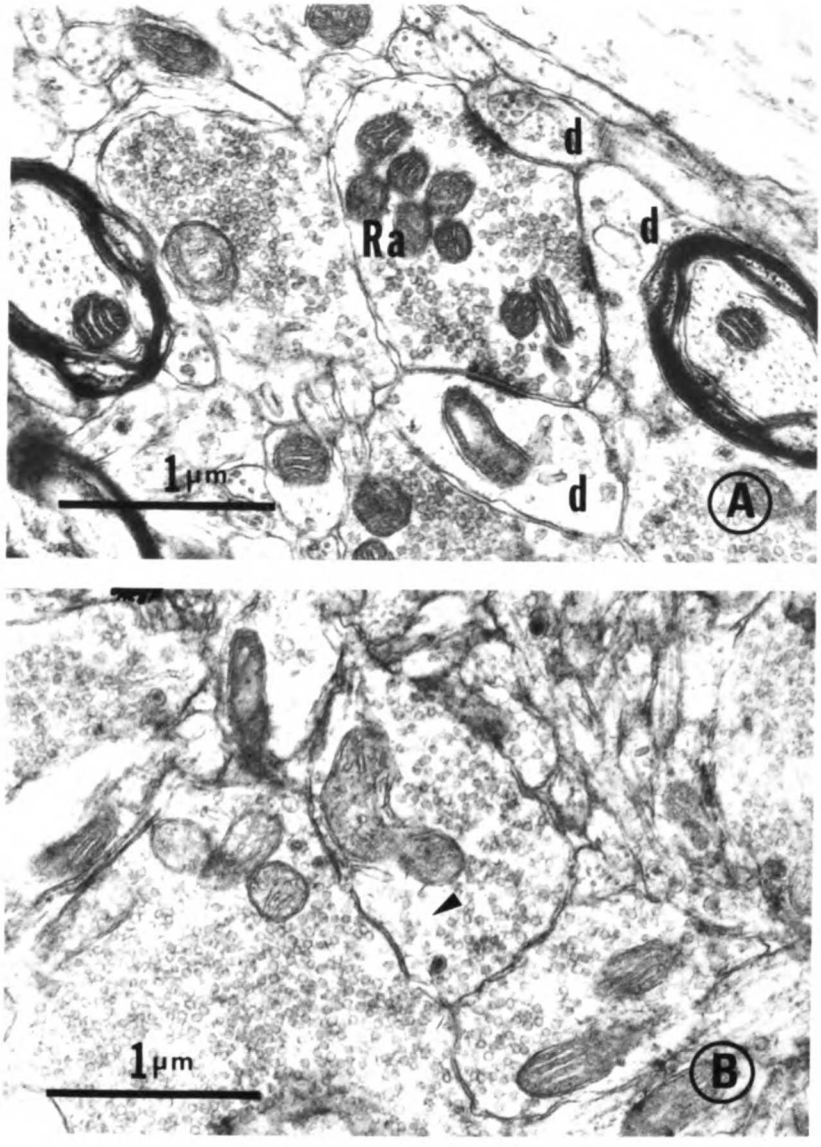

Ra
Fs
d
Fs
D
1μm
N

Fig. 6 A) Central axonal bouton in the ventromedial PAG presynaptic to three small dendrites. Axonal profiles making greater than 1 synaptic contact in a single plane of section are seen infrequently. Note the presence of a multivesicular body in the smallest, spine-like dendrite. B) Possible axoaxonic synapse in the dorsolateral PAG. Arrow indicates presumed presynaptic element. R = round vesicle, A = asymmetric density, d = small dendrite.


Ra
d
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d
1μm
A
1μm
B

Fig. 7 Vesicle-containing dendrites in the caudal ventral PAG. A) Presynaptic dendrite (PSD) forming a dendrodendritic synapse. Note pale cytoplasm, vesicle clumping and size of profile. B) axonal bouton presynaptic to a vesicle-containing profile, presumably a dendrite. Compare this to figure 13 of immunoreactive ENK-labelled material. Fs = flattened vesicles, symmetric density.

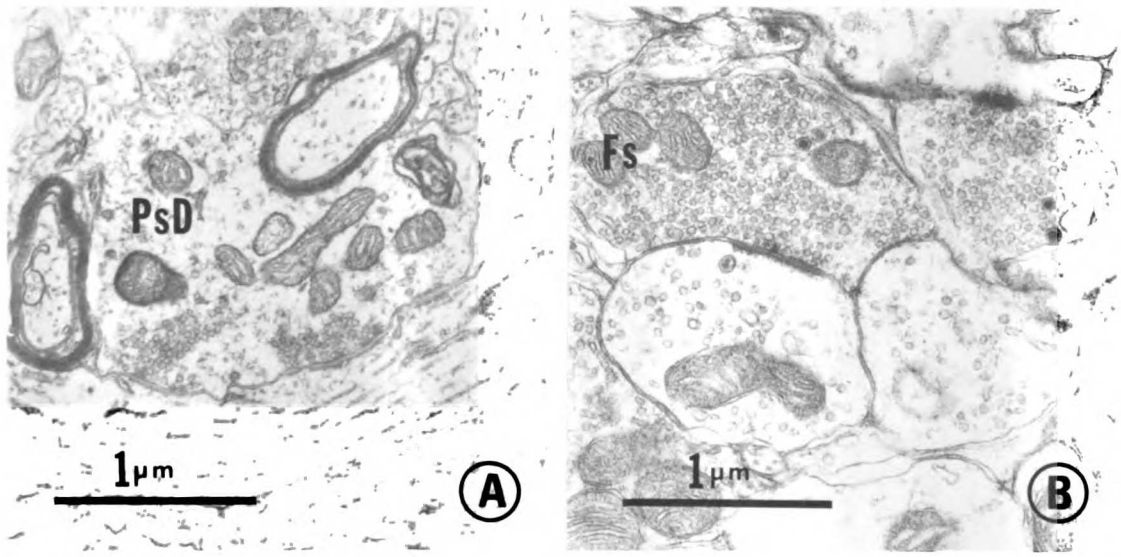

PsD
1μm
A
Fs
1μm
B

Fig. 8 Montage of an immunoreactive ENK-labelled proximal dendrite in the caudal ventral PAG. The dendrite is surrounded by many vesicle-containing profiles, but only a few make synaptic contact (arrows). Insert: higher magnification of synaptic contacts made by an axonal bouton and an unclassified presynaptic element (Uc). Fs = flattened synaptic vesicles, symmetric density. (colchicinized animal).

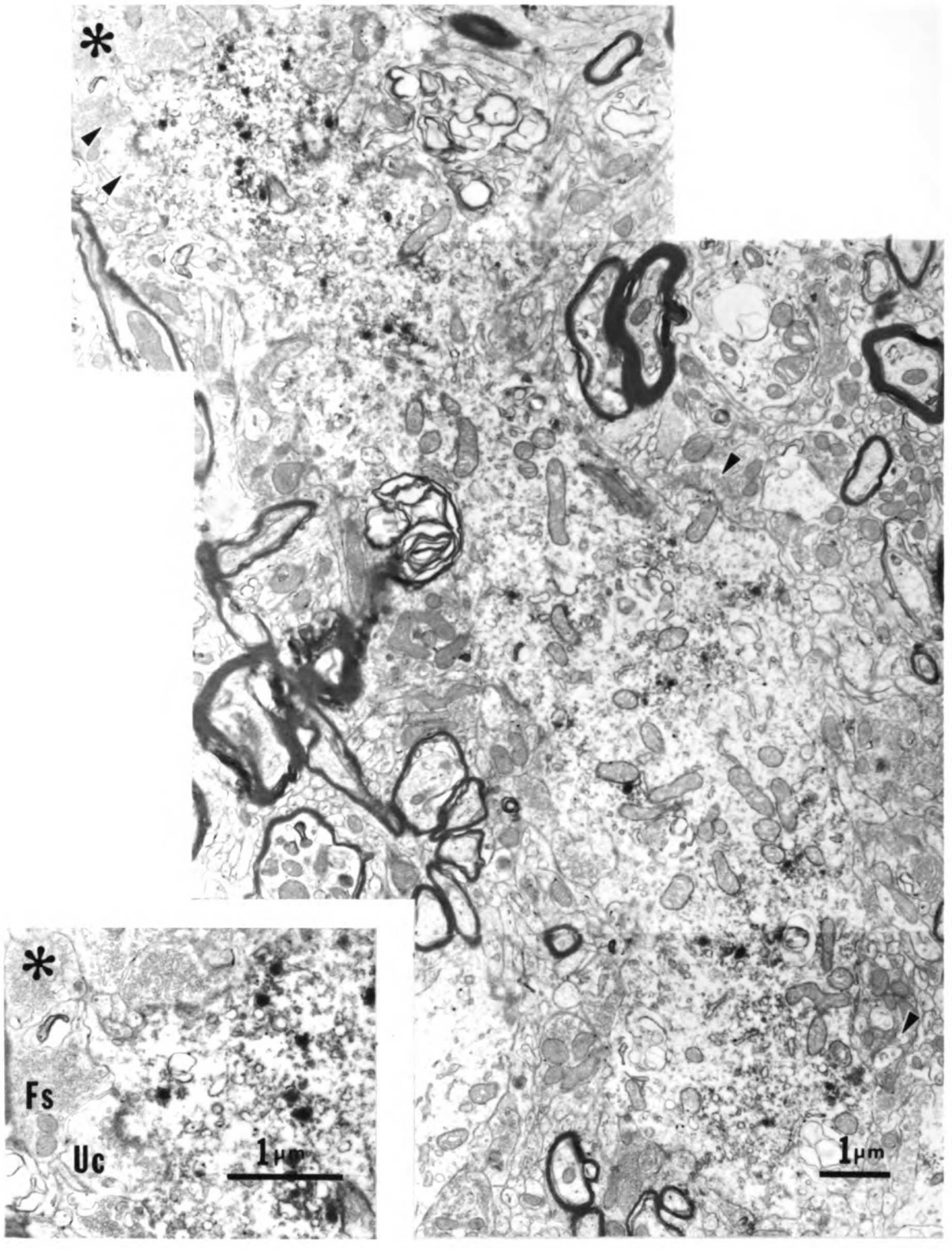

*
1μm
*
Fs
Uc
1μm

Fig. 9 Immunoreactive ENK-labelled central dendrite (D). A) and B) are from a series taken through the same dendrite. Note the numerous synaptic contacts made by convergent axonal boutons of differing synaptic morphology. (colchicinized animal).

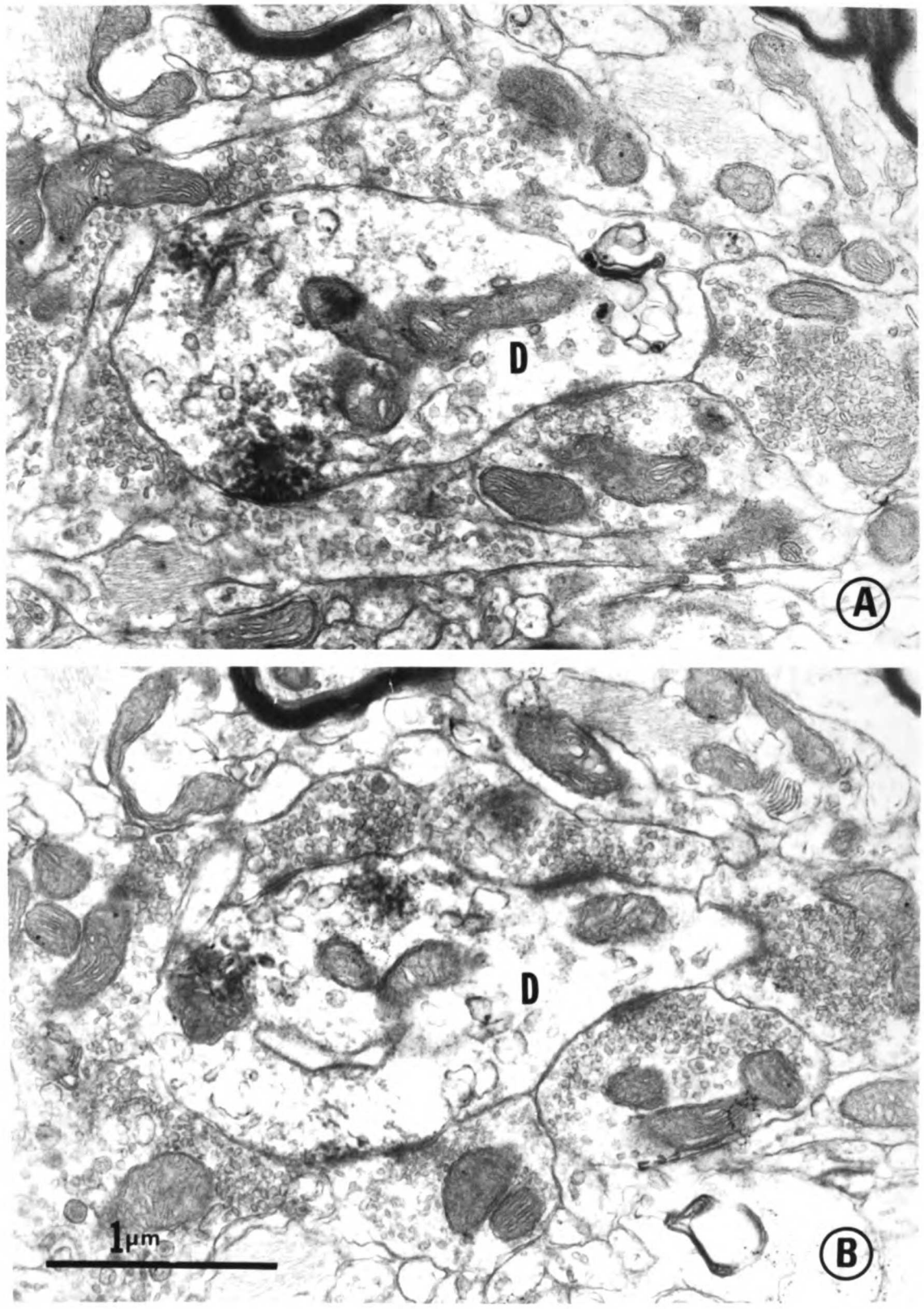
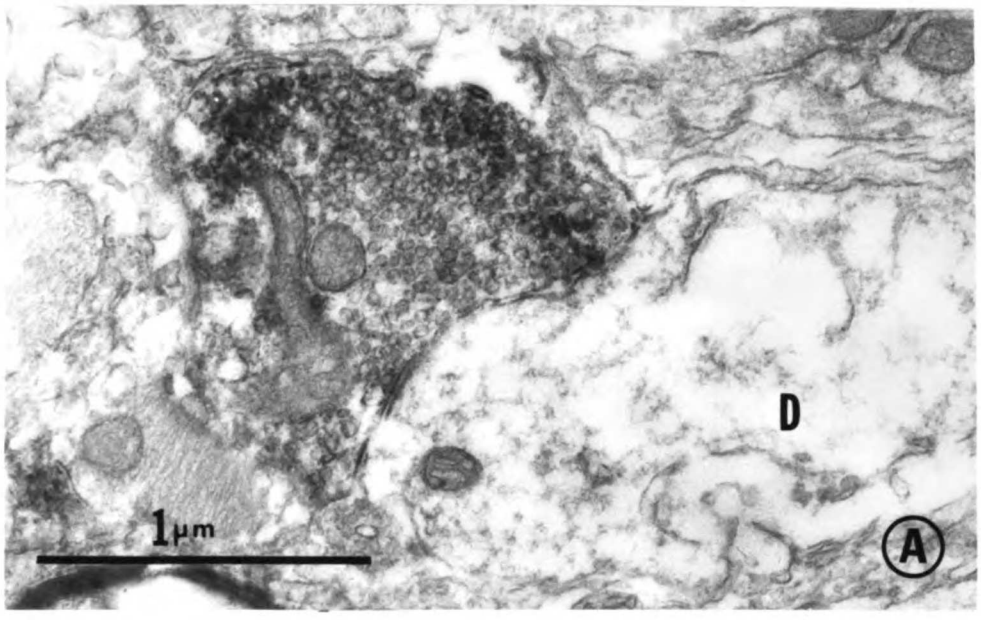

D
A
D
1μm
B

Fig. 10 Synaptic morphology of ENK-labelled boutons presynaptic to unlabelled dendrites. Note the round vesicles and polarization of reaction product. Neither of these terminals contain large dense core vesicles. A) symmetric synaptic density onto a medium dendrite (D). B) asymmetric synaptic density onto a small dendrite (d).


D
1μm
A

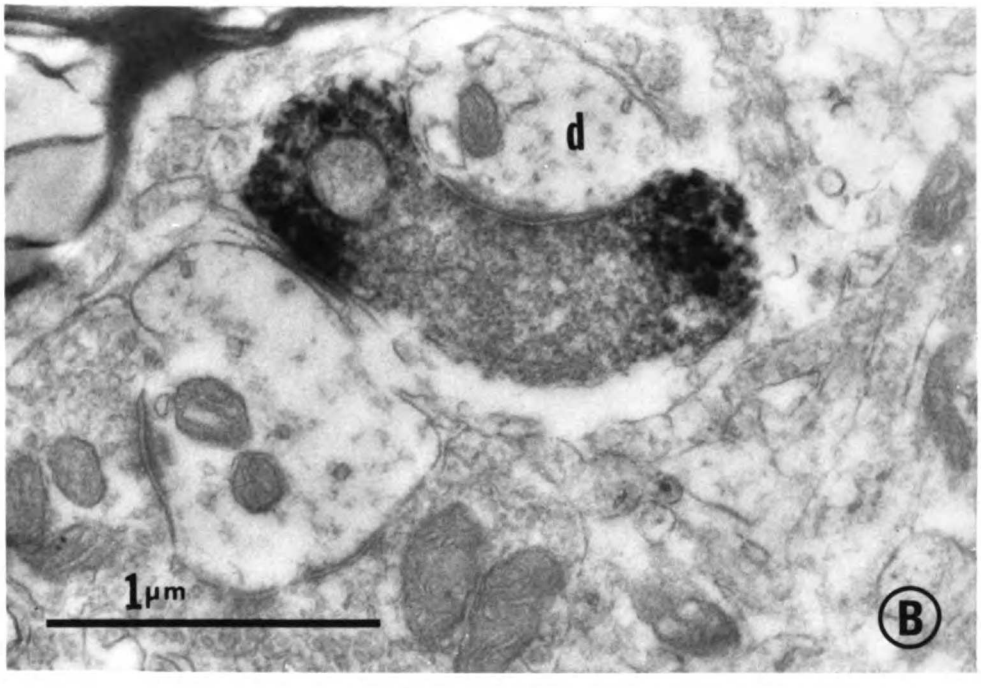

d
1μm
B

Fig. 11 Postsynaptic unlabelled dendrites (d) receiving convergent input from immunoreactive ENK labelled and unlabelled axonal boutons. A) and B) are from a series taken through the same bouton. C) Two ENK containing boutons, possibly branches from the same axon, presynaptic to a small dendrite. D) ENK-labelled and unlabelled input onto a spine-like dendrite. In all the above examples the ENK-labelled axons form symmetric densities. Arrows indicate large dense core vesicles.

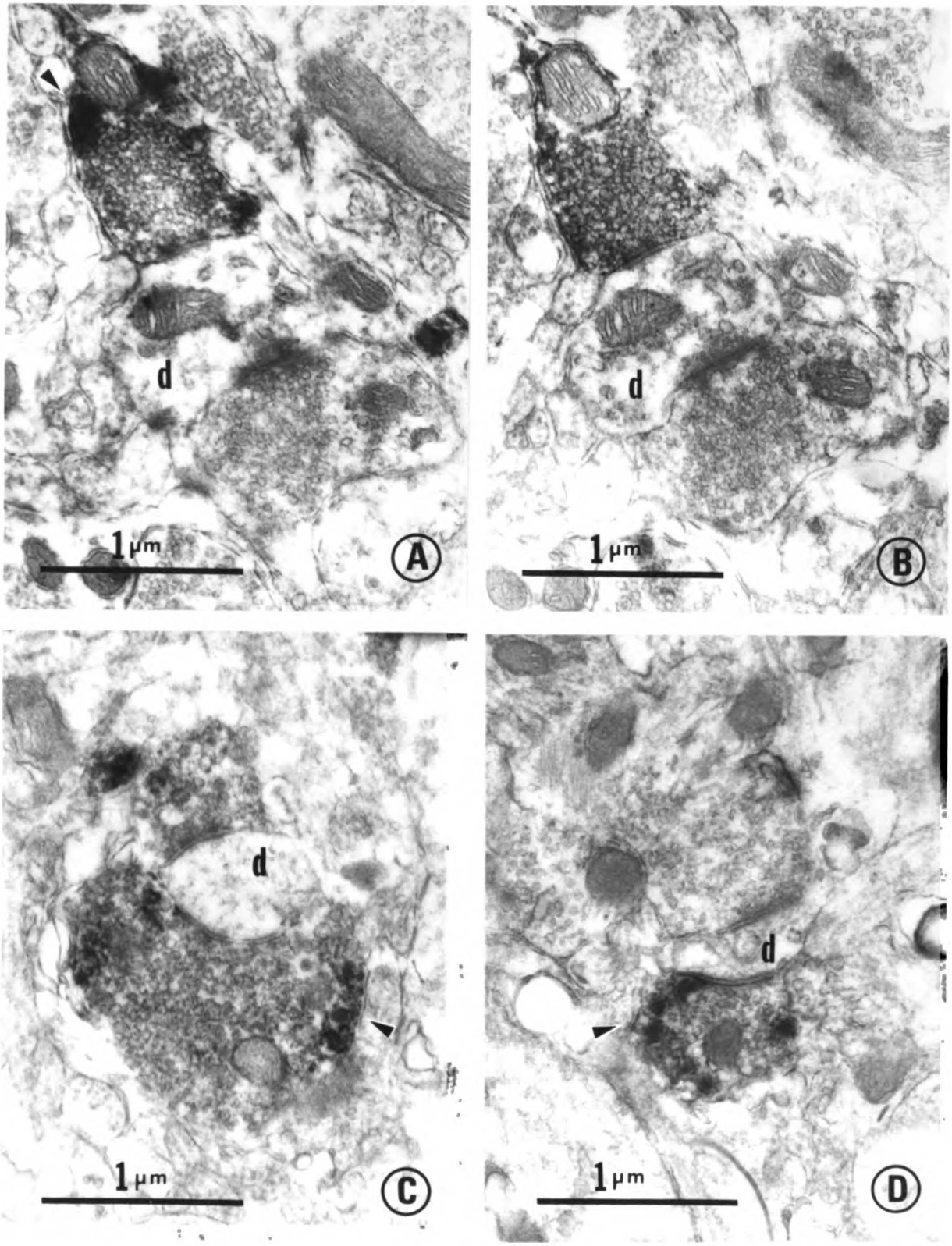

d
1μm
A
d
1μm
B
d
1μm
C
d
1μm
D

Fig. 12 Central unlabelled, medium sized dendrite (D), receiving convergent synaptic inputs from one ENK-labelled and numerous unlabelled axonal boutons. Note the presence of large dense core vesicles (arrows) in the ENK-labelled bouton.

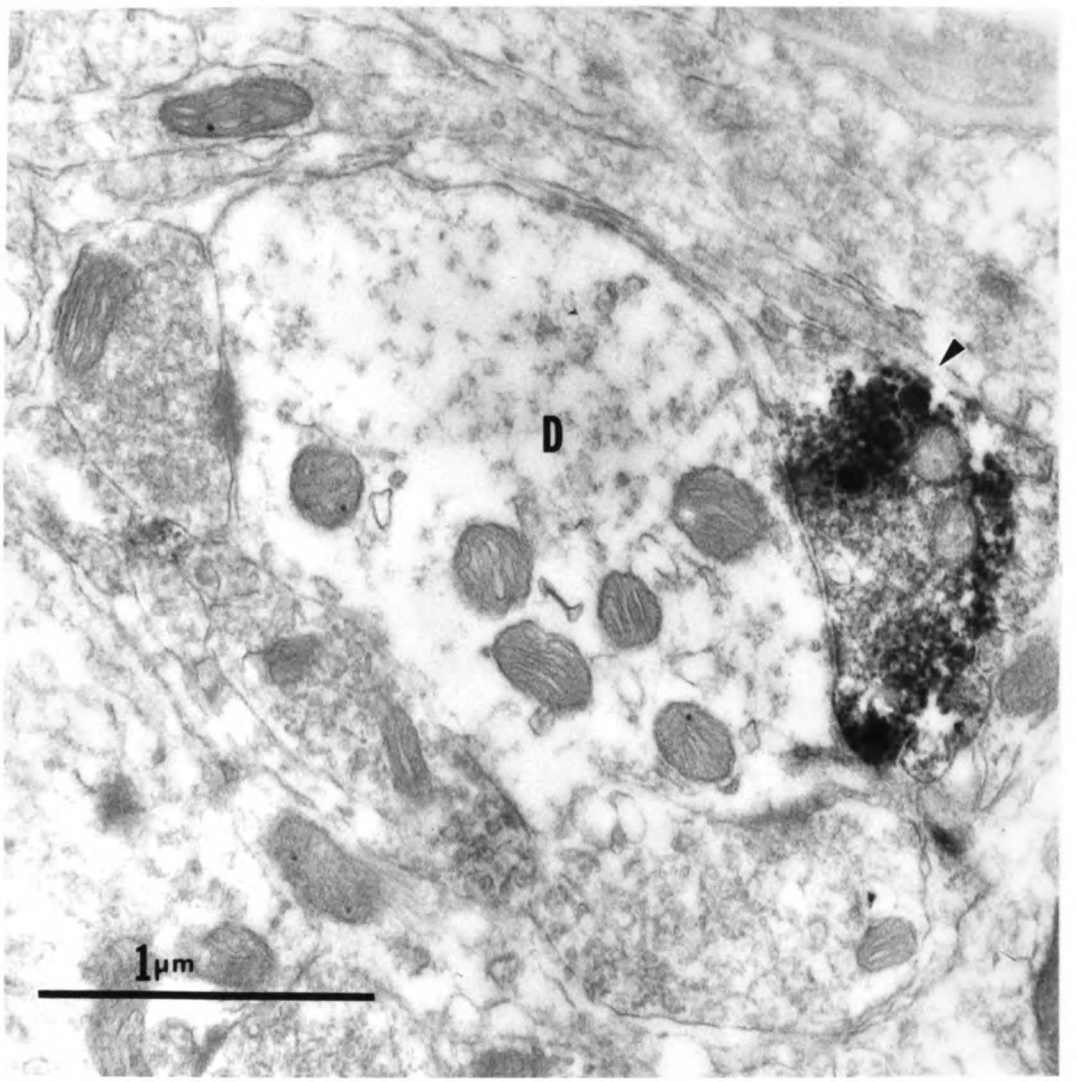
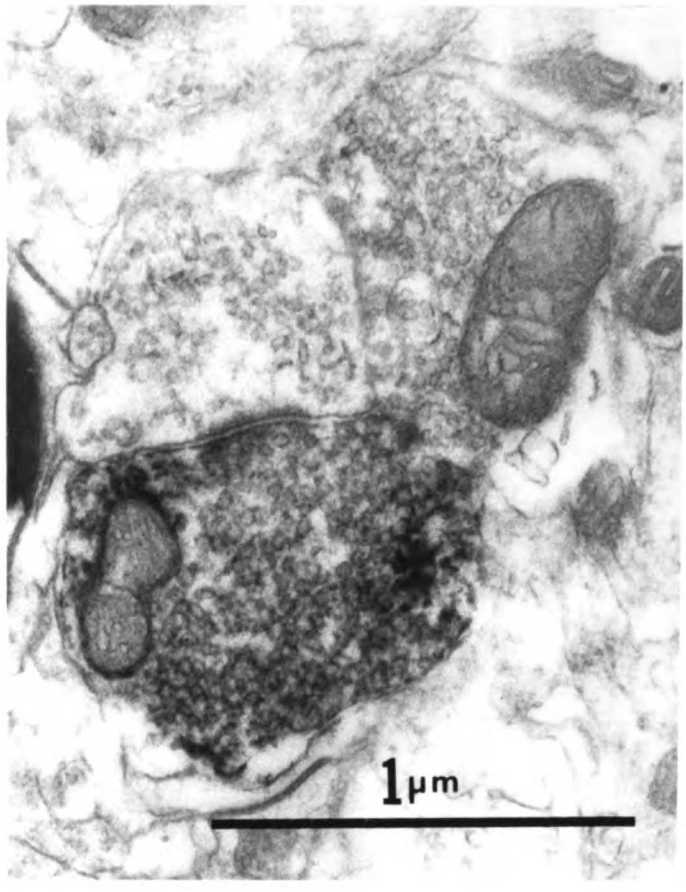

D
1μm

Fig. 13 Immunoreactive ENK-containing axonal profile presynaptic to a vesicle-containing profile. This type of interaction is rarely seen. Compare this synapse with that seen in figure 7 of normal material. See text for discussion.


1μm

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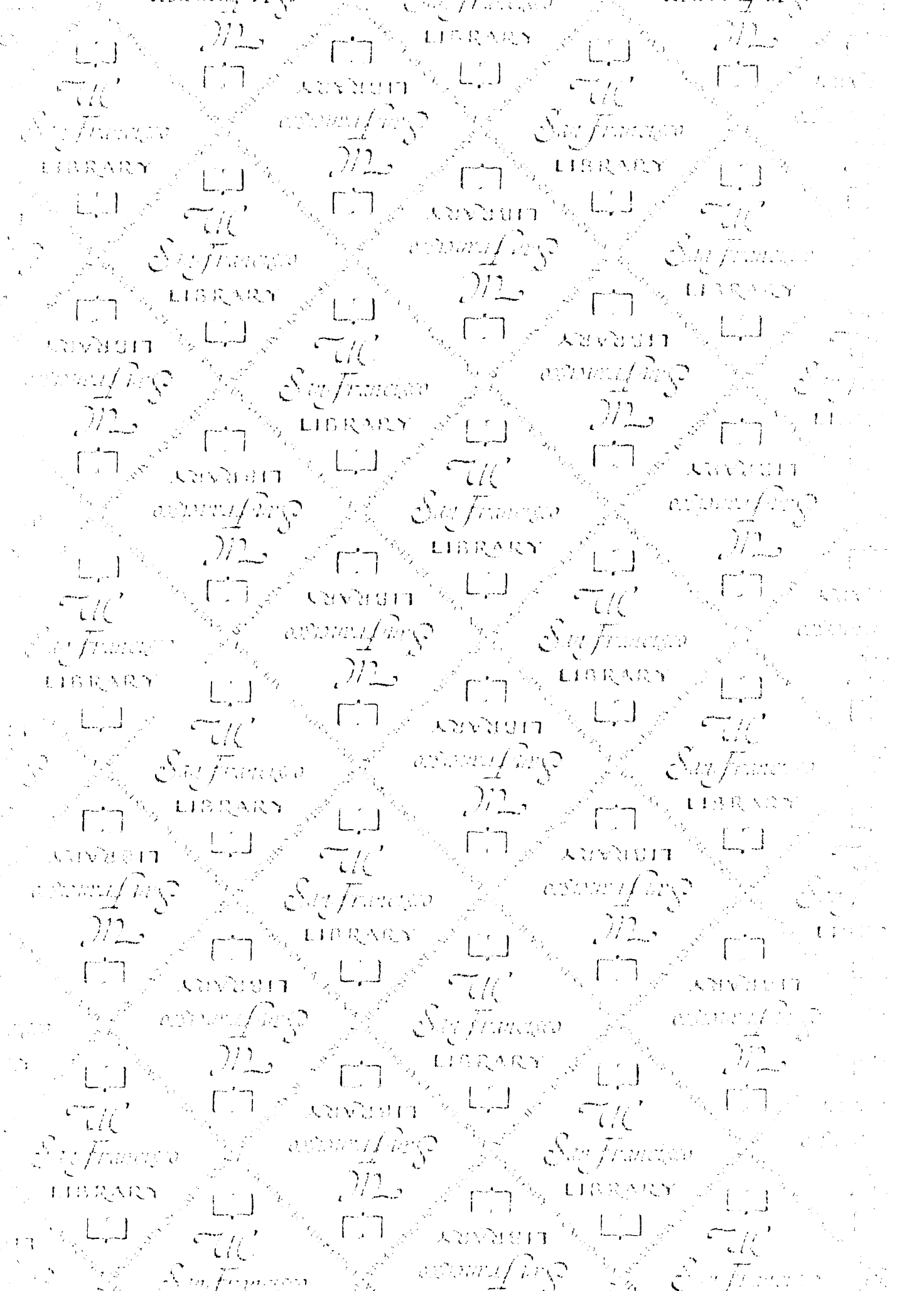
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