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Anatomical Relationship Between Opioid Peptides and Receptors in Rhesus Monkey Brain

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LEWIS, M. E., H. KHACHATURIAN, H. AKIL AND S. J. WATSON. *Anatomical relationship between opioid peptides and receptors in rhesus monkey brain.* BRAIN RES BULL 13(6) 801–812, 1984.—To determine whether opioid peptide-receptor pharmacological associations found *in vitro* (e.g., enkephalin-δ, dynorphin-κ) predict anatomical relationships *in situ*, immunocytochemical and receptor autoradiographic studies were carried out on adjacent sections from the same brains of formaldehyde-perfused rhesus monkeys. Apparent μ and κ opioid receptors (labeled, respectively, by [^{3}H] naloxone and [^{3}H]bremazocine under different incubation conditions), but not δ opioid receptors (labeled by [^{3}H]D-Ala^2, D-Leu^5-enkephalin), survived the fixation procedure, and were found to be colocalized throughout the brain. We have observed complex associations between these binding sites and one, two, or all three opioid peptide systems (i.e., proopiomelanocortin, proenkephalin, and prodynorphin) in different brain regions. These multiple opioid peptide-receptor subtype associations are apparent, for example, in neural systems involved in the processing of pain stimuli, and may be important for mediating different types of analgesia. Since differential processing of proenkephalin and prodynorphin can give rise to opioids of varying receptor selectivities, the colocalization of opioid receptor subtypes may signify that such processing is a key regulatory event in determining which receptor subtype is activated and, thus, the physiological consequences of opioid neurotransmission.

Opioid receptor autoradiography [^{3}H]naloxone [^{3}H]bremazocine Proopiomelanocortin Enkephalin Dynorphin Rhesus monkey brain

THE existence of three precursors for opioid peptides has been clearly established by molecular biological studies [8, 16, 24, 45, 51–53, 62, 63]. These precursors are: (1) proopiomelanocortin (POMC), which gives rise to β-endorphin and its NH_2-terminally acetylated and/or COOH-terminally cleaved forms; (2) proenkephalin, which contains four copies of Met-enkephalin and single copies of Leu-enkephalin, Met-enkephalin-Arg^6-Phe^7, and Met-enkephalin-Arg^6-Gly^7-Leu^8, and gives rise to extended enkephalin peptides such as BAM-22P; and (3) prodynorphin, which gives rise to α-/β-neo-endorphin, dynorphin A, and dynorphin B, all of which contain the Leu-enkephalin sequence at the NH_2 terminus. The peptides derived from these three precursors are contained within three distinct sets of neural systems, as determined by numerous immunocytochemical studies (see [1, 28, 37, 75] for reviews). Although the precursors are neuronally segregated, the enkephalin and dynorphin systems, in particular, exhibit substantial anatomical contiguities in many brain regions [17, 25, 30, 48, 77]. Given the anatomical complexity and biochemical relatedness of the multiple opioid systems, it seems worth considering how the "messages" of these systems are differentiated at the receptor level. Despite a wealth of pharmacological and biochemical evidence for the existence of multiple opioid receptors (see [64] for review), the relationship of these receptors to multiple opioid systems is poorly understood. Nevertheless, pharmacological studies of the endogenous ligands have provided some rationale for making anatomical predictions of ligand-receptor associations. While the pentapeptide enkephalins appear to bind preferentially to the δ opioid receptor subtype [44], prodynorphin-related peptides are usually reported to exhibit selectivity for the κ receptor subtype [7, 9, 57, 81]. In contrast, β-endorphin binds equally well to the μ and δ opioid receptor subtypes [44], but not to the κ subtype [34,57].

To begin to explore whether *in vitro* ligand-receptor associations predicted anatomical relationships *in situ*, we found that it was possible to carry out opioid immunocytochemical and receptor autoradiographic studies on adjacent sections from formaldehyde-perfused rat brain [38]. This initial study revealed an association, in many brain areas, between Leu-enkephalin, a putative δ ligand, and [^{3}H]naloxone binding sites, which appear to correspond to μ opioid receptors under the conditions used. Further studies pointed to a complex relationship between [^{3}H]naloxone binding sites and all three opioid systems in rat brain [40], a finding also obtained in preliminary studies of rhesus monkey brain [39]. In the present report, we extend the study of rhesus monkey brain to describe the relationship between the multiple opioid peptide systems and apparent μ and κ opioid receptors.

LIST OF ABBREVIATIONS

ac	anterior commissure	ot	optic tract
aq	cerebral aqueduct	pc	posterior commissure
at	anterior thalamus	pn	pons
bst	bed n. of stria terminalis	ps	presubiculum
cau	caudate	put	putamen
cc	corpus callosum	pv	periventricular n. of thalamus
ci	internal capsule	pvn	paraventricular n.
cl	claustrum	s	subiculum
cp	cerebral peduncle	sc	superior colliculus
dg	dentate gyrus	sgc	substantia grisea centralis
ip	interpeduncular n.	sn	substantia nigra
ls	lateral septum	son	supraoptic nucleus
m	molecular layer	st	stria terminalis
ma	mammillary n.	v	ventricle
me	median eminence	vta	ventral tegmental area
oc	optic chiasm	VI	cortical layer VI

METHOD

Adult rhesus monkeys *(Macaca mulatta)* were anesthetized with sodium pentobarbital (30 mg/kg, IV), respirated through an endotrachial tube, and perfused through the aorta with 2 liters of saline followed by 16 liters of 0.1 M phosphate-buffer 4% formaldehyde. Some animals were treated, under anesthesia, with colchicine (1–3 mg intracerebroventricularly) 48 hours prior to perfusion. Following perfusion, the head was severed from the trunk, placed in a stereotaxic apparatus, and the top of the calvarium was removed. Within the skull, the brain was cut into 1 cm thick slices in the frontal or parasagital planes using a Kopf stereotaxic device with blades attached. The brain slices were removed from the skull, postfixed in buffered 4% formaldehyde for 2–4 hours at 4°C, and then transferred into 15% sucrose in 0.02 M phosphate-buffered saline (PBS) for overnight incubation at 4°C. The tissues were then frozen by immersion into isopentane at −50°C followed by encasement in pulverized dry ice, and stored in a freezer at −80°C. The frozen tissue blocks were sectioned at 20 μm in a Bright cryostat, and the sections were thaw-mounted onto subbed slides and stored in a freezer at −80°C.

Immunocytochemistry

Sections to be processed for immunocytochemistry were air-dried at room temperature and incubated at 37°C with normal goat serum (NGS, from GIBCO) at a dilution of 1/30 for 10 minutes, followed by primary rabbit antisera (diluted with PBS containing 0.3% Triton X-100) against ACTH (to label POMC neurons), Leu-enkephalin, or dynorphin B(1–13) for one hour at 37°C and overnight at 4°C. Control sections were incubated with primary antiserum preabsorbed with 1–20 μM concentrations of peptide against which the antiserum was raised, as well as with other peptides with amino acid sequences in common with the original peptide. For these cross-blocking studies, the primary antisera were preabsorbed with Leu-enkephalin, BAM-22P, dynorphin B(1–13), dynorphin A(1–17), β-endorphin and ACTH (see [26, 27, 29–33] for antisera characteristics). The next day, the sections were washed in PBS (3× 10 minutes) and incubated with NGS for 10 minutes at 37°C followed by goat antirabbit serum (Arnel) at 1/100 dilution for 30 minutes at 37°C and overnight at 4°C. On the third day, the sections were washed in PBS and incubated with NGS for 10 minutes at 37°C, followed by anti-horseradish peroxidase (anti-HRP) serum at 1/200 dilution for 40 minutes at 37°C. The sections were again washed with PBS and incubated with PBS containing 4 μg/ml HRP enzyme (Sigma, Type VI) for 40 minutes at 37°C. The peroxidase reaction was carried out by incubating the sections in PBS containing 0.03% H_2O_2 and 0.125 mg/ml diaminobenzidine (Sigma) for 15 minutes at room temperature with constant stirring. The reacted sections were washed in distilled water (3× 10 minutes), briefly osmicated (2% OsO_4), washed, dehydrated through ascending concentrations of ethanol and exylenes, and coverslipped in Permount.

Receptor Autoradiography

Sections adjacent or near to those processed for immunocytochemistry were processed for opioid receptor autoradiography [21, 22, 38–40, 42]. In rhesus monkey cerebral cortex, [^{3}H]naloxone and [^{3}H]D-Ala^2, D-Leu^5-enkephalin, under carefully selected assay conditions, bind to different populations of sites which appear to correspond, respectively, to μ and δ opioid receptor subtypes [4, 21, 22]. In addition, [^{3}H]bremazocine, in the presence of unlabelled μ and δ ligands, appears to bind selectively to κ opioid receptors [9]. To label apparent μ sites, slide-mounted sections were incubated in 0.05 M Tris HCl (pH 7.55) containing 1 nM [^{3}H]naloxone (New England Nuclear, 37.7 Ci/mmol) and 100 mM NaCl at 4°C for 60 minutes. To label apparent δ sites, adjacent or nearby slide-mounted sections were incubated in 0.05 M Tris HCl (pH 7.4) containing 1 nM [^{3}H]D-Ala^2,D-Leu^5-enkephalin (New England Nuclear, 39.5 Ci/mmol), 30 mM NaCl, 3 mM $Mn(OAc)_2$, 2 μM guanosine triphosphate, 0.2% bovine serum albumin, and 80 μg bacitracin/ml at 25°C for 30 minutes. To label apparent κ sites, adjacent or nearby sections were incubated in 0.05 M Tris HCl (pH 7.4) containing 2 nM [^{3}H]bremazocine (New England Nuclear, 32.3 Ci/mmol), 100 nM D-Ala^2-MePhe^4-Gly-ol^5-enkephalin (DAGO), and 100 nM D-Thr^2-Leu^5-enkephalin-Thr^6 (DTLET) at 25°C for 60 minutes. Incubations were terminated by transferring the slides through dishes containing 200 ml (at 4°) of 0.1 M phosphate-buffered saline (pH 7.4) for the [^{3}H]naloxone-incubated slides (6× 20 second washes) or 0.05 M Tris HCl buffer (pH 7.55) for the [^{3}H]D-Ala^2,D-Leu^5-enkephalin-incubated slides (6× 20 second washes) and [^{3}H]bremazocine-incubated

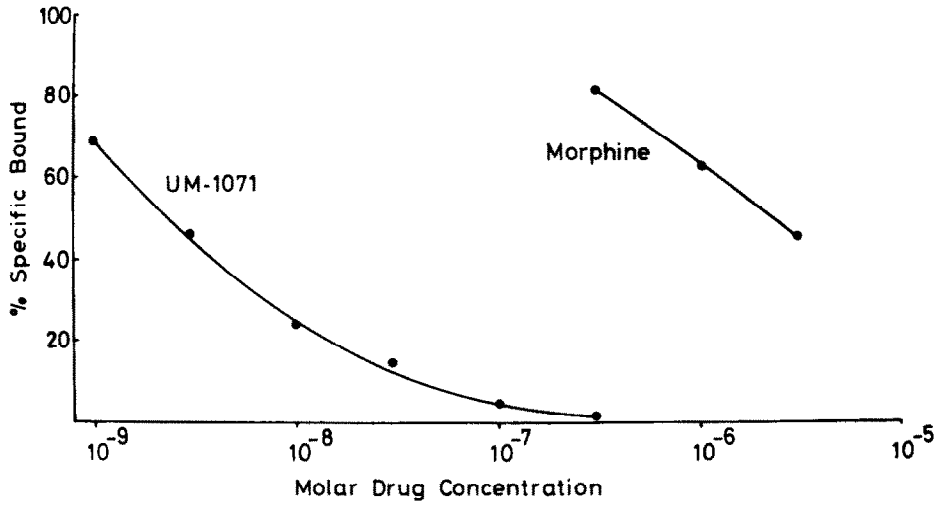

FIG. 1. Competition of UM-1071 (MR 2034) and morphine for specific [^{3}H]bremazocine binding sites (in the presence of 100 nM DAGO and DTLET) in brain sections from formaldehyde-perfused rhesus monkey (determinations in quadruplicate). Note the three order-of-magnitude difference in potency, indicating the κ selectivity of the binding.

slides (4× 4 minute washes). The slide-mounted sections were then dried under a stream of cold air.

Nonspecific binding was determined by adding 1–10 μM levorphanol (for μ and δ binding) or UM-1071 (for κ binding) to the incubation media for some sections, and then measuring the bound radioactivity to elution into scintillation fluid (Safety-Solve; Research Products Int. Corp.) followed by liquid scintillation counting. Other sections prepared for nonspecific binding were saved for autoradiography.

The slide-mounted sections were processed for autoradiography using one of two methods. Some slides were arranged in X-ray cassettes (Spectroline) and exposed to tritium-sensitive film ([^{3}H]Ultrofilm, LKB) for 8–16 weeks. The film was developed in D-19 (Kodak) for 4 minutes at 19°C, washed in 2% acetic acid for 30 seconds, fixed in Rapidfix (Kodak) for 5 minutes, rinsed in water for 20 minutes, dried, and then photographed using an enlarger. Other slides were exposed to paraformaldehyde vapors *in vacuo* at 80°C for 2 hours, demyelinated through ascending concentrations of ethanol, xylenes, and descending concentrations of ethanol, dried, and coated, under safelight illumination, with liquid emulsion (Kodak NTB2, diluted 1:1 with 0.1% Dreft detergent) [22]. The emulsion-coated sections were exposed in light-proof boxes for 8–16 weeks at 4°C and then developed in D-19 for 2 minutes at 16°C, rinsed in water for 15 seconds, fixed in Rapidfix for 3 minutes, rinsed in water for 45 minutes, counterstained lightly with cresyl violet, and coverslipped in Permount. These sections were compared, using darkfield optics, to adjacent or nearby sections which had been processed for immunocytochemistry.

RESULTS

Scintillation counting studies demonstrated that [^{3}H]naloxone binding to rhesus monkey brain sections was approximately 90% specific, which is comparable to results obtained in previous studies of both formaldehyde-fixed [38,40] and unfixed [42] rat brain sections. In contrast, virtually no specific binding of [^{3}H]D-Ala^2,D-Leu^5-enkephalin could be detected in the formaldehyde-fixed rhesus monkey brain sections. The efficacy of the labelling method was confirmed by demonstrating 85–90% specific binding of the tritiated peptide in unfixed rhesus monkey brain sections (data not shown). Binding of [^{3}H]bremazocine to both fixed and unfixed rhesus monkey brain sections was 90–95% specific. To examine the κ selectivity of this binding, competition studies were carried out with UM-1071 (MR 2034), a potent κ agonist, and morphine, the prototypical μ agonist (Fig. 1). Under the conditions used, UM-1071 was three orders of magnitude more potent than morphine (IC_{50}=2.4 nM and 2.2 μM, respectively) in competition with [^{3}H]bremazocine binding to fixed rhesus monkey brain sections.

The LKB Ultrofilm and Kodak wet emulsion autoradiographic methods gave the same results for [^{3}H]naloxone localization, as reported before for rat brain [22,42]. However, bound [^{3}H]bremazocine was not successfully vapor-fixed (see [35]), and was therefore localized using only Ultrofilm.

The anatomical loci of [^{3}H]naloxone and [^{3}H]bremazocine binding sites, using the incubation conditions described, were indistinguishable at every level of the central nervous system which was examined (e.g., Fig. 2). Thus, in the following anatomical descriptions, these sites are referred to as opioid binding sites.

The anatomical distribution of opioid binding sites is particularly pronounced in brain regions which also contain substantial amounts of opioid immunoreactive structures [17, 25, 26]. As an exception, however, the cerebral cortex and hippocampus/dentate gyrus contain a readily visualized population of opioid binding sites (Fig. 6), but very little or no apparent opioid immunoreactive perikarya or fibers. In the striatal complex, distinct patches of [^{3}H]opioid binding sites are seen scattered throughout the caudate (Fig. 2). Both enkephalin and dynorphin immunoreactivity also exist in neuronal perikarya as well as fibers in caudate. Furthermore, POMC-immunoreactive fibers are seen in the rostral-ventral aspect of the head of caudate and putamen. The exact anatomical relationship of these opioid systems to opioid

receptors is not clear at preseennt, and as is the case in the rat brain [40], no apparent correspondence is noted between receptor localization and peptide distribution. Enkephalin and dynorphin perikarya in caudate may project to the globus pallidus and the substantia nigra in monkey as they do in rat [71], but only modest levels of [^{3}H]opioid binding sites are present in either of the latter two regions. Elsewhere in the telencephalon, the bed nucleus of stria terminalis contains a dense concentration of POMC fibers, enkephalin perikarya and terminals, and [^{3}H]opioid binding sites (Fig. 3). Furthermore, in amygdala, the central and medial nuclei receive POMC projections, and contain enkephalin and dynorphin perikarya and fibers. Dense [^{3}H]opioid binding is also seen in all regions of the amygdala.

In the preoptic area, the location of [^{3}H]opioid binding sites (Figs. 3 and 4) corresponds to the distribution of a distinct bundle of immunoreactive POMC fibers coursing through this region. Numerous enkephalin and dynorphin neuronal perikarya and terminals also are seen scattered in the medial preoptic area.

Within the diencephalon, a particularly good correspondence can be seen between opioid peptides and binding sites (Figs. 4 and 5). Unlike in the rat brain, the rhesus monkey hypothalamus is very rich in [^{3}H]naloxone and [^{3}H]bremazocine binding sites. For example, exceptionally heavy labelling is seen in the external layer of the median eminence. Here also, a particularly rich network of enkephalin and POMC terminals is seen surrounding the portal capillaries (Fig. 5). Opioid binding is also heavy in the magnocellular paraventricular (Fig. 4) and supraoptic (Fig. 2) nuclei, as well as periventricular and infundibaular nuclei (Fig. 4). The magnocellular nuclei contain high quantities of dynorphin (Fig. 2) synthesized by the same neurons that also produce vasopressin [74]. These cells may also contain Leu-enkephalin; however, the biosynthetic source of this peptide in the magnocellular neurons is not clear at present. The paraventricular, and to a lesser extent, the supraoptic nucleus, are both innervated by POMC fibers. The infundibular nucleus is very rich in both POMC perikarya and fibers (Fig. 4). This region also contains scattered enkephalin and dynorphin neurons some of which may contribute fibers to the median eminence. In contrast, the lateral and posterior hypothalamic areas contain both lower levels of opioid peptides and [^{3}H]opioid binding sites. In the thalamus, many nuclei are rich in [^{3}H]opioid binding sites, and most of these nuclei also contain enkephalin immunoreactivity. The correspondence is particularly striking in the midline periventricular nucleus, which exhibits a dense accumulation of both binding sites as well as POMC and enkephalin fibers (Fig. 5).

In the region of midbrain, the interpeduncular nuclear complex contains a very dense population of [^{3}H]opioid binding sites (Fig. 3). This nucleus also contains scattered enkephalin immunoreactive perikarya and fibers, yet is virtually devoid of any POMC immunoreactivity. Dorsally in midbrain, there is a clear correspondence in the relative densities of opioid binding sites and opioid immunoreactivity in the substantia grisea centralis (or periaqueductal gray). Here also, a distinct projection of POMC fibers is seen surrounding the cerebral aqueduct (Fig. 3).

In the brainstem, the parabrachial nucleus is richly innervated by POMC and enkephalin fibers and terminals and contains a high density of opioid binding sites. Other pontine nuclei and regions are apparently devoid of opioid peptides and binding sites. In the medulla, a good correspondence between all three opioid peptides and opioid binding sites is seen only in the nucleus tractuas solitarius and the spinal trigeminal nucleus. The latter nucleus, which is in effect the rostral extension of the spinal cord dorsal gray, is particularly rich in enkephalin immunoreactivity distributed in both perikarya and terminals, as well as [^{3}H]opioid binding sites.

DISCUSSION

The present study has described anatomical relationships between multiple opioid immunoreactive systems and [^{3}H]naloxone and [^{3}H]bremazocine binding sites in adjacent or nearby sections from formaldehyde-perfused rhesus monkey brain. The preservation of [^{3}H]naloxone binding sites following formaldehyde perfusion was also found for rat brain [38,40], and we report here that [^{3}H]bremazocine binding sites are also maintained after fixation. These [^{3}H]bremazocine binding sites, labelled in the presence of 100 nM concentrations of DAGO, a selective μ agonist [34], and DTLET, a selective δ agonist [82] appear to correspond to κ opioid receptors. Bremazocine is a very potent κ ligand [65] which should be κ-selective in the presence of excess concentrations of μ and δ ligands [9], as confirmed here in the competition experiment with UM-1071 (MR 2034) and morphine. The anatomical loci of [^{3}H]bremazocine binding sites corresponded exactly with the distribution of [^{3}H]naloxone binding sites. Although the [^{3}H]naloxone incubations were carried out in the absence of δ and κ ligands, there is substantial evidence that low concentrations of [^{3}H]naloxone, under the incubation conditions used, selectively label μ opioid binding sites ([14, 21, 22, 56] but see [43]). Even if [^{3}H]naloxone labelled κ as well as μ opioid receptors in rhesus monkey brain, the identical distribution of [^{3}H]naloxone and [^{3}H]bremazocine binding sites would still indicate the colocalization of μ and κ receptors. Otherwise, the "μ component" of [^{3}H]naloxone binding sites would not correspond to the distribution of [^{3}H]bremazocine binding sites, and some anatomical discrepancy would be apparent. Colocalization of μ and κ opioid binding sites has also been reported in rat brain [56], but not in guinea pig [14] or human [46] brain. The extent to which these findings reflect authentic species differences [10,58] or simply variation in the ways subtypes are labeled remains to be determined. However, in primates, a clear species difference does appear to be present: [^{3}H]bremazocine, in the presence of μ and δ ligands, binds heavily to interpeduncular nucleus in rhesus monkey brain (see Results), but not at all to this structure in human brain [46].

The apparent colocalization of μ and κ opioid receptors throughout rhesus monkey brain is in contrast to the differential distribution of apparent μ and δ opioid receptors in monkey cerebral cortex [41]. A similar pattern also holds for rat brain [15, 42, 59]. We were unable to examine the distribution of δ opioid receptors in the present study since virtually all [^{3}H]D-Ala^2, D-Leu^5-enkephalin binding was abolished by formaldehyde perfusion. This fixation-induced loss of δ binding was also observed for rat brain [40], and may be indicative of a chemical difference in the opioid receptor subtypes [23] or possibly a shift in receptor conformation [4,5], possibilities which we cannot presently discriminate.

The distribution of [^{3}H]naloxone and [^{3}H]bremazocine binding sites in formaldehyde-perfused rhesus monkey brain corresponds very closely to the localization previously reported in studies of unfixed brain with [^{3}H]naloxone [79] and

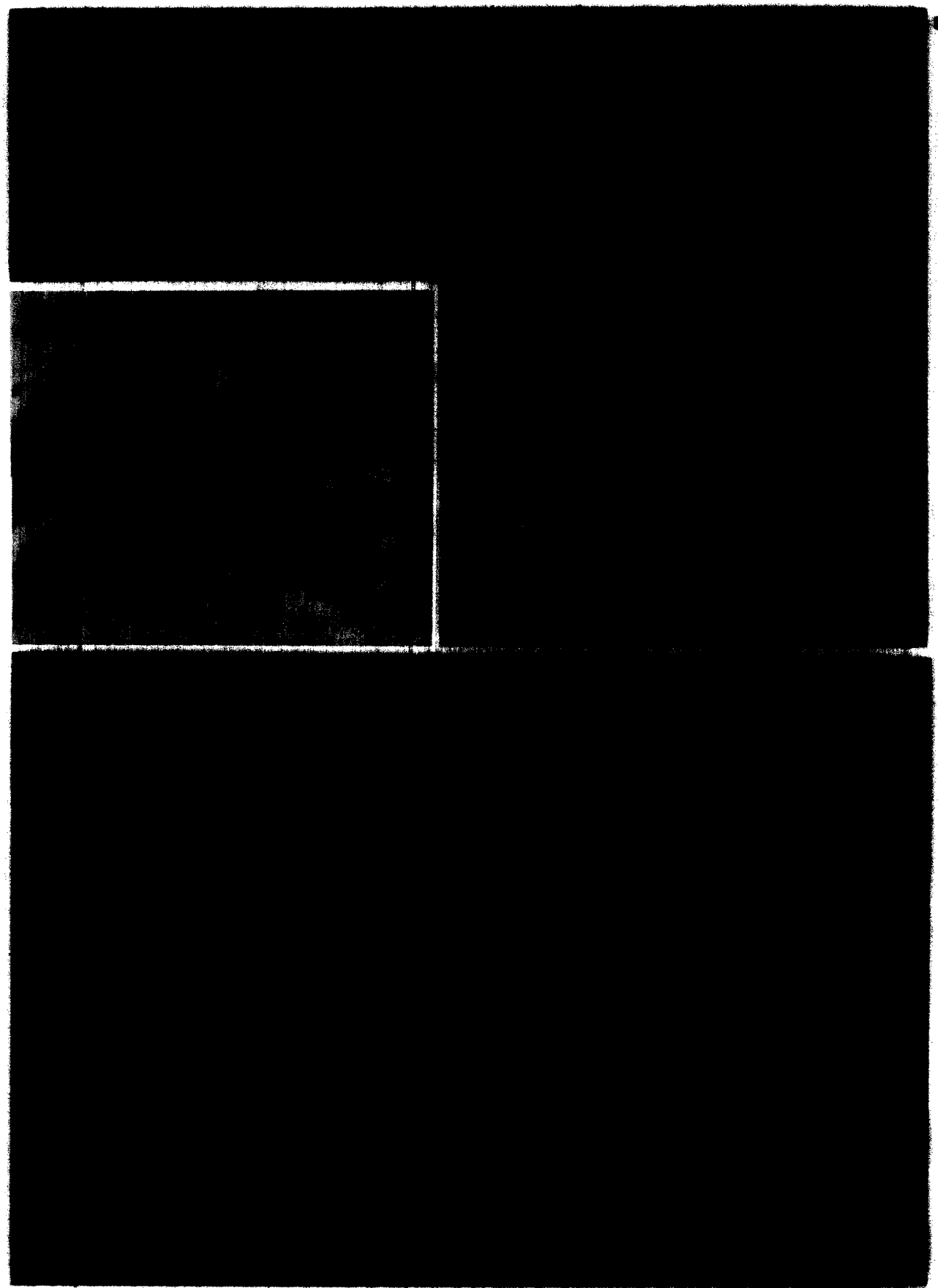

FIG. 2. A and B are near-adjacent parasagittal sections showing [^{3}H]naloxone and [^{3}H]bremazocine binding sites, respectively. Note the similar distribution of binding sites in all regions; e.g., arrows point to identical patches of sites in the caudate. Note also the intense binding in the supraoptic nucleus, and its correspondence with dynorphin B(1–13) immunoreactive neurons in this nucleus (A: inset).

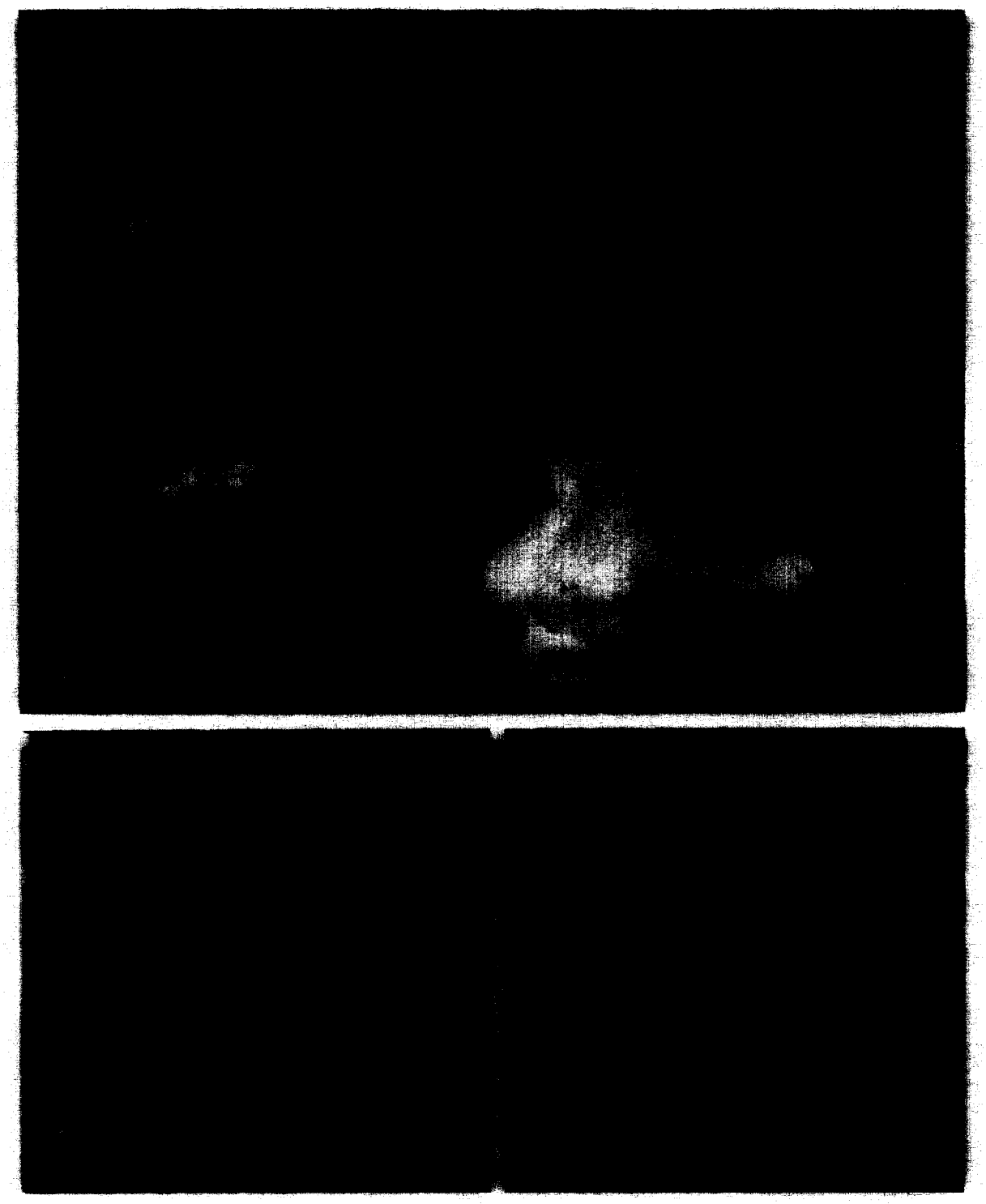

FIG. 3. A shows [^{3}H]bremazocine binding sites in a parasagittal section. In adjacent sections B and C (see arrows in A), immunoreactive ACTH fibers are shown in the substantia grisea centralis (B) and the bed nucleus of stria terminalis (C).

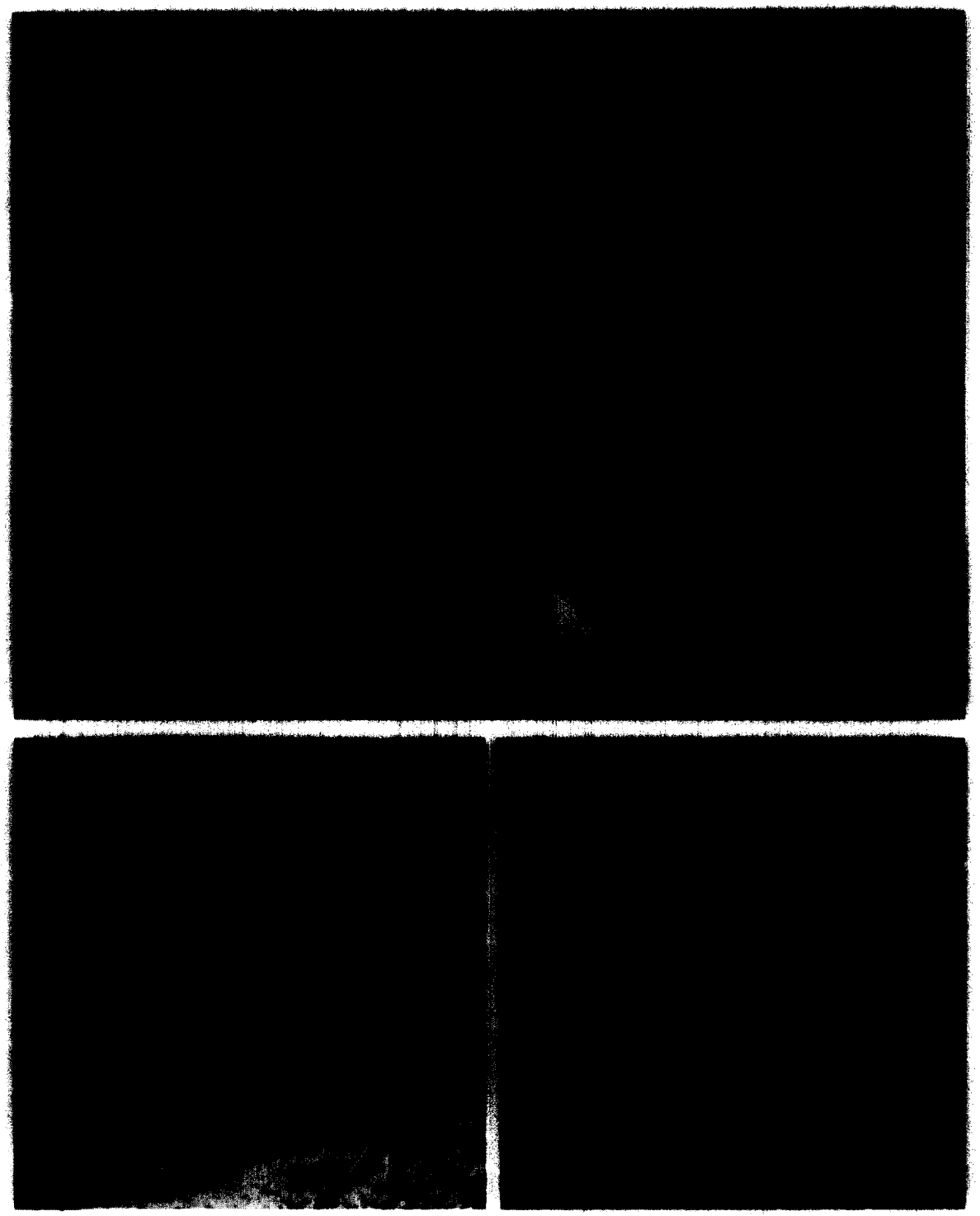

FIG. 4. In A, [^{3}H]bremazocine binding sites are seen in a parasagittal section close to midline. B and C (see arrows in A) are adjacent sections which show the distribution of ACTH immunoreactive fibers in the infundibular nucleus (B) and the bed nucleus of anterior commissure (C).

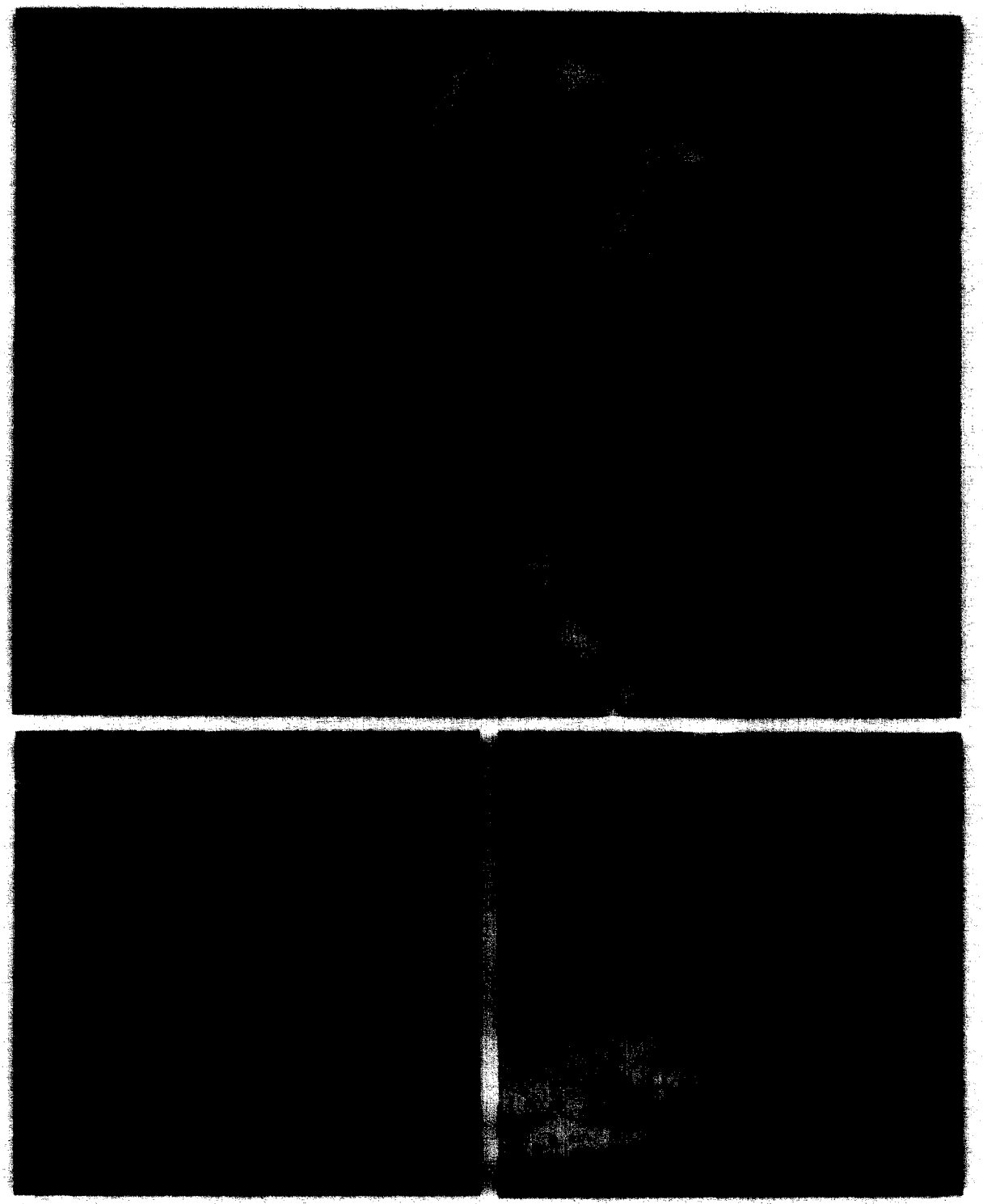

FIG. 5. [^{3}H]bremazocine binding sites are seen in a mid-sagittal plane (A). In adjacent sections B and C (see arrows in A), ACTH immunoreactive fibers are seen in the periventricular nucleus of thalamus (B), and diffuse Leu-enkephalin immunoreactivity is shown in the median eminence (C).

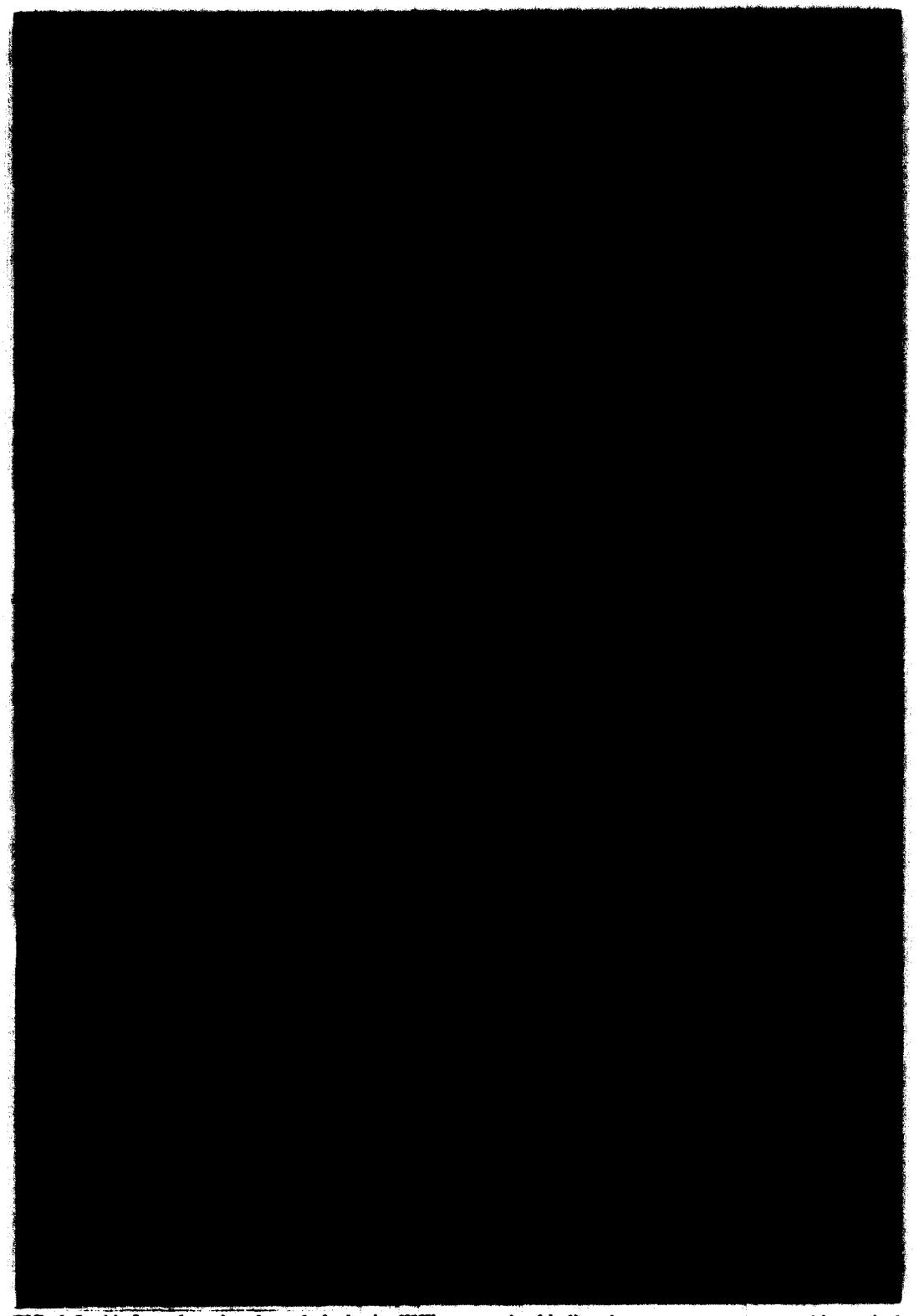

FIG. 6. In this frontal section through the brain, [^{3}H]bremazocine binding sites are seen concentrated in cortical layer VI (arrow points to area TE, visual association cortex), claustrum, presubiculum and subiculum, as well as stratum granulosum (g) and stratum moleculare (m) of the dentate gyrus.

[^{3}H]dipremorphine [73]. The preservation of the anatomical distribution of [^{3}H]naloxone and [^{3}H]bremazocine binding sites following formaldehyde perfusion has allowed us to perform receptor autoradiographic and opioid peptide immunocytochemical studies on adjacent sections from the same brain. Although general anatomical relationships between endogenous ligands and receptors can be discerned by comparing tissue sections from brains processed differentially for immunocytochemistry and autoradiography [69,70], such comparisons are difficult due to problems in section alignment, the complexity of receptor distribution, and the anatomical relatedness of the multiple opioid systems in many regions.

Using adjacent section analysis, we have detected associations between apparent multiple opioid receptors and one, two, or all three opioid systems in many regions of rhesus monkey brain [39]. The neural systems involved in the processing of painful stimuli [1, 3, 36] provide a good example of these multiple associations. The spinal trigeminal nucleus, the rostral continuation of the spinal cord dorsal gray, is particularly rich in both enkephalin and dynorphin perikarya and terminals and apparent μ and κ opioid receptors. These peptide systems and receptors appear thoroughly interdigitated, at least at the light microscopic level. The periaqueductal gray, or substantia grisea centralis, contains substantial enkephalin and POMC (and some dynorphin) immunoreactivity which occurs in complex relation to the multiple opioid receptors. Stimulation of this region produces profound analgesia in monkeys [47,54] and humans [60,61], and the role of the multiple opioid systems and receptors in mediating antinociception requires further study. Thalamic nuclei involved in pain processing (e.g., midline periventricular nucleus) also show a correspondence between POMC and enkephalin immunoreactivity and apparent μ and κ opioid receptors. While much attention has been focused on the role of μ receptors in mediating opioid analgesia, κ agonists also have significant antinociceptive actions, though apparently on different types of pain [6, 19, 55, 67, 68]. It is also worth noting that μ and κ ligands produce centrally discriminable effects in rhesus monkeys [20,80]. Given the apparent colocalization of μ and κ opioid receptors in the species used in the antinociception tests (rats) and drug discrimination paradigms (rhesus monkeys), the behavioral differentiation of μ and κ effects has interesting implications. These opioid receptor subtypes, although colocalized at the light microscopic level, may have different synaptic loci (e.g., pre- vs. post- vs. non-synaptic) which would determine their actions. This question can perhaps be explored to some extent by electron microscopic localization of opioid receptors [18]. Still another possibility for μ-κ differentiation is at the level of receptor-effector relationships [4, 50, 78].

Despite the close correspondence of opioid peptides and receptors in many areas, there is no apparent relationship in other regions (see also [37,40]). For example, in cerebral cortex and hippocampus/dentate gyrus, no immunoreactivity could be detected (see also [17]), despite clear lamination of opioid binding sites in these areas. This discrepancy may be due to a relative lack of sensitivity of immunocytochemistry, since rhesus monkey cerebral cortex appears to contain measurable levels of met-enkephalin-like immunoreactivity as determined by radioimmunoassay (unpublished results). In rat, also, autoradiographic procedures had demonstrated cortical opioid receptors [2] at a time when immunocytochemical studies failed to detect significant cortical opioid peptide immunoreactivity [11, 69, 76]. However, as a result of technical improvements, later immunocytochemical studies did show enkephalin and dynorphin perikarya and fibers in rat cerebral cortex [12, 13, 27, 28, 32, 33, 48, 49, 66, 72].

The purpose of the studies described here was to determine whether the anatomical relationship of opioid peptide systems and receptors was related to peptide-receptor affinities *in vitro*. The complexity of the association between the multiple opioid systems and apparent μ and κ opioid receptors evidently precludes a straightforward one-to-one view of opioid peptide-receptor relationships. Thus, the anatomical associations predicted by *in vitro* receptor studies (e.g., enkephalin-δ or dynorphin-κ) are too simplistic. Indeed, the prediction itself was too simplistic, since it is now known that the dynorphin and enkephalin precursors can give rise to a diversity of opioids with widely differing receptor selectivities. For example, some extended proenkephalin peptides have μ and κ activity [57], while the κ selectivity of dynorphin A is lost upon COOH-terminal cleavage [9,57]. Since differential processing of proenkephalin and prodynorphin can give rise to opioids of varying receptor selectivities, the colocalization of opioid receptor subtypes may signify that such processing is a key regulatory event in determining which receptor subtype is activated and, thus, the physiological consequences of opioid neurotransmission.

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