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OPIATE RECEPTOR BINDING—ENHANCEMENT BY OPIATE ADMINISTRATION *IN VIVO**

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Abstract—Administration of opiate agonists and antagonists to mice produces a dose-dependent 50–100 per cent enhancement of stereospecific [^{3}H]dihydromorphine or [^{3}H]naloxone binding to brain homogenates within 5 min. Three opiate antagonists are 10–1000 times more potent in eliciting this increase in binding than their structurally analogous agonists. Naloxone, the antagonist with the least agonist activity, is the most potent drug in producing receptor enhancement. Implantation of morphine pellets in mice increases receptor binding 30–100 per cent for 2–108 hr with no time-dependent trend. Drug-induced receptor binding enhancement appears to involve an increase in number of binding sites rather than a change in receptor affinity. Sodium, which increases binding of opiate antagonists in normal mouse brain homogenate, fails to increase binding of [^{3}H]naloxone in homogenates derived from naloxone-injected mice.

A number of theories have been proposed to explain the mechanism of tolerance and physical dependence to opiates [1]; some propose that these phenomena reflect the same underlying process, while others suggest that they are distinct. Certain theories of opiate addiction have related tolerance and/or physical dependence to an alteration in the number of opiate receptors [2, 3].

Based on the criteria of Goldstein *et al.* [4] that opiate receptor binding to brain homogenates should be stereospecific, we have utilized a rapid filtration method to identify reversible opiate-receptor binding interactions [5, 6], with results like others [7, 8]. These interactions appear to be pharmacologically relevant, because many nonradioactive opiates bind to the receptor with an affinity that reflects their potency *in vivo*. Radiolabeled opiate agonists such as etorphine [8] and levorphanol, oxymorphone and dihydromorphine [9], as well as antagonists such as naloxone [5, 6], levallorphan and nalorphine, bind specifically and appear to compete for the same population of sites [9].

Recently, we reported differential enhancement of opiate receptor binding in mouse brain by treatment *in vivo* with opiate agonists and antagonists [9]. In the present study, we have examined in greater detail the influence of administration *in vivo* of opiate receptor binding. In addition, we have monitored alterations in receptor binding in mice rendered tolerant to and physically dependent on morphine.

MATERIALS AND METHODS

Drugs were donated by the following companies: naloxone, naltrexone and oxymorphone from Endo Laboratories, Garden City, N.Y.; levorphanol, dextrorphan and levallorphan from Roche Laboratories, Nutley, N.J.; pentazocine from Winthrop, Rochester, N.Y.; and nalorphine was purchased from the Merck Chemical Co.

Morphine pellets (75 mg) were implanted in male Jackson mice (30–35 g) under light anesthesia in the dorsal subcutaneous space [10]. Control mice were implanted with placebo pellets composed of the identical inert ingredients [11]. In later experiments, sham-operated mice in which a small incision had been made under anesthesia were used as controls. Values for placebo-implanted mice, sham-operated mice and naïve mice are indistinguishable.

At various times after drug injection intraperitoneally, or pellet implantation, mice were killed by cervical dislocation and each brain was individually homogenized in 14 ml of 0·05 M Tris–HCl buffer, (pH 7·4 at 37°) for 20 sec (Polytron PT-10 homogenizer, 3000 rev/min). Before assaying for opiate receptor binding, it was imperative to remove virtually all the nonradioactive morphine, since low concentrations inhibit binding [5, 6]. Brain homogenates from control and treated mice were centrifuged at 18,000 *g* for 10 min, the supernatant fluid was discarded and the pellet was resuspended in 14 ml of cold Tris buffer. This washing procedure was repeated and after a third centrifugation each homogenate was suspended in 75 ml of the buffer for individual assay.

Homogenates (1·9 ml) were incubated in triplicate in the dark for 30 min at 25° in the presence of 10^{-7} M levorphanol or 10^{-7} M dextrorphan after the addition of [^{3}H]dihydromorphine (1×10^{-9} M final concentration) or [^{3}H]naloxone (1×10^{-9} M final concentration). Assays conducted in 100 mM sodium employed levallorphan and (+)-hydroxy-*N*-allyl-morphinan instead of levorphanol and dextrorphan. Samples were filtered as previously described [5, 6] and washed with two 5-ml portions of ice-cold Tris buffer. After extraction, the filters were counted by liquid scintillation [5, 6]. Specific opiate receptor

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binding was calculated by subtracting binding of [^{3}H]dihydromorphine or [^{3}H]naloxone in the presence of 10^{-7} M dextrophan or (+)-3-hydroxy-*N*-allylmorphinan, their analgetically inactive enantiomers. Incubations were carried out in triplicate on freshly prepared 0·66-mg protein portions of mouse brain homogenates, which is within the range of linearity with protein concentration (0·3 to 1·8 mg). Typically, about 2400 and 600 cpm bind to 1·8 mg of mouse brain protein in the presence of 10^{-7} M dextrorphan and 10^{-7} M levorphanol respectively. [^{3}H]naloxone (6·1 Ci/m-mole) and [^{3}H]oxymorphone (5·0 Ci/m-mole) were obtained from New England Nuclear Corp., Boston, Mass., and purified as described previously for [^{3}H]naloxone [6]. [^{3}H]dihydromorphine (55 Ci/m-mole) and [^{3}H]naloxone (23·6 Ci/m-mole) were purchased from New England Nuclear Corp.

RESULTS

Effect of morphine pellet implantation on [^{3}H]dihydromorphine binding. As early as 2 hr after pellet implantation [^{3}H]dihydromorphine binding is significantly enhanced (Table 1). Increased binding, varying between a 30 and 100 per cent elevation, is present at a variety of time intervals up to 108 hr after pellet implantation. There appears to be no marked difference or time-dependent trend in the extent of elevation between 2 and 108 hr. Reasons for variations in

Table 1. Enhancement of opiate receptor binding by morphine injection or pellet implantation*

Time after morphine pellet implantation (hr)	Stereospecific [^{3}H]dihydromorphine binding (fmoles/mg tissue)		Per cent increase	P value
	Control ± S. E. M.	Experimental ± S. E. M.		
2	2·09 ± 0·18	3·00 ± 0·17	43	< 0·02
2	2·77 ± 0·21	3·85 ± 0·30	39	< 0·01
3	2·09 ± 0·21	3·07 ± 0·12	47	< 0·01
3	2·52 ± 0·21	3·89 ± 0·12	55	< 0·001
6	2·52 ± 0·21	4·48 ± 0·27	78	< 0·001
12	3·41 ± 0·26	5·39 ± 0·48	58	< 0·01
12	2·03 ± 0·26	4·24 ± 0·59	108	< 0·01
12	2·59 ± 0·15	4·25 ± 0·14	69	< 0·001
24	1·92 ± 0·15	2·96 ± 0·23	54	< 0·01
36	2·31 ± 0·26	3·27 ± 0·33	40	< 0·05
60	2·17 ± 0·20	3·03 ± 0·39	40	< 0·02
60	2·36 ± 0·32	3·64 ± 0·32	54	< 0·01
60	2·52 ± 0·08	3·35 ± 0·12	32	< 0·02
84	2·73 ± 0·18	3·69 ± 0·28	35	< 0·05
84	2·13 ± 0·15	2·84 ± 0·22	33	< 0·05
108	2·63 ± 0·16	3·75 ± 0·15	43	< 0·02
156	2·81 ± 0·27	3·23 ± 0·34	NS	
Time after morphine pellet removal (hr)				
4·5	2·52 ± 0·08	2·95 ± 0·17	17	< 0·02
10·5	2·52 ± 0·08	2·49 ± 0·18	NS	
24	2·74 ± 0·18	2·57 ± 0·23	NS	
Reserpine†	2·18 ± 0·12	2·04 ± 0·13	NS	
Na-pentobarbital‡	2·48 ± 0·13	2·61 ± 0·11	NS	
d-Amphetamine SO_4§	1·96 ± 0·11	1·92 ± 0·30	NS	
Chronically injected morphine SO_4§	1·96 ± 0·10	2·56 ± 0·13	23	< 0·05
Two sequential morphine pellets¶	2·35 ± 0·12	3·75 ± 0·21	60	< 0·01
Time after morphine SO_4 injection (50 mg/kg, i.p.) (min)				
5	2·06 ± 0·17	3·61 ± 0·21	75	< 0·01
10	2·06 ± 0·17	3·65 ± 0·11	77	< 0·01
20	2·41 ± 0·20	4·02 ± 0·22	67	< 0·02
60	2·41 ± 0·20	3·18 ± 0·36	32	< 0·02

* Control treatment for morphine pellets is described in the text. Control mice for injected drugs received equal volumes of 0·9% NaCl. Each control or experimental group contains seven to ten mice each assayed in triplicate for stereospecific [^{3}H]dihydromorphine binding to brain homogenates.

† Single 15 mg/kg injection daily for 3 days, assay day 3.

‡ Single 100 mg/kg injection, assay 12 hr later.

§ One 10 mg/kg injection on day 1; two 5 mg/kg injections on day 2; assay day 3.

Day 1: three 10 mg/kg injections; day 2: two 20 mg/kg and one 30 mg/kg injection; days 3 and 4: three 30 mg/kg injections each; day 5: three 80 mg/kg injections; days 6 and 7: three 100 mg/kg injections each; day 8: one 100 mg/kg injection, killed 2 hr later.

¶ Day 1: first pellet implanted; day 4: second pellet implanted; killed on day 7.

the proportional increase in binding in different experiments are not clear. At 156 hr no increase in receptor binding is detectable.

Because apparent maximal increases in receptor binding occur at the earliest time point studied after morphine pellet administration, earlier times were examined by administering morphine intraperitoneally. As early as 5 min after injection, morphine elicits a 75 per cent enhancement in receptor binding (Table 1).

Way *et al.* [10] have closely examined the development of tolerance and physical dependence in mice implanted with morphine pellets using conditions identical to those of the present study. Tolerance to the analgesic effects of morphine and withdrawal symptoms precipitated by naloxone increase gradually and peak 3 days after pellet implantation, when animals are tolerant to doses five times those required to elicit analgesia in naïve mice. At 156 hr, no analgesia or physical dependence is apparent [10], since, at this time, the pellets are isolated by connective tissue and no longer release morphine. The early enhancement of receptor binding in our experiments fails to correlate with the slower development of tolerance and physical dependence in mice treated with morphine pellets [10].

Four hr after pellet removal, there is a lesser enhancement of opiate receptor binding, and 10·5 and 24 hr after pellet removal no elevation in binding can be detected. Animals which have two sequential morphine pellets exhibit no greater enhancement of binding than animals with only one pellet. Moreover, chronic injections of morphine for 1 week produce no greater increase in binding than pellet administration (Table 1).

To evaluate the possibility that enhanced receptor binding is related to general behavioral excitation or depression, we measured [^{3}H]dihydromorphine binding in the brains of mice treated with large doses of reserpine for 3 days, sodium pentobarbital for 12 hr or *d*-amphetamine for 2 days (Table 1). None of these treatments alters specific receptor binding.

The enhanced receptor binding after morphine treatment might reflect an increase in the number of receptors or a change in affinity with no alteration in total number of apparent receptor sites. These possibilities were compared by measuring binding at nine different concentrations of [^{3}H]naloxone, which gives similar results to [^{3}H]dihydromorphine. Twelve hr after morphine pellet implantation the extent of increase in receptor binding, about 55–70 per cent, is similar between 0·15 and 2 nM [^{3}H]naloxone. The affinity constant, determined by double-reciprocal analysis (Fig. 1) for [^{3}H]naloxone, is about 1 nM both for control and treated mice. The apparent total number of binding sites is increased 60 per cent in morphine-treated animals. Thus, morphine treatment appears to increase the number of opiate receptor binding sites in the brain.

Receptor binding was measured in several regions of mouse brain 2 hr after morphine implantation. The extent of increased binding is not significantly different in telencephalon, midbrain-diencephalon and hindbrain (Table 2A). The enhancement of receptor binding by opiate treatment *in vivo* is similar in all subcellular fractions examined (Table 2B). A 64–74 per cent enhancement of binding is observed in crude nuclear (P_1), crude mitochondrial (P_2) and crude microsomal (P_3) fractions. As in naïve animals, the greatest total amount of binding occurs in the P_2 fraction, while the highest specific activity is noted in P_3, consistent with earlier studies of the subcellular distribution of stereospecific [^{3}H]naloxone and [^{3}H]dihydromorphine binding [5, 12].

Comparison of opiate agonists and antagonists. Three opiates and their corresponding antagonists were administered in a variety of doses, and receptor binding was examined 20 min later (Table 3). The opiate antagonists are 10–1000 times more potent than their corresponding agonists in enhancing receptor binding. Thus, nalorphine increases receptor binding 37 per cent at 0·2 mg/kg, while morphine has no effect at 2 mg/kg and evokes a 35 per cent enhancement at 10 mg/kg. Naloxone appears to be yet more potent than oxymorphone, its corresponding agonist. At 0·02 mg/kg naloxone elicits a statistically significant, 11 per cent, elevation in receptor binding with a 60 per cent increase at 0·2 mg/kg. By contrast, oxymorphone produces no effect at 2 or 10 mg/kg and a 59 per cent rise at 20 mg/kg. Thus, while nalorphine is 10–50 times more potent than morphine, naloxone appears to be 100–1000 times more potent than oxymorphone in enhancing receptor binding. Levallorphan increases binding 27 per cent at 0·1 mg/kg, while levorphanol fails to alter binding at 0·5 mg/kg. Dextrorphan, the analgetically inactive enantiomer of levorphanol, fails to alter binding at 20 mg/kg.

Pentazocine, a partial agonist which is an effective clinical analgesic, is also able to enhance receptor binding in relatively low doses.

Like morphine, naloxone exerts its effects rapidly. Ten min after injection, binding enhancement is as great as after 20 min. At 60 min there seems to be a somewhat lesser increase in receptor binding, while at 120 min no elevation of binding can be detected.

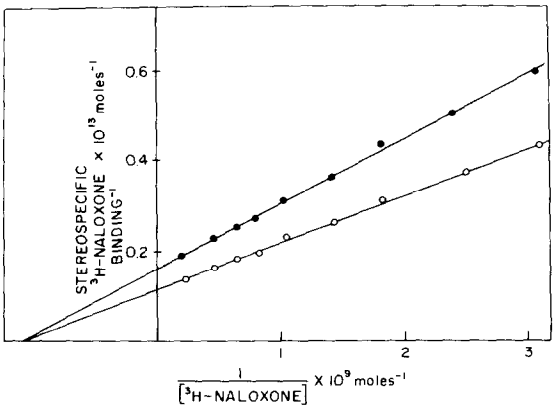

Fig. 1. Influence of naloxone injection on stereospecific binding of varying concentrations of [^{3}H]naloxone. Five mice were injected with 0·2 mg/kg of naloxone or 0·9% NaCl and killed 20 min later. After 'washing' as described in Methods, the experimental (○—○) and control (●—●) homogenates were assayed at nine concentrations of [^{3}H]naloxone (0·15 to 2 nM). Each point represents stereospecific binding from triplicate determinations of binding in the presence of levallorphan (0·1 μM) or its (+)-isomer (0·1 μM). The experiment was replicated three times.

Table 2. Morphine-elicited enhancement of [^{3}H]dihydromorphine binding in different brain regions and subcellular fractions

	Stereospecific [^{3}H]dihydromorphine binding (fmoles/mg protein)			
	Controls ± S. E. M.	Morphine pellet implantation	Increase (%)	P value
(A) Brain regions*				
Telencephalon	35·8 ± 2·4	42·4 ± 5·9	34	<0·01
Midbrain-diencephalon	44·8 ± 8·5	62·8 ± 7·1	40	<0·02
Hindbrain	19·0 ± 1·6	24·4 ± 2·5	29	<0·02
(B) Subcellular fractions†				
P_1	12·9 ± 1·0	22·4 ± 2·0	74	<0·01
P_2	39·5 ± 3·4	64·8 ± 5·3	64	<0·01
P_3	69·5 ± 5·0	115·3 ± 9·4	65	<0·01
Whole brain	25·1 ± 1·4	42·9 ± 21	71	<0·01

* Two hr after implantation of 75 mg morphine pellets or placebos, 12 experimental and 12 control mice were killed alternately and their brains rapidly dissected into three regions. After washing away residual morphine by three centrifugations as described in methods, each region was assayed after final suspension in 300 vol. of 0·05 Tris buffer.

† Twenty min after injection of 0·2 mg/kg of naloxone HCl or 0·9% NaCl, mice were killed and their brains individually homogenized in 0·32 M sucrose with a loosely fitting Teflon pestle. To assess binding enhancement in whole brain, an aliquot was removed and washed as described. P_1 was obtained by centrifugation for 10 min at 1000 g. The supernatant fluid from P_1 was centrifuged for 20 min at 17,000 g to obtain P_2. The resulting supernatant fluid was centrifuged for 60 min at 100,000 g to obtain P_3. All pellets were resuspended twice in 12 ml of 0·05 M Tris buffer for washing before a final resuspension yielding 0·4 to 0·6 mg protein/ml. Aliquots (1·9 ml) were assayed as described in Methods.

Table 3. Relative abilities of opiate antagonists and agonists administered *in vivo* to enhance stereospecific [^{3}H]dihydromorphine binding*

Drug	Dose (mg/kg)	Stereospecific [^{3}H]dihydromorphine binding (fmoles/mg tissue) ± S. E. M.		Increase (%)	P value
		0·9% NaCl	Drug		
Morphine SO_4	0·2	2·23 ± 0·15	1·69 ± 0·24	NS	
	2·0	2·69 ± 0·26	2·23 ± 0·17	NS	
	10·0	2·69 ± 0·21	3·64 ± 0·11	35	<0·02
	20·0	2·09 ± 0·32	3·26 ± 0·18	56	<0·01
	50·0	2·06 ± 0·17	3·44 ± 0·25	67	<0·02
	60·0	2·43 ± 0·33	3·41 ± 0·25	40	<0·02
Nalorphine SO_4	0·05	2·23 ± 0·15	2·44 ± 0·52	NS	
	0·2	2·69 ± 0·21	3·67 ± 0·33	37	<0·02
	2·0	2·69 ± 0·26	3·80 ± 0·29	42	<0·02
	10·0	2·23 ± 0·15	3·63 ± 0·25	85	<0·02
Oxymorphone HCl	2·0	2·57 ± 0·11	2·94 ± 0·15	NS	
	10·0	2·23 ± 0·15	2·38 ± 0·21	NS	
	20·0	2·69 ± 0·21	4·28 ± 0·32	59	<0·02
Naloxone HCl	0·01	2·50 ± 0·18	2·30 ± 0·15	NS	
	0·02	2·40 ± 0·05	2·68 ± 0·14	11	<0·01
	0·2	2·40 ± 0·05	3·85 ± 0·25	60	<0·01
	2·0	2·93 ± 0·20	4·78 ± 0·24	63	<0·01
	10·0	1·90 ± 0·12	3·51 ± 0·22	85	<0·01
Levorphanol tartrate	0·5	1·98 ± 0·10	2·33 ± 0·12	NS	
	1·0	2·15 ± 0·02	2·41 ± 0·11	12	<0·05
	2·0	1·98 ± 0·10	2·57 ± 0·22	30	<0·02
Levallorphan tartrate	0·1	2·15 ± 0·02	2·72 ± 0·23	27	<0·02
	0·5	1·98 ± 0·10	2·84 ± 0·17	43	<0·01
	2·0	1·98 ± 0·10	2·66 ± 0·17	34	<0·01
Dextrorphan tartrate	20·0	2·09 ± 0·32	2·40 ± 0·35	NS	
	200·0	2·44 ± 0·51	2·10 ± 0·43	NS	
Pentazocine	0·5	2·15 ± 0·02	2·53 ± 0·20	8	<0·02
	2·0	2·15 ± 0·02	2·95 ± 0·29	37	<0·001
	10·0	1·88 ± 0·80	3·14 ± 0·22	56	<0·05

* Drugs were injected intraperitoneally and mice were decapitated 20 min later. Control and experimental mice were killed alternately. Statistical significance was by the Mann Whitney U rank test. Each experiment involved groups of seven control mice and seven drug-treated mice with individual mouse brains assayed in triplicate.

Effect of reserpine on naloxone-induced enhancement of opiate binding. Numerous investigators have postulated that opiate action, tolerance and physical dependence may be related to influences of these drugs upon the neurotransmitters serotonin and the catecholamines [13]. Doses of reserpine which massively deplete norepinephrine, dopamine and serotonin from the brain do not alter opiate receptor binding (Table 1). To determine whether endogenous levels of catecholamines and serotonin are required for the enhanced receptor binding, we examined the influence of reserpine (5 mg/kg) on the naloxone-induced increase of binding (Fig. 2). This dose of reserpine, which depletes brain catecholamines and serotonin by 90 per cent or more, does not affect the naloxone-induced increase in binding.

Interactions between agonists and antagonists. To determine whether antagonists and agonists administered together increase receptor binding to a greater or lesser extent, mice were injected with doses of naloxone or morphine or both drugs simultaneously (Table 4). Naloxone and morphine alone produce a 30–60 per cent increase in binding. No further increase is obtained when the two drugs are combined.

When mice are rendered tolerant and physically dependent to morphine after pellet implantation, they become exquisitely sensitive to naloxone. At the time of maximal tolerance, withdrawal symptoms can be precipitated by much smaller doses of naloxone than are required to precipitate withdrawal when the animals are less tolerant [10]. To determine whether the naloxone-induced increase in receptor binding is correlated with these changes in sensitivity to naloxone, we assessed the ability of two doses of naloxone to affect opiate receptor binding in naïve as well as highly tolerant mice 2·5 to 3 days after pellet implantation (Table 4). The total elevation of opiate receptor binding produced by naloxone in morphine-pelleted animals is not significantly different from the elevation of receptor binding in naïve animals injected with naloxone. Moreover, the highly tolerant mice do not appear to have been 'sensitized' to naloxone. Both in naïve mice and in mice 3 days after morphine pellet implantation, 0·01 mg/kg of naloxone does not significantly alter receptor binding, although this dose did produce diarrhea, a symptom associated with withdrawal, in the morphine-pelleted animals treated with naloxone.

Some workers have suggested that opiate antagonists enter the brain more readily than agonists [14], which conceivably could account for the greater potencies of opiate antagonists in enhancing receptor binding. Accordingly, we injected mice intraperitoneally with 1 mg/kg of [^{3}H]oxymorphone and [^{3}H]naloxone and measured brain tritium levels for the drugs 20 min later, when receptor binding is enhanced (Table 5). Greater than 80 per cent of brain tritium represents unmetabolized [^{3}H]naloxone and [^{3}H]oxymorphone as determined by thin-layer chromatographic analysis. No difference can be detected between brain tritium levels for the two drugs. In these experiments it was also possible to assess the efficacy of the washing procedure employed for removing injected agonists and antagonists prior to binding assay. Virtually all the tritium in the brains of the mice treated with [^{3}H]naloxone or [^{3}H]oxymorphone is removed by this washing procedure (Table 4).

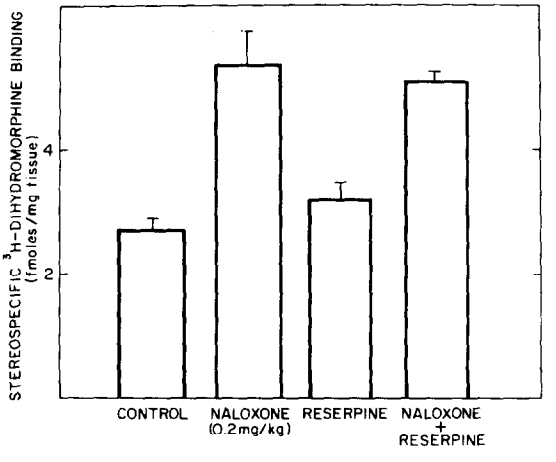

Fig. 2. Effect of reserpine on naloxone-induced receptor binding enhancement. On 3 successive days, eight mice were injected with 15 mg/kg of reserpine or an equal volume of vehicle. On day 4, control and reserpine-treated mice were injected with 0·9% NaCl or 0·2 mg/kg of naloxone and killed 20 min later. [^{3}H]Dihydromorphine binding was assessed individually on the eight mice in each group as described in Methods. The lines above each bar depict the magnitude of the S. E. M.

Table 4. Influence of combined treatment with naloxone and morphine on opiate receptor binding*

	Stereospecific [^{3}H]dihydromorphine binding (fmoles/mg tissue)					
	Control	Naloxone	Dose (mg/kg)	Morphine	Dose	Naloxone and morphine
(1)	2·84 ± 0·30	4·57 ± 0·31†	20	4·10 ± 0·44†	20 mg/kg	4·27 ± 0·21
(2)	2·31 ± 0·32	3·61 ± 0·16†	6	3·24 ± 0·24†	6 mg/kg	3·45 ± 0·18†
(3)	2·93 ± 0·21	4·44 ± 0·24†	2	3·52 ± 0·17†	75 mg pellet (2·5 days)	4·78 ± 0·24†
(4)	2·02 ± 0·19	1·86 ± 0·18	0·01	2·69 ± 0·25†	75 mg pellet (3 days)	2·35 ± 0·22†

* In Expts. 1 and 2, mice received intraperitoneal injections of naloxone, morphine, the two drugs together or 0·15 M NaCl and were killed after 20 min. In Expts. 3 and 4, mice were implanted with morphine or placebo pellets and after 60 hr (Expt. 3) and 72 hr (Expt. 4) received intraperitoneal injections of naloxone or 0·15 M NaCl and were killed 5 min later. Data are the mean values ± S. E. M. for groups of six to ten mice.

† Differs from control values, $P < 0.05$.

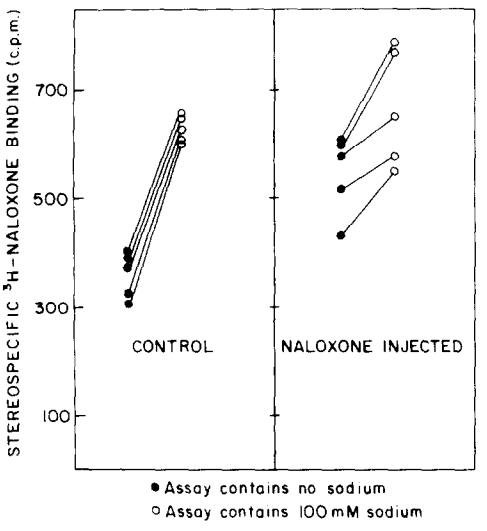

Fig. 3. Effect of sodium on opiate receptor binding in brain homogenates from mice injected with naloxone. Two groups of five mice each were injected with 0·2 mg/kg of naloxone or 0·9% NaCl and killed 20 min later. Each mouse brain was individually homogenized, 'washed' as described in Methods and assayed in triplicate in the presence or absence of 100 mM NaCl. In these assays, specific receptor binding was detected as the difference between [^{3}H]naloxone bound in the presence of 0·1 μM levallorphan or 0·1 μM (+)-3-hydroxy-*N*-allyl-morphinan, the (+)-isomer of levallorphan. Levorphanol and dextrorphan could not be employed, because in 100 mM NaCl the ability of 0·1 μM levorphanol to displace [^{3}H]naloxone binding is greatly diminished [7, 9]. Each point represents stereospecific binding from an individual mouse assayed with (○) or without (●) sodium.

Effect of sodium. We have previously reported that sodium increases the number of [^{3}H]naloxone binding sites [9]. We examined the ability of sodium to increase [^{3}H]naloxone binding in mice injected with naloxone (Fig. 3). Although naloxone injection produces the expected 50 per cent increase over control mice, homogenates from the naloxone-treated mice show a greatly diminished stimulation by sodium.

DISCUSSION

Enhanced opiate receptor binding is elicited by administration *in vivo* of opiate agonists and antagonists. Similar enhancement is observed whether [^{3}H]naloxone or [^{3}H]dihydromorphine is used to assess binding. In no experiments were we able to detect more than a doubling of binding. The inability of Klee and Streaty [15] to observe these changes in rats may be related to differences in tissue preparation, since we have observed increased opiate receptor binding in rats after morphine pellet implantation or naloxone injection (C. Pert and S. Snyder, unpublished observations). Hitzemann *et al.* [16] reported small but significant receptor enhancement after morphine pellet implantation, although the number of mice in each group is unclear.

Some theories of addiction have related tolerance and/or physical dependence to alterations in the number of opiate receptors [2, 3]. Our findings do not support such relationships. After morphine implantation, the degree of enhanced binding appeared to be the same between 2 and 108 hr after pelleting, a period during which tolerance increases 5-fold [10]. Naloxone, the 'pure' antagonist which is unable to produce physical dependence [16], is the most potent enhancer of receptor binding. Moreover, a maximum increase in binding occurs only a few minutes after morphine injection, long before maximal development of tolerance and physical dependence.

All drugs tested by injection *in vivo* produced an effect that fell within a relatively small range (30–100 per cent enhancement). Thus, in comparing drugs, the most informative analysis appears to be an 'all or none' judgment for a range of doses with each drug. What differentiates the drugs is their relative milligram potencies. Interestingly, naloxone, which is the 'purest' antagonist [17], is the most potent of the antagonists examined in enhancing receptor binding, even though its affinity *in vitro* for the receptor is not greater than that of levallorphan and nalorphine [5, 6, 16]. This fact, together with the greater potencies of the antagonists nalorphine and levallorphan than the agonists morphine and levorphanol in increasing receptor binding, suggests that 'receptor

Table 5. Penetration of naloxone and oxymorphone into mouse brain*

	[^{3}H]oxymorphone (pg)	[^{3}H]naloxone (pg)
Whole brain content	74·0 ± 18·5	70·5 ± 7·0
Discarded with first wash	72·6 ± 13·5	70·0 ± 9·5
Discarded with second wash	7·3 ± 1·9	5·4 ± 0·5
Discarded with third wash	0·7 ± 0·2	0·6 ± 0·1
Total recovery	80·6	76·0

* Mice were injected i.p. with 1 mg/kg (14 μCi) of [^{3}H]oxymorphone or [^{3}H]naloxone. After 20 min mice were killed, their brains were homogenized in 14 ml of 0·05 M Tris buffer, and a 0·5-ml aliquot was counted (1 ml NCS solubilizer + 14 ml toluene fluor) at 35 per cent efficiency to determine brain tritium. Only 0·1 per cent of the injected drug could be recovered in brain after 20 min. Brain homogenates were centrifuged at 18,000 *g* for 10 min as described in Methods. Five ml of each discarded supernatant fluid was counted at 42 per cent efficiency in 12 ml of PCS scintillation fluor. Greater than 80 per cent of brain tritium represents unmetabolized [^{3}H]naloxone and [^{3}H]oxymorphone as determined by thin-layer chromatographic analysis of a methanol extract in two solvent systems [*N*-butanol glacial acetic acid H_2O (4:1:2) and ethanol dioxane benzene NH_4OH (5:40:50:5)]. Values are means ± S. E. M. for groups of four mice.

enhancement, whatever its mechanism, is closely correlated with the properties which determine narcotic antagonist effects.

We recently reported that low concentrations of sodium enhance antagonist binding and inhibit agonist binding [18] and suggested that this 'sodium effect' occurs because the opiate receptor can interconvert between an agonist and antagonist conformation [18, 19]. The relation between our molecular model of opiate receptor function [18] and the current finding is unclear. Apparently, administration *in vivo* of opiates somehow makes more receptors available for measurement *in vitro*. The ability to obtain this effect, however, is highly dependent on assay conditions, i.e. the absence of sodium, multiple centrifugations and maintenance at a low temperature. In any case, we wish to emphasize that, utilizing binding studies *in vitro*, we were unable to detect alterations in opiate receptors which are related to the development of tolerance and physical dependence. It is conceivable, however, that alterations in opiate receptors may occur which could only be detected by binding methods *in vivo* [20]. For example, if the equilibrium of opiate receptor interconversion shifts to favor the antagonist conformation [21], this qualitative change would have gone undetected in the present study. The markedly reduced ability of sodium to increase receptor binding by brain homogenates of naloxone-injected mice suggests that the receptor enhancement observed after administration *in vivo* of opiate agonists and antagonists is highly dependent on assay conditions. Indeed, the 'receptor enhancement' effect is entirely obliterated if the assay is performed in the presence of sodium. The most probable explanation for the receptor enhancement reported here is that the opiates displace endorphin, an endogenous morphine-like factor [22–24], from opiate receptors, which are then more accessible for labeling *in vitro* by [^{3}H]opiates.

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REFERENCES

1. J. Cochin, *Fedn Proc.* **29**, 19 (1970).
2. H. O. J. Collier, *Adv. Drug. Res.* **3**, 171 (1966).
3. S. Ehrenpreis and D. N. Teller, in *Chemical and Biological Aspects of Drug Dependence* (Eds. S. J. Mule and H. Brill), pp. 177–217. The Chemical Rubber Co., Cleveland, Ohio (1972).
4. A. Goldstein, L. I. Lowney and B. K. Pal, *Proc. natn. Acad. Sci. U.S.A.* **68**, 1742 (1971).
5. C. B. Pert and S. H. Snyder, *Science, N.Y.* **179**, 1011 (1973).
6. C. B. Pert and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **70**, 2243 (1973).
7. L. Terenius, *Acta pharmac. tox.* **32**, 317 (1973).
8. E. J. Simon, J. M. Hiller and I. Edelman, *Proc. natn. Acad. Sci. U.S.A.* **70**, 1947 (1973).
9. C. B. Pert, G. Pasternak and S. H. Snyder, *Science, N.Y.* **182**, 1359 (1973).
10. E. L. Way, H. H. Loh and F.-H. Shen, *J. Pharmac. exp. Ther.* **167**, 1 (1969).
11. R. D. Gibson and J. E. Tingstad, *J. pharm. Sci.* **54**, 426 (1970).
12. C. B. Pert, A. Snowman and S. H. Snyder, *Brain Res.* 75, 356 (1974).
13. E. L. Way and F.-H. Shen, in *Narcotic Drugs, Biochemical Pharmacology* (Ed. D. Clouet), pp. 229–50. Plenum, New York (1971).
14. W. R. Martin, *Pharmac. Rev.* **19**, 463 (1967).
15. W. A. Klee and R. A. Streaty, *Nature, Lond.* **238**, 61 (1974).
16. R. J. Hitzemann, B. J. Hitzemann and H. H. Loh, *Life Sci.* **14**, 2393 (1974).
17. H. Blumberg and H. B. Dayton, in *Agonist and Antagonist Actions of Narcotic Analgesic Drugs* (Eds. H. W. Kosterlitz, H. O. J. Collier and J. E. Villarreal), pp. 110–19. University Park Press, Balt., Md. (1973).
18. C. B. Pert and S. H. Snyder, *Molec. Pharmac.* **10**, 868 (1974).
19. C. B. Pert and S. H. Snyder, *Neurosci. Res. Prog. Bull.* **13**, 73 (1975).
20. C. B. Pert and S. H. Snyder, *Life Sci.* **16**, 1623 (1975).
21. S. H. Snyder, *Neurosci. Res. Prog. Bull.* **13**, 141 (1975).
22. J. Hughes, *Brain Res.* **88**, 1 (1975).
23. G. W. Pasternak, R. Goodman and S. H. Snyder, *Life Sci.* **16**, 1765 (1975).
24. L. Terenius and A. Wahlstrom, *Acta physiol. scand.* **94**, 74 (1975).