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Differentiation of delta and mu opiate receptor localizations by light microscopic autoradiography

(multiple receptors/enkephalin)

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ABSTRACT **We have observed two discrete populations of opiate receptors that are differently localized in rat brain. Morphine-like (mu) receptors, labeled by ^{125}I-labeled [D-Ala-^{2}MePhe^{4}Met(*O*)^{5}-ol]enkephalin, are concentrated selectively in lamina IV of the cerebral cortex, certain thalamic nuclei, and the periaqueductal grey, while delta receptors, labeled by ^{125}I-labeled [D-Ala^{2}D-Leu^{5}]enkephalin, are more diffused, having high densities in cerebral cortex, corpus striatum, amygdala, and olfactory tubercle. Because of similarities in their localizations, we propose that mu and delta receptors are respectively the physiologic receptors for [Met]- and [Leu]enkephalin neurons. These distributions reflect the different physiological functions attributed to mu and delta receptors and thus represent discrete functions of [Met]- and [Leu]enkephalin neurons.**

Based on pharmacologic effects in humans and spinally transected dogs, Martin *et al.* (1) have distinguished three opiate receptor effects that they designated mu, kappa, and sigma. Opiate binding studies also show multiple receptor sites; the properties of these, however, do not correspond to the pharmacologic distinctions described by Martin *et al.* Several opiates are less potent displacers of [^{3}H]enkephalin binding than of [^{3}H]opiate binding (2–4). The different drug affinities for enkephalin and opiate binding sites correspond to different pharmacological potencies in the mouse vas deferens (termed delta or enkephalin-like) and the guinea pig ileum (termed mu or morphine-like) (2). Protection experiments also distinguish mu- and delta-receptor binding sites in brain tissue (5). Differential tolerance of the mouse vas deferens to mu- and delta-specific drugs strongly supports the pharmacological relevance of the two receptors (6). Chang *et al.* (7, 8) labeled mu and delta receptors selectively with two ^{125}I-labeled enkephalin analogs. A variety of opiates show different rank orders of potency for the two receptors, which also differ in regional distribution in rat brain (7, 8). Various pharmacologic effects of certain enkephalin derivatives suggest that mu receptors mediate analgesic actions of opiates (9), while delta receptors may be responsible for epileptic (10, 11), sedative (1), and behavioral (10, 12) effects.

Larsson *et al.* (13) recently showed that met- and leu-enkephalin are contained, at least in part, in separate neuronal populations (13). We (14) suggested that mu and delta receptors, respectively, are physiologic receptors for met- and leu-enkephalin neurons.

In the study reported here, we used a generally applicable, *in vitro* autoradiographic procedure developed by Young and Kuhar (15) to localize the two opiate receptors in rat brain. Mu and delta receptors are labeled preferentially with ^{125}I-labeled enkephalin analogs (8). We describe distinct differences in the localizations of mu and delta sites that correspond to differences in [Met]- and [Leu]enkephalin neuronal localizations.

MATERIALS AND METHODS

In our autoradiographic procedure, the opiate receptors are labeled in intact, slide-mounted tissue sections on which binding can be assayed biochemically. Autoradiograms are generated by apposition of emulsion-coated coverslips (15).

Sections (4–6 μm) of rat (male, Sprague–Dawley, 150–200 g) brain were thaw-mounted onto microscope slides. After a 15-min preincubation with 50 μM GTP and 100 mM NaCl and a 5-min wash, both at room temperature in 0.17 M Tris·HCl, pH 7.6, to help remove endogenous opioids, the slide-mounted tissue sections were transferred to the same buffer containing 0.1–0.2 nM ^{125}I-labeled [D-Ala^{2}D-Leu^{5}]enkephalin (^{125}I-DADL) or [D-Ala^{2}MePhe^{4}Met(*O*)^{5}-ol]enkephalin (FK 33-824, Sandoz Pharmaceutical; ^{125}I-FK) (4–8 $\times$ 10^{5} dpm/ml; 1 dpm = 16.7 mBq) for 40 min. After two consecutive 5-min washes in buffer at ice-water temperatures, the sections were wiped off the slides, and their radioactivity was assayed by liquid scintillation spectrometry (in the biochemical studies), or they were placed on a cold plate and dried under a stream of cold, dry air (for the autoradiographic studies). Adjacent sections were incubated with 1 μM naloxone to produce blanks or with 1 nM FK 33-824 in the ^{125}I-DADL incubations for more-selective labeling of the delta receptors by ^{125}I-DADL. Autoradiograms were exposed for 2–3 weeks, and the developed sections were fixed, stained, and examined as described (15).

Assays of the binding of ^{125}I-labeled enkephalin to brain membranes were conducted as reported (8, 16). Reaction mixtures at 25°C for 40 min (final volume was 0.25 ml) contained tissue suspension (final tissue concentration was 10 mg/ml), one of the ^{125}I-labeled enkephalins (4–8 $\times$ 10^{5} dpm/ml; about 0.1–0.2 nM), and buffer or 1 μM levallorphan.

^{125}I-DADL and ^{125}I-FK (both 1–2 Ci/μmol; 1 Ci = 3.7 $\times$ 10^{10} becquerels) were prepared and purified as described (17).

RESULTS

Biochemical Studies. In our experiments, membrane binding properties of ^{125}I-DADL and ^{125}I-FK resemble those reported earlier (7, 8). The concentration that produces 50% of maximal inhibition (IC_{50}) values of morphine at ^{125}I-FK and ^{125}I-DADL sites are 0.8 nM and 60 nM, respectively, and the IC_{50} values of unlabeled DADL are 6 nM and 3 nM, respectively. The binding ratios of ^{125}I-DADL and ^{125}I-FK in the thalamus and frontal cortex are 1:3.8 and 1:1.4, respectively.

In tissue sections, morphine displays higher affinity for ^{125}I-FK sites than for ^{125}I-DADL sites (IC_{50} values of 2 nM and 30 nM, respectively). The IC_{50} values of unlabeled DADL for ^{125}I-FK and ^{125}I-DADL sites are the same as in membranes.

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Abbreviations: FK 33-824, [D-Ala^{2}MePhe^{4}Met(*O*)^{5}-ol]-enkephalin; DADL, [D-Ala^{2}D-Leu^{5}]-enkephalin; IC_{50}, half-inhibition concentration.

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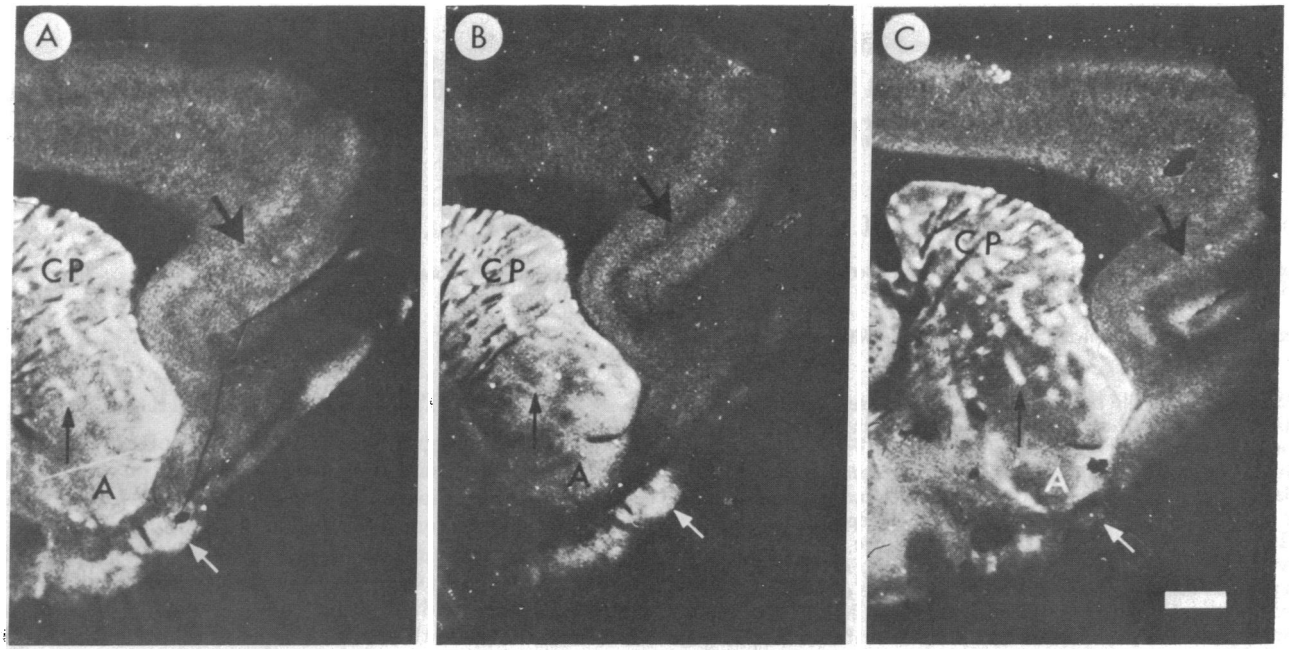

FIG. 1. Distribution of delta and mu opiate receptors in the frontal cortex, corpus striatum (CP), nucleus accumbens (A), and olfactory tubercle (white arrow), as seen in dark-field micrographs of adjacent sagittal sections. Bar = 500 μm. (*A*) Delta and mu receptors labeled by 0.2 nM of ^{125}I-DADL. (*B*) Delta receptors labeled more selectively by 0.2 nM ^{125}I-DADL in the presence of 1 nM FK 33-824 (to block most of the mu receptors). (*C*) Mu receptors labeled by 0.2 nM of ^{125}I-FK. Lamina IV of the cerebral cortex (wide black arrow) is enriched in mu receptors; compare the abundant labeling by ^{125}I-FK (*C*) with the slight labeling by ^{125}I-DADL (*A*) which is essentially eliminated by unlabeled FK displacement (*B*). Similar labeling of many of the clusters in the corpus striatum (example of one such cluster is labeled by thin black arrow) indicates that they contain mostly mu receptors. Areas rich in delta receptors include laminae II, III, and V of the cerebral cortex, the corpus striatum (diffusely labeled), the nucleus accumbens, and the olfactory tubercle; these areas are well labeled by ^{125}I-DADL (*A*), even in the presence of unlabeled FK (*B*), but are nearly unlabeled by ^{125}I-FK (*C*). Adjacent control sections (1 μM naloxone added) show low levels of grains, similar to white matter areas in experimental sections. Controls in other areas produced similar low levels.

Similar regional differences in the binding ratios of the iodinated enkephalins occur in tissue sections as in membranes (^{125}I-DADL/^{125}I-FK is 1:2.3 for thalamic sections and 1:1.3 for frontal cortex sections).

Autoradiographic Studies. The percentage occupancy of the mu and delta receptors by the ^{125}I-labeled ligand in each of the three conditions used can be estimated from the apparent dissociation constants (K_ds) of the ligands for the two receptors (8). The K_ds of FK 33-824 for mu and delta receptors are approximately 0.8 and 10 nM, respectively; for DADL, they are about 4.0 and 1.5 nM (8). The percentage occupancy (% Occ) is $L/(L + K_d)$, where L = concentration of ligand (0.2 nM). Thus, ^{125}I-FK should occupy about 20% of the mu receptors and about 2.0% of the delta receptors and ^{125}I-DADL should occupy approximately 4.8% of the mu receptors and 12% of the delta receptors. Occupancy by ^{125}I-DADL in the presence of 1 nM FK 33-824 can be calculated by using the formula $RL = RL^0 [1 + (K_A \times L)]/[1 + (K_A \times L) + (K_A' \times L')]$, where RL = % Occ, RL^0 = % Occ without unlabeled ligand present, K_A = affinity constant of ^{125}I-labeled ligands, L = concentration of ^{125}I-labeled ligand, K_A' = affinity constant of FK 33-824 and L' = concentration of FK 33-824 (1 nM). In this case, ^{125}I-DADL will occupy only about 2.2% of the mu receptors, but 11% of the delta receptors.

Table 1. Mu and delta opiate receptors in rat brain regions

Predominantly mu	Predominantly delta	Mu and delta
Laminae I and IV of cerebral cortex	Laminae II, III, and V of cerebral cortex	Lamina VI of cerebral cortex
Streaks and clusters in corpus striatum	Diffuse grains in corpus striatum	Nucleus tractus solitarius
Thalamus (dorsomedial, ventral)	Amygdala	Vagal fibers
Hypothalamus	Nucleus accumbens	Nucleus ambiguus
Hippocampus (pyramidal cell layer)	Olfactory tubercle	Substantia gelatinosa of spinal cord trigeminal
Periaqueductal gray	Pontine nuclei	
Interpeduncular nucleus		
Inferior colliculus		
Midbrain median raphe		

Adjacent sections labeled with the two different ^{125}I-labeled ligands were used to compare localizations of mu and delta receptors. In all studies, mu receptors are operationally defined as sites labeled by ^{125}I-FK, and delta receptors designate sites labeled by ^{125}I-DADL in the presence of 1 nM of unlabeled FK 33-824. The localizations of the mu and delta receptors in the cerebral cortex are strikingly different (Figs. 1 and 2). In both sagittal and coronal sections, mu receptors are selectively concentrated in laminae I and IV, whereas delta receptors are preferentially localized to laminae II, III, and V and show very little if any labeling in laminae I and IV.

In the caudate putamen, mu receptors are highly localized to discrete clusters and a subcallosal streak observed in earlier autoradiographic studies (15). In contrast, delta receptors are more diffusely distributed throughout the caudate putamen; there is significant ^{125}I-DADL labeling in areas between the mu-receptor clusters. In the caudate putamen and cerebral

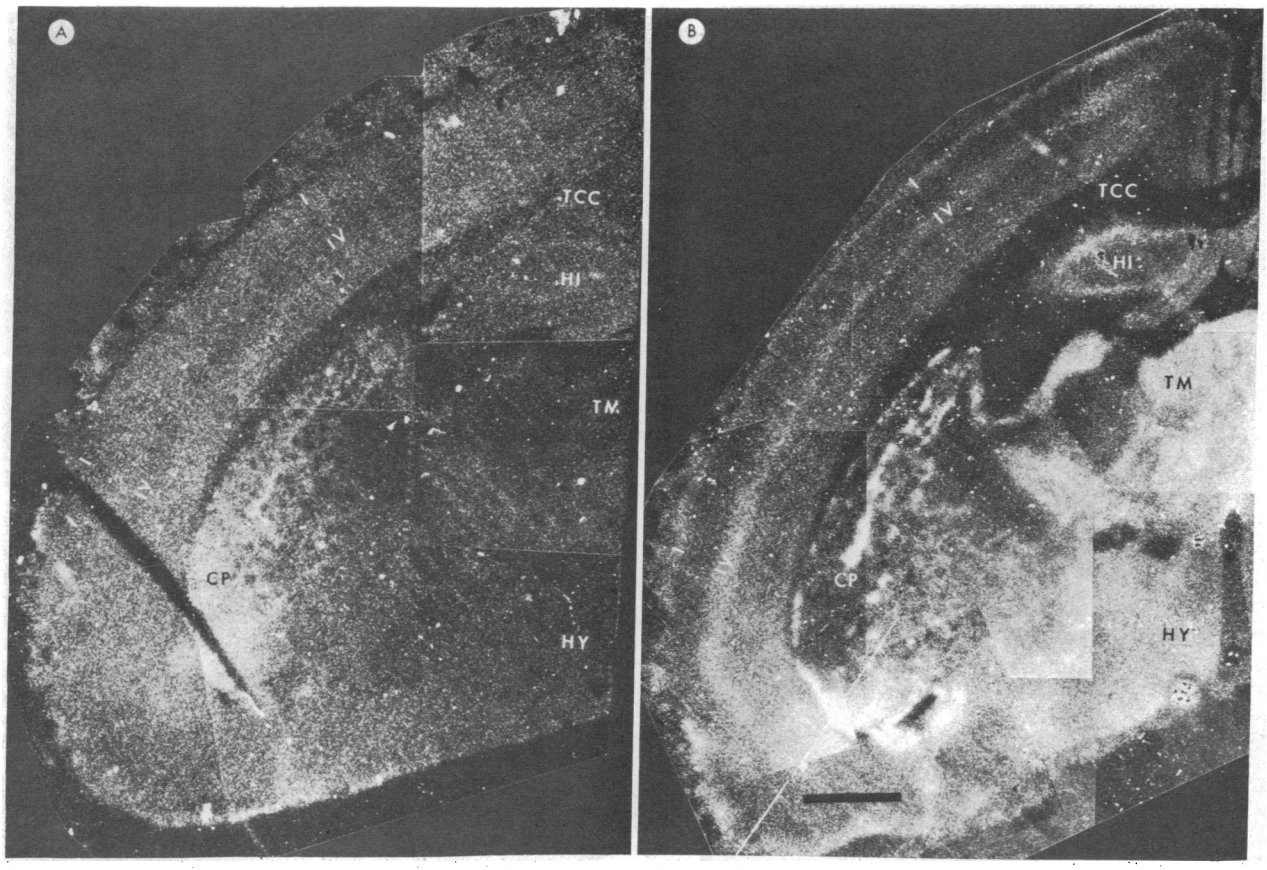

FIG. 2. Distribution of delta and mu opiate receptors as shown by dark-field micrographs of adjacent coronal sections of cerebral cortex, corpus striatum (CP), hippocampus (HI), thalamus (TM = dorsomedial thalamus), and hypothalamus (HY). Bar = 1000 μm. (*A*) Selective labeling of delta receptors by 0.2 nM of ^{125}I-DADL in the presence of 1 nM of FK 33-824. (*B*) Mu receptors labeled by 0.2 nM of ^{125}I-FK. The receptor distribution seen in the cerebral cortex (laminae I and IV, labeled I and IV) and corpus striatum corresponds to that seen in Fig. 1. For instance, the subcallosal streak and clusters appear to be mostly due to mu receptors. Labeling of mu receptors in the pyramidal cell layer of the hippocampus can be seen (*B*), but little labeling of delta receptors in hippocampus is seen (*A*). (Note that the overall intensity of the labeling in the corpus striatum is similar in *A* and *B*.) Comparison of the intensity of labeling in the hypothalamus and the dorsomedial and ventral thalamus with that in the corpus striatum shows that these areas are greatly enriched in mu receptors.

cortex, as well as all other areas, sections incubated with ^{125}I-DADL only display autoradiographic grains associated with both mu and delta receptors (Fig. 1*A*).

The most striking contrast between mu- and delta-receptor localization occurs in the thalamus and hypothalamus (Fig. 2). The dorsomedial and ventral thalamus, as well as the hypothalamus, display very high concentrations of mu receptors and extremely few delta receptors, resembling the biochemical results (8). The hippocampal mu receptors are highly localized to the pyramidal cell layer, a localization not observed earlier (15), presumably because of lesser sensitivity. Neurophysiologic effects of opiates in hippocampus are localized to interneurons adjacent to the pyramidal cells (18). Delta receptors are more diffusely distributed and low in density in the hippocampus. In contrast to the thalamus and hypothalamus, which are selectively enriched in mu receptors, the olfactory tubercle, nucleus accumbens (Fig. 1), and amygdala (not shown) have high densities of delta receptors and relatively few mu sites. In most brainstem areas, densities of mu receptors greatly exceed those of delta sites (Fig. 3). Areas most enriched in mu receptors include the interpeduncular nucleus, midbrain median raphe, inferior colliculus, and periaqueductal grey; the concentration of mu receptors in the pontine nuclei is fairly low. Delta receptors are enriched only in the pontine nuclei. Apparent delta receptor labeling in the interpeduncular nucleus and inferior colliculus may represent "spill over" from the greatly enriched mu receptor densities in this region.

In the medulla oblongata and spinal cord, mu- and delta-receptor densities are similar, which agrees with biochemical data in spinal cord (19). There are high levels of both receptors in the nucleus tractus solitarius, vagal nerve fibers, and nucleus ambiguus and moderate levels in other grey matter areas (Fig. 4). In the spinal cord, there are high densities of both mu and delta receptors in the substantia gelatinosa and moderate densities throughout the grey matter (Fig. 5).

Adjacent sections produced by incubations in the presence of 1 μM naloxone (Fig. 5*C*) show background levels in all of the areas discussed above, comparable to levels in white matter. GTP (10 μM) greatly decreases the binding of both ^{125}I-labeled ligands, consistent with their agonist properties.

DISCUSSION

The major finding of this study is that mu and delta opiate receptors are differentially localized throughout rat brain (Table 1). The localization of mu receptors found resembles that found for opiate receptors in previous autoradiographic studies in this laboratory, in which the conditions used resulted in labeling of mu receptors primarily (15, 20). The evidence that the two receptors are labeled preferentially by ^{125}I-DADL and ^{125}I-FK derives in part from biochemical studies (2–4, 8).

The autoradiographic data reinforce other evidence that mu and delta receptors have different functions. For instance, mu-selective enkephalin analogs are more potent analgesics than delta-selective peptides (9), which agrees with our obser-

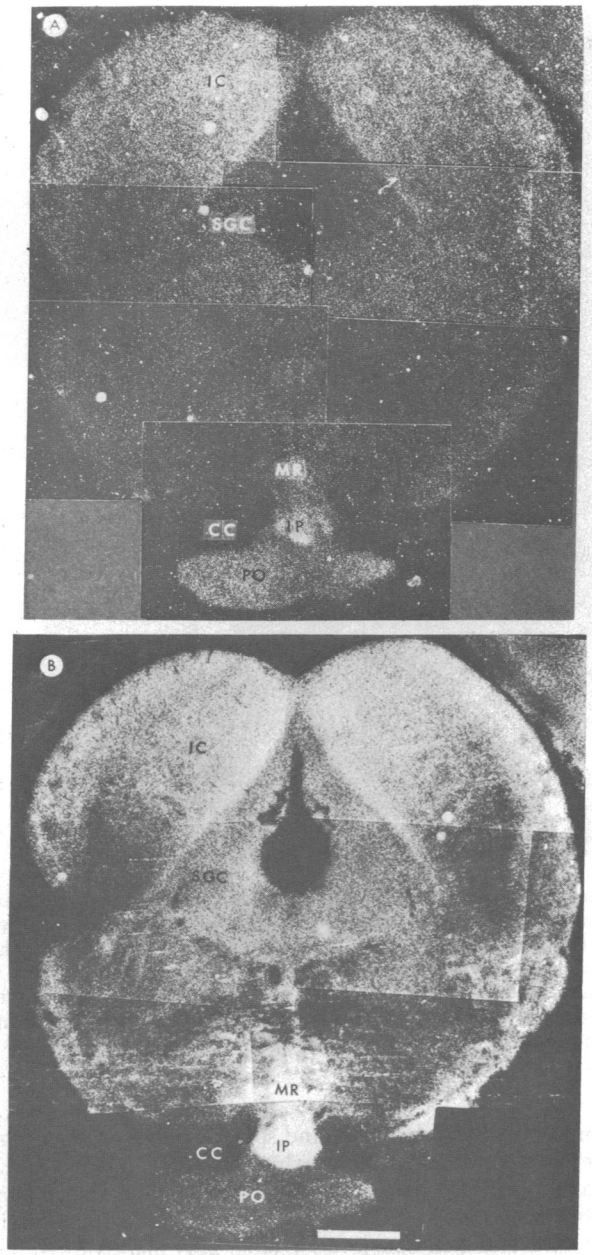

FIG. 3. Dark-field micrographs of delta and mu opiate receptor distribution in adjacent coronal sections of rat midbrain. Bar = 1000 μm. (*A*) Delta receptors selectively labeled by 0.2 nM ^{125}I-DADL in the presence of 1 nM unlabeled FK 33-824. (*B*) Mu receptors labeled by 0.2 nM ^{125}I-FK. High concentrations of mu receptors are seen in the inferior colliculus (IC), the periaqueductal grey (SGC), the median raphe nucleus (MR), and the interpeduncular nucleus (IP). A relatively high density of grains is found in the pontine nuclei shown in *A*. The ratio of this density to that in other areas shown in *A* is much greater than the same ratio shown in *B*, indicating an enrichment of delta receptors in the pontine nuclei. The moderate labeling of the inferior colliculus and interpeduncular nucleus (*A*) occurs in areas that have very high levels of mu receptors and may be secondary to residual labeling of mu receptors by ^{125}I-DADL.

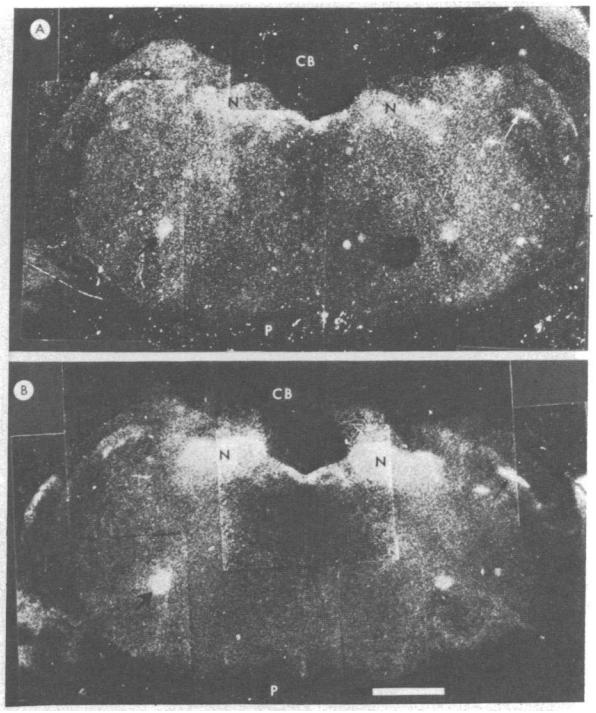

FIG. 4. Dark-field micrographs of coronal sections of rat medulla oblongata, showing delta and mu opiate receptor distribution. Bar = 1000 μm. (*A*) Delta receptors labeled selectively by 0.2 nM ^{125}I-DADL in the presence of 1 nM FK 33-824. (*B*) Mu receptors labeled by 0.2 nM ^{125}I-FK. The highest concentrations of delta and mu receptors are found in the same areas at this brain level. These high concentrations are seen in the nucleus tractus solitarius (N), nucleus ambiguus (thick black arrows), and fibers of the vagus nerve (thin arrows). Moderate grain density is seen in all grey matter areas.

vation that mu receptors are more localized to brain regions subserving pain perception (such as the dorsomedial thalamus and the periaqueductal grey). The existence of both mu and delta receptors in the substantiae gelatinosae of the spinal cord and trigeminal nucleus suggests that the drug selectivity for eliciting analgesia at a spinal level may differ somewhat from analgesic actions at a supraspinal level. Delta-specific enkephalins are more effective in eliciting limbic seizures (10, 11) and in facilitating reward behavior (10, 12) than mu selective peptides. Interestingly, some limbic structures (such as the amygdala, nucleus accumbens, and olfactory tubercle) are enriched in delta receptors.

The mu- and delta-receptor dichotomy does not seem to fit with the pharmacologic classification of mu, kappa, and sigma receptors (1). The distinct high- and low-affinity opiate receptors, which differentially regulate opiate analgesia and respiratory depression (21), may represent mu and delta sites, respectively, because regional variations in high- and low-affinity sites parallel those for mu and delta receptors (22).

Evidence for distinct mu and delta receptors is strong.

(*i*) Drug specificity (2, 4, 7, 8, 14): mu—morphine > FK 33-824 > β-endorphin > Met^5-enkephalin > Leu^5-enkephalin > D-Ala^{2}D-Leu^5-enkephalin vs. delta—D-Ala^{2}D-Leu^5-enkephalin ≅ Leu^5-enkephalin > Met^5-enkephalin > β-endorphin > FK 33-824 > morphine.

(*ii*) Mu receptors are more markedly regulated by sodium and GTP than are delta receptors (7, 14, 23).

(*iii*) Regional variations in biochemical binding studies (8): ratio of mu to delta—thalamus = hypothalamus > brain stem = hippocampus > cerebral cortex = corpus striatum (ratio for cerebral cortex = 1).

(*iv*) Pharmacologic responses: predominantly mu in guinea pig ileum (2) vs. delta in mouse vas deferens (2), rabbit intestine ion flux (24), and neuroblastoma adenylate cyclase (25).

(*v*) Lack of cross tolerance between mu- and delta-specific drugs in regulation of mouse vas deferens contractions (6).

(*vi*) Mu-selective enkephalin analogs are more analgesic (9) vs. delta-selective peptides are more epileptogenic (10, 11) and involved in reward systems (12).

(*vii*) Relationship between receptor localization and function: mu receptors—lamina IV of cerebral cortex, medial and ventral

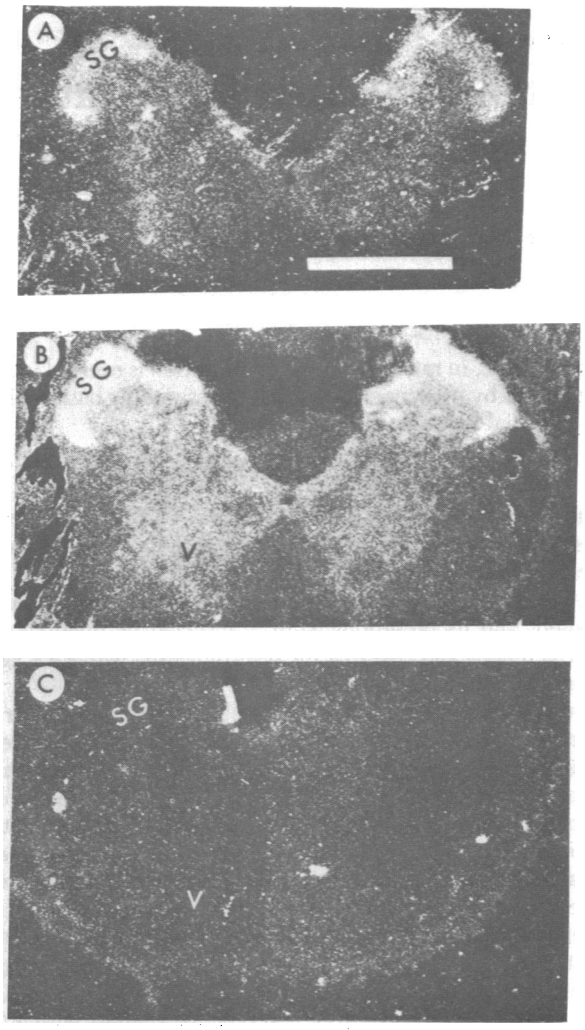

FIG. 5. Delta and mu opiate receptors in adjacent coronal sections of rat spinal cord. Bar = 1000 μm. (*A*) Labeling of delta receptors by 0.2 nM ^{125}I-DADL in the presence of 1 nM FK 33-824. (*B*) Mu receptors labeled by 0.2 nM ^{125}I-FK. High concentrations of both delta and mu receptors are found in the substantia gelatinosa of the spinal cord. Moderate grain density is seen throughout the spinal cord grey matter; few grains are seen over white matter structures. (*C*) Example of a control section having 0.2 nM ^{125}I-FK and 1 μM naloxone. Note that the low grain densities seen in this section are the same as or lower than those found over the white matter areas in *A* and *B*.

thalamus, and periaqueductal grey—and their respective functions—sensory integration, sensory integration, and analgesia; delta receptors—limbic structures (amygdala, nucleus accumbens, and olfactory tubercle) and their functions—reward behavior; and both mu and delta receptors—nucleus tractus solitarius, vagal fibers, and nucleus ambiguus and substantia gelatinosa of spinal cord and trigeminal and their respective functions—visceral functions (respiration and nausea) and spinal-brainstem pain perception.

Because [Met]- and [Leu]enkephalins tend to be mu and delta specific in receptor binding affinities, we suggested differential physiologic roles of the two enkephalins at the two receptors (14). The differential localization of [Met]- and [Leu]enkephalin neurons fits the mu–delta dichotomy. Rat brain regions that have more mu than delta receptors (hippocampus, thalamus) also possess more [Met]- than [Leu]enkephalin neurons in guinea pig brain (L. Larsson, personal communication). Conversely, the central amygdala, which possesses more delta than mu receptors, has more [Leu]- than [Met]enkephalin neurons. The substantia gelatinosa of spinal cord and the caudate, which have similar total levels of mu and delta receptors, also have similar numbers of [Met]- and [Leu]enkephalin neurons. Thus, we propose that mu and delta receptors serve respectively as the physiological receptive sites for synaptic actions of [Met]- and [Leu]enkephalin neurons. It follows, then, that the different pharmacological effects associated with mu and delta receptors, respectively, reflect the physiological actions of [Met]- and [Leu]enkephalin neurons.

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