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Physiology

Opiate receptor: Autoradiographic localization in rat brain

([^{3}H]diprenorphine/locus coeruleus/substantia gelatinosa/caudate-putamen)

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ABSTRACT **Opiate receptor sites in rat brain can be labeled *in vivo* by [^{3}H]diprenorphine, a potent opiate antagonist. Using techniques to minimize diffusion in fresh, frozen, unfixed brain, we have localized [^{3}H]diprenorphine by autoradiography to visualize the distribution of opiate receptors. Silver grains indicative of the binding of labeled [^{3}H]diprenorphine are discretely localized in numerous areas of the brain with very high densities in the locus coeruleus, the substantia gelatinosa of the spinal cord, and in clusters within the caudate-putamen, amygdala, and parts of the periventricular gray matter.**

Binding of opiates to their pharmacologically relevant receptor *in vitro* shows dramatic regional variations in monkey (1), human (1, 2), rat (3), and calf (4) brain, with highest localizations in limbic regions. Autoradiographic studies, which can provide detailed cellular localizations, require labeling of receptors in intact animals. Recently we demonstrated *in vivo* labeling of the opiate receptor in which 30–60% of total radioactivity in various brain regions was specifically associated with the opiate receptor after [^{3}H]naloxone administration (5); with the much more potent opiate antagonist [^{3}H]diprenorphine, almost all radioactivity was selectively bound to receptors *in vivo* (6). [^{3}H]Diprenorphine and histochemical techniques designed to minimize diffusion of drug from receptor sites facilitated autoradiographic studies that elucidated the cellular localization of opiate receptors (6). The present report describes the detailed autoradiographic localization of [^{3}H]diprenorphine associated with opiate receptor sites in rat brain.

METHODS

The procedure for the autoradiographic localization of opiate receptors involves injecting a suitable radioactive opiate antagonist, sacrificing the animal at a time when the bulk of the drug is specifically bound to receptors, and processing the tissue in such a way as to prevent diffusion of drug away from its binding site. The suitability of [^{3}H]diprenorphine is due to its very high affinity for the opiate receptor ($K_D = 1\text{–}2 \times 10^{-10}$ M) and its corresponding slow dissociation rate from the receptor, especially at low temperatures (6). The data presented here (see *Results*) and in our preliminary study (6) indicate that 1 hr after injection the bulk of the drug in brain was associated with opiate receptors. We processed the tissue by thaw-mounting thin sections of fresh, frozen, unfixed tissue to emulsion-coated slides to prevent or minimize diffusion of drug from binding sites (7–10). After exposure, the localization of opiate receptors was inferred from the distribution of silver grains, which are indicative of [^{3}H]diprenorphine binding sites. These techniques have also been utilized in our laboratory to localize the cholinergic muscarinic receptor by autoradiographic methods (11).

Twenty-two male Sprague-Dawley rats (180–220 g) each received 125 μCi of [^{3}H]diprenorphine (13.0 Ci/mmol, Amersham/Searle) by tail-vein injection in 0.3 ml of saline, and were decapitated 1 hr later, when essentially all tritium in the brain can be accounted for as unmetabolized [^{3}H]diprenorphine (C. Pert, unpublished). Brains were removed rapidly from the skulls and 2- to 5-mm slices of various regions were mounted on copper block supports and quickly frozen by partial immersion in liquid nitrogen slush (7). Frozen, 4 μm sections of fresh, unfixed tissue were thaw-mounted (8–10) onto emulsion-coated (Kodak NTB-3) microscope slides and exposed for 5 weeks at 2°. After exposure, the slides were brought to room temperature, developed, fixed by immersion in Carnoy's solution, and stained by pyronine Y. After drying, the sections were mounted with Permount and examined in a Zeiss Universal microscope.

Control slides prepared for negative and positive chemography showed no fading of latent images or spurious generation of grains. Measurements of autoradiographic grain densities utilized photographs or slides with a grid superimposed to

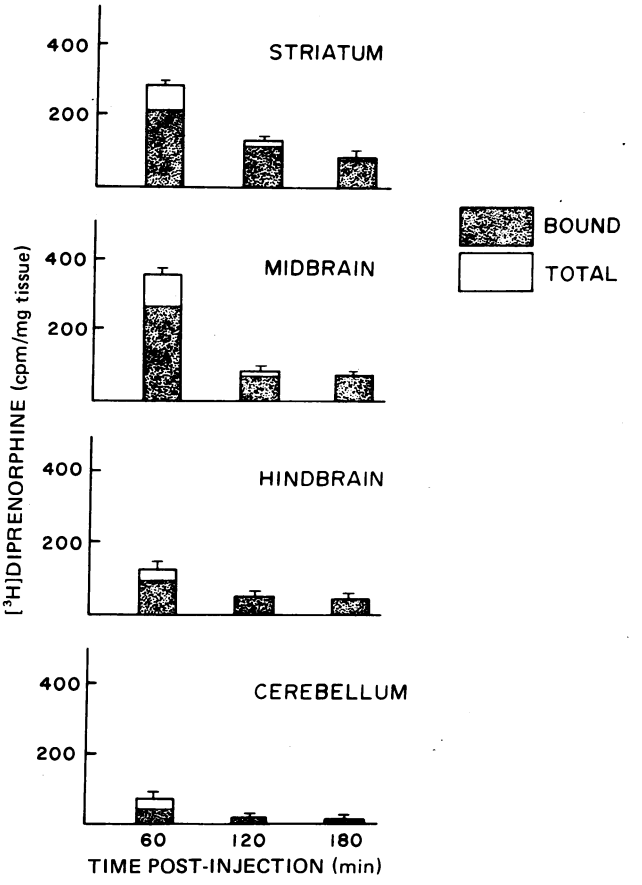

FIG. 1. Regional localization of total and bound [^{3}H]diprenorphine in rat brain after intravenous administration.

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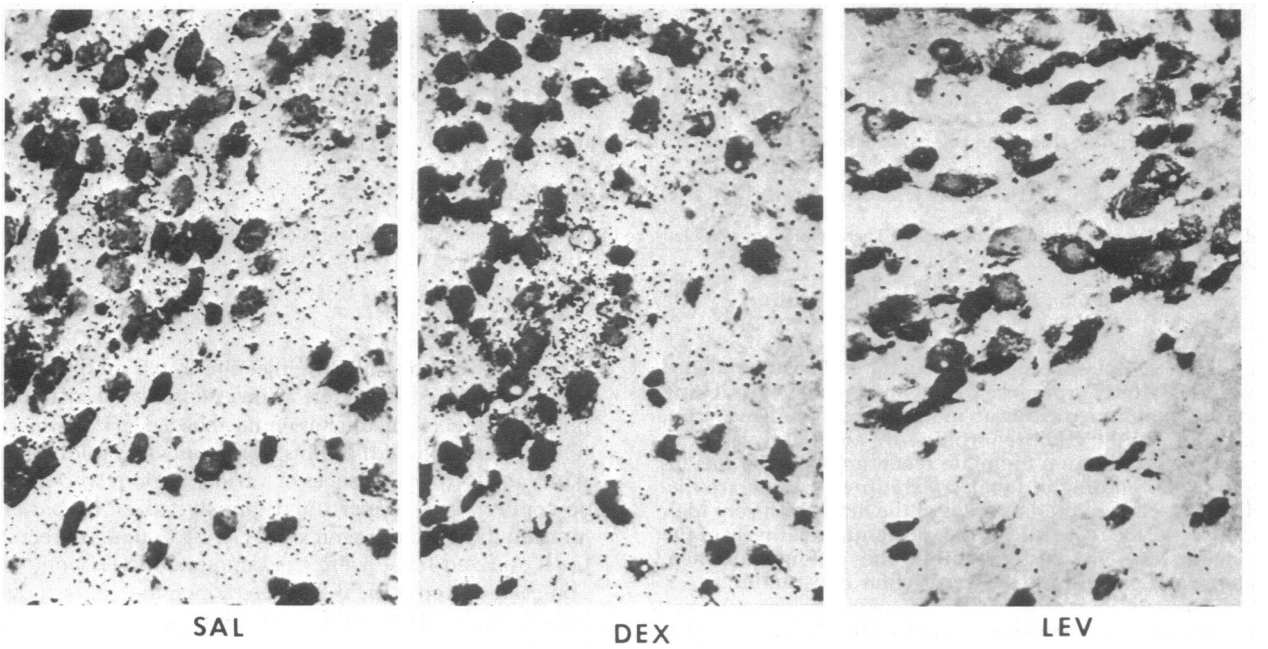

FIG. 2. Localization of [^{3}H]diprenorphine in the lateral edge of medial habenula: effect of cotreatment with saline (SAL; controls), dextrallorphan (DEX), or levallorphan (LEV). See *text* for details.

quantify grains per unit area. Particle-bound and total [^{3}H] diprenorphine were assayed as described (5, 6).

RESULTS

Intravenously administered [^{3}H]diprenorphine is most concentrated in brain regions that *in vitro* assays reveal to be enriched in opiate receptors (6). Brain levels of [^{3}H]diprenorphine are reduced 75% by prior injections of nonradioactive levallorphan (5 mg/kg) but are unaffected by the same doses of the drug's inactive isomer, dextrallorphan (6). In the present study we have examined [^{3}H]diprenorphine levels in particulate fractions of various brain regions at various intervals after injections (Fig. 1). Total and bound tritium distribution 1–3 hr after [^{3}H]diprenorphine administration parallels reported regional variations in opiate receptor density (3, 6), with highest levels in midbrain and corpus striatum, intermediate values in the hindbrain, and lowest levels in the cerebellum (Fig. 1). At 1 hr, 60–75% of tritium in all regions is particulate, while at 2 and 3 hr essentially all radioactivity is "bound." Levallorphan (5 mg/kg) administered intravenously with [^{3}H]diprenorphine abolishes the accumulation of radioactivity in the particulate fraction at 1–3 hr in noncerebellar regions with no effect in the cerebellum, while the same dose of dextrallorphan is ineffective. These data indicate that about 75–80% of radioactivity in noncerebellar regions of the rat after [^{3}H]diprenorphine administration is associated with opiate receptors.

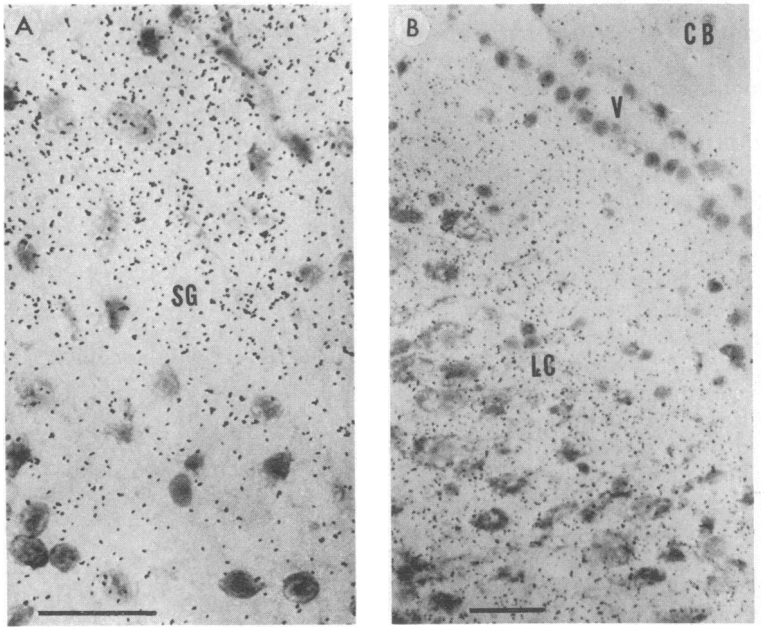

FIG. 3. Localization of [^{3}H]diprenorphine. (A) Substantia gelatinosa (SG) of the spinal cord. (B) The locus coeruleus (LC) and some adjacent areas. Fourth ventricle = V; cerebellum = CB; bars = 100 μm.

Table 1. Autoradiographic grain densities in various brain regions

Area	Autoradiographic grain density	Area	Autoradiographic grain density
Cerebral cortex (A 6860)		Midbrain-pons	
"Deep" areas	12 ± 2	Interpenduncular nucleus (A 1610)	
Dorsal areas	5 ± 1	Dorsal	54 ± 5
Pyriform cortex (A 3990)	9 ± 2	Ventral	15 ± 3
Hippocampus (A 3990)	9 ± 4	Nucleus linearis caudalis (A 1610)	12 ± 4
Dentate gyrus (A 3990)	9 ± 3	Periaqueductal gray (P 290)	24 ± 5
Medial septum (A 7190)	8 ± 1	Periaqueductal gray (−7.2*)	30 ± 5
Striatal area (A 6860)		Dorsal raphe nucleus (P 290)	13 ± 2
"Streak" under corpus callosum	65 ± 6	Median raphe nucleus (P 290)	15 ± 4
"Patches"	53 ± 5	Superior cerebellar peduncle (P 290)	6 ± 2
Areas adjacent to patches	9 ± 2	Nucleus tegmenti pontis (P 290)	3 ± 1
Corpus callosum	2 ± 1	Inferior colliculus (−7.2*)	24 ± 3
Internal capsule bundles	3 ± 1	Reticular formation (−7.2*)	
Anterior commissure	5 ± 2	Midline (narrow vertical areas)	42 ± 6
Globus pallidus	20 ± 4	Ventral area	14 ± 3
Thalamic area (A 3990)		Lateral areas	8 ± 2
Medial areas	29 ± 4	Pontine nuclei (−7.2*)	5 ± 2
Lateral areas	20 ± 5	Decussation of cerebellar peduncle	4 ± 1
Habenula		Substantia nigra	
Medial aspect of medial nucleus	11 ± 4	Zona reticulata	14 ± 2
Lateral aspect of medial nucleus	63 ± 5	Zona compacta (central)	30 ± 5
Medial aspect of lateral nucleus	11 ± 2	Zona compacta (remainder)	15 ± 3
Lateral aspect of lateral nucleus	15 ± 6	Cerebellum	
Periventricular substance	29 ± 5	Vermis, lobes I-IV (−7.2*)	3 ± 1
Hypothalamic area (A 3990)		Deep nuclei (−9.6*)	3 ± 1
Area dorsal to ventricle III	14 ± 3	Other cortical areas (−7.2, −9.6*)	3 ± 1
Area ventral to ventricle III	15 ± 4	Medulla (−8.2*)	
Area lateral to ventricle III	15 ± 3	Locus coeruleus	43 ± 5
Ventricle III	6 ± 2	Ventricle IV	4 ± 2
Preoptic area (A 6860)	14 ± 4	Nerve VII	7 ± 2
Amygdala (A 3990)		Nucleus nerve V	10 ± 2
Basolateral nucleus	64 ± 7	Spinocerebellar tract	4 ± 1
Cortical nucleus	45 ± 8	Medial longitudinal bundle	4 ± 1
		Reticular gray	15 ± 3
		Periventricular gray	28 ± 4
		Spinal cord	
		Substantia gelatinosa	51 ± 6
		Remainder of dorsal horn	9 ± 3
		Ventral horn	7 ± 1
		White matter	3 ± 1

Autoradiographic grain counts are mean ± SEM grains/400 μm^2. Number of measurements involved three separate slides from two different animals. Numbers in parentheses indicate coordinates according to Konig and Klippel (25) or, with asterisk, to Pellegrino and Cushman (26).

To assess stereospecificity of the labeling, we examined rats pretreated with levallorphan or its inactive isomer dextrallorphan (Fig. 2). In the lateral edge of the medial habenular nucleus, as well as in all other brain areas examined, levallorphan pretreatment reduces grain density to background levels, abolishing selective local enrichments of grains, while dextrallorphan treatment has no detectable effect. Stereospecificity as well as the selective localization of grains argues against the possibility that the distribution of grains represents a nonspecific process (Table 1).

In the locus coeruleus, dorsal interpeduncular nucleus, and lateral edge of the medial habenular nucleus grain density was compared in areas overlying and between cells. In the sections examined, the grain densities between cells are 11.4, 13.4, and 15.6 grains/100 μm^2, respectively, while the corresponding grain densities overlying cells are 4.6, 4.4, and 6.8, respectively. Thus, in these areas, grain density is two to three times greater between than over cells.

Confirming preliminary observations (6), we observe a marked heterogeneity of autoradiographic grain distribution (Figs. 2–4, Table 1). Within the spinal cord the greatest density of grains occurs dorsally in a sharp, narrow band (laminae I and II; S. Atweh and M. J. Kuhar, in preparation) that includes the substantia gelatinosa, whereas other regions of the dorsal gray contain a much lower grain density. The ventral gray and dorsal and ventral white matter contain negligible grain densities, confirming biochemical studies of opiate receptor distribution within the spinal cord (12).

The dorsal part of the interpeduncular nucleus is one of the most heavily labeled areas, while labeling in the ventral part of the nucleus is only ¼ as high. In the substantia nigra, grain density is two times higher in the central zona compacta, which contains dopamine cells, than in the rest of the zona compacta and the zona reticulata, which are largely devoid of dopamine cells.

The locus coeruleus, which contains *almost exclusively* nor-

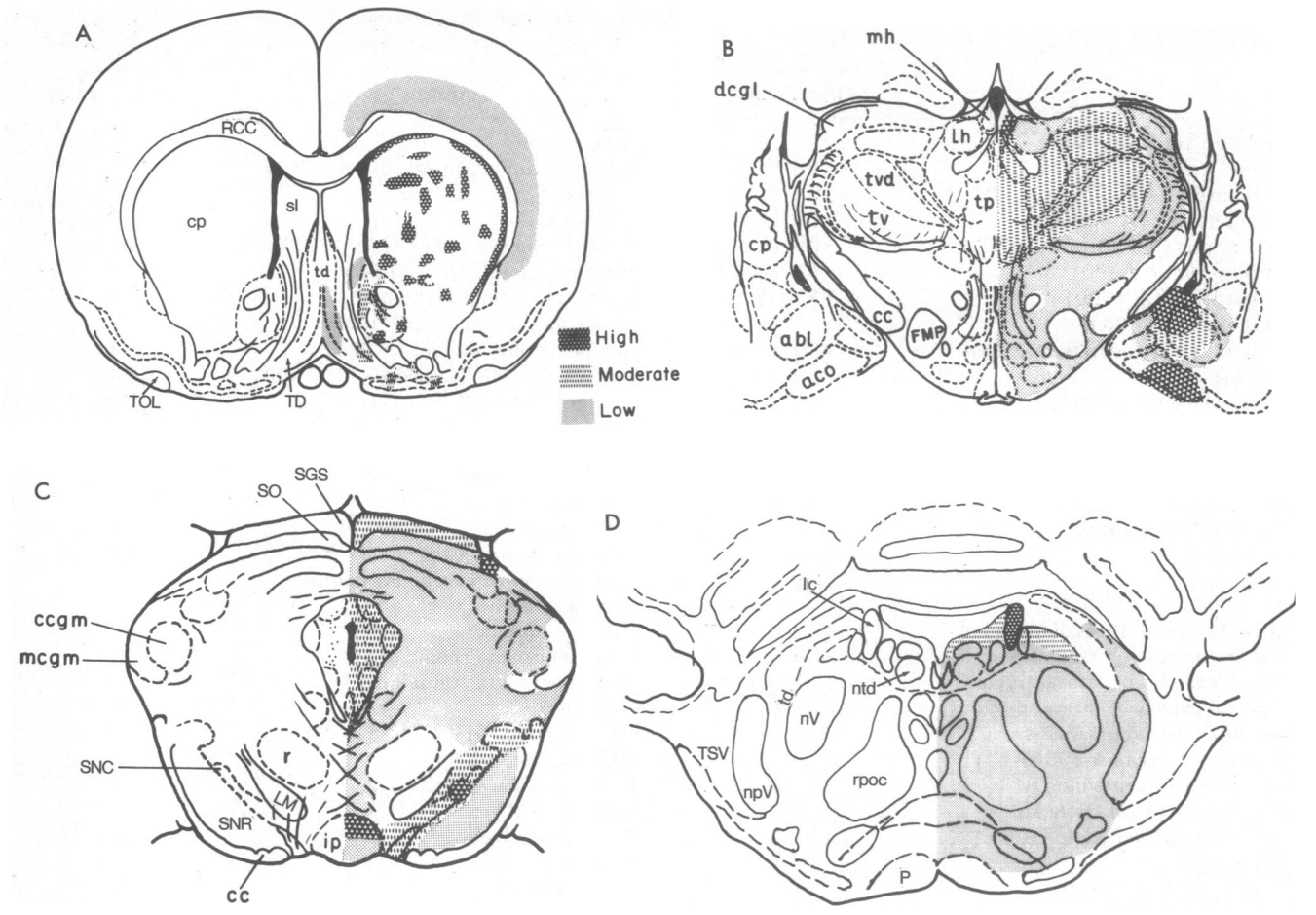

FIG. 4. Variations in autoradiographic grain density in various brain regions. High densities were 30–70 grains/400 μm^2, moderate were 15–30 grains/400 μm^2, and low were 10–15 grains/400 μm^2. Open areas were less than 10 grains/400 μm^2. Levels A, B, and C correspond to levels A 8380, A 3750, and A 1760, respectively, of Konig and Klippel (25). Level D corresponds to level P 2.3 as described by Palkovits and Jacobowitz (27). Abbreviations are as follows: abl, nucleus amygdaloideus basalis, pars lateralis; aco, nucleus amygdaloideus corticalis; ccgm, nucleus centralis corporis geniculati medialis; cp, nucleus caudatus putamen; dcgl, nucleus dorsalis corporis geniculati lateralis; ip, nucleus interpeduncularis; lc, nucleus locus coeruleus; lh, nucleus habenulae lateralis; mcgm, nucleus marginalis corporis geniculati medialis; mh, nucleus medialis habenulae; npV, nucleus principalis nervi trigemini; ntd, nucleus tegmenti dorsalis Gudden; nV, nucleus originis nervi trigemini; r, nucleus ruber; rpoc, nucleus reticularis pontis caudalis; sl, nucleus septi lateralis; td, nucleus tractus diagonalis (Broca); tp, nucleus posteromedianus thalami; tv, nucleus ventralis thalami; tvd, nucleus ventralis thalami, pars dorsomedialis; CC, crus cerebri; FMP, fasciculus medialis prosencephali; LM, lemniscus medialis; P, tractus corticospinalis; RCC, radiatio corporis callosi; SGS, stratum griseum superficiale colliculi superioris; SNC, substantia nigra, zona compacta; SNR, substantia nigra, zona reticulata; SO, stratum opticum colliculi superioris; TD, tractus diagonalis (Broca); TOL, tractus olfactorius lateralis; TSV, tractus spinalis nervi trigemini.

epinephrine cell bodies, is one of the most heavily labeled areas. The locus coeruleus is located at the lateral edge of the periaqueductal gray, which also contains high levels of grains. The floor of the fourth ventricle in the pons-medulla contains a grain density nearly as great as that of the locus coeruleus. The more ventrally located dorsal tegmental nuclei, not immediately adjacent to the fourth ventricle, contain much lower grain densities. While in the medulla gray matter surrounding the fourth ventricle is heavily labeled, in the midbrain the periaqueductal gray contains a lower but still moderate grain density. Negligible grain density is detected in the fourth ventricle.

In the habenular nuclei, highest grain density occurs in the lateral edge of the medial nucleus, more in its anterior than posterior portion. By contrast, the medial edge of the medial habenular nucleus, both rostrally and caudally, and the lateral habenular nucleus contain few grains. Selectivity of localization is further emphasized by the fact that the portion of the hippocampus that is dorsally apposed to the habenular nucleus contains virtually no grains, nor does the third ventricle adjacent to the habenular nucleus. By contrast, the periventricular nucleus close to the habenular nucleus has moderate to low grain densities.

Unlike the marked differences within the relatively small habenular area, little variation in grain density is detected throughout a series of thalamic and hypothalamic nuclei located dorsally, ventrally, anteriorly, and posteriorly. The bulk of the thalamus shows a moderate grain density, while the hypothalamus is lower.

The basal ganglia present a dramatic picture of grain variations. Within the caudate-putamen, grains are highly concentrated in a streak immediately ventral to the corpus callosum, presumably the subcallosal fasciculus, and in clusters throughout the nucleus. Cellular areas between these clusters and fibers homologous to the internal capsule passing through the caudate-putamen contain very few grains. A similar but less intense clustering of grains is observed in the nucleus accumbens and olfactory tubercle, which are closely adjacent to the basal ganglia. Grain densities at different levels of the caudate-putamen vary inversely with the amount of "internal capsule"

fibers so that few grains can be detected posteriorly where these fibers are most concentrated. The globus pallidus contains a fairly low density of evenly distributed grains.

The amygdala, which in biochemical studies contains the greatest opiate receptor binding in monkey brain (1), displays variable levels of grains in different portions, with particularly high densities in the baso-lateral nucleus.

In biochemical studies (1–3), opiate receptor binding is fairly low in the cerebral cortex. Autoradiographic examination shows a tendency for greater grain density in deeper areas of the cortex adjacent to the corpus callosum than in more superficial regions of the cerebral cortex. Extremely few grains are detected in the cerebellum, with no marked variations throughout the cerebellar cortex or deep nuclei.

High levels of grains are observed in the choroid plexus, but in contrast to all other areas enriched in autoradiographic grains, those in the choroid plexus remain after pretreatment of rats with 5 mg/kg of levallorphan and presumably are not associated with opiate receptors.

DISCUSSION

Evidence presented in this and a previous paper (6) that [^{3}H] diprenorphine administered *in vivo* labels the opiate receptor includes regional variations and stereospecificity. The marked heterogeneity of grain distribution in various brain areas, the tendency for grains to occur between rather than over cells, and the sharp boundaries of grain densities in certain areas support the notion that observed localizations are not determined by diffusion, but in fact represent the distribution of opiate receptor sites.

The wide anatomical distribution of opiate receptors observed in this study suggests that opiates can influence a large variety of functional pathways. For example, the high receptor densities in laminae I and II of the spinal cord may be involved in modulation of painful stimuli, since these areas appear related to processing of nociceptive stimuli (13, 14). Also, analgesia is elicited in animals by application of morphine to the region of the periventricular gray matter and medial thalamus, areas enriched in opiate receptors as evidenced by their high grain densities (15, 16). Opiate receptor grains are highly localized to the substantia gelatinosa of the spinal trigeminal nucleus and to components of the vagus neuronal system, including the vagus nerve itself, the dorsal motor nucleus of the vagus, the nucleus tractus solitarius, nucleus ambiguus, and nucleus commissuralis (17). The vagal system is involved in visceral reflexes, such as coughing and vomiting, which are influenced by opiates. Interestingly, the nucleus raphe magnus, which can modulate nociceptive stimuli, projects to the periaqueductal gray, the substantia gelatinosa of the spinal cord and spinal nucleus of the trigeminal, the nucleus tractus solitarius, and dorsal motor nucleus of the vagus, all of which are heavily labeled with opiate-receptor-associated grains (18).

The locus coeruleus, with a high grain density, responds selectively to intravenous (19) or iontophoretic (20) administration of morphine by a slowed firing rate, while closely adjacent areas are unresponsive to morphine. The existence of opiate receptors in the locus coeruleus does not reflect a uniform association of opiate receptors with all norepinephrine cell bodies, because norepinephrine cell bodies of group A-5 (21) have negligible opiate receptor grains.

Light microscopic autoradiography does not permit identification of the ultrastructural site of opiate receptors. Dorsal root lesions and vagotomy, respectively, elicit a loss of opiate receptor binding in the dorsal gray of the spinal cord and in the nucleus tractus solitarius and commissuralis ipsilateral to the lesions (ref. 12, S. Atweh and M. J. Kuhar, in preparation). Because of its relatively rapid time course, the loss of opiate receptors after such lesions is not likely to be due to transneuronal degeneration, so that opiate receptors appear to be associated with axon terminals. Accordingly, in these regions neurons containing the opiate-like peptide enkephalin (4, 22–24) may form axoaxonal synapses with opiate receptors on afferent nerve terminals.

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