Table 2. The effects of central monoamine receptor blockers on the estrous behavior of estrogen-primed female rats.

Treatment	Medial preoptic–anterior hypothalamus $N=3$		Posterior hypothalamus $N=5$		Caudate nucleus $N=5$	
	Mean LQ	Median quality	Mean LQ	Median quality	Mean LQ	Median quality
Blank	.09	1	.09	1	.02	1
Methysergide	.82	4	.57	4	.10	1
Cinanserin	.44	4	.73	4		
Phentolamine	.02	1	.13	1		

ity rating taken for each animal at the time of optimum drug action. For the carrier group the maximum score for each animal regardless of the time of testing was also used. As indicated in Table 1, lordotic behavior was activated by the administration of the ovarian hormone progesterone, by blockade of the synthesis of serotonin with PCPA, or by blockade of serotonergic receptors with methysergide. Kruskal-Wallis tests indicated that the treatment groups were significantly different in both LQ ($P<.001$) and quality scores ($P<.01$). Subsequent Mann-Whitney U tests showed that the LQ ($P<.01$) and quality scores ($P<.001$) of all three experimental groups were significantly larger than those of the carrier control group. The groups treated with PCPA and methysergide had significantly lower LQ ($P<.001$) and quality scores ($P<.002$) than did the progesterone group. There were no significant differences between the PCPA and methysergide groups on either measure. The difference in the quality rating between the progesterone and the PCPA and methysergide groups was due largely to a total absence of soliciting behavior in the latter two groups. While all generally displayed high quality lordotic postures, characterized by an arching of the neck and rump which was often held after the male had dismounted, the active component of the estrous behavior pattern of the female rat was seen only with progesterone treatment.

We also have administered methysergide or cinanserin hydrochloride directly into the brain of EB-primed, ovariectomized female rats. Dual wall cannulas were chronically implanted into sites in the medial preoptic area, the anterior hypothalamus, or the posterior hypothalamic nucleus. These sites are known to contain serotonergic terminals (*10*) or to have the capacity to take up estrogen from the bloodstream (*11*), or both. We have some evidence (Table 2) which indicates that unilateral treatment of any of these three sites with 10 μg of either methysergide or cinanserin significantly increased the LQ and quality scores (*12*). Soliciting behavior was not activated by either of these manipulations. Administration of methysergide to control sites in the caudate nucleus did not produce lordotic behavior. Moreover, placement of empty cannulas or application of phentolamine hydrochloride, an alpha noradrenergic receptor blocker (*13*), failed to elicit lordosis at any of the above-mentioned hypothalamic sites where serotonergic blockers had succeeded.

Our results indicate that lordotic behavior can be elicited in EB-primed female rats by the administration of drugs that interfere with serotonergic activity in the brain. Because all animals had been adrenalectomized, the effects cannot be attributed to the liberation of adrenal steroids. Whereas the passive lordotic reflex was induced by the inhibition of serotonergic activity, the active soliciting component of the female estrous pattern was not released. Soliciting behavior may be under the control of nonserotonergic neurons. The absence of soliciting cannot be attributed to an impairment in general activity since neither methysergide, PCPA, nor cinanserin caused sedation, general debilitation, or fatality.

In summary, the finding that lordotic behavior can be elicited by a variety of antiserotonergic manipulations supports Meyerson's hypothesis of an inhibitory serotonergic system for female sexual behavior. Moreover, we have some evidence that serotonin exerts this action at receptors in the hypothalamus and the preoptic area. However, since the soliciting pattern was not evoked by either systemic or central administration of these drugs, activation of the total estrous behavior pattern must also involve transmitters other than serotonin.

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Opiate Receptor: Demonstration in Nervous Tissue

Abstract. *Tritiated naloxone, a powerful opiate antagonist, specifically binds to an opiate receptor of mammalian brain and guinea pig intestine. Competition for the opiate receptor by various opiates and their antagonists closely parallels their pharmacological potency. The opiate receptor is confined to nervous tissue.*

Pharmacological evidence for the existence of a specific opiate receptor is compelling, but heretofore it has not been directly demonstrated biochemically. We report here a direct demonstration of opiate receptor binding, its

localization in nervous tissue, and a close parallel between the pharmacologic potency of opiates and their affinity for receptor binding.

Rats, guinea pigs, and mice were killed by cervical dislocation. Their brains were homogenized in six volumes of 0.05*M* tris-HCl buffer, *p*H 7.4, and diluted to 18 times the original volume of the same buffer. Portions (2 ml) were incubated for 5 minutes at 35°C with various drugs, and the incubation was continued for 15 minutes after the addition of (—)-[^{3}H]naloxone ($5 \times 10^{-9}M$ final concentration). Samples were cooled to 4°C, filtered through Whatman glass fiber circles (GF-B), and the filters were washed under vacuum with two 8-ml portions of ice-cold tris buffer. The filters were extracted by shaking with 1 ml of 10 percent sodium dodecyl sulfate in counting vials for 30 minutes, and radioactivity was determined by liquid scintillation spectrometry after the addition of 12 ml of PCS counting fluid (phase combining system; Amersham/Searle). Other tissues were assayed for opiate receptor binding in homogenates or minces by filtering (Tables 1 and 2). All determinations of binding capacity were performed under conditions in which binding was linear with tissue concentration.

(−)-Naloxone was labeled by tritium exchange at the New England Nuclear Corporation as follows. A portion (50 mg) was dissolved in 0.3 ml of trifluoroacetic acid with 50 mg of 5 percent Rh on Al_2O_3 to which was added 25 curies of [^{3}H]water; the mixture was then incubated for 18 hours at 80°C. In our laboratory, a 70-mc portion of the [^{3}H]naloxone was evaporated to dryness twice, purified by thin-layer chromatography (*n*-butanol, glacial acetic acid, H_2O, 4 : 1 : 2), and its purity was verified in three other thin-layer systems. The specific activity of [^{3}H]-naloxone was 6.1 c/mmole as determined by comparison with the ultraviolet absorption of standard solutions of naloxone.

The analgesic activity of opiates is highly stereospecific, with almost all activity residing in those isomers with a configuration analogous to that of D(−)-morphine. Accordingly, on the assumption that analgesic potency parallels affinity for binding to the opiate receptor, the D(−) isomers should have much higher affinity than the L(+) isomers. We found that $10^{-7}M$ and $10^{-6}M$ levorphanol, a potent opiate that has the D(−) configuration, decreased the total binding of [^{3}H]naloxone to homogenates of rat, guinea pig, and mouse brain by 70 percent, while at these concentrations, dextrorphan, the analgesically inactive L(+) isomer failed to influence binding. Therefore, in all experiments, incubations with $10^{-7}M$ concentrations of levorphanol and dextrorphan were included, and the radioactivity in the presence of levorphanol was subtracted as a "blank value" representing nonspecific binding; the failure of dextrorphan to reduce binding in all tissues ensured that the effect of levorphanol was related to its affinity for the specific opiate receptor. In a typical experiment in which $5 \times 10^{-9}M$ [^{3}H]naloxone (65,000 count/min) was incubated in a 2-ml volume with a homogenate derived from whole rat brain (18 mg, wet weight), the amount bound was 2000 and 800 count/min in the absence and presence of $10^{-7}M$ levorphanol, respectively. The term "specific receptor binding" is used to mean the binding of [^{3}H]naloxone in the presence of $10^{-7}M$ dextrorphan minus its binding in the presence of $10^{-7}M$ levorphanol.

Table 1. Relative potencies of drugs in reducing stereospecific [^{3}H]naloxone binding to rat brain homogenates and minced guinea pig intestine. Stereospecific [^{3}H]naloxone binding to rat brain homogenates was assayed as described. Longitudinal muscle with adherent myenteric (Auerbach's) plexus from the small intestine of guinea pigs was minced and suspended in Krebs-Ringer-tris solution, *p*H 7.4, at 37°C. Portions (0.4 ml) were transferred to 20-ml beakers containing Krebs-Ringer-tris solution and the drug (2 ml total volume) and incubated in the same way as brain homogenates. After being cooled, the tissue was filtered over Whatman 540 paper circles and washed with two 8-ml portions of the cold Krebs-Ringer-tris solution. The filters were transferred to counting vials and shaken for 1 hour at 40°C with NCS (Nuclear-Chicago solubilizer; Amersham/Searle); the radioactivity was determined by liquid scintillation spectrometry after the addition of 15 ml of toluene phosphor. No metabolites of [^{3}H]naloxone in rat brain and guinea pig intestine could be detected after thin-layer chromatographic analysis of ^{3}H-labeled material eluted from tissue by methanol extraction. The ED_{50} values for inhibition of stereospecific [^{3}H]naloxone binding were defined as the concentration of drug which inhibited by 50 percent the stereospecific binding of [^{3}H]naloxone ($5 \times 10^{-9}M$) to whole rat brain homogenate (18 mg, wet weight of tissue) or minced guinea pig intestine (48 mg, wet weight of tissue). Under these conditions binding was linear with tissue protein. The ED_{50} values were derived from log-probit plots of three to five concentrations of the drug, each assayed in duplicate. Values are the means of three separate determinations.

Drug	ED_{50}
Brain homogenate	
(−)-Naloxone	$1 \times 10^{-8}M$
(−)-3-Hydroxy-*N*-allylmorphinan (levallorphan)	$1 \times 10^{-9}M$
Levorphanol	$2 \times 10^{-9}M$
(−)-Nalorphine	$2 \times 10^{-9}M$
(−)-Morphine	$6 \times 10^{-9}M$
(−)-Methadone	$2 \times 10^{-8}M$
(±)-Pentazocine	$5 \times 10^{-8}M$
(+)-Methadone	$2 \times 10^{-7}M$
(±)-Propoxyphene	$1 \times 10^{-6}M$
(+)-3-Hydroxy-*N*-allylmorphinan	$5 \times 10^{-6}M$
Dextrorphan	$8 \times 10^{-6}M$
(−)-Codeine	$2 \times 10^{-5}M$
Phenobarbital	*
Serotonin	*
Norepinephrine	*
Carbamylcholine	*
Choline	*
Atropine	*
Histamine	*
Colchicine	*
Intestine, minced preparation	
Levorphanol	$8 \times 10^{-8}M$
(−)-Morphine	$3 \times 10^{-6}M$
Dextrorphan	$4 \times 10^{-5}M$
Codeine	$1 \times 10^{-4}M$

* No effect at $10^{-4}M$

Specific binding of [^{3}H]naloxone occurred rapidly at 37°C, attained half-maximum values by 2 minutes, and reached equilibrium by 15 minutes. The ratio of specific to nonspecific binding at this time in whole rat brain homogenates was between 2.5 and 3.0 to 1. At $1 \times 10^{-8}M$ specific binding was half-maximum. With 15-minute incubations specific binding was temperature dependent with maximum binding occurring at 35°C; a Q_{10} (change of rate of reaction for each 10°C of temperature) value of 1.5 between 25° and 35°C, and about 90 percent of the specific binding was eliminated at 4°C. Specific binding was totally prevented by heating homogenates at 55°C for 10 minutes. We found a sharp *p*H optimum at 7.4 for specific binding with no binding below *p*H 5 and above *p*H 10. At 1 m*M*, calcium and magnesium inhibited stereospecific binding by 50 percent; up to 500 m*M* sodium had no influence on binding. Specific binding was not significantly reduced when homogenates were maintained at −20°C for 3 weeks (*1*).

Dissociation of [^{3}H]naloxone from its binding site was examined at various temperatures by adding $10^{-5}M$ nonradioactive naloxone after a 15-minute incubation with [^{3}H]naloxone. Approximate half-lives for dissociation at 5°, 15°, and 25°C, respectively, were 5 minutes, 2 minutes, and 1 minute (*1*).

The affinity of drugs for the [^{3}H]-naloxone binding sites was examined by incubating tissues with five concentrations of various nonradioactive drugs prior to the addition of [^{3}H]naloxone.

The concentration of drug that decreased the specific binding of $5 \times 10^{-9}M$ [^{3}H]naloxone by 50 percent was determined by log-probit analysis. The slopes of lines describing the percentage of inhibition plotted against drug concentration were the same for all opiates in the brain and guinea pig intestine, respectively (*1*). The binding affinities of various opiates and antagonists closely paralleled their pharmacologic potencies. Naloxone had an effective dose (ED_{50}) of $1 \times 10^{-8}M$. Morphine and nalorphine, its corresponding antagonist, showed similar affinities as did levorphanol and its antagonist levallorphan, although in both cases the opiate antagonist was somewhat more potent than the opiate itself. Levorphanol had about 4000 times the affinity of its nonanalgesic enantiomer dextrorphan. Interestingly, the (−) isomer of methadone, which had 1/10 the affinity of levorphanol, was only ten times more potent than its less analgesically active (+) isomer, conceivably because the methadone molecule has greater conformational mobility than levorphanol (*2*). Moreover, (−)-levallorphan, whose binding affinity is 20 times that of (−)-methadone, was 5000 times as potent as its enantiomer in specific receptor binding. Propoxyphene, a weak analgesic (*3*), had only 1/200 the affinity of morphine for receptor binding. Codeine, which in animals and humans is about 1/10 as active as morphine, bound only weakly to the opiate receptor, being only 1/3000 as potent as morphine. Since codeine is the methyl ether of morphine and since liver microsomal enzymes are capable of *O*-demethylation of codeine, codeine may exert its analgesic activity only after conversion in the body to morphine (*4*). Interestingly, while codeine is 1/10 as potent as morphine when administered to the intact animals, it is only 1/100 as potent as morphine in the isolated guinea pig ileum, where the probability of its enzymatic conversion to morphine is greatly diminished (*5*).

Drugs that are neither opiates nor their antagonists failed to influence [^{3}H]naloxone binding to whole brain homogenates at concentrations of $10^{-4}M$. These include phenobarbital, serotonin, norepinephrine, choline, carbamylcholine, atropine, colchicine, and histamine.

The relative potencies of opiates in reducing [^{3}H]naloxone specific binding in minced guinea pig intestine roughly paralleled results obtained from brain, although the drugs were less potent in the intestine to a variable degree. Levorphanol and morphine were, respectively, 40 and 500 times more active in the brain than in the intestine. In contrast to the 4000-fold difference in potency between levorphanol and its enantiomer dextrorphan in the brain, these drugs differed only 500-fold in the intestine. This finding suggests that the opiate "receptor" may be qualitatively different in various tissues. Nonetheless, in both brain and intestine, the close parallelism of pharmacological potency and specific binding, as well as the high affinity and stereospecificity of the potent opiates indicate that [^{3}H]naloxone binds specifically to physiologically significant receptor sites.

Our studies of the tissue distribution of opiate receptor binding provide evidence for the locus of the pharmacologic actions of opiates (Table 2). The greatest amount of opiate receptor binding occurred in the brain, while guinea pig small intestine displayed one-half as much opiate receptor binding. However, different binding assays are used for brain and intestine. To ascertain whether the opiate receptor was confined to nervous tissue, we obtained strips of intestinal longitudinal muscle to which the myenteric (Auerbach's) plexus had adhered along portions of its length. Plexus-free portions of the muscle obtained in this way are completely free of innervation (*6*). When comparable minced samples of plexus-free muscle and innervated muscle were assayed simultaneously, no specific binding at all was detected in the plexus-free muscle. Removal of its innervation in this way abolished all opiate receptor binding from the guinea pig intestine, indicating that the opiate receptor is confined to nervous tissue. Furthermore, no opiate receptor binding could be detected in human red blood cells and baker's yeast, which are not nerve tissues.

Table 2. Stereospecific [^{3}H]naloxone binding in various tissues. For subcellular fractionation, three whole rat brains were homogenized in 20 volumes of 0.32*M* sucrose in a glass homogenizer with a loosely fitting Teflon pestle and a portion was removed for receptor-binding assay. The remaining homogenate was centrifuged at 1000*g*, this sediment being the crude nuclear fraction. The crude mitochondrial-synaptosomal fraction was derived by centrifuging the supernatant from the crude nuclear fraction at 20,000*g* for 20 minutes. The microsomal fraction and the soluble supernatant fraction were obtained by centrifuging the supernatant from the mitochondrial-synaptosomal pellet at 100,000*g* for 90 minutes. The sedimented pellets were suspended in 0.05*M* tris-HCl buffer, *p*H 7.4 at 37°C, and assayed at five concentrations. After incubation with [^{3}H]naloxone, protein of the soluble supernatant was precipitated either by ice-cold absolute ethanol, saturated ammonium sulfate solution, or 5 percent polyethylene glycol; the precipitate was then harvested on a Millipore filter. Values [disintegrations per minute (dpm)] are means of four separate experiments in which specific receptor binding was linear with protein concentration. For ascertaining the regional distribution of binding: Brains from three rats were pooled, and dissected rapidly; the homogenates were assayed at dilutions for which specific receptor binding was linear with protein concentration. The guinea pig intestine was dissected by a modification of the method of Paton and Zar (*6*). Values presented are the means of quadruplicate determinations from two separate experiments in which innervated and denervated muscles were assayed at the same tissue concentrations. Erythrocytes from freshly drawn human blood were assayed both as cell suspensions and as homogenates. The sensitivity of the binding assay is such that 250 dpm of [^{3}H]naloxone per milligram of protein is approximately the minimum amount of binding detectable.

Fraction	[^{3}H]naloxone specifically bound (dpm/mg)	Percent of total
Rat brain		
Whole brain homogenate	2381 ± 235	
Subcellular		
Crude nuclear	896 ± 28	21 ± 3
Mitochondrial-synaptosomal	2214 ± 215	48 ± 1
Microsomal	3807 ± 246	32 ± 2
Soluble supernatant	< 250	
Regional distribution		
Striatum	8993 ± 483	
Midbrain	2246 ± 382	
Cortex	2089 ± 395	
Brainstem	1282 ± 63	
Cerebellum	< 250	
Guinea pig small intestine		
Myenteric plexus + longitudinal muscle	1400 ± 115	
Longitudinal muscle only (7 mg of protein)	< 250	
Human erythrocytes (11 mg of protein)	< 250	
Baker's yeast (4 mg of protein)	< 250	
Rat liver (6 mg of protein)	< 250	

Subcellular fractionation of rat brain showed the greatest enrichment of opiate receptor binding in the microsomal fraction, which is rich in membrane fragments (Table 2). About half of the total opiate receptor activity could be recovered in the crude mitochondrial-synaptosomal fraction. The crude nuclear fraction contained the least opiate receptor binding of all fractions examined. No opiate receptor binding could be demonstrated in the soluble supernatant fraction (Table 2).

In what type of nervous structures is the opiate receptor localized? Effects of opiates have been demonstrated on acetylcholine (*7*), serotonin, and norepinephrine (*8*). Thus, as with most psychoactive drugs, effects can be demonstrated on several neurotransmitter systems so that it is impossible to ascertain which of these represents the primary action of the drug. Our regional study of opiate receptor binding in rat brain revealed the most binding in the corpus striatum, whose binding exceeded that of the cerebral cortex more than fourfold. No opiate receptor binding was detectable in the cerebellum. Of the known neurotransmitters only dopamine and acetylcholine are highly concentrated in the corpus striatum. Dopamine concentrations are low in most other regions of the brain, and its regional variations do not parallel those found for the opiate receptor (*9*). By contrast, regional differences in acetylcholine concentrations more closely parallel the variations we have observed in opiate receptor binding. Both for opiate receptor binding and acetylcholine concentrations, there is a marked difference between corpus striatum and cerebral cortex, and negligible concentrations of acetylcholine and opiate receptor binding occur in the cerebellum (*10*). If the opiate receptor is associated with a known neurotransmitter, acetylcholine is the most likely candidate.

Goldstein *et al.* (*11*) reported binding of radioactive levorphanol to mouse brain homogenates, about 2 percent of which showed stereospecific differences between levorphanol and dextrorphan. It is unlikely that the binding of these two compounds was pharmacologically relevant for the following reasons. Maximum differences between levorphanol and dextrorphan were obtained at $3.9 \times 10^{-3} M$, concentrations at which we found that the specific opiate receptor sites are fully saturated for both drugs so that no differences can be detected. Moreover, Goldstein *et al.* (*11*) reported that the degree of stereospecificity increased with increasing concentrations of levorphanol and dextrorphan, whereas we found that stereospecific differences decrease with increasing concentrations. Our results differ from those of Goldstein *et al.* (*11*) in several other ways. They reported the greatest amount and activity per unit weight of binding in the crude nuclear fraction, whereas we observed the highest specific activity of receptor binding in the microsomal fraction and the greatest recovery in the crude mitochondrial-synaptosomal fraction (Table 2). Moreover, they failed to find any regional variations of binding in the brain.

As A. Burgen (*12*) has pointed out, "Contemporary ideas of drug action and drug specificity are all based on the assumption that the initial process in drug action is the formation of a reversible complex between the drug and a cell component generally known as the drug receptor." Only drugs that present a high degree of molecular complementarity toward the site at which they act are believed to be able to form this drug-receptor complex (*12*). We have shown that a component of nervous tissue can selectively form complexes with opiate drugs at very low concentrations. Since this binding is highly stereospecific and corresponds well with the previously reported pharmacological potencies of opiates, we believe that our results represent a direct demonstration of "opiate receptor" binding.

Identification of the opiate receptor provides new insight into the mechanism of action of opiates. Our binding assay affords a rapid means of determining the relative potencies of potential narcotic agonists and antagonists, with attendant theoretical and practical implications.

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Genetic Mechanisms Determining the Central Visual Pathways of Mice

Abstract. *Albino mammals have abnormal visual pathways. "Flecked" mice have a variegated pigment distribution, with roughly half the cells normally pigmented and half albino. They do not show the albino abnormality in the visual pathways. The gene at the albino locus, which determines the course of the visual pathways must, thus, have an extracellular action.*

The central visual pathways of albino mammals are abnormal (*1*, *2*). Parts of the retina that normally give rise to uncrossed retinofugal axons send axons across the midline in albinos. These segments of abnormal retina, which have well-defined borders (*2*), must contain ganglion cells that are wrongly specified concerning the central course of their axons. If the primary abnormality involved a deviation of fibers at the level of the chiasm, where, normally, the retinofugal fibers are not arranged in strict retinotopic order, then the borders of the abnormally connected retina would not be well defined.

Since an abnormally small ipsilateral pathway has been found in albinos of