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Properties of Opiate-Receptor Binding in Rat Brain

(naloxone/opiate antagonist)

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ABSTRACT **[^{3}H]Naloxone, a potent opiate antagonist, binds stereospecifically to opiate-receptor sites in rat-brain tissue. The binding is time, temperature, and pH dependent and saturable with respect to [^{3}H]naloxone and tissue concentration. The [^{3}H]naloxone–receptor complex formation is bimolecular with a dissociation constant of 20 nM. 15 Opiate agonists and antagonists compete for the same receptors, whose density is 30 pmol/g. Potencies of opiates and their antagonists in displacing [^{3}H]naloxone binding parallel their pharmacological potencies.**

The elegant conceptualization of Goldstein *et al.* (1) that opiate-receptor binding should be stereospecific enabled us to demonstrate and quantify opiate-receptor binding (2). Specific-receptor binding of opiates and their antagonists closely parallels their pharmacological potency and is confined to nervous tissue. The present report describes the kinetics of specific opiate-receptor binding and the influences of temperature, pH, ionic concentrations, and several opiates and their antagonists.

MATERIALS AND METHODS

Male Sprague–Dawley rats, 7–12 weeks old, were killed by cervical dislocation and decapitated, and their brains were rapidly removed. After the cerebellum, which is devoid of receptor activity (2), was excised, each brain was homogenized in 10 ml of 0.05 M Tris·HCl buffer, pH 7.4 at 35°, by 10 strokes of a motor-driven ground-glass pestle, and the homogenate was diluted to 110 volumes of tissue with cold Tris buffer.

In the standard binding assay, 1.9-ml aliquots of this freshly prepared homogenate were incubated for 5 min in triplicate with either levorphanol or dextrorphan (0.1 μM). After cooling to 4°, [^{3}H]naloxone was added to a final concentration of 8 nM (65,000 cpm) and incubation at 35° in a final volume of 2 ml was resumed for 15 min. Samples were cooled to 4° in an ice bath and then filtered under reduced pressure through Whatman glass-fiber circles (GF-B) which were chosen because of their ability to hold relatively large quantities of tissue while maintaining an adequate flow rate. Filters were washed twice with 8 ml of cold Tris buffer. The entire filtration cycle consumed only 20 sec for each sample. The filters were shaken with 1 ml of 10% sodium dodecyl sulfate in counting vials for 30 min. After the addition of 12 ml of PCS (Amersham–Searle; Phase Combining System), radioactivity was determined by liquid-scintillation spectrometry, at a counting efficiency of 28%. Protein was measured by the method of Lowry *et al.* (3), with bovine-serum albumin as a standard.

(−)-Naloxone was labeled by tritium exchange at the New England Nuclear Corp. 50 mg of naloxone were dissolved in 0.3 ml of trifluoroacetic acid with 50 mg of 5% Rh/Al_2O_3 to which were added 25 Ci of ^3H_2O, and the mixture was incubated 18 hr at 80°. In our laboratory, a 70-mCi portion of [^{3}H]naloxone was evaporated twice to dryness, and purified by thin-layer chromatography on Silica gel G plates (MN-Kieselgel G/uv 254) of 0.25-mm thickness (*n*-butanol–glacial acetic acid–H_2O; 4:1:2). When the purified [^{3}H]naloxone was chromatographed in three additional solvent systems, the resulting single peak of radioactivity coincided with authentic naloxone, which was chromatographed beside it. The specific activity of [^{3}H]naloxone was 6.1 Ci/mmol of standard, as determined by comparison with the ultraviolet absorption of standard solutions of naloxone at 260 nm. Based upon this specific activity determination and a counting efficiency of 28%, 378 cpm is equivalent to 0.1 pmol of naloxone.

Drugs were generously donated by the following companies: Endo (naloxone, oxycodone); Roche [levorphanol, dextrorphan, levallorphan, (+)-3-hydroxy-*N*-allyl-morphinan]; Lilly [(−)- and (+)-methadone, (±)-propoxyphene]; Winthrop (pentazocine cyclazocine, meperidine); Reckitt and Colman, American Cyanamid Agricultural Division and Dr. William Martin, Lexington, Kentucky (etorphine); Knoll (hydromorphone); Ciba-Geigy (etonitazene).

RESULTS

The time-course of [^{3}H]naloxone binding to whole rat-brain homogenates was studied at 35° in the presence of 0.1 μM levorphanol or 0.1 μM dextrorphan (Fig. 1). For most opiates, analgesic activity is highly stereospecific with almost all activity residing in isomers with a configuration analogous to that of D-(−)-morphine. Levorphanol is a potent opiate with the D-configuration, while dextrophan is its enantiomer and is essentially devoid of analgesic activity.

Binding of [^{3}H]naloxone occurred rapidly, was linear for no more than 1 min, and plateaued between 10 and 30 min so that equilibrium was reached by 15 min (Fig. 1). After 30 min, [^{3}H]naloxone binding gradually decreased by about 25% and then remained constant up to 70 min. 0.1 μM dextrorphan did not reduce [^{3}H]naloxone binding at any time. By contrast, 0.1 μM levorphanol greatly reduced the binding of [^{3}H]naloxone at all time intervals examined. Increasing

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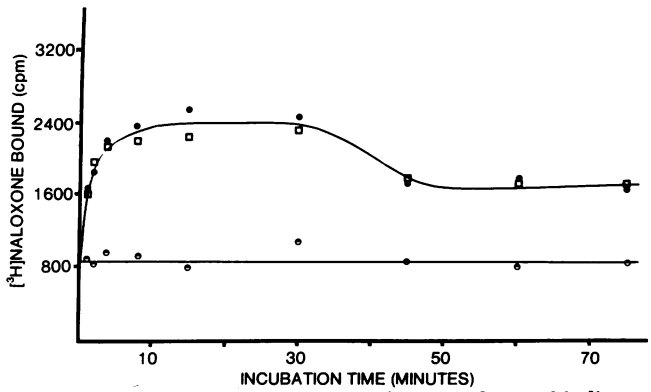

FIG. 1. Time-course of stereospecific [3H]naloxone binding to rat-brain homogenate. After 5 min preincubation in the presence of 0.1 μM levorphanol (◓), 0.1 μM dextrorphan (□), or no drug (●), homogenates were incubated at 35° in the standard binding assay from 1 to 75 min with [3H]naloxone. After samples were cooled to 4° they were filtered rapidly and the radioactive content of the tissue-laden filter was determined by liquid scintillation spectrometry. In some experiments binding declined continuously between 30 and 70 min.

the levorphanol concentration to 0.1 mM did not further diminish naloxone binding.

In all subsequent experiments, incubations with 0.1 μM concentrations each of levorphanol and dextrorphan were included and the radioactivity in the presence of levorphanol was subtracted as a "blank" value representing nonspecific binding, while the failure of dextrorphan to reduce binding ensured that the effect of levophanol was related to its affinity for the specific opiate receptor. In a typical experiment in which 65,000 cpm of [3H]naloxone (8 nM) were incubated in 2 ml with brain homogenate (18 mg wet weight equal to 1.8 mg of protein) 2000 cpm and 800 cpm were bound, respectively, in the absence and presence of 0.1 μM levorphanol. In the absence of tissue about 300 cpm were bound to the filters. Accordingly, only about 500 cpm were bound nonspecifically to brain tissue in the presence of levorphanol so that the ratio of specific to nonspecific binding in typical experiments would be about 3. Henceforth, "specific receptor binding" will refer to the binding of [3H]naloxone in the presence of 0.1 μM dextrorphan minus its binding in the presence of 0.1 μM levorphanol.

Specific [3H]naloxone binding was linear with brain-tissue protein over the range 0.2–2.0 mg of protein (Fig. 2). Specific binding displayed a sharp pH optimum at 7.4 (Fig. 2), with a steep decline at more acid pH values and a gradual decline at more alkaline pH values. In some experiments, the fall in binding at slightly acid pH was less sharp, presumably because of variable tissue aggregation.

The thermal stability of the opiate receptor was examined by prior incubation of the tissue for 10 min at various temperatures and then conducting the standard binding assay at 35°. Binding was not affected by prior incubation at temperatures from 20 to 40°, but was reduced by heating at higher temperatures. Heating for 10 min at temperatures in excess of 50° reduced specific naloxone binding by 90% or more (Fig. 2).

Specific binding was temperature dependent with maximal binding at 35° and a Q_{10} value of 1.5 when measured between 25 and 35° and 1.3 when measured between 15° and 25° after 15 min of incubation. At 4° binding was reduced to 25% of values at 35°.

Specific [3H]naloxone binding could be altered by various ionic manipulations (Fig. 3). The divalent cations calcium and magnesium lowered binding by about 40% at concentrations of 3 mM and further lowered binding to 30% of control values at 10 mM. In the absence of added sodium and potassium, specific binding was the same as in the presence of physiological concentrations of these ions. At concentrations in excess of 500 mM both lithium and sodium produced a gradual decrease of binding. Potassium, which at physiological concentrations of 5 mM did not affect binding, reduced specific binding 20% at 10 mM and 50% at 30 mM. Sodium, lithium, magnesium, and calcium did not alter the nonspecific binding of [3H]naloxone in the presence of nonradioactive levorphanol.

Naloxone binding was demonstrated to be saturable by two techniques (Fig. 4). In one approach, we measured the binding of increasing amounts of [3H]naloxone, while in other

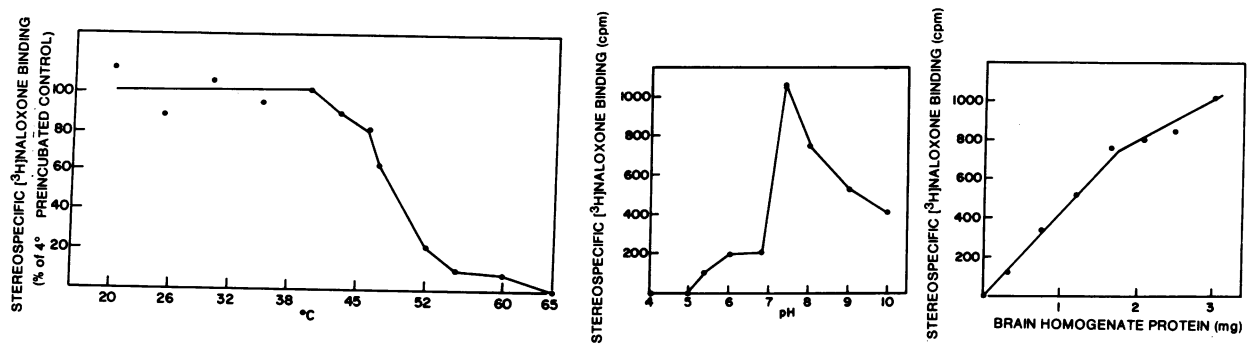

FIG. 2. Thermal sensitivity, pH dependence, and tissue linearity of stereospecific [3H]naloxone binding to rat-brain homogenate. *Middle:* Rat brains without cerebellum were divided longitudinally and each half was homogenized in 5 ml of 0.05 M citrate-phosphate buffer, pH 4, 5, or 5.4; 0.05 M sodium phosphate buffer, pH 6, 6.8, or 7.4; or 0.05 M glycine NaOH buffer, pH 8, 9, or 10. After dilution to 75 ml with the appropriate buffers, 1.9-ml aliquots of each homogenate were incubated with 0.1 μM levorphanol or 0.1 μM dextrorphan followed by [3H]naloxone in the standard binding assay. *Left:* Aliquots (1.9 ml) of rat-brain homogenate were maintained for 10 min at various temperatures before incubation with 0.1 μM levorphanol or 0.1 μM dextrorphan followed by [3H]naloxone in the standard-binding assay. Stereospecific [3H]naloxone binding is expressed as percent of the control samples, which were maintained at 4° for 10 min before assay. *Right:* One rat brain without cerebellum was homogenized in 10 ml of Tris buffer and diluted to a total volume of 120 ml with the same buffer. Various dilutions of this homogenate were incubated with 0.1 μM levorphanol or 0.1 μM dextrorphan followed by [3H]-naloxone in the standard-binding assay. All experiments were done twice.

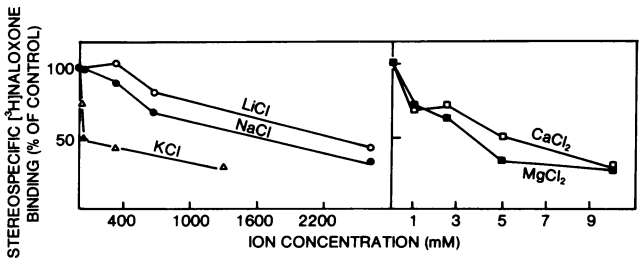

FIG. 3. Effect of increasing ionic concentrations on stereospecific [³H]naloxone binding to rat-brain homogenate. Various amounts of ions were included in the standard-binding assay. Data are presented from a typical experiment which was done twice.

experiments we studied the extent to which increasing amounts of nonradioactive naloxone decreased specific [³H]naloxone binding. Half saturation of tissue binding occurred at about 16 nM with 1.8 mg of brain-homogenate protein.

To determine the rate constant of opiate–receptor association, we examined the time-course of stereospecific [³H]-naloxone binding in detail (Fig. 5). Specific binding is a time-dependent process, whereas nonspecific binding in the presence of nonradioactive levorphanol was maximal even at incubation periods of 1 min (Fig. 1). From the data on the rate of binding, it was possible to calculate the bimolecular rate constant of opiate–receptor association, K_1, which is equal to $[2.303/t(a-b)] \log [b(a-x)/a(b-x)]$, where a = [[³H]naloxone], b = [receptors] and x = amount of tritiated naloxone reacting in time (t). The concentration of receptors in the standard incubation system was calculated to be 0.3 nM based upon the amount of [³H]naloxone specifically bound at saturation. The rate of association was $1.15 \pm 0.34 \times 10^6\ M^{-1}\ sec^{-1}$ at 25°.

The rate of dissociation was examined at four temperatures (Fig. 5). At 35°, [³H]naloxone specifically bound to the receptor had completely dissociated by 30 sec. At 25°, 15°, and 5°, dissociation appeared to be strictly a first-order process. The half lives for dissociation at 25°, 15°, and 5°, respectively, were 43 ± 7 sec, 113 ± 16 sec, and 4.7 ± 0.2 min. The rate constant at 25° for [³H]naloxone dissociation from the receptor (K_2) was calculated to be $1.61 \pm .24 \times 10^{-2}\ sec^{-1}$. The calculated value of K_2/K_1 based upon these direct kinetic data was 14.0 ± 4.1 nM, which corresponds to the experimentally determined concentration of 16 nM naloxone required for half-maximal saturation of receptor binding (Fig. 4).

We compared the affinity of various drugs for opiate-receptor binding by incubating several concentrations of nonradioactive drugs with tissue homogenate before the addition of [³H]naloxone. The concentration of drug that lowered specific binding of 8 nM [³H]naloxone by 50% was estimated (Table 1) by log-probit analysis (Fig. 6). For the 15 opiate-related drugs examined, the slopes of lines describing percent inhibition against drug concentration were all parallel. The pharmacologic potencies of opiates and opiate antagonists were related proportionally to their ability to compete with [³H]naloxone for binding to the opiate receptor. Etorphine, one of the most potent known analgesics, displayed the greatest affinity of any of the drugs examined with an ED_{50}† value

† ED_{50} is the concentration of drug that reduces specific [³H]-naloxone binding by 50%.

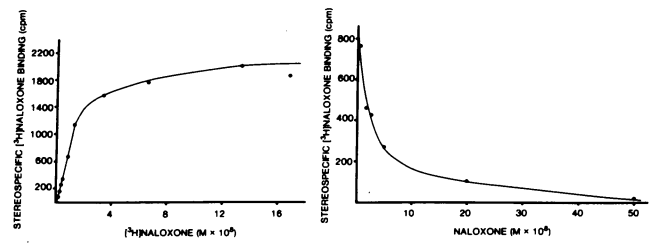

FIG. 4. Saturation of stereospecific [³H]naloxone binding to rat-brain homogenate (*left*) and the ability of nonradioactive naloxone to diminish stereospecific [³H]naloxone binding (*right*). *Left:* Standard aliquots of homogenate were incubated for 15 min at 35° with increasing concentrations of [³H]naloxone in the presence of 0.1 μM levorphanol or 0.1 μM dextrorphan. *Right:* Increasing concentrations of naloxone (plotted on the abscissa) were used to decrease the stereospecific binding of 8 nM [³H]-naloxone in the standard assay. Nonspecific binding in the presence of 0.1 μM levorphanol was subtracted from all samples.

of 0.3 nM, about 1/20th of the corresponding value for morphine. Etonitazene, an opiate whose potency *in vivo* is similar to that of etorphine (4), was almost as potent as etorphine in displacing [³H]naloxone binding.

Levorphanol, a potent opiate, had 4000-times the affinity of dextrorphan, its analgesically inactive enantiomer. Similarly, levallorphan, the opiate antagonist derived from levorphanol, was 7000-times as potent as its enantiomer. (−)-Methadone, the more analgesically active form of methadone, was only about 10-times as potent as (+)-methadone, perhaps because it has greater conformational mobility than levorphanol (5).

The opiates, morphine, and levorphanol, and their corresponding antagonists, nalorphine and levallorphan, had similar affinities for the opiate-receptor binding sites, al-

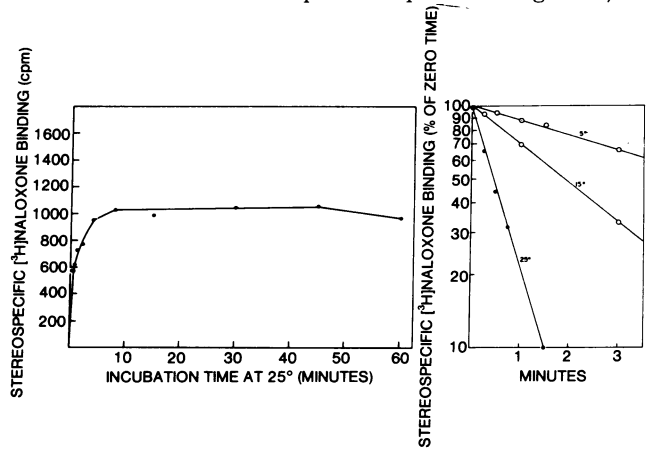

FIG. 5. Rate of stereospecific binding of [³H]naloxone at 25° (*left*) and semilog plot of dissociation of bound [³H]naloxone from rat-brain homogenate at 5°, 15°, and 25° (*right*). *Left:* Standard homogenates were incubated for intervals varying from 15 sec to 60 min with [³H]naloxone in the presence of 0.1 μM dextrorphan or 0.1 μM levorphanol at 25° before cooling to 4°. Samples were filtered immediately after cooling. *Right:* Brain homogenates were incubated for 15 min at 35° with [³H]-naloxone in the presence of 0.1 μM levorphanol or 0.1 μM dextrorphan in the standard binding assay. After cooling in an ice bath, nonradioactive naloxone (10 μM final concentration) was rapidly added and samples were filtered immediately (time 0) or allowed to incubate for various times at 5°, 15°, or 25° before rapid cooling and filtration.

TABLE 1. *Relative potencies of drugs in reducing stereospecific [^{3}H]naloxone binding to rat-brain homogenate*

Drug	ED_{50}(nM)	No effect at 0.1 mM
(−)-Etorphine	0.3	Phenobarbital
(−)-Etonitazene	0.5	Norepinephrine
Levallorphan	1	Atropine
Levorphanol	2	Pilocaprine
(−)-Nalorphine	3	Arecholine
(−)-Morphine	7	Colchicine
(−)-Cyclazocine	10	γ-Aminobutyric acid
(−)-Naloxone	10	Bicuculline
(−)-Hydromorphone	20	Serotonin
(−)-Methadone	30	Carbamylcholine
(±)-Pentazocine	50	Neostigmine
(+)-Methadone	300	Hemicholinium
Meperidine	1,000	Histamine
(±)-Propoxyphene	1,000	Glycine
(+)-3-Hydroxy-*N*-allyl-morphinan	7,000	Glutamic acid
Dextrorphan	8,000	Δ9-Tetrahydrocannabinol
(−)-Codeine	20,000	Acetylsalicylic acid
(−)-Oxycodone	30,000	Caffeine

Values represent means from 3 log-probit determinations each using five concentrations of drug.

though in both cases the antagonist had twice the affinity of the agonist.

Codeine, which is analgesically about 1/10 as potent as morphine, displayed less than 1/3000 the receptor affinity of morphine. Because codeine is *O*-demethylated by liver microsomal enzymes to morphine, it may exert analgesic activity only after metabolism to morphine (6–8).

Propoxyphene, which is a weak analgesic (9), had only 1/200 the affinity of morphine for receptor sites. (±)-Pentazocine, estimated to be from 1/6 as potent to equipotent to morphine in humans (10), had 1/8 the affinity of morphine for receptor sites. A wide variety of drugs that are not opiates had no significant affinity for the opiate-receptor binding sites (Table 1).

DISCUSSION

The pharmacological activity of opiates and their antagonists parallels their affinity for [^{3}H]naloxone-binding sites. Apparent discrepancies between potency *in vivo* and receptor affinity *in vitro* appear related to penetration of the blood–brain barrier or drug metabolism. Though etorphine exceeds morphine 1,000- to 10,000-times in analgesic potency but only 20-times in receptor potency, it enters the brain 200-times as readily as morphine, rationalizing a 4000-fold difference in potency *in vivo* (11). The moderate analgesic activity of codeine despite its negligible affinity for the opiate receptor may be explained by its conversion *in vivo* to morphine.

Our data support suggestions that opiates and their antagonists compete for the same receptor sites (12, 13). The kinetics of receptor binding indicate a strictly bimolecular process. Log-probit profiles for receptor affinity of a series of 15 opiates and their antagonists are parallel. [^{3}H]Levorphanol, [^{3}H]-levallorphan, [^{3}H]oxymorphone, [^{3}H]nalorphine, and [^{3}H]dihydromorphine bind stereospecifically to brain homogenates (manuscript in preparation). Because agonists and antagonists have similar affinities, their pharmacologic differences appear related to differences in "intrinsic activity" (14).

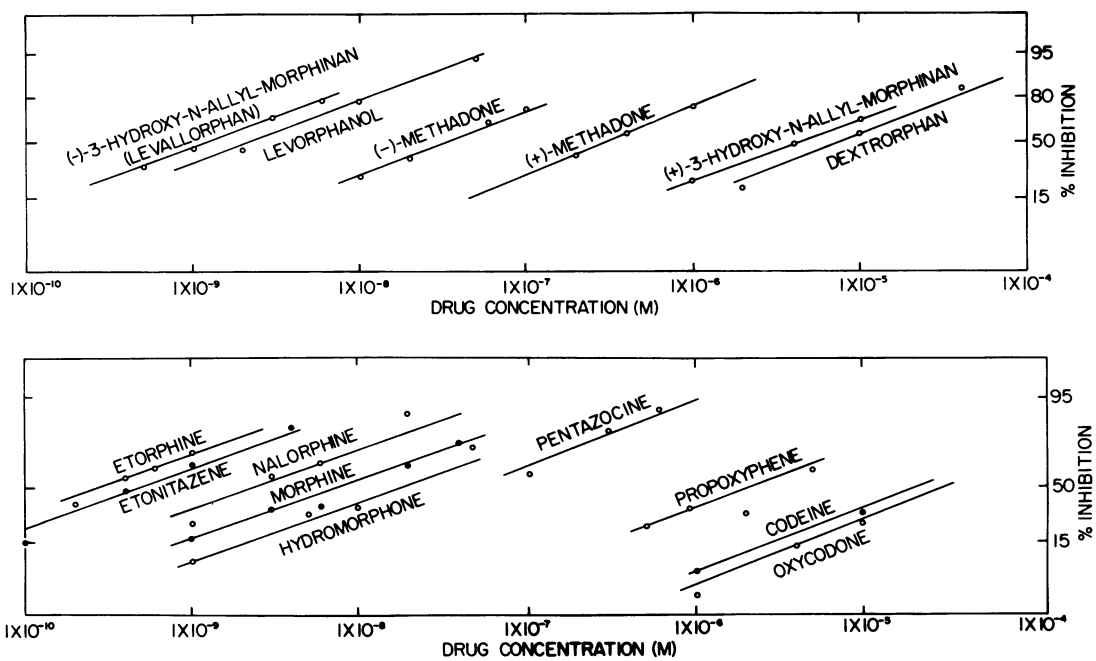

FIG. 6. Effects of opiates and opiate antagonists on stereospecific [^{3}H]naloxone binding. Five concentrations of each opiate were preincubated for 5 min with standard aliquots of rat-brain homogenate, and the incubation was continued for 15 min at 35° in the presence of [^{3}H]naloxone (8 nM). Percent inhibition of control stereospecific [^{3}H]naloxone binding was computed for each concentration of opiate, after subtracton of nonspecific binding from all experimental points, and plotted as a function of drug concentration on log-probit paper. *Above:* Three pairs of enantiomers are presented. *Below:* 11 Additional opiates and opiate antagonists that exhibit a wide range of inhibitory potencies have been plotted.

In our previous study (2), and recent experiments (unpublished observations) we demonstrated the presence of the opiate receptor in mouse, bovine, guinea pig, cat, chick, monkey, toad, and fish brain as well as rat brain, indicating a broad phylogenetic distribution.

Physiological concentrations of calcium significantly inhibit and the chelating agents EDTA, EGTA, and citrate enhance receptor binding (manuscript in preparation), suggesting that endogenous calcium plays a role in the action of opiates (15). The opiate receptor is extremely sensitive to digestion by trypsin and chymotrypsin as well as detergents such as Triton X-100, sodium dodecylsulfate, and deoxycholate (manuscript in preparation).

The number of opiate receptors increases in the brains of mice as early as 2 hr after implantation of a morphine pellet, and declines when pellets are removed in parallel with the fall in physical dependence (manuscript in preparation).

Other workers have studied the binding of opiates to brain tissue but failed to show specificity and pharmacologic relevance (16). Goldstein *et al.* (1) reported stereospecificity in 2% of [^{3}H]levorphanol binding to mouse-brain homogenates. However, unlike the saturable opiate receptor described here, the stereospecificity of binding reported by Goldstein *et al.* (1) increased with increasing concentration and accordingly seems to represent a different binding site.

It can be calculated from the direct measurement of [^{3}H]-naloxone bound at saturation that 1 g of rat brain without cerebellum has sufficient receptors to bind 30 pmol of naloxone. Assuming that each naloxone molecule interacts with one receptor unit, we calculate the number of receptor units in one rat brain (1.6 g) to be 2×10^{13}.

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