THE PEPTIDERGIC ORGANIZATION OF THE CAT PERIAQUEDUCTAL GRAY

II. The Distribution of Immunoreactive Substance P and Vasoactive Intestinal Polypeptide¹

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Abstract

Despite the important contribution of the midbrain periaqueductal gray (PAG) to endogenous pain suppression systems, little is known about the neuroanatomical basis of its functional organization. In a previous study of the distribution of the endogenous opiate leucine-enkephalin (ENK) in the PAG (Moss, M. S., E. J. Glazer, and A. I. Basbaum (1983) *J. Neurosci.* 3: 603-616), we found that immunoreactive ENK-containing neurons and terminals are clustered in discrete populations. In this study we have extended our analysis of the neurochemical organization of the PAG by using immunocytochemistry to map the distribution of two non-opiate peptides that produce potent analgesia when administered at central gray levels: substance P (Sub P) and vasoactive intestinal polypeptide (VIP).

Immunoreactive Sub P neurons and terminal fields are clustered in discrete populations throughout the PAG. The distribution pattern of these populations changes at different rostral-caudal levels of the PAG. For example, there is a ventral-to-dorsal shift in the location of Sub P-like immunoreactivity from the caudal to the rostral PAG. Few immunoreactive Sub P neurons are found in the nucleus raphe dorsalis although moderately dense terminal field staining is present.

The staining pattern of immunoreactive VIP is totally different from that of Sub P. Regardless of the rostral-caudal level examined, VIP-containing neurons are found tightly clustered in the subependymal neuropil of the ventromedial PAG. Only a few immunoreactive VIP-containing neurons are found in the ventral PAG or nucleus raphe dorsalis.

The striking differences between the distribution of Sub P- and VIP-like immunoreactivity in the PAG indicates that the neural circuitry underlying pain suppression by Sub P and VIP may also differ.

The periaqueductal gray (PAG) is a component of an endogenous pain suppression system that has been implicated in stimulation-produced and opiate analgesia (Basbaum, et al., 1976, 1977, 1978; Mayer and Price, 1976; Lewis and Gebhart, 1977; Yeung et al., 1977; Basbaum and Fields, 1978, 1979; Fields and Anderson, 1978; Oliv-

eras et al., 1979). Despite the important contribution of the PAG to pain suppression systems, studies are only beginning to dissect the complex neuroanatomical basis of the PAG's functional organization. In a previous paper (Moss et al., 1983) we described the PAG distribution of the endogenous opiate peptide, leucine-enkephalin (ENK). Immunoreactive ENK-containing neurons and terminal fields are clustered in discrete populations; the distribution of this clustered pattern changes at different rostral-caudal PAG levels. These results illustrated the heterogeneity of the PAG's neurochemical organization and provided clues to the differential sensitivity of the various regions of the PAG to opiate microinjection.

In addition to the endogenous opiates, the non-opioid peptides substance P (Sub P) (Stewart et al., 1976; Fredrickson et al., 1978; Malick and Goldstein, 1978; Mohrland and Gebhart, 1979; Sullivan and Pert, 1981; Naranjo

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et al., 1982) and vasoactive intestinal polypeptide (VIP) (Sullivan and Pert, 1981) also generate a profound analgesia when injected either intraventricularly or directly into the PAG. These two peptides are of particular interest because they generate, respectively, a naloxone-sensitive and a naloxone-insensitive analgesia. Thus a comparison of the degree of overlap of the distribution patterns of these two peptides may provide some information on the inter-relationships of opiate and non-opiate peptides in endogenous pain control mechanisms.

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

Materials and Methods

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

Sub P-like and VIP-like immunoreactivity was localized on representative sections at 150- to 400- μ m intervals using the peroxidase-antiperoxidase (PAP) method (Sternberger, 1974). To enhance antibody penetration, Vibratome and frozen sections were pre-incubated in a sodium ferricyanide/methanol solution (Straus, 1976) or 0.3% Triton X-100 was added to all solutions. Sections were incubated in primary antisera for 48 hr at 4°C. Bridging antisera and PAP incubations were at least 30 min in duration, at room temperature. The PAP reaction product was visualized using 3,3'-diaminobenzidine as the chromagen.

To establish histochemical specificity, adjacent control sections were incubated either in Sub P antisera (ImmunoNuclear Corp.) preabsorbed with an excess of Sub P (Sigma) (100 μ g/ml of diluted antisera) or in VIP antisera (Dr. J. Walsh, University of California, Los Angeles) preabsorbed with excess VIP (Sigma) (200 μ g/ml of diluted antisera). Both Sub P and VIP staining were abolished in the respective control sections. Although the terms "Sub P-like" and "VIP-like immuno-

reactivity" are not used consistently throughout this report, they are at all times implied. Some portion of the immunoreactivity demonstrated with the antisera to Sub P and VIP may be due to other unknown peptides with similar sequences.

Pretreatment with colchicine was necessary to visualize the Sub P perikarya in the PAG. Although colchicine was always administered via third ventricle injections, differences in cell labeling suggest that its penetration into the surrounding neuropil varied from animal to animal. This probably resulted from variations in cerebrospinal fluid (CSF) flow or from diffusion barriers set up by axonal tracts. Therefore, it was essential that we compare the perikaryal labeling from several animals. In contrast, some VIP perikarya in the PAG could be visualized without colchicine pretreatment. Colchicine pretreatment increased the number of immunoreactive VIP neurons at each level, but the distribution pattern of the neurons did not change.

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

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

Results

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

Sub P

Nucleus raphe dorsalis (Figs. 1 and 2). Several Sub P-containing perikarya are found within the cytoarchitectural boundaries (Taber, 1961) of the nucleus raphe dorsalis. Most are found in the lateral wings of the nucleus, at the level of the IV nerve nucleus. The densest Sub P terminal field staining is located in more dorsal regions of the nucleus and along its midline (Fig. 4C).

Caudal PAG (Fig. 1). In the caudal central gray, at the level of the dorsal tegmental nucleus of Gudden (DTN), Sub P perikarya are seen in the ventrolateral PAG. Just caudal to the IV nerve nuclei, this distribution changes; Sub P neurons are now predominantly found in the lateral PAG, adjacent to the aqueduct. Scattered Sub P-containing neurons are also located adjacent to the nucleus raphe dorsalis and in the dorsal PAG.

Sub P terminal field staining is densest in a ring around

Figure 1. Caudal PAG levels

Figures 1 to 3. Drawings of representative sections through the PAG illustrating the distribution of Sub P-containing perikarya (stars) and terminals (cross-hatching, ranging in density from 0 to 3). CS, centralis superior; Cun, nucleus cuneiformis; DTN, dorsal tegmental nucleus of Gudden; EW, Edinger-Westphal nucleus; Hb, habenulae; IP, interpeduncular nucleus; LGB, lateral geniculate; LI, linearis intermedius; LR, linearis rostralis; Mes V, mesencephalic tract of V nerve; MLF, medial longitudinal fasciculus; Post C, posterior commissure; Pulv, pulvinar; RD, raphe dorsalis; RN, red nucleus; VTN, ventral tegmental nucleus of Gudden; III, third nerve nucleus, IV, fourth nerve nucleus. Mes V, MLF, and the Hb-interpeduncular tract are indicated by diagonal hatching.

the aqueduct and in the ventrolateral (Fig. 4A) and dorsal PAG. The nucleus cuneiformis also contains terminal labeling, but no Sub P cell bodies are seen.

Mid-PAG (Fig. 2). At all mid-PAG levels, the population of Sub P neurons in the lateral PAG is still present. At the levels of the III and IV nerve nuclei (Fig. 5), this lateral cell population is still located adjacent to the aqueduct. With the appearance of the Edinger-Westphal (EW) nucleus, however, this Sub P cell cluster shifts outward, away from the aqueduct, toward the lateral perimeter of the PAG. Another prominent population of Sub P perikarya is located in the dorsal PAG. It extends dorsally, away from the aqueduct, along the midline, and then sweeps outward toward the dorsolateral PAG. Many Sub P neurons are also found in the EW nucleus (Fig. 6).

The distribution of Sub P terminal fields at mid-PAG levels is similar to that observed more caudally; dense patches of labeling are still found in the dorsal PAG and

in the dorsolateral PAG. However, the ventrolateral PAG no longer contains terminal staining. A ring of dense terminal staining around the aqueduct is also present (Fig. 4B) and is quite prominent in the lateral PAG, fanning outward from the aqueduct.

Rostral PAG (Fig. 3). The distribution of Sub P-containing neurons and terminal fields at the level of the posterior commissure is similar to that observed at mid-PAG levels. There are two principal Sub P neuronal populations: one is located in the lateral PAG (away from the aqueduct), and the other is found in the dorsal-dorsolateral PAG (Fig. 7). Sub P perikarya are also found in the cap of PAG neuropil situated dorsal to the posterior commissure. Terminal field staining is densest in the lateral PAG, adjacent to the aqueduct, and overlaps the distribution of the cell bodies in the dorsal-dorsolateral PAG.

Sub P neurons and terminals are also found in the

Figure 2. Mid-PAG levels

rostral continuation of the PAG, i.e., the periventricular gray. In some animals it seemed that the dorsal periventricular gray contained slightly more Sub P immunoreactivity than did the ventral periventricular gray.

VIP

Regardless of the rostral-caudal level examined, VIP-containing neurons are found tightly clustered in the subependymal neuropil of the ventromedial PAG (Fig. 8). The largest number of subependymal VIP perikarya are located in the mid-PAG, at the levels of the III and IV nerve nuclei (Fig. 8, B and C). Scattered VIP neurons are also found in the ventral PAG at these levels and in the raphe dorsalis. Although immunoreactive VIP-like cells are found in the rostral PAG and periventricular gray, no VIP cells were seen in the most caudal region of the PAG, at the level of the dorsal tegmental nucleus of Gudden.

Terminal field labeling, when present, was limited to the ventral and ventrolateral PAG. Staining was sparse and more often consisted of scattered beaded varicosities than well defined regions of terminal field staining. Many

VIP-labeled processes (possibly axonal or dendritic) arborize within the subependymal neuropil. Several fibers entered the ependymal layer, but they could not be followed to their termination.

Discussion

As we found previously for ENK (Moss et al., 1983), immunoreactive Sub P and VIP neuronal and terminal field staining is concentrated in several discrete regions throughout the rostral-caudal extent of the PAG. Although ENK and Sub P have similar distributions, VIP staining is completely different.

The cat PAG has been subdivided into various nuclear regions based on Nissl- and Golgi-stained material (Hamilton, 1973; Liu and Hamilton, 1980). Despite the variations in cell body shape and dendritic branching patterns exhibited by the immunoreactive Sub P- and VIP-labeled neurons, it was not possible to draw analogies to the previously described cytoarchitectural subdivisions. Nevertheless, the presence of discrete populations of Sub P-, VIP-, and ENK (Moss et al., 1983)-immunoreactive neurons in the PAG is consistent with the conclusions of

Figure 3. Rostral PAG levels

Figure 4. The distribution of Sub P terminal fields in (A) ventrolateral PAG at the level of the dorsal tegmental nucleus of Gudden ($\times 88$), (B) lateral PAG at the level of the III nerve nucleus ($\times 88$), and (C) raphe dorsalis at caudal PAG levels ($\times 63$). *Aq*, aqueduct of Sylvius; *Mes V*, mesencephalic tract of V nerve; *MLF*, medial longitudinal fasciculus.

Hamilton and co-workers that the PAG is not a homogeneous structure.

Several aspects of the staining pattern of Sub P and ENK perikarya (Moss et al., 1981, 1983) are remarkably similar. Particularly comparable are the ventral-to-dorsal shift in these peptide-containing neuronal populations from caudal to rostral PAG, and the shift away from the aqueduct, toward the perimeter of the PAG, which characterizes the lateral neuronal populations. The closest degree of overlap of Sub P- and ENK-immunoreactive neuronal populations occurs at the more rostral levels of the PAG. There are differences, however, in the staining pattern of ENK and Sub P cells. For example, although there are many ENK-containing neurons in the caudal ventrolateral PAG and the nucleus raphe dorsalis, few Sub P cells are present in these areas. In addition, at all PAG levels the individual populations of Sub P neurons

are more extensive than those of the ENK cells; that is, Sub P neuronal populations contain more cells and/or are more widely distributed.

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

Just as the distribution patterns of Sub P and ENK cell populations are similar, so the Sub P terminal field staining in the PAG follows the pattern seen for ENK terminal field labeling. This is evident at the level of the III nerve nucleus where the densest terminal field stain-

Figure 5. Sub P neurons and terminals in the lateral PAG at the level of the IV nerve nucleus ($\times 88$). Aq, aqueduct of Sylvius.

ing of Sub P immunoreactivity is located in the lateral PAG, adjacent to the aqueduct, and in the dorsal and dorsolateral PAG. At all levels, however, Sub P terminal field staining is consistently more dense around the aqueduct than is the ENK terminal staining. As described above, many immunoreactive ENK-labeled neurons are found in the caudal ventrolateral PAG and in the nucleus raphe dorsalis, whereas few Sub P cells are seen. Nevertheless, very dense Sub P and ENK terminal labeling is found in both areas.

It is of interest to note that the populations of immunoreactive Sub P- and ENK-labeled cells and terminals in the dorsal region of the mid-PAG overlap the distribution of PAG neurons that project to the nucleus raphe magnus (NRM) (Abols and Basbaum, 1981). The NRM is an important component of the descending pathway to the spinal cord dorsal horn through which activation of the PAG produces analgesia (Basbaum et al., 1976, 1977, 1978; Fields and Anderson, 1978). Whether the Sub P neurons and/or the ENK neurons in the dorsal PAG project directly to the NRM or whether their terminals locally modulate the activity of the neurons projecting to the NRM is unknown.

Unlike the extensive distribution of Sub P and ENK immunoreactivity in the PAG, immunoreactive VIP neurons are tightly clustered in the subependymal neuropil

of the ventromedial PAG. Although the cat raphe dorsalis contains numerous ENK perikarya and terminals along its midline (Moss et al., 1981, 1983), the labeling is located ventral to the VIP staining. Some VIP neurons are occasionally found in the raphe dorsalis and ventral PAG, but in much smaller numbers than either ENK or Sub P cells.

Although this is the first map of the distribution of Sub P and VIP immunoreactivity that focused on the cat PAG, previous generalized mappings of the distribution of VIP and Sub P in the rat central nervous system have included observations in the PAG. The distribution of Sub P neurons in the rat PAG resembles that of the cat (Hokfelt et al., 1977; Ljungdahl et al., 1978). In both species, Sub P cell populations are located in the lateral and ventral PAG (in and adjacent to the nucleus raphe dorsalis). However, except for the level of the posterior commissure, the large population of Sub P perikarya seen in the dorsal and dorsolateral cat PAG are not seen in the rat PAG. The functional significance of these species differences in the distribution of Sub P immunoreactivity is unknown.

Sub P terminal field labeling throughout the rat PAG is reported to be uniform (Cuello and Kanazawa, 1978), although a higher density of Sub P in the dorsal PAG has been described (Ljungdahl et al., 1978). The micro-

Figure 6. A, Sub P-containing neurons in the nucleus of Edinger-Westphal ($\times 88$). B, Absorption control ($\times 88$).

graphs of immunoreactive Sub P at more caudal rat PAG levels, however, clearly show denser staining in regions adjacent to the aqueduct. Because the action of Sub P presumably reflects the action of the peptide at a receptor, it follows that the terminal and not the cellular distribution of Sub P is a better indicator of its site of action. Thus, administration of Sub P into areas of the PAG with dense Sub P terminal labeling would have the most potent effects. Given the dense distribution of Sub P terminals around the aqueduct, it is likely that the peptide acts at this region. It follows that intraventricularly injected Sub P would have relatively easy access to Sub P receptors.

In the rat PAG, VIP-like immunoreactive cells were reported adjacent to the ventral and lateral aspects of the aqueduct; a moderately dense concentration of fibers was seen (Loren et al., 1979; Sims et al., 1980). The great majority of VIP cells in the cat PAG are also found adjacent to the aqueduct, but only in its ventral aspect. Scattered cells are also located farther ventrally, away from the aqueduct, for example, in the nucleus raphe dorsalis. The major difference between the rat and the cat in the distribution of immunoreactive VIP is the much reduced terminal field labeling in the cat. When present, it is restricted to more caudal, ventral regions of the PAG. Unlike the other peptides, immunoreactive VIP cells can be seen in the cat PAG without colchicine pretreatment, indicating that this peptide may be processed differently from ENK and Sub P in the PAG. Whether this difference contributes to the minimal staining of VIP terminal fields is unknown.

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

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

This paradox, namely that Sub P contributes both to nociception and to antinociception, is also evident if one examines the effects of Sub P at midbrain levels. In the majority of studies, intraventricular or intracerebral

Figure 7. The distribution of Sub P immunoreactivity in the dorsal PAG at the level of the posterior commissure ($\times 88$). Note the difference in size between the cells dorsal and ventral to the commissure. Aq, aqueduct of Sylvius.

(PAG) injection of Sub P in mice or rats generates a naloxone-reversible analgesia (Stewart et al., 1976; Frederickson et al., 1978; Malick and Goldstein, 1978; Naranjo et al., 1982). It has been reported, however, that intraventricular Sub P does not produce analgesia in mice (Hayes and Tyers, 1979), and it produces transient analgesia in rats only when administered at high doses (Sullivan and Pert, 1981). Apparently, Sub P's analgesic potency depends both on the dose administered (Frederickson et al., 1978) and on the initial responsiveness of the animal to a noxious stimulus (Oehme et al., 1980).

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

et al., 1978; Malick and Goldstein, 1978; Naranjo et al., 1982) indicates that, in the midbrain, Sub P may also activate opioid neurons. Thus, in both the spinal cord and midbrain, Sub P may cause the release of endogenous opiates. However, in the cord, the opiate neuron is part of a negative feedback loop, whereas in the midbrain it is part of a feedforward circuit, which generates analgesia.

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

Stimulation-produced analgesia elicited from the PAG has been reported to be both naloxone sensitive (Adams, 1976; Akil et al., 1976; Hosobuchi et al., 1977) and insensitive (Gebhart and Toleikas, 1978; Yaksh et al., 1978). Electrical stimulation in regions of the PAG containing ENK cell populations would be more likely to generate a naloxone-sensitive analgesia. On the other hand, stimulation of the PAG either in regions distant from ENK-containing neurons or in ENK terminal regions could

Figure 8. VIP neurons in the ventromedial PAG, at (A) a level just caudal to the III nerve nucleus ($\times 153$), (B) the level of the IV nerve nucleus ($\times 153$), and (C) the level of the III nerve nucleus ($\times 112$). In A, B, and C, note the arborization of VIP neuronal processes in the subependymal zone. D, Absorption control of a section adjacent to C. Arrows indicate nonspecific ependymal staining ($\times 112$).

produce analgesia that is unaffected by opiate antagonists. This would result from activation of neurons postsynaptic to the ENK-containing elements, bypassing the midbrain opiate link. However, the naloxone-insensitive analgesia that is sometimes produced by PAG stimulation may also indicate the existence of multiple endogenous analgesia systems involving the PAG; some opiate, others non-opiate in nature (see Watkins and Mayer, 1982, for review).

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

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

Also of interest is the close association of VIP cells and processes with the subependymal neuropil. Conceivably, this could underlie some type of CSF-VIP interaction in VIP-produced analgesia. In addition, although both immunoreactive Sub P and VIP have completely different patterns of distribution in the PAG, they share a common ability to act as cerebral vasodilators (Larsson et al., 1976; Duckles and Said, 1982; Edvinsson and Uddman, 1982). The possibility exists that their analgesic effects are related to a general physiologic effect on cerebral blood flow. It would be of interest to examine whether the analgesia produced by Sub P and VIP injection into the PAG is antagonized by concurrent administration of vasoconstricting drugs.

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

In conclusion, we have demonstrated that the two non-opiate peptides, which generate profound analgesia upon intracerebral microinjection, have totally different distributions within the PAG of the cat. The distribution of immunoreactive Sub P-containing neurons and terminals is quite similar to, although somewhat more extensive

than, that of immunoreactive ENK. In contrast, immunoreactive VIP cells and processes are tightly clustered in the ventromedial PAG, just ventral to the aqueduct. Since Sub P and VIP generate, respectively, a naloxone-sensitive and a naloxone-insensitive analgesia upon microinjection into the PAG, these data provide additional evidence for the existence of separate opiate- and non-opiate-mediated analgesia systems involving the midbrain central gray.

References

- Abols, I., and A. I. Basbaum (1981) Afferent connections of the rostral medulla of the cat: A neural substrate for midbrain medullary interactions in the modulation of pain. *J. Comp. Neurol.* 201: 281-297.
- Adams, J. E. (1976) Naloxone reversible analgesia produced by brain stimulation in the human. *Pain* 2: 161-166.
- Akil, H., D. J. Mayer, and J. C. Liebeskind (1976) Antagonism of stimulation produced analgesia by naloxone, a narcotic antagonist. *Science* 191: 961-962.
- Basbaum, A. I., and H. L. Fields (1978) Endogenous pain control mechanisms: Review and hypothesis. *Ann. Neurol.* 4: 451-462.
- Basbaum, A. I., and H. L. Fields (1979) The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: Further studies on the anatomy of pain modulation. *J. Comp. Neurol.* 187: 573-532.
- Basbaum, A. I., C. H. Clanton, and H. L. Fields (1976) Opiate and stimulus-produced analgesia: Functional anatomy of a medullospinal pathway. *Proc. Natl. Acad. Sci. U. S. A.* 73: 4685-4688.
- Basbaum, A. I., N. J. E., Marley, J. O'Keefe, and C. H. Clanton (1977) Reversal of morphine and stimulus-produced analgesia by subtotal spinal cord lesion. *Pain* 3: 43-56.
- Basbaum, A. I., C. H. Clanton, and H. L. Fields (1978) Three bulbospinal pathways from the rostral medulla of the cat. An autoradiographic study of pain modulating systems. *J. Comp. Neurol.* 178: 209-224.
- Berman, A. L. (1968) *The Brainstem of the Cat: A Cytoarchitectonic Atlas with Stereotaxic Coordinates*, University of Wisconsin Press, Madison.
- Cannon, J. T., G. J. Prieto, A. Lee, and J. C. Liebeskind (1982) Evidence for opioid and non-opioid forms of stimulation-produced analgesia in the rat. *Brain Res.* 243: 315-321.
- Cuello, A. C., and I. Kanazawa (1978) The distribution of substance P immunoreactive fibers in the rat central nervous system. *J. Comp. Neurol.* 178: 129-156.
- Cuello, A. C., M. del Fiocco, and G. Paxinos (1978) The central and peripheral ends of substance P-containing sensory neurons in the rat trigeminal system. *Brain Res.* 152: 499-509.
- Duckles, S. P., and S. I. Said (1982) Vasoactive intestinal peptide as a neurotransmitter in the cerebral circulation. *Eur. J. Pharmacol.* 78: 371-374.
- Edvinsson, L., and R. Uddman (1982) Immunohistochemical localization and dilatory effects of substance P in human cerebral vessels. *Brain Res.* 232: 466-471.
- Erichsen, J. T., A. Reiner, and H. J. Karten (1982) Co-occurrence of substance P-like and leu-enkephalin-like immunoreactivity in neurons and fibers of avian nervous system. *Nature* 295: 407-410.
- Fields, H. L., and S. D. Anderson (1978) Evidence that raphe-spinal neurons mediate opiate and midbrain stimulation produced analgesia. *Pain* 5: 333-349.
- Fields, H. L., A. I. Basbaum, C. H. Clanton, and S. D. Anderson (1977) Nucleus raphe magnus inhibition of spinal cord dorsal horn neurons. *Brain Res.* 126: 441-453.
- Frederickson, R. C. A., V. Burgis, C. E. Harrell, and J. D.

- Edwards (1978) Dual actions of substance P on nociception: Possible role of endogenous opioids. *Science* 199: 1359-1362.
- Gebhart, C. F., and J. R. Toleikas (1978) An evaluation of stimulation-produced analgesia in the cat. *Exp. Neurol.* 62: 570-579.
- Gilbert, R. F. T., P. C. Emson, S. P. Hunt, G. W. Bennett, C. A. Marsden, B. E. B. Sandberg, H. W. M. Steinbusch, and A. A. J. Verhofstad (1982) The effects of monoamine neurotoxins on peptides in the rat spinal cord. *Neuroscience* 7: 69-87.
- Glazer, E. J., and A. I. Basbaum (1981) Immunohistochemical localization of leucine-enkephalin in the spinal cord of the cat: Enkephalin-containing marginal neurons and pain modulation. *J. Comp. Neurol.* 196: 377-389.
- Hamilton, B. (1973) Cytoarchitectural subdivisions of the periaqueductal gray matter in the cat. *J. Comp. Neurol.* 149: 1-28.
- Hayes, A. G., and M. G. Tyers (1979) Effects of intrathecal and intracerebroventricular injections of substance P on nociception in the rat and mouse. *Br. J. Pharmacol.* 66: 488P.
- Henry, J. L. (1976) Effects of substance P on functionally identified units in cat spinal cord. *Brain Res.* 114: 439-451.
- Hokfelt, T., R. Elde, O. Johansson, R. Luft, G. Nilsson, and A. Arimura (1976) Immunohistochemical evidence for separate populations of somatostatin-containing and substance P-containing primary afferent neurons in rat. *Neuroscience* 1: 131-136.
- Hokfelt, T., A. Ljungdahl, L. Terenius, R. Elde, and G. Nilsson (1977) Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: Enkephalin and substance P. *Proc. Natl. Acad. Sci. U. S. A.* 74: 3081-3085.
- Hokfelt, T., A. Ljungdahl, H. Steinbusch, A. Verhofstad, G. Nilsson, E. Brodin, B. Pernow, and M. Goldstein (1978) Immunohistochemical evidence of substance P-like immunoreactivity in some 5-hydroxytryptamine containing neurons in the rat central nervous system. *Neuroscience* 3: 517-538.
- Hosobuchi, Y., J. E. Adams, and R. Linchitz (1977) Pain relief by electrical stimulation of the central grey matter in humans and its reversal by naxolone. *Science* 197: 183-186.
- Jessell, T. M., and L. L. Iversen (1977) Opiate analgesics inhibit substance P release from rat trigeminal nucleus. *Nature* 268: 549-551.
- Johnson, J. H., A. R. Swajkowski, and L. Anderhub (1982) Stimulation of the mesencephalic central grey increases blood prolactin in ovariectomized rats. *Exp. Neurol.* 74: 419-429.
- Jurgens, U., and R. Pratt (1979) Role of the periaqueductal grey in vocal expression of emotion. *Brain Res.* 167: 367-378.
- Lakoski, J. M., and G. F. Gebhart (1982) Morphine administration in the amygdala or periaqueductal grey depresses serum levels of luteinizing hormone. *Brain Res.* 232: 231-237.
- Larsson, L. I., L. Edvinsson, J. Fahrenkrug, R. Hakanson, C. H. Owman, O. Schaffalitzky de Muckadell, and F. Sundler (1976) Immunohistochemical localization of a vasodilatory polypeptide (VIP) in cerebrovascular nerves. *Brain Res.* 113: 400-404.
- Lewis, V. A., and C. F. Gebhart (1977) Evaluation of the periaqueductal central gray (PAG) as a morphine specific locus of action and examination of morphine-induced and stimulation-produced analgesia at coincident PAG loci. *Brain Res.* 124: 283-303.
- Liu, R., and B. Hamilton (1980) Neurons of the periaqueductal gray matter as revealed by Golgi study. *J. Comp. Neurol.* 189: 403-418.
- Ljungdahl, A., T. Hokfelt, and G. Nilsson (1978) Distribution of substance P-like immunoreactivity in the central nervous system of the rat. I. Cell bodies and nerve terminals. *Neuroscience* 3: 861-943.
- Loren, I., P. C. Emson, J. Fahrenkrug, A. Bjorklund, J. Alumets, R. Hakanson, and F. Sundler (1979) Distribution of vasoactive intestinal polypeptide in the rat and mouse brain. *Neuroscience* 4: 1953-1976.
- Malick, J. B., and J. M. Goldstein (1978) Analgesic activity of substance P following intracerebral administration in rats. *Life Sci.* 23: 835-844.
- Marley, P. D., P. C. Emsen, S. P. Hunt, and J. Fahrenkrug (1981) A long ascending projection in the rat brain containing vasoactive intestinal polypeptide. *Neurosci. Lett.* 27: 261-266.
- Mayer, D. J., and D. D. Price (1976) Central nervous system mechanisms of analgesia. *Pain* 2: 379-404.
- Mehler, W. R., and W. J. H. Nauta (1958) *A Stereotaxic Atlas of the Brainstem of the Cat*, Walter Reed Army Medical Center, Washington, D. C.
- Mohrland, J. S., and G. F. Gebhart (1979) Substance P-induced analgesia in the rat. *Brain Res.* 171: 556-559.
- Moss, M. S., and A. I. Basbaum (1982) The peptidergic organization of the periaqueductal grey of the cat: Substance P and VIP. *Soc. Neurosci. Abstr.* 8: 767.
- Moss, M. S., E. J. Glazer, and A. I. Basbaum (1980) Enkephalin neurons in the raphe dorsalis and periaqueductal grey of the cat: A comparison with substance P. *Anat. Rec.* 196: 131A.
- Moss, M. S., E. J. Glazer, and A. I. Basbaum (1981) Enkephalin immunoreactive perikarya in the nucleus raphe dorsalis of the cat. *Neurosci. Lett.* 21: 33-37.
- Moss, M. S., E. J. Glazer, and A. I. Basbaum (1983) The peptidergic organization of the cat periaqueductal grey. I. The distribution of immunoreactive enkephalin-containing neurons and terminals. *J. Neurosci.* 3: 603-616.
- Mudge, A. W., S. E. Leeman, and G. D. F. Fischbach (1979) Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. *Proc. Natl. Acad. Sci. U. S. A.* 76: 526-530.
- Naranjo, J. R., F. Sanchez-Franco, J. Garzon, and J. del Rio (1982) Analgesic activity of substance P in rats: Apparent mediation by met-enkephalin release. *Life Sci.* 30: 441-446.
- Oehme, P., H. Hilse, E. Morgenstein, and E. Gores (1980) Substance P: Does it produce analgesia or hyperalgesia? *Science* 208: 305-307.
- Oliveras, J. L., F. Redjemi, G. Guilbaud, and J. M. Besson (1975) Analgesia induced by electrical stimulation of the inferior central nucleus of the raphe in the cat. *Pain* 1: 139-145.
- Oliveras, J. L., G. Guilbaud, and J. M. Besson (1979) A map of serotonergic structures involved in stimulation producing analgesia in unrestrained, freely moving cats. *Brain Res.* 164: 317-322.
- Pearson, J., L. Brandeis, and A. C. Cuello (1982) Depletion of substance P-containing axons in substantia gelatinosa of patients with diminished pain sensitivity. *Nature* 295: 61-63.
- Pelletier, G., H. W. M. Steinbusch, and A. A. Verhofstad (1981) Immunoreactive substance P and serotonin present in same dense core vesicles. *Nature* 293: 71-72.
- Rivot, J. P., A. Chaouch, and J. M. Besson (1980) Nucleus raphe magnus modulation of response of rat dorsal horn neurons to unmyelinated fiber inputs: Partial involvement of serotonergic pathways. *J. Neurophysiol.* 44: 1039-1057.
- Sakuma, Y., and D. W. Pfaff (1980) LH-RH in the mesencephalic central grey can potentiate lordosis reflex of female rats. *Nature* 282: 566-567.
- Seybold, V. S., L. L. K. Hylden, and G. L. Wilcox (1982) Intrathecal substance P and somatostatin in rats: Behaviors indicative of sensation. *Peptides* 3: 49-54.
- Sims, K. B., D. L. Hoffman, S. I. Said, and E. A. Zimmermann (1980) Vasoactive intestinal polypeptide (VIP) in mouse and rat brain: An immunocytochemical study. *Brain Res.* 186: 165-183.
- Skultety, M. (1963) Stimulation of the periaqueductal grey and hypothalamus. *Arch. Neurol.* 8: 608-620.
- Sternberger, L. (1974) *Immunocytochemistry*, Ed. 2, John Wiley

- & Sons, Inc., New York.
- Stewart, J. M., C. J. Getto, K. Neldner, E. B. Reeve, W. Krivoy, and E. Zimmermann (1976) Substance P and analgesia. *Nature* 262: 784-785.
- Straus, W. (1976) Use of peroxidase inhibitors for immunoperoxidase procedures. In *Immunoenzymatic Techniques*, P. Pruet, J. Bignar, and S. Avrameas, eds., pp. 117-124, Elsevier-North Holland Publishing Co., Amsterdam.
- Sullivan, T. L., and A. Pert (1981) Analgesic activity of non-opiate neuropeptides following injections into the rat periaqueductal grey matter. *Soc. Neurosci. Abstr.* 7: 504.
- Taber, E. (1961) The cytoarchitecture of the brainstem of the cat. I. Brainstem nuclei of the cat. *J. Comp. Neurol.* 116: 27-69.
- Watkins, L. R., and D. J. Mayer (1982) Organization of endogenous opiate and non-opiate pain control systems. *Science* 216: 1185-1192.
- Yaksh, T. L., J. C. Yeung, and T. A. Rudy (1978) An inability to antagonize with naloxone the elevated nociceptive thresholds resulting from electrical stimulation of the mesencephalic central gray. *Life Sci.* 18: 1193-1198.
- Yaksh, T. L., T. M. Jessell, R. Gamse, A. W. Mudge, and S. E. Leeman (1980) Intrathecal morphine inhibits substance P release from mammalian spinal cord *in vivo*. *Nature* 286: 155-156.
- Yeung, J. C., T. L. Yaksh, and T. A. Rudy (1977) Concurrent mapping of brain sites for sensitivity to the direct application of morphine and focal electrical stimulation in the production of antinociception in the rat. *Pain* 4: 23-40.
- Zorman, G., G. Belcher, J. E. Adams, and H. L. Fields (1982) Lumbar intrathecal naloxone blocks analgesia produced by microstimulation of the ventromedial medulla in the rat. *Brain Res.* 236: 77-84.