

Combined Autoradiographic- Immunocytochemical Analysis of Opioid Receptors and Opioid Peptide Neuronal Systems in Brain

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LEWIS, M. E., H. KHACHATURIAN AND S. J. WATSON. *Combined autoradiographic-immunocytochemical analysis of opioid receptors and opioid peptide neuronal systems in brain.* PEPTIDES 6: Suppl. 1, 37-47, 1985.—Using adjacent section autoradiography-immunocytochemistry, the distribution of [³H]naloxone binding sites was studied in relation to neuronal systems containing [Leu]enkephalin, dynorphin A, or β -endorphin immunoreactivity in rat brain. Brain sections from formaldehyde-perfused rats show robust specific binding of [³H]naloxone, the pharmacological (μ -like) properties of which appear unaltered. In contrast, specific binding of the δ ligand [³H]D-Ala², D-Leu⁵-enkephalin was virtually totally eliminated as a result of formaldehyde perfusion. Using adjacent section analysis, we have noted associations between [³H]naloxone binding sites and one, two, or all three opioid systems in different brain regions; however, in some areas, no apparent relationship could be observed. Within regions, the relationship was complex; for example, in caudate-putamen, patches of opioid receptors did not correspond to the distribution of enkephalin immunoreactivity, but there was a correspondence between subcallosal streaks of binding sites and enkephalin. The complexity of the association between [³H]naloxone binding sites and the multiple opioid systems, and previous reports of colocalization of μ and κ receptors in rat brain, are inconsistent with a simple-one-to-one relationship between a given opioid precursor and opioid receptor subtype. Instead, since differential processing of the three precursors gives rise to peptides of varying receptor subtype potencies and selectivities, the multiple peptide-receptor relationships may point to a key role of post-translational processing in determining the physiological consequences of opioid neurotransmission.

Opioid receptor autoradiography Enkephalin Dynorphin β -Endorphin

THE pharmacological and biochemical evidence for multiple subtypes of opioid receptors has reached overwhelming proportions [5, 17, 19], but the physiological significance of this multiplicity remains obscure. One simple and elegant possibility is that the receptor subtypes serve to discriminate between the multiple forms of endogenous opioid peptides. Thus, the multiple receptors could be differentially coupled to neural systems containing one of the three opioid peptide precursors: (1) proopiomelanocortin [32, 47, 48], which gives rise to β -endorphin and its NH₂-terminally acetylated and/or COOH-terminally shortened forms; (2) proenkephalin [7, 13, 38], which contains four copies of [Met]enkephalin and single copies of [Leu]enkephalin, [Met]enkephalin-Arg⁶-Phe⁷ and [Met]enkephalin-Arg⁶-Gly⁷-Leu⁸, and gives rise to longer enkephalin-containing peptides, such as BAM-12P, BAM-22P, and Peptides E and F; (3) prodynorphin [20], which gives rise to three peptides containing the [Leu]enkephalin sequence: α - β -neo-endorphin, dynorphin A, and dynorphin B. Since these peptides all share the NH₂-terminus tetrapeptide sequence (Tyr-Gly-Gly-Phe) coding for opioid receptor activation, the COOH-terminus sequence may code for which receptor subtype is to be occupied. Since *in vitro* studies have shown the pentapeptide

enkephalins to preferentially bind the δ subtype [31], while prodynorphin-related peptides have usually been reported to exhibit selectivity for the κ subtype [16, 8, 64; but see [44]], it might be predicted that enkephalin- and dynorphin-containing neurons would be associated, respectively, with δ and κ opioid receptor subtypes. However, since β -endorphin binds equally well to the μ and δ subtypes *in vitro* [31], but not to the κ subtype [46], an association of proopiomelanocortin neurons with both μ and δ sites would be predicted.

Direct testing of these predictions, using presently available techniques, requires that all three classes of opioid neurons be discriminated immunocytochemically in anatomical relation to autoradiographically detected opioid receptor subtypes. Since enkephalin and dynorphin neurons, in particular, are anatomically contiguous or relatively nearby in many brain regions [21,58], it is critical that the antisera used discriminate between these neurons and that the receptors are localized in close relationship to the immunoreactive neuronal structures. We have recently reported that adjacent sections from formaldehyde-perfused rat brain can be processed for immunocytochemical localization of [Leu]enkephalin and autoradiographic detec-

tion of [^3H]naloxone-labeled opioid receptors [27]. This finding was confirmed for rhesus monkey brain [28], and an anatomical relationship was shown between [^3H]naloxone binding sites and all three opioid systems in different brain regions. We now present findings showing that the pharmacological properties of [^3H]naloxone binding sites in rat brain are unchanged by formaldehyde perfusion, and describe the anatomical relationship of these sites to neuronal systems immunoreactive for molecules derived from the three opioid precursors.

METHOD

Adult male Sprague-Dawley rats were anesthetized with sodium pentobarbital and given stereotaxic microinjections of colchicine (150–300 $\mu\text{g}/10\ \mu\text{l}$ 0.9% saline) into the lateral ventricle, or left untreated. Forty-eight hours later, the rats were anesthetized with sodium pentobarbital (50 mg/ml, intraperitoneally), packed in ice, and the cardiovascular system was flushed with 50 ml of ice-cold 0.9% saline through the left ventricle, with the right atrium cut open. The ventricles were excised and a cannula was inserted into the aorta, through which the animal was perfused with ice-cold 0.1 M phosphate-buffered 4% formaldehyde (pH 7.4, prepared from depolymerized paraformaldehyde) at 140 mm Hg for 30 minutes. The brains were removed, blocked in coronal or parasagittal planes, and incubated overnight at 4°C in 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 15% sucrose. The brain pieces were frozen by immersion in -40°C isopentane for 30–40 seconds, embedded in powdered dry ice, and stored at -70°C . Sections were cut at 20 μm thickness in a cryostat at -20°C and thaw-mounted onto subbed slides which were stored at -70°C until use. Sections were similarly cut from the brains of unperfused rats, but were vacuum-dried at 4°C for 2 hours to facilitate their adhesion to the slides [16].

For peroxidase-antiperoxidase immunocytochemistry [25], frozen tissue sections were allowed to air-dry at room temperature and were then incubated at 37°C with normal goat serum (NGS, from GIBCO) at a dilution of 1/30 for 5 to 10 minutes, followed by primary rabbit antiserum (i.e., anti- β -endorphin, anti-[Leu]enkephalin, and anti-dynorphin A sera), diluted with 0.02 M PBS containing 0.3% Triton X-100, for 1 hour at 37°C and overnight (approximately 24 hours) at 4°C. Possible cross-reactivity of each antiserum was tested by incubating control sections with antiserum preadsorbed with 1–20 μM concentrations of the peptide used as antigen as well as with peptides having related amino acid sequences (see [57] for antiserum specificities). The following day, the sections were washed in three changes of PBS (10 minutes each) and incubated with NGS for 10 minutes at 37°C, followed by goat-antirabbit serum (Sternberger-Meyer) at 1/100 dilution for 30 minutes at 37°C and then overnight at 4°C. The next day, the sections were washed in PBS and incubated with NGS for 10 minutes at 37°C, and then with rabbit antihorseradish peroxidase serum at 1/200 dilution for 40 minutes at 37°C. The sections were then washed in PBS and incubated with 4 $\mu\text{g}/\text{ml}$ horseradish peroxidase (Sigma, Type VI) for 40 minutes at 37°C. The histochemical reaction for peroxidase was carried out by incubating the sections in 200 ml of PBS containing 0.03% H_2O_2 and 0.125 mg/ml diaminobenzidine (Sigma) for 15 minutes at room temperature, with constant stirring. The sections were then washed in distilled water, briefly osmicated (2% OsO_4 , 1–2 seconds), rewashed, dehydrated

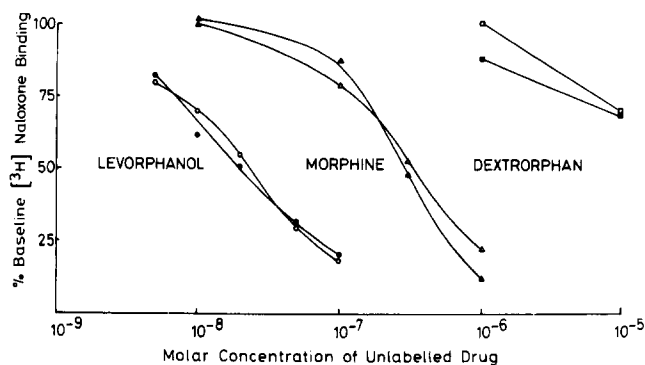


FIG. 1. Effect of formaldehyde perfusion on the pharmacological characteristics of specific [^3H]naloxone binding in striatal sections. Filled symbols: sections from unperfused rat brains. Open symbols: sections from 4% formaldehyde-perfused rat brains.

through ascending concentrations of ethanol and xylenes, and coverslipped in Permount.

Sections adjacent to those processed for immunocytochemistry, or sections from unperfused rat brain, were processed for opioid receptor autoradiography using the method of Herkenham and Pert [14,16]. The slide-mounted sections were incubated in (a) 0.05 M Tris·HCl (pH 7.4) containing 1 nM [^3H]naloxone (40 Ci/mmol; New England Nuclear) and 100 mM NaCl for 2 hours at 4°C, or (b) 0.05 M Tris·HCl (pH 7.4) containing 1 nM [^3H]D-Ala²-D-Leu⁵-enkephalin (40 Ci/mmol; Amersham), with or without 3 mM Mn (OAc)₂, 100 mM NaCl and 2 μM guanosine triphosphate, for 30 minutes at 25°. These incubation conditions are similar to those used to label apparent μ and δ opioid receptors, respectively, in previous studies of rhesus monkey [29] and rat [30] cerebral cortex, and also rat striatum [4, 15, 36]. To evaluate nonspecific binding, some slides were incubated in the the above media which also included 1 μM levorphanol. The pharmacological properties of the binding sites were evaluated by carrying out displacement studies with different unlabeled opiates, followed by liquid scintillation counting of the sections. The incubations were terminated by transferring the slides through five rinses (20 seconds each, in 200 ml at 4°C) of 0.1 M PBS (pH 7.4) for the [^3H]naloxone-incubated slides or 0.05 M Tris·HCl (pH 7.4) for the [^3H]D-Ala²-D-Leu⁵-enkephalin-incubated slides, and then drying the sections under a stream of cold air.

RESULTS

As reported before [27], brain sections from formaldehyde-perfused rats exhibited high specific binding (85% of total binding) of [^3H]naloxone. When striatal-level brain sections from formaldehyde-perfused and unperfused rats were labelled with [^3H]naloxone, specific binding was inhibited equivalently in both tissues by levorphanol, and less potently by morphine, while dextrophan was ineffective (Fig. 1). Despite some loss of [^3H]naloxone binding sites [27], the pharmacological properties of the remaining sites do not appear to have been dramatically altered. However, virtually all specific binding of [^3H]D-Ala²-D-Leu⁵-enkephalin was eliminated in the perfused tissue, preventing an adjacent section analysis of δ sites in relation to the opioid immunoreactive systems.

Anatomically, a large number of brain regions contain opioid peptides and [^3H]naloxone binding sites. By analyzing

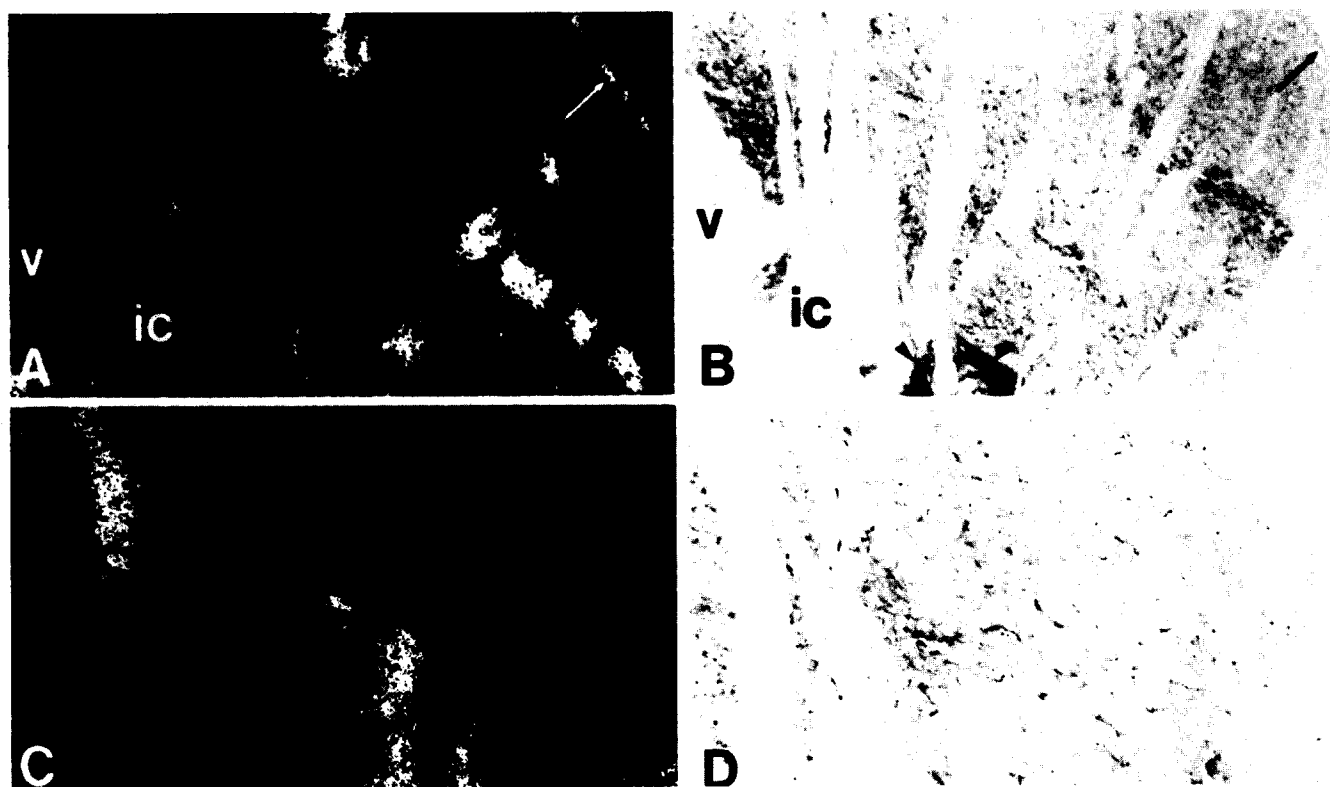


FIG. 2. Caudate-putamen. Patches of $[^3\text{H}]$ naloxone binding sites are shown in A and in higher magnification in C. Corresponding adjacent sections B and D depict $[\text{Leu}]$ enkephalin immunoreactivity. Arrows in A and B indicate the subcallosal streak; arrowheads in B demarcate terminal immunoreactivity in the globus pallidus. v: ventricle; ic: internal capsule. Mag. A, B: $64\times$; C, D: $160\times$.

adjacent brain sections, we have noted many associations between the distribution of these binding sites and one, two or all three opioid peptides. In contrast, we have also noted a distinct lack of such associations in other brain regions. In the following paragraphs, we will present some of our observations on the distributions of β -endorphin, $[\text{Leu}]$ enkephalin, and dynorphin A immunoreactive neurons and $[^3\text{H}]$ naloxone binding sites at several levels of the rat brain and spinal cord.

Telencephalon

In the cerebral cortex, $[^3\text{H}]$ naloxone binding sites show striking regional differences in laminar distribution, although most areas have concentrations of sites in the most superficial part of layer I and in layer VI, with an intermediate band of sites having a region-specific laminar localization (see [30] for a detailed, quantitative analysis). The prelimbic and cingulate cortex exhibits a distinctive trilaminar pattern of $[^3\text{H}]$ naloxone binding sites in layers I, III, and VI. $[\text{Leu}]$ enkephalin immunoreactive perikarya are present in the submolecular laminae, including layer VI, which contains a particularly high density of $[^3\text{H}]$ naloxone binding sites. Motor and sensory cortical areas generally exhibit a lower density of sites and correspondingly fewer enkephalin immunoreactive perikarya, which are present in layers II–III and to a lesser extent in layers V–VI (see [24,33]). In piriform cortex, $[^3\text{H}]$ naloxone binding sites are concentrated in layer III at the transition area to claustrum, while enkephalin immunoreactive perikarya are mostly present in layer II and may give rise to medially coursing immunoreac-

tive fibers. A similar relationship of enkephalin perikarya and $[^3\text{H}]$ naloxone binding sites is seen in entorhinal cortex. Dynorphin immunoreactive perikarya were observed mainly in layer II of entorhinal cortex, although immunoreactive fibers were often detected traversing layers I–III in most areas of cerebral cortex.

A very dense distribution of $[^3\text{H}]$ naloxone sites has been noted in the striatal nuclear complex. These are for the most part in the form of large patches in the caudate-putamen, as well as a distinct subcallosal streak within the latter complex (Fig. 2). Also within caudate-putamen, there exist both enkephalinergic and dynorphinergic neurons which project to the globus pallidus and possibly elsewhere in the brain, notably the substantia nigra. $[\text{Leu}]$ enkephalin-containing processes form distinct, yet small immunoreactive patches distributed in ventromedial regions of the caudate-putamen, adjacent to the globus pallidus. Additionally, a distinct subcallosal streak of $[\text{Leu}]$ enkephalin immunoreactivity is seen in this nuclear complex. Upon analyzing adjacent sections processed for $[^3\text{H}]$ naloxone autoradiography and $[\text{Leu}]$ enkephalin immunoreactivity, no correspondence was noted between the large receptor-rich patches and the relatively small enkephalin-rich patches (Fig. 2). In fact, in some areas a negative correlation was noted between the distribution of binding sites and peptide. By contrast, the subcallosal streak of binding sites corresponds precisely to that of $[\text{Leu}]$ enkephalin immunoreactivity in the same regions (Fig. 3). The globus pallidus, an area of particularly rich $[\text{Leu}]$ enkephalin and dynorphin A terminal immunoreactivity, is virtually devoid of $[^3\text{H}]$ naloxone binding sites.

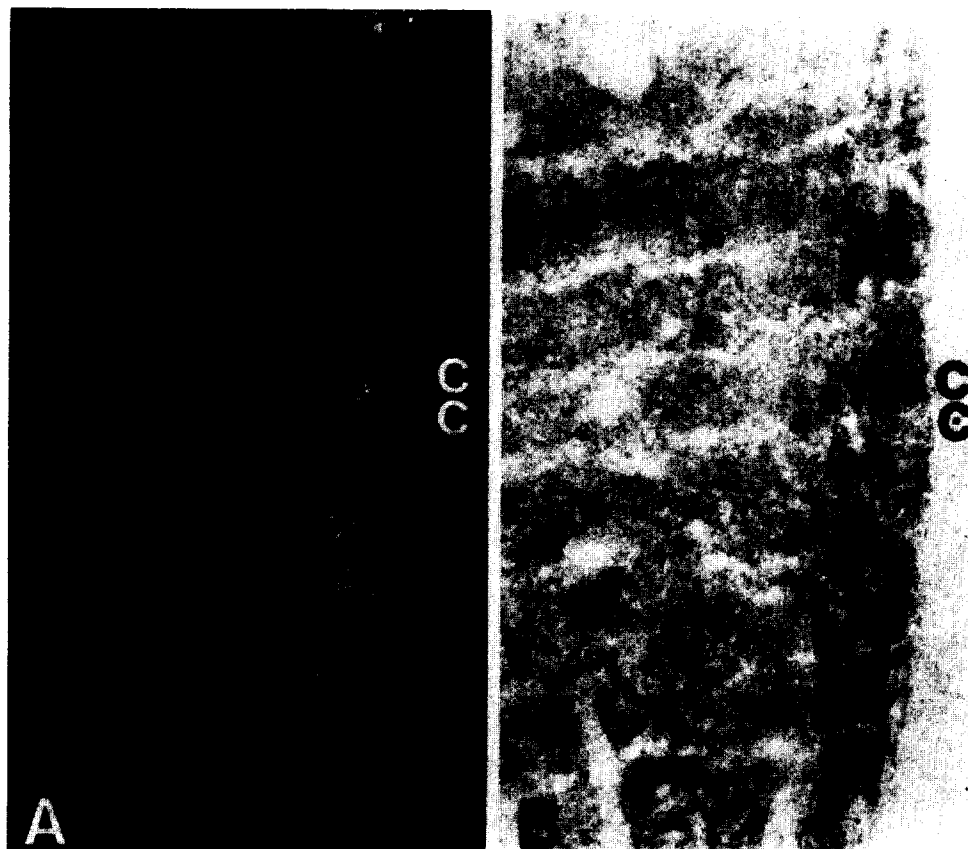


FIG. 3. Caudate-putamen. These adjacent sections illustrate [^3H]naloxone binding (A) and [Leu]enkephalin immunoreactivity (B). Note the correspondence between the thick streak of binding sites and immunoreactivity in the subcallosal streak. cc: corpus callosum. Mag.: 200 \times .

Elsewhere in the telencephalon, the central nucleus of amygdala is particularly rich in all three opioid peptides. Both [Leu]enkephalin and dynorphin A neuronal perikarya and processes are seen in this nucleus, and a moderately dense distribution of β -endorphin immunoreactive processes also innervate the central nucleus. In adjacent sections, the latter nucleus also appears to be rich in [^3H]naloxone binding sites, corresponding well with the distribution of the opioid peptide immunoreactivities.

In the hippocampal formation, [Leu]enkephalin and dynorphin A immunoreactivity was seen in the mossy fiber projections from the dentate gyrus to the hippocampus proper. Interestingly, the dentate gyrus granule cell layer and the pyramidal cell layer of hippocampus contain a relatively high density of [^3H]naloxone binding sites. Since the mossy fibers innervate the pyramidal cells, the existence of opiate binding sites on these cells correlates well with the opioid nature of the mossy fibers.

Diencephalon

Compared to the striatum, thalamus, and inferior colliculus, the hypothalamus is not an area particularly enriched in [^3H]naloxone binding sites. However, moderate levels of [^3H]naloxone binding are noted in the medial basal hypothalamus of the rat, concentrated especially in the median eminence. Various hypothalamic nuclei contain neuronal

perikarya which produce β -endorphin, [Leu]enkephalin, or dynorphin A. In fact, opioid peptide-producing neurons are concentrated heavily in the hypothalamus. It is important to note that these opiateergic hypothalamic neurons project either to the median eminence or to extrahypothalamic brain regions, while only a few hypothalamic nuclei contain moderately dense innervation patterns. These include the dorsomedial and periventricular nuclei which contain β -endorphin projections from the arcuate perikarya, and ventromedial nucleus which contains [Leu]enkephalin and dynorphin A perikarya as well as terminals. None of these nuclei are particularly rich in [^3H]naloxone binding sites. However, the median eminence, as mentioned, contains moderate levels of these sites. Immunoreactive processes of all three opioid peptides are also seen in the median eminence surrounding the portal capillaries. Therefore, the existence of [^3H]naloxone sites in this region corresponds well with the known neuroendocrine functions of the opioid peptides.

In the thalamus, the distribution of [^3H]naloxone binding sites is particularly dense in specific nuclear regions. Although in general the distribution of opioid peptides is not particularly heavy in thalamic nuclei, some striking correlations can be noted. A distinct bundle of immunoreactive β -endorphin fibers traverses and perhaps innervates the periventricular thalamic nucleus, which also contains moderate levels of immunoreactive [Leu]enkephalin terminals.

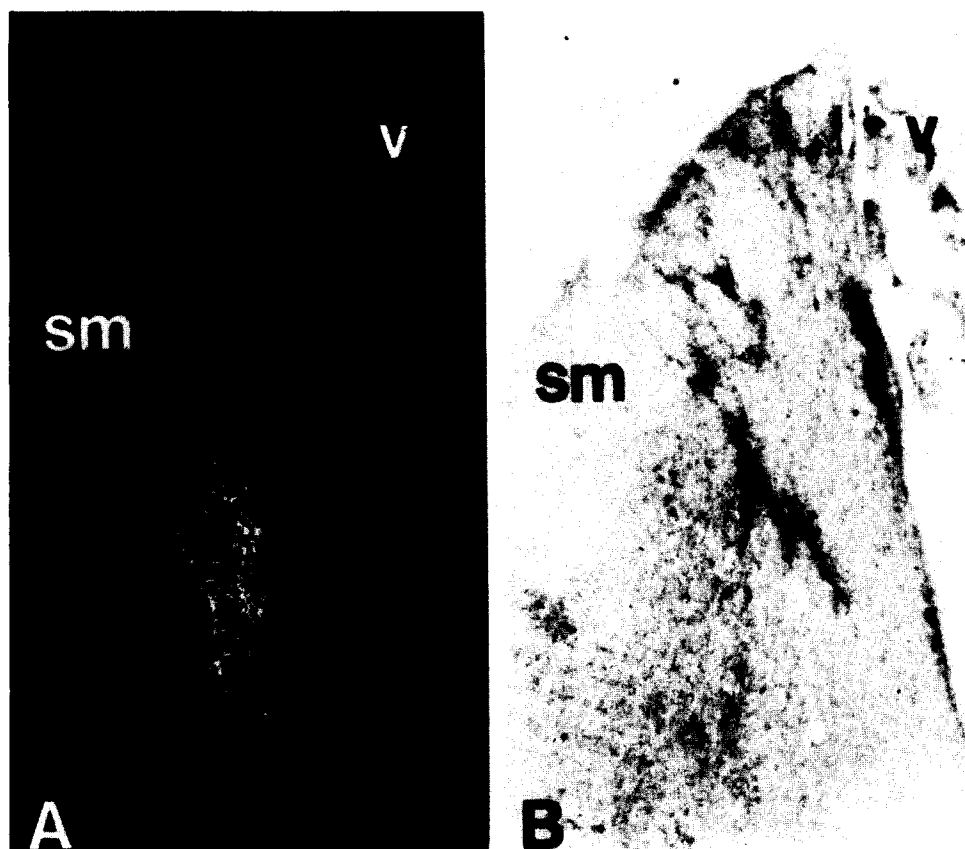


FIG. 4. Medial habenula. A and B are adjacent sections depicting $[^3\text{H}]$ naloxone binding and $[\text{Leu}]$ enkephalin immunoreactivity, respectively. Dashed lines in A outline the tissue boundaries corresponding to B. sm: stria medullaris; v: ventricle. Mag.: 200 \times .

Likewise, $[^3\text{H}]$ naloxone site density in this nucleus is pronounced and corresponds well with peptide distribution in adjacent sections. Other thalamic regions which contain both binding sites and $[\text{Leu}]$ enkephalin fibers in good correspondence include the anterodorsal and central medial nuclei. Other areas of dense binding site distribution do not contain β -endorphin or dynorphin A, but are sparsely populated by $[\text{Leu}]$ enkephalin fibers. In the habenula there is an excellent correlation between the distribution of enkephalin immunoreactive fibers and $[^3\text{H}]$ naloxone binding sites (Fig. 4).

Mesencephalon

Several areas of the mesencephalon contain a dense distribution of $[^3\text{H}]$ naloxone binding sites. Some of these areas are also rich in opioid peptide immunoreactivity. Ventrally, the substantia nigra pars compacta is rich in binding sites, but poor in opioid peptide content. Only sparse $[\text{Leu}]$ enkephalin immunoreactivity is seen in the pars compacta. In contrast, the pars reticulata of substantia nigra, which contains a low density of $[^3\text{H}]$ naloxone sites, is particularly rich in dynorphin A immunoreactivity.

The interpeduncular nuclear complex is an area of exceptional correspondence between the distribution of $[^3\text{H}]$ naloxone binding sites and $[\text{Leu}]$ enkephalin immunoreactive perikarya and fibers (Fig. 5). In adjacent sections, almost identical, dense patterns of distribution of bind-

ing sites and immunoreactive fibers and terminals are seen in several, but not all, subdivisions of this nucleus. Generally, this correspondence is best in the rostral and central subnuclei (see Fig. 5). Interestingly, the interpeduncular nucleus is devoid of β -endorphin and dynorphin A immunoreactivity.

More dorsally, a moderately dense distribution of $[^3\text{H}]$ naloxone sites is noted in the periaqueductal gray (Fig. 6). This distribution is uniform and upon serial section analysis corresponds well with the distribution of β -endorphin fibers and terminals ventrally, but not dorsally. Likewise, immunoreactive $[\text{Leu}]$ enkephalin and dynorphin A neurons also exist in ventral periaqueductal gray as well as laterally in the mesencephalic reticular formation. Immunoreactive fibers and terminals of $[\text{Leu}]$ enkephalin and dynorphin A neurons are distributed somewhat more uniformly throughout the periaqueductal gray, but also tend to be more concentrated ventrally.

Within the colliculi, a laminated pattern of $[^3\text{H}]$ naloxone binding sites is noted in the superior colliculus with the most superficial layer being particularly rich in sites. The inferior colliculus contains a uniformly dense distribution of binding sites. Immunoreactive fibers belonging to all three opioid peptide systems are also seen in both the superior and inferior colliculi, with the latter receiving a relatively denser innervation by β -endorphin and $[\text{Leu}]$ enkephalin fibers and terminals. However, the laminations in binding site distribution in the superior colliculus do not correspond to the pat-

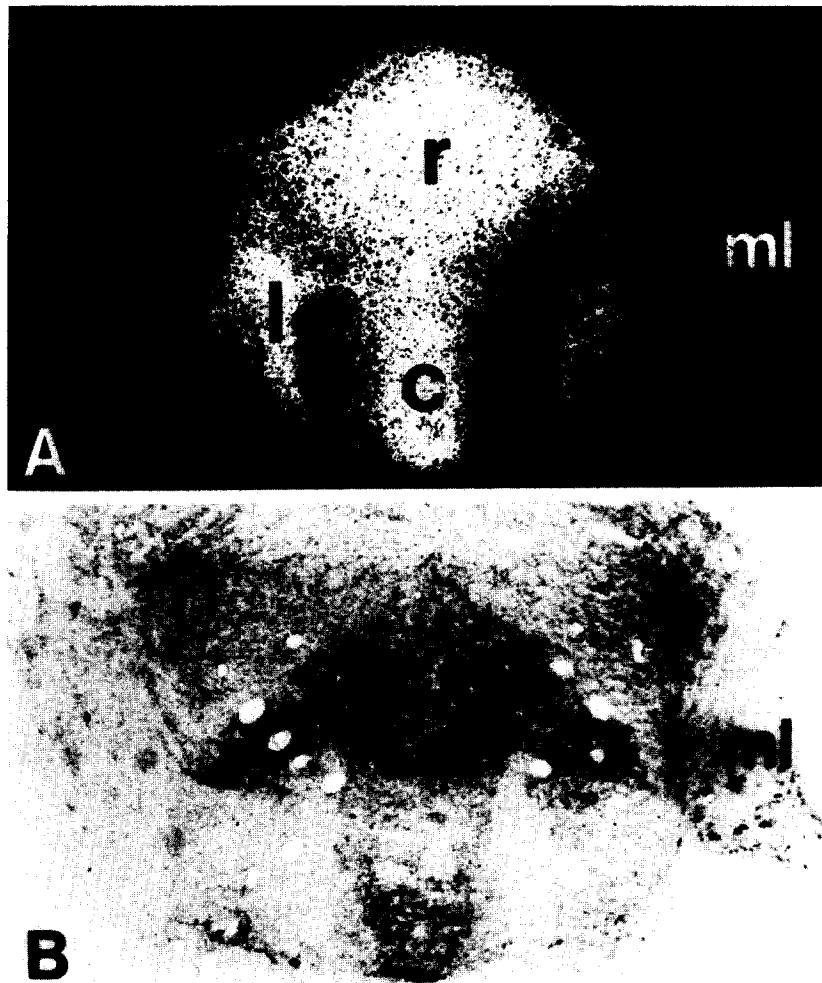


FIG. 5. Interpeduncular nuclear complex. Binding sites for [^3H]naloxone are shown in A, while immunoreactive [^3H]enkephalin perikarya and fibers in an adjacent section are shown in B. ml: medial lemniscus. Mag.: $80\times$. Note the correspondence between immunoreactivity and binding sites in the rostral (r) and central (c) subnuclei, but the relative abundance of sites and paucity of immunoreactivity in the lateral (l) subnucleus.

tern of immunoreactivity seen with any of the opioid peptides.

Pons and Medulla

The densest areas of [^3H]naloxone binding site distribution in the pons occur in the dorsal tegmental regions. The parabrachial nucleus is rich in binding sites as is the nucleus locus coeruleus. The parabrachial nuclei also exhibit a dense pattern of β -endorphin fiber immunoreactivity. Furthermore, both [^3H]enkephalin and dynorphin A containing neuronal perikarya and processes are also seen in the parabrachial nuclei, which upon serial section analysis appear to form separate neuronal populations. Interestingly, [^3H]naloxone binding site density in this nucleus is uniform and corresponds well with the distribution of all three opioid peptides (Fig. 7). The locus coeruleus is also very rich in [^3H]naloxone sites. Yet this nucleus contains only a light density of β -endorphin and [^3H]enkephalin immunoreactivity.

In the medulla oblongata, the nucleus tractus solitarius

shows a dense distribution of [^3H]naloxone binding sites. All three opioid peptides are also seen in this nucleus in both neuronal perikarya and fibers. The correspondence between the distribution of [^3H]enkephalin immunoreactivity and binding site distribution is particularly well outlined in the more caudal areas of this nucleus (Fig. 8). Another medullary area of particularly good correlation between the distributions of binding sites and [^3H]enkephalin is the nucleus of the spinal tract of the trigeminal nerve (Fig. 8). Upon serial section analysis, both peptide immunoreactivity and binding sites are concentrated in the outermost laminae of this nucleus which in actuality are the rostral continuation of the spinal cord dorsal horn laminae I and II. These laminae also exhibit dense [^3H]enkephalin and [^3H]naloxone binding site distribution.

DISCUSSION

We have described anatomical relationships between multiple opioid immunoreactive neural systems and [^3H]naloxone binding sites in adjacent sections from

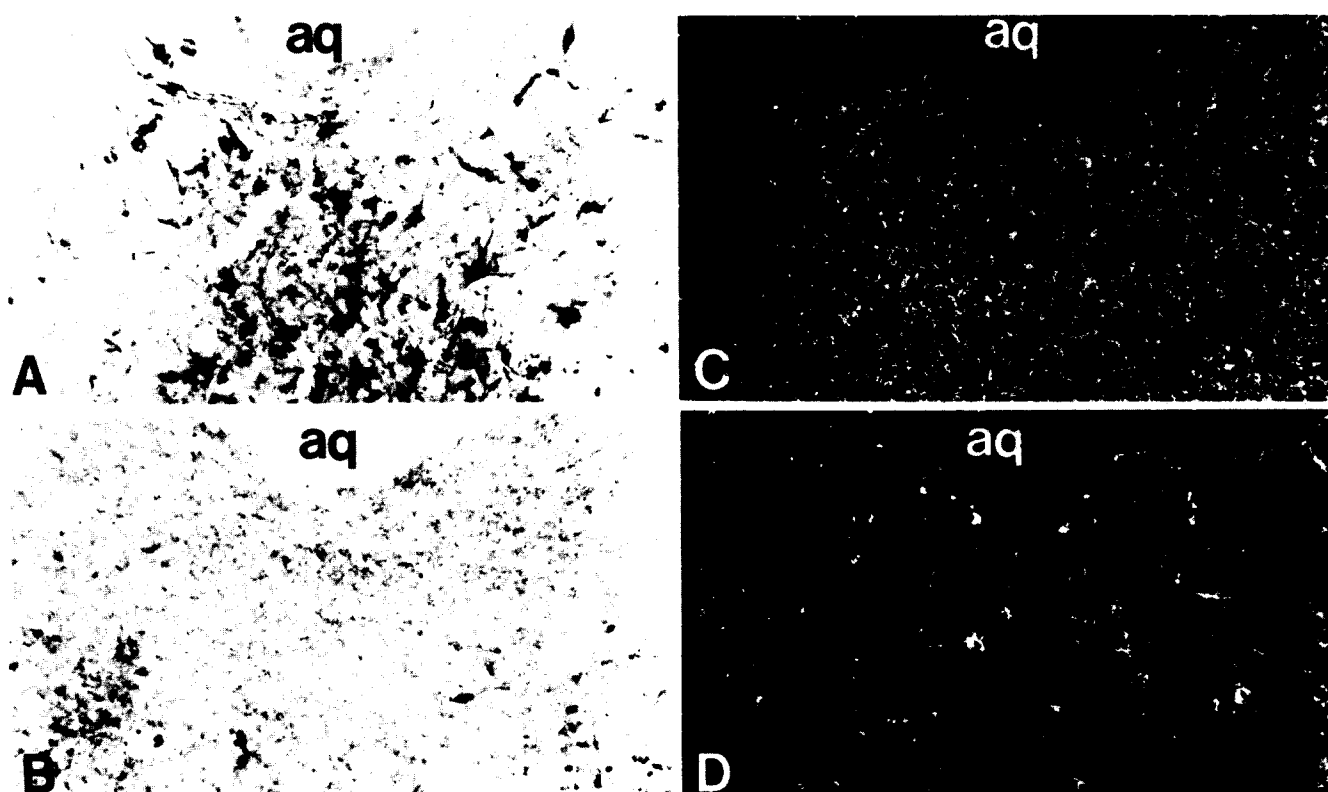


FIG. 6. Periaqueductal gray. This series of four adjacent sections demonstrates the relationship between serotonergic dorsal raphe neurons (A), [Leu]enkephalin immunoreactive perikarya and fibers (B), [^3H]naloxone binding sites (C) and β -endorphin immunoreactive fibers (D). aq: aqueduct. Mag.: 160 \times .

formaldehyde-perfused rat brain. The pharmacological properties of these binding sites were evidently unchanged by perfusion, as indicated by unaltered displacement potencies of several compounds. The pharmacological relevance of the binding sites is indicated by the excellent correlation between the pharmacological efficacy of a series of opiate compounds and their potency in displacing [^3H]naloxone binding from brain tissue sections [4,16]. The identity of these binding sites as μ rather than δ or κ in rat brain has been indicated by appropriate structure-activity studies [4, 16, 42].

The survival of [^3H]naloxone binding sites following formaldehyde perfusion is in contrast to the virtually total loss of [^3H]D-Ala²,D-Leu⁵-enkephalin binding. The differential sensitivities to formaldehyde of the binding of these two ligands may reflect a difference between the chemical properties of the μ and δ - opioid receptors, as suggested by Hiller *et al.* [18], who reported differential inhibition of μ and δ ligand binding in the presence of alcohols. Alternatively, it is possible, if μ and δ sites occur as interconverting forms of a single receptor [4], that formaldehyde has "fixed" opioid receptors into their μ conformation. We cannot discriminate these possibilities at present, but suggest that formaldehyde may prove to be a useful reagent for investigators who wish to explore the physicochemical properties of opioid receptors.

The survival of [^3H]naloxone binding following formaldehyde perfusion has permitted us to carry out receptor autoradiographic and opioid peptide immunocytochemical studies on adjacent sections from the same brain. While it is possible to obtain general information about the anatomical

relationships between endogenous ligands and their receptors by comparing tissue sections from brains processed differentially for immunocytochemistry and autoradiography [52,54], such comparisons are intrinsically difficult due to complexities in receptor distribution, and, in the case of the opioid systems, the anatomical proximity of proenkephalin and prodynorphin neurons. Also, while the use of nonselective opioid ligands, such as [^3H]diprenorphine [1-3, 52] and [^3H]D-Ala²,Met-enkephalinamide [54] may be useful in providing a global picture of opioid receptor distribution, the use of more selective ligands should aid in determining relationships between different receptor subtypes and multiple opioid systems. In this aim, it is especially critical to use antisera which clearly discriminate opioid systems containing different precursors, since these precursors give rise to opioids of varying receptor subtype selectivities (see Introduction). The use of antisera which crossreact with peptides from two different opioid precursors (e.g., [54]) can confuse the issue of the differential localization of neurons containing these precursors (see [21]) as well as the issue of the relationship of these neurons to different opioid receptors. The present study utilized [^3H]naloxone, a relatively μ -selective ligand in rat brain, and [Leu]enkephalin, dynorphin A, and β -endorphin antisera which were previously shown to be non-crossreactive with peptides from the other precursors [21-25, 57, 58].

Using adjacent section analysis, we have noted associations between [^3H]naloxone binding sites and one, two or all three opioid systems in many brain regions; in others, no

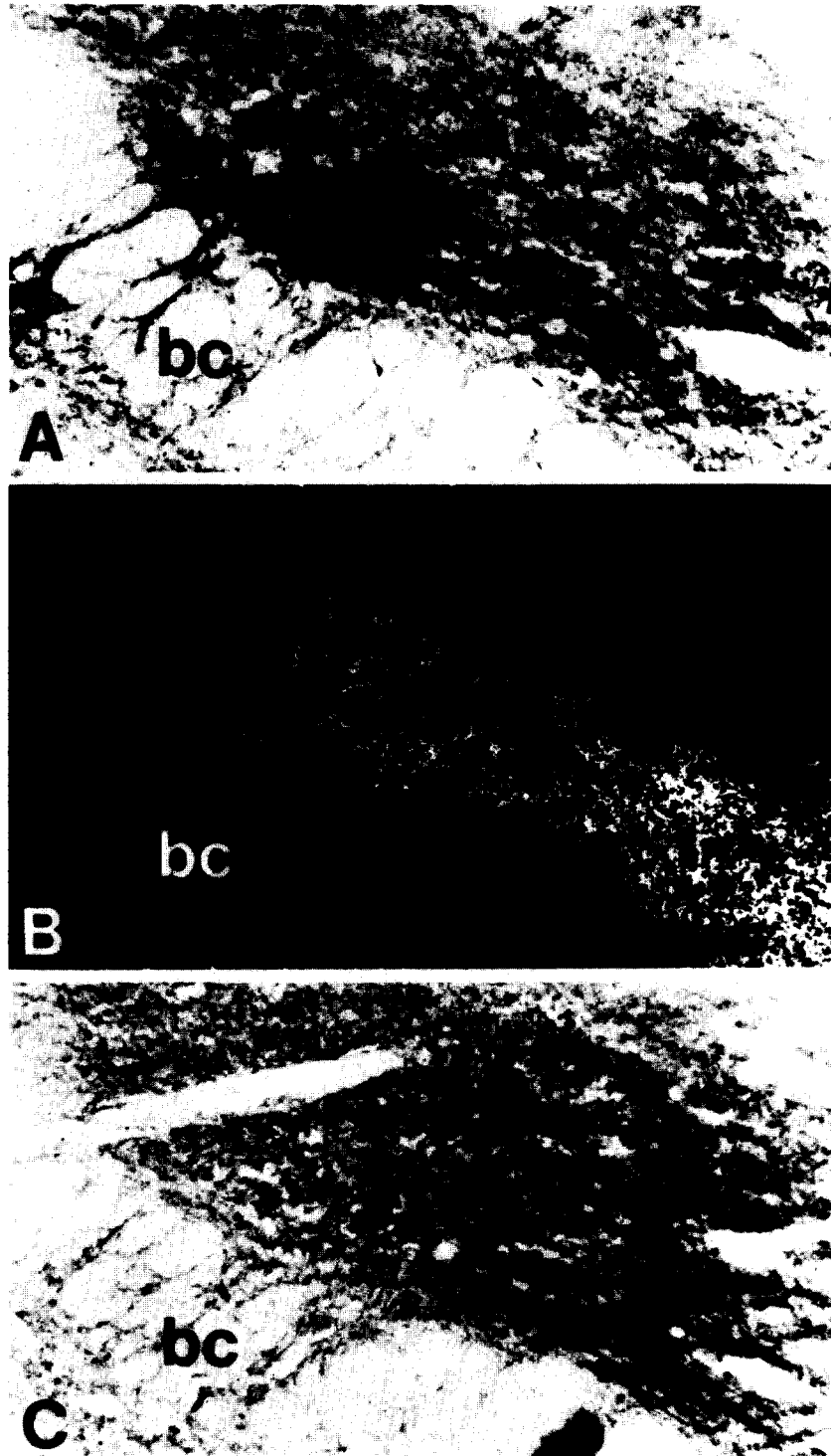


FIG. 7. Lateral parabrachial nucleus. These three adjacent sections show immunoreactive [Leu]enkephalin perikarya in A, [^3H]naloxone binding sites in B, and dynorphin A immunoreactive perikarya in C. The positions of [Leu]enkephalin and dynorphin A immunoreactive perikarya are separate in this nucleus, while the terminal immunoreactivity of both peptides corresponds to the distribution of [^3H]naloxone binding sites. bc: brachium conjunctivum. Mag.: 200 \times .

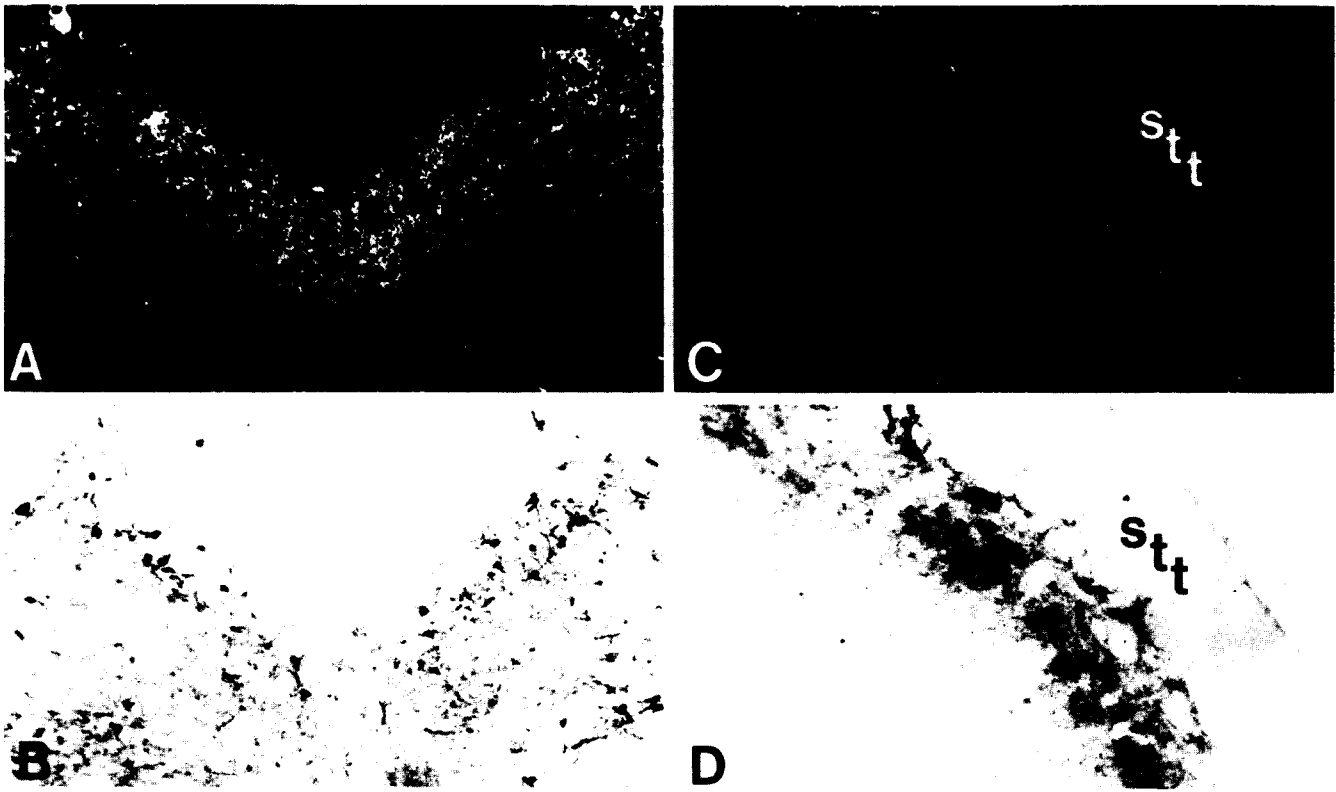


FIG. 8. Nucleus tractus solitarius and the spinal nucleus of the trigeminal nerve. A and B are adjacent sections showing the correspondence between [^3H]naloxone binding and [Leu]enkephalin-positive perikarya in nucleus tractus solitarius. C and D demonstrate the relation between a dense streak of [^3H]naloxone binding sites and [Leu]enkephalin immunoreactive terminals in the spinal nucleus of the trigeminal nerve. stt: spinal tract of the trigeminal nerve. Mag.: $64\times$.

relationship between binding sites and opioid systems was discernible. Even within a region, the relationship can be complex; for example, in caudate-putamen, there was no apparent correspondence between the large receptor-rich patches and the smaller [Leu]enkephalin patches of immunoreactivity. In contrast, a good correspondence was seen between the subcallosal streaks of binding sites and [Leu]enkephalin immunoreactivity. These relationships could not have been determined by comparing sections obtained from different brains. Adjacent section analysis also revealed the distribution of immunoreactive perikarya in some opioid receptor-rich areas of midbrain and brainstem (e.g., interpeduncular nuclear complex, parabrachial nucleus, and nucleus tractus solitarius). Such observations point to the possible existence of opioid local circuits in these regions, although the contribution of distal opioid neurons to these receptor fields also requires evaluation.

Despite the existence of impressive opioid peptide-receptor associations, as described in the Results section, we, as well as other investigators, have observed discrepancies between the distribution of opioid peptides and receptors. Both types of discrepancy have been noted—ligands in the apparent absence of receptors and vice versa—and may be due to relatively trivial technical reasons, or reflect authentic phenomena. In the case of a relative absence of receptors (e.g., in globus pallidus), it is possible that the physiological receptor has not been labelled, due to postmortem lability or the use of inappropriate ligands and/or binding conditions. As an example of the latter, it is

possible, in the case of complex polypeptide precursors (e.g., the three opioid precursors, the substance P precursors, etc.), that the postsynaptic cell may express receptors for some products, but not others (e.g., ACTH, but not β -endorphin, or substance K, but not substance P). In the case of an abundance of receptors in the absence of endogenous ligand, it is possible that the histochemical procedure for detecting the latter may not be sufficiently sensitive. As an example, the first immunocytochemical studies of enkephalin distribution detected very little or no immunoreactivity in cerebral cortex [9, 52, 56] despite the existence of radioimmunoassayable quantities of enkephalin-like peptides in this tissue [49,62]. However, as a result of technical improvements, later immunocytochemical studies did succeed in localizing these peptides in cortex [10, 11, 23, 24, 33, 34, 50, 55]. Other causes of discrepancies include the detection of receptors in axonal transit before their insertion into the membrane, or simply "spare" receptors which may be genetically expressed, but are physiologically unimportant or uncoupled to appropriate effector mechanisms.

Discrepancies between the localization of opioid neuronal systems and opioid receptors are not unique to these systems; such anomalies have also been observed for substance P [45,51], neurotensin [43,63], angiotensin II [35], norepinephrine [39], and GABA [40,41]. In the case of GABA, at least, some of the anatomical discrepancies have been resolved due to the discovery of a GABA receptor subtype not labelled in the previous autoradiographic studies [61].

The complexity of the association between [³H]naloxone binding sites and the multiple opioid systems precludes any simple view of opioid peptide-receptor relationships. Although the [³H]naloxone binding sites appear to reflect mostly μ receptors, there is evidence that κ sites are distributed identically in rat brain [42]. Thus, there does not appear to be a unique association between the multiple opioid neuronal systems and opioid receptor subtypes in brain, although *in vitro* studies of the receptor selectivity of opioid peptides (e.g., dynorphin A for the κ receptor; [6, 8, 64]) gave rise to a prediction of selective anatomical associations (e.g., δ sites with [Leu]-enkephalin-containing neurons; [12]). Despite the apparent theoretical attractiveness of such selective associations, the picture which has emerged is much richer in possibilities for regulation of opioid neurotransmission. The availability of multiple opioid receptors for interaction with the products of each opioid precursor should not be surprising; multiple receptors have already been identified for individual ligands, including dopamine, norepinephrine, serotonin, GABA, and acetylcholine [53]. For some multiple receptor groups, it is clear that the subtypes are differentially coupled to effectors (e.g., adenylate cyclase), and this appears to be the case for opioid receptor subtypes, as well [4,59]. Given the presence of multiple opioid receptor subtypes which are differentially coupled to effectors, and the occurrence of endogenous ligands which

have varying affinities for these receptor subtypes, it appears that the qualitative and quantitative parameters of opioid neurotransmission will be a function of the relative ratios of opioid forms released and receptor subtypes present. Posttranslational processing thus emerges as a key regulatory event; for example, the release of dynorphin A would presumably affect mainly κ receptors (unless they were scarce), but processing of the peptide to dynorphin A (1-8) would improve its relative affinity for μ and δ receptors [8,46]. The relative mix of μ , δ , and κ receptors at the synapse would determine the physiological outcome of this particular processing step (see [60]). Bearing on this issue, we have shown a clear relationship between the ratios of dynorphin A: dynorphin A (1-8) and κ : μ opioid receptors in rat and guinea pig substantia nigra [26]. Such findings point to the need for correlative anatomical/biochemical studies using multiple antisera and receptor ligands as tools to uncover some of the subtleties of opioid neurotransmission in the brain.

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