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Pharmacological and Anatomical Evidence of Selective μ, δ, and $\varkappa$ Opioid Receptor Binding in Rat Brain

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Key words: Opioid receptors, μ, δ, $\varkappa$ — Autoradiography — Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO) — 2-D-Penicillamine-5-D-penicillamine-enkephalin (DPDPE) — Bremazocine

While the distribution of opioid receptors can be differentiated in the rat central nervous system, their precise localization has remained controversial, due, in part, to the previous lack of selective ligands and insensitive assaying conditions. The present study analyzed this issue further by examining the receptor selectivity of [^{3}H]DAGO (Tyr-D-Ala-Gly-MePhe-Gly-ol), [^{3}H]DPDPE (2-D-penicillamine-5-D-penicillamine-enkephalin), [^{3}H]DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) and [^{3}H](−)bremazocine, and their suitability in autoradiographically labelling selective subpopulations of opioid receptors in rat brain. The results from saturation, competition, and autoradiographic experiments indicated that the three opioid receptor subtypes can be differentiated in the rat brain and that [^{3}H]-DAGO and [^{3}H]DPDPE selectively labelled μ and δ binding sites, respectively. In contrast, [^{3}H]DSLET was found to be relatively non-selective, and labelled both μ and δ sites. [^{3}H]Bremazocine was similarly non-selective in the absence of μ and δ ligands and labelled all three opioid receptor subtypes. However, in the presence of 100 nM DAGO and DPDPE, concentrations sufficient to saturate the μ and δ sites, [^{3}H]bremazocine did label $\varkappa$ sites selectively. The high affinity [^{3}H]bremazocine binding sites showed a unique distribution with relatively dense $\varkappa$ labelling in the hypothalamus and median eminence, areas with extremely low μ and δ binding. These results point to the selectivity, under appropriate conditions, of [^{3}H]DAGO, [^{3}H]DPDPE and [^{3}H]bremazocine and provide evidence for the differential distribution of μ, δ, and $\varkappa$ opioid receptors in rat brain.

INTRODUCTION

Several lines of evidence have suggested the existence of multiple opioid receptor subtypes in the central and peripheral nervous systems[1,25,28,31,33]. These receptor subtypes have been referred to in various ways but, are commonly known as μ, δ, and $\varkappa$. The existence of multiple forms or subtypes of opioid receptors was first suggested in the chronic spinal dog preparation by Martin and his colleagues[5,19], and has since been supported by numerous behavioral, pharmacological, and receptor binding studies[6,7,11,12,32]. One line of evidence is support of multiple opioid receptors in their differential distribution in the nervous system[1,3,9,16,20,23,27]. This has been demonstrated either with membrane homogenates from dissected regions of brain and spinal cord or with the use of in vivo and in vitro autoradiographic techniques. The use of the latter methods, while more time consuming, has provided otherwise unobtainable anatomical detail and resolution.

While the overall autoradiographic results suggest that the μ and δ opioid subtypes are differentially localized in the nervous system, the precise distributions of these receptors has remained controversial. For instance, some investigators have argued that δ receptors are diffusely distributed throughout cortex[16], while others suggest that they vary with cortical laminae[9,27]. Similarly, some studies have reported μ and δ sites to be densely distributed in the hypothalamus[3,23], while others have reported either the presence of predominantly μ sites[9] or sparse, if any, opioid binding[27]. Inconsistencies have also been found in other limbic structures such as the amygdala

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and hippocampus, as well as midbrain periaqueductal grey area[3,9,27].

The recent autoradiographic localization of $\varkappa$ receptors has only added to the confusion. Goodman and Snyder[8] have reported that $\varkappa$ sites are uniquely distributed in the guinea pig, with dense labelling in the deep layers (V and VI) of cortex and diffuse binding in the caudate-putamen. Quirion et al.[26] confirmed these findings in the guinea pig, but failed to find similar results in the rat. They reported that, in contrast to their findings in the guinea pig, μ and $\varkappa$ receptors were similarily distributed in the rat. These results in the rat were not, however, confirmed by more recent studies[17].

While there are several reasons for these conflicting results, the following are perhaps the most likely. First, some studies provide little evidence to suggest they are selectively labelling a single population of sites. Often there is a reliance on data from other laboratories, and if competition studies are performed, they are often done on membrane homogenates which may have different properties than the slide-mounted brain sections normally used for receptor autoradiography. Second, many studies have arbitrarily used a 1–2 nM concentration of tritiated ligands or 0.1–0.2 nM concentration of iodinated ligands to map the receptor sites. Often little or no information is given as to percentage of sites occupied by this labelling concentration or why this concentration was used. At times, the rationale for using these low concentrations of ligands has been to assure that the drug is selectively labelling the desired receptor site, but this may not be necessary with the use of more selective ligands. The choice of labelling concentration is, in fact, a major consideration when comparing the distribution of several receptor subtypes which have varying affinities, since it would otherwise be impossible to determine whether the results were due to differences in the percentage of receptors occupied or anatomical differences in their distributions. To properly compare the receptor subtypes one needs to perform saturation studies on slide-mounted sections and on the basis of these results vary the ligand concentration in order to label an equivalent percentage of sites. To our knowledge this has not been done in comparing to opioid subtypes.

A third problem that has plagued investigators has been the lack of selective ligands and this too has led to some confusion as to the distribution of the multiple opioid receptor subtypes. Recently, this problem has been reduced with the development of ligands that selectively label μ and δ sites. DAGO (Tyr-D-Ala-Gly-MePhe-Gly-ol) and DPDPE (2-D-penicillamine-5-D-penicillamine-enkephalin) have been shown to be selective μ and δ agonists, respectively, in receptor binding assays using brain homogenates and peripheral organ bioassays[10,12,13,21,22]. While a selective agonist has been developed for $\varkappa$ receptors[30], its radiolabelled form is still not widely available.

In this paper we address the issues outlined above and have determined the binding kinetics and selectivities of various opioid ligands under autoradiographic conditions. Once established, these parameters will guide us in designing experiments aimed at examining the distribution and modulation of the opioid receptor subtypes. One of the strategies we have employed involves performing saturation studies using slide-mounted brain sections in the presence and absence of a competing ligand. If, for instance, a ligand is a selective δ agonist, then the addition of a saturating concentration of a selective μ ligand should have no effect on its binding capacity or affinity. If, however, the μ and δ ligands competitively inhibited one another, then one would conclude that the ligands were not selectively labelling a uniform population of receptors. The radioligands examined in this study were DAGO, DPDPE, DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr)[2,4], and bremazocine[29]. As bremazocine has been reported to have a high affinity for all three (μ, δ, $\varkappa$) opioid receptor subtypes[18], we compared its binding characteristics in the presence and absence of both μ and δ agonists. This was done in one of two ways; either a constant final concentration of DAGO and DPDPE was added to all the concentrations of [^{3}H]bremazocine examined, as is conventionally done, or a constant ratio of DAGO and DPDPE to [^{3}H]bremazocine was maintained across all the concentrations of [^{3}H]bremazocine studied.

Once the binding kinetics had been established for each of the ligands, a series of competition studies was performed using slide-mounted brain sections to characterize the binding observed under autoradiographic conditions. Following the conclusion of these studies, autoradiograms were produced with

these ligands at concentrations designed to label an equivalent percentage of receptor sites to determine if they indeed labelled anatomically discrete subpopulations of receptors.

MATERIALS AND METHODS

Adult male Sprague–Dawley rats were sacrificed by decapitation and their brains were quickly removed and frozen in liquid isopentane (−30 °C) for 30 s. Frozen brains were sectioned on a Bright cryostat (25 μm) and thaw-mounted on precleaned and subbed microscope slides. The brain sections were then dried overnight at 4 °C under reduced pressure in a glass desiccator and stored in a −80 °C freezer. The saturation and competition results presented were derived from rat forebrain and midbrain sections. The slide-mounted sections were gradually brought up to room temperature and incubated with various tritiated opioid ligands in 50 mM Tris buffer (pH 7.5 at 25 °C). The tritiated opioids used were DAGO (45 Ci/mmol) and DPDPE (33 Ci/mmol) purchased from Amersham, and DSLET (30.5 Ci/mmol) and (−)bremazocine (41.4 Ci/mmol) purchased from New England Nuclear.

The concentrations used in the saturation studies varied from 0.16 to 40 nM for DPDPE, DSLET and bremazocine, and 0.08–20 nM for DAGO. The slides were placed in incubation chambers designed to maintain ambient temperature (25 °C) and 60–80% relative humidity. The brain sections were incubated with 200 μl of [^{3}H]ligand and buffer. A minimum of nine concentrations was used for each of the ligands in performing the saturation studies. Following a 60 min incubation period, the slides were drained and washed in four consecutive 250 ml 50 mM Tris washes (pH 7.6, 4 °C). Slides incubated with DAGO, DPDPE and DSLET were given four 30-s washes, while those incubated with bremazocine were given four 4 minute washes. Following the Tris washes, all slides were rinsed (2 s) in a 250 ml wash of distilled water (4 °C) and quickly dried with a portable hair dryer set to cool. Non-specific binding was evaluated by treating a parallel set of slides with the same concentrations of [^{3}H]ligand with a 1 μM final concentration of an unlabelled competitor: levorphanol to displace [^{3}H]DAGO, DSLET to displace [^{3}H]DSLET or [^{3}H]DPDPE, and UM 1071 ((−)-(1R,5R,9R,2′′S)-5,9-dimethyl-2-tetrahydrofurfuryl-2′-hydroxyl-6,7-benzomorphan) to displace [^{3}H]bremazocine.

Saturation experiments with [^{3}H]DAGO were performed in the presence and absence of 100 nM final concentration of either DPDPE or DSLET. This concentration of unlabelled ligands was determined by pilot studies to be sufficient to saturate the delta sites. Conversely, [^{3}H]DSLET and [^{3}H]DPDPE saturations were performed in the presence and absence of 100 nM DAGO, a concentration previously determined to be sufficient to saturate the available μ sites. Saturation studies with [^{3}H]bremazocine were performed either in the presence and absence of a constant final 100 nM concentration of DAGO and DPDPE or with a constant ratio of [^{3}H]bremazocine and unlabelled DAGO and DPDPE (approximately 1:160). With this latter procedure only 7 concentrations of [^{3}H]bremazocine were used ranging from 0.1–6.2 nM. All of the above saturation experiments were performed at least twice.

The binding was quantitated by placing the brain sections with the underlying glass in scintillation vials containing 10 ml of scintillant and vigorously shaking them for 30 min in a metabolic shaker. Each data point is an average of a minimum of two brain sections. The data were graphed as Scatchard plots and the K_d and B_{max} values were determined with the LIGAND program developed by Munson and Rodbard[24].

Since specific binding varied with ligand and concentration, we found in preliminary studies that a concentration three times the K_d value provided acceptable levels of specific binding with all the [^{3}H]ligands used while occupying 75% of each of the available sites. In other words, these concentrations provided the maximal equivalent receptor occupancy without sacrificing specific binding. To characterize these binding sites further, competition studies were performed with a series of μ (DAGO, morphine, levorphanol) δ (DSLET, DPDPE, DADL (D-Ala-D-Leu-enkephalin)), and κ (EKC (ethylketocyclazocine), UM 1071, U50,488H (methanesulfonate, hydrate), bremazocine) agonists. In each case, a [^{3}H]ligand concentration equal to three times the K_d was used in order to evaluate the sites that would be labelled under our autoradiographic mapping conditions. The brain sections were incubated, washed,

and dried and the amount of binding was quantified as described above.

Following the completion of the competition studies, brain sections from three rats at the level of the hypothalamus and periaqueductal grey were selected for autoradiographic mapping. The slides were incubated, washed, and dried as described above and apposed to tritium-sensitive LKB Ultrofilm in X-ray cassettes. The films were exposed at room temperature and developed in Kodak D-19 (4 min, 19 °C), agitated in 2% acetic acid (30 s), and fixed in Kodak Rapidfix (5 min). The films were then washed in running water (30 min) before air drying. Film exposure time varied with ligand; slides treated with [^{3}H]-DAGO, [^{3}H]DSLET, and [^{3}H]DPDPE were exposed to LKB film for 34 days, while those treated with [^{3}H]bremazocine were exposed for 92 days.

RESULTS

Saturation studies

As shown in Fig. 1A, the presence of 100 nM DPDPE produced only minor changes in the binding parameters of [^{3}H]DAGO, the putative μ-selective ligand. The K_d and B_{max} of [^{3}H]DAGO were 1.4 nM and 180 pM, respectively, in the absence of DPDPE as compared to 1.6 nM and 176 pM in the presence of DPDPE. Similarly, the presence of 100 nM DAGO did not appreciably alter the binding parameters of [^{3}H]DPDPE, the putative δ-selective ligand (Fig. 1B). The K_d and B_{max} of [^{3}H]DPDPE were 8.0 nM and 349 pM, respectively, in the absence of DAGO, as compared to 11.1 nM and 317 pM in the presence of 100 nM DAGO. The saturation data for both [^{3}H]DAGO and [^{3}H]DPDPE were best fitted by a single-site model as determined using the LIGAND program. Taken together, these results indicate that DAGO and DPDPE label two separate populations of sites which presumably correspond to the μ and δ sites, respectively.

The presence of 100 nM DSLET in the [^{3}H]DAGO binding assay did, however, substantially alter its binding constants. As shown in Fig. 2A, DSLET competitively inhibited the binding of [^{3}H]DAGO. The presence of DSLET substantially altered the K_d of [^{3}H]DAGO (1.2 nM in absence of DSLET and 7.6 nM in the presence of DSLET), but did not change its binding capacity. Similarly, the presence of 100 nM

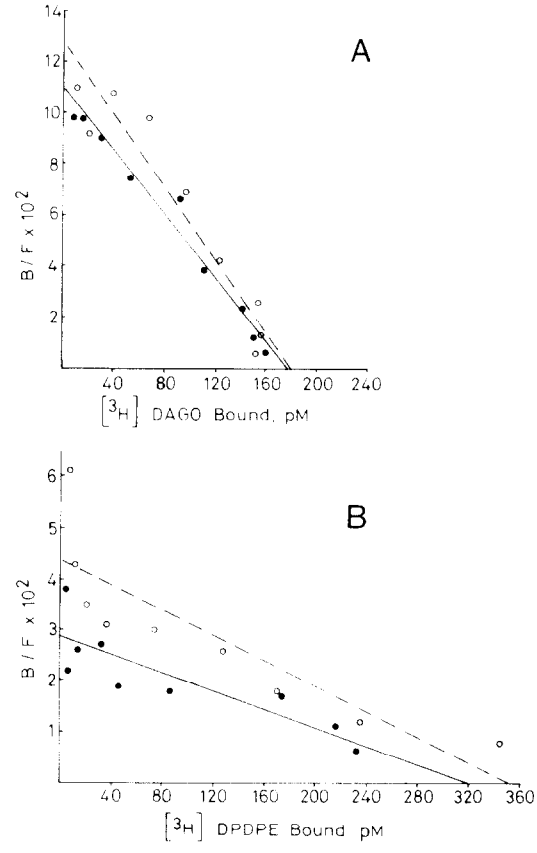

Fig. 1. A: Scatchard plot of [^{3}H]DAGO binding in the presence (●——●) and absence (○---○) of 100 nM DPDPE. B: Scatchard plot of [^{3}H]DPDPE binding in the presence (●——●) and absence (○---○) of 100 nM DAGO. The B_{max} data are expressed as pM (picomolar) instead of fmol/mg tissue conventionally used in homogenate binding because tissue weights vary somewhat from one series of brain sections to another. The pM estimates can be converted to fmol/brain section by dividing by 5. The mean tissue weight of sections used in this and in the other Scatchards was 692 μg ± 103. Within a series of sections the variation in tissue weight is substantially reduced and varies from 0.5–9.6% depending on the forebrain region used. The design of the experiments was such that adjacent sections were used in performing the saturations in the presence or absence of selective agonists in order to minimize variations in brain region and tissue weight.

DAGO in the [^{3}H]DSLET binding assay altered its binding constants. The K_d and B_{max} of [^{3}H]DSLET were 7.0 nM and 378 pM, respectively, in the absence of DAGO, as compared to 4.4 nM and 185 pM in the presence of 100 nM DAGO (Fig. 2B). Also as shown in Fig. 2B, DAGO appeared to non-competitively inhibit the binding of [^{3}H]DSLET. These results suggest that DSLET, unlike DPDPE, labels at least two sites: a presumed μ site for which DAGO has

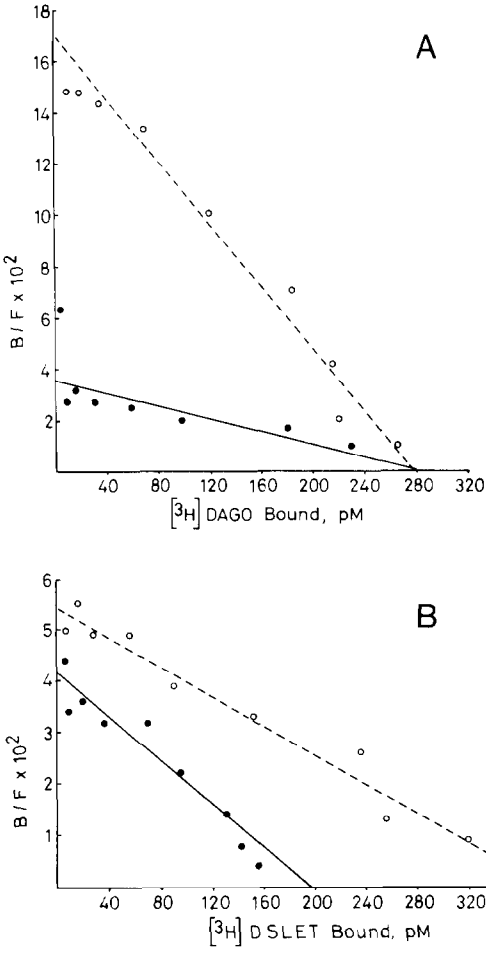

Fig. 2. A: Scatchard plot of [^{3}H]DAGO binding in the presence (●——●) and absence (○---○) of 100 nM DSLET. B: Scatchard plot of [^{3}H]DSLET binding in the presence (●——●) and absence (○---○) of 100 nM DAGO.

high affinity and a presumed δ site which does not bind DAGO.

In agreement with earlier studies[18], [^{3}H]bremazocine showed a lack of selectivity and its binding parameters were substantially altered in the presence of 100 nM DAGO and DPDPE. The binding data for [^{3}H]bremazocine alone were best fitted by a single-site model with a K_d of 0.9 nM and B_{max} of 447 pM (Fig. 3A). The presence of 100 nM DAGO and DPDPE in the binding assay altered the resulting Scatchard plot so that the data were best fitted by a two-site model. The high affinity site had a K_d of 0.2 nM with a B_{max} of 34 pM, while the low affinity site had a K_d of 18.6 nM with a B_{max} of 428 pM. If, however, a constant ratio of [^{3}H]bremazocine and unlabelled μ and δ blockers is used in performing the saturation study, only a single high affinity (0.3 nM) site is observed (Fig. 3B).

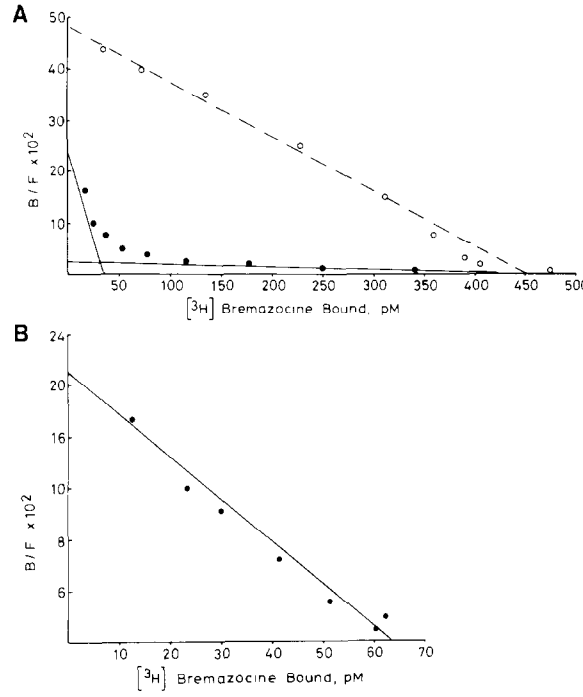

Fig. 3. A: Scatchard plot of [^{3}H]bremazocine binding in the presence (●——●) and absence (○---○) of 100 nM DAGO and DPDPE. B: Scatchard plot of [^{3}H]bremazocine in the presence of a constant ratio (1:160) of unlabelled DAGO and DPDPE to block μ and δ sites.

Competition studies

In order to further characterize the pharmacological profile of the ligands, a series of competition studies were conducted. The results from these studies are presented in Table I. All three μ compounds examined were more potent at sites labelled by [^{3}H]DAGO than sites labelled by [^{3}H]DPDPE. DAGO was the most selective of the μ compounds showing a ratio of IC_{50}s for DAGO/DPDPE of 0.003, followed by morphine (0.045) and levorphanol (0.081). The δ compounds, on the other hand, showed a greater affinity for those sites labelled with [^{3}H]DPDPE. DPDPE was the most selective with an IC_{50} ratio for DAGO/DPDPE of 115.8, followed by DSLET (10.6) and DADL (4.0). Thus, DPDPE appears to be an order of magnitude more selective than DSLET, which is consistent with the saturation studies presented above. Both the μ and δ com-

TABLE I

IC_{50} concentrations (nM) of various compounds competing for μ, δ, and κ sites

	[^{3}H]DAGO	[^{3}H]DPDPE	[^{3}H]Bremazocine*
Levorphanol	3.4	42.2	100.0
DAGO	7.5	2371.0	–
Morphine	25.1	562.0	2985.0
DADL	89.1	22.4	11,220.0
DSLET	149.6	14.1	5,623.0
DPDPE	3000.0	25.9	–
Bremazocine	9.4	11.2	3.8
UM 1071	6.9	50.1	18.8
EKC	28.2	39.8	35.5
U50,488H	11,220.0	>10,000.0	681.0

* [^{3}H]Bremazocine binding was done in the presence of 100 nM DAGO and DPDPE.

pounds, however, showed a low affinity for those sites labelled by [^{3}H]bremazocine in the presence of 100 nM DAGO and DPDPE.

Consistent with previous reports[18], bremazocine, UM 1071 and EKC were non-selective and competed for the sites labelled by all three [^{3}H]ligands at relatively low nanomolar concentrations. U50,488H, on the other hand, did show a selectivity for those sites labelled with [^{3}H]bremazocine with an IC_{50} ratio for DAGO/bremazocine of 16.5. These results, in conjunction with those reported above, indicate that under our autoradiographic conditions, [^{3}H]DAGO, [^{3}H]DPDPE, and [^{3}H]bremazocine (in the presence of 100 nM DAGO and DPDPE) selectively label μ, δ, and κ sites, respectively.

Autoradiographic studies

The labelling of brain sections with [^{3}H]bremazocine (in the presence of unlabelled 100 nM DAGO and DPDPE), [^{3}H]DAGO, or [^{3}H]DPDPE produced three distinct anatomical patterns of opioid binding that presumably correspond to κ, μ, and δ receptors. At the level of the hypothalamus (Fig. 4), μ binding was prominent in cortical layers 1 and 4, hippocampus, medial habenula, certain thalamic nuclei (e.g. mediodorsal, paracentral, rhomboid and reunions), and the lateral and medial nuclei of the amygdala. Only extremely low levels of μ binding were observed in the hypothalamus and in the caudal part of the caudate-putamen. In contrast, delta binding as measured by [^{3}H]DPDPE was concentrated in cortical layers 2, 3, 5 and 6, caudate-putamen, and the lateral and basolateral nuclei of the amygdala. Only low levels of diffuse delta binding were observed in the thalamic and hypothalamic nuclei. [^{3}H]Bremazocine in the presence of μ and δ agonists labelled a third anatomically distinct subpopulation of receptors, with relatively dense areas of binding in the amygdala and midline thalamic nuclei (particularly the periventricular nucleus), the lateral hypothalamic area, the

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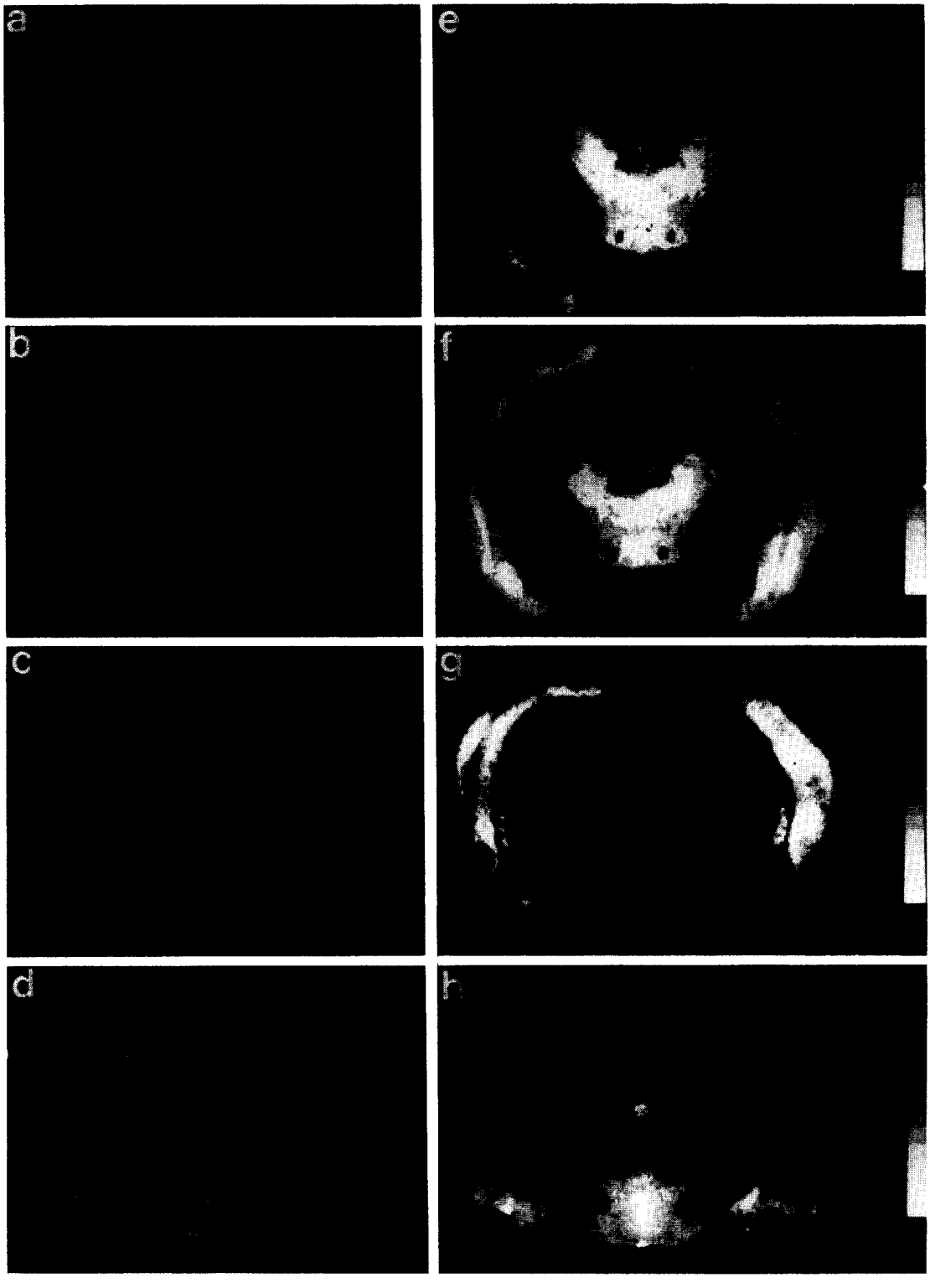
Fig. 4. [^{3}H]DAGO (a), [^{3}H]DSLET (b), [^{3}H]DPDPE (c), and [^{3}H]bremazocine (d) dark-field autoradiograms at the level of the hypothalamus. Sections treated with [^{3}H]bremazocine (0.9 nM) were incubated in the presence of 100 nM DAGO and DPDPE. The nanomolar concentrations of the other ligands were as follows: DAGO 4.5, DSLET 23.3, and DPDPE 31.4. These concentrations of radioligands are 3 times the K_d value of each of the ligands as determined from averages of 2–6 experiments. The amount of specific binding observed in producing the autoradiograms varied with the ligands and binding conditions. As determined by scintillation counting, [^{3}H]DAGO showed the highest amount of specific binding (91%), followed by [^{3}H]DSLET (81%), [^{3}H]bremazocine (79%), and [^{3}H]DPDPE (66%). The structures identified in the autoradiograms include the amygdala (am), hippocampus (hp), hypothalamus (hy), median eminence (me), striatum (st), and thalamus (th). Since there were relatively few κ sites in the rat, computer-enhanced images produced by the Loats Imaging System (Westminster, MD) are provided in the adjacent panels (e–h). The computer-enhanced images are meant to be viewed qualitatively and are not designed to imply an absolute number of sites. The gray tones produced in these computer images can be converted to relative optical densities by reference to the color bar on the side. The optical density bars to the right of the figures are computer generated and are designed to give a relative measure of binding density within a section. Note that the selective δ ligand [^{3}H]DPDPE (c, g) does not label any medial regions but does label cortical regions, whereas the less selective [^{3}H]DSLET (b, f) yields a mixed pattern.


a
e
b
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zona incerta, and the median eminence.

Consistent with the biochemical results presented above, [^{3}H]DSLET showed a composite anatomical pattern of μ and δ binding. [^{3}H]DSLET densely labelled cortical layers 1 and 4, the thalamic nuclei, and habenula, a pattern identical to that observed with [^{3}H]DAGO. In addition, as observed with [^{3}H]DPDPE, [^{3}H]DSLET also densely labelled cortical layers 2, 3, 5 and 6 and the caudal region of caudate-putamen. [^{3}H]DSLET binding in cortex appeared, in fact, to be diffuse because of its labelling of both μ and δ sites in complementary layers. The binding pattern of [^{3}H]DSLET had no similarity, however, to that seen with [^{3}H]bremazocine.

A similar set of results emerged with more caudal sections taken at the level of the inferior colliculus and periaqueductal grey (Fig. 5). [^{3}H]DAGO binding was high in the ventral periaqueductal grey, the raphe nuclei, and the inferior colliculus. In contrast, [^{3}H]DPDPE binding was more restricted, with relatively dense labelling of cortex and pontine nuclei. No [^{3}H]DPDPE binding was observed, however, in the raphe and periaqueductal grey. The [^{3}H]DSLET autoradiogram was a composite of the [^{3}H]DAGO and [^{3}H]DPDPE pattern with heavy labelling of the inferior colliculus and raphe (DAGO) as well as the pontine nuclei (DPDPE). Taken together, these results indicate that [^{3}H]DAGO, [^{3}H]DPDPE, and [^{3}H]bremazocine in the presence of μ and δ ligands are sufficiently selective to label anatomically discrete populations of μ, δ and $\varkappa$ receptors. [^{3}H]-DSLET, however, proved not to be as selective as [^{3}H]DPDPE and labelled both μ and δ sites.

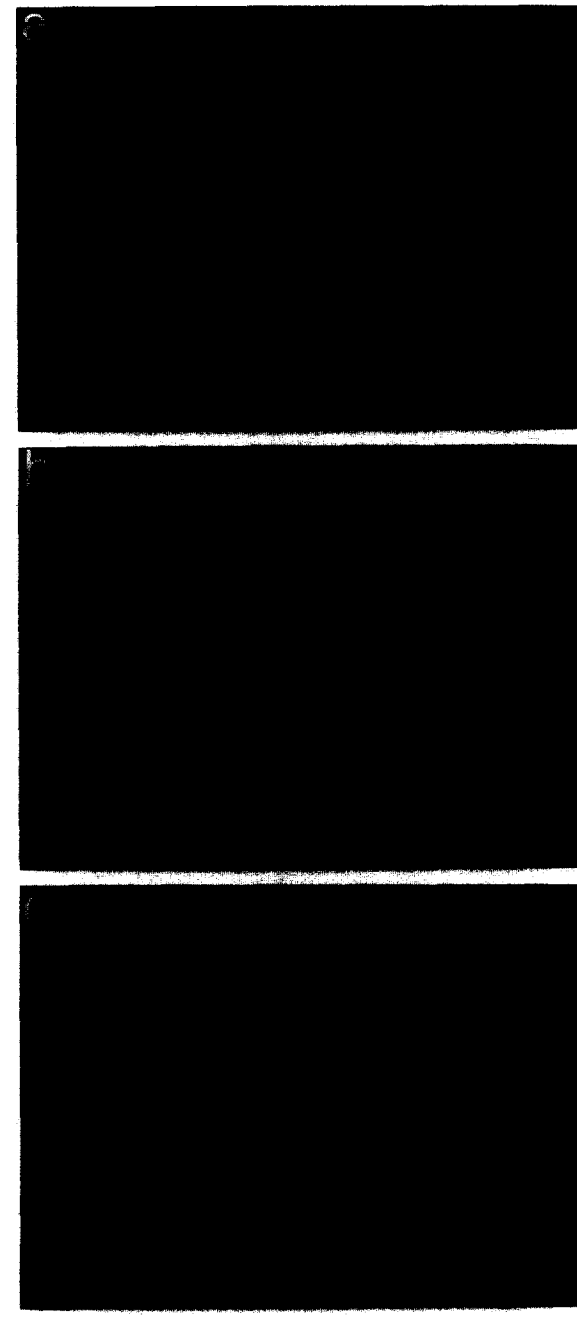

Fig. 5. [^{3}H]DAGO (a), [^{3}H]DSLET (b), and [^{3}H]DPDPE (c) dark-field autoradiograms at the level of the inferior colliculus. The labelling concentrations of radioligands were the same as those noted in Fig. 4. The structures identified in the autoradiograms include the aqueduct (a), hippocampus (hp), inferior colliculus (ic), raphe (r), and pontine nuclei (pn).

DISCUSSION

The results clearly demonstrate that the three opioid receptors can be differentiated in the rat and that previous failures to dissociate the three suptypes in this species may have been due to insensitive procedures. In each case an equivalent proportion of receptor sites have been labelled which has facilitated the comparison of each of the distributions. Furthermore, competition studies designed to evaluate our autoradiographic labelling conditions strongly suggest that the sites labelled with [^{3}H]DAGO, [^{3}H]DPDPE and [^{3}H]bremazocine in the presence of μ and δ blockers, correspond to the μ, δ and $\varkappa$ sites, respectively. In contrast, [^{3}H]DSLET was found not to be as selective as [^{3}H]DPDPE and labelled both μ and δ sites. These conclusions were further sup-

ported by the saturation studies in which the binding parameter of [^{3}H]DAGO was not substantially altered by the presence of a concentration of DPDPE sufficient to saturate the δ sites. Similarly, the presence of a concentration of DAGO sufficient to saturate the μ sites did not appreciably alter the binding parameters of [^{3}H]DPDPE.

In agreement with these biochemical results, [^{3}H]DAGO and [^{3}H]DPDPE binding sites had distinctive anatomical distributions. [^{3}H]DAGO binding was dense in cortical layers 1 and 4, thalamus, amygdala, ventral periaqueductal grey, and median raphe. In contrast, [^{3}H]DPDPE binding was heaviest in cortical layers 2, 3, 5 and 6, caudate-putamen, and amygdala. The complementary nature of the μ and δ distributions in parietal cortex is of interest and implies that the opioid subtypes may have different functional roles within the cortical circuitry. μ receptors because of their localization in layers 1 and 4 may play a role in thalamocortical functioning, while δ receptors may modulate intracortical functions because of their diffuse distribution within layers 2, 3, 5 and 6. More detailed analysis is needed, however, in order to evaluate this hypothesis.

While both DAGO and DPDPE binding sites were localized in the amygdala their distributions differed with nuclear group. DAGO binding was primarily in the lateral, basolateral and medial amygdala, while DPDPE binding was limited to the two former nuclear groups. Little, if any, μ or δ binding could be observed in the central nucleus of the amygdala despite its rich opioid innervation[14].

The binding pattern produced with [^{3}H]DSLET, on the other hand, was not unique and appeared to be a composite of the μ and δ sites labelled with [^{3}H]DAGO and [^{3}H]DPDPE, respectively. While the concentration of [^{3}H]DSLET used to produce the autoradiograms was relatively high (23 nM), it was proportional to the [^{3}H]DPDPE and [^{3}H]DAGO concentrations. In all cases the labelling concentrations chosen were three times the K_d value for each of the ligands and represented a 75% receptor occupancy. In addition, this concentration of [^{3}H]DSLET is within the range of concentrations routinely used in saturation studies. Conclusions concerning the affinity and number of delta receptors that are based on the binding of [^{3}H]DSLET may, therefore, be erroneous because of its affinity for both μ and δ sites. Lower concentrations of [^{3}H]DSLET may selectively label δ sites given proper conditions such as the addition of selective μ receptor blockers.

In agreement with the autoradiographic results, 100 nM DSLET competitively inhibited the binding of [^{3}H]DAGO, indicating a common site of action. Given the selectively of DAGO and DPDPE described above, one would conclude that DAGO and DSLET are competing at a μ site. Despite the affinity of [^{3}H]DSLET for both μ and δ receptors, its Scatchard plot showed a single component and was fitted best by a straight line. These results indicate the danger of drawing conclusions concerning ligand selectivity entirely on the basis of Scatchard analysis. These conclusions are supported by the results of the competition studies in which DSLET was found to be at least 10-fold less selective for δ receptors than DPDPE.

As demonstrated by both the saturation and competition studies, [^{3}H]bremazocine in the absence of μ and δ blockers was also found to be non-selective. The presence of 100 nM DAGO and DPDPE dramatically altered the binding parameters of [^{3}H]bremazocine; Scatchard analysis demonstrated that [^{3}H]bremazocine in the absence of μ and δ ligands showed a single site, while both a high and low affinity site was demonstrated in the presence of 100 nM DAGO and DPDPE. While the addition of a constant concentration of μ and δ blockers has been conventionally used in saturation experiments with non-selective $\varkappa$ ligands[18,26], it may not be the best procedure in determining a ligand's binding parameters. For when a constant ratio of [^{3}H]bremazocine and μ and δ blockers was used, one observes a single high affinity site. The apparent low affinity site observed with [^{3}H]bremazocine in the presence of 100 nM DAGO and DPDPE is most likely due to an insufficient blockade by the μ and δ sites at the higher bremazocine concentrations. Also, one tends to underestimate the capacity of the high affinity site when constant concentrations of μ and δ blockers are used. The B_{max} of the high affinity site was found to be nearly twice as high when a constant ratio of [^{3}H]bremazocine and unlabelled μ and δ blockers was used. Even with this estimate of receptor capacity, it is apparent that there are approximately 3–5 times as many μ or δ sites as $\varkappa$ sites in the rat.

Despite the relatively low number of high affinity $\varkappa$

sites, their distribution was quite different from the μ and δ pattern observed with [^{3}H]DAGO and [^{3}H]DPDPE. Relatively dense $\varkappa$ binding was observed in the amygdala, caudate-putamen, hypothalamus, and median eminence. The labelling of the hypothalamic nuclei is of particular interest in view of the localization of all three opioid precursors within different neurons in these nuclei[14] and provides an anatomical framework for understanding the effects of $\varkappa$ drugs on the regulation of the hypothalamic-pituitary axis.

While we sought to selectively label $\varkappa$ sites by the addition of 100 nM DAGO and DPDPE to the [^{3}H]bremazocine assay, it is possible that not all the μ and δ sites have been occupied by these agonists. Given the K_d of DPDPE, a 100 nM concentration will occupy approximately 91–93% of the available δ sites. Therefore [^{3}H]bremazocine will label both these remaining sites as well as 75% of the $\varkappa$ sites. Higher nanomolar concentrations of DPDPE were not used in order to minimize any possible loss of $\varkappa$ sites. The competition data and the autoradiographic results, however, suggest that we are primarily labelling a $\varkappa$ receptor site. Anatomical areas where there is an apparent overlap with δ sites, such as in the amygdala, should be viewed with caution, however, as this binding may not represent only $\varkappa$ sites. More selective $\varkappa$ ligands are needed to further evaluate this issue.

The anatomical distribution of the $\varkappa$ sites, while in agreement with those of Lynch et al.[17], markedly differ from those reported by Quirion et al.[26] who found no difference between the distribution of μ and $\varkappa$ sites in the rat forebrain. A possible explanation for these differences may be due to the different incubation conditions that Quirion et al.[26] reported with their autoradiographic data. They found that [^{3}H]EKC in the presence of a constant final concentration of μ and δ blockers labelled a single receptor site with a K_d of approximately 5 nM. This is substantially different from our observations with [^{3}H]bremazocine which demonstrated a high affinity site with a K_d of 0.2–0.3 nM.

In summary, the results demonstrate that [^{3}H]-DAGO, [^{3}H]DPDPE, and [^{3}H]bremazocine (in the presence of μ and δ ligands) provide a means of selectively labelling μ, δ, and $\varkappa$ receptors, respectively. These results are of importance in designing rational anatomical and pharmacological experiments to provide a better understanding of the distribution of these receptors and their possible functional significance. The localization of relatively dense $\varkappa$ binding in the hypothalamus, for example, suggests a possible role of $\varkappa$ receptors in neuroendocrine control and homeostatic regulation. On an anatomical level, the development of these selective ligands and binding conditions will allow the evaluation of the co-distribution of the multiple opioid peptides and receptors[15] providing further information as to their functioning, modulation, and circuiting.

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Congophilia in Cerebral Amyloidosis is Modified by Inactivation Procedures on Slow Transmissible Pathogens

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Cerebral tissues with amyloid deposits were treated by various chemicals which inactivated the agent of subacute spongiform encephalopathy (SSE). We discovered Congophilia in the amyloid plaques in cases of Creutzfeldt–Jakob disease (CJD) and Gerstmann–Sträussler syndrome (GSS) correlated to the chemical inactivation profiles of SSE. After incubation with trichloroacetate, guanidine-SCN, guanidine-HCl, formic acid, phenol and autoclaving, amyloid plaques in unfixed frozen sections of human brains with CJD or GSS, lost the affinity of Congo red and green birefringence under polarized light. In formalin-fixed, paraffin-embedded tissue sections, amyloid plaques of CJD and GSS lost the affinity of Congo red after most of these treatments. On the other hand, senile plaques in the aged, patients with Alzheimer's disease and with senile dementia of the Alzheimer type did not lose the affinity of Congo red after most of these treatments. Therefore, the amyloid deposits in the amyloid plaques differ from those in senile plaques. The methods we used facilitate differentiation of amyloid and senile plaques in formalin-fixed, paraffin-embedded tissues.

INTRODUCTION

Creutzfeldt–Jakob disease (CJD) is caused by a slow transmissible infectious pathogen or 'prion'[20]. Prusiner et al. reported that the pathogenetic agent 'prion' possessed the nature of amyloid fibrils[20] and that this agent was inactivated by chemicals such as chaotropic ions[17–19]. Masters et al.[14] purified the cerebral amyloid plaque core protein from patients with Alzheimer's disease and those with Down's syndrome and compared the core protein solubility profile with the inactivated scrapie agent. We reported that the autoclave method applied to tissue sections enabled classification of amyloid fibril proteins of various origins[10] and we suggested that the Congophilia in amyloid plaques (AP) and infectivity of brain homogenates of CJD may correlate. To compare the infectivity of subacute spongiform encephalopathy (SSE) with the Congophilia of AP, we applied certain chemicals which inactivate SSE agents to unfixed and formalin-fixed tissue sections of CJD, Gerstmann–Sträussler syndrome (GSS), Alzheimer's disease (AD) and senile dementia of Alzheimer type (SDAT). We found that the differentiation between AP of CJD and GSS, and senile plaques (SP) of SDAT and AD was greatly facilitated.

MATERIALS AND METHODS

Brain tissues were obtained from 9 autopsied patients (Table I). In most instances, the diagnosis had been made at the time of routine postmortem examination.

Subacute spongiform encephalopathy

This group included two patients with CJD and one with GSS. Case 1 was the first isolate named Fukuoka-1 strain in our laboratory[23]. Case 2 was a CJD patient with typical clinical and pathological findings. Case 3 (GSS) was one of familial occurrence, with a

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