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ANATOMICAL STUDIES OF BRAINSTEM-EVOKED ANTINOCICEPTIVE CONTROLS

by

DAVID B. REICHLING

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

NEUROSCIENCE

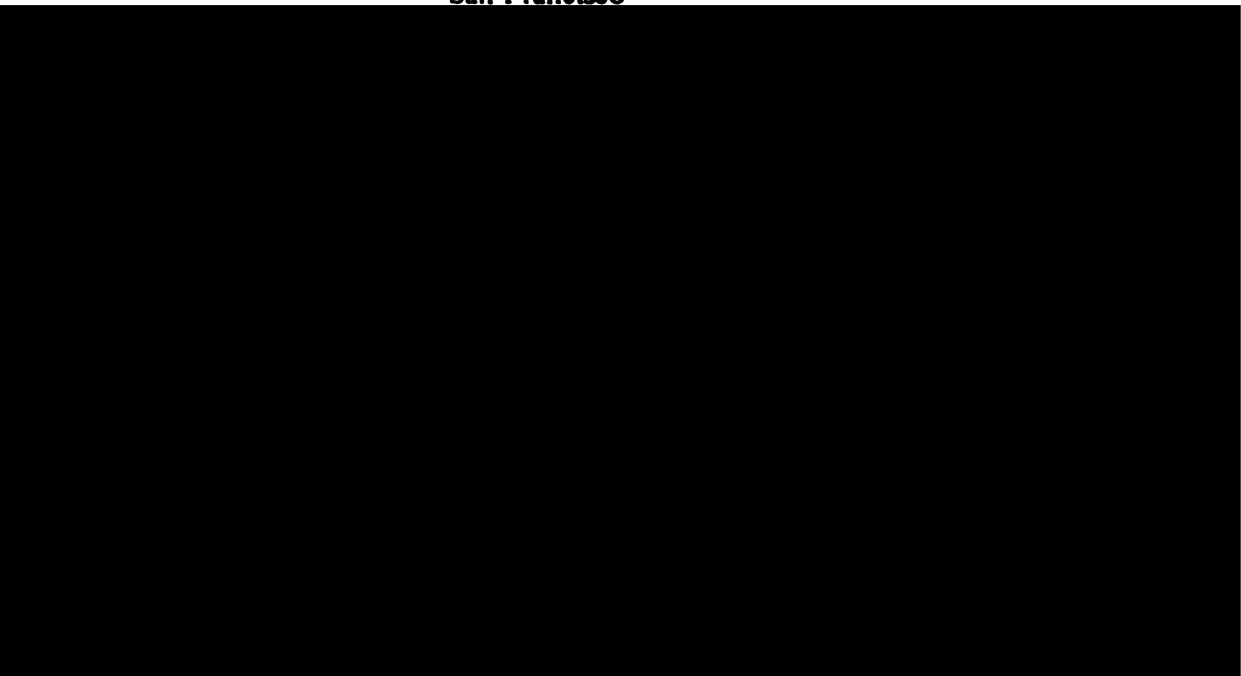
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David B. Reichling

Dedication

This thesis is dedicated to Vandana, for everything; and to my mother, who has contributed to all my work from its very conception.

Acknowledgements

Dr. Allan Basbaum has been my advisor and teacher, generously sharing with me his knowledge, experience, enthusiasm, wit, and wine. It is my privilege to say that my association with Allan will probably always be the single greatest influence on my career in science, and I sincerely thank him for the experience. I would also like to thank all the past and present members of the Pain Research Group at UCSF, who, under the leadership of Allan Basbaum, Howard Fields, Peter Ralston, and Jon Levine, have created an exciting research atmosphere, full of ideas. Thanks, also, to Peter Ohara and Mary Heinricher for long discussions about science and camping, and also for warning me. Finally, I would like to thank Allison Gannon, Bonnie Lord, and Margaret Mayes for their technical, and friendly, assistance.

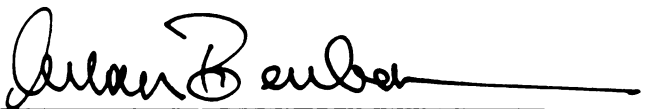
Anatomical Studies of Brainstem-Evoked Antinociceptive Controls

David B. Reichling

ABSTRACT

Although the midbrain periaqueductal gray matter (PAG) is a focal point of analgesia research, its internal circuitry has not been characterized. This research project used anatomical techniques to study the neuronal circuitry that mediates antinociceptive effects elicited from the PAG. Since it has been suggested that tonically active GABAergic interneurons are interposed between opioid elements in the PAG and the neurons that project to the medullary nucleus raphe magnus (NRM), a series of experiments were performed to characterize the relationship between GABAergic neuronal elements and PAG-NRM projection neurons. First, the distributions of relevant transmitters (GABA, β -endorphin, enkephalin, and dynorphin) were mapped by light-microscopic immunocytochemistry. These maps showed that the transmitters were confined to distinct regions of the PAG and that the predicted GABA/opioid interactions were likely to occur in the ventrolateral PAG. Second, combined electron-microscopic immunocytochemistry and retrograde tracing established the neural substrate for a GABAergic control. Approximately 50% of synaptic contacts onto PAG neurons retrogradely labeled from the NRM were GABA-immunoreactive. A related electron-microscopic immunocytochemistry double-labeling study demonstrated that serotonin-immunoreactive neurons in the dorsal raphe nucleus also receive GABA-immunoreactive synaptic contacts. Third, it was determined that although a significant proportion of projection neurons in the medulla are GABA-immunoreactive, few GABA-immunoreactive neurons in the PAG project to the NRM. Finally, a series of neuronal tract tracing studies were performed

to characterize the relationship between ascending and descending collaterals of PAG neurons. Many PAG neurons retrogradely labeled from the NRM were double-labeled by retrograde tracer injections into medial thalamic nuclei or ventrobasal hypothalamus. In contrast, the ventrobasal thalamus, lateral hypothalamus, amygdala, and cerebral cortex receive afferent projections from the dorsal raphe nucleus, but not from the PAG. Together these studies have provided information on the characteristics of the PAG-NRM projection and on the nature of GABAergic controls that influence its activity.



Signature of Advisor

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INTRODUCTION

The midbrain periaqueductal gray matter (PAG) has been implicated in a wide range of behaviors and physiological functions including antinociception and analgesia¹ (Basbaum and Fields 1984; Fields and Basbaum 1978; Mayer and Price 1976), pain (Mehler 1969; Nashold C 1969; Valenstein 1965), lordosis (Sakuma and Pfaff 1979), vocalization (Jurgens and Pratt 1979; Kanai and Wang 1962; Yajima *et al.* 1980), cardiovascular regulation (McDougall *et al.* 1985), regulation of hormone secretion (Aulsebrook and Holland 1969), feeding (Jenck *et al.* 1987; Skultety and Gary 1962; Waldbillig 1975), gut motility (Skultety 1959), thermoregulation (Tseng *et al.* 1980; Shen *et al.* 1986) rage reactions (Hunsperger 1956; Kiser and Lebovitz 1956), fear, anxiety (Nashold *et al.* 1969). This abundance of putative functions has attracted investigators from a number of fields to study the PAG. Electrical stimulation is commonly used to study PAG function, and for the most part, this approach treats the PAG as a "black circuit box" from which efferents to numerous areas may be activated. Thus, despite interest in the functions of PAG projections to other parts of the brain, very little is known about the neuronal circuitry within the PAG itself that controls activity in its efferent pathways. The relative paucity of data on PAG internal circuitry probably derives, in part, from features inherent to the PAG that do not lend it to study by methods that have been applied successfully to other areas of the brain. In

¹ The term "analgesia" means a reduction in the perception of pain. Animal studies estimate analgesia by measuring a reduced response to noxious stimuli. This effect is properly referred to as "antinociception." Although the occurrence of antinociception and analgesia are often correlated, the phenomena (and the terms) are not identical. I will use terms like "stimulation-produced analgesia" that have come into general use, advisedly, without intending to imply true analgesia.

the following pages I describe some of these factors, and outline strategies that the present research program has employed to begin characterizing the antinociception-related circuitry of the PAG.

Anatomical Heterogeneity of PAG

Predictable spatial organization of neuronal elements in a region can greatly facilitate analysis of neuronal circuits. For example, our present understanding of circuitry in the cerebellum, cerebral cortex, and spinal cord is founded on knowledge of the laminar organization of recognizable classes of neurons within these regions. In contrast, the morphology of neurons in the PAG ranges from small/fusiform to large/multipolar and these neurons are not clustered into obvious morphological classes. Furthermore, PAG neurons are arranged in a reticular fashion -- not organized into readily apparent anatomical subdivisions. In fact, historically, atlases of the brain have either represented the PAG without subdivisions (Berman 1968; Brown 1943; Cajal 1909) or have divided it into dorsal and ventral halves on embryological grounds (Castaldi 1923), or into quadrants (Konig and Klippel 1963; Gillilan 1943). In fact, some recent studies that were specifically designed to identify cytoarchitectural features in the PAG, did not detect cytoarchitectural subdivisions, although a gradual increase in cell density, soma and dendrite size, and degree of myelination from the aqueduct outward was reported (Mantyh 1982; Gioia *et al.* 1984).

In contrast to the view that the PAG cannot be regionated, some studies report the existence of cytoarchitectural subdivisions. The subdivision schemes are not identical, but they propose a similar combination of concentric and radially oriented boundaries. Hamilton (1973) mapped the distribution of three classes of neurons in Nissl-stained cat PAG, and detected three major subdivisions. The nucleus medialis is an annulus around the

aqueduct containing sparse, small neurons. This region was recognized in human by Olszewski and Baxter (1954), and later in rat by Beitz (1985a,b). In addition, Hamilton's nucleus medialis extends in a band outwards to the ventrolateral border of the PAG. Another subdivision, located directly dorsal to the aqueduct, is also commonly recognized (Beitz 1985a,b; Castaldi 1923; Hamilton 1973; Oleszewski and Baxter 1954). This subdivision contains densely packed, medium-sized neurons. The lateral region of the PAG contains densely packed large neurons and may be divided into dorsal and ventral parts (Beitz 1985a,b; Oleszewski and Baxter 1954). The pattern of cytochrome oxidase in the PAG seems to agree with this type of subdivision scheme (Conti *et al.* 1988). Although together, these studies argue convincingly that the PAG contains at least three major regions, the lack of agreement on exact boundaries, even among these very detailed studies, makes it clear that natural boundaries do not exist in the PAG. Thus, the cytoarchitectural approach to parcellating the PAG has probably been played out nearly to its limit.

As an alternative to cytoarchitectural mapping of morphological classes of neurons, the immunocytochemical approach can generate maps of transmitter distributions; these maps may have more direct functional significance. The earliest recognized, and most carefully mapped histochemical locus within the PAG is the dorsal raphe nucleus (DRN; Dahlström and Fuxe 1964). The DRN consists of a dense cluster of serotonergic neurons located in the ventral PAG; it extends from the most caudal aspect of the PAG to the level of the third nerve nucleus. Since these serotonergic neurons are physiologically distinctive (Aghajanian *et al.* 1978), and their axons project much more widely throughout the brain and spinal cord than do axons of PAG neurons (Chapter III), the DRN is properly considered a nucleus

separate from, but embedded within the PAG. Therefore, my use of the term "PAG" excludes the DRN.

Differential distributions of other transmitters in the PAG have been noted by immunocytochemical surveys. Briefly, somatostatin (SS) immunoreactive fibers are dense in the medial and ventrolateral PAG, in a pattern similar to the peptides mentioned above, and a few SS cell bodies are on the dorsal midline (Spangler and Morley 1987, cat; Krisch 1978, rat). Adrenocorticotrophic hormone (ACTH) and FMRF-amide fibers, which originate in the mediobasal hypothalamus, are also found in the medial and ventrolateral PAG (Romagnano and Joseph 1983; Triepel and Grimmelikhuijzen 1984). Substance P immunoreactive neurons and fibers are concentrated lateral to, but near the aqueduct; this pattern shifts dorsolaterally and dorsally in the rostral PAG (Gioia *et al.* 1988; Moss and Basbaum 1983). Vasoactive intestinal polypeptide (VIP) immunoreactive neurons are found only subependymally, in the ventromedial PAG; sparse VIP immunoreactive fibers are found in the ventral and ventrolateral PAG (Moss and Basbaum 1983, cat). Cholecystikinin (CCK) immunoreactive neurons and fibers are found lateral to the aqueduct at the level of the fourth nerve nucleus, and a dense group of cells is in the Edinger-Westphal nucleus (Innis *et al.* 1979). 5-hydroxytryptamine (5-HT) immunoreactive fibers are distributed evenly throughout the PAG (Clements *et al.* 1985), as are histamine (histidine decarboxylase immunoreactive) fibers, which derive from the posterior hypothalamus (Watanabe *et al.* 1984). Glutamate and aspartate immunoreactive fibers are spread evenly throughout the PAG. Although glutamate cells are found throughout the PAG, aspartate cells are concentrated near the outer edges of the rostral PAG (Clements *et al.* 1987). A few choline acetyltransferase and tyrosine hydroxylase immunoreactive cell bodies are found in the

ventrocaudal PAG (Jones and Beaudet 1987). The results of these mapping studies emphasize the fact that the PAG is composed of many superimposed mosaics, which can never be represented by any single scheme of intranuclear subdivisions.

Recently, five transmitters that have been implicated in antinociception have been mapped in the PAG. These maps are particularly relevant to the present series of studies. Shipley *et al.* (1987) produced a map of the distribution of immunoreactive neurotensin in the rat, and Chapter II reports our observations on the distribution of GABA, β -endorphin, enkephalin, and dynorphin in the rat. Immunoreactive staining for all five transmitters is most intense in the region corresponding to Hamilton's (1973) nucleus medialis. This pattern must, in part, reflect the greater density of terminals versus cell bodies in this region, but since some transmitters (described above) are not found in this pattern, the relationship to Hamilton's scheme is probably significant. Beyond this overall likeness, however, each pattern of staining has some prominent unique features (see discussion in Chapter II). As described later in this Introduction and also in Chapter II, these observations are useful in making predictions about antinociception-related neuronal interactions and their localization in the PAG.

Light microscopic observations, however, can be easily overinterpreted. For example, it may not be safe to assume that the distribution of transmitters in a region is a good indication of the distribution of their postsynaptic targets. There is evidence that this commonsense arrangement is sometimes violated in the brain (for review see Herkenham 1987), and in this respect the PAG is a major trouble spot, particularly in regard to transmitters that are of special interest to the present series of studies. As Chapter II describes in detail, prominent "mismatches"

exist between the distribution of opioid peptides and their receptors in the PAG. I have used light microscopic immunocytochemical maps to hypothesize certain neuronal interactions and to suggest where conditions are permissive for them to occur. Demonstration that neural connections, in fact, exist requires the use of much more difficult techniques.

Electrophysiology

The anatomical heterogeneity of the PAG might result in variations in the electrophysiological properties of neurons according to their locations. a problem that must be confronted by such physiological studies is that PAG neurons exhibit a wide variety of responses to peripheral stimuli, and it is not clear how these responses should be divided into physiological classes. This problem is compounded by the general lack of consensus over how PAG neurons respond to peripheral stimuli or to opiates (for review see Gebhart 1982). Using a simple means of classifying responses to heat stimuli, Sanders *et al.* (1980) reported that most responsive neurons in the PAG are excited, while inhibitory responses predominate in the DRN. Schurr *et al.* (1981) reported that neurons in the ventral PAG are distinctive in that they have much higher spontaneous activity than other PAG neurons, in the morphine dependent rat. Liebeskind and Mayer (1971) found evidence of somatotopy in PAG evoked potentials. This somatotopy might be a subtle property distributed over the population of PAG neurons, since single-unit recording studies do not indicate somatotopic organization of the large receptive fields of PAG neurons.

One approach to this problem of defining physiological classes of neurons in the PAG analyses the relationship between their activity and nocifensive behaviors, instead of concentrating on somatosensory responses. Many neurons in the midbrain change their firing rate immediately before such behavior (Handwerker and Sack 1982; Korzeniewska *et al.* 1986), and

these neurons are most common in ventral than in dorsal PAG (Heinricher *et al.* 1987). This approach is promising, but it is clear that our present understanding of PAG electrophysiology is still sketchy.

Functional Heterogeneity -- Electrical Brain Stimulation

In view of the intermixture of anatomical and electrophysiological characteristics in the PAG, it is optimistic to expect to find discrete localization of functions within the PAG. In fact, functional studies of the PAG have not, for the most part, found distinct zones, but instead reported gradual changes across relatively large distances. For example, Skultety (1963) reported that stimulation of rostral PAG produced rage reactions in cats, while stimulation at caudal sites produced escape behavior. Most reports of functional heterogeneity have described differences along the dorsoventral dimension. In general, the electrical stimulation in the dorsal PAG is aversive (Cosyns and Gybels 1979; Delgado 1955; Fardin *et al.* 1984b; Hunsperger 1956; Kelly *et al.* 1946; Kiser and Lebovitz 1975; Magoun *et al.* 1937; Nashold *et al.* 1969; Olds and Olds 1962; Valenstein 1965; Wada *et al.* 1970; Wolfle *et al.* 1971). On the other hand, electrical stimulation in the ventral PAG is rewarding (Belluzzi and Stein 1977; Schenberg and Graeff 1978; Schmitt *et al.* 1974). Motor phenomena are also associated mainly with ventral PAG stimulation (Blackburn *et al.* 1980; Fardin *et al.* 1984a; Kiser *et al.* 1978; Nicolau *et al.* 1979; Skultety 1962). This localization of reward and motor effects of stimulation in the ventral PAG may reflect the presence of the DRN there -- serotonergic pathways from the DRN have been implicated in both phenomena.

Numerous studies have mapped the PAG for sites that support stimulation-produced analgesia (SPA). Soper and Melzack (1982) reported that dorsal (and rostral) electrical stimulation produced antinociception against pinch applied to the rostral body surface, while ventral (and caudal)

stimulation was more effective for caudal stimuli. Generally, it is agreed that although antinociception may be elicited from most parts of the PAG (especially in rats), the most effective region is the ventral PAG (Oliveras *et al.* 1974; Yeung *et al.* 1977). More specifically, some studies have singled out a region at the ventrolateral edge of the PAG (Dennis *et al.* 1980; Gebhart and Toleikis 1978; Lewis and Gebhart 1977). In addition, the nature of antinociception elicited from dorsal and ventral sites may differ. Liebeskind and coworkers demonstrated that tail-flick analgesia elicited from ventral, but not dorsal, PAG sites is reduced by naloxone pretreatment or NRM lesions (Cannon *et al.* 1982; Prieto *et al.* 1983; however, see Klatt *et al.* 1988). Furthermore, post-stimulation antinociception is more often elicited from dorsal, than ventral, PAG (Cannon *et al.* 1982; Fardin *et al.* 1984b,c). Fardin *et al.* (1984b,c) also noted that antinociception can be elicited from the ventral PAG unaccompanied by behavioral effects, while dorsal PAG analgesia was not separable from aversive side effects. This linkage of aversion and antinociception in the dorsal PAG suggested that antinociception from this region might result indirectly from stress induced by the aversive effects of stimulation. Contrary to this proposal is the observation by Morgan *et al.* (1987) that systemic benzodiazepine can, in fact, dissociate the antinociceptive and aversive effects of stimulation in the PAG.

Much emphasis has been placed on the discrete zones in the ventral PAG where SPA can be elicited in the absence of any aversive side effects. While the location of these "pure analgesia areas" has obvious clinical importance, its relevance to the analysis of antinociceptive circuitry is questionable. From the foregoing discussion of the intermingled systems in the PAG, it seems very unlikely that any site in the PAG will be found exclusively dedicated to a single physiological function. The relevance of

neuronal circuitry at a site should not be underestimated only because it is intermingled (and perhaps interrelated) with other functional systems.

Since some minimum number of relevant neurons must be activated in order to produce an observable effect by electrical stimulation, the technique is inherently limited in its resolution. By exciting all neurons within this volume of tissue, electrical stimulation overrides local synaptic circuitry, exciting axons that project from, and through, the stimulated area. These axons are uniformly excited (orthodromically and antidromically) in non-physiological patterns. In this way, electrical stimulation maps only the distribution of projection neurons (and axons of passage) that mediate an effect, and in this manner it is very nearly the inverse of making a lesion. For these reasons, electrical stimulation is very poorly suited to task of analyzing synaptic circuitry local to an area.

Functional Heterogeneity -- Microinjection

A more physiological means of activating PAG circuitry is the microinjection of drugs and transmitters. Glutamate has often been considered a non-selective excitant of neuronal cell bodies, and is used in lieu of electrical stimulation to avoid stimulating axons of passage, or antidromically activating other axons. Although glutamate microinjection in the PAG causes potent antinociception (Behbehani and Fields 1978) and many components of the defense reaction (for review see Bandler and Depaulis 1988), as yet this technique has not been used to map the region. Interestingly, N-methyl-D-aspartate, a specific NMDA receptor ligand, also causes antinociception when microinjected in the PAG (Jacquet 1988). Thus, it is risky to assume that the antinociceptive effect of glutamate results from a non-selective excitation of neurons in the PAG.

Opiate microinjection is another more physiological means of activating PAG circuitry. It is often used to elicit antinociception from the PAG, and some regional differences have become apparent. In general, the most efficacious sites for producing antinociception by opiate microinjection lie in the ventrocaudal PAG (for review see Yaksh and Rudy 1976). More specifically, Lewis and Gebhart (1977) found that microinjected morphine produced the most potent antinociception when microinjected in the ventromedial PAG. Similarly, Sharpe *et al.* (1974) reported that antinociception was elicited with the shortest latency by morphine microinjected in the ventromedial PAG. The greater efficacy of medial, compared to lateral, morphine injections might be caused, in part, by spread into the ventricle, giving the drug faster access to a greater area of PAG tissue.

Together, the physiological and functional studies discussed above suggest that the major spatial variation of function in the PAG occurs between its dorsal and ventral parts. As mentioned previously, these differences are probably due, in part, to the presence of the DRN in the ventral PAG. In addition, however, neuronal tracing studies reveal that the dorsal and ventral parts of the PAG have markedly different afferent connections with other regions of the brain. The ventral PAG receives projections from limbic, autonomic, and motor systems, while the dorsolateral PAG receives predominantly sensory inputs (Beitz 1982). While this segregation of inputs suggests that different functions are mediated by these regions, it also raises suspicions that some of the apparent functional differences demonstrated with electrical stimulation may be due entirely to antidromic activation of afferents to the PAG.

Strategic Considerations

Compared to the practical difficulties with PAG research, described above, a more conceptual obstacle to progress is the poor understanding of PAG functions. Although electrical stimulation of the PAG influences a wide range of brain processes and associated behaviors, it is not clear how these artifacts of stimulation are related to actual physiological functions of the PAG. The results of electrical stimulation are particularly difficult to interpret for an area that has afferent and efferent connections with so many other areas of the brain. (Chapter III discusses some serious difficulties in interpreting the results of electrical stimulation in the PAG, that were revealed by new anatomical data.) As a result, most functional proposals concerning the PAG have been formulated only in the most general terms, e.g. "control of feeding." In fact, many cannot be considered proper functional descriptions, but rather statements of suspected involvement of the PAG in some process or behavior. This problem results, in part, from the very nature of these phenomena -- many are diffusely distributed processes in the brain (e.g. arousal), and the individual participation of the PAG may not yield to simple verbal descriptions.

Further progress in sorting out and defining functions of the PAG will require a more sophisticated understanding of the neuronal circuitry within the PAG itself. One logical approach to the problem of characterizing local neuronal circuitry is to first determine which efferents from the structure mediate a particular behavior or physiological effect, and which inputs, upstream in the local circuitry, are able to activate these efferents. From knowledge of the input/output relationship between these two points, it should be possible to make testable predictions about the interposed neuronal circuitry. This strategy is most practical if the relevant afferent and efferent pathways are well-characterized and accessible. Sorting out pathways to and from the PAG, however, can be a difficult task because of the great number of

interconnections between this and other regions of the brain.² Because of these extensive interconnections between the PAG and other regions of the brain, relevant afferent and efferent pathways are only poorly characterized for most of the phenomena induced by electrical stimulation of the PAG.

Model System -- Lordosis

One putative function of the PAG that has been described in relatively specific terms relates to reproductive behavior in female rats. Electrical stimulation of the PAG markedly facilitates lordosis, a dorsiflexion reflex of sexually receptivity that is elicited in estrogen-treated female rats by somatic stimulation of the hindquarters (Sakuma and Pfaff 1979a). Conversely, lesions of the PAG abolish the reflex (Sakuma and Pfaff 1979b). A series of experiments by Pfaff and others have begun to outline the neuronal circuitry involved in this phenomenon. Electrical stimulation of the ventromedial nucleus (VMN) of the hypothalamus facilitates the lordosis reflex; this effect is abolished by PAG lesions (Sakuma and Pfaff 1979b). Based on these data, it has

² The PAG receives major afferent projections, in various species, from medial prefrontal cerebral cortex, amygdala, diagonal band of Broca, hypothalamic nuclei (dorsal premammillary, ventromedial, lateral, posterior, anterior, perifornical, preoptic, and tubercinereum), cuneiform n., subcuneiform n., substantia nigra, ventral tegmental area, locus coeruleus, parabrachial area, mesencephalic, pontine, and medullary reticular formation, n. raphe magnus, pallidus, obscurus, and centralis, trigeminal n. and spinal cord (dorsal horn, lateral cervical n.) (Beitz 1982; Grofová, *et al.* 1978; Liu 1983; Marchand and Hagino 1983; Meller and Dennis 1986; Swett, *et al.* 1985; Wiberg, *et al.* 1987).

The PAG sends major efferent projections, in various species, to lateral habenula, zona incerta, thalamic nuclei (n. reticularis, mediodorsal n., laterodorsal n., lateral posterior n., midline group, and intralaminar group), hypothalamic nuclei (preoptic n., lateral n., posterior n., anterior n., dorsal n., periventricular n., ventromedial n., periarcurate area), superior colliculus, cuneiform n., dorsal tegmental n., locus coeruleus, lateral parabrachial n., n. *reticularis*, pontine reticular n. oralis and caudalis, medullary gigantocellular n., paragigantocellular n., n. raphe magnus and pallidus, n. ambiguus, (Abols and Basbaum 1981; Beitz, *et al.* 1983; Chi 1970; Fardin, *et al.* 1984a; Gallager and Pert 1978; Hamilton 1974; Hamilton and Skultety 1970; Mantyh 1983a,b; Ruda 1975).

been proposed that the VMN initiates lordosis via its efferent connection with the PAG. Since neurons in the VMN are sensitive to estrogen levels, this pathway is probably responsible for the estrogen-sensitivity of the behavior. A second necessary stimulus for lordosis is pressure or noxious stimuli to the hindquarters. The PAG receives such input directly via spinopetal projections, and indirectly from the reticular formation, and perhaps from the thalamus. These inputs are presumably integrated in the PAG and activity in some efferent pathway is accordingly modified. Evidence suggests that the relevant efferent target of the PAG is the nucleus reticularis gigantocellularis (NGc) of the medulla. Lesions in the lateral medullary reticular formation disrupt lordosis behavior (Modianos and Pfaff 1979), and electrical stimulation of the NGc monosynaptically excites motor neurons that innervate muscles of the ipsilateral back which perform the dorsiflexion (Peterson *et al.* 1979).

Demonstrating links within the PAG that interconnect the afferent and efferent pathways for lordosis has, however, been problematical. Consistent with their model, Sakuma and Pfaff (1980a) showed that more than half the units they recorded in the PAG responded both to electrical stimulation of the VMN and to cutaneous noxious or pressure stimuli. The same investigators, however, were unable to demonstrate somatosensory responses in any of 57 PAG neurons antidromically activated from the gigantocellular and magnocellular nuclei of the medulla (Sakuma and Pfaff 1980b). As a result, it was concluded that these data "preclude the involvement of these cells in the [stimulus-bound] reflexive control of lordosis," and that the involvement of the PAG in the reflex is probably limited to "transmit[ting] hormonal effects on the forebrain and hypothalamus to the reticulospinal system."

Model System -- Antinociception

Compared to lordosis, the phenomenon of antinociception may be more amenable to the kind of input/output approach suggested above. The hypothesis that the PAG is involved in an endogenous pain inhibitory system has been reviewed extensively (Basbaum and Fields 1984; Fields and Basbaum 1978; Liebeskind *et al.* 1976; Mayer and Price 1976). Stated simply, the PAG is thought to integrate information about internal and external states and, under the appropriate conditions, it activates bulbospinal inhibitory pathways via direct projections to the medullary reticular formation. Activation of these pathways specifically inhibits noxious-evoked activity in spinal cord dorsal horn neurons, including spinothalamic tract neurons. This apparent function of the PAG has received far more attention than any other this antinociceptive system here are several features of that make it a relatively convenient paradigm for studying the neuronal circuitry of the PAG.

First, the system is activated by opiates microinjected in PAG. Therefore, one important input to PAG antinociceptive circuitry can be readily identified in anatomical studies (by immunocytochemistry), and physiological studies can mimic and manipulate this input using a wide range of specific ligands. Three classes of opioid peptides and receptors are present in the PAG (see discussion, Chapter II). It is also fortuitous that β -endorphin terminals derive exclusively from the hypothalamic arcuate nucleus, and so this input to the PAG is accessible to stimulation or lesions. In fact, experiments in which the arcuate nucleus was lesioned have implicated β endorphin in analgesia produced by stimulation in the PAG (Millan *et al.* 1986).

Secondly, it is well-established that the projection from the PAG to the nucleus raphe magnus (NRM) of the rostral ventromedial medulla mediates, in part, antinociceptive effects elicited from the PAG (see Basbaum and Fields

1984). This is the single largest descending projection from the PAG, and so using anatomical or electrophysiological methods it is relatively easy to identify neurons from which it originates. This pathway is not, however, homogeneous. It contains a mixture of transmitters: approximately 32% of neurons retrogradely labeled from the NRM contain neurotensin, 13% somatostatin, 9% serotonin, and 2% GABA (Beitz *et al.* 1983; Chapter III), and nearly half the transmitter content of the pathway is still unaccounted for. The neuronal tracing experiments described in Chapter III reveal an interesting anatomical heterogeneity in the pathway. A large proportion of PAG neurons at the origin of the projection to the NRM also send collaterals to the ventrobasal hypothalamus and to the medial thalamus. Thus, activation of this pathway necessarily activates projections to other areas of the brain that have been implicated in nociception and antinociception.

Circuit Hypotheses

Although relevant inputs to, and outputs from, the PAG have been identified, very little is known about the neuronal circuitry within the PAG through which antinociceptive effects are generated. Based on available information, it is possible to make specific, testable hypotheses. Since electrical stimulation of, or glutamate microinjection into, the PAG does produce analgesia (Behbehani and Fields 1978), and since electrolytic lesion of the PAG does not (Deakin and Dostrovsky 1977), it follows that PAG neurons that project to the NRM must be excited in order to produce analgesia. Opioidergic synapses, however, are believed to be exclusively inhibitory (Nicoll and Alger 1979). Thus, for opiates to excite the PAG output neuron, it has been proposed that opioid peptides inhibit an interneuron that tonically inhibits the output neuron (Yaksh *et al.* 1976). In other words, opiates disinhibit the PAG output neuron. GABA has been suggested as a likely

transmitter for the inhibitory interneuron (Basbaum and Fields 1984). As discussed in Chapter V, numerous pharmacological, physiological, and anatomical observations are consistent with this proposal.

Recent technical developments in electron microscopic immunocytochemical and tracing techniques have made it possible to begin verifying that the anatomical substrate for this hypothetical circuitry, in fact, exists. The experiments in Chapter V were designed to establish that there are GABA-immunoreactive synaptic contacts onto some PAG neurons that project to the NRM. The latter were identified by injection of the colloidal gold labeled tracer, WGA-apoHRP-Au, into the NRM. Putative GABAergic terminals were identified using an antibody directed against glutaraldehyde-fixed GABA. These experiments not only demonstrated the existence of this synapse, but also characterized the morphology of GABAergic synapses in the PAG, in general.

The DRN has also been implicated in the antinociceptive effect of opiates, and there is evidence that the serotonergic projection neurons in this nucleus mediate part of the antinociception induced by opiates. In addition, interactions between GABA and serotonergic neurons in the DRN have been implicated in cardiovascular regulation and in the anxiolytic action of benzodiazepines (see discussion, Chapter VI). Therefore, I also used electronmicroscopic immunocytochemical techniques to verify that GABA immunoreactive axon terminals are in synaptic contact with serotonin-immunoreactive cell bodies in the DRN. These experiments are described in Chapter VI.

Conclusions

This series of experiments have contributed toward beginning the characterization of antinociception-related circuitry in the PAG by:

- 1) providing detailed information about the topography of antinociception relevant transmitters in the PAG (Chapter II);
- 2) characterizing the NRM-PAG projection pathway by:
 - b) describing major collateral pathways deriving from the PAG-NRM projection, that are likely to mediate effects related to antinociception (Chapter III), and
 - a) investigating the contribution of GABAergic projection neurons to the pathway (Chapter IV);
- 3) providing evidence for GABAergic control over the PAG-NRM projection, and also over the serotonergic neurons of the DRN (Chapters V and VI);

These experiments are obviously just a beginning in the effort to characterize the circuitry in the PAG that mediates antinociception. The results leave many questions unanswered, but also suggest a number of new experiments to help move our understanding of PAG circuitry further along upstream.

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CHAPTER II

**β -endorphin, enkephalin, dynorphin, and GABA immunoreactivity in
the rat midbrain periaqueductal gray.**

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SUMMARY

It has been suggested that the antinociceptive action of opiates and endogenous opioid peptides, is mediated, in part, through inhibition of GABAergic neurons in the midbrain periaqueductal gray matter (PAG). To begin the anatomical study of the underlying opioid-GABAergic circuitry, we have mapped the distribution of immunoreactive β -endorphin, enkephalin, dynorphin, and GABA cell bodies and terminals in the PAG of the rat. Immunoreactivity for each compound forms a distinct pattern of staining which changes significantly over the rostral-caudal extent of the PAG. In general, terminal staining for all four putative transmitters is densest near the aqueduct; opioid terminal staining is also dense in the ventrolateral PAG. Enkephalin and dynorphin-immunoreactive cell bodies cluster in distinct parts of the PAG; GABA-immunoreactive cell bodies are more widespread. These results suggest that opioid-GABAergic interactions that contribute to antinociceptive controls are most likely to occur in the medial and ventrolateral PAG.

INTRODUCTION

Systemically administered opiates initiate powerful descending antinociceptive controls, in part, through the activation of neurons in the midbrain periaqueductal gray (PAG) that project to the medullary nucleus raphe magnus (NRM). The PAG microcircuitry through which this control is generated, however, is poorly understood. Since the effect of opiates on CNS neurons is exclusively inhibitory, it has been proposed that opiates do not act directly on the PAG output neuron. Rather, opiates are hypothesized to disinhibit PAG output neurons, by inhibiting a tonically active inhibitory interneuron (Yaksh *et al.* 1976). It has been suggested that the tonically active interneuron is GABAergic (Basbaum and Fields 1984). This hypothesis is supported by the observations that PAG microinjection of the GABA antagonist, bicuculline, produces analgesia (Moreau and Fields 1986) and that microinjection of the GABA agonist, muscimol, attenuates opiate

analgesia (Zambotti *et al.* 1982). Furthermore, Depaulis *et al.* (1987) showed that microinjection of the GABA agonist, THIP, into the ventral PAG, reverses the analgesic effects of morphine microinjected into the same site. Despite these pharmacological data, there is no anatomical evidence in support of the proposed endorphin-GABAergic interactions in the PAG.

A variety of techniques have been used to examine the distribution of GABA-containing cells and terminals in the PAG. One study reported that glutamic acid decarboxylase- (GAD) immunoreactive neurons are distributed throughout the PAG (Mugnaini and Oertel 1985); others found that [³H]GABA-accumulating neurons and neurons immunoreactive for the GABA degradative enzyme, GABA transaminase (GABA-T), are common only in the region of the dorsal raphe (Belin *et al.* 1979; Nagai *et al.* 1983). Because of this differing results, and due the fact that the regional distribution of the different opioid peptide subtypes has not been studied in detail throughout the rostral-caudal extent of the PAG, it is difficult to evaluate the likely location of opioidergic-GABAergic interactions in the PAG.

With the introduction of antisera directed against glutaraldehyde conjugates of the GABA molecule, it is now possible to more directly examine the distribution of presumptive GABA-containing cells and terminals. In this study we used a GABA-directed antiserum to study the distribution GABA-immunoreactive neurons and terminals over the rostral-caudal extent of the PAG. Since our interest is in the relationship of endorphin elements and GABA neurons, we have also produced detailed maps of enkephalin, dynorphin and β -endorphin immunoreactivity in the PAG.

The PAG is spatially heterogeneous with respect to its antinociceptive functions. For example, stimulation-produced analgesia (SPA) that is elicited from ventral PAG is reduced by either systemic injection of the opiate antagonist, naloxone, or by lesions of the nucleus raphe magnus (Cannon *et al.* 1982; Prieto *et al.* 1983); SPA from dorsal PAG is not affected by those manipulations. Furthermore, Fardin *et al.* (1985) reported that

although electrical stimulation throughout the PAG may produce analgesia, only that elicited from the DRN and a subregion of the caudal, ventrolateral PAG is free of motor or aversive side effects. The ventrolateral and medial PAG is also the region from which microinjection of low doses of opiates elicits the most potent analgesia (Lewis and Gebhart 1977; Sharpe *et al.* 1974; Yaksh *et al.* 1976). To be useful for hypotheses of neural circuitry, light microscopic cytochemical maps must resolve details at least as small as any relevant functional zones. For these reasons, the present study describes in detail the immunocytochemistry of GABA, β -endorphin, enkephalin, and dynorphin at four rostral-caudal levels of the rat midbrain PAG.

METHODS

Male Sprague-Dawley rats (250-300 gram) were used in this study. Three rats received stereotaxic microinjections of colchicine (100 μ g; 6 μ l) into the third ventricle in order to increase staining of cell bodies. Forty-eight hours after administration of colchicine, the animals were perfused intracardially with 0.1 M phosphate-buffered saline (PBS) followed by one of two fixative solutions. For opioid peptide immunocytochemistry the fixative was 4% paraformaldehyde in 0.1 M phosphate buffer with 4% sucrose, and for GABA immunocytochemistry it was 4% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M PBS. Each rat was perfused with 500 ml of fixative, and the brain was removed and placed in the same fixative for an additional two hours at 4° C. Three rats were pretreated with drugs that inhibit GABA uptake; four hours prior to perfusion, these rats received a third ventricle injection (300 μ g in 3 μ l) of either β -alanine or nipecotic acid. The former drug inhibits neuronal and glial uptake of GABA; the latter preferentially inhibits neuronal GABA uptake (Johnston 1975; Schon and Kelly 1974).

Thirty- or fifty-micron coronal sections of the rostral pons and midbrain were cut serially on a freezing microtome or Vibratome. Adjacent sections were immunostained using the peroxidase-antiperoxidase method (Sternberger 1979). To identify

immunoreactive products of the proenkephalin and prodynorphin precursors we used antisera directed against leucine enkephalin and dynorphin B, respectively. A few sections were stained using an antiserum directed against methionine enkephalin-arg-gly-leu; no significant differences were observed between the distribution of staining for this peptide and for leucine enkephalin. These antisera were kindly provided by Dr. Ekhard Weber of the University of Oregon. Radioimmunoassay characterization of each antiserum demonstrated high specificity for the peptide to which it was directed and almost no cross-reactivity with other peptides (Weber *et al.* 1982). To identify immunoreactive β -endorphin we used an antiserum kindly provided by Dr. Volker Höllt of the Max Planck Institute, FRG. That this antiserum does not cross-react with either dynorphin B or leu enkephalin is shown by the absence of staining of cell bodies in the PAG of colchicine-treated animals. Furthermore, the pattern of β -endorphin immunoreactivity was very different from that seen with the other antisera. Finally, absorption controls were performed for all three opioid peptide antisera. Adjacent sections were stained with antisera that had been preabsorbed with the antigen against which the antibody was directed, or with the one of other opioid peptides. Immunohistochemical staining was only abolished when the antisera were preabsorbed with 10 μ M of the appropriate opioid peptide.

GABA immunoreactivity was mapped using an antiserum kindly provided by Dr. Andrew Towle of the Cornell University. The antiserum was raised against a glutaraldehyde-linked GABA-bovine serum albumin (BSA) conjugate; radioimmunoassay shows no significant cross-reactivity with conjugated glutamate, β -alanine, or taurine (Lauder *et al.* 1986). The antiserum was used at a dilution of 1:3750. Immunoreactivity in tissue sections was completely abolished by preabsorption of the antiserum with 10 μ M of the GABA-BSA conjugate.

The Paxinos and Watson (1986) atlas of the rat brain was used to determine stereotaxic coordinates and as a reference for mapping the distributions of cell bodies and terminals in the PAG. To estimate the proportion of neurons that were GABA-

immunoreactive, the total number of neurons in the PAG was counted in Nissl-stained sections adjacent to immunoreacted sections.

RESULTS

Figures 1-5 show photomicrographs of opioid peptide and GABA immunoreactivity in sections of the PAG. Figures 6 and 7 schematically illustrate the distributions of immunoreactive fibers and cell bodies of the four compounds at three rostrocaudal levels of the PAG. In the following discussion the term, "rostral PAG," refers to levels including the third cranial nerve (oculomotor) nucleus, "middle" levels of PAG extend from the caudal pole of the third cranial nerve nucleus to the caudal pole of the superior colliculus, and "caudal" levels are caudal to the decussation of the superior cerebellar peduncle.

β-Endorphin

There are no β-endorphin-immunoreactive cell bodies in the PAG. In contrast to the enkephalin and dynorphin fiber staining, which is predominantly punctate in appearance, β-endorphin immunoreactivity consists of long, varicose fibers. At caudal levels of the PAG, β-endorphin fiber staining extends ventrolaterally, in a band from the aqueduct beyond the border of the PAG and into the nucleus cuneiformis (figure 1C). A second zone of β-endorphin staining, found at all levels of the PAG, extends laterally outward from the aqueduct, almost to the edge of the PAG. This lateral area of staining occurs at all levels of the PAG. Only sparse β-endorphin fibers are found in the dorsal PAG, ventral PAG, and dorsal raphe nucleus (DRN). The dorsolateral PAG is nearly devoid of β-endorphin immunoreactivity.

Enkephalin

Enkephalin-immunoreactive cells are found at all levels of the PAG, but are most concentrated in the caudal, ventrolateral PAG (figure 2). This cluster of enkephalin cells

does not extend beyond the lateral edge of the PAG. Rostrally, there is another small cluster of enkephalin-immunoreactive cells dorsal to the aqueduct (figure 3). Moderate numbers of enkephalin-immunoreactive cells are found in the DRN. The enkephalin-immunoreactive cell bodies are of small-diameter (approximately 12-25 μm). The population of cells may be classified into the following morphological groups: 70% fusiform/bipolar, 20% triangular, 10% multipolar. This distribution of cell types is typical of the general population of neurons in the medial and ventrolateral PAG (Beitz and Shepard 1985; Liu and Hamilton 1980).

Of the three opioid peptides examined, enkephalin-immunoreactive terminals are the most dense and most widespread in the PAG. The most intense enkephalin immunoreactivity generally follows the pattern described for β -endorphin. In addition, there are several intense patches of terminal staining unique to enkephalin. One patch is located in the dorsolateral PAG; it increases in size rostrally (figure 1E). This zone of dense staining is surrounded by areas of less intense enkephalin immunoreactivity that is characteristic of most of the dorsolateral PAG. A second bilateral patch of staining occurs in the lateral part of the DRN (figure 1F). We did not determine if this patch of terminals overlaps the location of serotonergic DRN neurons. A third notable zone of enkephalin terminal immunoreactivity is found on the midline, in the ventral part of the DRN; it is particularly conspicuous rostrally in the caudal linear raphe nucleus (between the oculomotor nuclei and ventral to Edinger-Westphal (figure 1D). Finally, there is a thin, subependymal layer of extremely intense enkephalin staining that completely surrounds the aqueduct.

Dynorphin

Dynorphin-immunoreactive cell bodies are neither as widespread nor as numerous as enkephalin cells in the PAG. A prominent group of dynorphin-immunoreactive cells overlaps somewhat with the ventrolateral group of enkephalin cells, but for the most part

they are clustered more ventrally (figure 2). In contrast to enkephalin cells, the cluster of dynorphin-immunoreactive cells in the ventrolateral PAG is continuous with a group of dynorphin cells in the nucleus cuneiformis. Another difference between enkephalin and dynorphin cell staining is the scarcity of dynorphin-immunoreactive cells in the dorsal PAG (figure 3) and in the DRN (figure 2). Dynorphin-immunoreactive neurons are about the same size as enkephalin neurons (12-25 μ m), but they tend to have more primary dendrites: 35% are fusiform/bipolar, 25% triangular, and 40% multipolar. This distribution of cell types is skewed toward multipolar morphologies compared to the general population of neurons in the same region of the PAG (Beitz and Shepard 1985; Liu and Hamilton 1980).

The PAG contains moderate levels of dynorphin terminal immunoreactivity. At caudal and middle levels of the PAG, the regions of most intense dynorphin staining are similar to the pattern of β -endorphin staining (figure 1H and I). Rostral to the fourth cranial nerve nucleus, however, dynorphin immunoreactivity is concentrated dorsolateral to the aqueduct, where it overlaps a region of dense enkephalin staining (figure 1G). Also at this level, dynorphin terminal staining is relatively sparse ventrolateral to aqueduct, where both β -endorphin and enkephalin staining are the most dense (figure 1G).

GABA

Figure 4 illustrates the distribution of GABA immunoreactivity in the PAG. Figure 4C shows that most GABA-immunoreactive cell bodies are small (1-15 μ m diameter) and exhibit very little dendritic staining. At caudal and middle levels of the PAG, GABA-immunoreactive cell bodies are most common in the ventrolateral and dorsolateral regions, accounting for approximately 15% of the total Nissl-stained population of neurons; more rostrally, only the dorsolateral population of intensely stained cells persists in large numbers. Moderate numbers of GABA-immunoreactive cell bodies are found in the DRN, particularly in its caudoventral aspect.

Figure 4A shows that GABA terminal staining is found throughout the PAG. Unlike the varicose appearance of opioid-immunoreactive fibers, GABA-immunoreactive fibers are punctate (figure 4B). GABA-immunoreactivity is most intense in a thin annulus that surrounds the aqueduct; this overlaps the region where opioid immunoreactivity is most dense. Surrounding this annulus, and extending into the ventrolateral PAG, is a wider zone of somewhat less intense GABA immunoreactivity.

Pretreatment with aminooxyacetic acid, an inhibitor of the GABA-degradative enzyme, GABA transaminase (GABA-T), produced more intense staining of cell bodies and proximal dendrites compared to untreated animals. Although the staining in other regions of the PAG was not changed, the number of labeled cell bodies in the DRN was increased after GABA-T inhibition (Figure 5). Neither the intensity nor the distribution of GABA immunoreactivity was changed by pretreatment with nipecotic acid, an inhibitor of neuronal uptake of GABA (Johnston 1975), or with β -alanine, an inhibitor of glial uptake of GABA (Schon and Kelly 1974). Although pretreatment with colchicine might cause glutamic acid decarboxylase, the GABA synthetic enzyme, to accumulate in cell bodies, this treatment did not improve staining of cell bodies.

DISCUSSION

The overall pattern of opioid immunoreactivity is consistent with previous immunocytochemical studies that have mapped β -endorphin (Finley *et al.* 1981a), enkephalin (Fallon and Leslie 1986; Finley *et al.* 1981b; Sar *et al.* 1978; Simantov *et al.* 1977; Uhl *et al.* 1979), and dynorphin immunoreactivity (Fallon and Leslie 1986; Khachaturian *et al.* 1982; Vincent *et al.* 1982). These studies surveyed large regions of the brain and thus revealed few of the details within the PAG. The present observations of enkephalin immunoreactivity in the rat PAG are very similar to our previous description in the cat (Moss *et al.* 1983), but disagree with the report by Goia *et al.* (1988) that enkephalin staining in rat does not vary along the midbrain axis.

We found that GABA-immunoreactive neurons are very common and widely distributed in the PAG. Mugnaini and Oertel (1985) reached the same conclusion using an antiserum directed against GAD. Our results, however, disagree with the reports of Clemens *et al.* (1985) and Ottersen and Storm-Mathisen (1984) who used anti-GABA antisera similar to the one used in this study. The former detected only a dorsal group of GABA-immunoreactive cells; while the latter reported "scarce" GABA-immunoreactive neurons in the PAG. It might be relevant that we found GABA-immunoreactive staining of cell bodies in the PAG to be less intense compared to other regions of the midbrain, especially the superior colliculus. Since the staining we observed was abolished by preincubation with a glutaraldehyde conjugate of GABA, it appears to represent genuine GABA immunoreactivity. Other studies might have understained neurons in the PAG if other regions of the midbrain were used as the basis for which to control the reaction time in DAB.

Our results also differ from studies that used other methods to stain putative GABAergic neurons. Cells that accumulate [^3H]GABA or that are stained histochemically for GABA-T, are found only in the ventromedial PAG and in the DRN (Belin *et al.* 1979; Nagai *et al.* 1983). Interestingly, this is the same region where we observed enhanced GABA immunoreactivity after pretreatment with AOAA, and AOAA has been used to increase the accumulation of [^3H]GABA by neurons (Schon and Iversen 1972). One possibility is that this ventral group of putative GABAergic neurons accumulate GABA, but do not synthesize it. This possibility has also been recognized in the goldfish retina and in the monkey striate cortex (Kisvárdy *et al.* 1986; Zucker *et al.* 1984).

The topography of opioid peptide immunoreactivity reflects previously identified cytoarchitectural subdivisions in the PAG. Hamilton (1973) described a "nucleus medialis" in the cat, a cell-poor region that surrounds the aqueduct and extends to the ventrolateral border of the PAG. This cytoarchitectural subdivision corresponds very closely to the prominent zone of opioid peptide-immunoreactive terminals in the caudal PAG which rings

the aqueduct and has bilateral extensions into the ventrolateral PAG (figure 1C and figure 6C). Beitz (1985) detected a smaller medial subdivision in the rat that contains small cell bodies that are sparsely distributed. The latter region appears to be coextensive with the narrow annulus of the most intense opioid peptide immunoreactivity which surrounds the aqueduct from caudal to mid-levels of the PAG (see figures 1 and 6).

Although the patterns of terminal immunoreactivity are similar for each of the three opioid peptides studied, there are important differences. β -endorphin terminals are much less densely distributed and are found in a far more restricted distribution than the other opioid peptides. Conversely, enkephalin is easily the densest and most widely distributed of the opioid peptides examined. Although enkephalin and dynorphin-immunoreactive cell bodies are located near one another in the lateral PAG, the enkephalin cell bodies are clustered more dorsally (figure 2). Thus, since dynorphin and enkephalin cell bodies are spatially segregated, and since β -endorphin cell bodies are found only in the hypothalamus, it is unlikely that opioid peptide transmitters coexist in PAG neurons. It follows that axon terminals containing the three opioid peptides may not only arise from different sources, but may also have different postsynaptic targets in the PAG.

There has been considerable discussion in the literature over the "mismatch" between opiate binding sites, which are densest in the dorsolateral part of the rat PAG and the relatively low level of opioid peptides there (*c.f.* Herkenham and Pert 1982). In fact, we found a distinct patch of intense enkephalin terminal immunoreactivity in dorsolateral PAG. It seems somewhat paradoxical, however, that binding is low in the ventrolateral PAG, where opioid immunoreactivity is dense, and where analgesia is most readily produced by opiate microinjection (Yaksh *et al.* 1976). It is also difficult to reconcile the patterns of opioid peptide staining we observed with the patterns of opioid peptide binding sites reported by Mansour *et al.* (1987) using selective μ , δ , and κ ligands. For example, binding of DAGO, a μ -selective ligand, is densest in the dorsolateral PAG, but immunoreactive β -endorphin (a putative endogenous μ ligand) is almost absent in the same region.

The selective δ ligand, DPDPE, does not bind in the PAG, and yet enkephalin-immunoreactive terminals are the most abundant. These data suggest that enkephalin effects in the rat PAG are not mediated via δ receptors. Consistent with this idea, Fang *et al.* (1986) concluded that an action at μ receptors is sufficient to explain analgesia produced by i.c.v. microinjection of putative δ ligands. This conclusion was based on the similarity of apparent pA₂ values for naloxone antagonism of morphine- and enkephalin analog-induced analgesia. Finally, although both κ binding (demonstrated with [³H]bremazocine), and immunoreactivity for the putative κ ligand, dynorphin, are dense and widely distributed in the PAG, whether κ ligands produce analgesia is unclear. In fact, rather than producing analgesia, i.c.v. dynorphin *antagonizes* the analgesic action of morphine or D-ala-enkephalin (Tulunay *et al.* 1981).

The analgesic action of electrical stimulation in the PAG clearly could result from direct activation of neurons projecting to the NRM, bypassing any opioid peptidergic circuitry in the PAG. On the other hand, a component of the analgesia produced by electrical stimulation or glutamate excitation of cell bodies in the PAG is naloxone-reversible (Akil *et al.* 1976; Cannon *et al.* 1982; Oliveras *et al.* 1977; Urca *et al.* 1980). This opioid-mediated component of electrical stimulation might be due to the direct activation of opioidergic elements in the PAG. Consistent with this idea, potent, aversion-free analgesia can be elicited by electrical stimulation in the ventrolateral PAG (Fardin *et al.* 1985), where opioid immunoreactivity is intense. In fact, electrical stimulation of the PAG produces a naloxone-reversible analgesia and concomitantly depletes pools of β -endorphin in that region (Millan *et al.* 1987). Interestingly, levels of enkephalin and dynorphin in the PAG were unchanged.

If stimulation-produced analgesia is, in fact, due to the direct excitation of opioidergic elements in the PAG then what is the downstream circuitry that results in activation of the NRM? Since lesions of the PAG do not produce analgesia (Dostrovsky and Deakin 1977; Kelly and Glusman 1968; Melzack *et al.* 1958; Rhodes 1979; Yeung *et*

al. 1975), it has been proposed that neurons of the PAG that project to the NRM must be excited in order to produce analgesia. Furthermore, since the only known postsynaptic effect of opioid peptides is hyperpolarization (Nicoll *et al.* 1980), it has been hypothesized that opioid peptides do not act directly upon the output (i.e. NRM-projecting) neurons of the PAG (Yaksh *et al.* 1976; Basbaum and Fields 1984). Rather, the opioid peptides are presumed to initiate antinociceptive controls by inhibiting interneurons in the PAG which tonically inhibit the PAG neurons that project to the NRM. In the hippocampus, opiates excite pyramidal neurons indirectly, by inhibiting a GABAergic interneuron (Nicoll *et al.* 1980). A comparable opioidergic inhibition of GABAergic interneurons might occur in the PAG.

In fact, there is considerable behavioral and pharmacological evidence for analgesia-relevant interactions between GABA and the opioid peptides in the PAG. Nisticó *et al.* (1979) reported that microinjection of β -endorphin into the third ventricle reduced levels of GABA in the chick brainstem by more than 50%. The hypothesized circuit predicts that GABA antagonists would mimic the effects of opioids in the PAG, by decreasing GABAergic transmission. Consistent with this hypothesis, injection of either morphine or of the GABA antagonists, bicuculline or picritoxin, similarly affect the noxious-stimulus evoked activity of physiologically defined classes of neurons in the NRM (Moreau and Fields 1986). In addition, intracisternal or PAG microinjection of bicuculline (Ueda *et al.* 1987; Moreau and Fields 1986) produces potent analgesia.

Conversely, a GABA agonist would be expected to antagonize opiate-induced analgesia. In fact, intracerebroventricular, PAG, or DRN microinjection of the GABA_A agonist, muscimol, antagonizes the analgesia produced by systemic morphine or β -endorphin (Zonta *et al.* 1981; Zambotti *et al.* 1982; Romandini *et al.* 1984). Similarly, i.c.v. administration of GABA antagonizes the analgesia produced by i.c.v. D-al²-met-enkephalinamide (Izumi *et al.* 1980) and inhibition of the uptake or breakdown of GABA antagonizes systemic morphine analgesia (Ho *et al.* 1976). Taken together, these results

suggest that analgesia-related actions of GABAergic interneurons in the PAG are mediated through postsynaptic GABA_A receptors.

Since GABA immunoreactivity is intense in regions which contain cells retrogradely labeled from the NRM, specifically, the lateral and ventrolateral PAG (Abols and Basbaum 1981; Beitz *et al.* 1983; Carlton *et al.* 1983; Fardin *et al.* 1984; Gallager and Pert 1978; Reichling *et al.* 1988), our findings are consistent with the hypothesis that GABAergic neurons contact the output neurons of the PAG. In fact, we have recently demonstrated that GABA-immunoreactive terminals make synaptic contact with PAG neurons that project to the NRM (Reichling and Basbaum 1987, Reichling *et al.* 1988). These data support the suggestion that local GABAergic interneurons inhibit the PAG-NRM projection neuron. The other main feature of the proposed circuit, *i.e.*, that opioid peptide terminals are presynaptic to the GABAergic interneurons remains to be demonstrated anatomically. To this end, it will be particularly important to determine whether terminals containing different classes of opioid peptides have different anatomical relationships with GABA-containing neurons of the PAG. Pharmacological data discussed above suggest that the three classes of opioid peptides have different effects on PAG antinociceptive circuitry. It is thus very likely that anatomical studies will reveal significant differences in their synaptic relationships with GABAergic neurons (and possibly with PAG output cells).

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Fig. 1. These color darkfield photomicrographs illustrate the distribution of β -endorphin- (A,B,C), enkephalin- (D,E,F), and dynorphin- (G,H,I) immunoreactive fibers in the PAG. In this darkfield illumination, immunoreactive fibers appear golden-orange. The top row of plates shows the rostral PAG at the level of the third nerve nucleus; the middle row shows the PAG at the level of the fourth nerve nucleus; the bottom row shows the caudal PAG at the level of the decussation of the superior cerebellar peduncle. Arrowheads indicate distinctive features of the staining patterns, which are described in the text.

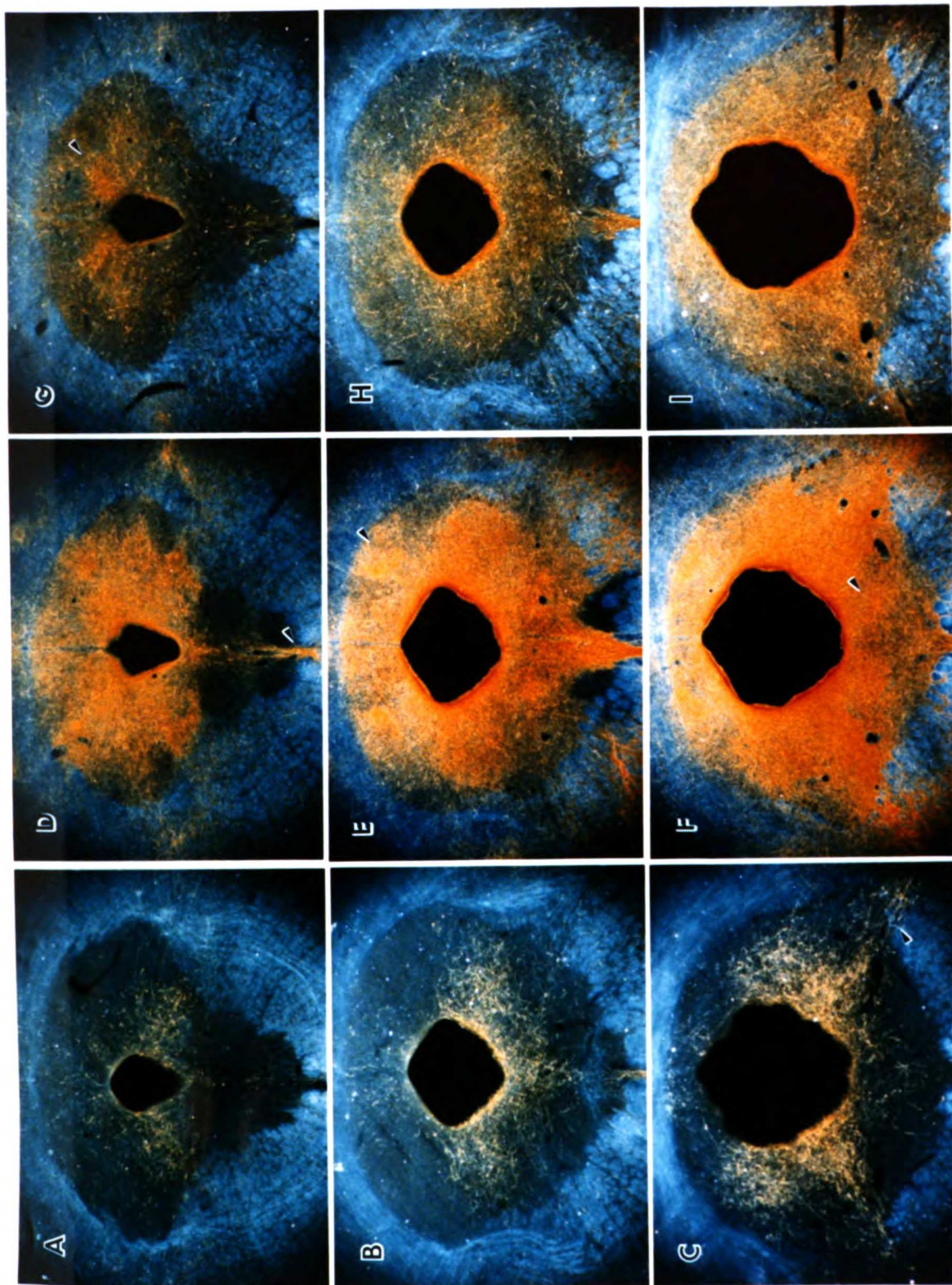
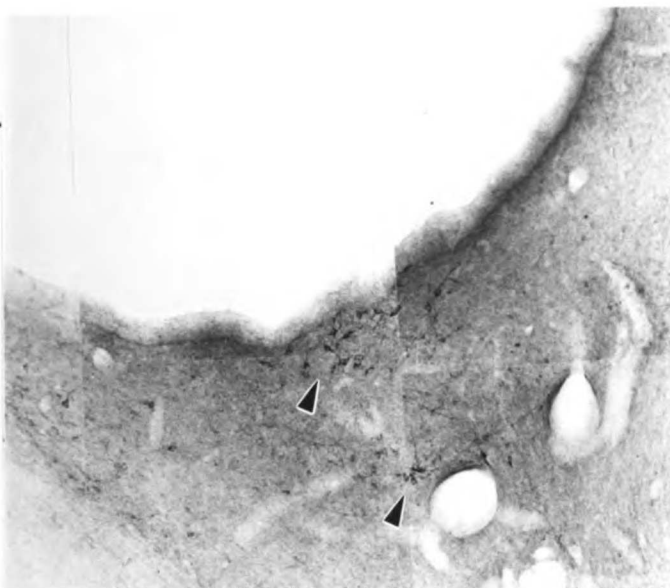
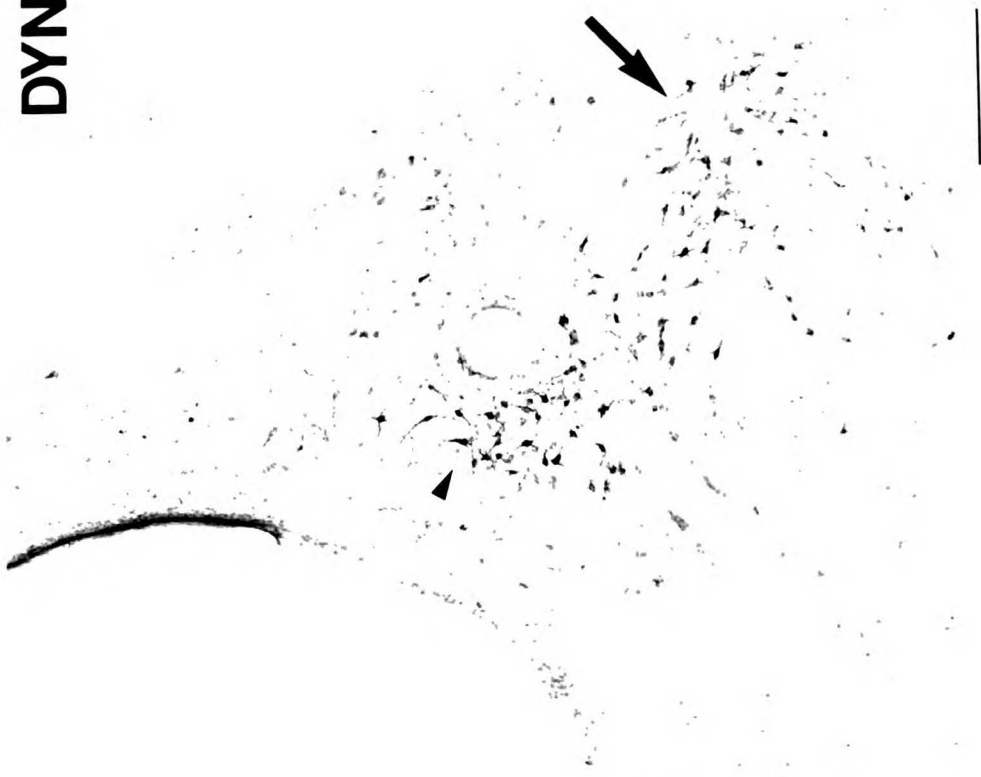


Fig. 2. These low-magnification bright-field photomicrographs illustrate the patterns of opioid-immunoreactive cell bodies in the caudal PAG. The left-hand plate shows enkephalin-immunoreactive cell bodies (arrowheads) in the ventrolateral PAG. Dynorphin-immunoreactive cell bodies, shown in the right-hand plate, are also found in the ventrolateral PAG (arrowheads), but they are located farther from the aqueduct and more ventrally than are enkephalin cells. The cluster of dynorphin cells also extends ventrolaterally into the nucleus cuneiformis (arrow). Scale bar = 0.5 mm.

DYN



ENK

Fig. 3. These photomicrographs show opioid-immunoreactive cell bodies in the dorsal PAG, at the level of the fourth nerve nucleus. Enkephalin-immunoreactive neurons (left-hand plate) are found in bilateral clusters dorsal to the aqueduct (AQ), but are rarely found on the midline. Dynorphin-immunoreactive cell bodies (right-hand plate) are rare in the dorsal PAG. Scale bar = 100 μ m.

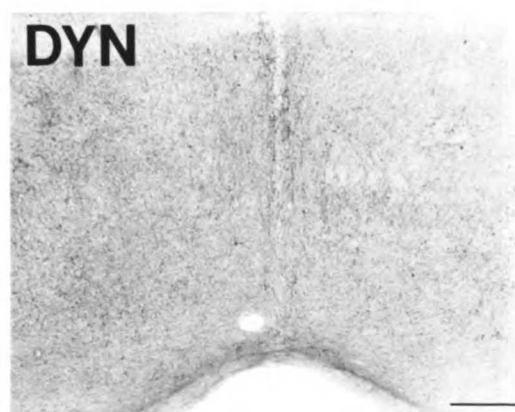
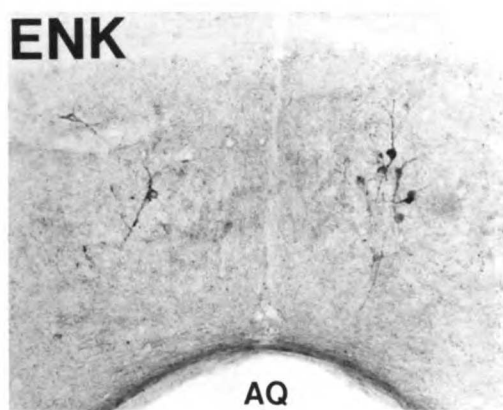


Fig. 4. These photomicrographs show GABA-immunoreactive elements in the PAG. **A.** Low-magnification photomicrograph of GABA-immunoreactive fibers in the PAG at the level of the decussation of the superior cerebellar peduncle (Bright-field illumination was used since dark-field illumination does not satisfactorily represent variations in the intensity of GABA fiber staining). GABA-immunoreactive fibers are most dense in the ventrolateral PAG, near the aqueduct (arrow). Scale bar = 1 mm. **B.** This higher-magnification photomicrograph of a region ventrolateral to the aqueduct (AQ) shows that individual GABA-immunoreactive fibers appear as numerous small, dark punctae (arrowheads). Scale bar = 100 μ m. **C.** This plate shows GABA-immunoreactive cell bodies (arrows) in the dorsolateral PAG at the level of the fourth nerve nucleus. Scale bar = 100 μ m.

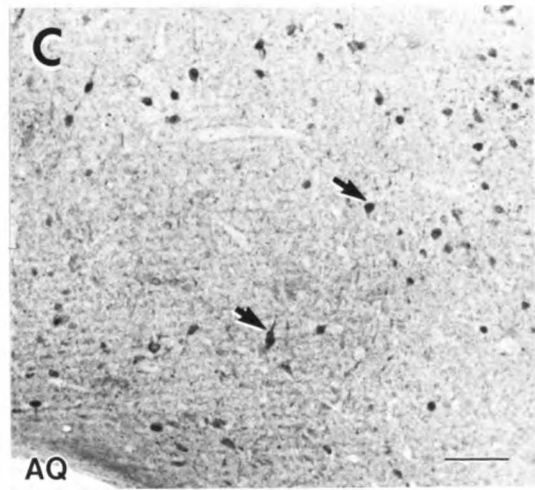
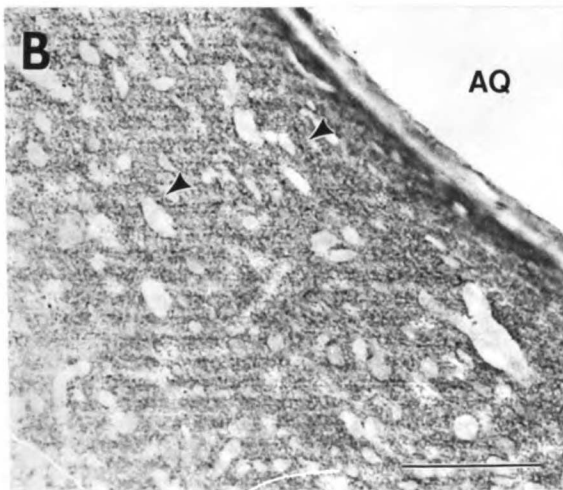
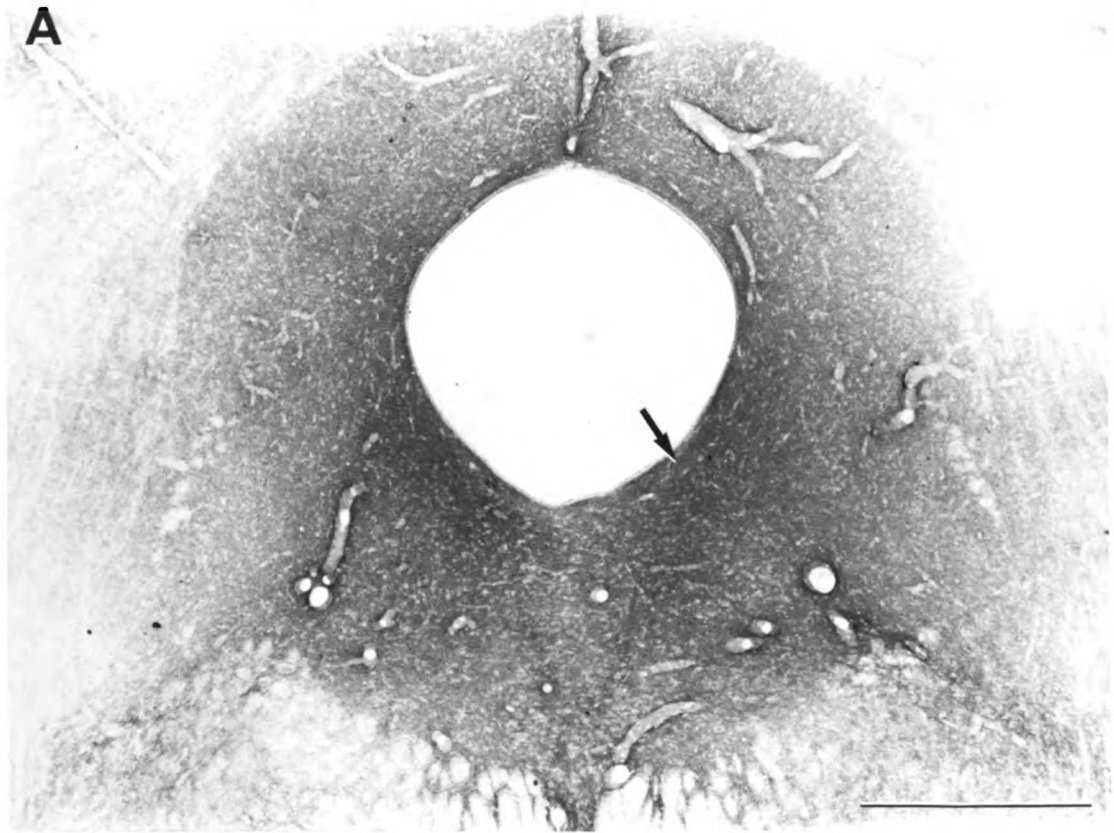
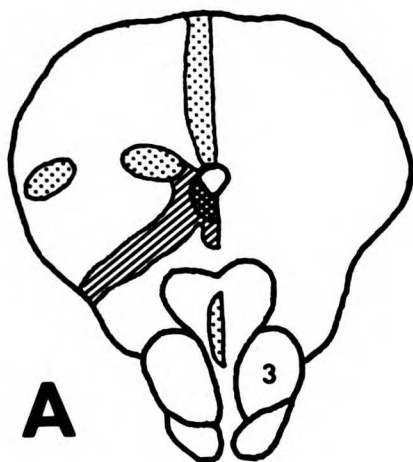


Fig. 6. Schematic illustration of the patterns of β -endorphin- and enkephalin-immunoreactive fibers and cell bodies at three rostrocaudal levels of the PAG. The density of cross-hatching in the left half of each drawing represents the relative density of immunoreactive fibers. Black dots in the right half of each figure represent 5 immunoreactive cell bodies in a typical 50 μ m section. The drawings are adapted from Paxinos and Watson (1986); numbers indicate the distance rostral to interaural zero according to that atlas.

β -END



ENK

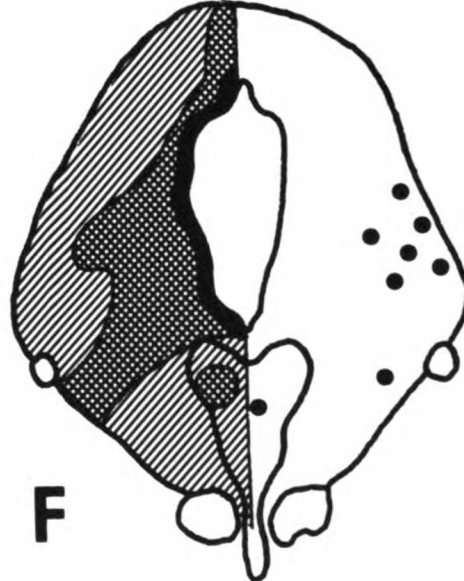
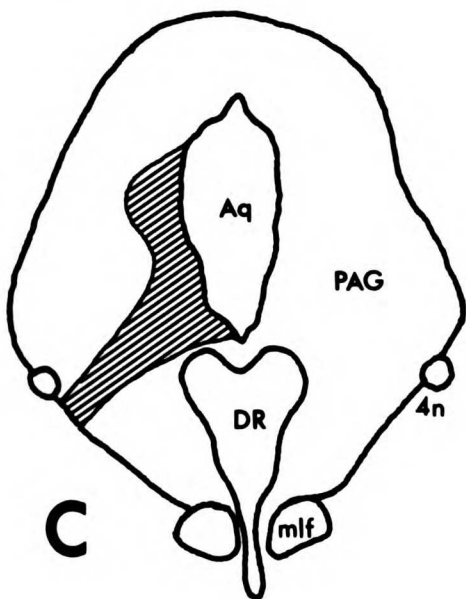
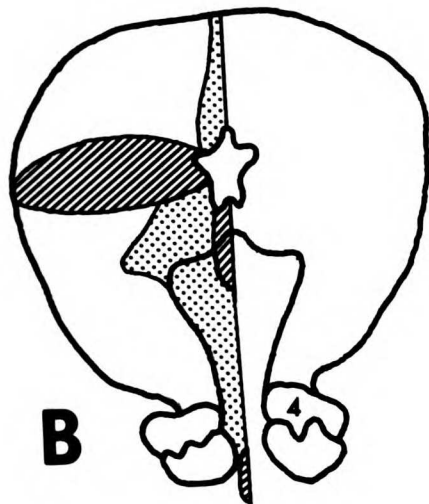
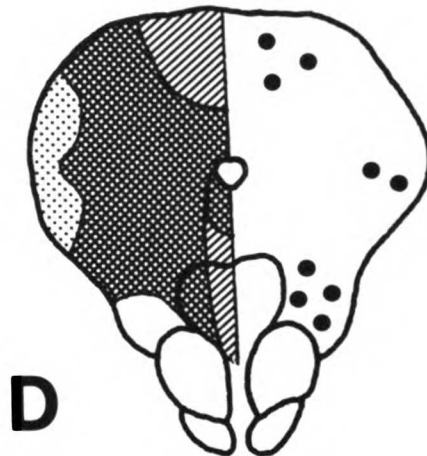
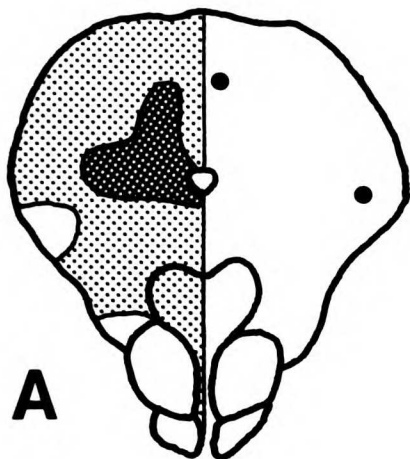


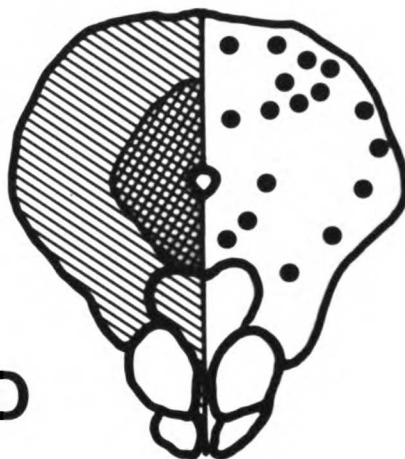
Fig. 7. Schematic illustration of the patterns of dynorphin- and GABA-immunoreactive fibers and cell bodies at three rostrocaudal levels of the PAG. The density of cross-hatching in the left half of each drawing represents the relative density of immunoreactive fibers. Black dots in the right half of each figure represent 5 immunoreactive cell bodies in a typical 50 μ m section.

DYN

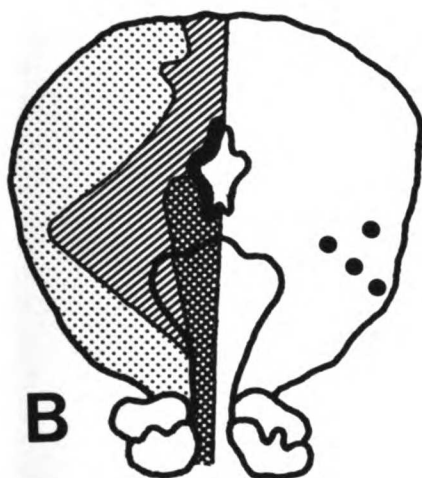


A

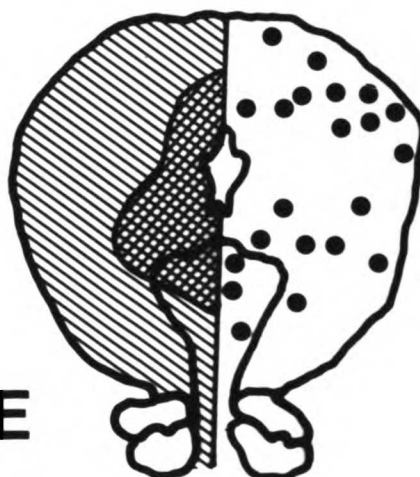
GABA



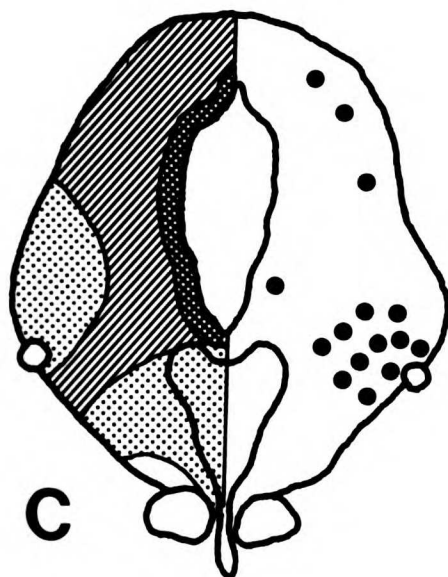
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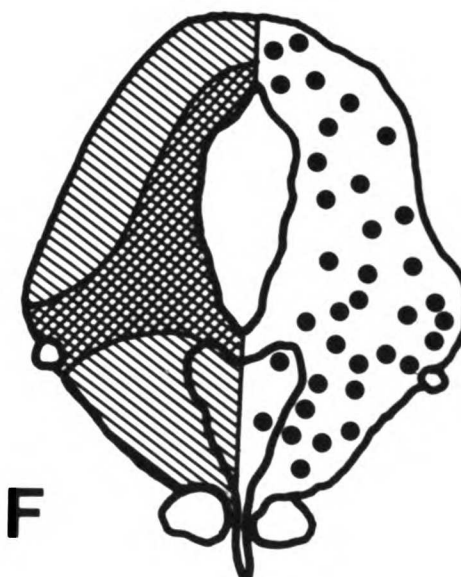
B



E



C



F

CHAPTER III

Axon Collaterals to the Hypothalamus and Thalamus from Periaqueductal Gray Neurons that Project to Raphe Magnus

David B. Reichling and Allan I. Basbaum

SUMMARY

Antinociceptive effects elicited from the midbrain may involve ascending, as well as descending, projections from the periaqueductal gray matter (PAG) and dorsal raphe nucleus (DRN). To investigate the relationship between these efferent pathways we performed a double-labeling retrograde tracer study using WGAapoHRP-Au and fluorescent tracers. One tracer was microinjected in the medullary nucleus raphe magnus (NRM), while another tracer was injected into one of several regions rostral to the PAG which have been implicated in nociceptive or antinociceptive processes. The results can be grouped into two categories according to the patterns of labeled neurons in the PAG and DRN. First, injections into the ventrobasal thalamus, lateral hypothalamus, amygdala, and cerebral cortex labeled neurons in the DRN but not in other regions in the PAG. Between 85 and 90% of these projection neurons were 5-HT-immunoreactive, and between 8 and 17% were also retrogradely labeled from the RVM. Second, only injections into the ventrobasal hypothalamus or medial thalamus labeled neurons in the PAG itself. The latter neurons were most numerous in the ventrolateral, lateral, and dorsal parts of the ipsilateral PAG. Injections in the medial thalamus, but not in the ventrobasal hypothalamus, also labeled neurons in the DRN. Between 13 and 20% of the neurons retrogradely labeled from these regions were also retrogradely labeled from the RVM. The existence of these rostrally-directed collaterals of the PAG-NRM projection complicate the interpretation electrical brain stimulation studies, and suggest the possibility that the medial thalamus and ventrobasal hypothalamus may be subject to antinociceptive controls ascending from the PAG.

INTRODUCTION

Most studies that address the antinociceptive function of the midbrain periaqueductal gray matter (PAG) emphasize its descending connections with neurons in the nucleus raphe magnus (NRM) of the rostral ventromedial medulla (RVM). Although

this view of the PAG as the upper tier in a descending antinociceptive system (Fields and Basbaum 1978; Basbaum and Fields 1984) has proved very useful in the study of bulbospinal inhibitory pathways, the PAG also has extensive rostral projections. For example, anterograde tracing studies demonstrated PAG projections to: hypothalamus (periventricular gray, dorsal area, dorsomedial, posterior, anterior, lateral, medial, ventromedial, mammillary, and supramammillary nuclei), thalamus (paracentral, centrolateral, paracentral, parafascicular, reticular, central medial, mediodorsal, rhomboid, and reuniens nuclei, and the ventrobasal complex), zona incerta, fields of Forel, pretectal area, and lateral habenula (Chi 1970; Hamilton 1973; Mantyh 1983a).

Since the PAG receives a variety of nociceptive inputs from the spinal cord dorsal horn and brainstem reticular formation, it has been suggested that ascending efferents from the PAG primarily relay nociceptive information (*c.f.* Mehler *et al.* 1969). Consistent with this idea, electrical stimulation of the PAG, in a number of species, including humans not only produces antinociception, but also a variety of pain-related reactions including hyperalgesia, vocalization, defense, fear, and panic, (Fardin *et al.* 1984b; Schmidek *et al.* 1971; Nashold *et al.* 1969). On the other hand, analgesia can be elicited from the PAG and from many of its more rostral targets either by electrical stimulation or narcotic microinjection. Conceivably, ascending projections from the PAG may contribute to the antinociceptive effects elicited by manipulations in the PAG itself. Such ascending controls might mediate effects such as anti-aversion or disruption of integrated reactions to painful stimuli.

The present study addresses the organization of ascending projections from the PAG and to relate these projections to the better characterized "antinociceptive" projection to the NRM. *In fact, some PAG neurons have axons that collateralize to both sides of the thalamus (Barbaresi et al. 1982), and some neurons in the PAG that project to the NRM collateralize to other parts of the medulla (Beitz et al. 1983).* Specifically, we used retrograde tracers to determine if the PAG-RVM pathway collateralizes to regions of

the forebrain that have been implicated in antinociceptive processes: the thalamic parafascicular nucleus, ventroposterior thalamus, ventrobasal hypothalamus, lateral hypothalamus, amygdaloid complex, medial or lateral frontal cortex, parietal sensorimotor cortex, or visual occipital cortex. Since the DRN has also been implicated in antinociception (Fardin *et al.* 1985b) and also projects to the NRM (in the rat) and to some of these forebrain areas, we assessed the contribution of dorsal raphe neurons to these pathways using 5-HT immunocytochemistry. A preliminary report of the results has been published (Reichling and Basbaum 1986)

METHODS

Adult male Sprague-Dawley rats (Bantin Kingman) were used in this study. Under pentobarbitol anesthesia the animals were placed in a stereotaxic frame, the skull was exposed, and a hole was drilled over the appropriate target for the tracer injection. The stereotaxic atlas of Paxinos and Watson (1986) was used to determine coordinates. Fourteen rats first received one stereotaxic microinjection (0.10-0.15 μ l) of a retrograde fluorescent tracer, either 5% aqueous True Blue (Sigma) or 5% Fluorogold in sterile saline (Fluoroprobe; Schmued and Fallon 1986), into the RVM; the injection was centered in the NRM. A second retrograde tracer, the colloidal gold-protein complex, WGAapoHRP-Au (Basbaum and Menetrey 1987), was then microinjected (0.05-0.15 μ l) into one site rostral to the PAG. The rostral target was either the thalamic parafascicular nucleus, ventroposterior thalamus, ventrobasal hypothalamus, lateral hypothalamus, amygdaloid complex, medial or lateral frontal cortex, parietal (sensorimotor) cortex, or occipital (visual) cortex.

To determine if smaller injections restricted to the NRM would produce results different from the larger injections of fluorescent tracer, three rats received the WGAapoHRP-Au tracer injection into the RVM. In these rats, the fluorescent tracer was injected into medial thalamus, ventromedial hypothalamus, or cortex.

Three to fourteen days after injection of the tracers, the rats were deeply anesthetized with pentobarbital and perfused transcardially with 100 ml 0.1 M phosphate buffered saline (PBS) followed by 500 ml of 4% paraformaldehyde in PBS. The brains were removed, cryoprotected in 30% sucrose PBS, and sectioned on a freezing microtome. The PAG was sectioned at 25 or 30 μm thickness; each injection site was sectioned at 100 μm .

Alternate sections of the PAG were silver-enhanced to make the colloidal gold tracer visible at the light microscopic level (Basbaum and Menetrey 1987). These sections were then mounted on uncoated glass slides and coverslipped with a medium that retards fading of fluorescent tracers (Dabco, Aldrich). By using a combination of ultraviolet epifluorescent illumination, and transmitted dark-field illumination, it was possible to simultaneously view the fluorescent tracers and the silver-enhanced WGAapoHRP-Au.

In some experiments, silver-enhanced sections of the PAG were stained by immunofluorescence for 5-hydroxytryptamine (5-HT). These sections were first incubated for 1/2 hr in a blocking solution of 3% normal goat serum (NGS) and 0.3% triton X-100 in PBS. The sections were next incubated overnight in rabbit anti-5-HT antiserum (IncStar) diluted 1:3000 in 1% NGS and 0.3% Triton X-100 in PBS. After several washes in PBS, the sections were incubated for 1 hour in fluorescein conjugated goat anti-rabbit IgG (Cappel), diluted 1:100. After washing, the sections were mounted and coverslipped. The locations of single- and double-labeled cells were plotted on *camera lucida* drawings.

RESULTS

Labeling from rostral ventromedial medulla injections

Tracer injections into the RVM consistently labeled neurons in the ventral, ventrolateral, lateral, and dorsal PAG. Very few retrogradely labeled neurons were found in the dorsolateral PAG. At levels of the PAG rostral to the fourth cranial nerve nucleus the dorsal group of cells persists, while cells in other parts of the PAG diminish in number.

Retrogradely labeled neurons in the dorsal raphe nucleus (DRN) were more common in the lateral part of the nucleus. The same pattern of retrograde labeling in the PAG was found when either WGAapoHRP-Au or fluorescent tracers were used. These results agree with previous reports of retrograde tracing experiments performed in rat (Beitz *et al.* 1983; Carlton *et al.* 1983; Gallager and Pert 1978) cat (Abols and Basbaum 1981) and monkey (Chung *et al.* 1983). *However, Fardin et al. (1984a) reported that neurons retrogradely labeled from the NRM were found only in the lateral DRN and dorsal to the aqueduct in the PAG of the rat. We found that these two regions where the largest-diameter (approximately 30 μ m) retrogradely labeled cell bodies*

Labeling from ventrobasal hypothalamus injections

Injection sites in the ventrobasal (VB) hypothalamus (figure 1) included the arcuate nucleus and ventromedial nuclei; there was usually some spread of tracer in the needle tract into the dorsomedial nucleus of the hypothalamus. The pattern of labeling produced by these injections was very similar to the distribution of neurons in the PAG that were retrogradely labeled from the RVM. Two thirds of the retrogradely labeled neurons were found ipsilateral to the injection site. Labeled neurons (figure 5) were found in the ventrolateral, lateral, and dorsal PAG, but few were found in the dorsolateral PAG. The dorsal group of cells was most prominent at rostral levels of the PAG. Finally, the DRN contained few labeled neurons -- less than 2% of neurons retrogradely labeled from the VB hypothalamus were 5-HT-immunoreactive.

Figure 2 illustrates examples of single- and double- labeled neurons. Approximately 13% of cell bodies retrogradely labeled from the VB hypothalamus were also labeled from the RVM. These double-labeled neurons appeared to be evenly distributed among the single-labeled neurons.

Labeling from lateral hypothalamus injections

Tracer injections in the lateral hypothalamus (figure 1) were limited to the lateral hypothalamic area. Neurons retrogradely labeled from these injections were found only in

the DRN. An example of 5-HT-immunoreactive retrogradely labeled neurons is shown in figure 4. Most retrogradely labeled neurons were found in the medial DRN, and in the lateral parts of the nucleus ipsilateral to the injection site. The more laterally situated cells are most common at mid rostrocaudal levels of the DRN (at the level of the fourth cranial nerve nucleus); at more rostral levels, retrogradely labeled cells are found only on the midline; 89% of the retrogradely labeled neurons were also 5-HT-immunoreactive. Approximately 10% of neurons retrogradely labeled from the lateral hypothalamus were also retrogradely labeled by tracer injections in the RVM. These double-labeled neurons were located randomly among the population of DRN neurons retrogradely labeled from the lateral hypothalamus.

Labeling from medial thalamus injections

Tracer injections in the medial thalamus (figure 1) were centered in the nucleus parafascicularis (Pf). Although the passage of the fasciculus retroflexus through the medial part of Pf is a convenient histological landmark, nuclear boundaries in the rat medial thalamus are somewhat difficult to distinguish. It is, thus, likely that some tracer spread into adjacent medial thalamic nuclei, particularly the central medial nucleus (CM) and centrolateral nucleus (CL) of the internal medullary lamina.

Approximately 75% of neurons retrogradely labeled from the medial thalamus were located in the PAG ipsilateral to the injection site (see figure 3, plate A, and figure 4). All regions of the PAG, except for the rostral dorsolateral sector, contained large numbers of labeled neurons. This distribution was similar to that produced by injections in the ventrobasal hypothalamus. In contrast to ventrobasal hypothalamus injections, however, we found many more retrogradely labeled neurons in the DRN -- after medial thalamus injections approximately 6% of all neurons labeled from the medial thalamus were immunoreactive for 5-HT

Approximately 20% of cell bodies retrogradely labeled from the medial thalamus were also labeled by injections into the RVM. Unlike the results after injections into the

ventrobasal hypothalamus, these double-labeled cell bodies are not evenly distributed among the population of single-labeled neurons. Among neurons retrogradely labeled from the medial thalamus, 22% were double-labeled on the side ipsilateral to the injection site; 10% were double-labeled on the contralateral side; the latter were most common in the lateral part of the PAG. There was also a pronounced change in the proportion of double labeled neurons along the rostrocaudal axis of the PAG. At the most caudal levels of the PAG only about 4% of cell bodies labeled from the medial thalamus were also labeled from the RVM; at the level of the fourth cranial nerve nucleus this number was greater than 30%.

Labeling from lateral thalamus injections

Tracer injections in the lateral thalamus (figure 1) were centered in the ventroposterior nuclei lateralis and medialis, but a small amount of tracer may have spread medially into the posterior nuclear group, or dorsally along the needle track into the lateral posterior and laterodorsal nuclei. These injections labeled a small number of neurons which were located in the DRN (figure 5); 85% of these neurons were 5-HT-immunoreactive. In the caudal DRN retrogradely labeled neurons were most common in the ventromedial and lateral parts of the nucleus. At the level of the fourth cranial nerve nucleus, the location of labeled neurons shifted to more dorsal parts of the DRN. Approximately 17% of neurons labeled from the lateral thalamus were also labeled from the RVM, and these double-labeled neurons appeared to be distributed evenly among the population of neurons labeled from the lateral thalamus.

Labeling from telencephalic injections

Tracer injections placed in the medial (figure 1) or lateral parts of the frontal cortex, amygdala, parietal cortex, and occipital cortex all produced very similar results. Retrogradely labeled neurons were found only in the DRN; between 87 and 93% were 5-HT-immunoreactive. In the caudal DRN, caudal to the decussation of the superior cerebellar peduncle, retrogradely labeled neurons were most common in the medial part of the DRN; at mid rostrocaudal levels of the DRN, labeled neurons were more common in

the lateral parts of the nucleus. The proportion of neurons projecting to these regions that were double-labeled by injections in the RVM ranged between 8 and 17%. Double-labeled neurons did not cluster within the distributions of single-labeled neurons.

DISCUSSION

Previous studies have demonstrated that single PAG neurons can have axon collaterals in two nearby areas of the brain (Barbaresi *et al.* 1982; Beitz *et al.* 1983), and the present results show that some PAG axons send collaterals in opposite, rostral and caudal, trajectories. Our results can be grouped into two categories according to the patterns of labeled neurons in the PAG and DRN after forebrain injections of retrograde tracer. First, injections into the ventrobasal thalamus, lateral hypothalamus, amygdala, and cerebral cortex labeled neurons in the DRN but not in other regions in the PAG. Between 85 and 90% of these projection neurons were 5-HT-immunoreactive, and between 8 and 17% of the total were also retrogradely labeled from the RVM. Second, only injections into the ventrobasal hypothalamus or medial thalamus labeled neurons in the PAG itself. These labeled neurons were most numerous in the ventrolateral, lateral, and dorsal parts of the ipsilateral PAG. Injections in the medial thalamus, but not in the VB hypothalamus, also labeled neurons in the DRN. Between 13 and 20% of the neurons retrogradely labeled from these regions were also retrogradely labeled from the RVM.

The presence of neurons in the PAG and DRN that send axon collaterals to the RVM and also to regions rostral to the PAG is a major complication for studies that use electrical brain stimulation to investigate antinociceptive systems. Electrical stimulation of areas rostral to the PAG, especially the medial thalamus and ventrobasal hypothalamus, could antidromically activate axons that originate from cell bodies in the PAG and DRN. Consequently, after reaching the PAG, action potentials could be propagated orthodromically in axon branches that terminate in the RVM. Based on these results, it seems that the use of electrical stimulation could lead to the incorrect conclusion that under

physiological conditions, the neuronal circuitry of these rostral regions can activate descending antinociceptive controls. Of course, stimulation-produced analgesia (SPA) elicited from the RVM might also be complicated by electrical activation of these bifurcating axons. Specifically, action potentials travelling antidromically from the NRM to the PAG could activate PAG projections to a variety of rostral targets.

MEDIAL DIENCEPHALON INJECTIONS

The PAG, medial thalamus, and ventromedial hypothalamus are part of a continuous periventricular zone in which morphine microinjection elicits analgesia (Herz *et al.* 1970; Mayer and Price 1976; Pert and Yaksh 1974; Yaksh *et al.* 1976; Yaksh and Rudy 1978). This zone overlaps considerably with the region which supports SPA in the rat (Mayer and Price 1976; Mayer and Liebeskind 1974). This region receives spinothalamic and spino-reticulo-thalamic somatosensory inputs, and probably contributes to the affective components of pain.

Ventrobasal hypothalamus

Retrograde tracer injections labeled neurons in the ventrolateral, lateral, and dorsal PAG, but few neurons were labeled in the DRN. This observation is consistent with a previous study in the cat (Smith and Flynn 1980).

The arcuate nucleus, in the ventrobasal hypothalamus (VBH), is the major source of β -endorphin neurons in the brain (Finley *et al.* 1981). Lesions of the VBH that deplete β -endorphin in the brain (Millan *et al.* 1984), lower nociceptive thresholds, reduce stress-induced analgesia, and block PAG stimulation-produced analgesia (Kelsey *et al.* 1986; Millan *et al.* 1983; Millan *et al.* 1986; Vidal and Jacob 1980). Conversely, electrical stimulation of the VBH produces analgesia (Goodman and Holcombe 1976; Rhodes and Liebeskind 1978; however, see Carstens 1986). The VBH projection to the PAG, which includes β -endorphin, enkephalin, and excitatory amino acid components (Beart *et al.* 1988; Bloom *et al.* 1978; Yamano *et al.* 1986) is the largest descending input to the PAG

(Beitz 1982; Marchand and Hagino 1983; Meller and Dennis 1986). Conceivably, activation of the VBH produces analgesia by initiating descending controls via the PAG. In fact, electrical stimulation of the VBH produces naloxone-reversible inhibition of PAG neurons (Strahlendorf *et al.* 1982) and excitation of neurons that project from the PAG to the NRM (Sakuma and Pfaff 1980). Furthermore, stimulation of the VBH excites NRM-spinal projection neurons in rat (Lumb and Morrison 1986), and inhibits spinal dorsal horn nociceptive neurons in rat and cat (Carstens 1982; Carstens 1986; Mokha *et al.* 1987).

Our observation that many PAG-NRM projection neurons send axon collaterals to the VBH suggests that, in addition to activating descending controls from the PAG, the VBH might, itself, be the target of nociception-related controls. In fact, the activity of many VBH neurons is modulated by systemically administered morphine (Dafny 1980; Kerr *et al.* 1974; Lakoski and Gebhart 1984). Since opiate binding sites (especially mu and delta) are relatively scarce in the VBH (Mansour *et al.* 1987), the opioid effect is probably mediated through PAG circuitry. In fact, microinjection of morphine in the PAG inhibits VBH neurons (Lakoski and Gebhart 1984).

Since neurons in the VBH do not have obvious peripheral receptive fields (Dafny *et al.* 1970; Sakuma and Pfaff 1982), PAG inhibition of VBH neurons probably does not simply interrupt nociceptive transmission. Rather, modulation of VBH activity might disrupt the affective reactions to noxious stimuli. A recent study by Pott *et al.* (1987) supports this hypothesis. Affective defense was elicited in cats by electrical stimulation of the VBH. Concurrent electrical stimulation in different zones of the PAG either suppressed or facilitated the behavior. Since the suppression was blocked by naloxone microinjected through the PAG electrode, and since microinjected D-Ala₂-Met₅-enkephalin also suppressed the behavior, endogenous opioids are probably involved. In this way, descending inhibition of spinal nociceptive transmission might be complemented by an ascending suppression of integrated reactions to noxious stimuli.

Medial thalamus

Much of the literature concerning the contribution of the medial thalamus to nociceptive processing has not distinguished among the individual nuclei. Therefore, when necessary, we use the term "medial thalamus" to refer to the intralaminar region that includes parafascicularis (Pf), central medial, centrolateral, and centre médian (in large mammals) thalamic nuclei, without implying that this is a homogeneous group of nuclei.

The intralaminar nuclei of the medial thalamus receive nociceptive inputs from the spinal cord and from the brainstemreticular formation. Most studies emphasize the contribution of intralaminar nuclei to aspects of nociception other than the spatial or temporal discrimination of noxious stimuli. Through projections to "limbic" structures, including much of the cerebral cortex, these medial thalamic sites are presumed to generate affective components of pain sensation rather than the discriminative components. Consistent with this idea, large lesions of the medial thalamus do not alter thresholds for nociceptive reflexes (Bevan and Pert 1975; Delacour 1971; Finger and Frommer 1970; Yaksh *et al.* 1977; Yeung *et al.* 1975), but may abolish escape reactions to electrical stimulation (Mitchell and Kaelber 1967), disrupt conditioned avoidance responses (Delacour 1971; Marburg 1973), and reduce chronic pain in human (Sano 1979; Sugita *et al.* 1972; Urabe and Tsubokawa 1965). Consistent with these lesion studies, microinjection of morphine in the medial thalamus can produce analgesia (Mayer and Price 1976; Pert and Yaksh 1974). In addition, ionophoretically applied morphine modulates the spontaneous activity Pf neurons in rat (Reyes-Vasquez and Dafny 1982), and iontophoretically applied D-ala-D-leu-enkephalin inhibits heat-evoked activity in the nucleus lateralis anterior of rat thalamus (Hill and Pepper 1978).

It is of interest, however, that analgesia also results from stimulation of medial thalamus, in rat and monkey (Mayer and Liebeskind 1974; Mayer and Price 1976; Oleson *et al.* 1980; Rhodes and Liebeskind 1978), and of centre median-Pf in humans for clinical pain (Andy 1980; Richardson and Akil 1977; Thoden *et al.* 1979). A possible explanation

for this somewhat paradoxical analgesic effect, is that medial thalamic SPA is an artifact of the antidromic activation of PAG neurons that send axon collaterals to both the medial thalamus and the NRM.

Since, electrical stimulation of the DRN inhibits spontaneous and tail pinch-evoked neuronal activity in Pf of rat through a serotonergic pathway (Andersen and Dafny 1983; Ishida and Kitano 1977), and since microinjection of 5-HT has a similar effect on Pf activity (Andersen and Dafny 1982), it has been proposed that Pf neurons are subject to antinociceptive controls from the DRN. However, these manipulations also inhibit spontaneous activity and therefore, this inhibition might not be specifically antinociceptive. We found that only a minority of PAG axons that project to medial thalamus are serotonergic; thus, the effects of PAG/DR stimulation are probably mediated predominantly by non-serotonergic mechanisms. In fact, Andersen (1986) found that stimulation in and around the PAG produces IPSPs in Pf neurons that suppress footshock-induced EPSPs. It was not determined if this effect was associated with behavioral antinociception.

TARGETS OF THE DORSAL RAPHE NUCLEUS

Consistent with previous reports, we found that neurons in DRN project to the lateral thalamus, lateral hypothalamus, amygdala, frontal cortex, sensorimotor cortex, and visual cortex (Azmitia and Segal 1978; Conrad *et al.* 1974; Moore *et al.* 1978); PAG neurons outside the boundaries of the DRN were not labeled by tracer injections into those regions. Between 80 and 90% of DRN retrogradely labeled from these injection sites were 5-HT immunoreactive; since only 25-50% of DRN neurons are serotonergic (Moore 1981; Descarries *et al.* 1982), a greater proportion of serotonergic, than non-serotonergic, neurons contribute to these rostral projections.

Consistent with previous studies, DRN neurons retrogradely labeled from the RVM injections were most common in the lateral DRN (Beitz 1982; Fardin *et al.* 1984a). In contrast, neurons retrogradely labeled by forebrain injections are, for the most part, located

more rostrally and more medially. Although different patterns of labeled neurons were produced in the DRN by the different forebrain injections, a regular "encephalotopic" organization of the DRN (Imai *et al.* 1986; Waterhouse *et al.* 1986) was not apparent.

Typically, axons originating in the DRN are highly collateralized (*c.f.* DeOlmos and Heimer 1980); our results indicate that at least 8-17% of DRN neurons that project to the forebrain regions studied, have axon collaterals that terminate in the RVM. Fallon and Loughlin (1982) found that neurons in the medial part of the DRN had more highly collateralized axons (terminating in forebrain structures) than did neurons in the lateral DRN. We did not find that double-labeled neurons were more common medially, perhaps because most neurons that project to the NRM are located in the lateral DRN.

It has been suggested that the DRN contributes to SPA elicited from sites in and near the PAG. For example, Fardin *et al.* (1984b) found that analgesia, unaccompanied by aversive side effects, could be elicited from the dorsal part of the DRN, a region that gives rise to an extensive system of ascending serotonergic projections. If ascending projections from the DRN are involved in antinociceptive processes, it seems likely that collaterals from the PAG-NRM would be involved. However, as discussed below, our data along with a variety of other studies, provide little evidence for a specific antinociceptive function of the DRN ascending projections.

Lateral Thalamus

Our observation that the lateral thalamus receives projections only from the DRN and not from the PAG itself is consistent with other studies in the rat (Mantyh 1983) and cat (Chi 1970; Hamilton and Skultety 1970), but differs from the observation by Barbaresi *et al.* (1982) of a large projection from the lateral PAG to ventrobasal complex in cat.

Electrical stimulation of the ventrobasal thalamic nuclei has been used to relieve clinical pain in humans (Dieckmann and Witzmann 1982; Hosobuchi *et al.* 1973; Mazars *et al.* 1979; Tsubokawa *et al.* 1982; Turnbull *et al.* 1980; Young *et al.* 1985). Stimulation of the ventral posterior lateral nucleus (VPL) excites medullary raphespinal neurons in cat

(Tsubokawa *et al.* 1981) and monkey (Willis *et al.* 1984), and inhibits spinothalamic tract (STT) neurons (Gerhart *et al.* 1983). This spinal inhibition could result from antidromic activation of STT neurons that have collaterals in the RVM or PAG (Kevetter and Willis 1983; Harmann *et al.* 1988), or from antidromic activation of neurons we observed in the DRN that have collaterals in the RVM. In fact, we found that the cell bodies of neurons retrogradely labeled from VPL are located in the dorsal part of the nucleus -- the region from which Fardin *et al.*, (1984b) elicited "pure" analgesia.

Since the VPL has no known descending projections, analgesic effects of electrical stimulation in the lateral thalamus stimulation might reflect the activation of intrathalamic or cortical projections of the ventrobasal complex (*c.f.* Benabid 1983). In fact, there are several differences in the character of analgesia elicited from the PAG and from the lateral thalamus in human (reviewed by Fields 1987). Another explanation for human lateral thalamic SPA, based on our data in rat, is that electrical stimulation in the lateral thalamus antidromically activates collaterals of the projection from the DRN to RVM. Such explanations, based on anatomical studies in animals, must be tentative since electrical stimulation of the lateral thalamus fails to produce analgesia in rat (Mayer and Liebeskind 1974; Morgan and Franklin 1988) or in monkey (Goodman and Holcombe 1976; Oleson *et al.* 1980; Schmidek *et al.* 1971).

Lateral Hypothalamus

Electrical stimulation of the lateral hypothalamus can produce analgesia in the rat (Balagura and Ralph 1973; Behbehani *et al.* 1988; however, see Mayer and Liebeskind 1974) and is antiaversive in rat (Cox and Valenstein 1965). Analgesia produced by electrical stimulation in the lateral hypothalamic is reduced by systemically administered opiate antagonist (Carr & Uysal 1985), by micronjection of a glutamate antagonist, or serotonin antagonist in the NRM (Aimone and Gebhart 1988). These observations implicate descending pathways in lateral hypothalamic SPA. However, since the lateral hypothalamus is traversed by the medial forebrain bundle (MFB), analgesic effects of

electrical stimulation in this region are particularly difficult to interpret. The MFB not only contains afferents and efferents of the lateral hypothalamus, but also of cerebral cortex, VB hypothalamus, parafascicularis, PAG, DRN, and NRM (Nieuwenhuys *et al.* 1982). Therefore, by activating fibers of passage in the MFB, electrical stimulation in lateral hypothalamus might activate any of these systems, including the collateral pathways we observed in this study. In fact, Aimone and Gebhart (1987) were able to elicit analgesia by electrical stimulation in the lateral hypothalamus, but not by microinjection of glutamate (however, see Behbehani *et al.* 1988).

Telencephalon

In humans, the affective-motivational aspect of pain sensation is apparently generated, in part, by telencephalic structures. Thus, in some chronic pain patients, frontal lobotomy or amygdectomy eliminates the objectional qualities of pain without reducing the sensation of noxious stimuli (Barber 1959; Freeman and Watts 1950; Hebben *et al.* 1985)). Some of these patients, in fact, have a lowered threshold for pain sensation. This latter effect is more readily observed in animals: functional lesion of the frontal cortex in rat, or amygdala in monkey, reduces the threshold for nociceptive responses (Bagshaw and Pribram 1968; Cooper 1976). Conversely, electrical stimulation of frontal cortex in rat, or amygdala in monkey, can produce antinociception (Chin *et al.* 1976; Hardy 1985). This effect could be produced by activation of descending inhibitory systems via orthodromic activation of projections from the cortex to the PAG, or antidromic activation of collaterals from the PAG-NRM pathway. The ability of somatosensory cortex stimulation to modulate spinal sensory transmission is well-known (Hagbarth and Kerr 1954; Lundberg *et al.* 1963). However, since such cortical stimulation does not specifically inhibit noxious-evoked activity in STT neurons (Coulter *et al.* 1974), it cannot be concluded that such descending inhibition is antinociceptive.

On the other hand, telencephalic structures might be the target of antinociceptive controls. Conceivably, the ascending collateral arm of the DRN-NRM pathway produces

antinociception. Wang and Aghajanian (1977) showed that electrical stimulation in the DRN does, in fact, inhibit activity of neurons in the amygdala. Since only spontaneous activity was studied, however, this inhibition may not have been specifically antinociceptive. Pucilowski *et al.* (1985) injected 5-HT into the amygdala and observed a suppression of aggressive behavior, but not of pain sensitivity. Thus, the relevance of this ascending pathway to antinociception is dubious.

To help further assess the possibility that ascending projections from the DRN are relevant to nociception, we made an injection of tracer into the occipital cerebral cortex. If collateral projections from the DRN-NRM pathway were specifically concerned with nociceptive processing, then we would expect that they would terminate only in regions that have been implicated in nociceptive processing. Presumably, the area of visual cortex we injected is not involved in nociception or antinociception. The results we obtained were very similar to the results of other cortical injections. Thus, despite the fact that these ascending projections from the DRN have collaterals that terminate in the RVM, we cannot conclude that they are involved in any function related specifically to nociception. Instead, it may be that these DRN projections mediate a less specific inhibition of sensory transmission. For instance, serotonergic projections from the DRN inhibit the transmission of visual information from the lateral geniculate nucleus to the cerebral cortex (Yoshida *et al.* 1984).

Conclusion

These experiments demonstrate that the projection from the PAG to RVM contains a significant number of axons that send collaterals rostrally. Thus, electrical stimulation of the neurons that project from the PAG to the NRM necessarily activates a variety of ascending collaterals. Those collaterals from the DRN-RVM projection are part of a divergent system of serotonergic fibers that probably mediate a rather general modulation of activity in the brain. On the other hand, in light of previous anatomical, pharmacological, physiological, and behavioral investigations, it seems that the ascending collaterals from the

PAG-RVM projection to ventrobasal hypothalamus and medial thalamus may be involved in modulating nociceptive transmission or in mediating some other nociceptive reactions. These projections probably contribute to a spectrum of effects elicited by any antinociceptive manipulation in the PAG.

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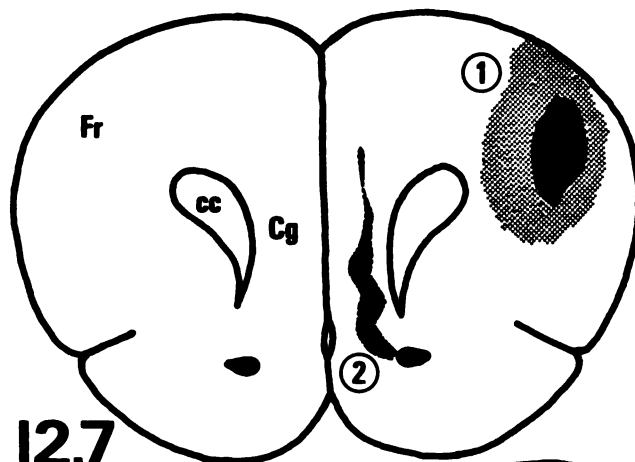
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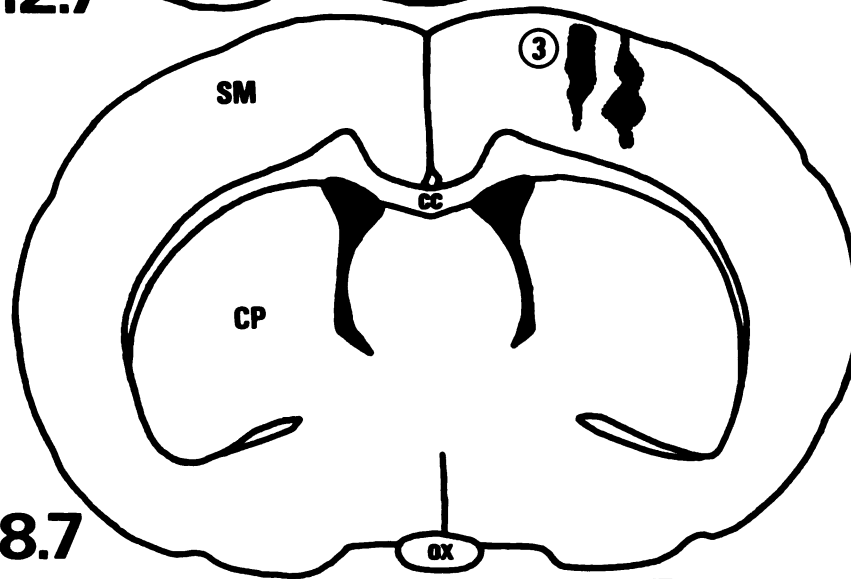
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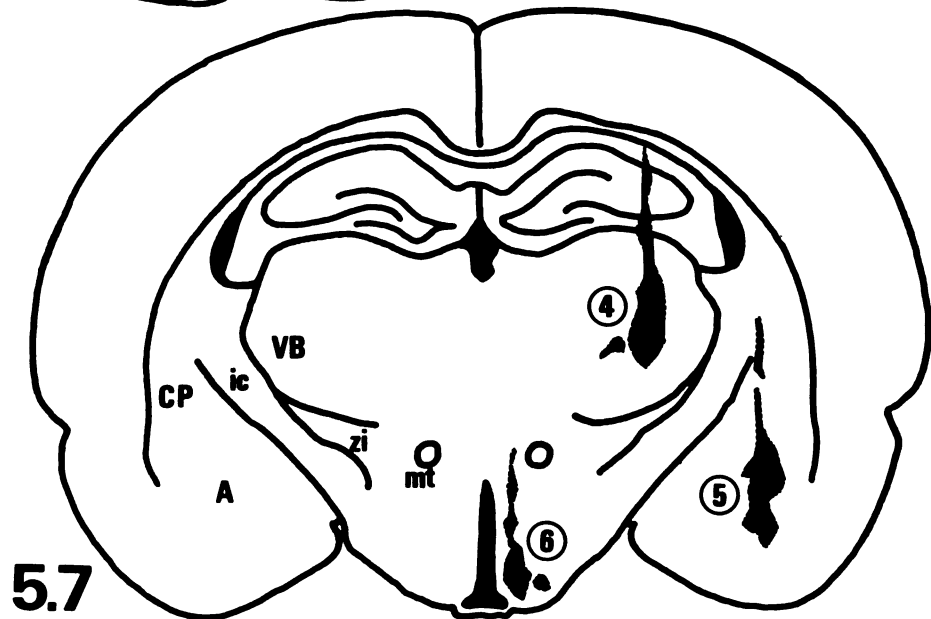
Fig. 1. This figure schematically illustrates the locations of typical injections of retrograde tracer into the forebrain of different rats. 1. frontal cortex (lateral); 2. frontal cortex (medial); 3. parietal cortex; 4. ventrobasal thalamus; 5. amygdala; 6. ventrobasal hypothalamus; 7. medial thalamus; 8. lateral hypothalamus; 9. occipital cortex. The fluorescent tracer, True Blue, was injected at site 1; WGAapoHRP-Au was injected at the other sites. Numbers next to each drawing indicate the distance (in mm) rostral to interaural zero according to the atlas of Paxinos and Watson (1986). Abbreviations: A, amygdala; cc, corpus callosum; Cg, cingulate gyrus; CP, caudate-putamen; fr, fasciculus retroflexus; Fr, frontal cortex; ic, internal capsule; IC inferior colliculus; mt, mammillothalamic tract; O, occipital cortex; ox, optic chiasm; PAG, periaqueductal gray; py, pyramidal tract; VB, ventrobasal thalamus; zi, zona incerta.



12.7



8.7



5.7

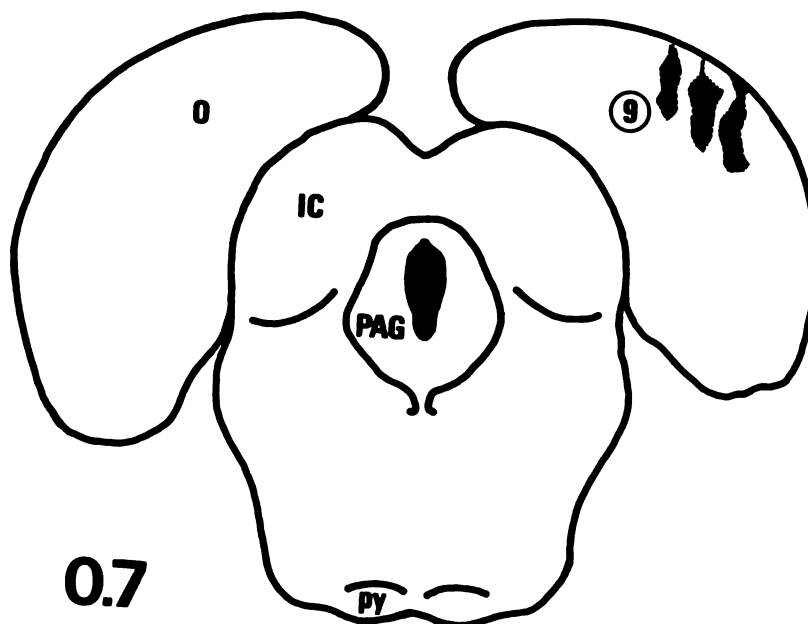
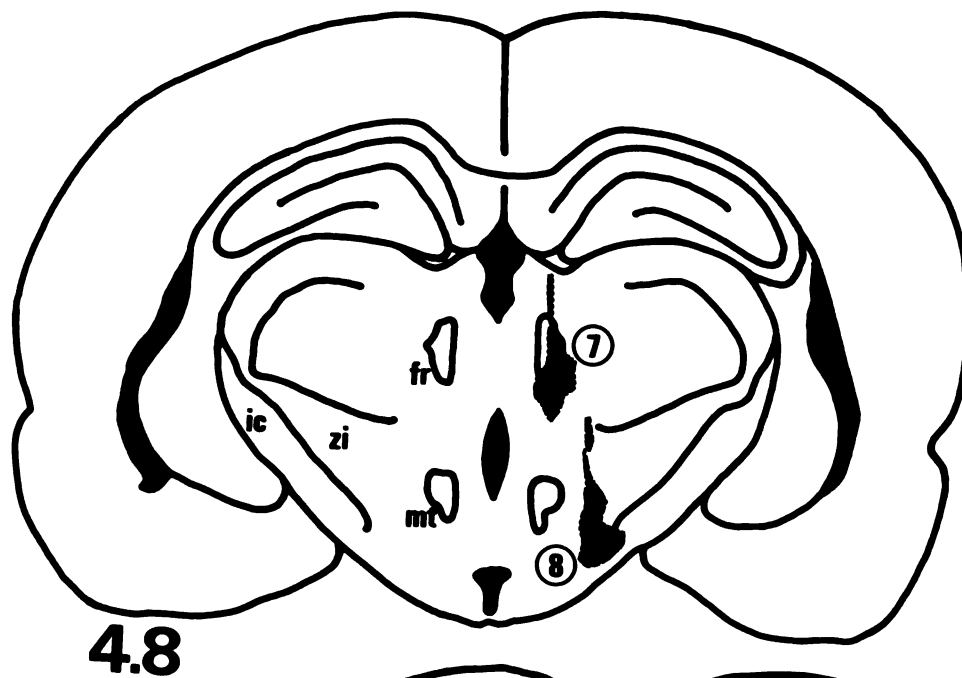


Fig. 2. This figure shows a photomicrograph of an injection of the fluorescent tracer, Fluoro-Gold in the RVM. The core of the injection, in the NRM, is outlined by arrowheads. Abbreviations: VII, facial nucleus; Gi, nucleus reticularis gigantocellularis; mlf, medial longitudinal fasciculus; py, pyramidal tract. Scale bar = 1mm.

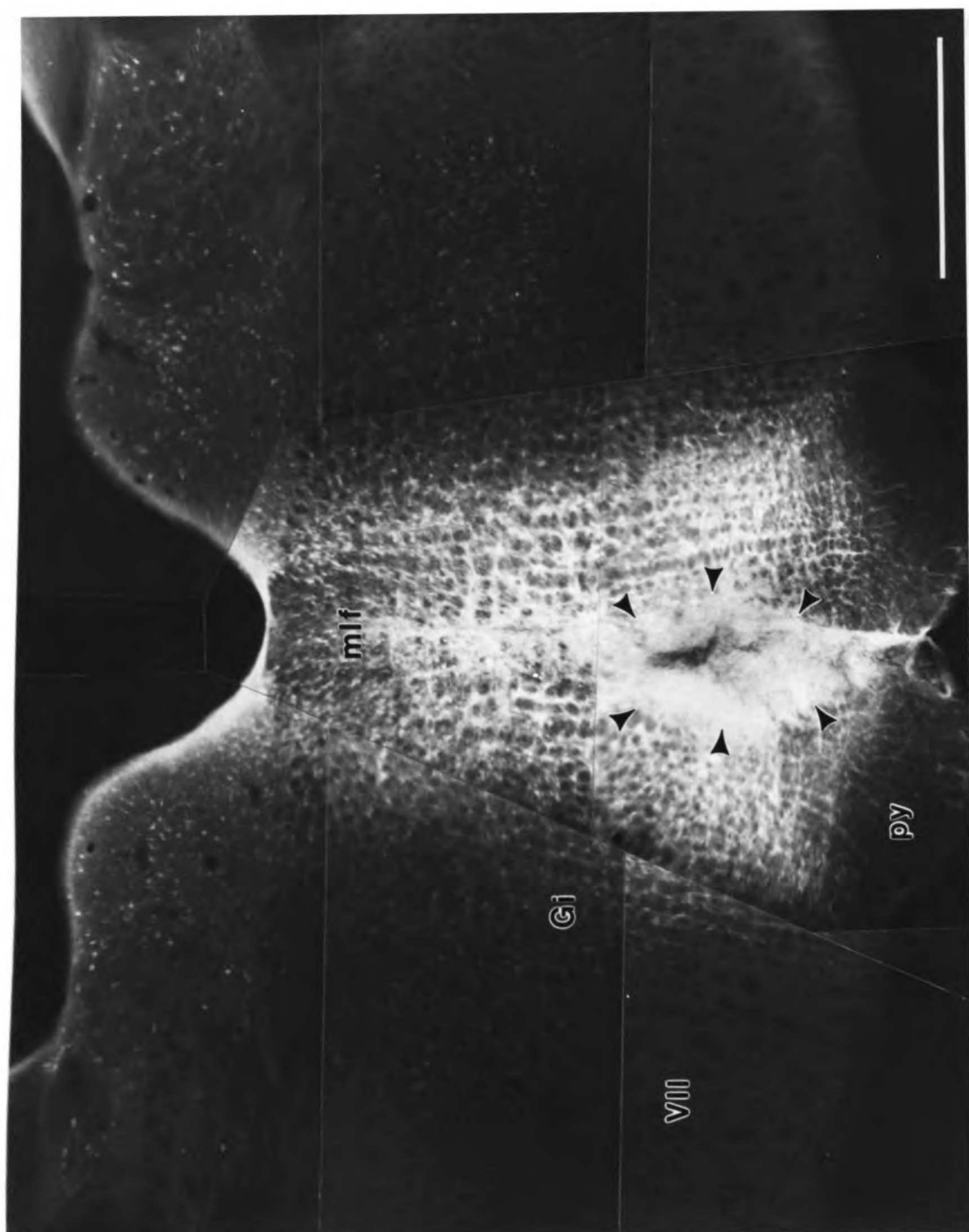


Fig. 3. This bright-field photomicrograph illustrates the central core of an injection of WGAapoHRP-Au in the medial thalamus (arrow). This injection is diagramed in Fig. 1. Abbreviations: III, third ventricle; fr, fasciculus retroflexus; Hb, habenula; ml, medial lemniscus; mt, mammillothalamic tract; Pf, nucleus parafascicularis of the thalamus. Scale bar = 1mm.

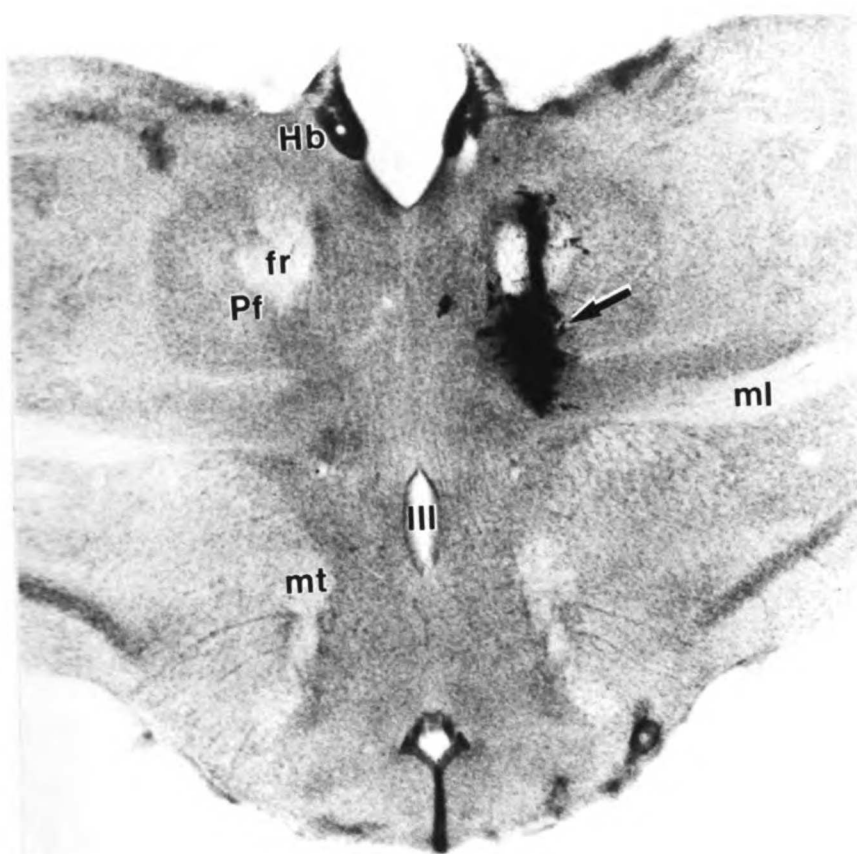


Fig. 4. This figure illustrates the appearance of single- and double-labeled neuronal cell bodies after an injection of WGAapoHRP into Pf and an injection of Fluoro-gold into the NRM. The fluorescence photomicrograph in plate A shows ventrolateral PAG neurons retrogradely labeled with Fluoro-gold from the NRM. Plate B shows the identical field of the section in dark field illumination. Neurons retrogradely labeled with WGAapoHRP-Au from Pf contain refractile particles of tracer. Double-labeled neurons are indicated by arrowheads. The photomicrograph in plate C shows neurons in the DRN retrogradely labeled with Fluoro-gold, and plate D shows neurons retrogradely labeled with WGAapoHRP-Au. Scale bars = 100 μ m.

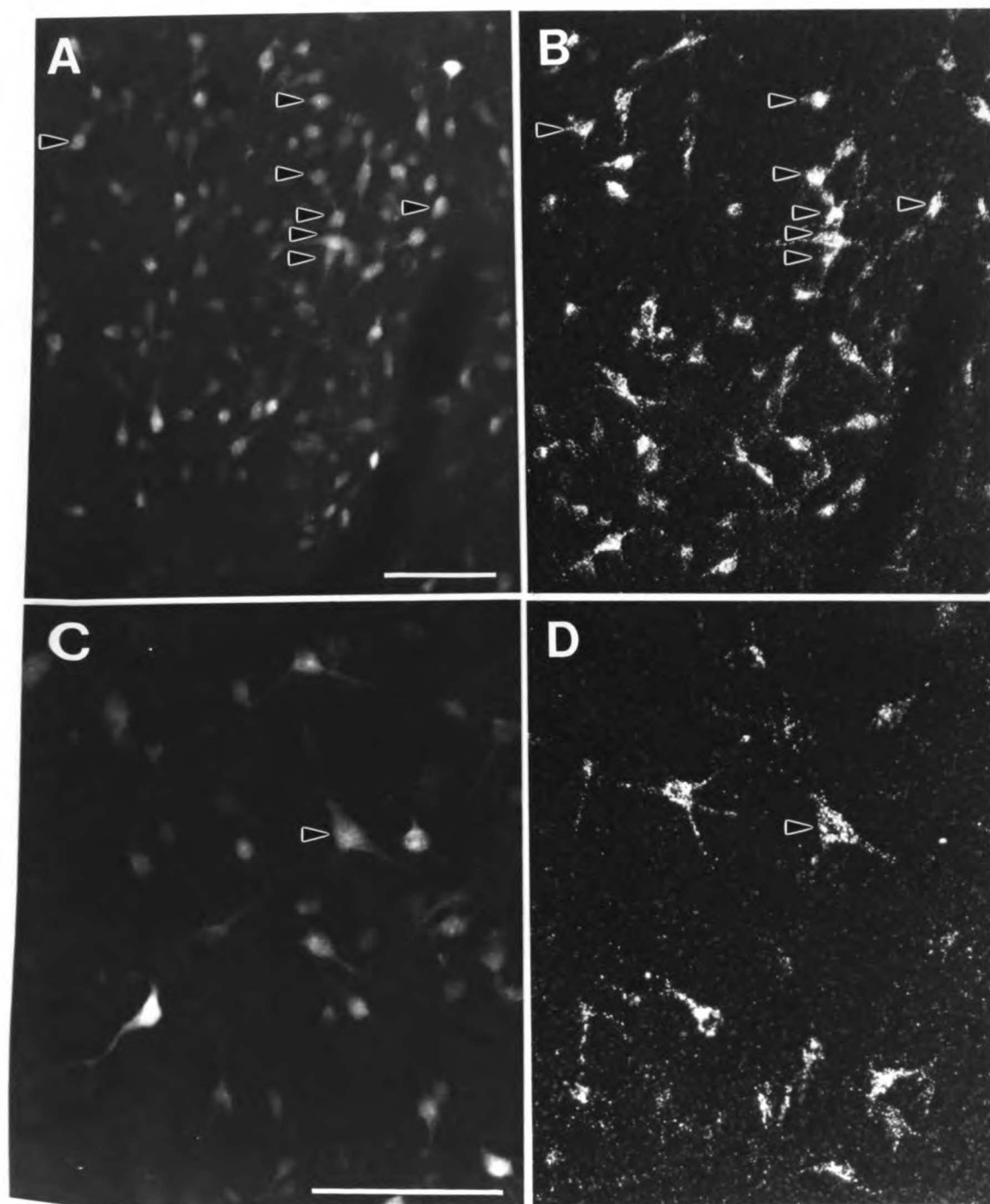


Fig. 5. These dark-field photomicrographs show examples of the two major distributions of retrogradely labeled neurons we observed in the PAG. In dark-field illumination, individual WGAapoHRP-Au labeled cell bodies are distinct, even at low magnification (arrows). The upper plate illustrates the distribution of retrogradely labeled neurons in the PAG after an injection into Pf. (the right side of the plate is ipsilateral to the injection site.) Retrogradely labeled neurons are most concentrated ventrolateral to the aqueduct (AQ). The lower figure shows neurons retrogradely labeled after a tracer injection into VB. In this case, retrogradely labeled cell bodies are found only in the DRN.

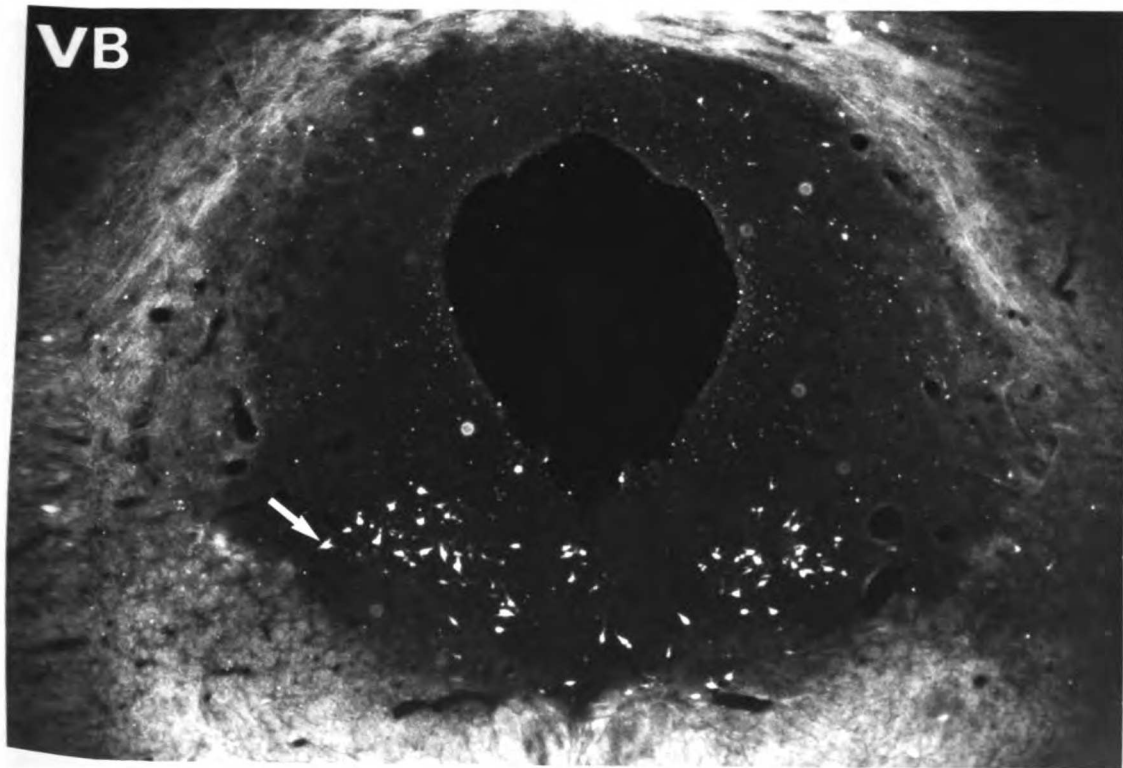
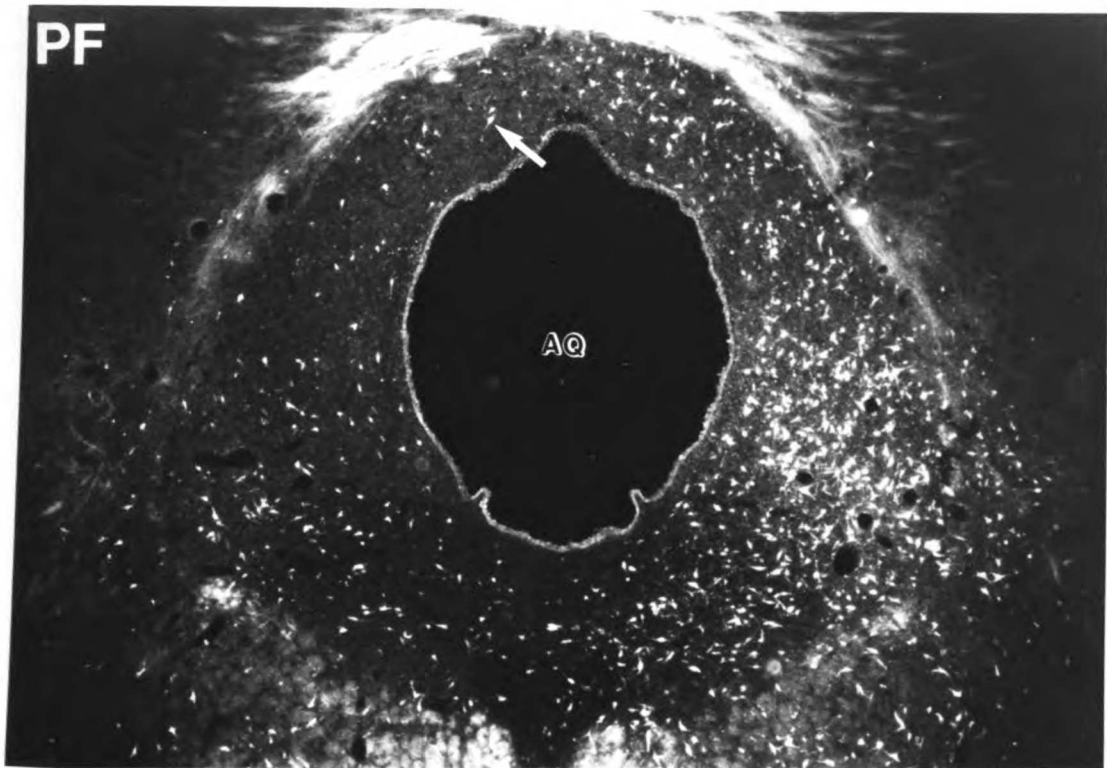


Fig. 6. Plate A shows 5-HT immunoreactive neurons in the lateral part of the DRN. Plate **B** is a dark-field photomicrograph of the same field. The neurons shown in B have been retrogradely labeled by an injection of WGAapoHRP-Au in Pf. Double-labeled neurons are indicated by arrows. It is possible to identify double-labeled neurons with bright-field illumination alone, since the immunostain is brown and the retrograde label is black, but this is not evident in black-and-white micrographs. Scale bar = 100 μm .

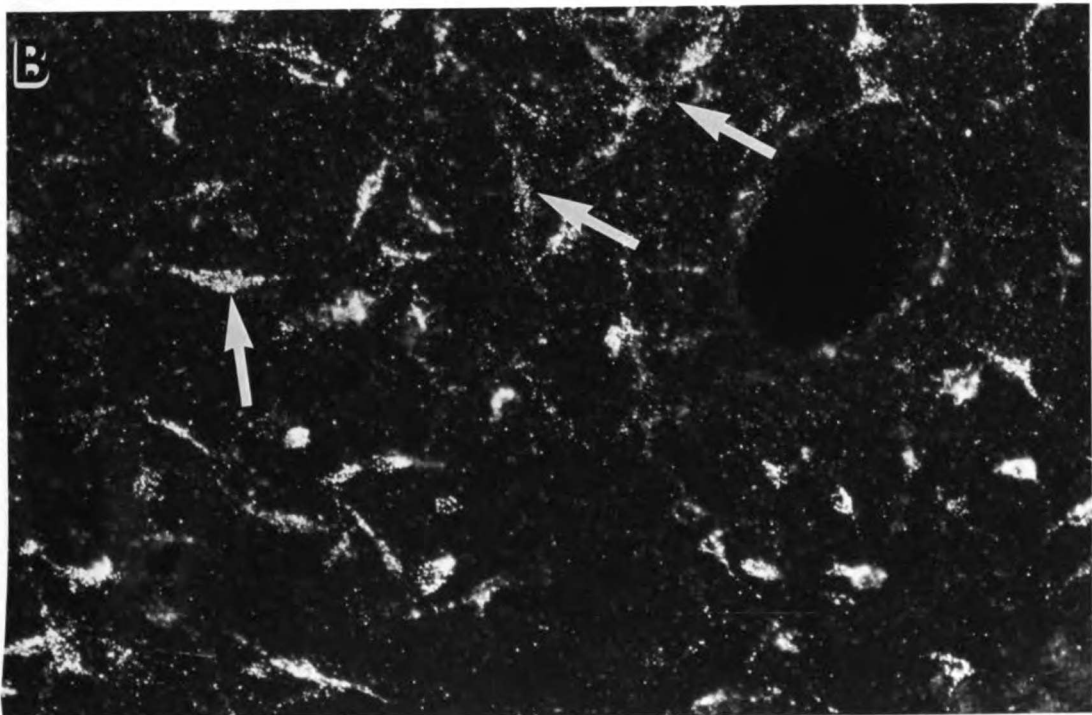
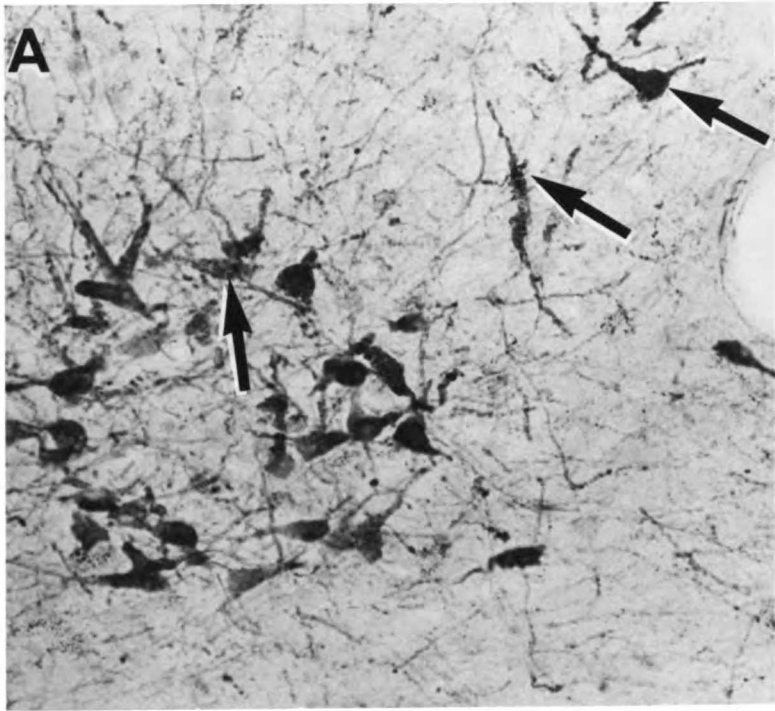
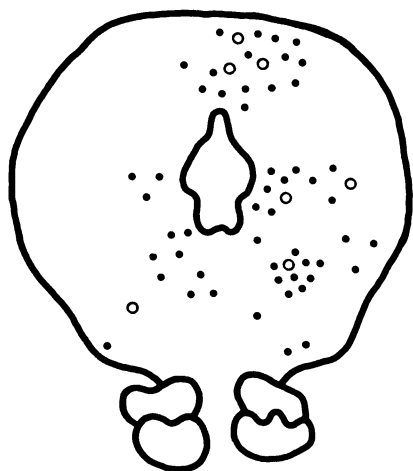
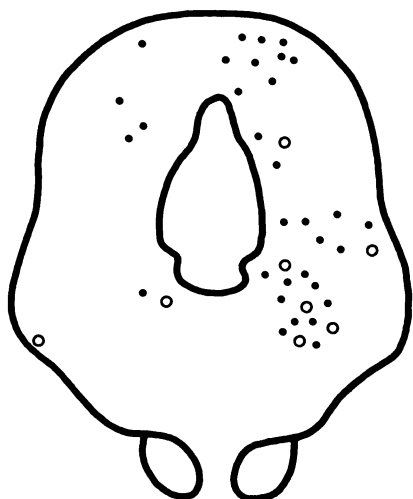


Fig. 7. These drawings illustrate the patterns of retrogradely labeled neurons (black dots) in the PAG after tracer injections (shown in figure 1) into regions of the brain rostral to the PAG. The right-hand side of each drawing is ipsilateral to the injection site. Open circles indicate neurons which were double-labeled by a tracer injection centered in the NRM. The drawings for medial thalamus and ventrobasal hypothalamus injections represent the number of cells labeled in a single 25 μm thick section; all other drawings indicate the total number of cells observed in four 25 μm sections. Numbers indicate the distance (in mm) rostral to interaural zero according to the atlas of Paxinos and Watson (1986).

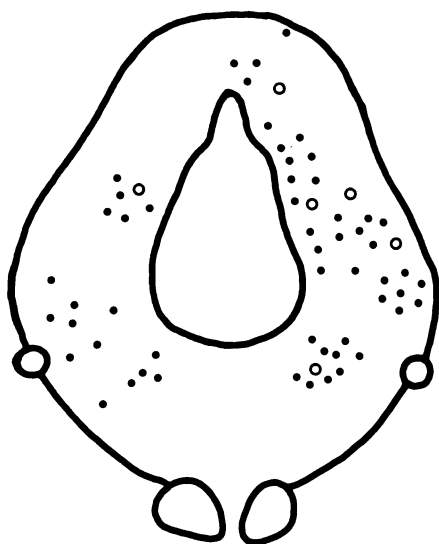
**VENTROBASAL
HYPOTHALAMUS**



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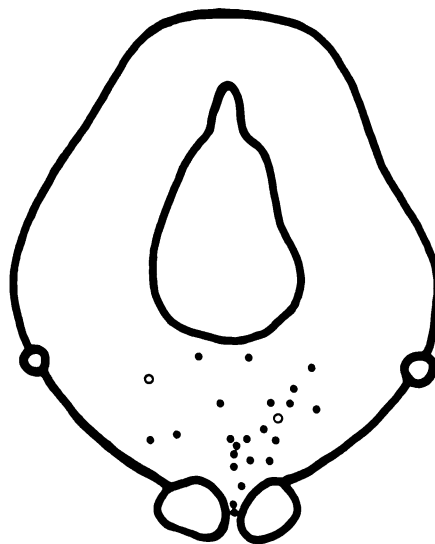
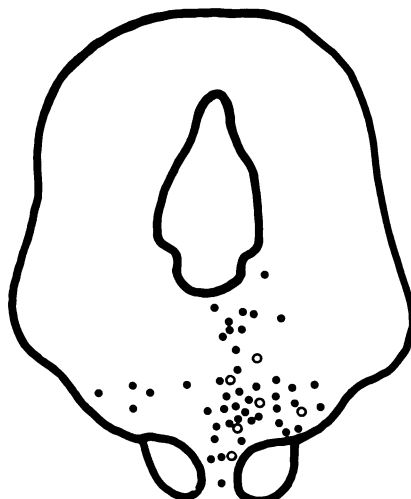
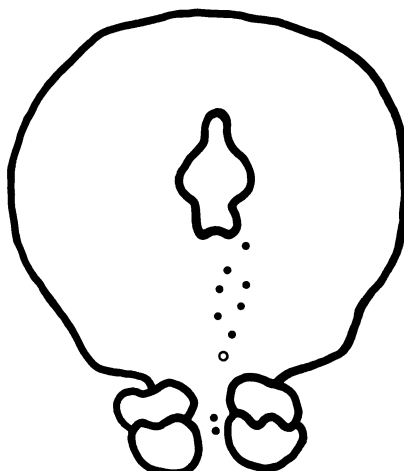


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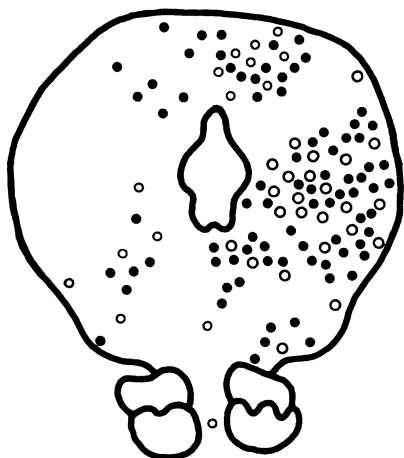


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**LATERAL
HYPOTHALAMUS**

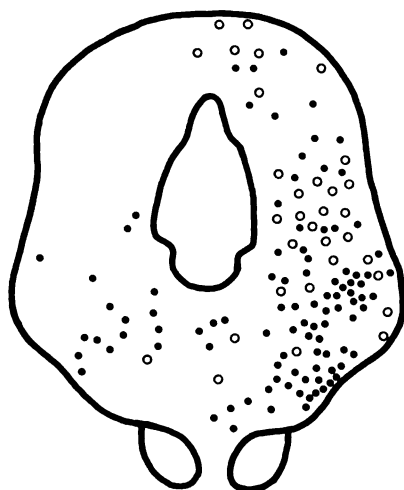
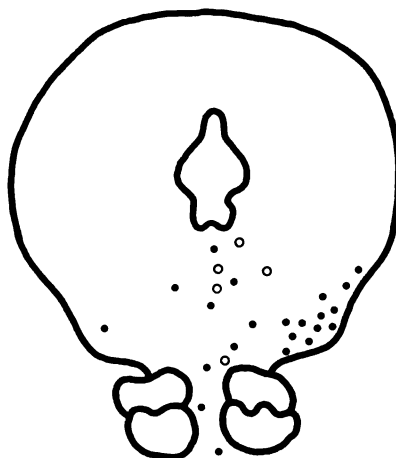


**MEDIAL
THALAMUS**

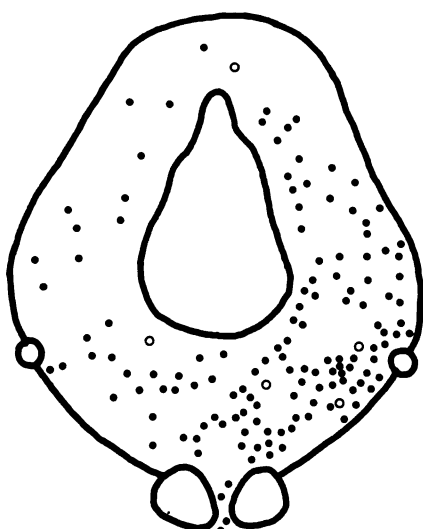
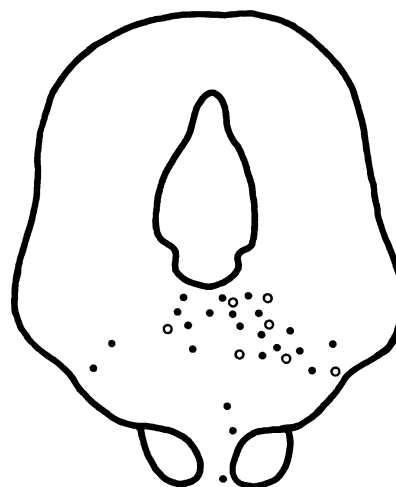


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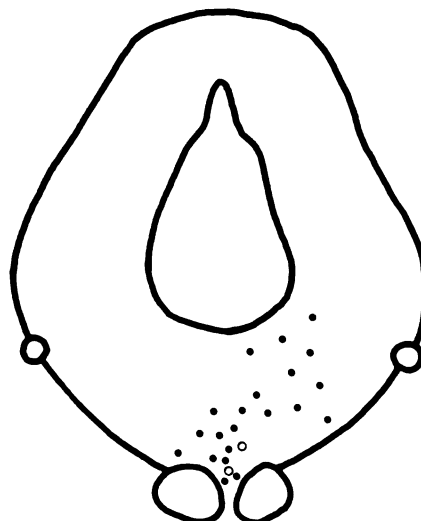
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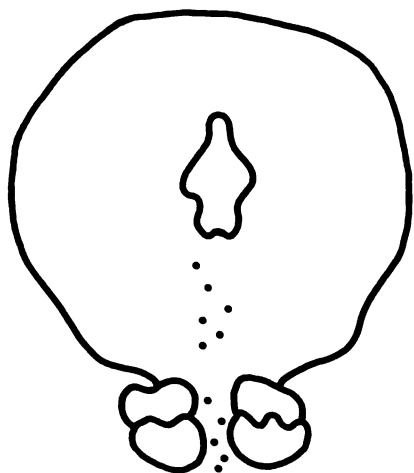
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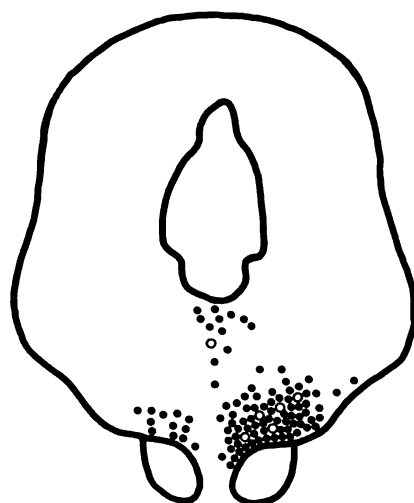
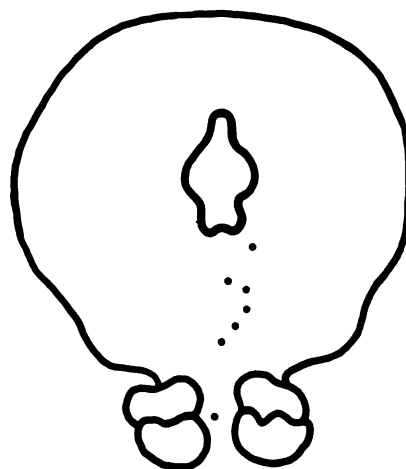
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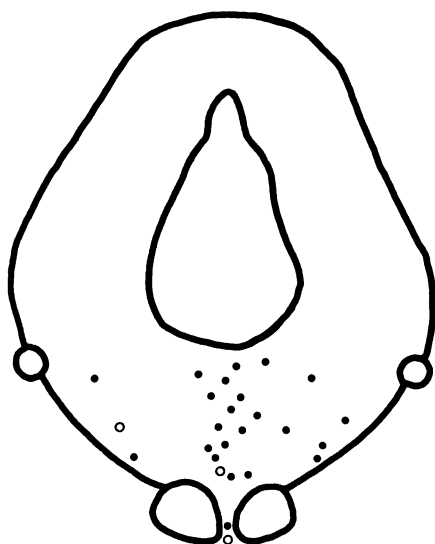
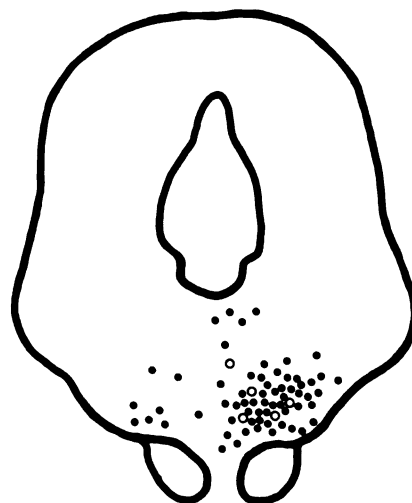
**FRONTAL
CORTEX**



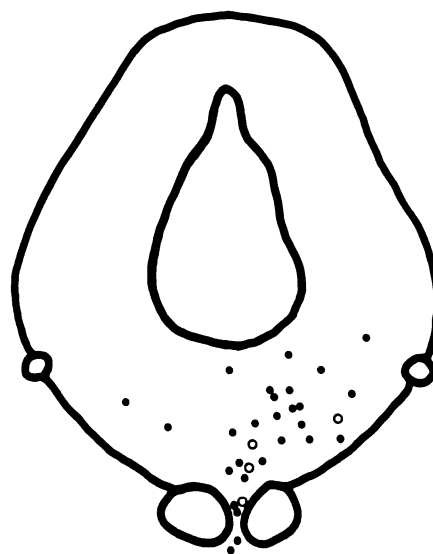
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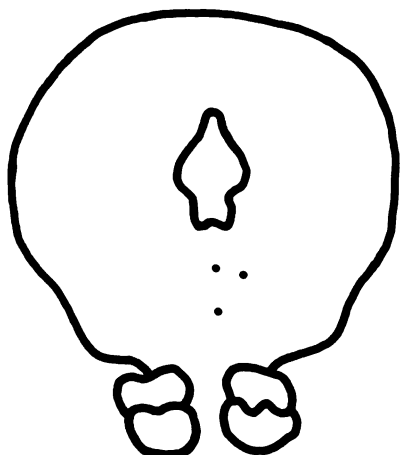
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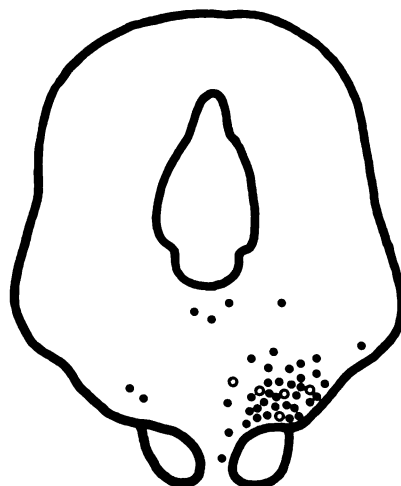
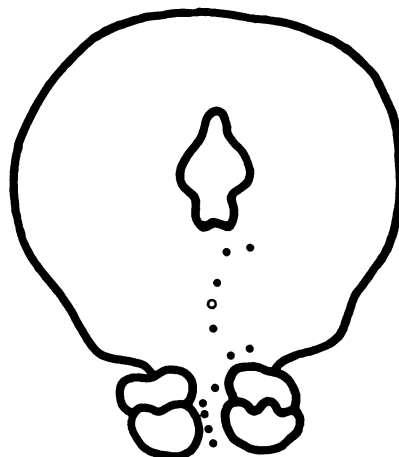


**PARIETAL
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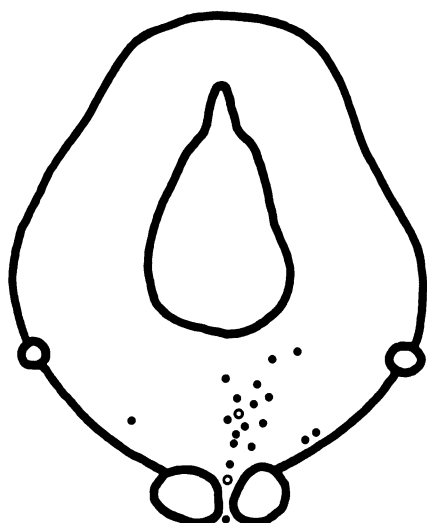
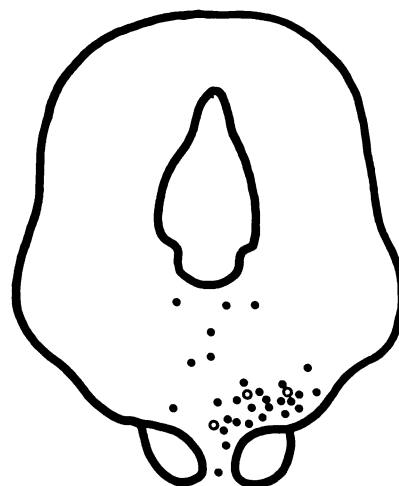


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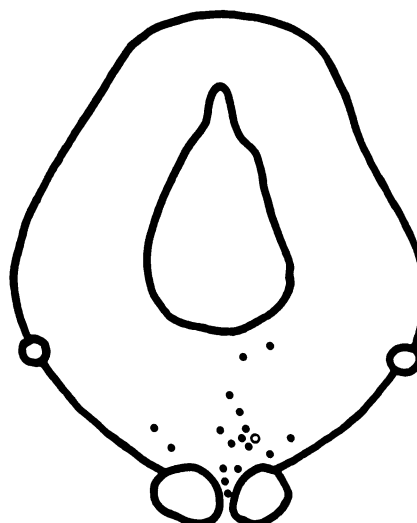
**OCCIPITAL
CORTEX**



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CHAPTER IV

The Contribution of Brainstem GABAergic Circuitry to Descending Antinociceptive Controls.

I. GABA-Immunoreactive Projection Neurons in the Periaqueductal Gray and Nucleus Raphe Magnus

David B. Reichling and Allan I. Basbaum

SUMMARY

The fact that GABA receptor ligands influence nociceptive thresholds when administered in the rostral ventral medulla or in the spinal cord, may reflect the involvement of GABAergic neuronal elements in endogenous antinociceptive pathways. The present study used immunocytochemistry and retrograde tract tracing (using WGAapoHRP-Au) to assess the possibility that GABAergic projection neurons contribute to the presumed antinociceptive pathway which descends from the midbrain periaqueductal gray matter (PAG) via the rostroventral medulla (NRM) to the spinal cord dorsal horn. In the PAG, very few GABA immunoreactive neurons were retrogradely labeled from the NRM. Similarly, in the NRM, very few GABA-immunoreactive neurons were retrogradely labeled from the spinal cord. A much higher proportion of GABA-immunoreactive neurons in the region lateral to the NRM, however, were retrogradely labeled from the spinal cord. Eighteen percent of GABA-immunoreactive neurons were retrogradely labeled in the nucleus reticularis paragigantocellularis; conversely, 15% of the retrogradely labeled neurons in this region were GABA-immunoreactive. These results indicate that GABAergic projections constitute a very minor component of the PAG-NRM-spinal cord pathway; however, they may be an important component of descending projections that originate in the medullary reticular formation lateral to the NRM. The majority of GABAergic neurons in the PAG and NRM are presumed to be inhibitory interneurons that directly or indirectly regulate activity in efferent pathways from these regions.

INTRODUCTION

The inhibitory amino acid, GABA, has been implicated in antinociceptive processes at both the brainstem and spinal cord levels. For

example, Moreau and Fields (1986) reported that microinjection of the GABA agonist, muscimol, into the midbrain periaqueductal gray matter (PAG) antagonizes analgesia produced by morphine microinjected in the same site; conversely, GABA antagonists produced analgesia. Similarly, in the rostroventral medulla (RVM), the GABA agonist, THIP, produces hyperalgesia, and the GABA antagonist, bicuculline, produces modest analgesia (Drower and Hammond 1988). Finally, the GABA_B agonist, baclofen, administered intrathecally, produces potent analgesia (Wilson and Yaksh 1978).

Millhorn *et al.* (1987, 1898) reported that some cells in the rostroventral medulla (RVM) that were immunoreactive for glutamic acid decarboxylase, the GABA synthetic enzyme, could be retrogradely labeled from the spinal cord. At some rostrocaudal levels, more than half the GABA-immunoreactive neurons in the RVM are also 5-HT immunoreactive. Since 85-90% of the 5-HT neurons in the medullary nucleus raphe magnus (NRM) project to the spinal cord (Bowker *et al.*, submitted), these data imply that many GABAergic neurons must project to the spinal cord.

Since the PAG contains a high density of putative GABAergic neurons (Mugnaini and Oertel 1985; Reichling and Basbaum 1987), it is of interest to determine if GABAergic projection neurons also contribute to the projection from the PAG to the NRM. The present study used a combination of retrograde tracing and GABA immunocytochemistry to identify putative GABAergic projection neurons in the PAG. In addition, we performed a detailed analysis of the distribution of GABA-immunoreactive projection neurons in the RVM. A preliminary report of a portion of this work has been published previously (Reichling and Basbaum 1987).

METHODS

Six adult male Sprague-Dawley rats (Bantin Kingman) were used in this study. The rats were anesthetized with pentobarbitol (60 mg/kg) during surgery. Three rats received stereotaxic microinjections of the gold conjugated retrograde tracer, WGAapoHRP-Au (Menetrey and Basbaum 1987), into the NRM. These rats were placed in a stereotaxic head frame, the skull was trephinated, and a 30 gauge 1 μ l Hamilton syringe was used to pressure inject 0.1 μ l of tracer over a period of 30 minutes. Three additional rats received injections of the retrograde tracer into the cervical enlargement of the spinal cord. Two injections, separated by about 2 mm rostrocaudally, were made on each side of the cord. These four 0.1 μ l injections were centered in the dorsal horn. After recovery from anesthesia the rats displayed no obvious motor or sensory deficits.

Seven to twenty-one days after surgery, the rats were deeply anesthetized with pentobarbitol and perfused transcardially with 100 ml of a fixative solution consisting of 0.05 M phosphate-buffered saline (PBS) followed by 500 ml of 1% glutaraldehyde and 4% paraformaldehyde in 0.05 M PBS. The brain was removed and sectioned on a Vibratome. The midbrain or medulla was sectioned at 30 μ m. Injection sites were sectioned at 100 μ m. Retrogradely transported colloidal gold particles were made visible by a silver-enhancement reaction (Basbaum and Menetrey 1987). For GABA immunocytochemistry the sections were first incubated for 30 minutes in a blocking solution of 3% normal goat serum and 0.3% Triton X-100 in 0.05 M PBS. The tissue was then incubated for 48 hours in the primary antiserum diluted 1:3750 in 0.05 M PBS containing 0.3% Triton X-100. This antiserum (kindly provided by Dr. Andrew Towle of Cornell University) was raised in rat against a glutaraldehyde conjugate of GABA and bovine serum albumin (BSA). Radioimmunoassay demonstrates that the antiserum is highly specific for

glutaraldehyde fixed GABA (Lauder *et al.* 1986). The tissue was rinsed, and incubated overnight in goat anti-rat IgG (1:100; Cappel). After another rinse the tissue was incubated in rat peroxidase-anti-peroxidase (1:500; ICN ImmunoBiologicals). Finally, the peroxidase reaction was developed with diaminobenzidine (DAB) as the chromogen. Preabsorption of the primary antiserum with 10 μ M of the GABA-BSA conjugate abolished all immunoreactivity in the tissue sections.

In bright-field illumination, retrogradely transported WGAapoHRP-Au appears as black punctae inside cell bodies. This retrograde label is readily distinguished from the diffuse light brown stain associated with GABA immunoreactivity. Thus, double-labeled neurons are easily recognized. Sections were photographed and the distribution of single- and double-labelled neurons was drawn by *camera lucida*.

RESULTS

Tracer injections in the RVM were centered in the NRM; there was a small amount of spread laterally into the nucleus reticularis paragigantocellularis pars α (Figure 1A, for example). Tracer injections in the spinal cord mainly involved the dorsal horn (Figure 1B, for example). **Figure 2 shows photomicrographs of single- and double-labeled neurons.** The WGA-apoHRP-Au retrograde label and DAB-visualized immunoreactivity can be observed simultaneously in the light microscope, allowing unambiguous identification of double-labeled neurons.

Periaqueductal gray matter

Our results for the PAG are presented schematically in figure 3. By comparing the number of retrogradely labeled cell bodies to the total number of cell bodies in Nissl-stained sections, we estimate that up to 18% of all

neurons in the PAG were retrogradely labeled from the NRM. Retrogradely labeled neurons were found throughout most of the PAG caudal to the oculomotor nuclei. The only regions of the PAG where retrogradely labeled neurons were rare were the ventromedial and dorsolateral PAG and the DRN. At the level of the level of the oculomotor nuclei and more rostrally, retrogradely labeled neurons were still common in the ventrolateral, lateral, and dorsal PAG, but the dorsolateral zone, which contained few labeled cells, enlarged. Most of the retrogradely labeled neurons were small (10-15 μm diameter), fusiform or oval; some labeled neurons in the dorsal and ventral parts of the PAG were larger (15-20 μm) and more multipolar. A similar distribution of cells retrogradely labeled from the NRM has been reported in previous studies in both the rat and cat, using a variety of tracers and chromogens (Abols and Basbaum 1981; Beitz *et al.* 1983; Carlton *et al.* 1983; Gallager and Pert 1978; however, see Fardin *et al.* 1984).

GABA-immunoreactive neurons comprised approximately 14% of the total number of PAG neurons located caudal to the oculomotor nuclei. Caudal to the oculomotor nuclei, GABA-immunoreactive neurons were common in all regions of the PAG and overlapped extensively with the distribution of neurons retrogradely labeled from the NRM. At more rostral levels, however, there were marked differences between the distributions of the two populations of neurons in the dorsal PAG. The distribution of both types of neurons overlapped in the ventrolateral part of the rostral PAG, however GABA-immunoreactive neurons were clustered in the dorsolateral PAG but were not common dorsal to the aqueduct and in the lateral PAG in a pattern almost reciprocal to the distribution of retrogradely labeled neurons in the rostradorsal PAG. This distribution of GABA-immunoreactive cell bodies agrees with the results obtained by Mugnaini *et al.* (1985), using an anti-glutamate

decarboxylase (GAD) antiserum.

Despite the intermingling of the populations of GABA-immunoreactive neurons and retrogradely labeled neurons in most parts of the PAG, we found few double-labeled neurons. Only 1.5% of all retrogradely labeled neurons in the PAG were also GABA-immunoreactive, and only 2.1% of all GABA-immunoreactive neurons in the PAG were retrogradely labeled. Although it is difficult to generalize about this small population of double-labeled neurons, it was our impression that double-labeled neurons were predominantly located in the ventral PAG. There was a small cluster of double labeled neurons dorsal to the PAG, at levels caudal to the oculomotor nuclei. The morphology of double-labeled neurons in the PAG was not distinctive.

Rostroventral medulla

Figure 4 illustrates the distribution of retrogradely labeled and immuno-labeled neurons in the RVM in relation to the boundaries of the reticular and raphe nuclei. These boundaries, adapted from Newman (1985) and Paxinos and Watson (1986), are approximations that were determined from dark-field examination of the tissue. We did not verify the delineations cytoarchitecturally. Consistent with the results of many previous studies, neurons retrogradely labeled from the spinal cord dorsal horn were common the RVM, including the NRM, nucleus raphe pallidus (RPa), nucleus raphe obscurus (ROb), nucleus reticularis paragigantocellularis (PGi), nucleus reticularis paragigantocellularis pars α (PGi α), nucleus reticularis paragigantocellularis lateralis (PGi l), and nucleus reticularis gigantocellularis (Gi; Bowker *et al.* 1981; Carlton *et al.* 1985; Kneisley *et al.* 1978; Kuypers and Maiskey 1975; Tohyama *et al.* 1979a,b; Zemlan *et al.* 1984). Rostral to the gigantocellular and paragigantocellular group of reticular nuclei, at the level of the lateral superior olive, the more laterally situated population of

retrogradely labeled cells shifts dorsally into the region of the caudal pontine reticular nucleus.

GABA-immunoreactive neurons were found in the midline NRM and RPa, and in the paragigantocellular reticular nuclei of the ventral medulla (PGi, PGi α , and PGi β). GABA-immunoreactive neurons were rarer in the more dorsally located ROb and Gi. Rostrally, at the level of the lateral superior olivary nucleus, the distribution of GABA-immunoreactive neurons expanded dorsally into the caudal pontine reticular nuclei. GABA-immunoreactive neurons in the RVM were typically small (10-25 μ m diameter) and multipolar. These results are consistent with those of previous studies using GABA immunocytochemistry (Bowker *et al.* 1988) and GAD immunocytochemistry (Millhorn *et al.* 1987; Mugnaini and Oertel 1984).

For the most part, the populations of retrogradely labeled and GABA-immunoreactive neurons were intermingled in the RVM. The two major exceptions are the nucleus reticularis gigantocellularis, which contains a moderate number of retrogradely labeled neurons, but very few GABA-immunoreactive neurons, and the lateral reticular formation at the level of the lateral superior olive, where the population of retrogradely labeled neurons is concentrated dorsal to the population of GABA-immunoreactive neurons.

Nine percent of all retrogradely labeled neurons in the RVM were also GABA-immunoreactive and 8% of all GABA-immunoreactive neurons in the RVM were retrogradely labeled. The degree of double-labeling, however, varied considerably among the regions studied. In the midline nuclei: NRM, RPa, and ROb, double-labeled neurons were very rare -- only 2% of retrogradely labeled neurons in the NRM were GABA-immunoreactive and only 3% of GABA-immunoreactive neurons in the NRM were retrogradely

labeled. In contrast, 30% of the sparsely distributed GABA-immunoreactive neurons in the nucleus reticularis gigantocellularis were retrogradely labeled. Other areas examined had intermediate proportions of double-labeled neurons: in the nucleus reticularis paragigantocellularis, 15% of retrogradely labeled neurons were GABA-immunoreactive and 18% of GABA-immunoreactive neurons were retrogradely labeled; in the nucleus reticularis paragigantocellularis pars α , 6% of retrogradely labeled neurons were GABA-immunoreactive and 7% of GABA-immunoreactive neurons were retrogradely labeled. Finally, we found no double-labeled neurons in the nucleus reticularis paragigantocellularis lateralis.

DISCUSSION

The results of this study suggest that many GABAergic neurons in the PAG are local interneurons -- although 14% of neurons in the caudal PAG were GABA-immunoreactive, only about 2% of these neurons were retrogradely labeled from the NRM. Based on their observation that lesions of major sources of afferents to the DRN (the dorsal pontine tegmentum, substantia nigra, or habenula) did not alter glutamic acid decarboxylase (GAD) activity in the ventral PAG, Belin *et al.* (1979) came to a similar conclusion. Although other possible sources of GABAergic inputs to the PAG have not been examined, it seems likely that most GABAergic neurons in the PAG are interneurons.

In contrast, GABAergic projection neurons in the RVM do make a significant contribution to medullo-spinal pathways. Although the populations of GABA-immunoreactive and retrogradely labeled neurons were intermingled in most regions of the RVM, the fraction of double-labeled neurons varied considerably among the different regions. Only a small

number of double-labeled neurons were found in the midline raphe nuclei; the more laterally placed reticular nuclei contained greater numbers of double-labeled neurons.

In light of studies by Millhorn *et al.* (1987,1988) we expected to find a relatively large proportion of retrogradely labeled GABA-immunoreactive neurons in the RVM. There are several possible explanations for the lower proportion of GABAergic projection neurons that we observed: (1) The presence of WGA-apoHRP-Au in a cell somehow interferes with GABA immunostaining. This is unlikely for several reasons: first, double-labeled cells were as intensely immunostained as were other GABA-immunoreactive cells, and second, we have used the tracer successfully in combination with many other antisera (c.f. Menetrey and Basbaum 1988). (2) The difference might relate to the site of retrograde tracer injection into the spinal cord. The estimate that 85-90% of serotonergic neurons in the RVM project to the spinal cord derives from studies in which large injections of tracer were made into unspecified regions of the gray and white matter of the spinal cord (Bowker *et al.* 1987, and submitted). Our relatively more focussed injections of WGAapoHRP-Au (which is picked up minimally by fibers of passage; Basbaum and Menetrey 1987) were limited to the dorsal horn of the cervical spinal cord. Conceivably, the population of RVM cells in which 5-HT and GABA coexist project preferentially to regions of the cord other than the dorsal horn, e.g. to the interomediolateral cell column (Loewy and McKellar 1981). (3) If the population of neurons in which 5-HT and GABA coexist includes all of the 10-15% of serotonergic neurons that apparently do not project to the cord (Bowker *et al.* 1987, and submitted), then the percentage of GABAergic projection neurons predicted from Millhorn and coworkers' data would be as low as 28%. In fact, at mid rostrocaudal levels of the nucleus reticularis

paragigantocellularis we found that approximately 30% of GABA-immunoreactive neurons were retrogradely labeled. (4) Finally, the anti-GABA antiserum which we used might have different binding specificity from that used by Millhorn *et al.* (1988). Since comparable absorption controls were performed in both cases, and since the two reported patterns of GABA-immunoreactive cell bodies are in agreement with each other, we conclude that explanation is unlikely.

Our results indicate that the lateral region of the RVM contains the largest number of GABA-immunoreactive spinal projection neurons. Although emphasis is usually placed on the NRM contribution to descending controls, the lateral RVM, in part, mediates analgesia elicited by narcotics or electrical stimulation of the PAG (Azami *et al.* 1982; Lovick 1985; Prieto *et al.* 1983; Sandkuhler *et al.* 1982). In light of reports that intrathecal administration of GABA_B agonists produces antinociception (Wilson and Yaksh 1978), it is conceivable that GABAergic projection neurons are involved in descending antinociceptive pathways. Since many GABA-immunoreactive neurons in the RVM lateral to the NRM are also 5-HT-immunoreactive (Millhorn *et al.* 1988), the GABA-immunoreactive projection neurons we observed might, in fact, contribute to descending antinociceptive serotonergic pathways.

In conclusion, we found that few GABA-immunoreactive neurons in the PAG project to the NRM. In contrast, a relatively greater number of GABA-immunoreactive neurons in the RVM project to the spinal cord dorsal horn. These GABAergic projection neurons were concentrated in PGi and PGi_α, but were rare in the midline raphe nuclei and in the more laterally located PGi_j. We conclude that GABAergic elements that contribute to the pathway descending from the PAG via the NRM are probably interneurons. On the

other hand, the parallel antinociceptive pathway that descends to the spinal cord from the lateral medullary reticular formation may have a significant GABAergic component.

ACKNOWLEDGEMENTS

The authors thank Ms. Katja Kohler for artistic assistance, and Ms. Simona Ikeda and Ms. Margaret Mayes for photographic assistance. This work was supported by NIH Public Health Service Grants NS 14627 and 21445.

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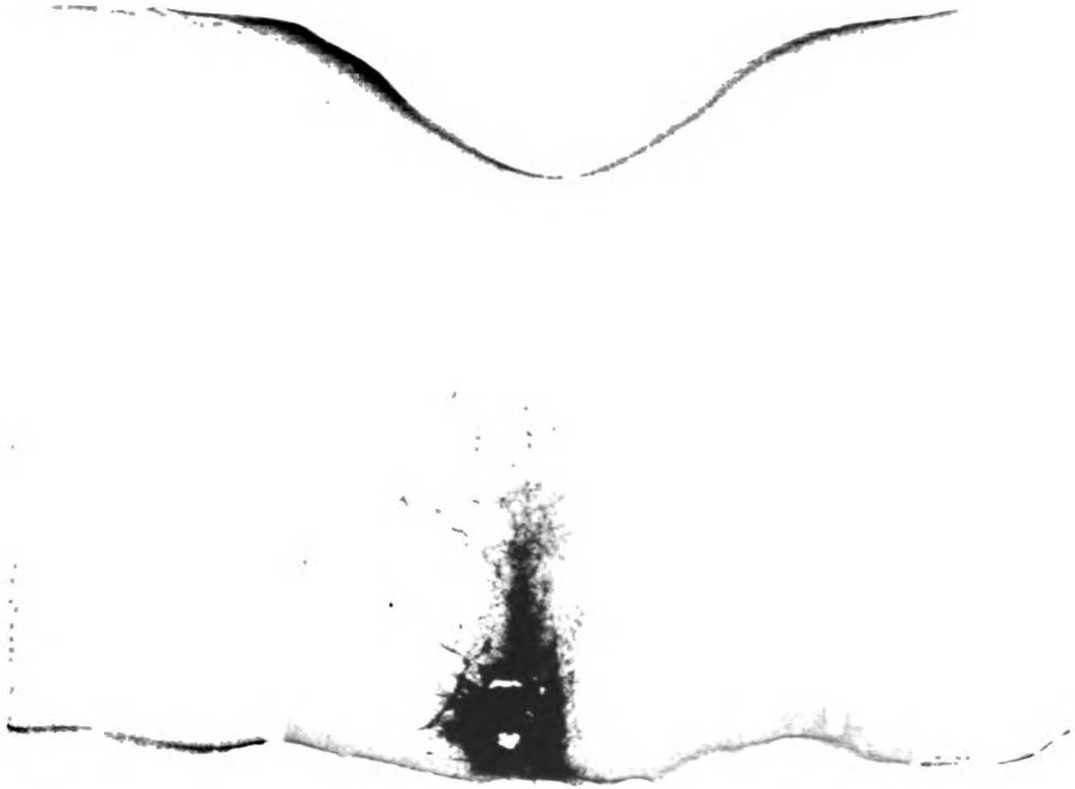
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Fig. 1. This figure shows typical injection sites of retrograde tracer. A shows a bright-field photomicrograph of an injection of WGAapoHRP-Au centered in the NRM. This 100 μm section contains the center of the injection site. B shows a diagram of the cervical spinal cord. The shaded region indicates the total extent of two nearby injections of WGAapoHRP-Au in the dorsal horn.

A



B

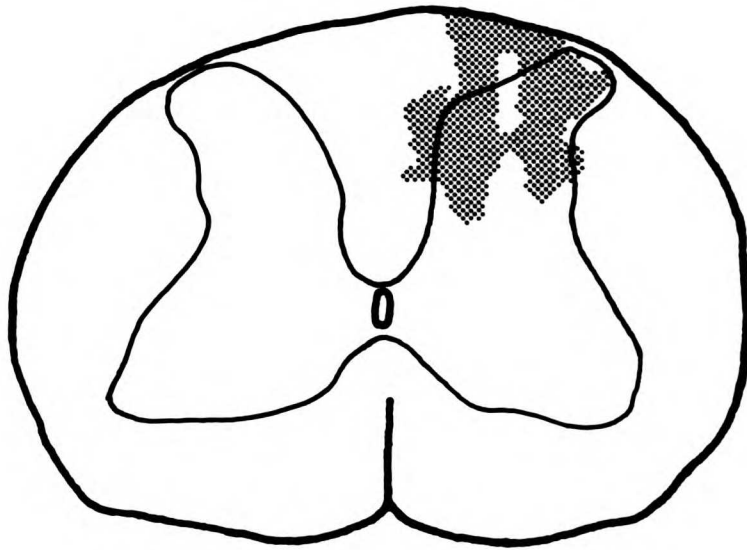


Fig. 2. This high-magnification photomicrograph illustrates the appearance of single- and double-labeled neurons in the nucleus paragigantocellularis. GABA-immunoreactive neurons (open arrow) contain diffuse, brown DAB reaction product. Retrogradely labeled cell bodies and their proximal dendrites (curved arrow) contain black, punctate particles of silver-enhanced WGAapoHRP-Au tracer retrogradely transported from the spinal cord. The two tracers are visible in double-labeled neurons in bright-field illumination. Although not apparent in this black-and-white micrograph, the difference in color between the two tracers greatly enhances the ability to identify double-labeled neurons. Scale bar = 100 μ m.

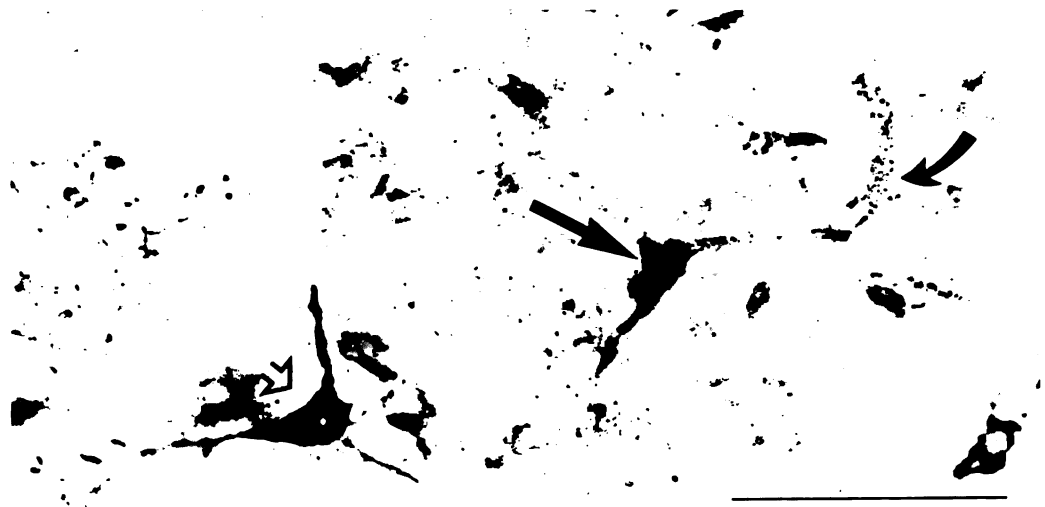
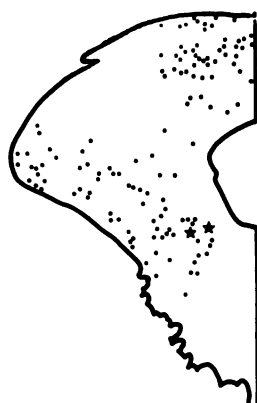
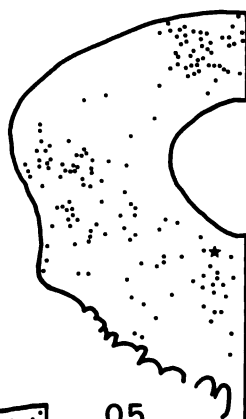


Fig. 3. This figure shows camera lucida drawings of typical 30 μm sections through three rostrocaudal levels of the PAG. In each pair of drawings of half of the PAG in a single section, the left-hand figure shows the distribution of retrogradely labeled neurons (black dots), and the right-hand figure shows the distribution of GABA-immunoreactive neurons (black dots). Double labeled neurons are indicated by stars. Numbers indicate the distance (in mm) rostral to interaural zero according to the rat brain atlas of Paxinos and Watson (1986).



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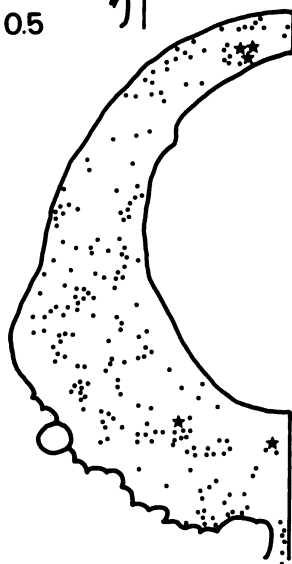
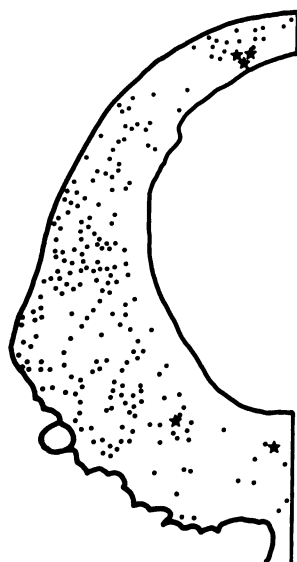
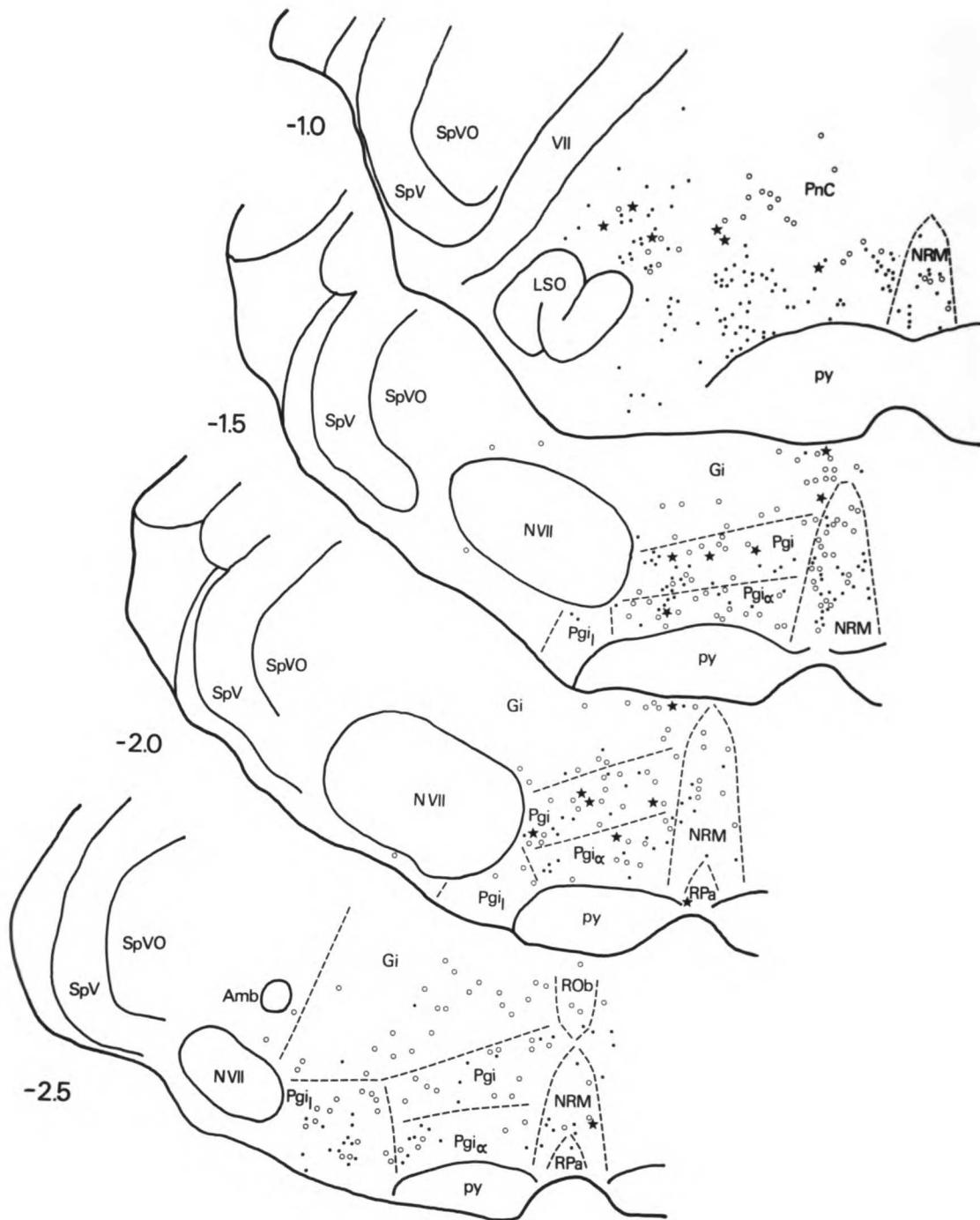


Fig. 4. This figure shows camera lucida drawings of the distribution of single- and double-labeled neurons in typical 30 μm sections of the RVM. Retrogradely labeled neurons are indicated by open circles, GABA-immunoreactive neurons are indicated by black dots, and double labeled neurons are indicated by black stars. Numbers indicate the distance (in mm) from interaural zero according to the rat brain atlas of Paxinos and Watson (1986). Abbreviations: VII, facial nerve; Amb, ambiguus nu.; Gi, nucleus reticularis gigantocellularis; LSO, lateral superior olivary nu.; NVII, facial nerve; NRM, nu. raphe magnus; PGi, nu. reticularis paragigantocellularis; PGi α , nu. reticularis paragigantocellularis pars α ; PGi λ , nu. reticularis paragigantocellularis lateralis; PnC, caudal pontine reticular nu.; py, pyramidal tract; RPa, raphe pallidus nu.; ROb, raphe obscurus nu.; SPV, spinal trigeminal tract; SPVO, spinal trigeminal nu. oralis.



CHAPTER V

The Contribution of Brainstem GABAergic Circuitry to Descending Antinociceptive Controls:

II. Electron Microscopic Immunocytochemical Evidence of GABAergic Control over the Projection from the Periaqueductal Gray to the Nucleus Raphe Magnus in the Rat.

David B. Reichling and Allan I. Basbaum

SUMMARY

Pharmacological, physiological, and behavioral studies suggest that inhibitory GABAergic neurons influence the projection from the midbrain periaqueductal gray matter (PAG) to the medullary nucleus raphe magnus (NRM). The present study used electron microscopic immunocytochemical techniques to examine the morphology and synaptic relationships of GABA-immunoreactive terminals in the ventrolateral PAG. These putative GABAergic terminals comprise 39% of all axon terminals in the PAG. GABA-immunoreactive terminals contain small, clear, pleomorphic or round, vesicles, and 46% also contain some dense-cored vesicles. In some experiments we also used a colloidal gold-conjugated retrograde tracer to label PAG neurons that project to the NRM. About half of the synaptic inputs onto the cell bodies and proximal dendrites of retrogradely labeled neurons are GABA-immunoreactive; these terminals make symmetrical contacts and rarely contain dense-cored vesicles. These putative GABAergic synapses, that directly control activity in PAG-NRM projection neurons, might mediate the antinociception-related effects of exogenous GABA_A receptor ligands.

INTRODUCTION

Many studies have implicated the inhibitory amino acid neurotransmitter, GABA, in CNS mechanisms of analgesia.(Defeudis 1983).

Electrical or chemical activation of the projection from the midbrain periaqueductal gray matter (PAG) to the medullary nucleus raphe magnus (NRM) produces antinociception (for review see Basbaum and Fields 1984). The opioid peptide transmitters are believed to have exclusively inhibitory effects in the CNS (Nicoll *et al.* 1980). Thus, for opiates to produce analgesia in the PAG, it has been proposed that opioidergic synapses inhibit an

interneuron that tonically inhibits the PAG-NRM projection neuron (Yaksh *et al.* 1976; Basbaum and Fields 1984). A number of pharmacological studies suggest that the projection from the PAG to the NRM is subject to GABAergic inhibition, and that this inhibition antagonizes the analgesic effect of opioid receptor ligands in the PAG (*c.f.* Moreau and Fields 1986). Therefore, the hypothetical inhibitory interneuron, through which opioids exert an antinociceptive effect in the PAG, might be GABAergic. The ventrolateral region of the PAG supports electrical stimulation-produced analgesia and, this region contains all the individual neural elements required by the circuitry model described above, namely, opioid peptide terminals, GABA cell bodies and terminals, and PAG-NRM projection neurons (see Reichling *et al.*, submitted).

The present study uses combined GABA immunocytochemistry and retrograde labeling at the electronmicroscopic level to test the hypothesis that neurons projecting from the ventrolateral PAG to the NRM receive GABAergic synaptic contacts. Preliminary reports of this work have been published previously (Reichling and Basbaum 1987; Reichling *et al.* 198).

METHODS

Preparation of animals

Male Sprague-Dawley rats weighing 280-320 grams were used in this study. The animals were anesthetized with pentobarbital (60 mg/kg, i.p.), placed in a stereotaxic frame, and the dura was exposed by a trephination. The retrograde tracer, WGAapoHRP-Au (0.2 μ l; Basbaum and Menetrey 1987) was stereotaxically microinjected through a 30 gauge Hamilton syringe into the NRM. After survival for 5-18 days the rats were perfused transcardially with 100 ml of 37° C 0.05 M phosphate buffered saline, pH 7.4 (PBS) followed by 500 ml of fixative (described below) at 4° C. After perfusion the brains were

dissected out, placed in cold fixative for 2 hour, and transferred to cold 0.05 M PBS.

Pre-embedding immunocytochemistry

For experiments in which immunocytochemistry was performed before the sections were embedded in plastic, the fixative was 0.5% glutaraldehyde and 4% paraformaldehyde in 0.05 M PBS. Thirty-micron Vibratome sections of the midbrain were collected, and then the tissue underwent a silver-intensification procedure to make the retrograde tracer visible at the light microscopic level and to facilitate its detection at the electron microscopic level. This reaction deposits a dense precipitate of silver ions on the colloidal gold particles. A detailed protocol has been published previously (Basbaum and Menetrey 1987). Briefly, the sections were first rinsed for 5 minutes in 0.1M sodium citrate buffer (pH 3.8), and then placed in a physical developer solution for 50 minutes in total darkness. Each 100 ml of developer consists of: 60 ml of 50% aqueous gum arabic, 10 ml of 1.0 M sodium citrate buffer (pH 3.8), 15 ml of 5.6% aqueous hydroquinone, and 15 ml of 0.7% aqueous silver lactate. After development, the sections were rinsed for 5 minutes in citrate buffer, rinsed 5 minutes in 0.05 M PBS, and then placed to a solution of 2.5% sodium thiosulfate in 0.05 M PBS for 5 minutes. Sections were rinsed at least 3 times in 0.05 M PBS before further processing.

To protect the silver-enhanced tracer against oxidation that occurs during osmication, the tissue next underwent a gold-toning reaction. The sections were washed three times in distilled water, and then was incubated, in the dark, in 0.05% aqueous chlorauric acid, followed by three distilled water washes. The gold chloride was then reduced by a two-minute rinse in 0.05% aqueous oxalic acid, followed by three distilled water washes. Three twenty-minute incubations in 1% aqueous sodium thiosulfate de-

impregnated excess silver. Finally, the tissue was washed three times in distilled water.

The sections were then immunostained according to the peroxidase anti-peroxidase (PAP) immunocytochemical technique (Sternberger 1979) using a rat polyclonal antiserum (kindly provided by Dr. Andrew Towle of Cornell University), that was raised against a glutaraldehyde conjugate of GABA and bovine serum albumin (BSA). Radioimmunoassay established that this antibody is highly specific for glutaraldehyde-fixed GABA (Lauder *et al.* 1986). Tissue staining was completely abolished by preabsorption of the antiserum with 10 μ M of the GABA-BSA conjugate. To improve penetration of the antibodies, incubation times were prolonged -- 48 hours in primary antibody (diluted 1:3750), 12 hours in goat anti-rat IgG (1:100; Cappel), and 1 hour in rat PAP (1:500; ICN Immunobiologicals). After the PAP step, the sections were rinsed in PBS and then reacted with the chromogen, diaminobenzidine. Small areas of the ventrolateral PAG were excised from the sections, osmicated (1% osmium in 0.05 M PBS for 1 hour on ice), dehydrated, and flat-embedded in Polybed (PolyScience).

Post-embedding immunocytochemistry

For experiments using post-embedding immunocytochemistry, the rats were perfused with a fixative solution containing 1.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M phosphate buffered saline. Thirty-micron Vibratome sections were silver intensified and gold-toned as described above. Small regions of tissue were excised from the ventrolateral PAG. This tissue was then fixed in 0.5% osmium in 0.05 M PBS for 1 hour on ice, dehydrated, and embedded in Polybed (PolyScience), a soft plastic embedding medium that is suitable for post-embedding immunocytochemistry. This procedure is a modification of that described by de Biasi and Rustioni (1988). After trimming

the blocks to size, ultrathin sections, were collected onto Formvar-coated 200-mesh nickel grids. For immunocytochemistry, the sections were etched in a saturated aqueous solution of NaIO_4 for 30-45 minutes to expose antigenic sites. This was followed by a five-minute wash in NaBH_4 to quench nonspecific staining. After a 30 minute incubation in normal goat serum diluted 1:30 in a buffer consisting of 0.1% bovine serum albumin and 0.01% sodium azide in 0.05 M Tris-buffered saline pH 7.2, the immunoreaction proceeded as follows: At room temperature, grids were placed for 1 hour in rabbit anti-GABA IgG (Immunonuclear) diluted 1:1000 in the buffer described above; rinsed in buffer; incubated 1 hour in goat anti-rabbit IgG conjugated to colloidal gold particles (10 nm; Janssen) diluted 1:25 in buffer; rinsed; and counterstained with lead citrate and uranyl acetate.

To study the synaptic organization of GABAergic elements in tissue undamaged by the silver intensification procedure, half of the sections for post-embedding immunocytochemistry were neither silver reacted nor gold toned.

Analysis

Quantitative ultrastructural data were derived from the immunogold-stained (post-embedding) tissue. To estimate the density of labeled and unlabeled GABA-immunoreactive terminals in the ventrolateral PAG, a montage was constructed of 15,000X photographs covering a $200\ \mu\text{m}^2$ area of tissue; the number of gold particles in each vesicle-containing profile was counted, and the density was calculated based on the cross-sectional area of the terminal. For analysis of the ultrastructural morphology of GABA-immunoreactive terminals, all 134 labeled terminals within five $40\ \mu\text{m}^2$ areas of the ventrolateral PAG were photographed at a magnification of 20,000X.

RESULTS

Retrograde label

Figure 1 illustrates a typical injection of WGA-apoHRP-Au centered in the NRM. To maximize the number of retrogradely labeled neurons in the PAG, we deliberately made large injections. Only a small amount of tracer spread laterally into the adjacent nucleus reticularis gigantocellularis pars α , presumably because the large size of the tracer complex limits its mobility in the tissue. Some tracer spread dorsally along the needle track into the medial longitudinal fasciculus.

At the light microscopic level, with bright field illumination, silver-enhanced WGA-apoHRP-Au is a black, punctate precipitate in cell bodies and proximal dendrites (figure 2). Retrogradely labeled neurons are most numerous in the ventrolateral and lateral regions of the PAG, comprising about one third of the total population of neurons in this region (figure 2.1). Retrogradely labeled neurons in the ventrolateral PAG are small (10-15 μ m long diameter), fusiform or multipolar, spherical cells. Another group of large (15-20 μ m long diameter), round or polygonal, multipolar labeled neurons was observed dorsal to the aqueduct. At the level of the inferior colliculus, another distinctive cluster of labeled, large (15-20 μ m), multipolar, round or polygonal neurons was seen in the lateral part of the ventromedial PAG, in a region that may correspond to the lateral part of the dorsal raphe nucleus. Since these neurons may be a subset of dorsal raphe serotonergic neurons, the blocks of tissue used in the present study did not include this cluster of cells.

Although gold-toning protects most of the silver-enhanced WGAapoHRP-Au from oxidation by osmium (which results in the loss of visible tracer particles), much of the labeling in proximal dendrites is still lost. For

this reason, all the present observations concern GABA-immunoreactive inputs onto neuronal cell bodies and large proximal dendrites. In the electron microscope, the retrograde label appears as large (up to approximately 0.3 μm diameter) electron-dense particles. These particles are observed only within neuronal cell bodies and, occasionally, in proximal dendrites. The particles are almost always seen in large (approximately 0.3-0.8 μm diameter) lysosomes (see figure 8), which otherwise have a fine granular content.

Most of the retrogradely labeled neurons received very few somatic synapses. We estimated the extent of synaptic inputs to cell bodies by making a linear measurement of the percent coverage of the perikaryal membrane by synapses and closely apposed terminals. This analysis was made in the plane of a single section. Consistent with the general scarcity of axo-somatic synapses in the ventrolateral PAG (Moss and Basbaum 1983), we measured coverage values of less than 5% for most neurons. Some neurons, however, including some that were retrogradely labeled had coverage values of up to 30%.

GABA Immunoreactivity

At the light microscopic level, GABA immunoreactivity in pre-embedding, DAB-reacted tissue, appeared as a diffuse stain throughout the PAG. Individual varicosities were only discernable at high magnification (figure 2). The GABA-immunoreactive fibers are densest in an annulus around the aqueduct and in the medial part of the ventrolateral PAG. For a complete report of our light-microscopic observations of GABA immunoreactivity in the PAG, see Reichling *et al.* (submitted).

Because of the small size of colloidal gold particles, and because cell bodies were not labeled, post-embedding immuno-gold labeling is only visible in the electron microscope. A low background density of gold particles

(approximately $0.5 \text{ particle}/\mu\text{m}^2$) is distributed over the entire tissue section. Since gold particles are found at much higher density within vesicle-containing profiles, immunolabeled profiles are readily distinguished from background. To establish a criterion to differentiate labeled from unlabeled profiles, we measured the densities of gold particles in 50 randomly selected terminals in the ventrolateral PAG. The results are presented graphically in figure 2.2. Two distinct populations are evident -- "unlabeled" profiles contain less than $10 \text{ particles}/\mu\text{m}^2$ and "labeled," or "immunoreactive," profiles contain more than $25 \text{ particles}/\mu\text{m}^2$. Using these criteria, 133 vesicle-containing profiles were classified as GABA-immunoreactive in a $2000 \mu\text{m}^2$ region of the ventrolateral PAG; 207 were unlabeled. Thus, approximately 39% of all terminals in the ventrolateral PAG are GABA-immunoreactive.

Colloidal gold particles do not preferentially cluster over the synaptic vesicles within immunoreactive terminals. Instead, single gold particles and/or clusters of 3-4 particles are spread evenly within the confines of the terminal. A higher density of gold particles was sometimes seen over mitochondria that were located in the immunoreactive synaptic terminals.

The mean area of GABA-immunoreactive profiles is $3.7 \mu\text{m}^2$. This figure does not differ significantly from the mean for unlabeled profiles. Immunoreactive profiles contain electronlucent cytoplasmic matrix and always contain small, agranular vesicles (40-50 nm diameter) that, under our fixation conditions, are round or occasionally pleomorphic (figure 3). The packing density of these vesicles varies widely, from sparse ($40 \text{ vesicles}/\mu\text{m}^2$), to very dense ($250 \text{ vesicles}/\mu\text{m}^2$). Dense-cored vesicles are also found in 46% of immunoreactive profiles (figures 3 and 6); in such terminals, 2-4 of these large (80-95 nm diameter) granular vesicles are usually seen in a single plane of section.

We could identify synaptic specializations in approximately 30% of the immuno-gold labeled profiles. A single section through the terminal usually revealed only a single zone of synaptic contact that typically measured 0.15-0.40 μm in length. The membranes in contact were usually straight or slightly curved. Synaptic contacts with details distinct enough to allow classification always appeared to be Gray's Type II, i.e. with "symmetrical" pre- and postsynaptic densities (figures 3 and 4).

The postsynaptic element of most of the GABA-immunoreactive synaptic contacts was identified morphologically as a dendrite ranging in area from 1-20 μm^2 with a median of 2 μm^2 . Only about 5% of the labeled terminals made synaptic contact with cell bodies. We found no evidence for GABA-immunoreactive axo-axonic synapses in the ventrolateral PAG.

Synaptic Contacts with Retrogradely Labeled Neurons

Some GABA-immunoreactive synaptic terminals made synaptic contact with retrogradely labeled cell bodies and proximal dendrites (figures 6 and 7). Retrogradely labeled neuronal cell bodies that were contacted by GABA-immunoreactive terminals were small (approximately 15 x 8 μm) and oval-shaped. These neurons probably correspond to the small, bipolar fusiform or oval, neurons seen in the light microscope. The cytoplasm of these cells had no remarkable features. Neither the morphology of these terminals nor their synaptic contacts distinguished them from the general population of GABA-immunoreactive terminals. These terminals, however, were distinctive with respect to their vesicle content: with only one exception, all of the GABA-immunoreactive terminals that were presynaptic to projection neurons contained densely-packed round or pleomorphic, small, agranular vesicles, but none contained dense-cored vesicles.

The necessity of performing electron microscopic studies to unequivocally establish the existence of synaptic contacts was evident in these studies. Specifically, we occasionally observed terminals that appeared to contact a dendritic profile, but higher magnification revealed that the apposed profiles were, in fact, separated by a very thin sheet-like process (figure 5). These processes, presumably astrocytic in origin, were in some cases as thin as 20 nm.

In order to estimate the relative contribution of GABAergic somatic inputs to these projection neurons, we counted the number of GABA-immunopositive and GABA-immunonegative synaptic contacts on 18 randomly selected, retrogradely labeled cell bodies and 3 proximal dendrites. Together, these profiles received 11 immunopositive contacts and 10 immunonegative synaptic contacts. Based on this small sample, it appears that putative GABAergic terminals constitute approximately half of the observed synaptic inputs to the cell bodies of some projection neurons in the PAG.

DISCUSSION

This study provides new information on the organization of putative GABAergic neuronal elements in the ventrolateral PAG of the rat. We found that 39% of axon terminals in the ventrolateral PAG were GABA-immunoreactive. These GABA-immunoreactive terminals contain numerous small, clear vesicles, and many also contain dense-cored vesicles. Importantly, neurons that were retrogradely labeled from the NRM received synaptic contacts onto their cell bodies and proximal dendrites. These contacts accounted for approximately half of the observed synaptic inputs to these neurons. These data provide support for the hypothesis that GABAergic interneurons control activity in PAG neurons that project to the NRM.

GABA immunoreactivity

Our initial studies characterized the overall distribution of GABA-immunoreactive terminals in the PAG using the pre-embedding PAP-reacted material. This distribution has been reported in detail previously (Reichling *et al.*, submitted). Briefly, GABA-immunoreactive terminals are moderately dense throughout the PAG, but are densest in an annulus surrounding the aqueduct and in the ventrolateral PAG.

The punctate nature of post-embedding immunogold stained material permits recognition of even very low density staining. GABA-immunoreactive material, may, however, result from metabolic (mitochondrial) processes in the cell or from accumulation of extracellular GABA by neurons and glia that do not produce GABA as a transmitter (Csillag *et al.* 1987; Izumi *et al.* 1980; Kisvárdy *et al.* 1986; Zucker *et al.* 1984). It is, therefore, important to establish quantifiable criteria to distinguish what might be non-transmitter-derived staining from specific staining of putative GABAergic synaptic terminals. Since there is such a clear separation between the populations of densely and lightly labeled terminals, we conclude that terminals with greater than 25 colloidal gold particles/ μm^2 are "labeled" and presumably GABAergic. The fact that a single thin section contains heavily labeled terminals that are immediately adjacent to unlabeled terminals (for example, see figure 3) provides some assurance that unlabeled terminals are not "false negatives" -- we believe that these two varieties of staining are due to properties of the terminals and are not due to vagaries of the staining process.

We observed that the anti-GABA antibody binds throughout the terminal profile, including the mitochondria. This distribution of antigenic sites may be related to the process of glutaraldehyde fixation, by which the amino group of the GABA molecule is cross-linked to proteins. Proteins rich in arginine

and lysine offer the most amino sites for cross-linkage, and so the staining pattern we observed might indicate a differential distribution of available cross-linkage sites in the terminal, rather than the distribution of GABA *in vivo*. There is no reason to believe that this fixation artifact reduces the reliability of our method to identify putative GABAergic terminals. In fact, Schon and Iversen (1972) offered a similar explanation for the preferential binding of [^3H]GABA to neuronal nuclei.

We found that 39% of all terminals in the ventrolateral PAG are GABA-immunoreactive, a figure that is consistent with previous counts in other regions of the brain. For example, Iversen and Bloom (1972) reported that 25-45% of synaptosomes in homogenates of rat brain accumulate [^3H]GABA, and using post-embedding immunocytochemistry, Cho and Basbaum (unpublished) found that 45% of terminals in the NRM are GABA-immunoreactive, and Beauvillain, et al. (1988) found that 40% of the boutons in guinea pig hypothalamus are GABA-immunoreactive.

Since 46% of GABA-immunoreactive terminals contain dense-cored vesicles, it is likely that coexistence with peptides is an important characteristic of GABAergic synaptic transmission in the PAG. Coexistence of GABA with putative peptide transmitters in the PAG has, however, not yet been demonstrated. In light of pharmacological evidence for antinociception-related interactions between GABA and opiates in the PAG (*c.f.* Moreau and Fields 1986) it is of interest that GABA coexists with enkephalin in some retinal amacrine cells (Watt et al. 1988), where it has been suggested that enkephalin exerts an autoregulatory influence over GABAergic transmission (Lam *et al.* 1986).

Retrograde Labeling

All injections of retrograde tracer were centered in the NRM. WGA-apoHRP-Au is poorly taken up by axons of passage, and is not transported anterogradely (Basbaum and Menetrey 1987). Thus, it is likely that all retrogradely labeled neurons observed in the PAG have axon terminals in the immediate vicinity of the injection site in the rostroventral medulla. Due to the large size of the WGAapoHRP-Au complex, which limits its mobility in tissue, only small amounts of tracer spread laterally from the NRM into the nucleus reticularis gigantocellularis pars α (NGc α), which receives a significant projection from the ventrolateral PAG (Mantyh 1983). Consistent with the idea that most neurons were retrogradely labeled from the NRM, the distribution of labeled neurons we found corresponds to that reported in other studies where different retrograde tracers were used (Abols and Basbaum 1981; Beitz *et al.* 1983; Carlton *et al.* 1983; Fardin *et al.* 1984a; Gallagher and Pert 1978). Nevertheless, since both the NGc α and NRM have been implicated in descending antinociceptive effects elicited from the PAG (Lovick 1985; Prieto *et al.* 1983; Sandkühler *et al.* 1984), the following discussion bears on PAG activation of both medullary sites.

GABA-immunoreactive synaptic contacts onto projection neurons

Background staining with WGAapoHRP-Au is almost nonexistent at the electron microscopic level. Thus, the ultrastructural identification of retrogradely labeled neuronal elements is unambiguous. This assertion is supported by the following observations: 1) silver-enhanced particles of retrograde tracer are found only in lysosomes in neuronal cell bodies and proximal dendrites; 2) labeled profiles usually contain more than one particle of the tracer.

We found that approximately half the somatic synapses on retrogradely labeled neurons were GABA-immunoreactive. The strategic location of

putative GABAergic synapses on cell bodies suggests that activity in some components of the PAG-NRM pathway are subject to GABAergic gating. Consistent with GABAergic terminals exerting an inhibitory action, all of the labeled terminals we observed formed symmetrical Gray type II synaptic contacts, and many contained pleomorphic vesicles. Importantly, we occasionally observed thin glial sheaths interposed between an immunolabeled terminal and the cytoplasmic membrane of a retrogradely labeled cell body. The existence of these near appositions emphasizes the danger in attaching much significance to reports of "apparent contacts" based on light microscopic observations -- confirmation of synaptic contact can only be made at the electronmicroscopic level.

The origin of GABA-immunoreactive terminals

Since GABA-immunoreactive neurons are very common in the PAG (Clements *et al.* 1985; Reichling *et al.* 1987; Reichling *et al.*, submitted), it is likely that the GABA-immunoreactive terminals we observed derive locally, from GABAergic cell bodies located within the PAG. In fact, other lines of evidence support the idea that GABAergic neurons in the PAG have local axon terminals. First, lesions of major sources of afferents to the dorsal raphe nucleus (the dorsal pontine tegmentum, substantia nigra, or habenula) do not alter glutamic acid decarboxylase (GAD) activity in the ventral PAG and dorsal raphe of the rat (Belin *et al.* 1979). Second, light microscopic Golgi studies (Tredici *et al.* 1983) show that PAG cell bodies often appear to be contacted by axons that originate from nearby neurons. These investigators also reported that all axo-somatic synapses in the PAG have symmetrical junctional morphology and contain pleomorphic vesicles, and inferred that these synaptic inputs comprise a uniformly inhibitory population of synapses. Our data contradict the conclusion that the population of axo-somatic synapses in the

PAG is homogeneous -- some are GABA-immunoreactive and some are unlabeled.

In other studies, Sandner *et al.* (1986) electrically stimulated and recorded at nearby sites in the rat PAG. Stimulation-induced inhibition of PAG neurons was often observed, and the latency of inhibition was consistent with mediation by a local inhibitory synapse. The post-stimulus patterns of activity suggested that both recurrent inhibition and local interneurons were involved. Since these inhibitory effects were most common when the electrodes were separated in the mediolateral, versus rostrocaudal, direction, local inhibitory connections in the PAG might be preferentially oriented in the coronal plane.

The contribution of GABAergic controls to antinociception

Although multiple pathways mediate antinociception elicited from the PAG, the most thoroughly characterized system involves the direct projection to the NRM. The latter region is the origin of an inhibitory serotonergic projection to the spinal cord dorsal horn (see Basbaum and Fields 1984). Since electrical stimulation in the PAG produces analgesia (for review see Mayer and Liebeskind 1974), and since lesions of the PAG do not produce analgesia (Dostrovsky and Deakin 1977; Kelly and Glusman 1968; Melzack *et al.* 1958; Rhodes 1979; Yeung *et al.* 1975), it has been concluded that the projection neuron from the PAG to the NRM must be activated for analgesia to result. Narcotics microinjected in the PAG also produce analgesia (for review see Yaksh and Rudy 1978) at least in part, via the same circuitry through which electrical stimulation produces analgesia (for discussion see Besson and Chaouch 1987, pp. 144-145). Opiates, however, are presumed to have exclusively inhibitory effects (Nicoll *et al.* 1980), and thus could not directly activate the PAG neurons that project to the NRM. It has, therefore, been

proposed that opiates indirectly activate the PAG-NRM projection neurons, via a disinhibition. Specifically, opiates are presumed to inhibit an inhibitory interneuron that tonically inhibits the PAG output neuron (Yaksh et al. 1976).

Zieglgänsberger *et al.* (1979) reported a similar disinhibitory action in the hippocampus that was mediated by opiate-sensitive, tonically active GABAergic interneurons. In view of this model, and the presence of putative GABAergic neurons in the PAG, Basbaum and Fields (1984) proposed that a similar tonically active, GABAergic interneuron is interposed between the opioidergic terminals in the PAG and the neurons that project to the NRM. This model predicts the existence of two synapses in the PAG: 1) opioid terminals that contact GABA neurons, 2) GABA terminals that contact PAG-NRM projection neurons. The present study demonstrates that GABA terminals do, in fact, synapse upon PAG neurons that project to the rostral ventral medulla.

Although we cannot conclude from our data that these GABAergic elements are subject to opioidergic controls, pharmacological studies support that hypothesis. For example, if opiates produce analgesia by reducing GABAergic controls over the PAG-NRM projection, then enhancing transmission at the GABAergic synapse should antagonize morphine analgesia. Ho *et al.* (1976), in fact, observed that inhibiting GABA uptake, or inhibiting the GABA-degradative enzyme, GABA transaminase, raised the ED₅₀ for morphine-induced antinociception in mice. Conversely, blocking GABAergic transmission should mimic the effects of morphine; Ho *et al.* (1976) indeed found that systemically administered bicuculline, a GABA antagonist, lowered the ED₅₀ for morphine. Other studies indicate that the GABA receptors that mediate these effects are supraspinal. Intracerebroventricularly (i.c.v.) injected GABA antagonizes antinociception produced by i.c.v. D-Ala²-Met-

enkephalinamide or morphine (Izumi *et al.* 1980; Mantegazza *et al.* 1979). Conversely, bicuculline has an antinociceptive action when administered intracisternally, but not intrathecally (Ueda 1987).

Microinjection studies indicate that the relevant GABA-sensitive circuitry is localized within the PAG. Thus, microinjection of GABA antagonists (bicuculline or picrotoxin) in the PAG produces antinociception (Depaulis, *et al.* 1987; Moreau and Fields 1986), and microinjection of the GABA agonist, muscimol, in the PAG antagonizes the antinociceptive effect of systemic morphine (Romandini and Samanin 1984; Zambotti, *et al.* 1982). Furthermore, since GABA agonists (muscimol or THIP) microinjected in the PAG antagonize antinociception produced by morphine microinjected at the same site (Depaulis, *et al.* 1987; Moreau and Fields 1986) it can be concluded that the relevant opiate-sensitive circuitry, as hypothesized, is also located in the PAG.

The same proposed circuit might also account for the antinociceptive effects of the benzodiazepines, drugs which enhance the inhibitory effect of GABA at the GABA_A receptor complex (for review see Haefele and Polc 1986). The benzodiazepine antagonist and inverse agonist, Ro15-1788, produces analgesia when administered systemically to the rat (Morgan *et al.* 1987). The PAG contains dense neuronal-type benzodiazepine binding sites (Young and Kuhar 1980). If Ro15-1788 were acting at the GABAergic synapse in the PAG which we observed, it would release the PAG-NRM projection neuron from (presumed) tonic GABAergic inhibitory control. This disinhibition would mimic the proposed antinociceptive action of opiates, but since benzodiazepine receptor ligands (and GABA receptor ligands) would act on circuitry in the PAG downstream from opioidergic synapses, analgesia produced by the former drugs should not be reversed by opiate antagonists in the PAG.

Autoradiographic receptor binding studies showed that GABA_A binding sites are much more dense than GABA_B binding sites in the PAG. This is particularly so in the caudal PAG, where analgesic manipulations are most effective (Bowery, et al. 1987; Yaksh et al. 1976). Since GABA_A receptors mediate post-synaptic, rather than presynaptic inhibition, it is significant that all of the GABA-immunoreactive terminals we observed in the caudal, ventrolateral PAG were either axo-dendritic, or, more rarely, axo-somatic; we never observed GABA-immunoreactive axo-axonic synapses. These observations suggest that actions of GABA at the synapses we observed are mediated by GABA_A receptors. In fact, all of the pharmacological studies described above, which are consistent with an action at those synapses, used ligands that act on the GABA_A receptor complex.

On the other hand, the analgesia-producing effects of GABA receptor agonists (Hynes et al. 1984; Vaught et al. 1985; Retz and Holaday 1986), might reflect an action on GABA_B receptors located in the spinal cord (Hammond and Drower 1984; Sawynok 1983; Vaught et al. 1985; Wilson and Yaksh 1978) or at supraspinal sites (Lim et al. 1985; Proudfit and Levy 1978; Zorn and Enna 1987).

In conclusion, these experiments demonstrate that a large proportion of axon terminals in the ventrolateral PAG are GABA-immunoreactive. Some of these putative GABAergic axon terminals make symmetrical synaptic contacts onto neurons that project to the NRM. Thus, exogenously applied GABA receptor ligands may produce antinociception-related effects by acting at these synapses, perhaps by binding to GABA_A receptors. Future studies will be directed towards characterizing the synaptic relationships between opioid terminals and GABAergic neurons in the PAG.

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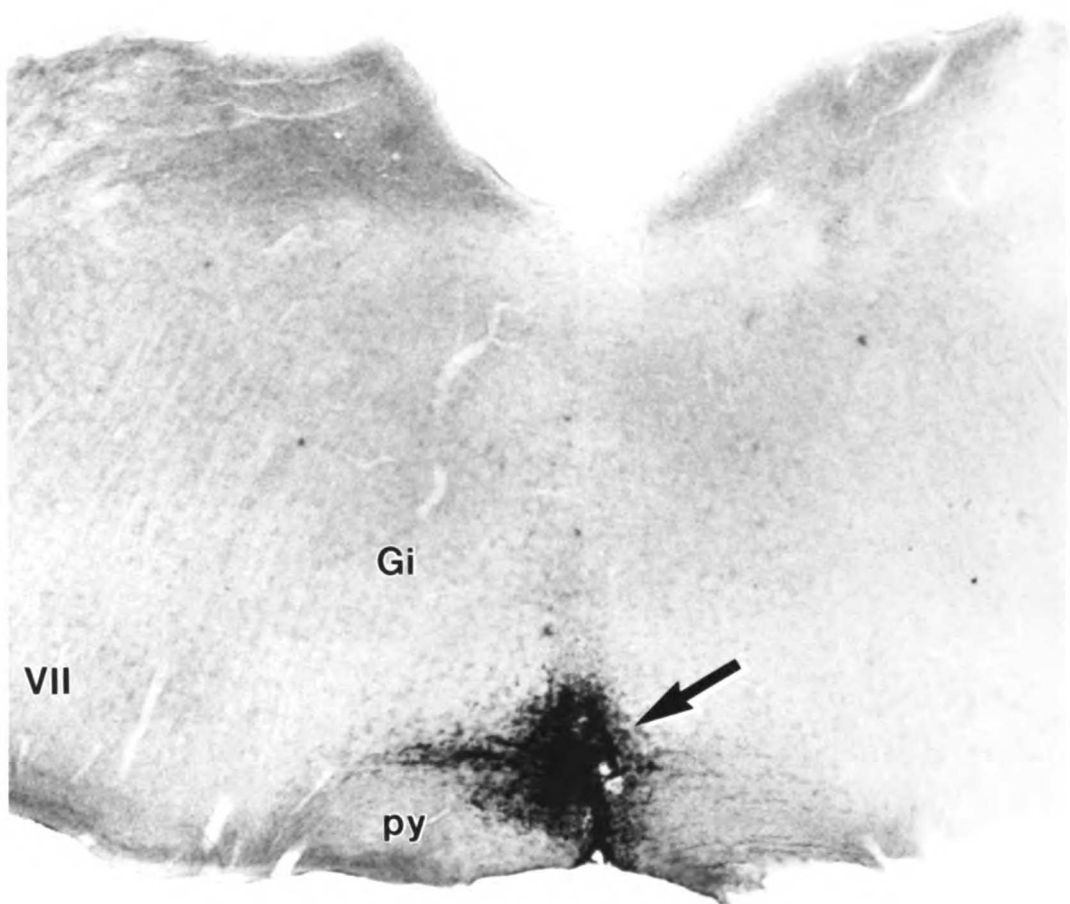


Fig. 2. This dark-field photomicrograph shows the distribution of retrogradely labeled neurons in the PAG after an injection of WGAapoHRP-Au in the NRM. Labeled neurons (small, white spots) are common in most of the PAG, but are rare in the dorsolateral sector (asterisk) and in the dorsal raphe nucleus (DRN). Scale bar = 0.5 mm.

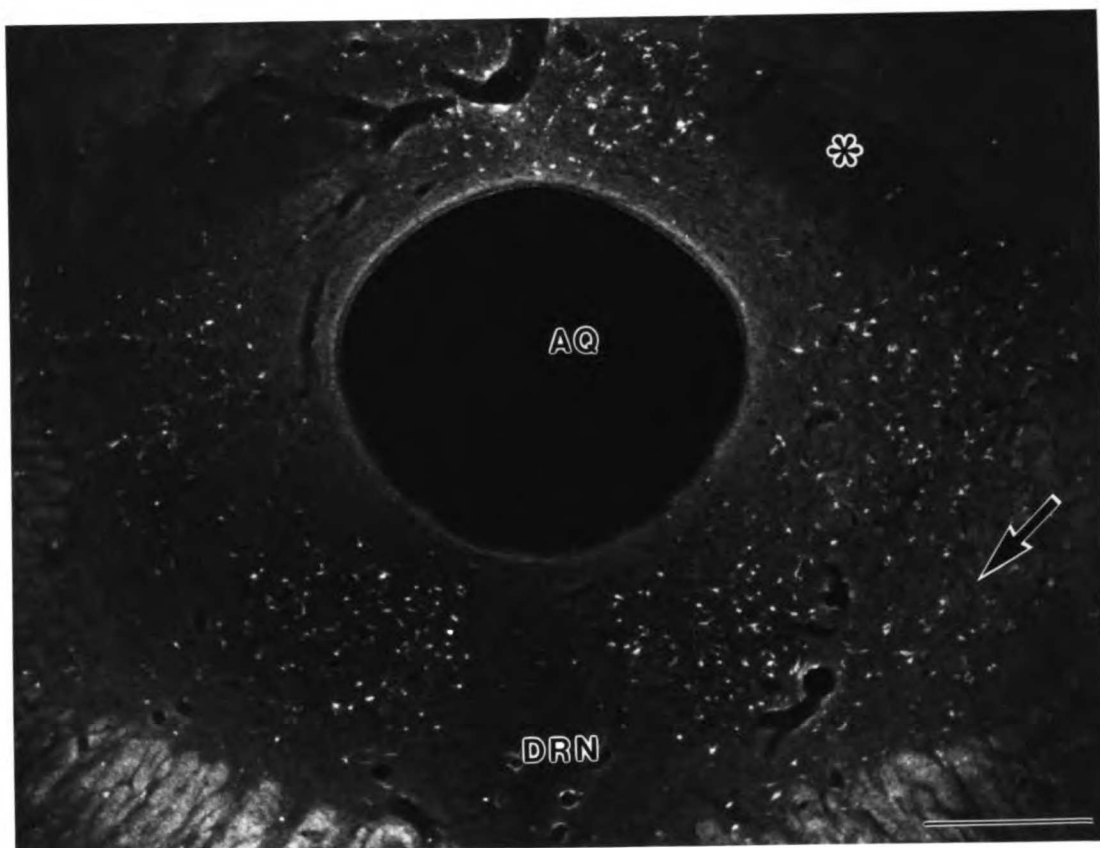


Fig. 3. This photomicrograph shows the light-microscopical appearance of retrograde tracer and pre-embedding GABA immunoreactivity in the ventrolateral PAG, before osmication and embedding. Retrogradely labeled neurons (arrows) contain numerous black particles of silver-enhanced tracer in their cell body and proximal dendrites. GABA-immunoreactive terminals (arrowheads) appear as small, brownish punctae in the DAB-reacted tissue. Scale bar = 100 μ m.

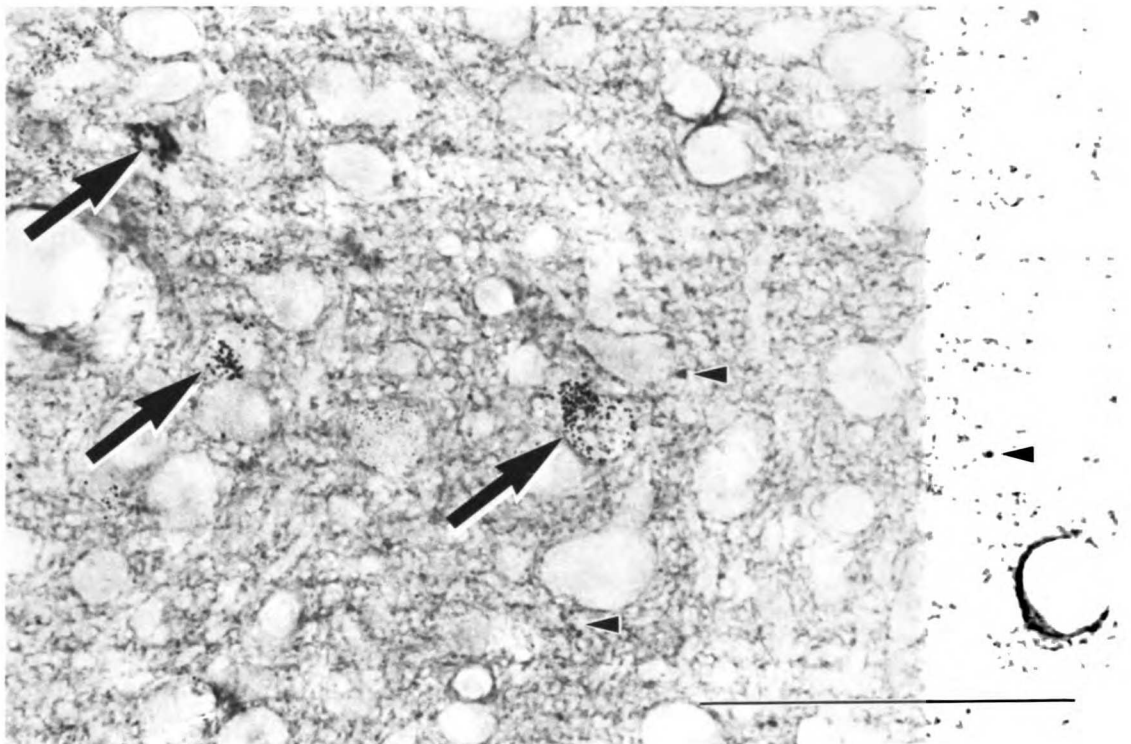


Fig. 4. This figure shows the electron microscopic appearance of post-embedding GABA immunoreactivity in the ventrolateral PAG. GABA-immunoreactive terminals (g_1 and g_2) contain numerous particles of colloidal gold (arrowheads). The GABA-immunoreactive terminal, g_1 , contains dense-cored vesicles (open arrows) in addition to small, clear, pleomorphic vesicles. This terminal forms a symmetrical synaptic contact (arrow) with an unlabeled dendrite (den). An adjacent unlabeled axon terminal (a) contains few particles of colloidal gold. Scale bar = 0.5 μ m.

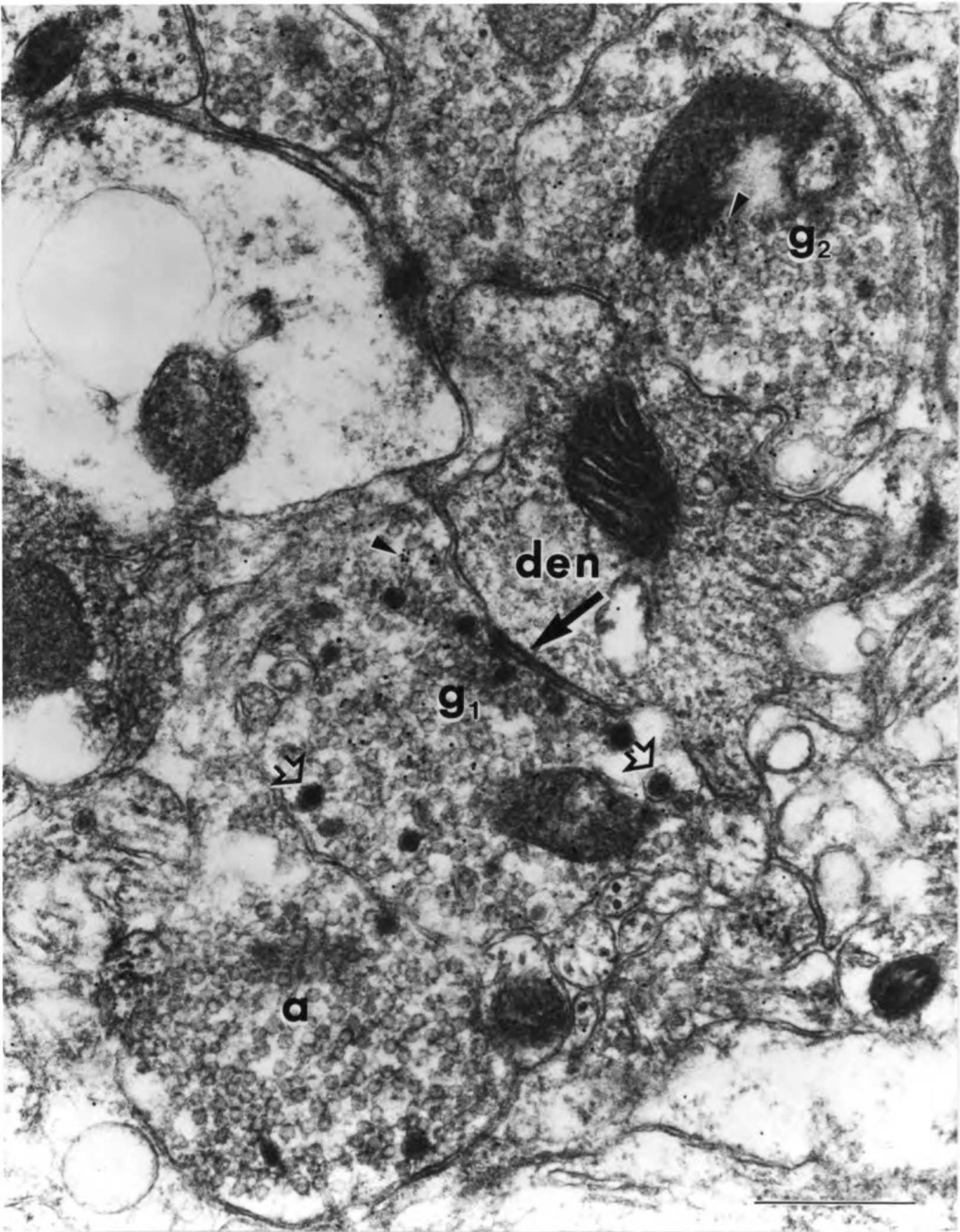


Fig. 5. This micrograph shows a thin dendrite (den) that receives particularly dense GABA-immunoreactive synaptic inputs. In this plate, five GABA-immunoreactive terminals (g) make symmetrical synaptic contacts (arrowheads) onto the dendrite. Other, nearby axon terminals (a) are unlabeled. Scale bar = 0.5 μ m.

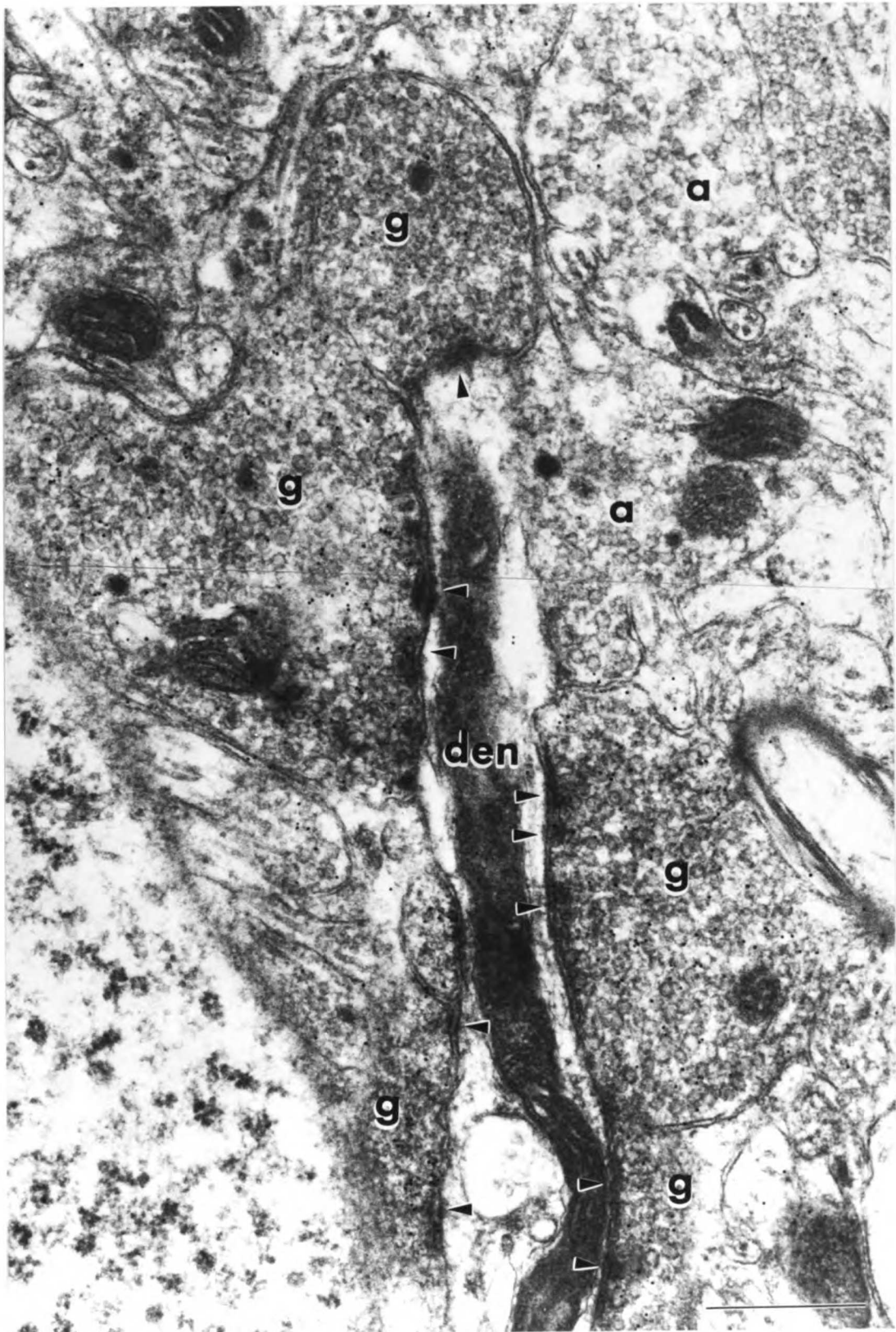


Fig. 6. Axon terminals in the ventrolateral PAG are often separated from other neuronal processes by glial processes. In this figure, a GABA-immunoreactive (g_1) and an unlabeled terminal (a) are separated from a cell body (cb) by a very thin astrocytic sheath (arrowheads). At the light-microscopic level, these terminals would appear to be directly apposed to the cell body. Also in this figure are another GABA-immunoreactive axon terminal (g_2) and a GABA-immunoreactive unmyelinated axon or preterminal segment (arrow). Scale bar = 0.5 μ m.

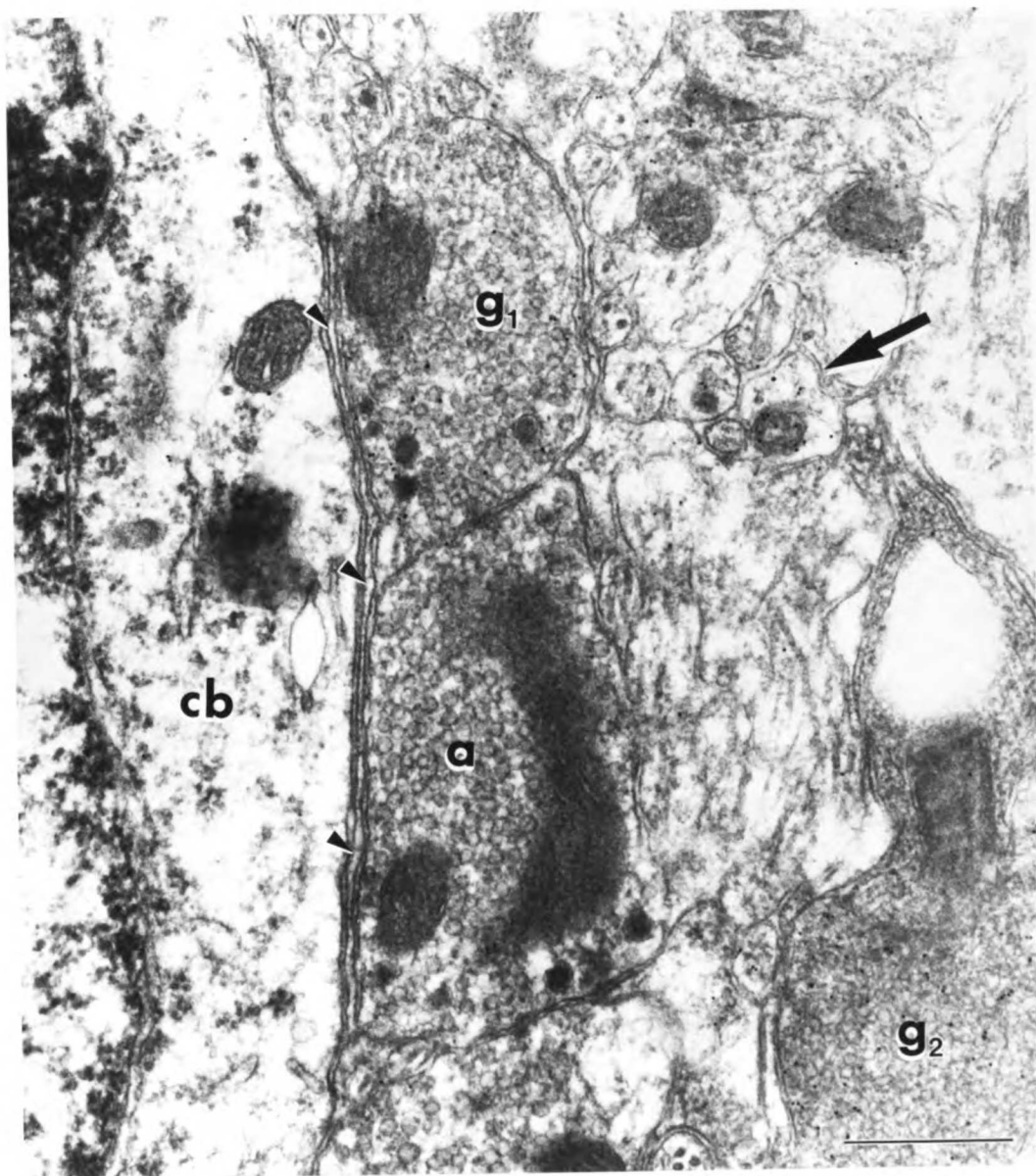


Fig. 7. These electron micrographs show tissue stained by pre-embedding immunocytochemistry. Neuronal cell bodies (cb) that contain silver-enhanced and gold-toned particles of WGAapoHRP-Au (arrows). This gold-conjugated tracer has been retrogradely transported from the NRM. The labeled cell bodies are synaptically contacted by GABA-immunoreactive axon terminals (arrowheads). These terminals are immunostained by a flocculent, electron-dense DAB reaction product. Scale bars = 1 μm and 0.5 μm in A and B, respectively.

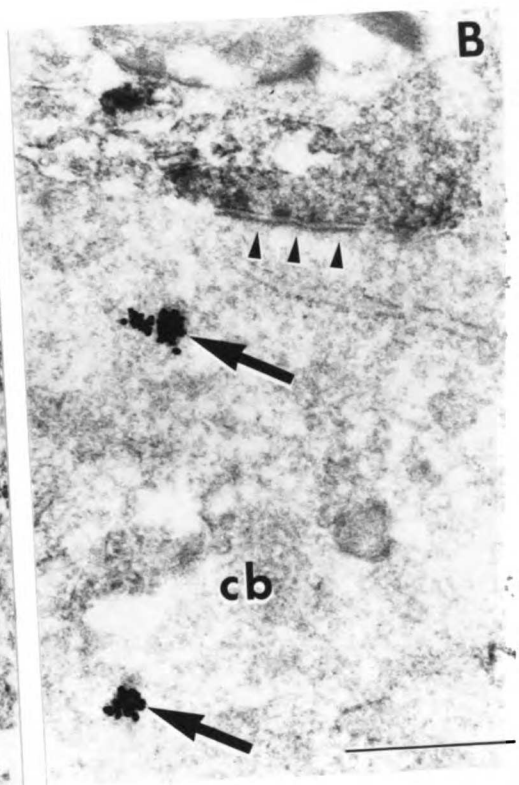
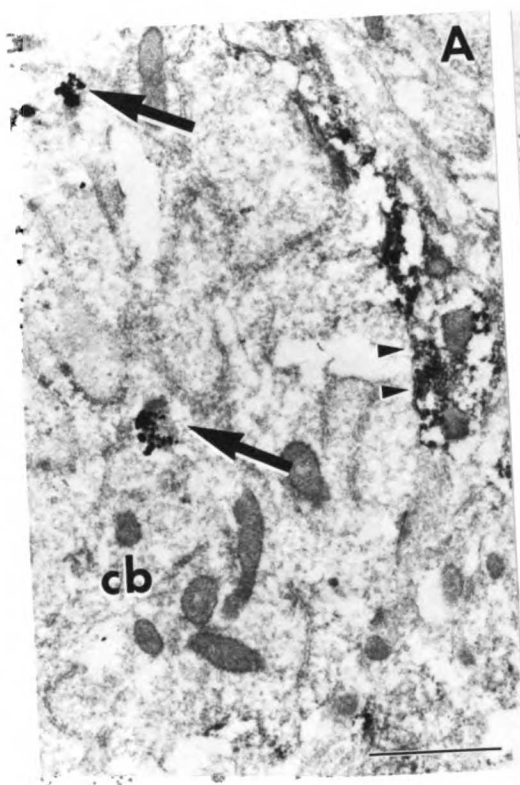


Fig. 8. These electron micrographs illustrate GABA-immunoreactive axon terminals (g), stained by a pre-embedding procedure, that are presynaptic to retrogradely labeled cell bodies in the ventrolateral PAG. The latter are identified by the presence of silver-enhanced and gold toned WGApoHRP-Au particles (arrows) within lysosomes. (The tracer was injected in the NRM.) Arrowheads point to synaptic specializations between the labeled terminals and cell bodies. Scale bars = 0.5 μ m.

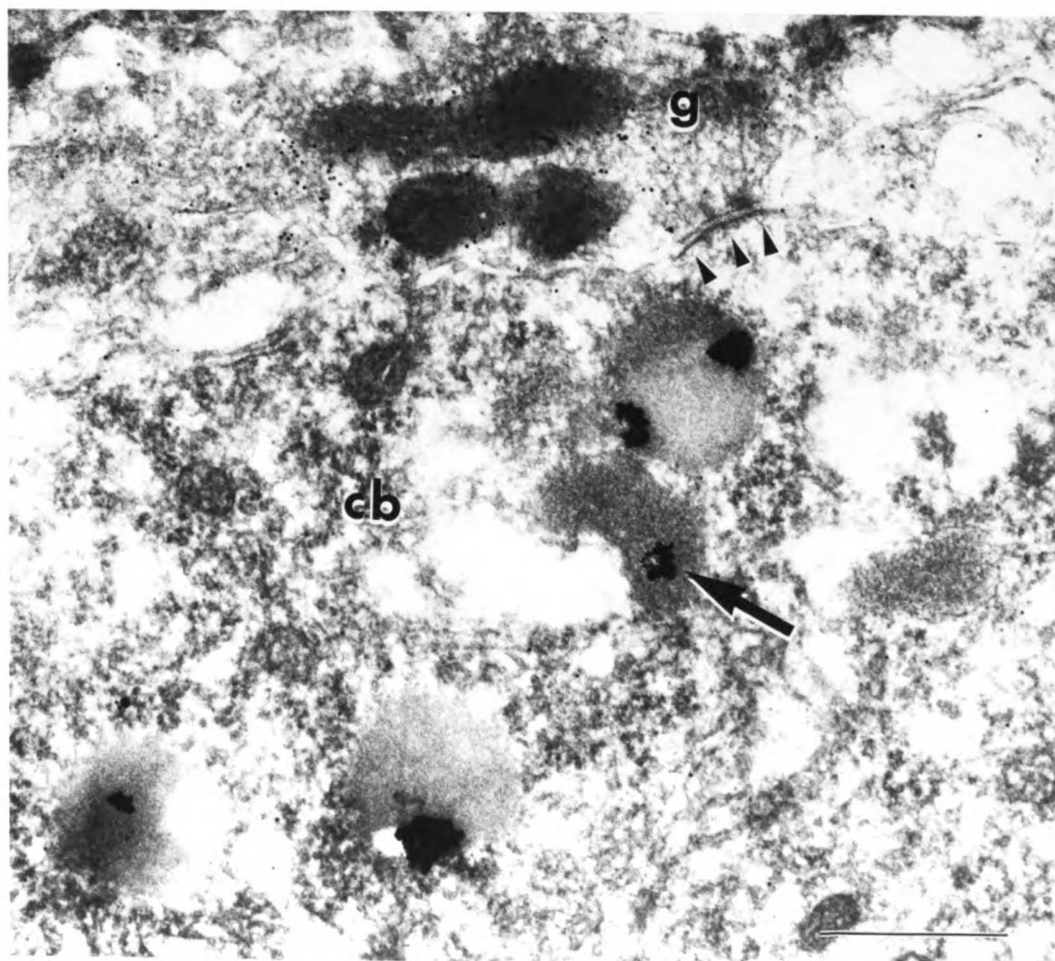
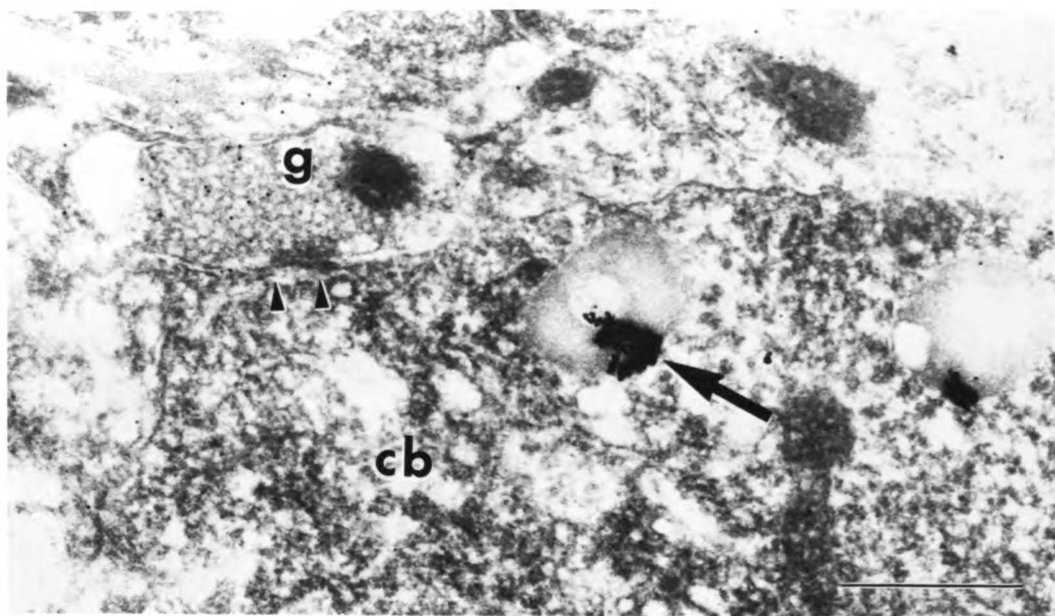


Fig. 9. This figure illustrates a neuronal cell body (CB) in the ventrolateral PAG which is post-synaptic to unlabeled axon terminals (arrowheads) and GABA-immunoreactive axon terminals (arrows). The latter were stained by post-embedding immunocytochemistry. The cell body contains silver-enhanced and gold-toned particles of WGAapoHRP-Au retrogradely transported from the NRM. The nucleus is labeled "Nu." The two regions outlined by squares are shown at higher magnification in figure 10. Scale bar = 1 μ m.

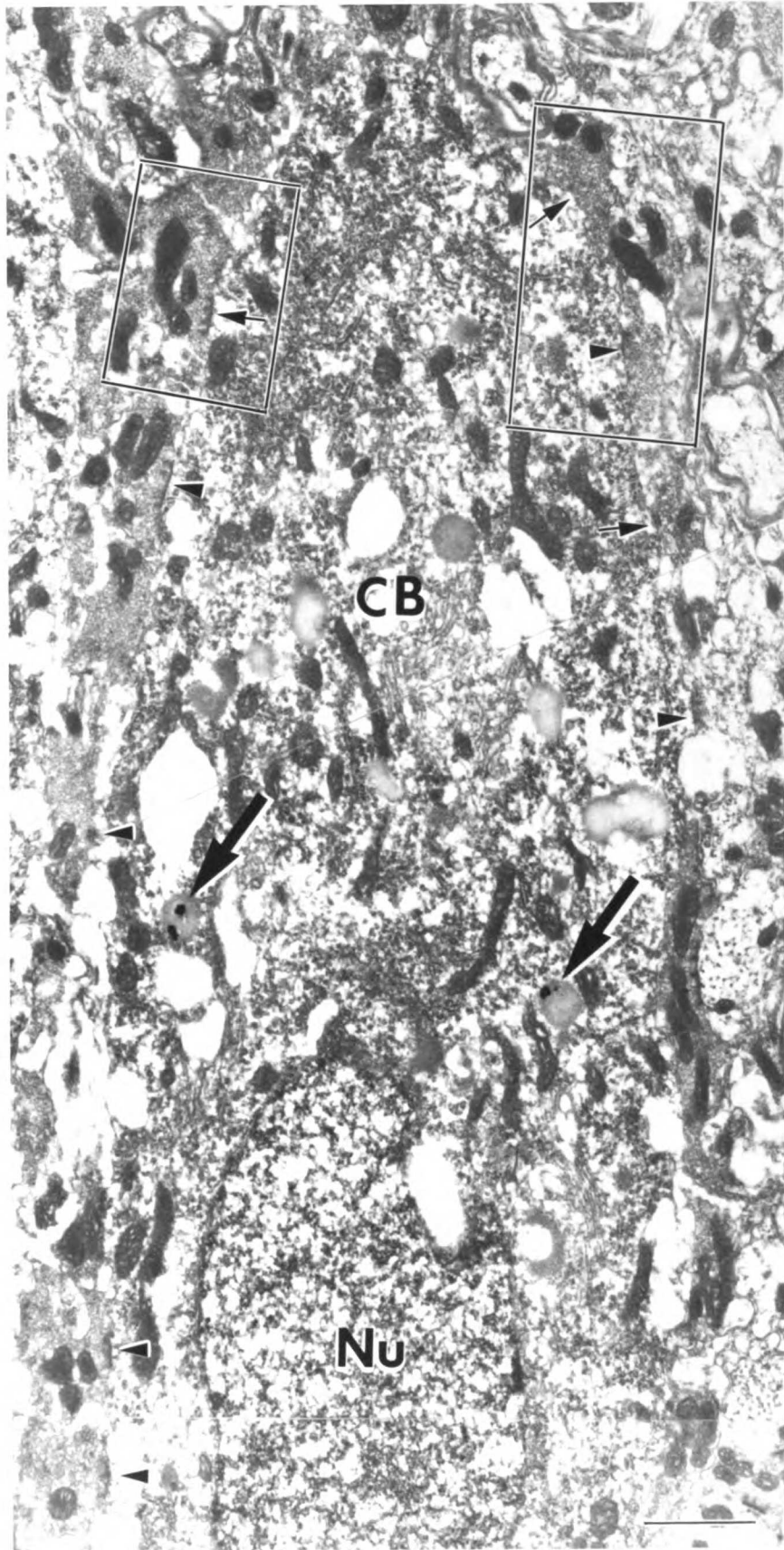
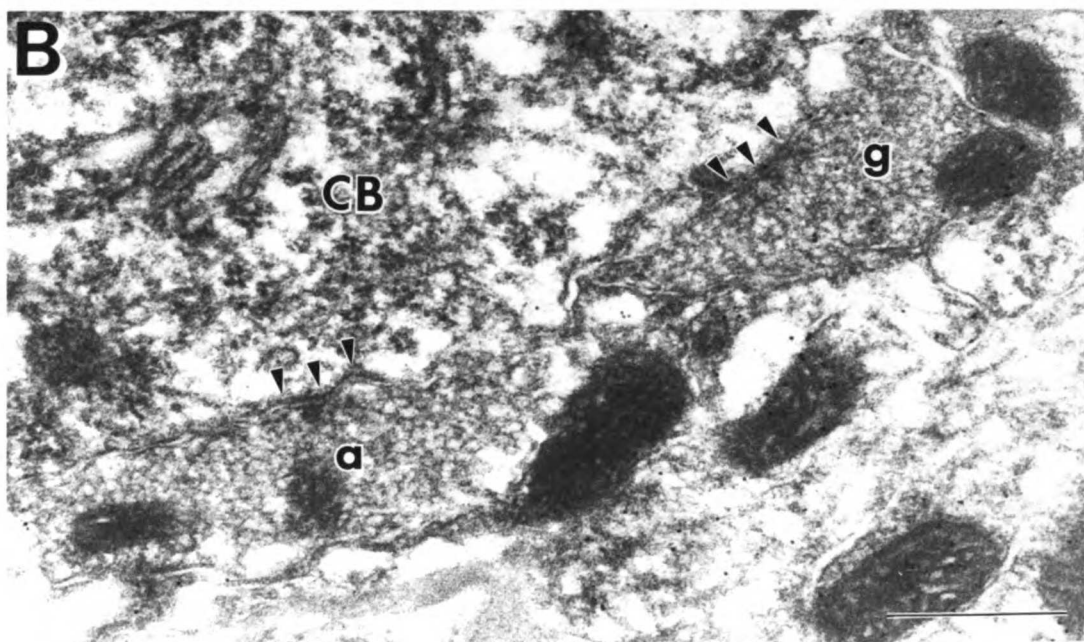
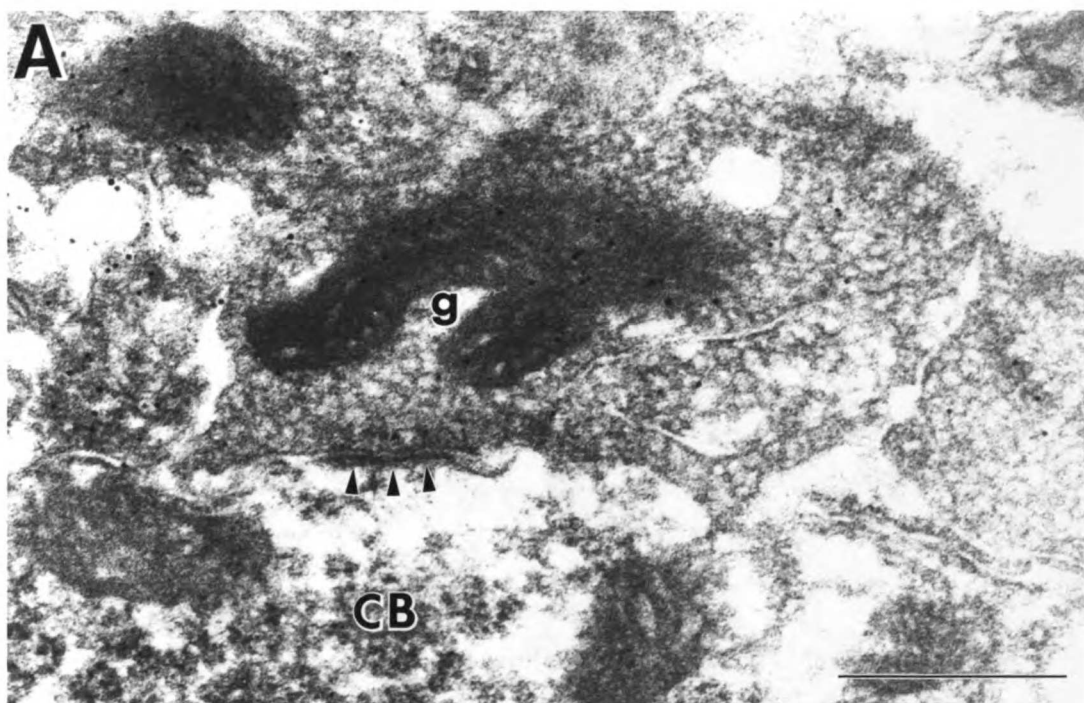


Fig. 10. This figure shows the regions outlined in figure 9 at higher magnification. GABA-immunoreactive axon terminals (g), stained by the post-embedding procedure, make synaptic contacts (arrowheads) onto the cell body (CB). Plate B also shows an unlabeled axon terminal (a) which is presynaptic to the cell body. Scale bars = 0.5 μ m.



CHAPTER VI

Electron Microscopic Immunocytochemical Evidence for Synaptic Interactions between GABAergic Terminals and Serotonergic Neurons in the Dorsal Raphe Nucleus of the Rat

David B. Reichling, Geneviève Chazal, and Allan I. Basbaum

Pharmacological and physiological evidence suggests that serotonergic neurons of the dorsal raphe nucleus (DRN) are under GABAergic control. For example, ionophoretic application of γ -aminobutyric acid (GABA) in the DRN inhibits units with firing patterns characteristic of serotonergic neurons [41]. Furthermore, the GABA antagonist, picrotoxin, blocks the inhibition of DRN serotonergic neurons that is produced by systemic administration of antipsychotic drugs or by electrical stimulation of the habenula or pontine reticular formation [11, 12, 40, 41]. Since presumptive GABAergic axon terminals in the DRN have been detected by GABA [7] or glutamic acid decarboxylase (GAD) immunocytochemistry [23], or by uptake of [3 H]GABA [4], the picrotoxin-sensitive suppression of activity in the DRN might be mediated by GABAergic terminals that synapse directly onto 5-HT-containing neurons. In fact, Harandi *et al.* [15] noted that some terminals that accumulate [3 H]GABA are in close apposition to, or synapse with, 5-HT-immunoreactive dendrites. The fact that a neuron accumulates extracellular GABA, however, does not assure that it synthesizes GABA as a transmitter -- neurons that are not GABA-immunoreactive can accumulate [3 H]GABA [8, 18, 43], and even glia possess high affinity GABA uptake mechanisms [17]. To avoid this problem, in the present study we used simultaneous electron microscopic immunocytochemical detection of GABA and 5-HT to provide direct anatomical evidence of synaptic contacts between presumptive GABAergic and serotonergic neuronal elements in the DRN. GABA-immunoreactive profiles were immunostained by the peroxidase-anti-peroxidase method [38]; 5-HT-immunoreactive profiles were immunolabeled with colloidal gold [19].

Male Sprague-Dawley rats were perfused with 100ml of 0.05M phosphate-buffered saline (PBS) followed by 500ml of fixative containing 4% paraformaldehyde and 1% glutaraldehyde in PBS. The brains were removed and 30 μ m sections were cut on a Vibratome. The sections were first incubated, for one hour, in a blocking solution of 3% normal goat serum in PBS, and then for 24 hours in a rabbit antiserum raised against a BSA conjugate of 5-HT (Incstar Corp.). The sections were next incubated in goat-anti-

rabbit IgG labeled with 15nm colloidal gold particles (Janssen). After extensive washing, sections were incubated in a polyclonal rat anti-GABA antiserum, diluted 1:3750. This antiserum (kindly provided by Dr. Andrew Towle of Cornell University) is directed against a glutaraldehyde conjugate of GABA and bovine serum albumin. Radioimmunoassay shows that the antiserum is highly specific for glutaraldehyde-fixed GABA [20]. Prolonged incubation times were used to improve penetration of antibody and avoid the ultrastructural damage caused by detergents. Thus, after 48 hours, the sections were incubated in goat-anti-rat IgG (1:100; Cappel) for 12 hours, followed by 1 hour in rat peroxidase-anti-peroxidase (1:500; ICN Immunobiologicals). GABA immunoreactivity was visualized by reacting the tissue with diaminobenzidine (DAB). Some of these sections were silver-enhanced and gold-toned to improve visibility of the colloidal gold particles [2]. The tissue was osmicated, dehydrated, and embedded in Polybed 812 (Polysciences). Ultrathin sections were cut from the surface of the tissue and lightly counterstained with lead citrate and uranyl acetate. Preincubation of either antiserum with 10 μ M of the appropriate immunogen completely blocked immunostaining in the DRN.

At the light microscopic level, 5-HT immunoreactivity appears as a light, pinkish stain over cell bodies and dendrites in the DRN. GABA-immunoreactive terminals are distributed throughout the DRN, and are densest in the dorsal part of the nucleus. At the electron microscopic level, 5-HT-immunoreactive terminals contain scattered clusters of electron-dense colloidal gold particles (Figure 1). GABA immunoreactivity consists of a dark, diffuse and flocculent stain, within vesicle-containing profiles and axons. Gold- and DAB-labeled profiles were considered positively labeled only if immunostaining was evident in serial sections. We detected very few GABA-immunoreactive cell bodies or dendrites, probably because the concentration of transmitter in these structures is below the detectable limit of our methods.

Most GABA-immunoreactive terminals were associated with unlabeled dendrites, but some contacted 5-HT immunoreactive dendritic profiles (Figure 1a). The presynaptic

GABA-immunoreactive terminals contained densely-packed, small, clear vesicles. Pre- and post-synaptic specializations were often present; when the presynaptic densities were not obscured by dense DAB reaction product, the synaptic morphology appeared to be symmetrical, Gray Type II [14]. In many cases, dendrites contacted by GABA-immunoreactive terminals were also contacted by other, unlabeled, terminals (Figure 1b); this indicates that chemically diverse inputs converge onto single dendrites of 5-HT neurons.

Previous studies reported that 5-HT and GABA coexist in some cell bodies, dendrites, and terminals in the DRN [5, 13, 24]. Those double-labeling studies used immunocytochemical localization of glutamate decarboxylase, GABA, or 5-HT, combined with autoradiographic detection of [^3H]GABA or [^3H]5-HT accumulation. In our studies, the vast majority of terminals were only single-labeled. However, in a few cases we found axons and terminals immunoreactive for both 5-HT and GABA (Figure 2). On the other hand, although dense-cored vesicles are found in 50% of GABA-immunoreactive terminals in the ventral PAG [30], we never observed dense-cored vesicles in GABA-immunoreactive terminals that contacted 5-HT-immunoreactive dendrites. It is, therefore, unlikely that GABA coexists with peptide transmitters at these synapses.

Although GABA cell bodies were not labeled in the present study, presumptive GABAergic neurons have been detected in the DRN [4, 23, 29, 31]. It is thus likely that at least some of the GABA-immunoreactive terminals that we observed originate from local interneurons. Consistent with this idea, lesions of major sources of afferents to the DRN (the dorsal pontine tegmentum, substantia nigra, or habenula) did not alter GAD activity in the DRN [4]. The fact that only a few GABAergic neurons contribute to the major efferent projection from the PAG to the ventromedial medulla [31] is also consistent with their being interneurons.

GABA-immunoreactive inputs to 5-HT-immunoreactive neurons probably contribute to the controls that influence ascending serotonergic projections from the DRN.

For example, systemic enhancement of GABAergic transmission reduces 5-HT synthesis and turnover in areas that receive 5-HT projections from the DRN, including striatum, frontal cortex, hypothalamus, and substantia nigra [25, 26, 35]. Furthermore, since infusion of GABA, or the GABA_A receptor agonist, muscimol, directly into the DRN reduces 5-HT metabolism in the striatum [28, 36], the effect of systemic GABA upon striatal 5-HT probably occurs, in part, within the DRN.

The anxiolytic action of benzodiazepines is thought to result, in part, from their enhancement of GABAergic inhibition of 5-HT neurons in the dorsal and median raphe nuclei [37]. Benzodiazepines interact with the GABA_A receptor complex to enhance the inhibitory effect of GABA [c.f. 16]. Benzodiazepine agonists applied iontophoretically in the DRN potentiate the inhibition of 5-HT neurons caused by iontophoretically applied GABA [10]. Furthermore, the observation that benzodiazepine agonists alone inhibit spontaneous activity of DRN 5-HT neurons implies a tonically active GABAergic input [21, 39]. On the other hand, the fact that picrotoxin does not alter the firing rate of these neurons, implies that the endogenous GABAergic inputs are not tonically active [10].

The GABA/5-HT interaction in the DRN might also be related to cardiovascular regulation. Electrical stimulation of the DRN elevates blood pressure in rats. Since pretreatment with 5,7-dihydroxytryptamine reduces this pressor effect, 5-HT neurons are probably involved [32]. Microinjection of muscimol, but not baclofen (a GABA_B agonist), into the DRN lowers heart rate and blood pressure [33]. Finally, since microinjected picrotoxin does not cause cardiovascular changes, the relevant GABAergic synapse in the DRN is probably not tonically active [33].

Serotonergic neurons of the DRN have also been implicated in antinociceptive mechanisms. Some serotonergic DRN neurons project to the medullary nucleus raphe magnus [3] from which originates a well-characterized descending inhibitory projection [c.f. 1]. The DRN may be the most efficacious region in the PAG for eliciting electrical stimulation-produced analgesia [27], and DRN lesions antagonize the analgesic effect of

systemic morphine [34, 42]. Since picrotoxin microinjected in this region produces analgesia, and muscimol reduces the analgesia produced by morphine [22], it is possible that this projection is subject to GABAergic controls. The ligands that produced effects in all the studies discussed in the paragraphs above, were specific for the GABA_A receptor. Thus, it seems likely that the effect of GABA upon serotonergic neurons in the DRN, are mediated by GABA_A receptors. Consistent with this idea, GABA_A binding sites are dense in the DRN, but GABA_B receptors are sparse [6].

In conclusion, the present study demonstrates that 5-HT-immunoreactive neurons in the DRN are contacted by GABA-immunoreactive terminals. There is evidence that this GABAergic control of serotonergic projections from the DRN is involved in the actions of benzodiazepines, cardiovascular regulation, and antinociception. In addition, since the widely divergent serotonergic projections from the DRN [9] have been implicated in a variety of other functions including hormone regulation, locomotion, sleep, eating, sexual activity, and aggression, it seems likely that many of these functions are also subject to GABAergic controls acting upon 5-HT neurons in the DRN.

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Fig. 1. A,B: Electronmicrographs of GABA-immunoreactive terminals (asterisks) in synaptic contact with 5-HT-immunoreactive dendrites (Den). The labeled dendrites contain scattered colloidal gold particles (arrowheads). B shows a dendrite contacted by three GABA-immunoreactive terminals and apposed by an unlabeled terminal (t). Scale bars = 1 μ m.

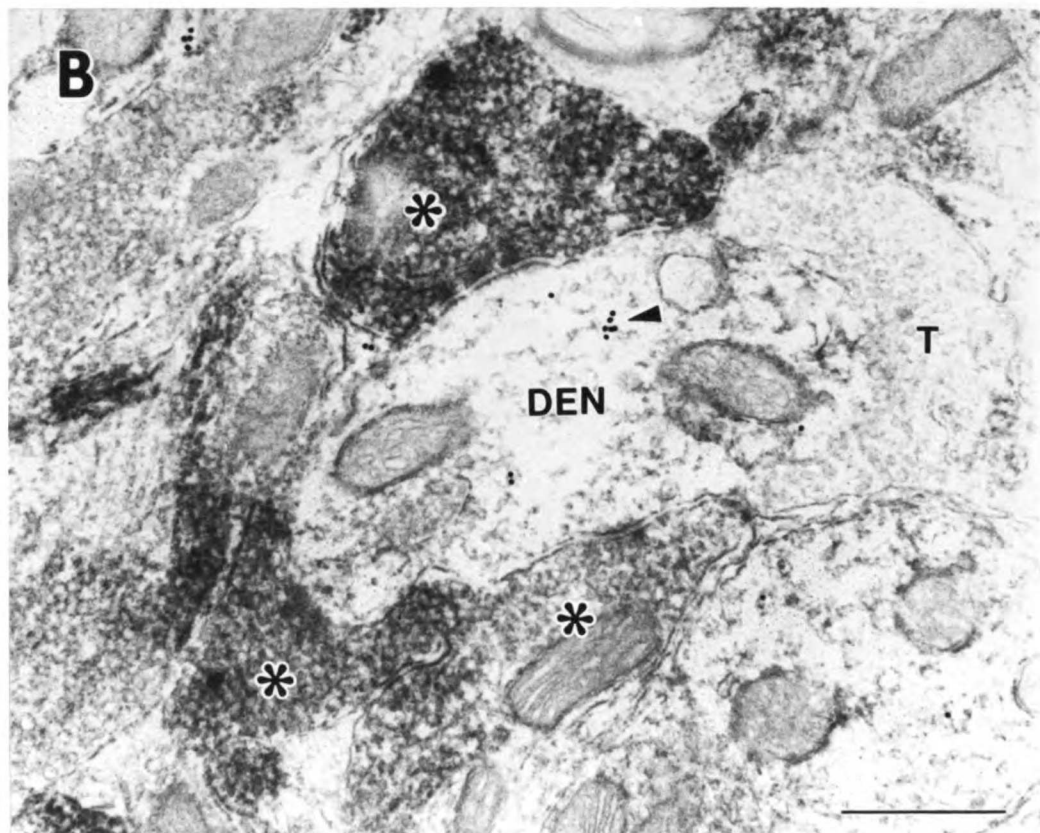
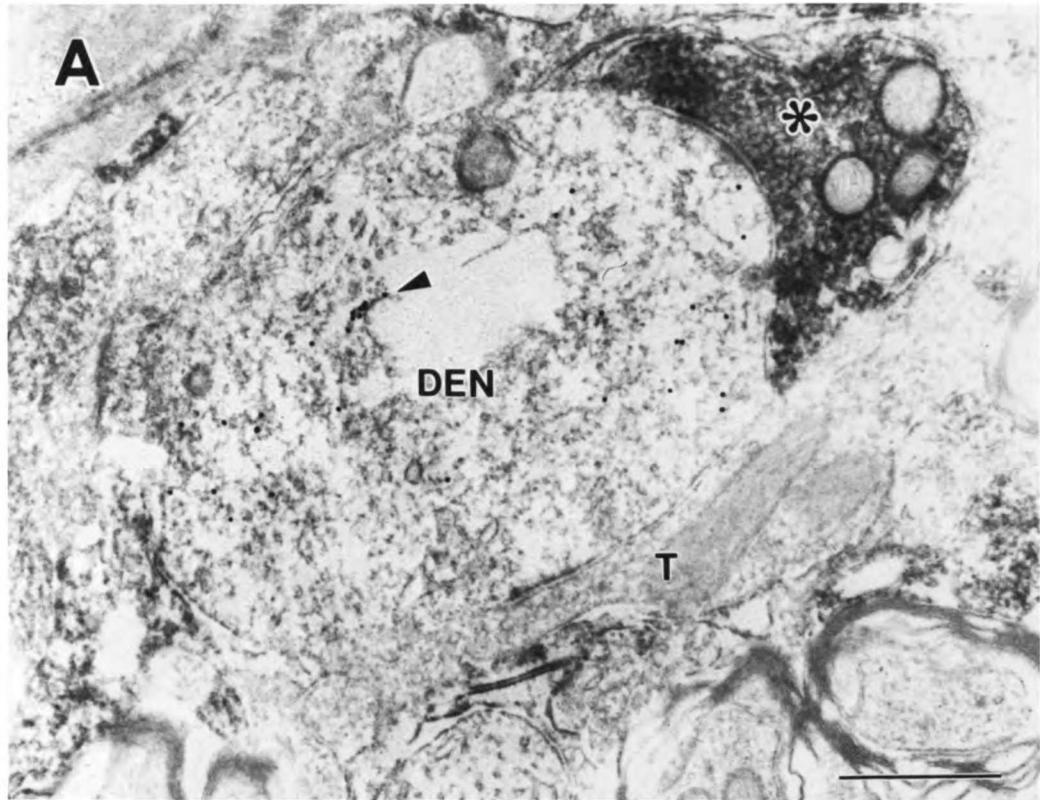
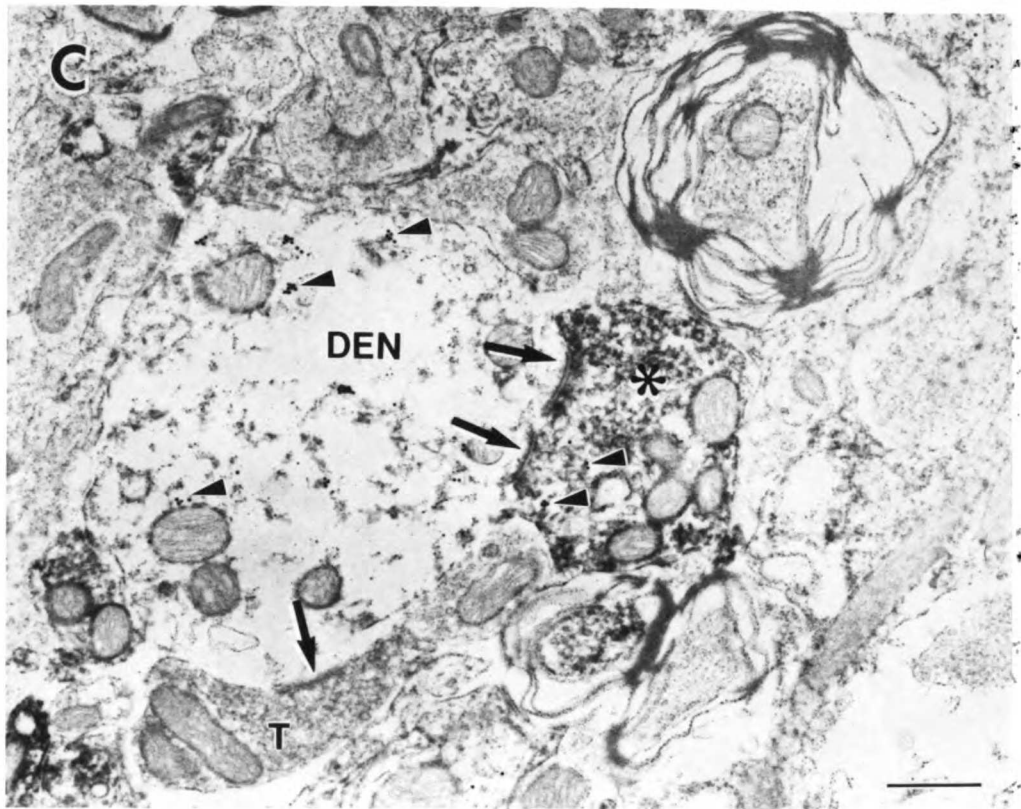
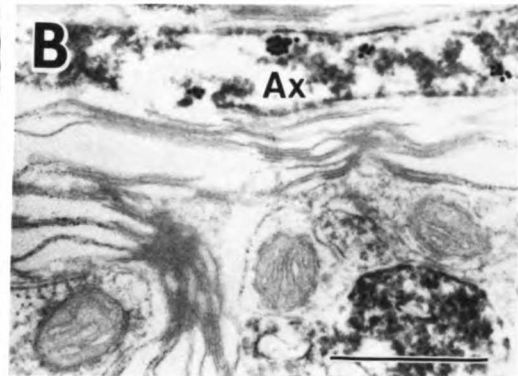
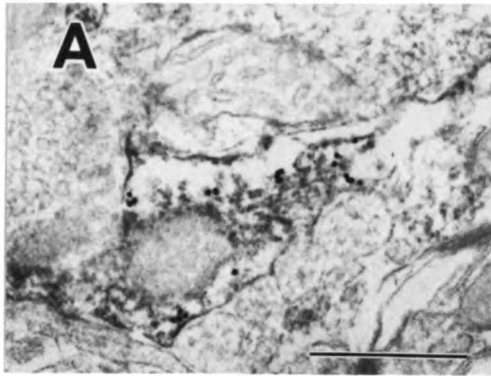
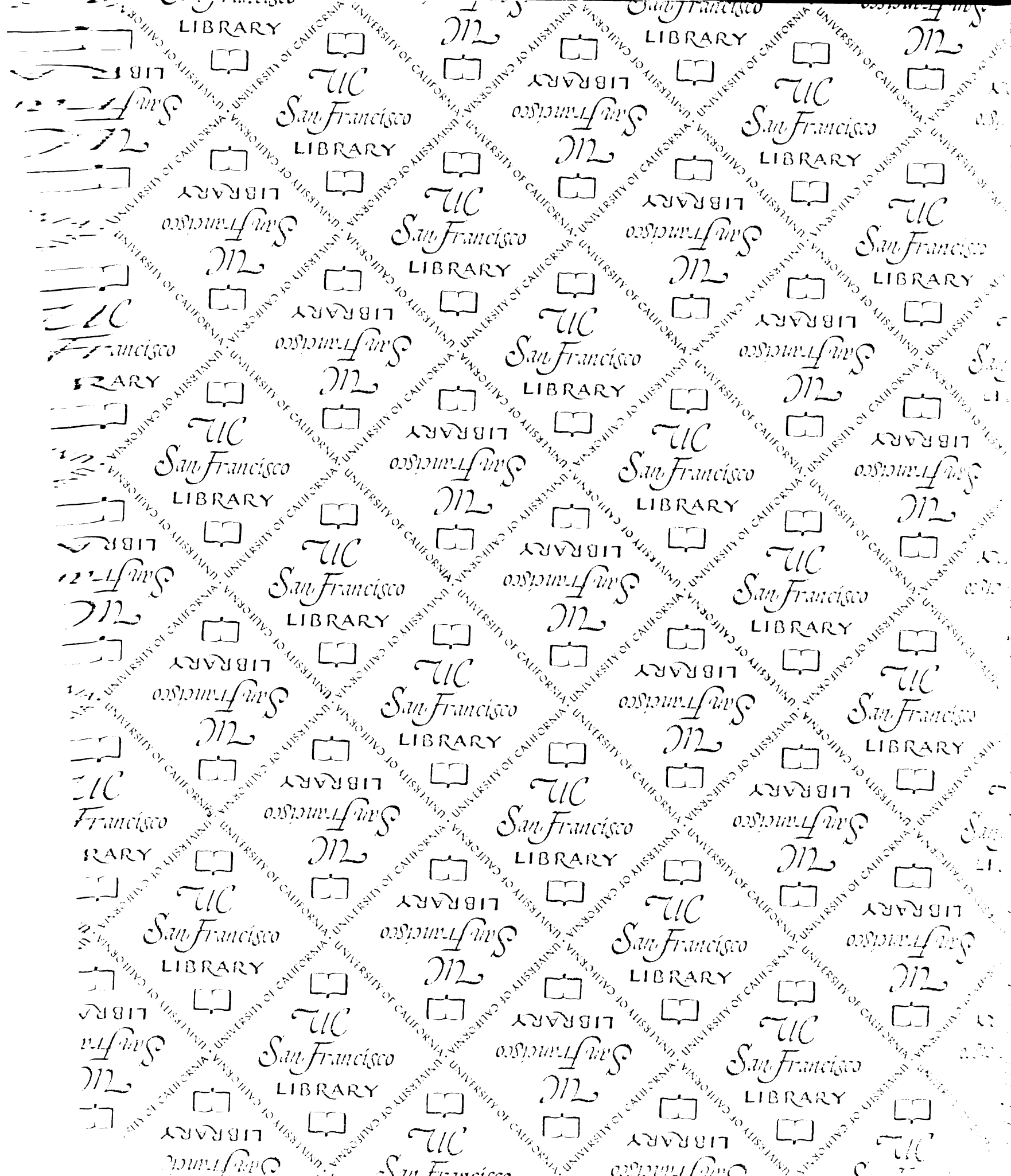


Fig. 2: Electronmicrographs of profiles labeled for both GABA and 5-HT. Some small-diameter unmyelinated (A) and myelinated axons (B) are double-labeled. C shows a double-labeled terminal (asterisk) in synaptic contact with a 5-HT-immunoreactive dendrite (Den). The dendrite is also contacted by two unlabeled terminals (arrows). Scale bars = 0.5 μ m.







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