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Supramedullary Afferents to the Nucleus Raphe Magnus in the Rat: A Study Using Transcannula HRP-gel and Autoradiography Techniques

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SUPRAMEDULLARY AFFERENTS TO THE NUCLEUS RAPHE MAGNUS IN THE RAT: A STUDY USING TRANSCANNULA HRP-GEL AND AUTORADIOGRAPHIC TECHNIQUES

by

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For my parents,

Douglas and Elizabeth Carlton

who gave me the most precious gifts any parent can give:

strong roots to grow and wings to fly.

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ABSTRACT

SUPRAMEDULLARY AFFERENTS TO THE NUCLEUS RAPHE MAGNUS IN THE RAT: A STUDY USING TRANSCANNULA HRP-GEL AND AUTORADIOGRAPHIC TECHNIQUES

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The nucleus raphe magnus (NRM) is known to play an important role in a descending antinociceptive system through its projections to the spinal cord dorsal horn and trigeminal subnucleus caudalis. Identifying those nuclei which project to NRM is essential in determining other CNS cell groups which could influence analgesic mechanisms by virtue of their connections with NRM.

In an attempt to define NRM afferents, 19 adult Sprague-Dawley rats were stereotaxically implanted with a pellet of HRP gel within NRM and in adjacent areas. A transcannula technique was specifically developed for the subcortical placement of HRP in a gel form. Use of a preimplanted stainless steel cannula for delivery of the gel to its target site, prevented contamination along the implantation tract, a factor which has compromised previous studies of this area. Employing tetramethylbenzidine neurohistochemistry, large numbers of retrogradely labelled cells were consistently found in the zona incerta, n. parafascicularis prerubralis (nPfPr), pretectum, dorsal and lateral periaqueductal gray, n. cuneiformis (nC), deep superior colliculus (dSC), and n. of Bechterew. Smaller numbers of cells were seen in the preoptic area, and the dorsomedial n. of the hypothalamus. Confirmational anterograde autoradiographic studies were done by injecting tritiated leucine into the nPfPr and into the area of the

dSC and nC. The HRP results are compared and contrasted with control HRP implants in the inferior olive, n. reticularis paragigantocellulais, medial facial n., spinal cord, and the dorsolateral funiculus. Comments are also made concerning the parcellation of the ventromedial medulla and the possible role of both NRM and its afferents in central analgesic mechanisms.

I. INTRODUCTION

The involvement of nucleus raphe magnus (NRM) in a descending pain inhibitory system has been well documented in the past decade. This nucleus, located in the ventromedial medulla, appears to play an important role in modulating noxious input at the spinal level. In exploring the various facets of this antinociceptive system, a considerable amount of the work has been done in the rat. There are several behavioral paradigms used in the rat which afford a reliable and reproducable measure of analgesia; also, the NRM spinal efferents which descend via the dorsolateral funiculus (DLF) in the rat have been demonstrated using a number of anatomical techniques. Finally, a body of literature does exist documenting electrophysiological recording in NRM in the rat following various manipulations including noxious stimulation of the periphery, stimulation of mesencephalic and diencephalic sites and, systemic, microinjected and iontophoresed morphine. These studies, in concert with the behavioral and anatomical data, stress the centricity of NRM in endogenous pain inhibitory mechanisms.

In an attempt to expand the existing anatomical framework of this system, we employed an original technique in a retrograde study, to document afferent systems of NRM; autoradiography was used in an orthograde study, to verify some of these afferent sites. The two previous studies of NRM afferents done in the rat were both compromised to a great extent by the axon of passage problem and by the fact that a comparatively insensitive neurohistochemical method was used to visualize the HRP. In the present study, both of these shortcomings are overcome with the use of the transcannula HRP gel implant technique, (eliminating to a great extent the problem of comtamination of transected

axons along the implant tract), and tetramethylbenzidine neurohistochemistry, (currently the most sensitive protocol for demonstrating HRP reaction product).

This dissertation is organized into several chapters, the last three of these chapters will later be published as separate manuscripts. The third chapter explains, in detail, the transcannula technique devised to accomplish the difficult task of "cleanly" implanting a pellet of HRP gel in a subcortical site, in this case the inferior olive. Included in this chapter is a discussion of inferior olive (IO) afferents. The forth chapter deals with the introduction of a new anatomical term which identifies an afferent source to NRM and the IO and clarifies its relationship to surrounding diencephalic structures. The fifth chapter presents the main body of work accomplished for my thesis: a complete analysis of NRM afferent sources in the rat as seen with retrograde HRP gel studies and orthograde autoradiographic studies. These results are compared and contrasted with all control cases (which includes implants in the IO, n. reticularis paragigantocellularis, facial n., spinal cord, and DLF).

II. LITERATURE REVIEW

A. General Information on the Raphe System

The raphe nuclei are a rostrocaudal column of serotonergic cell groups located on the midline (hence the term raphe) of the brain stem reticular formation (RF). This system extends from the mesencephalon, through the pons, to the medulla. Members of this monoaminergic system include the nucleus raphe (NR) pallidus, NR obscurus, NR magnus, NR pontis, NR medianus, NR dorsalis, NR linearis caudalis, intermedianus, and rostralis. The nuclei have been implicated in the mechanisms of morphine analgesia (Lewis and Gebhart, 1977; Sasa et al., 1977), stimulation produced analgesia (Samanin and Valzelli, 1971; Mayer and Leibeskind, 1974; Basbaum et al., 1977), feeding and drinking behavior (Asin and Wirthshafter, 1980). attention (Wirthshafter et al., 1980), and the induction and maintenance of sleep (Kostowski et al., 1969). Attention will be focused in this body of work on the nucleus raphe magnus, which is located in the rostral ventromedial medulla. Several lines of evidence suggest that the nucleus raphe magnus (NRM) is involved in central opiate and/or non-opiate analgesic mechanisms.

B. Nucleus Raphe Magnus Neuroanatomy

It becomes very evident when revieweing the literature concerning stimulation, ablation, and recording from NRM and the surrounding area that very few authors agree on the boundaries of this nucleus. Taber et al., (1960) are credited with the first in depth description of the cytoarchitecture of NRM employing serial Nissl stained sections. The NRM is described in the cat as extending from the rostral pole of the superior olive to the rostral pole of the inferior olive. These

rostral-caudal boundaries are more or less consistent from species to species. However, the lateral bounderies of NRM are not as well defined. There are no definite fiber tracts which separate it from the adjoining reticular formation. Taber's original description limited NRM to those cells lying on or immediately about the midline, (hence, the term 'raphe' meaning the seam of the brainstem). With the advent of the histochemical method for the cellular localization of catecholamines and indoleamines (Falck et al., 1962), the localization of serotonergic neurons within the midline nuclei of the brainstem including NRM was established. These studies however demonstrated that the serotonergic neurons in the ventromedial medulla (VMM) extended beyond the limits of NRM as originally described by Taber. Dahlstrom and Fuxe (1964), described serotonergic neurons lying not only in NRM but also just dorsal to the pyramids throughout the rostro-caudal extent of the facial n., and within an area just lateral to the pyramidal tract corresponding to the nucleus paragigantocellularis lateralis (PGC) in the cat (Taber, 1961). (This latter area corresponds to n. reticularis magnocellularis, RMC, as illustrated by Berman, 1969). This entire area containing serotonergic (5-HT) cells was nambed B3. Watkins and coworkers (1980) demonstrated that DLF-projecting cells from the VMM included RMC as well as midline NRM. They placed NRM and RMC into one functional category, renaming the combined nuclei "nucleus raphe alatus" ("winged nucleus of the raphe"). Palkovits and Jacobowitz (1974) take the "middle of the road" in their atlas of the rat brain representing NRM as extending out from the midline to cover half of each pyramid, and they do not illustrate the location of PGC or RMC.

1. Morphology/Cytology

NRM contains a heterogenous population of cells including large, and medium sized polygonal and small piriform or spindle shaped cells (Taber et al., 1960). The larger cells frequently have eccentric nuclei (Taber et al., 1969; Felten and Cummings, 1979; 1981). Cell sizes range from 20μ to 50μ. Using a Golgi-Cox stain, Felten and Cummings described NRM cells as having 4-8 primary dendrites of medium to large caliber radiating from the soma. The dendrites appeared to be oriented perpendicular to, or at an oblique angle to, the midline. Cell bodies in NRM are generally smooth, but, scattered short somatic spines may be present (Felten and Cummings, 1978; 1981). Some primary dendrites also have short spines. It was also noted (Felten and Crutcher, 1979), that a small percentage of monoaminergic cells in NRM possess neuronal-vascular relationships suggesting chemoreceptive or osmoreceptive functions. Valverde (1962), in his study of the reticular formation of the albino rat, makes an observation about the horizontal orientation of the largest axis of the NRM cells, which is typical of reticular formation cells.

2. Afferent Connections

The first systematic but rather crude anatomical study of NRM connections was attempted by Brodal et al., (1960), using the silver impregnation method of Glees (1946), to visualize degenerating axons and boutons. In the adult cat, three groups of afferents were described, namely fibers from the 1) spinal cord; 2) cerebral cortex; 3) cerebellum (including the fastigial nucleus and cortex). Ruda (1976) in her autoradiographic study concerning connections of central gray in the cat, described afferents to NRM from the ventral central

gray (CG). However, not until the evolution of horseradish peroxidase (HRP) as a retrograde neuronal marker, was a more detailed study of NRM afferents made possible. Gallager and Pert (1978) were the first to describe NRM afferents in the rat after iontophoresis of HRP into the brainstem raphe (NRM, nucleus raphe pallidus, and nucleus raphe obscurus). With diaminobenzidine (DAB) neurohistochemistry, labelled areas included: the dorsal and ventrolateral CG, dorsal and ventral tegmentum, deep layers of the superior colliculus, the dorsal and ventromedial prefrontal cortex and the medial vestibular nucleus. In contrast to Brodal's (1960) work, no labelled cells were found in the cerebellum. The NRM afferents in the cat were described by Abols and Basbaum (1979) with iontophoresis of HRP, however, their labelled sites were restricted to the dorsal, lateral and medial CG, n. cuneiformis, and n. subcoeruleus. Senba et al., (1981) repeated the study in the rat, again using HRP iotophoresed into NRM and reported a variety of different nuclei labelling retrogradely including the nucleus linearis rostralis, n. parafascicularis, zona incerta, midbrain RF. Senba confirmed but did not discuss the afferents of NRM arising from the prefrontal cortex, cerebellum or CG.

3. Efferent Connections

Again it was Brodal (1960) who first described some of the NRM efferents in the cat employing degeneration techniques. In this study, gross lesions were made in the spinal cord or mesencephalon and the NRM was scrutinized for chromatolytic cell changes. The NRM-spinal efferents were described although the funiculus in which they descended could not be determined. Ascending projections were also reported but due to the technique used, no terminal fields could be described.

Basbaum et al., (1976; 1978), using autoradiography in the cat, cited the following as NRM efferents: solitary n., dorsal motor n. of the vagus, spinal trigeminal n., CG, dorsal raphe, intermediate layers of the superior colliculus, centro-median n., paracentralis n., centralis lateralis, dorsal medial thalamus, zona incerta (ZI), and the lateral and dorsal hypothalamic n. Bobillier (1976) who also carried out an autoradiographic study of NRM efferents in the cat, produced quite a different but seemingly very complete list of efferent sites: n. intercalatus, facial n., inferior olivary complex, n. prepositus hypoglossi, NR obscurus, NR pallidus, n. reticularis gigantocellularis, n. reticularis lateralis, n. reticularis medulla oblongata, n. reticularis paramedianus, the inferior, lateral, medial and superior vestibular n., CG, cerebellar cortex, trapezoid body, locus coeruleus, motor n. of V., abducens n., dorsal and ventral parabrachial n., raphe centralis superior, NR dorsalis, NR pontis, n. reticularis parvocellularis, n. reticularis pontis caudalis and oralis (nRP), n. reticularis tegmenti pontis, spinal n. of V, pretectal area, mesencephalic reticular formation, n. posterior commissure, n. Darkschewitsch, Edinger-Westphal n., interstitial n. of Cajal, red n., deep layers of the superior colliculus (dSC), dorsal, lateral and posterior hypothalamic area, n. centralis (pars lateralis and pars medialis), centro-median n., parafascicularis n. (nPF), subparafascicular n., ventral posterolateral thalamus, and the diagonal band of Broca. Concerning the NRM efferent system in the rat Takagi et al., (1981), accomplished this study using HRP. Sites which contained labelled terminal fields included the cerebellar cortex, nRP, nCS, DR, dSC, ZI, nPF, medial thalamus and the preoptic

area. The NRM-spinal projections have been the subject of much study due to their proposed involvement in a descending antinociceptive system. In the cat, the majority of NRM-spinal projecting neurons course in the dorsolateral funiculus (Basbaum et al., 1976; Tohyama et al., 1979). The same is true in the rat (Dahlstrom and Fuxe, 1965; Leichnetz et al., 1978; Watkins et al., 1980). These NRM-spinal projections are purported to be serotonergic (Dahlstrom and Fuxe, 1965; Takagi et al., 1981; Bowker et al., 1981, Loewy and McKellar, 1981); however, recent findings challenge this concept (Johannessen et al., 1981; 1982).

C. NRM Transmitter Content

NRM has been demonstrated to contain both serotonergic (5-HT) and non-5-HT neurons (Hokfelt et al., 1977; Johannessen et al., 1981), and 5-HT terminals (Dahlstrom and Fuxe, 1965). Immunofluorescent and immunocytochemical techniques have localized enkephalinergic cells in NRM (Hokfelt et al., 1977; Finley et al., 1981). A substance P-like peptide is also reported to be present in some 5-HT neurons (Hokfelt et al., 1978), and it has been demonstrated that substance P iontophoresed onto NRM cells, excites the majority of the cells tested (Pomeroy and Behbehani, 1980). Cuello and Kanazawa (1978), failed to find an abundance of substance P fibers in NRM. Low opiate receptor density has been described in NRM (Atweh and Kuhar, 1977; Pert, 1975), and no noradrenergic (NA) neurons are seen in this region (Dahlstrom and Fuxe, 1964; Moore and Bloom, 1979; Palkovits and Jacobowitz, 1974), however, a NA system is believed to play a role in modulating NRM neurons (Yaksh and Rudy, 1978; Hammond et al., 1980; Behbehani et al., 1981).

D. NRM Involvement in Endogenous Pain Inhibitory Systems

Several lines of evidence suggest that the NRM is involved in pain inhibitory mechanisms. In the rat, it has been demonstrated that most neurons in NRM are excited principally by noxious stimuli including pinch, radiant heat, and bradykinin (Guildbaud et al., 1980). However, in the cat, very few neurons fell into this category since most respond to both noxious and non-noxious stimuli (Moolenaar et al., 1976; Anderson et al., 1978). Electrophysiological studies demonstrate that stimulation of NRM selectively inhibits responses to noxious stimuli of laminae I and V dorsal horn neurons (Basbaum et al., 1976; Fields et al., 1977; Guilbaud et al., 1977; Belcher et al., 1978; Duggan et al., 1979), including cells giving rise to the spinothalamic tract (Beall et al., 1976; Willis et al., 1977; McCreery et al., 1979; Gerhart et al., In Press; Pearson et al., 1979). Stimulation of NRM also inhibits the responses of dorsal horn cells to C-fiber volleys (Rivot et al., 1979; Gerhart et al., In Press), and to A delta and C afferent input (Rivot et al., 1980). Finally, it has been demonstrated that NRM stimulation can produce powerful behavioral analgesia (Oleson et al., 1978; Oliveras et al., 1975; Oliveras et al., 1977; Proudfit and Anderson, 1975; Basbaum et al., 1976; Satoh et al., 1980), which is naloxone reversible (Oliveras et al., 1977; Zorman et al., 1981). Systemic administration of morphine produces a significant elevation of the spontaneous multiple unit (Oleson et al., 1978) and single unit (Anderson et al., 1977; Deakin et al., 1977; Haigler, 1978) activity in NRM. However, when morphine is iontophoresed into NRM it is reported to either depress NRM activity (Wolstencroft et al., 1978) or have no effect (Haigler, 1978; Wolstencroft et al., 1978). The

data concerning morphine microinjectin (MM) into NRM present a confusing story since several investigators report that MM results in behavioral analgesia (Proudfit et al., 1977; Levy et al., 1978; Levy et al., 1979; Dickenson et al., 1979), which is attenuated by microinjection of naloxone into NRM (Dickenson et al., 1979), while others do not observe such actions (Takagi et al., 1976; Akaike et al., 1978; Takagi et al., 1980).

Lesions of the NRM block or attenuate analgesia produced by systemic morphine (Proudfit and Anderson, 1975; Yaksh et al., 1977; Chance et al., 1978; and Young et al., 1981), and also attenuate midbrain stimulation-produced analgesia (Behbehani et al., 1979; Cannon et al., 1980). Watkins et al., 1981 have recently demonstrated that lesions of NRM attenuate the analgesia produced by electrical foot shock. However Proudfit (1980), has since demonstrated that acute lesions of NRM do not alter the capacity of morphine to induce antinociception and suggests that NRM is not directly involved in mediating the antinociceptive actions of opiates. Bilateral DLF lesions result in a reduction of inhibition of the response of dorsal horn units to noxious stimulation (Fields et al., 1977; Willis et al., 1977), reduction in stimulation-produced analgesia from PAG and dorsal raphe (Basbaum et al., 1976; Basbaum et al., 1977), and decreased analgesia following foot shock (Watkins et al., 1981).

Further support of a role of NRM in antinociception is derived from pharmacological studies which manipulate central nervous system (CNS) levels of serotonin. Depletion of CNS serotonin blocks the antinociceptive effects of NRM stimulation (Proudfit and Anderson, 1975). Proudfit et al., (1980) also demonstrated that morphine

analgesia is dependent on the integrity of the descending bulbospinal serotonergic pathways. Stimulation of NRM induces an accelerated turnover of spinal serotonin (Bourgion et al., 1980), and microinjection of serotonin in NRM has been reported to result in behavioral analgesia as tested with tail flick but not paw pinch, (Llewelyn et al., 1981). However, Johannessen et al., (In Press) demonstrated that a descending serotonergic pathway is unnecessary to elicit analgesia by stimulation of, or morphine microinjection into, the midbrain PAG. Vogt (1973), reported a similar result using systemic morphine. Also, Johannessen has demonstrated that serotonin is not a component in the DLF projection which originates in the VMM.

Anatomical connections of NRM which circumstantially implicate it in opiate mechanisms are derived from the projections it receives from the PAG. Electrical stimulation of PAG (Lovick et al., 1978), or local application of morphine to this area (Behbehani et al., 1978), results in activation of a population of NRM neurons. Since a direct pathway from NRM descends down the DLF of the spinal cord and terminates in the dorsal horn laminae I and V, it is very plausible that the DLF represents the 'final common pathway' for most supraspinal inhibition of nociceptive stimuli. Classically conditioned analgesia (Watkins et al., In Press), analgesia produced by electrical foot shock (Watkins et al., 1980, Mayer and Watkins, In Press, Watkins et al., manuscript submitted), systemic and intra-cerebral morphine analgesia (Basbaum et al., 1976, 1977; Murfin et al., 1976; Hayes et al., 1978; Barton et al., 1980), and stimulation produced analgesia (Basbuam et al., 1976; Basbaum et al., 1977; Fields et al., 1977), are all dependent on the integrity of the DLF in the spinal cord. The fact

that NRM is one of the major contributors of axons to this tract (Basbaum et al., 1976; Leichnetz et al., 1978, Watkins et al., 1980), strongly suggests that this nucleus modulates nociceptive transmission at the spinal level.

III. A TRANSCANNULA METHOD FOR SUBCORTICAL HRP GEL IMPALNTS: INFERIOR OLIVE AFFERENTS IN THE RAT

Retrograde axoplasmic transport of horseradish peroxidase (HRP) has been extensively used to study connectivity in the central nervous system. Previous investigations employing the HRP technique to study the projections of subcortical areas were limited to the use of the enzyme in either a fluid or crystalline form. Both of these techniques have several disadvantages. First of all, it is very difficult to keep fluid HRP localized at the injection site. Depositing a volume of HRP solution sufficient for uptake and transport often results in diffusion of the fluid beyond the limits of the target site and contamination of adjacent tissue. In addition, uptake of the enzyme by fibers of passage along the needle tract compromises the results of many HRP studies by producing extraneous labelling of other cell groups whose axons are transected. The implantation of crystalline HRP in subcortical areas has been attempted (10) but the technique is involved and time consuming, and the amount of enzyme which comes in contact with the tissue is minimal. The injection or ionotophoresis of HRP fluid into the brain necessitates anesthetization of the animal, and since it has been reported that barbiturates decrease axoplasmic transport (9) their use in concert with any HRP study could result in less than maximal retrograde transport. Griffin et al., (3) introduced the technique for incorporating HRP into a polyacrylamide gel which provides several distinct advantages

over fluid or crystalline methods. This has been successfully employed for HRP placements in superficial cortical and spinal cord areas, (5,6,15) and when combined with tetramethylbenzidine neurohistochemistry (8) has been shown to produce outstanding visualization of both anterogradely and retrogradely-transported HRP. This present study evaluates the use of HRP gel for subcortical placements, using a cannula as a conduit for delivery of the gel to a medullary target site.

CANNULA APPARATUS

Cannula materials were obtained from Small Parts, Inc., Miami, Fla. (hypodermic, stainless steel tubing, type 304, HTX-26). The 26 gauge tubing had an outside diameter of .018" and inside diameter of .010". An appropriate length of cannula (in this case 21mm.) was cut and then each end has sanded with emory cloth to ensure an open, smooth orifice.

A separate carrier bar (stainless steel wire approx. 4" in length) was bent into an "L" shape and placed on a piece of paper. The cannula was placed on top of, and perpendicular to, the horizontal section of the "L" bar, such that 3/4 of the cannula was below the bar and 1/4 of the cannula was above it. The cannula was then cemented at its intersection with the "L" bar with a drop of dental acrylic. When the acrylic had dried, the carrier bar was removed from the paper by cutting around the acrylic, then rinsed with alcohol and sterilized at 250^{o} for one

hour prior to use. The long stem of the "L" bar was then used to mount the cannula in the electrode holder on a David Kopf stereotaxic instrument, (see Figure 1A).

Stylets for each cannula were made from stainless steel teflon coated wire (Medwire Corp. #316SS, 8T, .010"). The wire was stripped of its teflon coating and stretched slightly between two pliers until the diameter of the wire exactly fit the bore of the cannula.* Two stylets were made per cannula, one the exact length of the cannula (to prevent postimplantation clogging of the bore), and a second, 1mm. longer than the cannula (for implantation of the gel pellet). Both stylet wires were cut such that a 5 mm. length extended out of the top end of the cannula shaft. This segment was bent to a 90^{o} angle to create a short tab on the top of each stylet, allowing easy insertion and removal. The stylets were rinsed with alcohol and sterilized before use.

CANNULA IMPLANTATION

With the longer of the two stylets in place, the cannula apparatus was zeroed on a zeroing plate. The longer stylet was then removed, and the shorter stylet inserted prior to implantation

*Small Parts Inc. Miami, Fla. does make matching cannula and stylet materials which can be used in larger animals (cats, monkeys).

of the cannula. The shorter stylet ended exactly at the lower orifice of the cannula, so that when implanted, the lower tip of the cannula was 1 mm above the planned target site and thus the target was not damaged.

Four adult Sprague-Dawley rats (350-450 gm) were used in this study. The animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal), and placed in the stereotaxic frame. A midline incision was made over the calvarium, and the skin flaps were retracted. Three skull screws were placed in the calvarium in a triangular arrangement rostral to the lambdoidal suture. Using a Dremel drill, a small hole was made in the calvarium above the target site (coordinates for the inferior olive were taken from the atlas of Pellegrino and Cushman: AP, 1.8, ML, $\pm$ 0.4, D, 1.6). After lowering the cannula into the brain it was secured to the skull by flowing dental acrylic around it and the skull screws. The end of the tab of the stylet was also cemented to the calvarium so that the animal could not pull the stylet out. The incision was then closed with wound clips. The animal was allowed 5 days for recovery, during which time retraction of brain tissue and rudimentary scar formation occurred around the cannula shaft. The formation of this seal around the cannula prevented the upward spread of the implanted HRP along the cannula shaft.

GEL IMPLANTATION

Under a dissecting scope, a sliver of solid polyacrylamide gel (3) was cut with a #11 surgical blade into very minute pieces approxi-

mately equal in size to the diameter of the stylet tip. After hardening (approx. 10 min.), one of the gel fragments was attached by dipping the tip of the long stylet into cyanoacrylate (Krazy Glue) and touching it to the piece of gel. After approx. 5 min. drying time, the pellet was securely cemented onto the stylet tip.

On the 5th day postsurgery, the animal was anesthetized using Metaphane (Pitman-Moore). Snipping the tab adjacent to the top of the cannula, the stylet was cut free from the dental acrylic and removed. Under the dissecting scope, the longer stylet with the gel attached was advanced slowly into the cannula bore and the tab was cemented down (see Figure 1B).

Following 24-48 hours survival time, the animal was deeply anesthetized with pentobarbital and perfused through the left ventricle with 300ml of heparinized saline, followed by 500ml 1.0% paraformaldehyde 1.25% glutaraldehyde, in 0.1M phosphate buffer, followed by 300ml of cold 10% phosphate-buffered sucrose. The bone of the skull was ronguered away, leaving the implanted cannula, dental acrylic and underlying calvarium in place. The cannula apparatus and attached bone were then lifted straight up out of the brain so as not to damage surrounding tissue. (In many cases, the evacuated gel pellet was still adhered to the tip of the stylet.) The brain was placed in additional phosphate-buffered sucrose overnight at $4^{o}C$, frozen sectioned at 25 μ the next day, and processed according to the tetramethylbenzidine protocol of Mesulam (8).

RESULTS

In the case described here, which primarily involved the principal olivary nucleus (Fig. 2), the principal sources of afferents to the inferior olivary complex were very clearly the ipsilateral nucleus parafascicularis prerubralis and the contralateral lateral cerebellar and dorsal column nuclei (Fig. 3).

In the medial subthalamus, abundant numbers of retrogradely-labelled cells surrounded the fasciculus retroflexus (FR). We have suggested the term nucleus parafascicularis prerubralis (nPfPr, Fig. 3) be used for this cell group for several reasons (see Carlton and Leichnetz in press and discussion this paper). Some of the labelled cells of nPfPr lateral to the FR were intermingled among the rostralmost fascicles of the medial longitudinal fasciculus which led us to believe that they may represent the rat homologue of the primate rostral interstitial nucleus of the medial longitudinal fasciculus (rostralmost interstitial nucleus of Cajal). Rostrally, the nPfPr cells are continuous with cells in the periventricular gray of the third ventricle and nucleus of Darkschewitsch; laterally, they are continuous with a considerable number of labelled cells in the zona incerta; caudally, with a paramedian vertical group of labelled cells in the rostral oculomotor nucleus (Fig. 4) inside the fascicles of the MLF. Corollary injections of extraocular muscles suggested that the most caudal of the olive-projecting cells of this study lie within the somatic

columns of the oculomotor nucleus. Retrogradely-labelled cells were present bilaterally in the pretectal nucleus of the posterior commissure, but not other pretectal cell groups. Only an occasional cell was seen in the major part of interstitial nucleus of Cajal at the level of the oculomotor nucleus. There were labelled cells present in the ventral nucleus cuneiformis which extended laterally in the mesencephalic reticular formation to a cluster of cells just medial to the medial geniculate nucleus. Very few retrogradely-labelled cells were observed in the rostral superior colliculus, but in more caudal SC and at intercollicular levels the numbers increased in the stratum profundum, although still not abundant. Only an occasional cell was seen in the pontine reticular formation. Considerable numbers, however, were present in the deep cerebellar nuclei, particularly the lateral cerebellar nucleus. Just rostral to the level of the implant there were large numbers of labelled cells in the rostral ventromedial paramedian medullary reticular formation and in the nucleus raphe magnus. Just caudal to the level of the implant one could follow labelled axons (internal arcuate fibers) to large numbers of retrogradely-labelled cells in the contralateral nucleus gracilis and cuneatus (Fig. 3). A few cells were also present in the spinal nucleus of the trigeminal complex, very probably due to the labelling of trigeminothalamic projections that join the medial leminiscus. Likewise, cells in the contralateral inferior olive were labelled, and since the olivocerebellar tract was labelled bilaterally it was assumed that the implant involved

both the ipsilateral origin, and crossing fibers, of this precerebellar pathway. Occasional cells that were observed in the frontal cortex rostral to the level of the anterior commissure were attributed to the slight invasion of the implant into the pyramid. In this case, we did not observe labelled cells in the red nucleus (RN), Edinger-Westphal nucleus, spinal and medial vestibular nuclei, nucleus prepositus hypoglossi or the lateral reticular nucleus, which have been reported by other investigators (1).

Other HRP gel implants (not illustrated in this report) involving the medial accessory olive and/or the medial part of the dorsal accessory olive resulted in sparse labelling of the ipsilateral dorsomedial red nucleus and the Edinger-Westphal nucleus. The spinal cord was not examined in any of our inferior olive cases.

DISCUSSION

The use of HRP gel implanted through a cannula offers appreciably improved capabilities for studies of connectivity of subcortical nuclei. In the polyacrylamide gel form, the HRP molecules are trapped in the interstices of the gel matrix and are slowly released from the pellet into the tissue. The terminals within the target structure therefore have a prolonged exposure to a concentrated oasis of the enzyme, which results in maximum uptake. The spread of the HRP away from the gel (and hence the injection site) is minimal, allowing for very discrete, very localized placements. The animal is anesthetized during the actual gel implant phase with a short-acting inhalant, Metaphane, so that detrimental effects on HRP transport, characteristic

of barbiturate anesthesia (9), are avoided. Finally, the exposure of axons of passage transected along the injection tract is essentially eliminated when the HRP gel is delivered to the target site through the indwelling cannula.

Using this HRP-transcannula technique we were able to verify the majority of afferents of the inferior olive in the rat as reported by Brown et al. (1). There were, however, several afferent sites that they reported which were not labelled in any of our cases, including certain pretectal nuclei (nuc. of the optic tract, olivary pretectal nuc., anterior and posterior pretectal nuclei), spinal and medial vestibular nuclei, nucleus prepositus hypoglossi, and the lateral reticular n., which we felt might be attributed to the difference in approaches taken to reach the IO. Brown et al. (1) employed a ventral-lateral approach, whereas the procedure described in this study used a dorsal approach, so that it is possible that certain other fiber tracts may have been inadvertently involved in the former study. For example, we observed remarkably few retrogradely-labelled cells in the sensorimotor cortex because the implant only slightly impinged on the medullary pyramid.

This study confirmed that one of the principal sources of IO afferents is a cell group that surrounds the fasciculus retroflexus in the prerubral subthalamic region. Several investigators (1,4,12) have referred to this group as the subparafascicular or parafascicular nucleus which has led to confusion with the nuclei of the same name in the dorsal thalamus. We have preferred to call this subthalamic group the n. parafascicularis prerubralis (2). It is very likely

that some of the neurons in this cell group have been previously reported as being contained in the prerubral fields of Forel.

Results from silver degeneration studies have supported the existence of a substantial projection from the RN to the IO (7,13,14). However, more recent HRP studies question the existence of rubroolivary projections (1,4,12), which is corroborated by our data, in that we found no retrogradely labelled RN neurons.

A number of investigators have reported finding olive-projecting cells in the midline rostral paramedian mesencephalic reticular formation. We feel that in some instance these cells have been mistakingly interpreted as being within the nucleus linearis rostralis (lr), anteromedian nucleus (Am), Edinger-Westphal nucleus (EW), interstitial nucleus of Cajal, or nucleus of Darkschewitsch. It is very likely that much of the confusion is due to disagreement among the most widely used rat atlases. In this study it seemed evident that there were retrogradely-labelled cells in a vertical configuration off the midline in the paramedian mesencephalic reticular formation just rostral to the oculomotor nucleus which continued caudally with a smaller group of cells within the ventrolateral somatic cell columns of the oculomotor nucleus itself. We are not able to explain the finding or its possible functional implications in oculomotor mechanisms, but our corollary injections of extraocular muscles substantiated the fact that these cells do not belong to lr, Am, or EW nuclei.

In addition to casting doubt on the existence of rubroolivary projections, questions were raised in this study concerning other sources of inferior olive afferents. The tectoolivary projection, for example, appeared to be at best meager. Retrogradely-labelled cells in the pretectal nucleus of the posterior commissure were continuous with a very few cells in the rostralmost stratum profundum of the superior colliculus (SC/SP). At more caudal levels of the SC/SP there were a few more cells, but the numbers of cells were very small in contrast to other cases in our laboratory where we have implanted gel in the nucleus raphe magnus or cervical spinal cord (unpublished observations). We found no evidence for vestibuloolivary projections, although the case reported here involved primarily the principal olivary nucleus.

The present findings demonstrate the advantage of the HRP gel transcannula technique for subcortical HRP studies. Since the technique was perfected in the rat, adapting the same method for use in larger mammals should allow for even more restricted subcortical implants; that is to say, implanting subdivisions of nuclear groups would be facilitated since they would be proportionately larger than the structures we have successfully implanted. The ability to produce very discrete HRP placements with intense retrograde labelling of cell somata, with no appreciable contamination of surrounding tissue, offers exciting possibilities for the furture application of this technique in connectivity studies.

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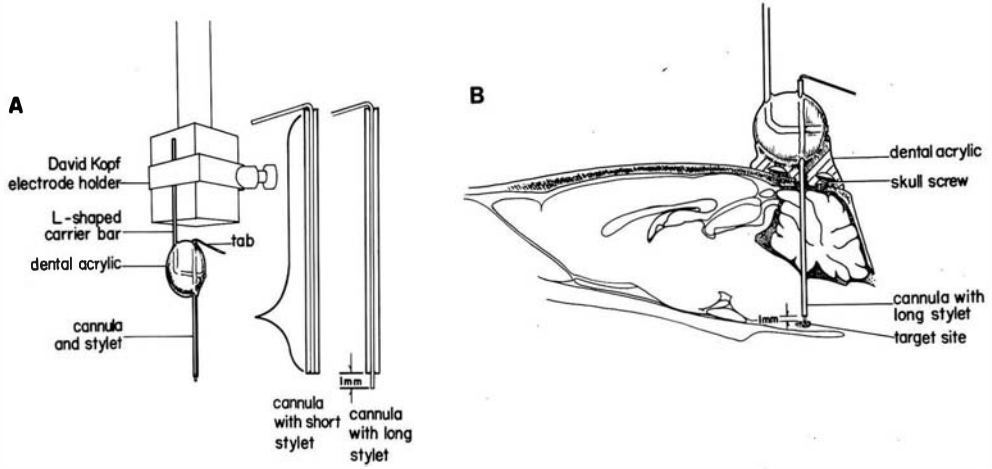
Figure 1. Illustration of the cannula apparatus for the transcannula subcortical HRP gel implantation procedure described in this report. See text for procedure. A. Assembled cannula apparatus mounted on electrode holder. Long stylet is in place when determining zeroing coordinates. Subsequent to zeroing, the long stylet is removed (and stored in a safe place), and short stylet is inserted in cannula. B. On 5th day post-implantation surgery, the short stylet is removed and replaced by long stylet with attached gel, (gel is not demonstrated in drawing).


A
David Kopf
electrode holder
L-shaped
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and stylet
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with short
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with long
stylet
B
dental acrylic
skull screw
cannula with
long stylet
1mm
target site

FIGURE 1

Figure 2. Bright field photomicrograph of the HRP gel implant in the inferior olive of the rat. The dark black implant site is centered in the principal IO nucleus, but there was slight spread into the dorsal and medial accessory olivary nuclei and ventrally into the pyramid (P). The hole above the IO was made by the end of the stainless steel cannula. Note that there is no spread around the cannula above IO. The bar represents 1 mm.

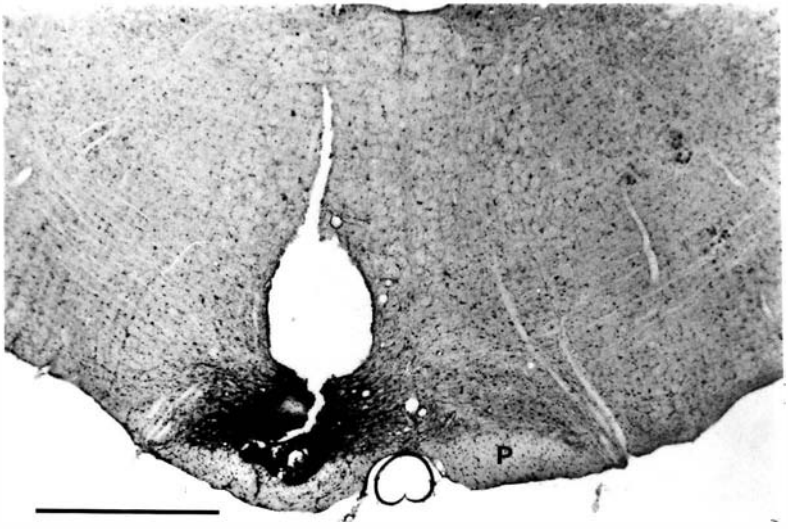

P

Figure 3.

A. Low power dark field photomicrograph of a coronal section of the brain at the mesen-dien-cephalic junction in a rat that received an HRP gel transcannula implant in the IO complex (see Fig. 1), showing retrogradely-labelled cells in the nPfPr surrounding the FR in the medial prerubral subthalamic region.

B. Higher power dark field photomicrograph of A. showing cells in the nPfPr surrounding FR. The cells lateral to FR are interdigitated among the rostralmost fascicles of the MLF (arrows).

C. Low power dark field photomicrograph of retrogradely-labelled internal arcuate fibers (arrows) and their cell bodies of origin in the dorsal column nuclei (nucleus gracilis and cuneatus) following an HRP transcannula gel implant in the contralateral IO complex.

D. Higher power dark field photomicrograph of retrogradely-labelled, olive-projecting cells in the nucleus gracilis and cuneatus in C.

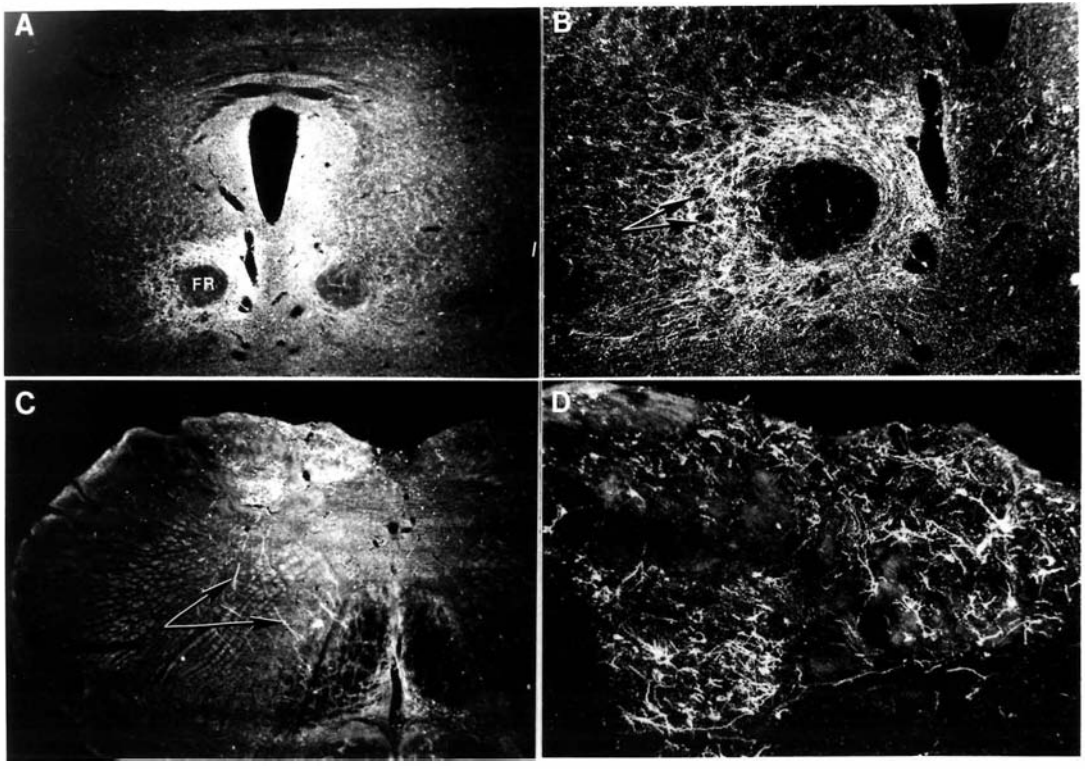
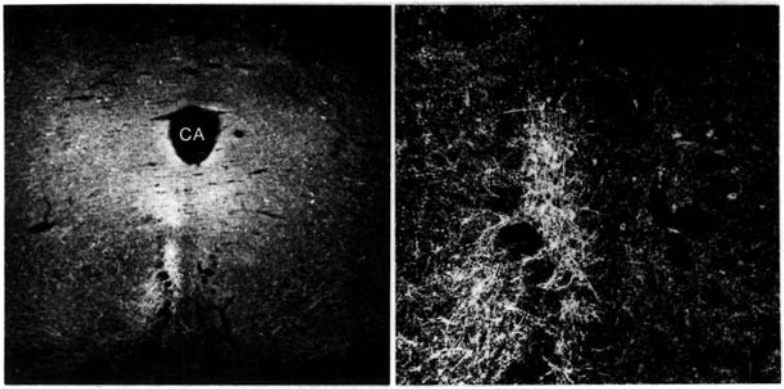

A
FR
B
C
D

Figure 4. A. Low power dark field photomicrograph of retrogradely-labelled, olive-projecting cells in the rostral oculomotor nucleus. CA = cerebral aqueduct

B. Higher power dark field photomicrograph of A. Note that the cells lie inside the MLF fibers within the somatic portion of the oculomotor nucleus.


CA

IV. PROJECTIONS FROM THE NUCLEUS PARAFASCICULARIS PRERUBRALIS TO MEDULLARY RAPHE NUCLEI AND INFERIOR OLIVE IN THE RAT: AN HRP and AUTORADIOGRAPHY STUDY

Interest in the connections of the nucleus raphe magnus (NRM) has been motivated by their importance in central nervous system analgesic mechanisms. Recent anatomical studies have demonstrated that both NRM (3,5,17) and the inferior olive, IO (1,3,4,16) receive supramedullary projections from nuclei at the diencephalic/mesencephalic junction. Difficulty in restricting experimental involvement to one or the other nucleus motivated us to determine which afferents represent common or independent inputs to the two structures. This report is part of a more global investigation of specific afferents to NRM.

Under sodium pentobarbital anesthesia (50 mg/kg IP), we implanted small pellets of solid polyacrylamide horseradish peroxidase (HRP) gel (10) through pre-implanted stainless steel cannulae (4) into NRM or IO in 14 adult Sprague-Dawley rats. In 4 additional animals, tritiated leucine was injected into the nucleus parafascicularis prerubralis (nPfPr), a principal source of afferents to the two structures. The HRP cases were processed according to the tetramethylbenzidine (TMB) neurohistochemical protocol of Mesulam (13), and the autoradiographic cases according to Edwards and Hendrickson (7). All of the reported observations were made using primarily dark field microscopy.

In both NRM and IO rat cases, the principal retrogradely-labelled cell group surrounded the fasciculus retroflexus (FR) in

the prerubral subthalamic region (Fig. 1, 2). In the NRM cases labelled cells were observed in a demilunar configuration around the dorsal, medial and ventral aspects of FR with a definite paucity of labelling on the lateral aspect (Fig. 2D), whereas in IO cases FR was completely surrounded with labelled cells (Fig. 2C, E). Several investigators have referred to this cell group as the nucleus subparafascicularis or parafascicular nucleus (1,5,11,16,17), which has led to confusion with the parafascicular and subfascicular nuclei of the dorsal thalamus. Since the nucleus in question is rostral to the red nucleus (prerubral) and surrounds FR (parafascicular), we suggest that the term nucleus parafascicularis prerubralis be used as a more appropriate designation.

Rostral to this level in both NRM and IO cases, the cells extended into the ventral periaqueductal gray and nucleus of Darkschewitsch. At the level of nPfPr, labelled cells extended laterally into the zona incerta (ZI) under the medial half of the medial lemniscus. Caudal to nPfPr, in the rostral mesencephalon, a vertically-oriented group of retrogradely-labelled cells was observed between the midline and the fascicles of the medial longitudinal fasciculus (MLF) in the rostralmost oculomotor nucleus, which we suggest may belong to the paraoculomotor medial accessory nucleus of Bechterew (8).

Although the transcannula HRP gel implant method tremendously restricted spread beyond the limit of the target nuclei, the finding of similar afferents to both NRM and IO required further substantiation using the anterograde autoradiographic technique. Following the injection of tritiated leucine into the nPfPr and

surrounding medial prerubral subthalamic region, the anterogradely-labelled projections descended through the MLF, central tegmental tract, and the paramedian brainstem reticular formation similar to the course of the medial tegmental tract described by Ogawa in the cat (14). In the caudal pons, the fiber system was oriented vertically such that one could observe a projection to the ipsilateral rostralmost nucleus raphe obscurus (NRO) that extended ventrolaterally into a rostrocaudally-running band over the ipsilateral transverse pontocerebellar fibers. In the rostral medulla, small clusters that appeared to represent terminations were observed in a vertical line in ipsilateral NRO and midline NRM (Fig. 3B, D), and laterally over the pyramidal tract in the nucleus reticularis magnocellularis (RMC). The overall distribution of labelling, from midline NRM through RMC, appeared to correspond to the combined NRM/RMC, spinal-projecting cell group of the ventromedial medulla referred to as the nucleus raphe alatus (NRA) by Watkins et al (21). At the mid-medullary level, a heavy projection was observed to the entire inferior olivary complex, including all subdivisions of the principal nucleus (PIO), dorsal (DAO) and medial accessory (MAO) nuclei (Fig. 3 C,E). The projection to the DAO was bilateral and labelled fibers were observed to decussate at the level of the nucleus. Thus it appeared that nPfPr projected to NRO, NRA (NRM/RMC) PIO, DAO and MAO.

Silver degeneration studies have reported a substantial projection from the parvocellular red nucleus (PRN) to the IO (12,19, 20), however, this study tends to support recent reports that the red nucleus does not project to IO (eg. 11). The

rostral limit of the PRN is particularly difficult to ascertain in the rat. Reid et al (15) in their Golgi study stated that "the fasciculus retroflexus lies just rostral to the rostral pole of the nucleus", and Gillilan (9) did not mention a parafascicular configuration of cells in her description of the rodent RN. Thus we feel that the nPfPr is more likely part of the prerubral field and not PRN, and that lesions of PRN reporting rubroolivary projections had inadvertently involved nPfPr. Since the organization of the nPrPr efferents in the brainstem is similar to that described by Ogawa (14), it is possible that the nucleus represents in part the rat homologue of the interstitial nucleus of Cajal.

Although we do not discuss all of the afferents to NRM and IO in this report, nPfPr appears to be a primary source of inputs to both cell groups. Since IO is established as a precerebellar extrapyramidal motor structure, this common input to both structures from the prerubral field raises the question, as others have already done (2,18), that NRM, rather than being involved solely in antinociceptive mechanisms, may influence a broad range of reflexes.

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ABBREVIATIONS

DAO - dorsal accessory olivary nucleus

FR - fasciculus retroflexus

IO - inferior olivary complex

MAO - medial accessory olivary nucleus

ML - medial lemniscus

ND - nucleus of Darkschewitsch

nPfPr - nucleus parafascicular prerubralis

NRA - nucleus raphe alatus

NRM - nucleus raphe magnus

NRO - nucleus raphe obscurus

P - pyramidal tract

PGC - nucleus paragigantocellularis

PIO - principal nucleus, inferior olivary complex

RMC - nucleus reticularis magnocellularis

ZI - zona incerta

LEGEND

Figure 1. Cresyl violet-stained coronal section through the prerubral subthalamic region in the rat. The nPfPr surrounds the FR (dotted lines) and is contiguous with periventricular gray dorsomedially and subthalamic areas laterally.

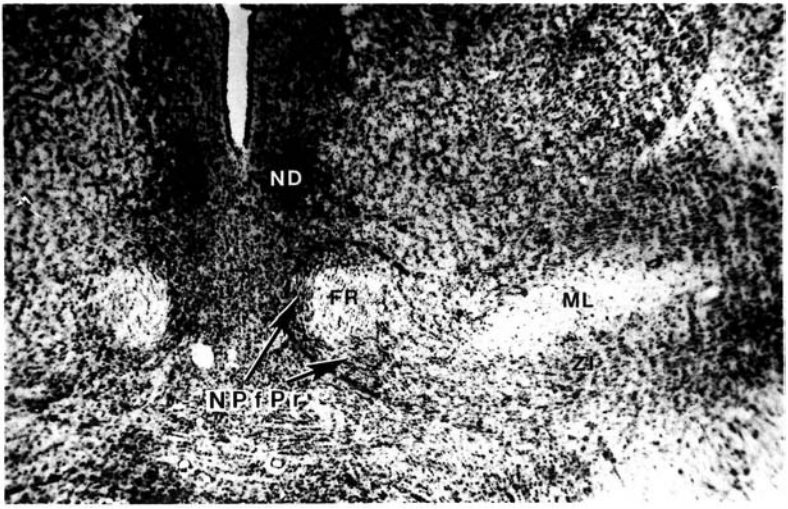

ND
FR
ML
ZI
NPf
PIF

Figure 2.

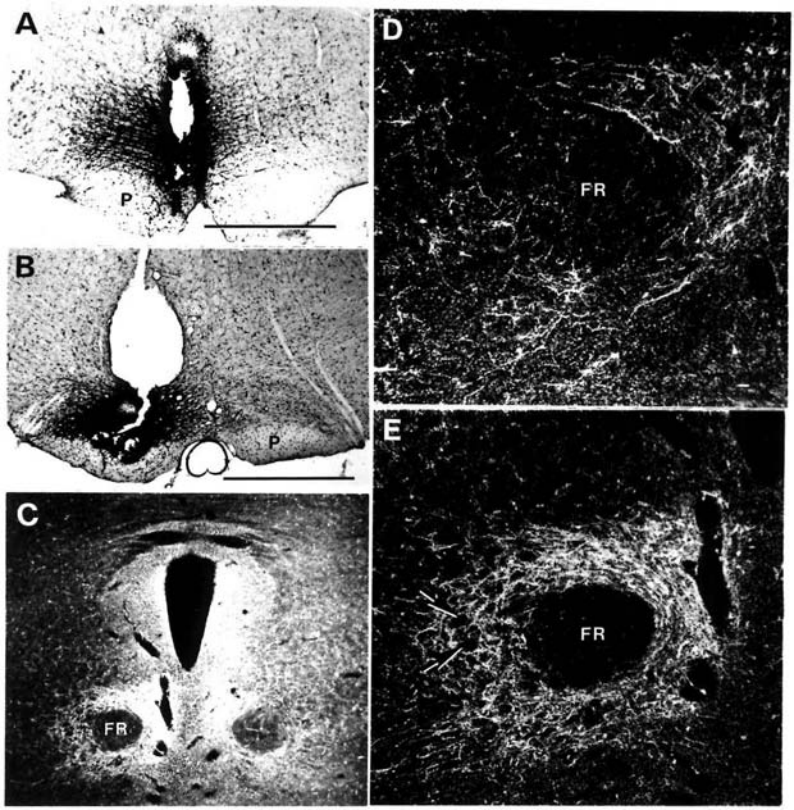
A. HRP gel implant site in paramedian NRM in the rostral ventromedial medulla above the pyramidal tract. Bar represents 1mm.

B. HRP-gel implant site in the IO at a mid-medullary level. Bar represents 1 mm. Hole above implant was made by pre-implanted cannula.

C. Low power dark field photomicrograph of the medial prerubral subthalamic region at the dien/mesencephalic junction showing retrogradely-labelled cells in nPfPr surrounding the ipsilateral FR resulting from HRP-gel implant in IO (in B.). A few cells are also labelled contralaterally in nPfPr.

D. Dark field photomicrograph of nPfPr following an HRP-gel implant in NRM (in A.). Note paucity of labelled cells lateral to FR as compared to an implant in IO (in C. & E.)

E. Higher power dark field photomicrograph of nPfPr (same section as in C.) showing that cells completely surround FR. The lateral cells are intermingled among the rostral-most fascicles of the MLF (arrows) suggesting that a constituent of nPfPr may represent the rat homologue of the interstitial nucleus of Cajal.


A
P
B
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C
FR
D
FR
E
FR

Figure 3.

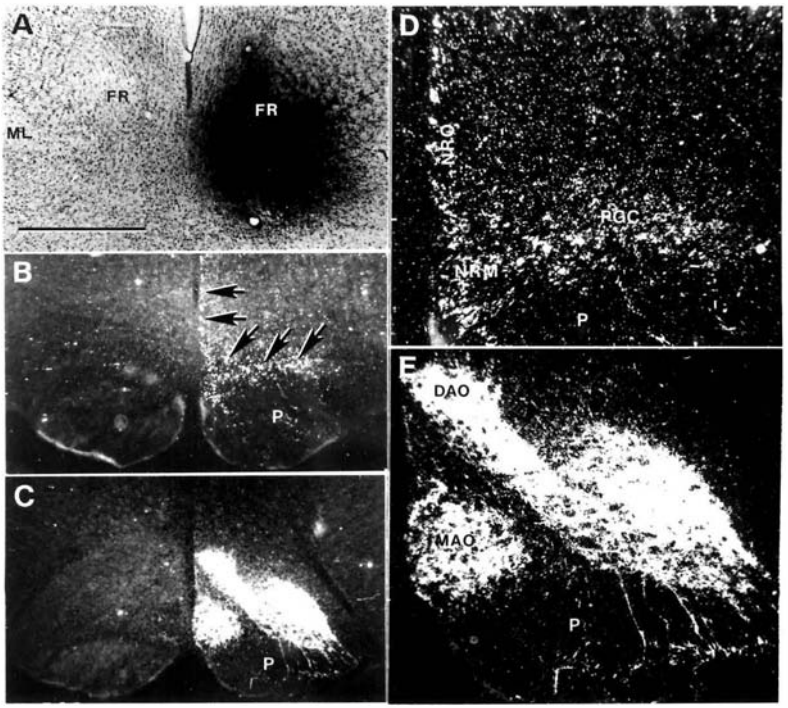
A. Autoradiographic injection site (tritiated leucine) in nPfPr and surrounding medial prerubral subthalamic region. Bar represents 1 mm.

B. Dark field photomicrograph showing anterogradely-labelled projections to the ipsilateral NRO and NRA (NRM/RMC) following injection in A. Clusters of label suggest terminations (arrows) amid fibers of passage which traverse the ventromedial medulla enroute to IO.

C. Anterogradely-labelled projections to ipsilateral IO.

D. Higher power dark field photomicrograph of B.

E. Higher power dark field photomicrograph of C. showing projections to DAO, MAO and PIO nuclei.


A
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DAO
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V. SUPRAMEDULLARY AFFERENTS OF THE NUCLEUS RAPHE MAGNUS IN THE RAT: A STUDY USING THE TRANSCANNULA HRP AND AUTORADIOGRAPHIC TECHNIQUES

Several lines of evidence suggest that the nucleus raphe magnus (NRM) in the rostral ventromedial medulla is involved in descending antinociceptive mechanisms, (for review see Mayer and Price, '76, '79; Messing and Lytle, '77; Fields and Basbaum, '78; Mayer, '79). Electrophysiological studies demonstrate that stimulation of NRM not only selectively inhibits the response to noxious stimuli of laminae I and V dorsal horn neurons (Basbaum et al., '76; Fields et al ., '77; Guildbaud et al., '77; Duggan et al., '79), but also produces a powerful behavioral analgesia (Proudfit and Anderson '75; Oliveras et al., '75; Basbaum et al., '76; Oliveras et al., '77; Oleson et al., '78; Satoh et al., '80), which is naloxone reversible (Oliveras et al., '77; Zorman et al., '81). Lesions of NRM block or attenuate midbrain stimulation-produced analgesia (Behbehani and Fields, '79; Cannon et al., '80). Since depletion of CNS serotonin blocks the antinociceptive effects of NRM stimulation (Proudfit and Anderson, '75), it has been widely held that these analgesic effects are serotonin-mediated; however, recent findings question this theory (Johannessen et al., '81).

The anatomical connections of NRM have been studied in the cat (Brodal et al., '60; Bobillier et al., '76; Tohyama et al., '79; Basbaum et al., '81) and rat (Gallager and Pert, '78; Senba et al., '81; Takagi et al., '81; Carlton et al., '81). Raphespinal

projections have been shown to traverse the dorsolateral funiculus (DLF) of the spinal cord in the rat (Leichnetz et al., '78; Watkins et al., '80), and have also been reported to terminate at all levels of the cord with the greatest density in the cervical area, (Basbaum et al., '76; Watkins et al., '80), and specifically in lamina I and V, the laminae known to contain neurons which are maximally responsive to noxious stimuli (Wagman and Price, '69; Christensen and Perl, '70; Liebeskind et al., '73; Burgess, '74; Price and Mayer, '75; Giesler et al., '76). Systemic and intracerebral morphine analgesia (Basbaum et al., '76; '77; Murfin et al., '76; Hayes et al., '78; Barton et al., '80), stimulation-produced analgesia (Basbaum et al., '76; '77; Fields et al., '77), and environmental stimuli such as front paw or hind paw shock (Watkins et al., '80; Mayer and Watkins, '81; Watkins et al., '82), are all dependent on the integrity of the DLF in the spinal cord. The fact that NRM is one of the major contributors of axons to this tract (Basbaum et al., '76; Leichnetz et al., '78; Watkins et al., '80), is further evidence that this nucleus plays an important role in potent supraspinal antinociceptive mechanisms and further raises the possibility that the DLF raphe-spinal system is a "final common pathway" for certain CNS analgesic mechanisms. Thus, we felt it necessary to identify those supramedullary structures which could influence analgesic mechanisms by virtue of their direct connections with NRM.

Due to its' location in the ventromedial medulla (VMM), NRM has proved to be a difficult area in which to carry out a

retrograde HRP study. The many fiber systems that traverse the medullary reticular formation are subject to transection by a needle tract and consequently also take up and transport the enzyme. However, with the development of a transcannula technique (Carlton et al., '81), we have been successful in implanting HRP-gel precisely into NRM, with little or no contamination of surrounding tissue. Combining this technique with the very sensitive tetramethylbenzidine (TMB) protocol of Mesulam ('78), we have been able to identify those afferent sites which project to NRM. Using autoradiography, pathways originating from the principal NRM afferent sources can be followed through the brainstem to their termination in NRM.

MATERIALS AND METHODS

Horseradish peroxidase gel implants

Eight adult male Sprague-Dawley rats (350-540 g) were stereotaxically implanted with a 26 gauge stainless steel cannula in NRM (AP -1.6, ML 0, V -1.0, based on the atlas of Pellegrino, Pellegrino and Cushman) under sodium pentobarbital anesthesia, 50 mg/kg IP. Control HRP gel implants were also made in the n. reticularis paragigantocellularis (Pgc, n=4), inferior olive, (IO, n=4), nucleus of VII, (n=3), and spinal cord (SC, n=6). On the fifth day after the cannula implantation surgery, a small pellet of HRP gel was delivered through the cannula to the target site (for a more complete description of the transcannula HRP-gel implant technique, see Carlton et al. '82). Spinal cord implants

were carried out according to the method of Griffin et al. ('79). Following two more days survival time, each rat was deeply anesthetized with sodium pentobarbital and perfused through the left ventricle with 300 ml heparinized saline, followed by 500 ml mixed aldehyde fixative (1% paraformaldehyde, 1.25% glutaraldehyde in 0.1M phosphate buffer), and then post-perfused with 300 ml 10% phosphate-buffered sucrose. The brain was immediately removed and placed in fresh 10% phosphate-buffered sucrose overnight at 4°C. The following day each whole brain was frozen sectioned at 25 µm and reacted according to the TMB protocol of Mesulam ('78). Alternate sections through each implant site were reacted with the benzidine dihydrochloride (BDHC) protocol of Mesulam ('76), to establish the center, or core uptake area, of the implant site. All sections were mounted on presubbed slides, counterstained with neutral red and studied under dark and bright field microscopy.

Autoradiography

In five additional animals, a confirmational anterograde study was carried out using autoradiography applied to sites found in the HRP studies to project to NRM. Each rat was first implanted with a 26 gauge stainless steel cannula in either the n. parafascicularis prerubralis (nPfPr), n. cuneiformis (nC), deep superior colliculus. On the fifth day post-cannula surgery, 0.5-0.10 µl of tritiated leucine (25 µCi/µl) was pressure injected through the cannula into the target site. The animals were allowed to survive for 12-24 hrs and then were deeply anesthetized

with sodium pentobarbital and perfused through the left ventricle with 300 ml heparinized saline, followed by 500 ml 10% formalin. The brains were removed and placed in 30% sucrose-formalin overnight at 4°C. The following day they were frozen sectioned at 50 μm and sections collected in 10% formalin. After three rinses in distilled water, the sections were mounted out of 1.5% alcoholic gelatin on acid cleaned, presubbed slides, and then were allowed to dry in a dust-free environment. After defatting in xylene for 24 hrs, the slides were rehydrated through descending graded alcohols to water, and then air dried again. Later they were coated with Kodak NTB-2 nuclear track emulsion according to the protocol of Cowan et al.,'72. Following 6 wks exposure time, all slides were developed, lightly counterstained with cresyl violet, and studied under dark field microscopy.

RESULTS

HRP studies: Afferents to the nucleus raphe magnus

Eight cases were used to compile the data in Table 1. The following description is of case RM 7, a case in which the gel implant (Fig. 2A) resulted in a representative retrograde labelling of NRM afferents. Viewed in coronal section, the restricted core of the implant was in midline NRM, spreading very slightly into the adjacent n. reticularis magnocellularis, and was approxi- 0.34 mm in width. The implant site extended rostrocaudal mately 2mm, from the caudal level of the facial n. to ' where the facial genu starts to form. The core of '

was at the widest extent of the facial n. (corresponds to figure 4c of Palkovits and Jacobowitz, P&J). Figure 1 is a charting of the distribution of labelled cells seen in this case.

The rostralmost level of the neuroaxis containing labelled cells was the sensorimotor cortex. Medium to large pyramidal cells in layer V were seen bilaterally labelled in the dorsal, dorsolateral, and lateral area of the frontal cortex (fig. 1A). In each of several sections, there were as many as 21 labelled cells in one hemisphere. Moving caudally, several labelled cells were seen in the ipsilateral preoptic area (POA) below the anterior commissure (fig 1B). There was an abundance of labelled cells seen in the ipsilateral dorsomedial n. of the hypothalamus (DMH, fig. 1C, corresponding to fig, 5b of J&P). At this same level, cells were seen in the ipsilateral zona incerta (ZI) lying just ventral to the full width of the medial lemniscus (ML), and extending caudally in ZI to the caudalmost level of the mammillary bodies (fig. 1D, corresponding to fig. 6b of J&P). At this level, the cells in ZI were continuous medially, with large numbers of labelled cells that surrounded the fasciculus retroflexus (FR) in the prerubral subthalamic region (fig. 1E, 2B), in a nucleus we have referred to as the n. parafascicularis prerubralis, nPfPr (Carlton et al., '82 in press). These cells were juxtaposed to the FR, forming a demilune around its dorsal, medial and ventral aspects, with a paucity of cells on the lateral side. At the level of the n. PfPr, and slightly caudal to it, labelled cells extended dorsally into the ventral peri(third)

ventricular gray and the n. of Darkschewitsch (nD fig. 1F). There were scattered cells in the pretectum in the n. of the posterior commissure (nPC). Retrogradely labelled cells were seen bilaterally in the rostralmost interstitial n. of Cajal (INC) at the dien-mesencephalic junction. Caudal to the level of n. PfPr, in the rostralmost mesencephalon, a group of retrogradely labelled cells formed a vertically-oriented band lying just off midline, and continuing into the dorsomedial parvocellular red nucleus, which is believed to correspond to the medial accessory n. of Bechterew (nB, fig 1G, 2C, corresponds to Fig. 1a of P&J). Moving caudally, this labelled group was seen in the oculomotor nucleus between the midline and the fascicles of the medial longitudinal fasciculus (MLF) and appeared to be cradled by the fiber bundles of the MLF. Labelled axons of these cells appeared to descend into the medial lemniscus in the rostralmost mesencephalon. Other midbrain areas which contained retrogradely labelled cells included the dorsal periaqueductal gray (PAG), lateral PAG (fig. 1G, H, 2D), deep layers of the superior colliculus (dSC, fig. 1G, 2E) and n. cuneiformis (nC, fig. 1G, H, 1, 2F). In the PAG, the dorsal and lateral groups labelled bilaterally and there was a very definite area devoid of cells (gap) between these groups (fig. 2D). In many sections, it appeared that those labelled cells in the lateral PAG were contiguous with a group labelled in nC located just lateral to the PAG in the midbrain RF, (fig. 1G, H). The labelled cells in the midbrain RF appeared to form an arc, not unlike the arching of the layers of the dSC.

The cells arched out ventro-laterally, lying in the nC and the more ventral midbrain RF. This formation was seen bilaterally. Contralaterally, at the same level, very heavily labelled cells were seen in the stratum intermedium and stratum profundum of the dSC. The labelling was most dense in the lateralmost dSC (fig. 1G, fig. 2E). This pattern of labelling in the PAG, nC, midbrain RF and dSC extended throughout the midbrain, and into the rostral pons.

In the rostral pons, there were a few scattered cells labelled bilaterally in the dorsal n. of the lateral lemniscus (dLL, Fig. 1I). There were also labelled cells scattered in the n. reticularis pontis oralis and caudalis (fig. 1J). In the very rostral medulla, there were a few labelled cells seen in the contralateral medial and lateral vestibular nuclei (MVN, LVN, fig. 1J, K).

At the level of the implant site, scattered labelled cells were again seen bilateral in the MVN and LVN. Caudal to the implant site, an abundance of heavily labelled cells were seen in the contralateral descending n. of V (dNV fig. 1L) and their labelled axons could be seen streaming out medially toward the midline. The dorsal column nuclei (DCN) contained several labelled cells on the contralateral side with associated labelling of some internal arcuate fibers (fig. 1M). A few labelled cells were juxtaposed to the dorsal tip of the medial longitudinal fasciculus (MLF), at the mid-medullary level (fig. 1L, corresponds to fig. 4e of P&J). These cells appeared to be a subdivision of the nucleus of XII.

In case RM 7, representative sections were taken from the cervical cord. A few labelled cells were found in the ipsilateral DLF, ipsilateral lamina II and III, and bilateral lamina VII, VIII and IX.

HRP Control Studies

In spite of the fact that the transcannula gel implant technique greatly restricted the site of uptake of HRP, we were still aware of the possibility that the HRP gel implants could have contaminated axons of passage which passed directly through NRM, descending to more caudal medullary or spinal levels. Therefore we did several controls studies to determine those sites which projected to the spinal cord, and those sites which projected to the inferior olive (IO). Table 2 illustrates those supraspinal sites which labelled following an HRP gel implant in a hemi-implant of the cord (C3, C4 level), or an HRP gel implant in the IO. Those areas which contained retrogradely labelled cells following a placement of HRP gel in one half of the spinal cord included the frontal cortex, ZI, nPC, dSC, INC, nC, dNV, pontine RF, and the vestibular n. Sources of afferents of the inferior olive included the frontal cortex, ZI, nPfPr, nD, INC, nB, nC, pontine RF, dNV, and DCN, (also see Carlton et al., '81).

We also studied the distribution of retrogradely labelled cells resulting from HRP gel implants in areas adjacent to, and at the same level as, NRM, including the Pgc, Pgc/medial facial n., and the medial facial n. Those areas which contained retrogradely labelled cells following a placement of HRP gel in Pgc

included the frontal cortex, ZI, nPfPr, nD, nPC, dorsal PAG, lateral PAG, nC, dSC, nB, INC, dNV, pontine RF, MVN, LVN, NRM, dLL, DCN, dorsal raphe. An HRP gel implant in the medial facial n. resulted in retrograde labelling in the frontal cortex, ZI, lateral PAG, nC, dSC, paralemniscal n, INC, dNV, pontine RF, NRM and dLL.

Autoradiographic Studies

Since nPfPr and dSC/nC appeared in the HRP studies to represent major sources of afferents to NRM, we undertook confirmational anterograde autoradiographic studies. Following an injection of tritiated leucine into the nPfPr and adjacent medial subthalamic region (fig. 3A and B, 4A), fibers were seen leaving the injection site taking one of three courses. They 1) entered the ipsilateral medial longitudinal fasciculus (MLF) and descended in a trajectory similar to that of the medial tegmental tract (MTT of Ogawa, '39, fig. 4B), 2) entered the central tegmental tract (CTT, fig. 4B), or 3) entered an ascending tract dorsal to the ML and travelled dorsolaterally toward the thalamus similar to the course of the field H_1 of Forel and thalamic fasciculus. As the fibers in the MTT descended through the midbrain, they fanned out ventrolaterally from the MLF into the RF (fig. 3C and D). Moving caudally through the midbrain and pons, these fibers gradually attain a paramedian orientation such that at the level of the caudalmost inferior colliculus, a group of labelled fibers could be seen lying just off midline and extending dorsoventrally from the MLF to the

pontocerebellar (PC) fibers (Fig. 3E and F). A triangular shaped terminal field is seen in the rostral medulla over the pyramidal tract. Small clusters that appear to represent terminations are seen in ipsilateral NRO, NRM and n. reticularis magnocellularis (RMC, fig. 3G, 4C), (the latter two were collectively called the nucleus raphe alatus, NRA, Watkins et al., '80). A major projection continues caudally, labelling terminal fields in the entire ispilateral inferior olivary complex, including all subdivisions of the principal nucleus (PIO), dorsal (DAO) and medial accessary (MAO) nuclei (fig. 3H). The projection to the DAO was bilateral and a few labelled fibers were observed to descussate at the level of the IO. The fibers coursing via the CTT form a fairly discrete bundle in the rostral midbrain (fig. 3C). However, as it progresses caudally through the brainstem, the medial portion tends to migrate toward, and eventually merge with, the MTT while the main portion of the bundle gradually disappears as fibers exit from the tract (fig. 3D). It appeared that the CTT projected primarily to rostral and caudal pontine RF, and a small terminal field was seen in the dorsal n. reticularis tegmenti pontis, nRTP). It appeared that the CTT did not contribute substantially to the terminal fields seen in the medulla. The ascending tract which projected into the thalamus was not studied.

In five cases, an injection of tritiated leucine was placed in the midbrain dSC (stratum profundum) and dorsal nC (see fig. 3I, J, K, 4D). Though the injections varied in size, the pattern of labelled fibers was similar in each case, and we have illustrated

a representative case (C 11). At the rostral most level of the injection site (fig. 3I), a fiber bundle could be seen entering the posterior commissure and distributing labelled axons into the contralateral posterior thalamic nuclei. On the ipsilateral side, fibers could be seen streaming ventrolaterally through the ventral thalamic n. and ML to reach the ZI and thalamic reticular n. where terminal fields were seen. Very light labelling was also seen in the pars compacta of the substantia nigra. A bundle of fibers was also seen leaving the injection site, coursing ventromedially to terminate in the area of the nD and nPfPr. At the major site of the injection (Fig. 3K), commissural fibers could be seen coursing dorsal to the PAG to distribute to the contralateral dSC and nC. Two other distinct fiber bundles arose from the injection site, taking pathways similar to those described by Edwards '75 and Harting '77. One fiber bundle coursed ventrolaterally from the injection site and entered the CTT in the lateral midbrain RF (fig. 3K). Labelled fibers could be followed caudally through this tract, giving off terminal fields into the nRTP, n. reticularis pontis oralis/caudalis (nRPO/C, fig. 3M). Very light terminal labelling was seen in the NRA (fig. 3O, 4F), and only a very light labelling of terminal fields was seen in the ipsilateral IO (fig. 3P). A second fiber system coursed ventromedially around the PAG with some fibers entering the ipsilateral MLF, and the majority continuing into the dorsal tegmental decussation (DTD, fig. 3K, 4E). The fibers in the ipsilateral MLF coursed in the dorsal part of this bundle until

the level of the IO where the fibers took a more ventral position along midline as the MLF elongated. The fibers which crossed in the DTD at midbrain levels, on the other hand, descended in the ventral part of the contralateral MLF and in the contralateral MTT to the level of the caudal pons, giving off terminal fields into the nRTP and to the nRPO/C, (fig. 3M, N).

DISCUSSION

The approach used in this study has led us to a more certain appreciation of the sources of afferents to the NRM. The trans-cannula technique has eliminated, to a great extent, the fiber of passage problem by preventing contamination through uptake of HRP along a needle tract (Carlton et al., '82). The HRP gel has been shown to have the advantages of restricting the spread of the enzyme at the implant site, and providing the axon terminals within the target structure prolonged exposure to a concentrated oasis of the enzyme, resulting in maximum uptake. Since the animal is anesthetized during the actual gel implant phase with a short-acting inhalant (Metaphane) the detrimental effects on HRP transport, characteristic of barbiturate anesthesia (Mesulam '80), are avoided. While it is still possible that certain fibers were damaged at the implant site itself (in this case less than 0.4 mm in diameter), we felt strongly that either direct projections to NRM exist from the retrogradely labelled sites or collaterals are given off to NRM from those systems projecting to more caudal sites. The TMB method is known to be the most sensitive

of HRP protocols which, when coupled with dark field microscopy, leads to excellent visualization of labelled elements. Using the results we have obtained in this study, we are able to present a more global evaluation of the inputs to NRM in the rat than presently exists in the literature.

Brodal et al. ('60) in their study of the afferent connections of the raphe nuclei, described NRM afferents arising from the cerebral cortex. In that degeneration study, very large areas of the cat cortex were ablated so that the origin of these terminals were only grossly determined. Gallager and Pert ('78) state that a few labelled cells were found in the ventromedial prefrontal cortex following iontophoresis of HRP into the brainstem raphe, and finally, Senba et al. ('80), confirmed, but did not illustrate, the afferents arising from the prefrontal cortex in the rat. In the present study, retrogradely labelled cells were found in the frontal cortex in all of the NRM cases. However, labelled cells were not observed in sulcal, dorsomedial and medial rostral frontal cortex, ie. in those areas considered to be the equivalent in the rat of the primate prefrontal (mediodorsal thalamic projection) cortex. As demonstrated in RM 7, figure 1K & 2A, the left dorsal pyramid was only slightly contaminated. It was felt that this minimal contamination could not account for the abundance of labelled cells found in the sensorimotor frontal cortex. Most likely, the majority of positive cells in the cortex resulted from the uptake of HRP at the core of the implant site which centered in midline NRM.

We confirmed Senba's et al., ('81), findings of retrogradely labelled neurons in the ZI, and nPfPr the dorsal PAG, and the midbrain RF (which includes the nC). Senba stated that a cell group on the midline of the rostral mesencephalon, the raphe linearis rostralis, was also retrogradely labelled. We believe that they incorrectly identified this cell group since the neurons which we found labelled at this same level were lying off the midline in a pattern more suggestive of the medial accessory nucleus of Bechterew, a cell group in the lateral rostral oculomotor complex in most mammals (see Fuse, '37). Gallager and Pert ('76), reported finding labelled neurons in the lateral and dorsal PAG, deep layers of the superior colliculus, pontine RF and medial vestibular nucleus, which is consistent with our data. Those areas which contained retrogradely labelled cells in our cases which have not been reported before in the rat include the POA, DMH, nPC, nD, dNV, dLL, a subdivision of the hypoglossal nucleus and the DCN. However, in an HRP study of NRM afferents in the cat, (Abols and Basbaum, '81), nD and dNV were reported as containing labelled cells.

With the exception of the cells in the subthalamic region, the midbrain clearly appears to contain the major sources of afferents to NRM. In all 8 cases the dorsal and the lateral PAG, nC and subjacent midbrain RF, and dSC, contained an abundance of retrogradely labelled cells bilaterally. From a physiological point of view, it is not surprising that the PAG contributes such heavy input to NRM since there is an abundance of data already

implicating the PAG in analgesic mechanisms. Stimulation of the PAG inhibits the firing of cells in the dorsal horn excited by noxious stimuli in both cats and rats (Mayer and Price, '76; Fields and Basbaum, '78). Lesions of the PAG reduce morphine analgesia (Deakin and Dostrovsky, '78) morphine microinjection into (Lewis and Gebhart, '77; Yeung et al., '77; Johannessen et al., '82) or stimulation of (Balagura and Ralph, '73; Mayer and Liebskind, '74; Mayer and Price, '76; Soper, '73; Rhodes and Liebeskind, '78; Mayer, '79) the PAG produces analgesia.

There is also evidence that the dSC could be involved in antinociceptive mechanisms based on the work of Stein and Dixon ('77, '78) who demonstrated that cells in the deep layers of the superior colliculus were preferentially or exclusively activated by noxious stimuli. Furthermore, responses to noxious stimuli were blocked by the analgesic drug etorphine and reinstated after administering the narcotic antagonist, naloxone.

Another interesting finding in this study was the pattern of retrograde labelling in the PAG. As described earlier and illustrated in figure 1G, H, & 2D, retrogradely labelled cells were found in the dorsal and lateral midbrain PAG with a very definite gap between these two labelled cell groups. This pattern is strikingly reminiscent of that produced by terminal efferent projections from the prefrontal cortex to the PAG, as demonstrated by Hardy and Leichnetz ('81). This apparent overlap of prefrontal-PAG projections with cell groups that project directly to NRM raises the possibility that the prefrontal cortex may influence

antinociceptive mechanisms through this disynaptic pathway, perhaps influencing some affective dimension of this system . In support of this theory, Kelly ('74) has shown that single units in the medial prefrontal cortex of the rat are activated by aversive electrical stimulation at PAG sites.

As mentioned previously, labelled neurons in the lateral PAG form a contiguous unit with labelled cells in the nC and subjacent midbrain RF. This arrangement is noteworthy since it has been reported by Simantov et al.('77), that substantial enkephalin immunofluorescence is present both in the lateral PAG and adjacent midbrain RF (eg. nC). They also observed that these same areas also display moderate numbers of autoradiographically-labelled opiate receptors, again suggesting that this area may play a possible role in pain mechanisms through both its inherent affinity for opioid substances and its projection to NRM.

The pattern of retrograde labelling in the deep layers of the superior colliculus observed in this study support the conclusion of Edwards ('80) that the stratum griseum intermedium (SGI) and the stratum griseum profundum (SGP) should be considered part of the reticular core, separate and distinct from the more superficial tectal layers. Following a gel implant in NRM, labelled cells were never seen in the superficial SC; however, the contralateral SGI, SGP, and underlying nC were always heavily labelled with somewhat less labelling of cells on the ipsilateral side. The number of labelled dSC cells were greater in NRM cases than in IO cases suggesting that the VMM target of tectobulbar

fibers is the NRM. Likewise, our autoradiographic studies demonstrated that the dSC/dorsal nC projects heavily through the ipsilateral CTT and reaches the VMM (NRA) with only a very light projection to the principal IO. Thus these data also question the supposed density of the tecto-olivary projection.

As discussed in a previous report (Carlton et al., '81), we have designated the labelled cells in the subthalamic prerubral area as the n. parafascicularis prerubralis. This terminology evolved out of the confusion caused by previous descriptions of this area as the subparafascicular n. (Brown et al., '77; Saint Cyr and Courville, '81) or the n. parafascicularis, (Senba et al., '81) which led to the impression that this area was somehow associated with nuclei of the same name in the dorsal thalamus. Those cells which retrogradely-label following an HRP gel implant in NRM are clearly in the subthalamus and lie just rostral to the red nucleus (hence prerubral). This same cell group has been reported to project to the IO in the rat (Brown et al., '77, Carlton et al., '81) and cat (Saint Cyr and Courville, '81). However, our autoradiographic injections in this area demonstrate terminal fields not only in the IO but also in NRO and NRA (fig. 4). Both HRP and autoradiographic results in this study supported the fact that rubro-olivary projections in the rat are meager, if they exist at all and that the descending projections to the IO probably originate in the prerubral field (including nPfPr) and reach the IO through the MTT, not CTT. There is some data which also implicates nPfPr in possible pain mechanisms. Atweh and

Kuhar ('77), for example, have demonstrated that the fasciculus retroflexus, which is in very close proximity to the nPfPr, has a moderate density of opiate receptors, and stimulation in the area of nPfPr in the rat demonstrates that this area is capable of supporting stimulation-produced analgesia (Mayer et al., '71).

In reference to control data, the purpose of implanting Pgc, Pgc/medial facial nucleus and medial facial nucleus was primarily to observe how the pattern of retrograde cell labelling changed as the implant site was placed progressively further from the midline in the VMM. From these control implants, we have established the following relationships: as the implant site moved laterally from midline NRM, through the medial facial n, there appeared to be a decrease in the number of retrogradely labelled cells in the ZI, nPfPr, POA, DMH, nD, nPC, nB, nD, dorsal and lateral PAG, the subdivision of XII, and the DCN. The number of retrogradely labelled cells in the paralemniscal nucleus and the INC appeared to increase as the implant site moved laterally from midline NRM. The number of retrogradely labelled cells in nC did not seem to change as the implant site moved laterally from the midline, and the labelled cells in nC were always seen bilaterally. A considerable number of labelled cells were seen in the frontal cortex and dSC following an implant in midline NRM and the medial facial nucleus. However, few cells were seen in either site following an implant in the intermediate site, the Pgc.

These data, in conjunction with the data gathered from the control IO and spinal cord implants, allow us to consider the

following as projecting directly to or sending collaterals into NRM: frontal cortex, POA, DMH, nPfPr, ZI, nD, nPC, dorsal and lateral PAG, nC, dSC, nB, and DCN. The labelling in the dLL and the dNV was probably a result of contamination of the trapezoid body and the fibers of the trigeminothalamic tracts respectively. The retrograde labelling of the few cells in the subdivision of XII also appeared to be due to an interruption of their fibers which coursed through the medullary RF in a paramedian position. The fact that the labelling in the INC increased tremendously as the implant site moved off midline indicated to us that NRM was not a principal target of INC efferents. Likewise, the sparse and irregular labelling of cells in the MVN and LVN suggest that these areas are not a likely source of NRM afferents. Also it is quite possible that the retrograde labelling seen in the DCN was a result of interruption of ML fibers since the ML traverses the VMM. In a cresyl violet stained section (figure 5), DLF projecting neurons in the NRA can be seen literally wrapped around ML fibers traversing the VMM, suggesting the possibility that the DCN may send collaterals into NRA, perhaps creating a somatosensory feedback loop with the final limb descending down the DLF.

It was demonstrated that a number of the sites considered to be sources of NRM afferents also labelled following control implants in the IO and spinal cord. These include the frontal cortex, ZI, nPfPr, nD, nPC, nC, dSC, and nB (see Table 2). Although it is not likely that all of these retrogradely labelled areas project to the cord or the IO through the restricted implant

area of the VMM involved in our HRP gel cases, caution must be used in declaring any of these sites as projecting only to NRM.

Until recently, the neuroanatomy of the VMM has not been so controversial. Taber's et al. ('60) classic description of the NRM represented the nucleus as a midline structure in the medullary reticular formation. A few years later Dahlstrom and Fuxe ('64) described area B3 as a serotonergic group of cells which included midline NRM, a horizontal band of cells dorsal to the pyramidal tract and an area lateral to the pyramidal tract corresponding to the nucleus paragigantocellularis lateralis (Pgc, Taber, '61) in the cat. The region between midline NRM and the facial nucleus has been termed the nucleus reticularis magnocellularis (Rmc) by Berman ('68) which includes the Pgc in its ventrolateral aspect. However, in a cresyl violet-stained section through the level of the facial nucleus of a rat (see figure 5), three cyto-architecturally distinct cell groups cannot be delineated in the VMM. There is no logical way to separate NRM from Rmc, or Pgc from the surrounding Rmc unless one draws arbitrary boundaries on this section. Such arbitrary handling of the anatomy in the VMM was of little importance until the VMM attracted considerable interest because of its proposed involvement in endogenous antinociceptive mechanisms.

Attempts at clarifying the neuroanatomy of the VMM were put forth by Leichnetz et al. ('78) and Watkins et al. ('80). Following

- the DLF of the rat spinal cord, retrogradely

- NRM but also in the

adjacent Rmc. In light of these new data, Watkins et al. ('80) suggested that this winged-shaped group of labelled cells over the pyramid be designated the nucleus raphe alatus, thereby using one term to identify the group of DLF projecting VMM cells found in both NRM and Rmc. The confusion arose when Watkins et al. ('80) equated the serotonergic cell group B3 of Dahlström and Fuxe with that same population of retrogradely labelled cells seen in NRM and Rmc (NRA). Johannessen et al. ('81; '82) using double labelling techniques (immunohistochemical staining for serotonin and retrograde labelling after spinal HRP gel implants) demonstrated that retrogradely labelled DLF-projecting cells of NRA exhibited a different distribution from those cells showing serotonin immunoreactivity. The serotonergic (5HT) cells lie on midline VMM and immediately above the pyramidal tract, giving the dorsal aspect of the pyramids a flattened, rather than convex, appearance whereas the DLF projecting neurons are located dorsal in a configuration corresponding to NRA (fig. 5). The 5HT cells lying just dorsal to the pyramid do label following an HRP spinal cord hemi-implant demonstrating that they do project down the cord, although the funiculus in which they travel has not yet been established. Johannessen further suggested that serotonin is not a major component in the DLF projection which originates in NRA. Thus the confusion which resulted from an attempt to logically parcelate the VMM into a functional unit is now dispelled and the use of the term NRA (to designate those cells in NRM and Rmc which retrogradely label following an HRP gel implant in the DLF) remains justified.

In light of these new facts, it is evident that we are dealing with at least two distinct populations in the VMM - the NRA which sends projections down the DLF, and a more ventral serotonergic population which does not travel via the DLF but does project to the cord, both of which are believed to play important roles in endogenous antinociceptive systems (for review see Mayer and Price, '76; Messing and Lytle, '77; Mayer, '79). Now that these groups have been anatomically defined, the next logical step is to differentiate their functions. Could it be that the serotonergic cells of B3 participate in antinociceptive mechanisms while the neurons in NRA may be involved with reflexes and visceral function (Sessle et al., '81; Cabot et al., '81)? Are the afferents to NRA different from the afferents to the more ventral serotonin cells? In the 8 NRM cases which were analyzed for this report, 3 showed no contamination of those cells ventral to NRA, 3 showed slight involvement of that area and 2 demonstrated heavy involvement. Yet, there were no striking differences in the retrograde labelling seen in any of the cases. A statement can be made however concerning nPfPr projections demonstrated in the autoradiography studies. The terminal fields resulting from an injection of tritiated leucine into the area of the nPfPr appeared to be on neurons in the NRA and not on the more ventral serotonergic cells. The dSC/nC autoradiographic injections led to labelling in NRA that extended to a very light terminal field over the ventral serotonergic cells above the pyramidal tract. At this point however, judgement must be withheld concerning common afferent sources to these two areas.

Neurotransmitters other than serotonin have been assocated with cell groups in the area of NRA and B3. These include enkephalin and substance P like peptides (Hokfelt et al., '79). In fact, these two neuromodulators have been identified in the same neurons in the VMM. It is yet to be established, however, what the relationship is of these cells to raphe-spinal DLF projecting neurons. But these findings coupled with those of Johannessen et al. ('81, '82) raises interesting questions regarding whether these neuromodulators could play a role in the antinociceptive system which originates in the VMM and descends in the DLF.

As mentioned previously, an extensive literature exists supporting the role of NRM in antinociceptive mechanisms. For that reason, the identification of nuclei supplying afferents to NRM could provide the next link in a complex chain of neural structures whose activation ultimately results in the inhibition of noxious input. One of the significant results of this study was the finding that none of the nuclei that project to NRM also project down the DLF (see Table 2), emphasizing the fact that the DLF may be a final common pathway for inhibition of noxious input, and that there are perhaps independent but parallel supraspinal pathways in the DLF which influence antinociception. The growing accumulation of data which supports the existence of multiple antinociceptive systems is thoroughly reviewed by Watkins and Mayer ('82). The data suggest that the DLF contains both a descending opiate and non-opiate system. It has been substantially demonstrated that the NRM is involved in opiate analgesic mechanisms

(for review see Fields and Basbaum '78; Mayer '79), while data supporting a role for NRM involvement in non-opiate analgesic systems is conflicting and sparse. It is possible therefore that some afferent sources to NRM are influencing opiate analgesic mechanisms while those nuclei which project directly down the DLF (red nucleus, paralemniscal n., locus coeruleus, ventral PAG), represent parallel but non-opiate analgesic systems. At this time there is little data to support the latter statement since manipulation of other DLF projecting nuclei (Watkins et al., '81), have not yet been attempted. However, data do exist implicating several NRM afferent sources as being involved directly in, or being influenced by, opiate mechanisms. As our knowledge of the anatomical substrate supporting analgesic systems expands, so will our understanding of how best to manipulate these systems for the relief of pain.

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Abbreviations

A - n. ambiguus

CA - anterior commissure

CO - optic chiasm

CP - cerebral peduncle

CT - trapezoid body

DBC - decussation of brachium conjunctivum

DCN - dorsal column nuclei

dLL - dorsal n. of lateral lemniscus

DMH - dorsomedial n. of hypothalamus

dNV - descending n. of V

DR - dorsal raphe

dSC - deep superior colliculus

FC - fasciculus cuneatus

FR - fasciculus retroflexus

FTM - mammillothalamic tract

GR - n. gracilis

IC - inferior colliculus

ICP - inferior cerebellar peduncle

III - oculomotor n.

INC - interstilial n. of Cajal

IO - inferior olive

IP - interpeduncular n.

LC - locus coeruleus

LVN - lateral vestibular n.

ML - medial lemniscus

MLF - medial longitudinal fasciculus

MR - median raphe

MVN - medial vestibular n.

NB - n. Bechterew

NC - n. Cuneiformis

ND - n. Darkschewitsch

NDT - dorsal tegmental n.

NMM- mammillary n.

NPFPR - n. parafascicularis prerubralis

NRA - n. raphe alatus

NRGC - n. reticularis gigantocellularis

NRO - n. raphe obscurus

NRP - n. raphe pallidus

NRO pars V - n. reticularis medullae oblongatae pars ventralis

NTM - mesencephalic tract n.

NTS - n. of solitary tract

NVT - ventral tegmental n.

P - pyramid

PAG - periaqueductal gray

PC - posterior commissure

PH - n. prepositus hypoglossi

PLRF - paralemniscal reticular formation

POA - preoptic area

PSC - superior cerebellar peduncle

PVH - periventricular hypothalamus

RN - red n.

RPCO - n. reticularis pontis caudalis

SGI - stratum griseum intermedius

SGP - stratum griseum profundus

SGS - stratum griseum superficialis

SN - substantia nigra

TP - n. reticularis tegmenti pontis

VII - facial n.

V - n. of spinal tract of V

VSP - spinal vestibular n.

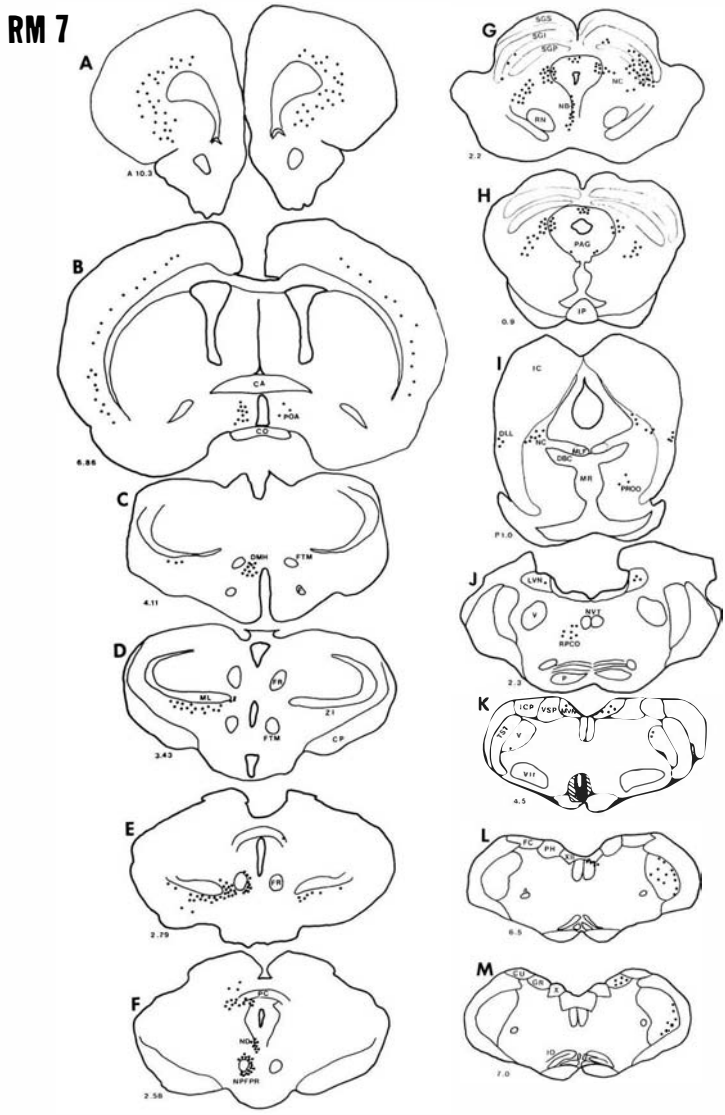
X - vagus

XII - hypoglossal

ZI - zona incerta

Figure 1. Charting of the distribution of labelled cells seen in HRP case RM7. Each dot represents one labelled cell.

RM 7


A
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B
CA
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F
PC
ND
NPF-PR
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SGS
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DRC
MR
PRDO
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IO
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Figure 2. A. Bright field photomicrograph of injection site in case RM7. Bar represents 1 mm.

B. Dark field photomicrograph of retrogradely labelled cells which surrounded the ipsilateral nPfPr in the subthalamus. The morphology of this area consisted of thin, elongated cells which wrap around the FR, giving the area a more fibrous than cellular appearance.

C. Dark field photomicrograph of retrogradely labelled cells in the nB. The cells were vertically oriented just off midline, with the caudal end sweeping out into the midbrain RF.

D. Dark field photomicrograph of the large, densely labelled cells in contralateral dSC (lateral aspect of stratum profundum).

E. Dark field photomicrograph of labelled cells in dorsal and lateral PAG, showing "gap", devoid of labelled cells.

F. Dark field photomicrograph of labelled cells in ipsilateral lateral PAG and adjacent nC.

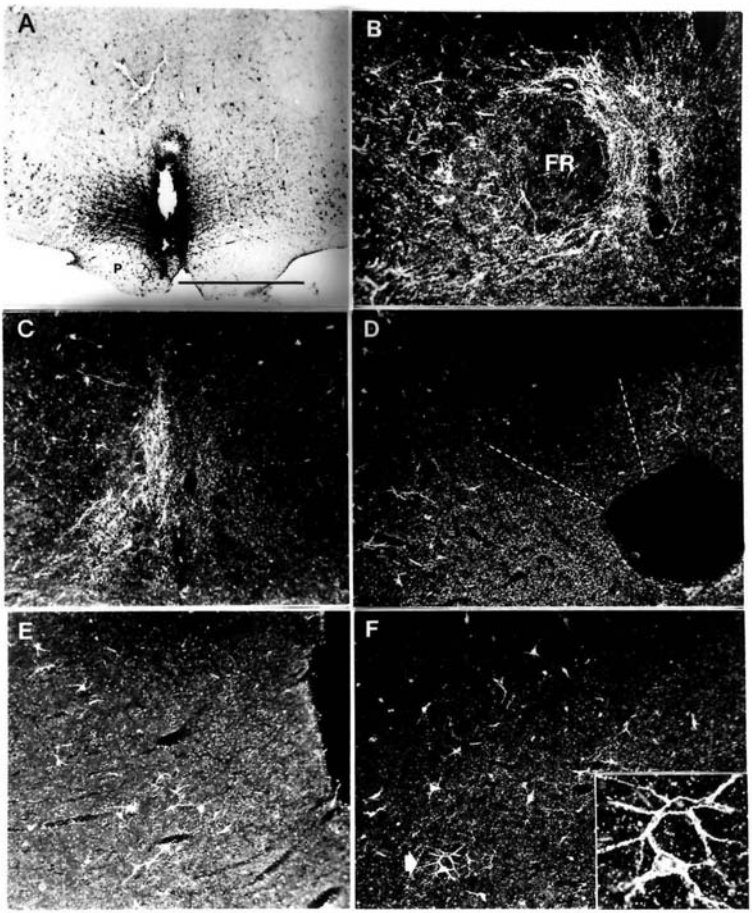
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Figure 3. Chartings of autoradiographic studies involving nPfPr (PF) and dSC/nC (C11). Dotted lines indicate fiber tracts, dots indicate terminal fields.

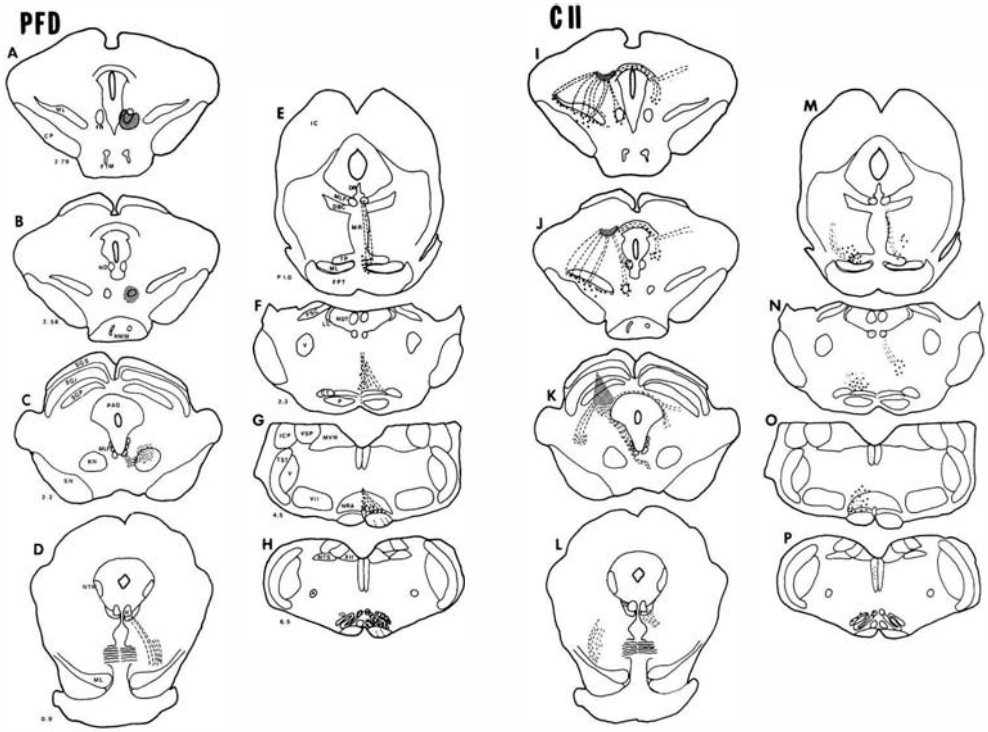
PFD
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Figure 4. A. Bright field photomicrograph of ^{3}H Leucine injection site in PFD (nPfPr and adjacent subthalamus).

B. Dark field photomicrograph of labelled fiber tracts in rostral midbrain following injection shown in A. The dotted line indicates the midline.

C. Dark field photomicrograph of labelled terminal fields in NRO and NRA, (NRM/RMC) at the level of the facial n. following injection shown in A.

D. Bright field photomicrograph of ^{3}H Leucine injection site in case C11 (dSC/dorsal nC).

E. Dark field photomicrograph of labelled fiber tracts in rostral midbrain following injection shown in D. The dotted line indicates the midline.

F. Dark field photomicrograph of the lightly labelled terminal field in NRA and over lying Rgc following injection shown in D.

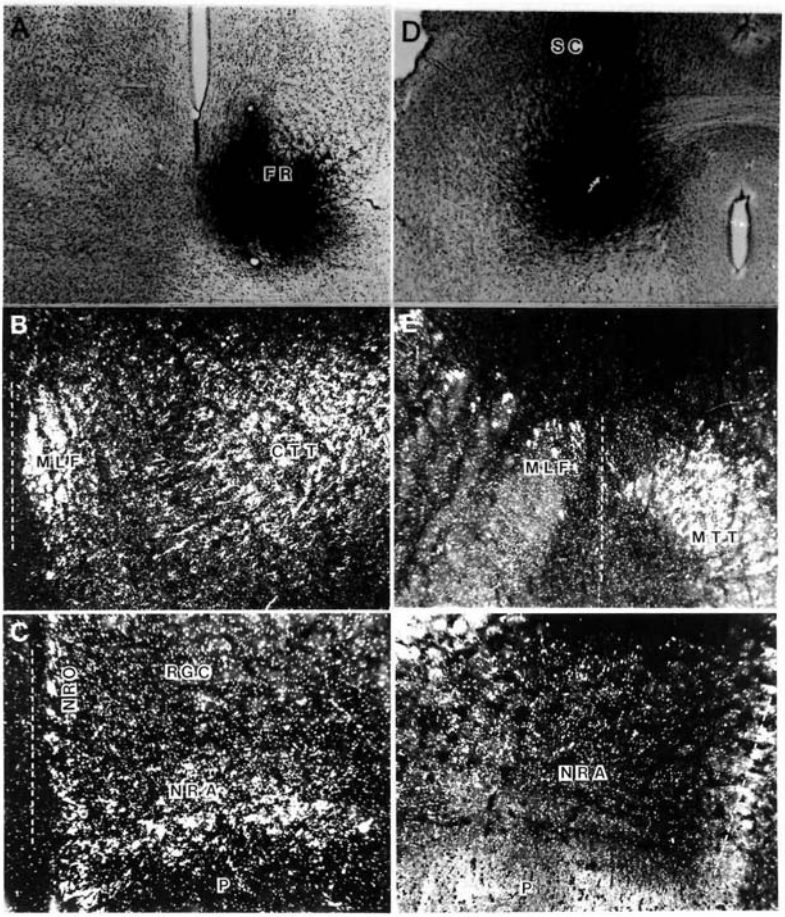
A
FR
D
SC
B
MLF
CTT
E
MLF
MTT
C
NRO
RGC
NRA
P
NRA
P

Figure 5. Parcellation of the VMM. NRA contains DLF-projecting neurons, B3 (dotted outline) contains 5-HT neurons. Notice there is considerable overlap of these populations only on midline. (Taken from Watkins et al., 1980 and Johannessen et al., 1982.)

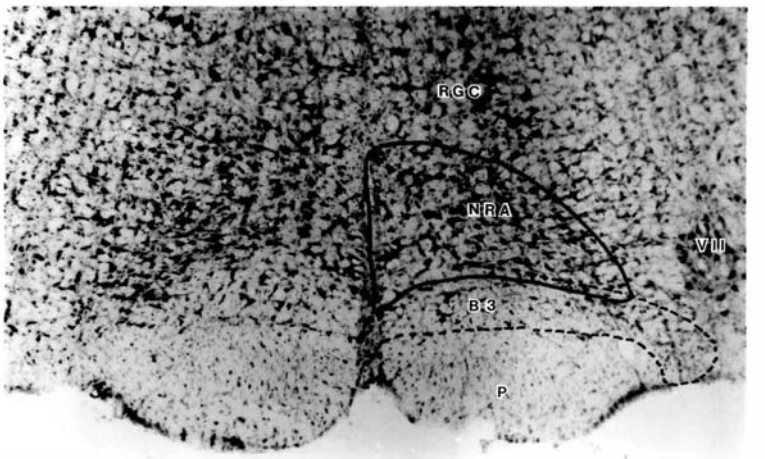
RGC
NRA
VII
B3
P

Table 1

Illustration of 8 NRM implantation sites and resulting retrograde labelling of supraspinal sites.

+ 1-5 labelled cells present in nucleus
0 no labelled cells present
- level not available for analysis

RM12*	RM11	RM10*	RM7	RM5*	RM4*	RM2	RM1	CASE
								
++	++	++	+++	+	++	+	+	FRONTAL CORTEX
o	I	+	+	I	+	I	I	POA
+	+	+	++	I	+	+	I	DMH
+	+	++	+++	+	++	+++	I	ZI
+	+	+	+	+	+	+	I	INC
++	+	+++	+++	++	++	++	I	n PtPr
++	+	++	++	++	++	++	I	n D
+	+	++	++	+	+	+	I	PRETECTUM (nPC)
+	+	+++	+++	++	++	++	+	Dorsal PAG
++	+	+	+++	++	+	+	+	Lateral PAG
++	+	++	+++	++	++	++	o	nC
++	+	++	+++	+	++	+++	+	dSC
++	+	++	+++	+	++	++	+	nB
+	+	+	+	+	+	+	o	dLL
+	+	+	+	+	+	++	+	Pontine RF
o	o	+	+	+	+	+	o	MVN, LVN
++	++	++	+++	+	++	+	+	dNV
+++	o	+	+	+	+	o	o	SUBDIV. of XII
+	++	+	++	+	+	I	o	DCN

Table 2

Comparison of NRM and IO afferents, also a listing of those supraspinal sites which project to the cord and specifically via the DLF to the cord.

DLF (Watkins et al. 1981)	Cord Hemi	IO	NRM	CASE
	+	+	+	FRONTAL CORTEX
			+	POA
			+	DMH
	+	+	+	ZI
		+	+	n PfPr
		+	+	nD
	+		+	Pretectum (nPC)
			+	Dorsal PAG
			+	Lateral PAG
	+	+	+	nC
	+	+	+	dSC
		+	+	nB
	+	+		INC
	+			dNV
	+	+		Pontine RF
	+			MVN, LVM
+	+			NRA
	+			NRP, NRO
+	+			N.RGC
		+	+	DCN
+	+			RN
+	+			PLRF
+	+			LC
+	+			PVH
+	+			N.R.O. pars V

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