

# Opioid peptide enkephalin: Immunohistochemical mapping in rat central nervous system

(opiate receptor/substantia gelatinosa/amygdala/endorphin)

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**ABSTRACT** Using specific antisera to methionine-enkephalin and leucine-enkephalin, we have visualized apparent enkephalin-containing neuronal fibers and terminals throughout the central nervous system of the rat. Immunoreactive enkephalin displays sharply defined localizations. Regions of highest immunofluorescent density include the laminae I and II of the spinal cord, the substantia gelatinosa of the caudal nucleus of nerve V, the vagal nuclei of the medulla, the periventricular and periaqueductal areas of the upper medulla and midbrain, dorsomedial thalamic regions, specific hypothalamic nuclei, the basal ganglia, particularly the globus pallidus and the central nucleus of the amygdala, and the lateral septum. In certain regions enkephalin immunofluorescence corresponds closely with the distribution of autoradiographic opiate receptor grains.

The opiate-like pentapeptides methionine-enkephalin (Met-enk) and leucine-enkephalin (Leu-enk) (1, 2) appear to be endogenous ligands for the opiate receptor. The regional localization of enkephalin in mammalian brain, determined biochemically, resembles that of opiate receptor binding (3-6). In subcellular fractionation studies, enkephalin is localized to synaptosomal fractions that contain nerve terminals (7). Autoradiographic studies of the opiate receptor reveal sharply defined localizations to structures mediating functions affected by opiates, such as pain perception (8-10). If the enkephalins are neurotransmitters or neuromodulators associated with opiate receptors, one might expect enkephalin to be localized microscopically to neuronal systems impinging on opiate receptors. Preliminary immunohistochemical studies show immunoreactive enkephalin fluorescence in nerve fibers and terminals with highest densities in areas enriched in opiate receptors (11). We now report a detailed mapping of the rat central nervous system for immunoreactive enkephalin.

## MATERIALS AND METHODS

**Antisera Preparation.** Met-enk or Leu-enk (20 mg) was coupled to keyhole limpet hemocyanin (10 mg) by incubation for 30 min in distilled water at room temperature with 150 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The material was dialyzed extensively against distilled water, lyophilized, suspended in 3 ml of distilled water, and stored. Guinea pigs were immunized with 1 mg of the hemocyanin-coupled enkephalin diluted 1:10 in saline and mixed 1:1 with Freund's complete adjuvant. Rabbits were injected with 1.6 mg of this conjugate. The immunization was repeated three to four times at 3 to 4 week intervals using incomplete Freund's adjuvant. The guinea pigs and rabbits were bled 7-10 days after the third and subsequent immunizations and the sera were tested for enkephalin binding in radioimmunoassays (ref. 12;

Simantov, Childers, and Snyder, unpublished data). Enkephalin binding by these antisera was not displaced by high concentrations of substance P, glucagon, insulin, neurotensin, angiotensin II, acetylcholine, norepinephrine, dopamine,  $\gamma$ -aminobutyric acid, 3':5'-cyclic AMP, or 3':5' cyclic GMP (12). Enkephalins were more than 200 times as potent as  $\alpha$ -endorphin and  $\beta$ -endorphin in displacing enkephalin binding to guinea pig or rabbit antisera against enkephalin. Met-enk was 6-8 times more potent than Leu-enk in displacing [ $^3$ H]Met-enk binding to guinea pig and rabbit antisera against Met-enk. Leu-enk was 8-10 times and 1000 times more potent than Met-enk in displacing [ $^3$ H]Leu-enk binding to guinea pig and rabbit antisera against Leu-enk, respectively. However, all antibodies could bind both enkephalins.

**Immunofluorescence.** Sprague-Dawley rats (150-200 g) were perfused through the aorta for 20 min with cold 4% depolymerized paraformaldehyde in 0.05 M sodium phosphate buffer, pH 7.3; the brains were dissected into four blocks, postfixed for 3 hr, and then stored at 4° in 0.24 M sucrose/0.05 M sodium phosphate buffer, pH 7.3. Sections of 12-16  $\mu$ m were cut at -10° with a Harris cryostat and stored at -20° for convenience until stained. No change in patterns of specific fluorescence has been noted after storage for up to 6 weeks. For staining, the sections were incubated for 30 min at 37° with 1:10-1:100 dilutions of rabbit or guinea pig antisera to Met-enk or Leu-enk diluted in 0.05 M phosphate-buffered saline, pH 7.4, containing 0.1-0.2% Triton X-100. The sections were washed three times (5 min each) with the same phosphate buffer containing 0.05% Triton X-100 and then incubated for 15 min at 37° with fluorescein-conjugated goat antibody against guinea pig IgG or fluorescein-conjugated goat antibody against rabbit IgG (IgG fraction, Cappel Laboratories) diluted 1:50 with phosphate buffer containing 0.05-0.1% Triton X-100. The sections were then washed three times (5 min each) in phosphate buffer containing 0.2% Triton X-100, dipped in H<sub>2</sub>O, mounted with 0.05 M sodium bicarbonate buffer, pH 8.4, diluted 1:1 with glycerol, and examined with a Zeiss Universal fluorescence microscope.

## RESULTS

Enkephalin immunoreactivity varies markedly among different structures in the central nervous system. Regional variations parallel variations in opiate receptor density determined either by autoradiography (Figs. 1-4) (8-10) or biochemical assays (13-15) and also parallel the regional distribution of endogenous enkephalin measured either by radioimmunoassay (12) or radio-receptor assay (3-6). Control experiments utilizing preimmune serum or serum previously incubated with Met-enk or Leu-enk (Fig. 1) show negligible fluorescence.

Throughout the rat central nervous system enkephalin-like immunofluorescence is localized to fiber-like structures and

Abbreviations: Met-enk, methionine-enkephalin; Leu-enk, leucine-enkephalin.

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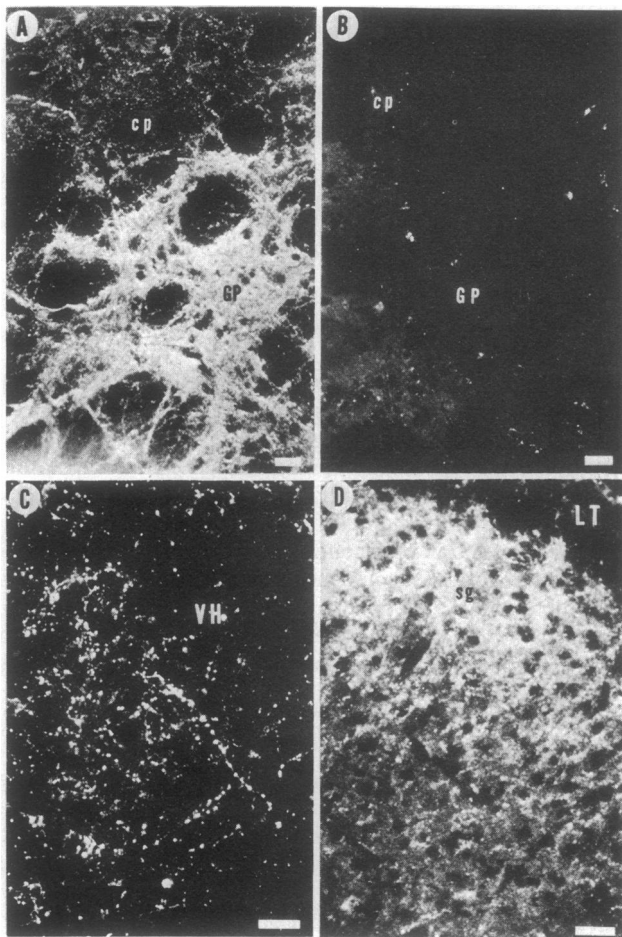


FIG. 1. Immunofluorescence micrographs of (A and B) the globus pallidus (GP) and nucleus caudatus-putamen (cp) and of the cervical spinal cord showing (C) the ventral horn (VH) and (D) Lissauer's Tract (LT) and substantia gelatinosa (sg). Micrographs A and B were taken from serial sections, but the primary serum used for staining in B was previously adsorbed overnight with 1 mM Leu-enk to establish a control. All sections were stained with rabbit antiserum against Leu-enk. Bars = 25  $\mu$ m.

small swellings that resemble biogenic amine-containing varicosities. This suggests a localization to fibers and nerve terminals. In some areas, such as the cerebral cortex, certain hypothalamic nuclei, lateral reticular formation, globus pallidus, and spinal cord, fluorescence is localized in nonnuclear portions of neuronal perikarya. Immunofluorescent patterns appear similar whether rabbit or guinea pig antisera to Met-enk or Leu-enk are utilized.

In the upper cervical spinal cord, a dense band of dot and fiberlike fluorescence is confined to laminae I and II (Figs. 1D and 3), resembling opiate receptor localization. Fluorescent fibers occur in the dorsal white matter adjacent to the lateral borders of the dorsal gray matter, within the ventral gray matter (Fig. 1C), and surrounding the central canal.

In the lower medulla, a dense immunofluorescence occurs in the substantia gelatinosa of spinal nerve V and the nucleus commissuralis; opiate receptor distribution shows a similar pattern (Fig. 3). A network of fibers also occurs in the reticular formation (Fig. 3), especially laterally.

At a more anterior portion of the medulla, corresponding to the level of the area postrema, immunofluorescent terminals and fibers are highly concentrated in the nucleus of the solitary tract, in the nucleus originis dorsalis of the vagus, and in the nucleus of nerve XII (Fig. 3). The nucleus ambiguus has a fairly

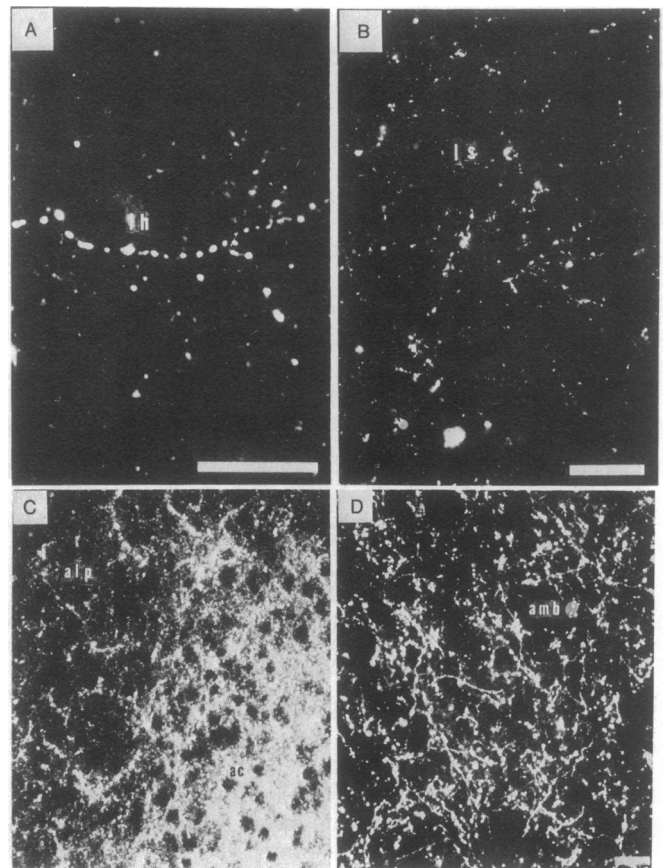


FIG. 2. Immunofluorescence micrographs of (A) the lateral hypothalamus (lh), (B) the lateral septum (ls), (C) the central (ac) and lateral posterior (alp) amygdaloid nuclei, and (D) nuclei ambiguus (amb). All sections were stained with rabbit antiserum against Leu-enk except for C, where guinea pig antiserum was used. Bars = 25  $\mu$ m.

dense reticular network of fibers and terminals (Fig. 2D). Less dense fibers occur ventrally to the hypoglossal nucleus, while a higher density system passes laterally from the nucleus of the solitary tract (Fig. 3).

In the brainstem at the level of the locus coeruleus, immunofluorescent terminals and fibers are most highly concentrated in the floor of the fourth ventricle, with a somewhat lesser density within and surrounding the locus coeruleus itself (Fig. 4). Just anterior to the locus coeruleus, immunofluorescence is most highly localized to the parabrachial nuclei and the floor of the fourth ventricle (Fig. 5).

In the lower midbrain, fluorescence is most pronounced in the gray matter surrounding the cerebral aqueduct with some lateral extensions into the tegmentum of the midbrain. Moderate fluorescence is also noted in a narrow band near the dorsal portion of the inferior colliculi and overlying the dorsal and median raphé nuclei (Fig. 5).

Within the thalamus, immunofluorescence is highest in medial dorsal areas, especially those surrounding the ventricles, while lateral thalamic regions are almost devoid of fluorescence. The midline nuclei of the thalamus have intense immunofluorescence, especially the periventricular nucleus rotundocellularis. At more anterior levels of the thalamus, fluorescence is high in the midline structures, notably the nucleus parataenialis and the medullary laminae of the thalamus, especially the internal laminae (Fig. 5). In contrast to the dense fluorescence of the dorsal and medial areas of the thalamus, most ventral and lateral regions exhibit sparse fluorescence. The zona

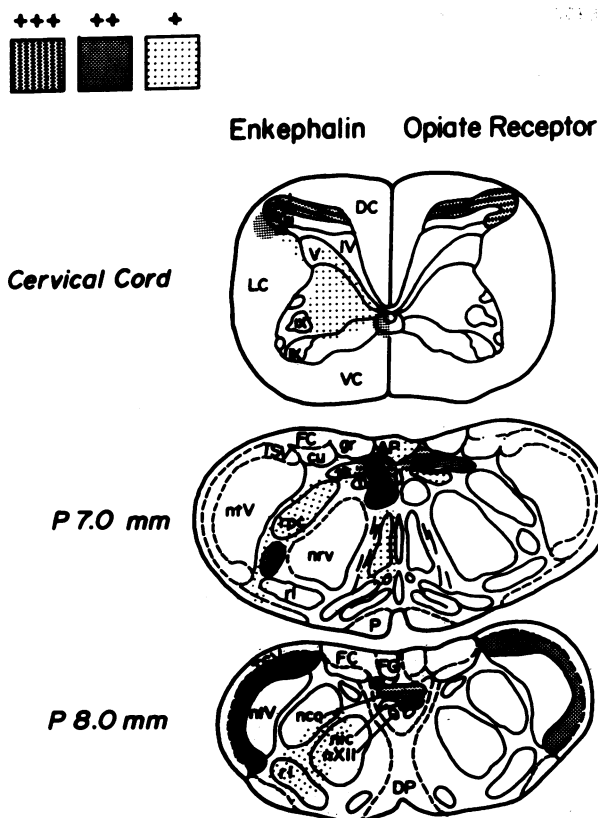


FIG. 3. Distribution of the opiate receptor and enkephalin in the cervical cord and lower medulla. Abbreviations: amb, nucleus ambiguus; AP, area postrema; cu, nucleus cuneatus; DC, dorsal column; DP, decussatio pyramidis; FC, fasciculus cuneatus; FG, fasciculus gracilis; gr, nucleus gracilis; io, nucleus olivaris inferior; LC, lateral column; nco, nucleus commissuralis; nic, nucleus intercalatus; nrv, nucleus reticularis medullae oblongatae pars ventralis; nts, nucleus tractus solitarius; ntV, nucleus tractus spinalis nervi trigemini; nX, nucleus originis dorsalis vagi; nXII, nucleus originis nervi hypoglossi; P, tractus corticospinalis; rl, nucleus reticularis lateralis; rpc, nucleus reticularis parvocellularis; sgV, substantia gelatinosa trigemini; ts, tractus solitarius; TSV, tractus spinalis nervi trigemini; VC, ventral column. Drawings and coordinates of lower medullary regions are after ref. 27. Cervical cord drawing is from ref. 28, and laminae are according to ref. 29. Opiate receptor densities are from ref. 9.

incerta displays substantial immunofluorescence (Fig. 5), while a network of fluorescent terminals covers the lateral edge of the medial habenula.

Several hypothalamic structures, especially periventricular and ventral portions, are rich in enkephalin immunofluorescence, while certain nuclei fail to display any fluorescence. The periventricular nucleus and a dense band along the ventral floor of the hypothalamus are highly fluorescent, while the supraoptic nucleus, mammillary bodies, arcuate nucleus, and suprachiasmatic nuclei display much sparser immunofluorescence. The only white matter of the hypothalamus displaying substantial fluorescence is the infundibulum; the fornix, optic tract, and optic chiasm lack immunofluorescence (Fig. 5).

Immunofluorescence in the amygdala is uneven. At all levels, the central nucleus of the amygdala displays dense immunofluorescence while the corticomедial and basolateral areas of the amygdala have lower levels (Figs. 2C and 5). Moderate to low fluorescence occurs lateral to the central amygdala in an area just ventral to the caudate-putamen, possibly in continuity with the immunofluorescent fibers of the ventral caudate-putamen.

Considerable variations in immunofluorescence occur within

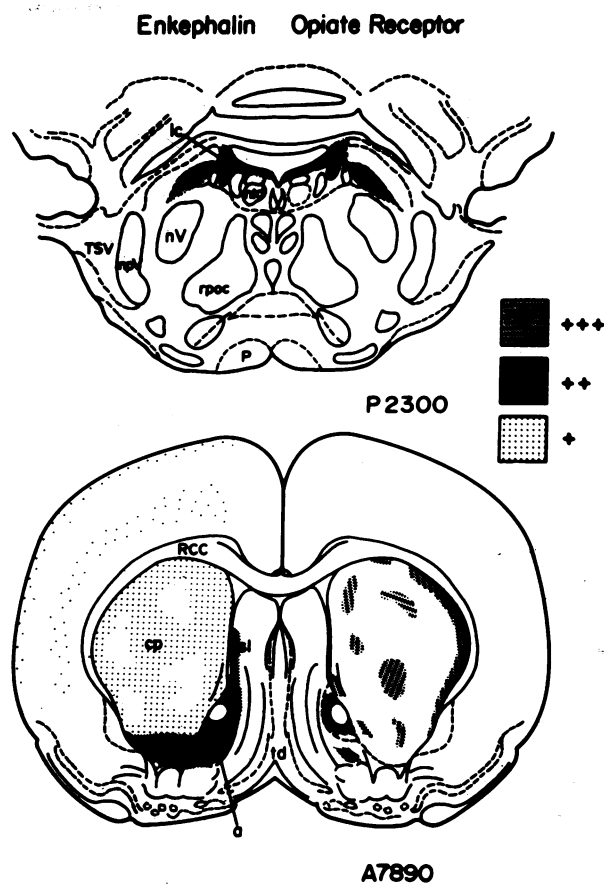


FIG. 4. Distribution of opiate receptors and enkephalin. Stereotaxic levels are after ref. 30. Opiate receptor distribution is from refs. 8 and 31. Abbreviations: a, nucleus accumbens; cp, nucleus caudatus-putamen; lc, locus coeruleus; npV, nucleus principalis nervi trigemini; ntd, nucleus tegmenti dorsalis Gudden; nV, nucleus originis nervi trigemini; P, tractus corticospinalis; RCC, radiatio corporis callosi; rDPC, nucleus reticularis pontis caudalis; sl, nucleus septi lateralis; td, nucleus tractus diagonalis (Broca); TSV, tractus spinalis nervi trigemini.

the basal ganglia. The globus pallidus is the most intensely fluorescent structure observed in the brain (Figs. 1 and 5). Within the globus pallidus, fluorescent fibers and terminals occur in a dense, reticular network surrounding the penetrating nonfluorescent fiber bundles (Fig. 1A). Within the caudate-putamen, a somewhat patchy immunofluorescence is largely confined to dorsal and ventral areas with relatively sparse fluorescence in the remainder of the caudate-putamen (Figs. 4 and 5). The nucleus accumbens and the interstitial nucleus of the stria terminalis show moderately dense networks of fibers, which are densest in regions adjacent to the anterior commissure. Densely fluorescent fibers and terminals are observed within the lateral septum (Figs. 2B and 4). A collection of fluorescent fibers appears to proceed ventrally from the lateral septum, possibly along the striohypothalamic tract. A limited amount of fluorescence is observed in the cerebral cortex, most marked in deeper layers, while hippocampal and cerebellar fluorescence is extremely sparse.

## DISCUSSION

In many regions of the central nervous system, enkephalin immunofluorescence and autoradiographic opiate receptor grains are intimately associated. Examples include the dorsal laminae of the spinal cord and brainstem, the nucleus of the

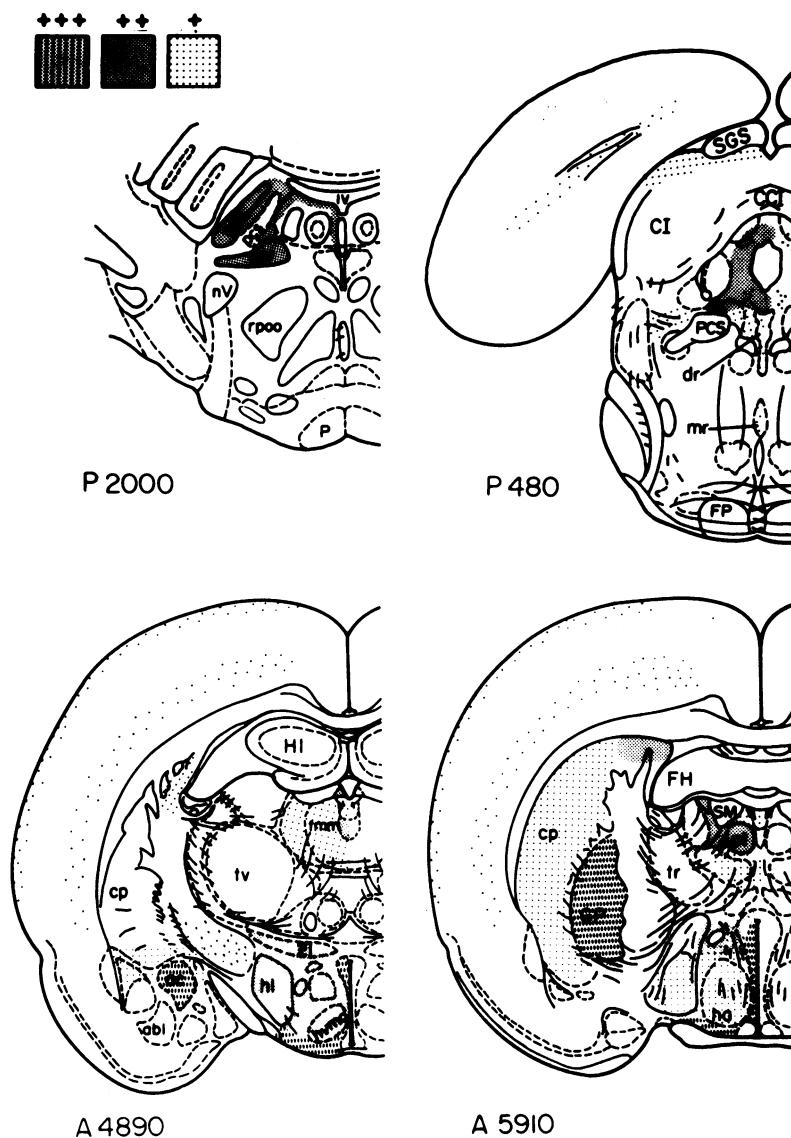


FIG. 5. Distribution of enkephalin. Stereotaxic levels are from ref. 30. Abbreviations: abl, nucleus amygdaloideus basalis, pars lateralis; ac, nucleus amygdaloideus centralis; cp, nucleus caudatus-putamen; dr, nucleus dorsalis raphés; ha, nucleus anterior (hypothalami); hl, nucleus lateralis (hypothalami); hvma, nucleus ventromedialis (hypothalami), pars anterior; mr, nucleus medianus raphés; npd, nucleus parabrachialis dorsalis; npv, nucleus parabrachialis ventralis; nV, nucleus originis nervi trigemini; pt, nucleus paratenialis; rpooc, nucleus reticularis pontis caudalis; tmm, nucleus medialis thalami, pars medialis; tr, nucleus reticularis thalami; tv, nucleus ventralis thalami; CCI, commissura colliculorum inferiorum; CI, colliculus inferior; FH, fimbria hippocampi; FP, fibrae pyramidales; GP, globus pallidus; HI, hippocampus; IV, nervus trochlearis; P, tractus corticospinalis; PCS, pedunculus cerebellaris superior; SGS, stratum griseum superficiale colliculi superioris; SM, stria medullaris thalami; ZI, zona incerta.

solitary tract, nucleus commissuralis, parabrachial nuclei, habenula, and globus pallidus (Figs. 3 and 4). There are discrepancies in the correlation between opiate receptor and enkephalin localizations. (a) Though the caudate-putamen displays high levels of opiate receptor binding in biochemical (14, 15) and autoradiographic (9) experiments as well as substantial endogenous levels of enkephalin in both radioreceptor assays (5, 6) and radioimmunoassay (12), immunofluorescence is not dense in the caudate-putamen. (b) Opiate receptor grains occur in patches throughout the substance of the caudate-putamen, but immunoreactive enkephalin tends to be localized mainly to dorsal and ventral regions. (c) The cerebral cortex contains substantial levels of opiate receptor binding (14, 15), demonstrates specific electrophysiological responses to opiates and enkephalin (16-18), and possesses moderate levels of enkephalin by radio-receptor and radioimmunoassay (5, 6, 12). However, we have detected relatively sparse enkephalin fluorescence

within the cerebral cortex. (d) In the spinal cord, the ventral gray matter and an area surrounding the central canal display fluorescent enkephalin fibers but no detectable opiate receptor autoradiographic grains. (e) In the amygdala, immunofluorescence is most highly concentrated in the central nucleus, whereas autoradiographic receptor grains are more evenly distributed throughout the amygdala.

These discrepancies between densities of enkephalin-like immunofluorescence and of opiate receptors may be rationalized in several ways. Brain areas where receptor density is disproportionately higher than fluorescence density might contain concentrations of "presynaptic" opiate receptors. These receptors could be localized to axons of neurons whose cells or dendrites receive enkephalin input (9, 19, 20). Alternatively, our technique may fail to visualize enkephalin within certain axons. Brain areas where fluorescence is higher than receptor density could conceivably contain concentrations of axons of

"enkephalinergic" neurons that pass through these areas and synapse on opiate receptors elsewhere.

Similar to the distribution of opiate receptors (8-10), the distribution of immunoreactive enkephalin corresponds to regions that mediate functions that are influenced by opiates. Enkephalin in laminae I and II of the spinal cord and the intralaminar nuclei of the thalamus may relate to integration of pain perception. Enkephalin within vagal nuclei of the medulla might play a role in visceral reflexes affected by opiates, such as the cough and vomiting reflexes. Within the periaqueductal gray, enkephalin may mediate electrical stimulation-induced analgesia (21-23). Enkephalin in the infundibulum and periventricular nuclei of the hypothalamus may reflect loci where opiates influence endocrine functions. Within certain limbic and cortical areas, especially the central nucleus of the amygdala, enkephalin might be associated with the euphoric effects of opiates.

The distribution of enkephalin immunofluorescence that we have observed is similar to that noted by Elde *et al.* (11). Though we have detected immunofluorescence in all regions where Elde *et al.* (11) reported fluorescence, we have also observed networks of fibers and terminals not reported by Elde *et al.* (11), such as the locus coeruleus and the floor of the fourth ventricle, where we observed dense staining.

The enkephalin antisera we have used display little cross-reactivity with larger opioid peptides,  $\alpha$ -endorphin and  $\beta$ -endorphin (12). Since radioimmunoassay has shown that rat brain levels of  $\alpha$ -endorphin and  $\beta$ -endorphin are lower than those of the enkephalins (J. Rossier, R. Guillemin, and F. Bloom, personal communication), it is unlikely that the immunofluorescence observed in the present study is attributable to these endorphins. These larger opioid peptides occur in very high concentrations in the pituitary (24-26).

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