THE SPECIFIC BINDING OF OPIATE AGONIST AND ANTAGONIST TO BRAIN TISSUE IN VITRO: ITS RELATIONSHIP TO OPIATE RECEPTORS AND THE INFLUENCE OF CHRONIC MORPHINE TREATMENT

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ABSTRACT

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By

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The primary aims of this investigation were to further characterize the properties of specific binding sites for an opiate agonist dihydromorphine and an opiate antagonist naloxone in vitro and to demonstrate that these specific binding sites are consistent with what is known about the pharmacologic receptor. Studies also were performed to determine if the development of tolerance and physical dependence during chronic morphine treatment is associated with changes in the concentration or affinity of specific binding sites for dihydromorphine and naloxone.

The binding in vitro of (^{3}H)-dihydromorphine was studied using particulate fraction obtained from rat brain homogenates and compared with that of (^{3}H)-naloxone. Tissue preparations were incubated with or without 10 μM levorphanol, unless otherwise indicated, at 35°C for 5 minutes in 50 mM Tris-HCl buffer (pH 7.4), artificial cerebrospinal fluid (CSF), or simulated intracellular fluid (ICF). Subsequently, (^{3}H)-dihydromorphine or (^{3}H)-naloxone was added

to the incubation mixture in final concentrations of 2 to 40 nM and incubated for an additional 15-minute period at 35°C. Bound drug was collected on Millipore filters (pore size, 0.8 μm) and washed immediately with 18 ml of ice-cold Tris-HCl buffer, CSF or ICF. Radioactivity of bound drug was assayed using liquid scintillation counting method.

Levorphanol as well as its pharmacologically inactive stereo-enantiomer dextrorphan inhibited (^{3}H)-dihydromorphine binding but dextrorphan was approximately three orders of magnitude less potent than levorphanol. The binding of (^{3}H)-dihydromorphine may be separated into two components: one saturable and stereospecific and the other non-saturable. The saturable, stereospecific binding may be calculated from the difference in binding assayed in the absence and presence of high concentrations of levorphanol. The use of dextrorphan resulted in an artifactual separation of the saturable binding component. The apparent Km value of the saturable, stereospecific binding sites for dihydromorphine in brainstem, estimated from Scatchard plot, was 7.9 ± 1.2 nM in 50 mM Tris-HCl buffer. The maximal specific binding was 0.25 ± 0.01 pmoles/mg protein. Based on Ki values estimated from Dixon plots of specific (^{3}H)-dihydromorphine binding in the presence of several non-labelled opiate analogs, levorphanol had the highest affinity for the specific dihydromorphine binding sites, followed by naloxone, morphine and d,l-methadone. Dextrorphan had an affinity 2000 times lower than that for levorphanol. Codeine and thebaine had

the lowest affinities. Apomorphine, dopamine, chlorpromazine, xylazine (Bayer 1470) and N-methylnicotinamide did not affect specific (^{3}H)-dihydromorphine binding at concentrations up to 10^{-5}M. SKF-525A inhibited specific dihydromorphine binding but this inhibition appeared to be resulted from the non-specific effects of this compound on the membranes. Thus, the saturable dihydromorphine binding sites appear to be specific for active opiate analogs.

Specific (^{3}H)-naloxone binding, assayed under the same conditions, also appeared to have two components. The apparent Km value of saturable, stereospecific binding sites in brain-stem for naloxone was 24.0 ± 6.6 nM in 50 mM Tris-HCl buffer and the maximal specific binding was 0.57 ± 0.01 pmoles/mg protein.

In CSF, as well as in ICF, the apparent affinity of specific binding sites for dihydromorphine was decreased while that for naloxone was increased as compared to those in Tris-HCl buffer. In CSF and ICF, apparent affinity of the specific binding sites for naloxone was significantly higher than that for dihydromorphine. The maximal specific binding for dihydromorphine and naloxone were both decreased in CSF and ICF as compared to those in Tris-HCl buffer.

There were marked regional differences in the distribution of specific (^{3}H)-dihydromorphine binding in the brain. It appeared that the specific binding sites in various brain regions had similar affinities for dihydromorphine except those binding sites in the cerebral cortex which had higher

affinity. In contrast, specific binding sites for naloxone in varous brain regions had different affinities. It appeared that naloxone has at least two types of specific binding sites, one of which is not available to dihydromorphine. This is based on observations that (1) the total concentration of sepcific binding sites for naloxone was greater than those for dihydromorphine in each brain region studied, except in the striatum, irrespective of the assay medium used and that (2) non-labelled dihydromorphine inhibited the specific (^{3}H)-naloxone binding in the striatum but failed to alter it significantly in the cerebellum whereas non-labelled naloxone reduced specific (^{3}H)-naloxone binding significantly in both brain regions. The differences in total binding sites for naloxone and dihydromorphine were relatively small in the striatum but large in the cerebellum, indicating that the specific binding sites in the cerebellum are predominantly naloxone-specific whereas those in the striatum are capable of binding both naloxone and dihydromorphine.

Two weeks after an intraventricular injection of 75 µg of 5,7-dihydroxytryptamine creatinine sulfate, specific (^{3}H)-dihydromorphine binding to preparations obtained from diencephalon and midbrain-low brain-stem of treated animals were not significantly different from specific binding to comparable preparations obtained from control animals. Similarly, pretreatment of rats with two intraventricular injections of 250 µg of 6-hydroxydopamine HBr also failed to

significantly alter specific (^{3}H)-naloxone binding to preparations obtained from cerebral cortex and brain-stem.

Chronic morphine treatment of rats or subsequent withdrawal failed to alter the concentration of either specific dihydromorphine or naloxone binding sites in the brain-stem when binding was assayed in 50 mM Tris-HCl buffer or in CSF. Chronic morphine treatment also failed to alter the affinities of the specific binding sites for naloxone and dihydromorphine. During withdrawal from morphine, there was a tendency toward a reduced affinity of specific binding sites for dihydromorphine, which returned toward control level upon the dissipation of the withdrawal syndrome.

It was concluded that the specific binding sites for dihydromorphine and naloxone could be demonstrated using low concentrations of radiolabelled opiate analogs. These binding sites appear to be saturable, stereospecific, specific for active opiate analogs and closely related to the pharmacologic receptors. Naloxone appears to have at least two types of specific binding sites, one of which is not available to dihydromorphine. It appears that these specific binding sites are not associated with central monoaminergic pre-terminal axons and nerve terminals which have been postulated to play an important role in the pharmacologic actions of opiate analgesics and the development of narcotic tolerance and physical dependence. Chronic morphine treatment failed to alter the concentration and affinity of specific binding sites for dihydromorphine and naloxone in particulate fraction obtained from brain-stem.

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INTRODUCTION

A. Opiate receptors

A-1. Properties of opiate receptors

It has long been believed by many investigators that there are specific receptors for opiate analgesics. Inferential data about opiate receptor were first derived by Beckett and Casy (1954) from studies on structure-activity relationships in several series of opiate analgesics. A receptor model has been formulated, containing a flat surface, a cavity and an anionic group in the proper spatial relationship to accommodate the active compounds. These studies called attention to the stereochemical requirements for analgesic activity. Portoghese and his colleagues [see Portoghese, 1965, 1966] have made major advances in understanding the stereospecificity inherent in the analgesic and addiction-producing actions of opiate analgesics. For morphine and for the various natural and synthetic morphine-type analgesics, it is always the D(-)-stereoisomer which is active, while the L(+)-isomer is essentially devoid of activity.

Further evidence for the existence of receptors is the fact that minor structural changes can result in the formation of potent and specific antagonists of many of the actions of morphine and its congeners. Thus, the

replacement of the methyl group on the tertiary nitrogen atom of morphine molecule by a large group, e.g., an allyl group, results in a potent morphine antagonist, nalorphine. However, this drug, as well as many other morphine antagonists, retains some analgesic potency and physical dependence-producing potential. Also they have some other undesirable psychotomimetic effects [see Casy, 1971]. Recently, it has been found that naloxone, an allyl analog of a potent opiate analgesic, oxymorphone, is a potent antagonist which is devoid of measurable agonistic properties [Blumberg et al., 1965; Harris and Dewey, 1966].

Changes in substituents on the nitrogen atom, however, are not consistently correlated with changes in analgesic activity. For example, N-allyl analogs of methadone and meperidine do not possess opiate antagonistic action [Costa and Bonnycastle, 1955; Portoghese, 1966]. Furthermore, as more compounds have been synthesized, a large variety of structurally-unrelated compounds have been found to possess morphine-like activity. Therefore, Portoghese introduced a new concept on the mode of interaction of opiate analgesics with their receptor in order to accommodate all the data obtained from extensive structure-activity relationship studies. The possibility of induced fit [Belleau, 1964] as a factor contributing to receptor binding of diverse analgesics was also recognized [Portoghese, 1965, 1966].

One of the possible modes of interaction is that different analgesics may interact with different sites on

the same receptor macromolecule. In this theory, it is assumed that the steric environment required for different analgesic molecules in different binding positions on a receptor are not identical. Smits and Takemori [1970], based on studies of pA_2 values (the negative logarithm of the molar dose of the injected antagonist which reduce the effect of a double dose of an agonist to that of a single dose) of naloxone for a series of opiate agonists and agonist-antagonists[1], concluded that opiate agonists and agonist-antagonists have different apparent pA_2s with naloxone. Takemori and his associates further showed that the apparent pA_2 value changed significantly from 6.96 in control mice to 7.30 in mice 2 hours after morphine treatment and further changed to 7.80 in morphine tolerant and dependent mice [see Takemori, 1974]. Takemori [1974] then postulated an opiate receptor similar to that suggested by Portoghese. The postulated receptor has different binding sites for opiate agonists, agonist-antagonists, and antagonists (naloxone); with a common site of attachment for the protonated nitrogen of these drug molecules. It is assumed that naloxone also interact at agonist site competitively and agonist-antagonists also interact at naloxone binding site. An opiate agonist is assumed to be able to induce a "better fit" at naloxone binding site. It is also assumed that

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[1]Agonist-antagonists are drugs with morphine-like action that have capacity to counteract the morphine-like action of other drugs under certain circumstances [see WHO Technical Report Series 495:11-12, 1972].

drugs interacting with different sites on the same receptor macromolecule could trigger different sequences of biochemical events and thus produce different pharmacologic effects.

Portoghese also pointed out that two or more species of receptors might mediate a similar analgesic response. Different receptors might interact with a single opiate analgesic, with somewhat different steric requirements. Also, different receptors could interact specifically and exclusively with different opiate analgesics. The last possibility is compatible with Martin's hypothesis [Martin, 1967].

Interaction studies between nalorphine and morphine on analgesia in man indicated that the dose-response curve was biphasic, with increasing antagonism as the dose of nalorphine was increased to a certain level and then reemergence of analgesia as the dose was further increased [Houde and Wallenstein, 1956; Houde et al., 1960]. Pentazocine also had similar effects [Jasinski et al., 1970]. When cyclazocine was chronically administered to man, tolerance developed to its sedative, psychotomimetic and ataxia-producing properties. Cross tolerance to these actions by morphine was observed [Martin et al., 1965]. Patients tolerant to either cyclazocine or nalorphine were refractory to the effects of morphine [Martin and Gorodetzky, 1965]. However, tolerance had not developed to the antagonistic properties of cyclazocine or nalorphine [Martin et al., 1965, 1966]. Patients tolerant to morphine were not cross tolerant to the psychotomimetic effects of cyclazocine. Physical dependence of

nalorphine-type drugs has not been associated with drug-seeking behavior [see Martin and Jasinski, 1972]. Thus, Martin hypothesized that both morphine-type agents and nalorphine-type agents act as agonists and that their agonistic actions are responsible not only for the desirable therapeutic effects but for dependence and tolerance. He further hypothesized that there are at least two types of receptors, the "morphine" and "nalorphine" type [see Martin, 1967]. These two receptors are responsible for two distinguishable types of agonistic activity and two types of dependence. Nalorphine binds to nalorphine-type receptors, that are not available to morphine, in addition to morphine-type receptors.

It has been argued that pharmacologic effects produced by morphine-type and nalorphine-type drugs can also be explained by postulating that different sequences of biochemical events could be triggered after the drug-receptor interaction. This possibility was discussed by Dole [1970] who has suggested that the pharmacological activity of opiate analgesics is an expression of an allosteric interaction [Koshland, 1958; Monod et al., 1963] in which a change in configuration of the receptor is essential to the biological action if the receptor occupancy theory [Clark, 1933] is to be retained. Both potency of the drug and the nature of its effect would be determined, not by the goodness of fit, but by the biochemical effects of the drug to cause a deformation of receptor molecules.

So far, there is no way of critically differentiating these hypotheses. However, it appears that there are some problems in the one receptor hypothesis. The basic point is the assumption that this receptor species has a common site of attachment for protonated nitrogen. According to this assumption, one receptor can only interact with one drug molecule at a time. Thus, the action of the drug molecule would depend entirely on its affinity with its receptor. However, this assumption could not explain the biphasic actions of nalorphine-type drugs. Presumably, one has to assume that nalorphine-type drugs have higher affinities for naloxone binding site than their affinities for agonist-antagonist binding site because these drugs produce opiate antagonistic effects at low concentrations. Following this assumption, then, one has to postulate that there is another species of receptor with a low affinity binding site for nalorphine-type drugs in order to explain the agonistic effects which are produced at high concentrations. If there is only one type of receptor, nalorphine-type drugs have no opportunity at all to interact with a low affinity binding site on the same receptor species. Since psychotomimetic effects and analgesic effects of nalorphine-type drugs can be separated, more types of receptors may have to be postulated. It is possible that different types of receptors are located close to one another and that allosteric effects could be induced by opiate agonists. Since naloxone can antagonize the analgesic effects of both the morphine-type

and nalorphine-type opiate drugs [Blumberg et al., 1966; McClane and Martin, 1967a; Jasinski et al., 1968], naloxone appears to have a high affinity for several types of receptors.

In addition to these anti-analgesic effects, naloxone also antagonizes the depressant effects of cyclazocine on the flexor reflex [McClane and Martin, 1967b] as well as the respiratory depressant and psychotomimetic effects of cyclazocine in man [Martin et al., 1966]. These data would indicate that the receptors responsible for the analgesic, respiratory depressant and psychotomimetic effects could be stereochemically quite similar and in some instances identical to the morphine-type receptor [Martin, 1967]. Alternatively, naloxone may have its own specific receptor(s) in various brain regions.

A-2. Attempts to localize opiate receptors in the central nervous system

In earlier studies, many investigators studied the selective distribution of various opiate analgesics in the central nervous system of laboratory animals in an attempt to correlate the physiological disposition of the drug with the localization of pharmacologic receptors. Efforts to achieve this, however, have largely been unsuccessful. No selective localization of labelled opiate analgesics has been found in any region of the central nervous system [Miller and Elliott, 1955; Mulé and Woods, 1962; Chernov and Woods, 1965]. Generally, the cerebral cortical gray matter contained higher

concentrations of free morphine. In cerebral and cerebellar white matter, levels of free morphine were lower than those in gray matter when sampled at early time intervals. This relationship tended to reverse itself when tissues were sampled at later time periods. It is interesting to note that while the overt response to morphine in the cat is stimulation in contrast to depression in the dog, comparative disposition studies have yielded no clue to explain the difference in responses between the cat and the dog [Chernov and Woods, 1965].

Studies of the intracellular distribution of (^{3}H)-dihydromorphine in the brain have shown that most of the radioactivity was in the soluble fraction. The neuclear fraction, which contains some cell membranes in addition to nuclei, contained 10 to 20% of the radioactivity of the homogenate. However, the lack of effect of non-labelled nalorphine administration on the radioactivity found in the nuclear fraction made it unlikely that the (^{3}H)-dihydromorphine found in the nuclear fraction represented the binding of the drug to pharmacologically active sites [Van Praag and Simon, 1966]. A high dose of dihydromorphine (100 mg/kg) was used in this study using high concentrations of non-labelled carrier dihydromorphine. Therefore, it is possible that the pattern of distribution demonstrated by these investigators presented non-specific binding (see Section A-3).

Ingoglia and Dole [1970] were the first to use the principle of stereospecificity in an attempt to identify opiate receptor sites. They studied the localization of

^{14}C-labelled d- and l-methadone after intraventricular injection into rat brain. Radioactivity was higher in the ipsilateral lateral ventricle and hypothalamus but there was no significant difference in the accumulation of the two isomers in the hypothalamus and in the other brain regions they studied. In experiments of this kind, most of the drug that diffuses into the tissue appears to be present unbound in tissue water or dissolved in tissue lipid. The amount of drug bound to receptors could only have been a minute fraction of the total drug. Seeman et al. [1972] also failed to observe any stereospecific binding, or selective distribution, with d- and l-methadone. In these investigations [Ingoglia and Dole, 1970; Seeman et al., 1972], the failure to demonstrate stereospecific binding may have been partly due to a poor choice of drugs, i.e., the isomers of methadone, since differences in pharmacologic effective doses of d- and l-methadone are not so great as with other stereoisomeric pairs. This probably reflects a higher degree of conformational flexibility of the methadone molecule than may occur with other pairs of stereoisomers [Portoghese, 1966].

Clouet and Williams [1973] have studied the localization of radio-labelled dihydromorphine, morphine, l-methadone, levorphanol, naloxone and nalorphine administered intracisternally to rats. In general, the levels of morphine and dihydromorphine were higher in regions rich in cell bodies such as cerebellum and hypothalamus, and lower in regions

containing many lipid structures such as midbrain and medulla. Concentrations of l-methadone and meperidine, on the other hand, were higher in midbrain. There seemed to be a positive correlation between the relative lipid solubility of the drugs and their abundance in anatomical and subcellular lipid-rich regions of brain. Subcellular studies indicated that these drugs were localized in the synaptosomal as well as in the soluble fractions. The administration of inactive isomers dextrorphan and d-methadone, had no effect on the amount of (^{3}H)-levorphanol and (^{3}H)-l-methadone distributed to the synaptosomal fraction. It should be pointed out that pharmacologically active doses of these drugs (equianalgesic with 60 mg/kg morphine, i.p.) were used and thus the concentrations of these drugs in the brain [Sanner and Woods, 1965] were much higher than the Km values (10^{-9}M to 10^{-7}M) of specific binding sites for these drugs estimated recently with *in vitro* studies [Lee *et al.*, 1973; Pert and Snyder, 1973b; Wong and Horng, 1973]. Thus, the major portion of binding they observed was probably nonspecific and would have masked the relatively small stereospecific binding. Diffusion of drug throughout the brain water would produce apparent localization in synaptosomes, because drug molecules dissolved in the acqueous interior of the nerve terminals would be trapped there when synaptosomes were formed during homogenization, whereas molecules in the axons and perikarya would be freed into the surrounding medium. This is demonstrated by the observation that about 70% of the radioactivity

in the synaptosomes could be released after osmotic lysis [Clouet and Williams, 1973].

Foster et al. [1967] observed that microinjections of morphine into the periventricular gray matter of the rostral hypothalmus caused a marked analgesia in a majority of the rats studied. Buxbaum et al. [1970] demonstrated that analgesic dose-response relationships could be observed in rats if microinjections were made into the anterior thalamic nuclei. They also noted analgesic effects when morphine was injected into other thalamic and hypothalamic areas. More recently, Jacquet and Lajtha [1973] injected morphine via fine-guage cannulas permanently implanted in various subcortical sites in the rat brain and observed that 10 μg of morphine injected into the posterior hypothalamus resulted in a significant analgesia, while the same dose injected into the medial septum, the caudate, or the periaqueductal gray matter yielded hyperalgesia. Thus, in rats, the main site of analgesic action of morphine appears to be in the periventricular structures of the third ventricle.

Tsou and Jang [1964], based on their investigation of analgesic effects after microinjections of morphine into various parts of rabbit brain, have concluded that the main site of morphine resides in the periventricular gray matter of the third ventricle. Herz et al. [1970] developed a method by which they were able to inject drugs into various specific and limited portions of the ventricular system in rabbits. Using this technique, the authors concluded that

the main sites of analgesic action of morphine were located in the periventricular gray matter surrounding the aqueduct and structures on the floor of the fourth ventricle. Whether these different conclusions are due to the techniques utilized remains to be investigated.

It is relevant to note from the above discussion that the intraventricular injections of morphine have been shown to produce tolerance and physical dependence in rats [Watanabe, 1971] and rabbits [Herz and Teschemacher, 1973].

Sites of action of opiate antagonists also have been studied. It was shown that the concurrent injection of nalorphine into the periventricular gray matter or into the aqueduct and the fourth ventricle in rabbits was effective in antagonizing the analgesic effects of morphine [Tsou and Jang, 1964; Albus et al., 1970]. In tolerant animals, the intraventricular injection of an opiate antagonist also precipitated withdrawal signs [Watanabe, 1971; Herz and Teschemacher, 1973]. Using a more elegant stereotaxic approach, Wei et al. [1972, 1973] introduced crystals of naloxone into various parts of the brain in morphine-dependent rats through concisely placed cannulas. Withdrawal signs were most frequently observed when the naloxone was placed in the medial thalamus and medial areas of the diencephalic-mesencephalic junctures. These are, therefore, presumed to be the primary sites of naloxone action.

These data would suggest that receptors responsible for the analgesia differ from that responsible for

precipitation of withdrawal syndrome or the primary sites of action of morphine-type analgesics and naloxone are located in different brain regions.

A-3. *In vitro* studies of opiate receptors

Recently, Goldstein *et al*. [1971] demonstrated *in vitro* that approximately 2% of radioactive levorphanol binding to mouse brain homogenate was saturable and stereospecific. The stereospecific binding was defined as the difference in labelled levorphanol binding observed in the presence of 100-fold excess of non-labelled levorphanol and its pharmacologically inactive enantiomer, dextrorphan. The fact that levorphanol is pharmacologically active whereas the L(+) isomer, dextrorphan is inactive may not require that the receptors be stereospecific. It is possible that both compounds combine with the receptors with the same affinity but only levorphanol makes the right molecular interaction to produce a pharmacologic effect. If the latter statement is true, however, it would be predicted that dextrorphan should be an antagonist; yet this is not so. Moreover, it is known that L(+) enantiomers of allyl-substituted antagonists are inert, having neither agonistic nor antagonistic effects. Therefore, it seems reasonalbe to conclude that only, D(-) enantiomers of opiate analogs can bind to the opiate receptors [Goldstein, 1974].

More recently, Pert and Snyder [1973a, 1973b] have demonstrated that more than 70% of (^{3}H)-naloxone binding is

saturable, stereospecific and can be displaced by other opiate agonists or antagonists. Moreover, the opiate receptor was found only in neuronal tissues. The quantitative differences observed by these two groups of investigators appears to result from differences in the concentration of radio-labelled compounds rather than from the specific compounds used, namely agonist or antagonist. The low concentration (4×10^{-9}M) employed by Pert and Snyder has been found to reduce the nonsaturable binding drastically and to affect the high affinity specific binding to a lesser extent [Lee et al., 1973]. The percentage of the specific binding, therefore, was dependent on the concentration of the labelled compound used in the study. Similar specific binding was observed with (^{3}H)-dihydromorphine [Terenius, 1973; Lee et al., 1973; Wong and Horng, 1973] and with (^{3}H)-etorphine [Simon et al., 1973] using low concentration of labelled compounds and a combination of either levorphanol-dextrorphan or l- and d-methadone to determine stereospecificity.

It was shown that specific naloxone binding had a Q_{10} (change in the reaction rate caused by a 10°C change in temperature) value of 1.5 between 25°C and 35°C, and about 70% of the specific binding was totally eliminated at 4°C [Pert and Snyder, 1973a, 1973b]. However, specific (^{3}H)-etorphine binding was not affected by high concentrations of sodium azide or sodium fluoride [Simon et al., 1973]. Specific (^{3}H)-dihydromorphine binding was not affected by 10^{-5}M ouabain [Wong and Horng, 1973]. These data would

suggest that the specific binding of naloxone, etorphine and dihydromorphine is not dependent upon energy from oxidative metabolism or glycolysis.

Specific (^{3}H)-naloxone binding had a sharp pH optimum at 7.4 [Pert and Snyder, 1973a, 1973b]. Specific (^{3}H)-etorphine binding, on the other hand, had a broad pH optimum between 6.5 and 8. Calcium and magnesium had no effect on specific naloxone binding [Pert and Snyder, 1973b]. Sodium decreased specific binding of (^{3}H)-etorphine and other opiate agonists [Simon et al., 1973; Pert et al., 1973] while it enhanced specific binding of (^{3}H)-naloxone and (^{3}H)-levallorphan [Pert et al., 1973]. Pert et al. [1973] have concluded that sodium increases the number of binding sites with no change in affinity for naloxone. No data was provided to support this conclusion.

Valinomycin and monensin, which can function as mobile carriers for monovalent cations in biological membranes in general, had no effect on the binding of (^{3}H)-dihydromorphine [Wong and Horng, 1973]. Since ouabain also has no effect on (^{3}H)-dihydromorphine binding, the authors interpreted their data as the absence of coupling between sodium transport and the uptake of the opiate analgesics.

Specific binding of (^{3}H)-naloxone and (^{3}H)-etorphine was proportional to the amount of protein over the range of 0.2 - 4.0 mg of protein. Specific binding of (^{3}H)-naloxone, (^{3}H)-etorphine and (^{3}H)-dihydromorphine was most rapid at 37°C and reached equilibrium in 15 minutes [Pert and Snyder,

1973a; Simon et al., 1973; Wong and Horng, 1973]. The specific binding of these agents were also shown to be reversible and the specific binding sites were saturable. For specific (^{3}H)-naloxone binding, the association constant (K_1) was 1.15 ± 0.34 x $10^6 M^{-1}$ sec^{-1} and the dissociation constant (K_2) was 1.16 ± 0.24 x 10^{-2} sec^{-1}, at 25°C [Pert and Snyder, 1973b].

Specific binding of (^{3}H)-naloxone, (^{3}H)-etorphine and (^{3}H)-dihydromorphine could be competitively inhibited by opiate analogs but not by putative neurotransmitters, prostagladin E_1 or E_2, acetylsalicylic acid, phenobarbital or Δ^9-tetrahydrocannabinol [Pert and Snyder, 1973a, 1973b; Simon et al., 1973; Wong and Horng, 1973].

Pert and Snyder [1973a, 1973b] were the first to study the relationship between the affinity of the specific naloxone binding sites for opiate analogs and the pharmacologic potency of these compounds. Based on the ID_{50} (the concentration of drug that reduces specific (^{3}H)-naloxone binding by 50%) of various opiate analogs to inhibit the specific binding of 8 nM (^{3}H)-naloxone, they demonstrated that etorphine has the greatest potency, the ID_{50} being about 1/20 of morphine. Levorphanol had 4000 times the potency of dextrorphan. Similarly, l-levallorphan was 5000 times as potent as its d-enantiomer. However, l-methadone was only about 10 times as potent as d-methadone, perhaps because it has greater conformational mobility than levorphanol [Portoghese, 1966]. Codeine, which is analgesically about

1/4 - 1/10 as potent as morphine, displayed less than 1/3000 of the potency of morphine. Because codeine is o-demethylated by liver microsomal enzyme to morphine, this drug may exert analgesic activity only after metabolism to morphine [Johannesson and Schou, 1963]. Naloxone was slightly less potent than morphine. On the specific etorphine binding observed in the presence of 3 nM (^{3}H)-etorphine, etorphine was about 60 times more potent than morphine. Dextrorphan was 4000 times less potent than morphine. However, naloxone was 6 times more effective than morphine [Simon et al., 1973]. On the specific (^{3}H)-dihydromorphine binding observed with 2 nM (^{3}H)-dihydromorphine; dihydromorphine, l-morhpine and levorphanol had about similar potency. Naloxone and l-methadone were slightly less effective. d-Methadone was about 50 times less potent than l-methadone while dextrorphan was 4000 times less effective than levorphanol [Wong and Horng, 1973]. Thus, these authors concluded that the affinity (ID_{50}) of various opiate agonists and antagonists generally parallels the known pharmacologic potency of these drugs. Opiate agonists and their antagonists compete for the same receptor sites. However, in these studies, a low concentration of labelled compounds were used. It should be noted that drug binding at a certain concentration is determined by both affinity and maximal binding capacity of binding sites. Moreover, it has been demonstrated that the Ki is not the same as the ID_{50} when competitive inhibition kinetics apply [Cheng and Prusoff, 1973]. Therefore, it is not

appropriate to assess the affinity of opiate analogs from a comparison of their ID_{50}s.

Goldstein et al. [1971] reported that the major regions of mouse brain (cerebrum, cerebellum, medulla, pons, diencephalon) did not differ greatly in their capacity for specific (^{14}C)-levorphanol binding. On the other hand, Pert and Snyder [1973a] reported that specific (^{3}H)-naloxone binding in mouse brain homogenates was high in striatum and low in midbrain, cortex, and brain-stem. According to these investigators, no specific (^{3}H)-naloxone binding was detectable in the cerebellum. In further studies, Kuhar et al. [1973] have demonstrated that the limbic system, thalamus and hypothalamus in monkey and human brain had highest specific (^{3}H)-dihydromorphine binding. Extrapyramidal areas, midbrain, and cerebral cortical white areas had lower specific binding. No specific (^{3}H)-dihydromorphine binding was detectable in the cerebellum-lower brain-stem and spinal cord (thoracic). Using (^{3}H)-etorphine similar results have been reported by Hiller et al. [1973]. They grouped the specific (^{3}H)-etorphine binding levels into four categories. The specific binding to most structures of human limbic system was grouped as the highest binding (0.44 - 0.23 pmole/mg protein). Caudate nucleus, putamen, hypothalamus, periaquaductal gray matter, etc., had moderate binding while hippocampus, globus pallidus, colliculi, substantia nigra, area postrema, cerebellar cortex, etc., had low binding. Cerebral white matter, dentate nucleus of cerebellum, pineal gland, pituitary gland,

etc., had very low binding. Kuhar et al. [1973] have concluded that regional differences in stereospecific (^{3}H)-dihydromorphine binding reflected variations in total number of receptor sites (Vmax) rather than in affinity (Km). No kinetic data was provided to support their conclusions. Whether the properties of specific (^{14}C)-levorphanol binding are different from the properties of specific (^{3}H)-etorphine and (^{3}H)-dihydromorphine binding remains to be studied.

Pert and Snyder [1973a] suggested that the regional differences in acetylcholine concentration paralleled the observed regional differences in specific naloxone binding and proposed a relationship with the action of opiates in diminishing acetylcholine release [see Weinstock, 1971]. In a subsequent study [Kuhar et al., 1973], electrolytic lesions resulting in the destruction of cholinergic, noradrenergic or 5-hydroxytryptaminergic pathways did not affect specific (^{3}H)-dihydromorphine binding in regions where the lesioned pathways terminate. The authors thus concluded that the opiate receptor is not a unique component of axons or nerve endings of any one of these neuronal tracts. Both Snyder and his associates and Simon and his associates have emphasized the importance of the limbic system in the mode of action of opiate analgesics.

Studies with subcellular particles indicated that specific (^{14}C)-levorphanol binding was high in the crude mitochondrial/cytoplasmic membrane fraction and not in the soluble supernatant [Goldstein et al., 1971]. Studies of

specific binding of (^{3}H)-naloxone to subcellular fractions gave similar results but the crude microsomal fraction appeared to have relatively higher specific binding [Pert and Snyder, 1973a]. Specific (^{3}H)-dihydromorphine binding was high in synaptosomes and low in mitochondria and microsomes [Wong and Horng, 1973].

Goldstein et al. [1971] demonstrated that nearly all stereospecific (^{14}C)-levorphanol binding in the crude nuclear fraction was accounted for in nuclear membranes. Terenius [1973] also demonstrated specific dihydromorphine binding in the synaptic plasma membrane fraction of rat cerebral cortex. Goldstein et al. [1971] reported that the membranes retained their stereospecific binding capacity for (^{14}C)-levorphanol after extraction of 70% of the protein by Triton X-100 or sodium dodecyl sulfate, providing that detergent was removed by dialysis. This binding in such preparations was largely abolished by treating with neuraminidase or pronase but not by trypsin. p-Chloromercuribenzoate, mercaptoethanol and iodoacetic acid failed to affect specific binding capacity. The binding capacity was retained nearly quantitatively in material extracted into chloroform-methanol. Simon et al. [1973] reported that specific (^{3}H)-etorphine binding was sensitive to trypsin and pronase and to N-ethylmaleimide, p-hydroxymercuribenzoate or iodoacetamide treatment. It was unaffected by phospholipase A and C. More recently, Pasternak and Snyder [1974] reported that specific (^{3}H)-naloxone binding was reduced by low concentrations of

trypsin and chymotrypsin, low concentrations of phospholipase A, high concentrations of phospholipase C and relatively insensitive to phospholipase D and neuraminidase. It should be pointed out that in these three studies, the specific binding was assayed under different experimental conditions. A series of studies on the effects of various treatments on the specific binding of a radiolabelled compound under the various experimental conditions utilized in studies cited above would be necessary before evaluating the results of the above studies.

In summary, the interaction of opiate analgesics with receptor may be interpreted in several ways. Takemori, based on pA_2 studies, favored the one receptor proposal while Martin favored the multi-receptor proposal. Attempts to correlate the localization of opiate receptors with selective physiological disposition of opiate analgesics have largely been unsuccessful. Using stereotaxic techniques, opiate receptors have been shown to be associated with periventricular structures of the third ventricle. Naloxone appears to have its primary sites of action in the medial thalamus and medial areas of the diencephalic-mesencephalic junctures.

Specific binding <u>in vitro</u> of opiate agonists and antagonists have been demonstrated with low concentrations of radiolabelled compounds. The specific binding is reversible and saturable at relatively low concentrations. In general, the ID_{50} of various opiate agonists and antagonists for specific opiate binding parallels the known pharmacologic

potency of these drugs. Specific binding assayed with a low concentration of radiolabelled opiate analog was different in various brain regions of laboratory animals as well as in man. This regional variation in specific opiate binding does not correlate with the regional distribution of any known neurotransmitter or its neuronal axons or nerve endings. It should be pointed out that the Ki rather than the ID_{50} should be estimated in order to assess the affinity of various opiate analogs for specific binding sites. Specific binding observed at a certain concentration of an opiate analog is determined by two independent variables, namely, maximal binding capacity and affinity. Thus, one cannot ascertain whether the regional differences in specific binding observed in previous studies are due to the differences in the concentration of specific binding sites, differences in affinity of specific binding sites for an opiate analog, or both. Therefore, in order to further understand the properties of the specific opiate binding sites, maximal binding and affinity of the specific binding sites should be studied.

B. Narcotic Tolerance and Physical Dependence

Development of tolerance and physical dependence are well known consequences of frequent, repeated administration of morphine and various natural and synthetic opiate analgesics. After repeated dosage, these drugs lose depressant activities while retaining stimulant potency [Seevers and

Woods, 1953]. The sedative, analgesic and respiratory effects become so attenuated that doses fatal for a normal individual can be taken without consequences. When an appropriate level of opiate analgesic is maintained, subjects appear functionally normal. However, an abrupt cessation of drug input or interruption of its action with an antagonist precipitates a set of excitatory abstinence (withdrawal) syndrome. Thus a physical dependence can be developed. In susceptible persons, opiate analgesics produce both physical and psychological dependence on the drug of such an intensity that the drive for drugs displaces all other desires. In many laboratory animals such as chimpanzee, monkey, dog, rat, mouse, cat, rabbit and guinea pig, tolerance to, and physical dependence on, opiate analgesics can also be developed to a greater or lesser degree [see Seevers and Deneau, 1963]. The riddle of the biochemical nature of such unique and hazardous effects of opiate analgesics has fascinated many researchers and many hypotheses have been advanced to explain these phenomena.

B-1. Development of narcotic tolerance and physical dependence

A majority of the investigators of opiate analgesics have assumed that tolerance and physical dependence are inseparable parts of a common mechanism since both syndromes develop and disappear concurrently [see Way et al., 1969]. The intimate relationship of tolerance with physical dependence is indicated by the fact that, as the animals become

more tolerant to morphine, the dose of naloxone required to precipitate the withdrawal syndrome becomes progressively less, and, conversely, more naloxone is required after physical dependence to morphine has largely subsided [Way et al., 1969]. The finding that tolerance and physical dependence can both be prevented by an antagonist also supports this concept. Nalorphine, administered either systemically or directly into the anterior hypothalamus, blocked the development of tolerance to the hypothermic and the analgesic effects of morphine in rats [Orahovats et al., 1953; Lomax and Kirkpatrick, 1967]. The development of physical dependence and tolerance to morphine could be prevented by a simultaneous administration of levallorphan in monkeys [Seevers and Deneau, 1968]. Furthermore, a pure narcotic antagonist naloxone, which lacks physical dependence liability, failed to produce tolerance [Jasinski and Martin, 1967], whereas an agonist-antagonist, like cyclazocine or nalorphine produces very mild physical dependence and weak tolerance [Martin et al., 1965; Martin and Gorodetzky, 1965].

Other investigators believe that narcotic tolerance and physical dependence may originate via different mechanisms. Cochin and Kornetsky [1964] demonstrated a persistence of morphine tolerance during a 15 month period without a manifestation of withdrawal syndrome following a single injection of the analgesic.

Nevertheless, it is generally believed that narcotic tolerance and physical dependence are inseparable parts of a

common mechanism and many hypotheses have been postulated to explain both phenomena simultaneously.

B-2. Dual action hypothesis

Tatum, Seevers and Collins [1929], based on classic observations of acute and chronic effects of morphine in several laboratory animals, have concluded that morphine simultaneously stimulates certain parts of the central nervous system and depresses others. Irritability increases with repeated administration of morphine because of the increment of stimulant effects. The increased nervous irritability thus required a larger dose of morphine to counteract and produce depressant effects. This increased dosage further augments the nervous excitability; hence a vicious cycle is developed. Addiction is largely a question of physiological balance between stimulation and depression at any given level of irritability of the integrated nervous system. Abstinence syndrome may result when increased irritability outlasts the depression. This concept has been termed the "dual action" theory because it visualizes a simultaneous existence of depression and stimulation in different parts of the nervous system. Twenty five years later, Seevers and Woods [1953] further postulated that the action of morphine is either depressant or stimulant depending on the location of the receptor. They postulated that the binding of morphine to certain sites on or near the surface of axons results in central depression, while that

to other intracellular sites in the cell body of the same or other neurons results in central stimulation. Morphine binding on the axon is visualized to be essentially a surface phenomenon dependent upon physicochemical forces, the pharmacologic response occurring only at the time of receptor occupation by the drug (see Section B-7). Morphine binding in the cell body is visualized to require intracellular penetration, to be slow in onset, firm in combination and long-lasting, the action being proportional (within limits) to the quantity present.

As time passed by, evidence has accumulated which apparently renders this hypothesis untenable as the sole explanation of the mechanism of morphine tolerance and physical dependence.

(1) In the first place, morphine must be present in nervous tissue during the entire period of withdrawal to elicit an excitatory response. However, it is known that free morphine disappears from the brain within 48 hours of a single injection [Mulé and Woods, 1962; Misra et al., 1971]. Thus, at the time of the maximal intensity of abstinence, 48 to 72 hours, only traces of morphine remain in the body. Several conjugated forms of ^{14}C-N-methyl morphine, on the other hand, could be detected in brain 3 weeks after a single subcutaneous injection of 10 mg/kg of ^{14}C-N-methyl morphine [Misra et al., 1971]. These investigators suggested that repeated administration of morphine could lead to a cumulative deposition of conjugated morphine in brain and

could bring about a biochemical alteration of a specific neuronal structure in the central nervous system and produce hyperexcitability if the site happens to be one where a receptor-neurotransmitter interaction occurs. It should be noted, however, that the small quantities of conjugated morphine reported by these investigators was within the range of error (±10%) and it has been shown that there was no signigicant difference in the quantity of conjugated morphine in brains of non-tolerant and tolerant dogs [Woods, 1954; Richter and Goldstein, 1970]. Furthermore, one of the conjugated morphine derivatives, morphine glucuronide has been shown to be pharmacologically inactive [Woods, 1954; Schulz and Goldstein, 1972]. Two contradictory reports [Hosoya and Oka, 1970; Sasajima, 1970] which claimed that the intracerebral injection of morhpine-3-glucuronide produced analgesia in mice, have been criticized on the basis that the observed action was caused by the free base resulting from hydrolysis of the conjugate [Schulz and Goldstein, 1972].

(2) According to this hypothesis the syndrome elicited by the direct stimulant action of morphine and morphine-like analgesics must be qualitatively similar to the abstinence syndrome. Although similarities are obvious in that both involve increases in reflex hyperexcitability, the two syndromes are by no means identical [see Seevers and Deneau, 1968].

(3) If the stimulant phase of morphine action is identical with the abstinence syndrome, a drug such as thebaine

which possesses only stimulant properties should either induce a high degree of physical dependence following chronic administration or it should produce the classical abstinence syndrome following acute administration. No physical dependence of any kind is developed to thebaine and the signs of drug action are not similar to those of abstinence.

(4) Shuster et al. [1963] and Goldstein et al. [1968] have demonstrated tolerance development to the stimulatory effects of morphine in mice, a finding that makes it very difficult to accept the concept that imbalance from the stimulatory effects elicited by morphine could account for the abstinence syndrome. However, depression of an inhibitory pathway may produce similar results to the unopposed stimulation of an excitatory pathway, and hence the classification of depressant and stimulatory actions of opiate analgesics becomes ambiguous.

B-3. Altered metabolism and distribution of opiate analgesics

The most obvious explanation for tolerance phenomena to many drugs is to propose the protection of susceptible tissue by the diminished absorption of the drugs, accelerated metabolism, or exclusion of drugs from the region of sensitive cells. However, in an extensive review of studies on distribution and the fate of morphine and its surrogates, Way and Adler [1960] concluded that any changes in the inactivation processes of morphine and its surrogates in vivo were disproportionately small when compared to the magnitude

of the loss of analgesic activity. The differences in the physiological disposition of morphine between nontolerant and tolerant dogs did not appear to be of sufficient magnitude to account for tolerance development [Mulé and Woods, 1962]. Similarly brain concentration of morphine after intraperitoneal injection in tolerant and nontolerant rats were not significantly different [Johannesson and Schou, 1963].

N-Dealkylation is the metabolic pathway common to most, if not all, opiate analogs [see Axelrod, 1968]. An interesting theory of cellular tolerance was based on the diminution of N-demethylating enzyme activity in the liver with repeated doses of opiate analgesics [Cochin and Axelrod, 1959]. This enzyme with its specific protein-drug interaction has been proposed to be an analog of the opiate receptor within brain tissue. If receptors in the brain are also decreased with chronic exposure to opiate analgesics, the neurons might diminish in reactivity to opiate analgesics and so the animal becomes tolerant. However, there was a lack of consistency and specificity with respect to the loss of demethylating ability by the liver microsomal enzymes and development of tolerance to opiate analgesics [see Way and Adler, 1960]. Tolerance to morphine and codeine analgesia and decreased drug-metabolizing activities in the liver microsomes were coincidental phenomena [Johannesson et al., 1965]. Various agents which altered demethylase activity did not always alter the rate of development of tolerance in the rat in a

similar fashion [Clouet and Ratner, 1964]. Although this hypothesis is now only of historical interest, Goldstein et al. [1973] reported recently that although the principal basis of tolerance of levorphanol in mouse running activity was a loss of sensitivity to levorphanol at the cellular level in brain, metabolic tolerance was also present. They indicated that this is due to increased conjugation and excretion of levorphanol.

More recently, Wang and Takemori [1972] have demonstrated that morphine is actively transported into the ventricular system via the choroid plexus and that the concentrations of morphine in cerebrospinal fluid can be two to three times higher than those in plasma. Since analgesic receptors appear to be readily accessible to cerebrospinal fluid [Tsou and Jang, 1964; Herz et al., 1970; Herz and Teschemacher, 1973], the concentration of opiate analgesics in the cerebrospinal fluid may be more important than average brain concentrations in determining the response to opiate analgesics, and hence changes in the capabilities of the active transport mechanism may play a role in tolerance development. However, the active transport mechanism dose not appear to be altered during chronic morphine treatment [Craig et al., 1971].

B-4. Redundancy hypothesis

Basically, this hypothesis states that there are two or more alternative pathways for mediating a physiological

function and that these pathways differ from the other in that they have different spectra of vulnerability to opiate analgesics [Martin, 1968]. It is assumed that the opiate analgesic interrupts one of the redundant pathways (pathway A), but not the other (pathway B). The next assumption is that pathway B will eventually hypertrophy with the continuous presence of opiate analgesic and takes over all or an increasingly large portion of the function mediated by pathway A. Thus, tolerance that developed to the opiate analgesic is a consequence of hypertrophy of the redundant and opiate-insensitive pathway B, not a decrement in effect on pathway A. When the opiate analgesic is withdrawn, pathway A returns to its normal level of excitability. However, because pathway B is now functioning at a higher level than prior to the chronic administration of the opiate analgesic the total system functions at much higher levels than it did in the pre-drug state. This exaggerated function is the hypersensitivity seen during abstinence [Andrews, 1943; Mulé et al., 1968; Kayan and Mitchell, 1968; Kayan et al., 1971; Tilson et al., 1973]. According to this hypothesis, the nature of the agonistic action of the opiate analgesic on a given functional system, as well as the rate at, and degree to which tolerance and physical dependence develops, depends on at least two factors: (1) the importance of the opiate-sensitive pathway in mediating the physiological response, and (2) the capacity of the opiate-insensitive pathway to hypertrophy. Thus, for some functions the

opiate-sensitive subpathway may represent only a small part of the total pathway, whereas in other functional systems it may represent a major portion of the pathway. Further, the opiate-insensitive pathway's capacity for hypertrophy may vary among the different functional systems independent of its role in mediating the function. Therefore, this hypothesis not only provides an explanation for both tolerance and physical dependence but also for the coexistence of partial and complete tolerance, as well as acute and chronic tolerance [Martin, 1968].

This attractive hypothesis, however, lacks supporting evidence. It was initially formulated to explain some of the actions of atropine on the ascending activating system and the descending vasomotor pathways emanating from the midbrain reticular formation [see Martin, 1968]. In these studies it was hypothesized that there is an alternative pathway mediating these physiological responses that did not contain muscarine synapses. Neurophysiological studies have shown that there are indeed atropine-sensitive pathways in parallel with atropine-insensitive pathways in the descending vasomotor and ascending activating systems. It has also been shown that there are independent cholinergic and adrenergic pathways that are facilitatory to the flexor reflex of the chronic spinal dog and whose activation produces spinal cord signs that are similar to those seen in abstinence [see Martin, 1968]. However, the hypothetical redundant pathways responsible for narcotic tolerance and

dependence have not been identified.

Another controversial point relates to the changes in the nervous system occurring during the chronic exposure to an opiate analgesic. Martin suggested that nervous pathway will hypertrophy when the pathway is "exercised"; on the other hand, Jaffe and Sharpless [1968] suggested that "disuse", not use, will strengthen the nervous pathway (see Section B-7). No direct evidence supports either suggestion.

B-5. Specific proteins and immune mechanisms

Apart from the previous hypotheses, it is possible that metabolic processes are involved in the development of tolerance. Actinomycin D given with repeated doses of morphine, impairs the development of tolerance in mice and rats [Cohen et al., 1965; Cox et al., 1968]. When given to animals not previously exposed to morphine, actinomycin D does not diminish the analgesic effect of morphine, and when given for a short period to animals with established tolerance, it does not lessen the tolerance. Thus, actinomycin D appears to affect a process which occurs only while tolerance is developing. Similarly, 8-azaguanine [Spoerlein and Scrafani, 1967; Yamamoto et al., 1967], puromycin [Smith et al., 1966] and cycloheximide [Way et al., 1968; Loh et al., 1969] also have been shown to inhibit the development of narcotic tolerance without blocking the action of the opiate analgesics in non-tolerant mice. In such experiments, it is

essential to establish that the opiate is indeed preventing the *in vivo* incorporation of radioactive precursors into brain proteins or nucleic acids. The half-life of at least some mammalian messenger RNAs are such that there is little change in proteins for several days after an injection of actinomycin D [Appleman and Kemp, 1966]. Most brain proteins that have been examined turn over slowly. Half-life for various fractions of rat brain proteins range from 12 to 22 days [Lajtha and Toth, 1966; von Hungen *et al.*, 1968]. By contrast, the half-life of many liver proteins is a few days or, in some cases, a few hours [Schimke *et al.*, 1968]. Because narcotic tolerance and physical dependence can be induced within a day or less [Lotti *et al.*, 1965; Cox *et al.*, 1968; Cheney and Goldstein, 1971], brain proteins that may increase in this process should have a rapid turnover. On the other hand, increases in proteins with a long half-life may account for the long persistence of some forms of narcotic tolerance. In this case, there may be permanent alterations produced in some manner by the opiate analgesics.

The above findings have been interpreted as evidence that synthesis of some specific proteins is required for development of tolerance. Since tolerance to opiate analgesics may represent a rise in the threshold to a chemical stimulus that acts to depress specific nervous pathways, Smith [1971] suggested that introduction of specific protein molecules somewhere in specific pathways may reduce the

sensitivity of synapses or neurons involved in transmission of the impulse in question.

Supporting the involvement of specific proteins in the development of morphine tolerance and dependence, Spoerlein and Scrafani [1967] noted an increase in the microsomal protein fraction from morphine tolerant mice. Clouet and Ratner [1968] also demonstrated a slight enhancement of _in vitro_ ribosomal protein synthesis from rats receiving 5 daily injections of 30 mg/kg morphine sulfate. However, in an earlier study [1967], they observed a greater inhibition of leucine incorporation into brain proteins _in vivo_ after the fifth daily injection of morphine than after the first, although there was no analgesic response on the fifth day. More recently, efforts to detect increased amounts or rates of synthesis of brain proteins using radioactive amino acid precursors and acrylamide gel electrophoresis also have failed [Hahn and Goldstein, 1971; Franklin and Cox, 1972]. Perhaps present methods are not sensitive enough to detect small changes which may occur in a specific protein in the brain of tolerant-dependent animals.

Not all inhibitors of protein synthesis block tolerance. Failure of ethionine to impair development of narcotic tolerance has been reported [Kato, 1967], although in the same experiments ethionine blocked the development of tolerance to phenobarbital and meprobamate, presumably by impairing synthesis of the hepatic drug-metabolizing enzymes. The lack of ethionine effect on morphine tolerance may be

due to the inability of this drug to alter protein synthesis in the brain.

Some of the protein synthesis inhibitors are also potent immunosuppressants. Cochin and Kornetsky [1964] have found that narcotic tolerance in rats can persist as long as 15 months after the termination of morphine treatments. Pretreatment of cycloheximide blocked the development of such tolerance [Feinberg and Cochin, 1969]. Since tolerance can be extremely persistent and since it does take a finite time to develop, it was proposed that tolerance might involve immune mechanisms. Morphine and its surrogates may stimulate the formation of antibody-like substances. Cycloheximide, therefore, should block the development of tolerance by blocking the synthesis of protein which can sequester or antagonize opiate analgesics. The possible immune mechanisms in the development of narcotic tolerance have been tested by experiments designed to transfer the tolerance factor from tolerant to non-tolerant animals. Successful transfer of narcotic tolerance by cell-free extracts had been claimed by Cochin and Kornetsky [1968] who reported a transfer of tolerance by blood serum from morphine-treated rabbits to naive mice. Kiplinger and Clift [1964], however, have reported that serum from tolerant humans and dogs potentiated, rather than inhibited, the analgesic effect of morphine in mice. Ungar and Cohen [1966] reported that extracts of brain from morphine tolerant rats and dogs conferred tolerance on mice. Other investigators, however, do not find

support for this hypothesis [Tirri, 1967; Smits and Takamori, 1968; Tilson et al., 1972].

Since it has been shown that brain levels of morphine are not different in tolerant and non-tolerant animals (see Section B-3), sequestering morpine in the blood cannot be the mechanism of tolerance. However, an antibody-like substance may be located near the opiate receptor sites and thus divert morphine from opiate receptors without altering the brain level of morphine. The specific antibody-like substance may be specific to opiate analgesics and may be common to different species of animals, but it would be overly optimistic to expect that the antibody-like substance administered systemically would reach the desired site within the central nervous system.

As pointed out earlier, efforts to detect new proteins in the brains of acutely morphine-treated and morphine tolerant and dependent mice and rats were unsuccessful [Hahn and Goldstein, 1971; Franklin and Cox, 1972]. Since morphine tolerance and physical dependence can be induced within a day or less [Lotti et al., 1965; Cox et al., 1968; Chenny and Goldstein, 1971] and since antibody formation in peripheral system would take about a week to develop, acute morphine tolerance and dependence appears not to involve immune mechanisms.

B-6. Alterations in central synaptic transmission

Since synapses are likely sites of the action of many drugs, attempts have been made to explain narcotic tolerance and physical dependence by alterations in synaptic transmission. Theories involving feedback control of neurotransmitter concentration have been put forward to explain both narcotic tolerance and physical dependence [Goldstein and Goldstein, 1961]. Opiate analgesics are assumed to inhibit an enzyme E that catalyzes the formation of product C, an enzyme reaction essential to a neuronal function or synaptic transmission. If C mediates an excitatory function, the response of the central nervous system to an opiate analgesic is a depression. If at the same time the level of C controls the synthesis and/or the breakdown of enzyme E, a decrease in the concentration of C will increase the synthesis or depress the breakdown of enzyme E. With continued exposure to the opiate analgesic, the quantity of active enzyme will be restored by an increase in synthesis and/or a stabilization of the enzyme E, resulting in tolerance development. If the opiate analgesic is suddenly withdrawn, the excess quantity of the enzyme, no longer inhibited, produces an excess of substance C which leads to excitation and an abstinence syndrome. A similar hypothesis was proposed independently by Shuster [1961]. According to this hypothesis, the steady state concentration of some central neurotransmitters would not change or would rather decrease during the tolerant state and would increase after the withdrawal of opiate

analgesics. This concept however, has not been supported by evidence.

Chronic administration of morphine has been shown to increase, rather than decrease, brain norepinephrine levels [Freedman et al., 1961; Maynert and Klingman, 1962; Sloan et al., 1963; Gunné, 1963; Akera and Brody, 1968]. Chronic morphine treatment failed to alter brain dopamine levels in monkeys [Segal and Deneau, 1962] and in dogs [Gunné, 1963]. Although Segal and Deneau [1962] have reported an increase in dopamine levels in caudate nucleus 24-48 hours after morphine withdrawal or after nalorphine precipitated withdrawal in monkeys, Gunné [1963] reported a decrease in brain dopamine levels 72 hours following morphine withdrawal in dogs when the animals were exhibiting moderate to severe withdrawal symptoms. Acute or chronic morphine treatment failed to alter brain 5-hydroxytryptamine (5-HT) levels in several animal species. Withdrawal of morphine in chronically treated animals failed to affect brain 5-HT levels [see Way and Shen, 1971]. Brain acetylcholine (ACh) levels increased after a single morphine administration [Maynert, 1967; Large and Milton, 1970]. Although an increase in brain ACh levels has been observed during morphine withdrawal [Large and Milton, 1970], brain ACh levels were either unchanged [Large and Milton, 1970] or increased [Hano et al., 1964; Maynert, 1967] during chronic morphine treatment. Thus, changes in brain levels of known neurotransmitters during the cycle of narcotic addiction do not follow the pattern

predicted from this hypothesis.

As pointed out earlier, a depression of an excitatory pathway may produce similar results to the unopposed stimulation of an excitatory pathway, and hence the classification of depressant and stimulatory functions of neurotransmitters is ambiguous.

The hypothesis also predicts that the development of narcotic tolerance may be blocked by treatment with inhibitors of protein or nucleic acid synthesis. As pointed out previously, there have been claims that the development of narcotic tolerance can be blocked by treatment with actinomycin D, azaguanine, puromycin and cycloheximide. However, as discussed previously (see Section B-5) efforts to detect increased amount or rates of synthesis of brain proteins were not successful.

In recent attempts to elucidate mechanisms involved in the genesis of morphine-induced analgesic tolerance, Shen et al. [1970] directed their attention to the effects of morphine on 5-HT turnover. Mice rendered tolerant by a morphine-pellet implantation procedure exhibited a significant increase in 5-HT turnover compared to placebo-implanted controls. Attenuation of these augmented responses upon the concurrent administration of either cycloheximide or p-chlorophenylalanine (PCPA) with morphine [Loh et al., 1969; Shen et al., 1970; Ho et al., 1972] suggested a plausible relationship between the synthesis of biologic macromolecules and morphine tolerance and physical dependence. Although an

intimate relationship between tolerance or physical dependence and brain 5-HT metabolism has been reported by several investigators to support this hypothesis [Tennen, 1968; Haubrich and Blake, 1969; Fennessy and Lee, 1970; Burks and Ducharme, 1971; Iwamoto et al., 1971; Thornburg et al., 1971; Bower and Kleber, 1971], other investigators have failed to observe an increased 5-HT turnover in morphine tolerant animals [Marshall and Grahame-Smith, 1970; Maruyama et al., 1971; Cheney et al., 1971; Algeri and Costa, 1971; Schechter et al., 1972].

Recently, the involvement of biogenic amines in the analgesic action of morphine has been approached by inducing degeneration of central monoaminergic nerve terminals with drugs such as 6-hydroxydopamine and 5,6-dihydroxytryptamine. 6-Hydroxydopamine, which produces a prominent and long-lasting depletion of catecholamines in the brain [Uretsky and Iverson, 1969] without significantly affecting the brain 5-hydroxytryptamine level, has been shown to decrease morphine analgesia [Ayhan, 1972; Bläsig et al., 1973]. 5,6-Dihydroxytryptamine, which produces a prominent and long-lasting depletion of brain 5-hydroxytryptamine [Baumgarten et al., 1972; Daly et al., 1973] did not affect morphine analgesia [Bläsig et al., 1973].

Midbrain raphé lesions, which induced a great reduction of forebrain 5-hydroxytryptamine, also did not affect morphine analgesia [Bläsig et al., 1973]. Samanin et al. [1973] did observe decreased morphine analgesia after raphé lesions,

but analgesia produced by methadone, meperidine, codeine or propoxyphene was not affected. These results thus suggested that catecholamines play a more important role in morphine analgesia. Whether the involvement of catecholamines suggested for the analgesic effect of morphine can be generalized to other opiate analgesics remains to be investigated.

It has been shown that 5,6-dihydroxytryptamine, but not 6-hydroxydopamine, inhibited the development of tolerance to and physical dependence on morphine [Frielder et al., 1972; Ho et al., 1973]. These results would suggest that 5-HT plays a more important role in the development of narcotic tolerance and physical dependence. Since there is evidence suggesting that the analgesic effect produced by morphine is closely related with the development of narcotic tolerance and physical dependence (see Section A-1 and B-8) this conclusion appears not to be consistent with previous conclusion which stated that catecholamines play a more important role in morphine analgesia. Further supporting evidence, therefore, is necessary before evaluating the results of the above studies.

B-7. Pharmacological supersensitivity hypothesis

Pharmacological or "disuse" supersensitivity of the central cholinergic system has been described by Friedman et al. [1969]. These authors treated mice with scopolamine, a centrally acting anticholinergic drug. After several days or weeks, scopolamine was withdrawn and mice were tested for

the hypothermic effect of pilocarpine, a cholinergic drug that can act on the peripheral and central nervous systems. The scopolamine pretreatment increased the degree and duration of the hypothermic response to pilocarpine. Moreover, development of tolerance was observed to the pilocarpine-blocking action of scopolamine. The development of tolerance followed the same time course as the development of supersensitivity. Both phenomena were ascribed to an increase in the number of central cholinergic receptors. This is supported indirectly by the finding that either surgical or pharmacological denervation (produced by botulinum toxin) increases the concentration of cholinergic receptors on the membrane of a striated muscle cell [Axelsson and Thesleff, 1959; Thesleff, 1960].

Jaffe and Sharpless [1968] suggested that most clinically important narcotic withdrawal syndrome represent some form of rebound hyperexcitability in central nervous pathways. According to this suggestion and based on the findings of Friedman et al. [1969] on central cholinergic systems, Jaffe and Sharpless [1968] postulated that the primary cause of the rebound hyperexcitability occurring during narcotic withdrawal is the result of disuse or depression of nervous pathways rather than the presence of the drug entity itself. In identifying the drug-induced depression rather than the opiate analgesic itself as the primary causative mechanism, they further suggested that drugs that act on different sites or occupy different receptors

might still produce the same abstinence syndrome by causing, directly or indirectly, a diminution in the flow of impulses along the same nervous pathway. On the other hand, classes of drugs that directly produce patterns of depression on different systems might be expected to produce distinct patterns of withdrawal hyperexcitability.

The primary controversy is the postulated central supersensitivity itself. Stolk and Rech [1968] have demonstrated that chronic treatment with reserpine made rats more sensitive to locomotor stimulation by d-amphetamine. This would be another example of pharmacological supersensitivity in the central nervous system. However, events observed in animals are an integrated activity of the total nervous system. Pharmacological effects of drugs such as scopolamine or reserpine on the central nervous system are undoubtedly a summation of complex interactions of the drug with different neuronal components, and hence the interpretation of results obtained with these agents may be multivariant. Moreover, supersensitivity with morphine or other opiate analgesics still has not been clearly demonstrated.

B-8. Receptor occupation hypothesis

Based on classic observations of acute tolerance to the vascular effects of morphine, Schmidt and Livingston [1933] have suggested that tolerance is the result of a change which occurs in depressible cells as soon as the concentration of morphine in contact with them has reached

a certain critical level, and that the change is reversed as soon as the concentration falls below this level. Abstinence syndromes may be the external manifestations of the reversal of the cell tolerance reaction when morphine is withheld. This hypothesis is compatible with the hypothesis which states that the tolerance is the result of receptor occupation [Clark, 1933], and the rate theory postulated by Paton [1961]. These hypotheses postulated that drug molecules exert their action at the time of initial attachment to the receptor sites and that, while the receptor sites are occupied or the dissociation rate of drug from receptor is very slow, the original molecules exert no further effect, but do prevent the initiation of response by receptor interaction with additional molecules of the same or similar drugs. Although many aspects of morphine action could be explained successfully by this theory, it would not explain the long-term persistence of tolerance shown by Cochin and Kornestsky [1964]. There is a lack of evidence that a significant amount of morphine residue could persist in brain for several weeks after a single injection (see Section B-2). Moreover, it has been shown that the chronic administration of naloxone, a potent narcotic antagonist, does not produce physical dependence or tolerance development to its antagonistic action [Jasinski and Martin, 1967]. These findings would indicate that receptor occupation itself is not sufficient to produce narcotic tolerance and physical dependence. Agonistic action of opiate analgesics

appears to be necessary for their development.

B-9. Alterations in the receptor

Induction of protein synthesis also is a central feature of the theory proposed by Collier [1965]. According to this theory, it is not necessary for the neurotransmitter concentration to be altered during the development of physical dependence to an opiate analgesic. Instead, it was proposed that there is an increase in the amount of protein which binds opiate analgesics. The opiate-induced synthesis of "silent receptors," i.e., macromolecules that interact with drugs but do not produce any detectable pharmacological effect, would reduce the amount of the drug bound by "active receptors" and hence result in tolerance development. However, efforts to detect such an increase in the amount of brain proteins have failed (see Section B-5).

The possibility remains that narcotic tolerance may be related to a change in the quality rather than quantity of certain brain proteins. In this case, opiate analgesics need not alter the overall rate of turnover of brain proteins but the development of tolerance still could be blocked by inhibitors of protein synthesis. For example, opiate analgesics may cause ambiguity in genetic coding. If the resulting altered proteins cause a central stimulation that could antagonize the depressant effects of an opiate analgesic, the result would be tolerance. Upon withdrawal of the opiate analgesic, the unopposed action of the altered proteins

could give rise to a withdrawal syndrome [Shuster, 1971]. If opiate analgesic did produce miscoding, they may also act as inhibitors of protein synthesis. Morphine and related compounds in high concentrations do inhibit protein synthesis in mammalian cells. However, there is no correlation between the analgesic activity of various derivatives or their potency to produce narcotic tolerance and their potency as inhibitors of protein synthesis [see Shuster, 1971].

A novel approach to detect anomalous protein content in brain homogenate of morphine-tolerant mice, however, was unsuccessful. Antibodies to naive brain homogenates were used to precipitate unaltered protein in homogenates obtained from brains of morphine-tolerant animals. After a precipitation of normal protein by antibodies no protein remained (Hosoya, personal communication). The failure to detect anomalous protein would indicate several possibilities: (1) no altered protein exists in the tolerant brain (change is quantitative), (2) altered protein is a part of larger particles and precipitates with other proteins, (3) the quantities of altered protein are too small to be detected, or (4) the altered protein is still recognizable by antibodies.

In summary, the biochemical basis for the development of narcotic tolerance and physical dependence appears to involve protein synthesis mechanisms in the central nervous system. Tolerance to and physical dependence on opiate analgesics may develop as a result of quantitative changes

in brain proteins if chronic morphine treatment induces changes in concentration of opiate receptors, changes in amounts of enzymes related to central neurotransmitters, or the synthesis of new proteins which could affect the opiate-receptor binding or affect the neurotransmitter-receptor interactions which are involved in pharmacologic manifestation of opiate analgesics. Tolerance and physical dependence may also develop as a result of qualitative changes in brain proteins if chronic opiate treatment alters the binding affinity of the existing receptors for morphine and its congeners. A number of investigators have failed to detect quantitative changes in brain proteins associated with the development of narcotic tolerance and physical dependence. The failure to detect increased amounts or alterations in the rate of synthesis of brain proteins could be due to the fact that currently available techniques do not allow the detection and accurate measurement of the turnover of minor constituents.

C. Summary and objectives

The elucidation of the site of action of drugs is one of the main concerns of pharmacology. Despite extensive efforts by numerous investigators, we have little positive knowledge about the site of action of opiate analgesics. It has long been believed by many investigators of opiate analgesics that receptors for these drugs do exist in the central nervous system. Based on the results of extensive structure-

activity relationship studies, Portoghese [1966] suggested the existence of three types of interaction of opiate analgesics with their receptor. Although Takemori [1974] favored the one receptor hypothesis, Martin's multi-receptor hypothesis [1967] in general appears to be more adequate for explaining the mode of actions of various opiate analgesics (see Section A-1). Attempts to correlate the localization of pharmacologic receptors with selective physiological disposition of opiate analgesics were unsuccessful (see Section A-2). Recently, it was suggested that the receptor for the analgesic action of opiates might be distributed within periventricular gray matter. Using elegant stereotaxic techniques, Jacquet and Lajtha [1973] concluded that the main site of morphine analgesia was in the posterior hypothalamus of the rat brain. Wei et al. [1972, 1973] concluded that the primary sites of action of naloxone, an opiate antagonist, are in the medial thalamus and the medial area of the diencephalic-mesencephalic junctures of rat brain. The first successful demonstration of opiate receptors in vitro was reported by Goldstein and his associates [1971] using radiolabelled levorphanol. In last two years, specific binding of (^{3}H)-naloxone, (^{3}H)-dihydromorphine and (^{3}H)-etorphine to brain tissue of laboratory animals and man has also been demonstrated (see Section A-3). However, in most of these studies, only one concentration of radiolabelled compound was utilized. Since specific binding observed at a certain concentration of an opiate analog is determined

by affinity and maximal binding capacity of specific binding sites for the particular opiate, it is apparent that these two kinetic parameters should be determined in order to understand the properties of the specific binding sites observed *in vitro*. The demonstration of specific opiate binding *in vitro* requires (^{3}H)-labelled compounds with high specific radioactivity. Since the only radiolabelled compounds available are dihydromorphine, etorphine, morphine and naloxone, the ID_{50} has been used to assess the affinity of other opiate analogs for the specific binding sites. However, the ID_{50}, particularly assayed in a simple buffer solution, is not an appropirate assessment of affinity for opiate analogs. the inhibiton constant (Ki) should be used for this purpose.

Narcotic tolerance and dependence, both physical and psychological, are well known consequences of frequent, repeated administration of morphine and various natural and synthetic opiate analgesics. Because of these undesirable effects, morphine and its congeners have been abused widely around the world. Many hypotheses have been proposed to explain these phenomena but none of them has been generally accepted. In general, some of the hypotheses postulated that a direct adaptation takes place in which the concentration and/or the affinity of receptor for the opiate analogs are(is) altered after repeated administration of opiates. Since the specific binding sites for several opiate agonists and antagonists in brain tissue assayed *in vitro* could be

candidates of pharmacologic opiate receptor, it is of interest to test these possibilities by examining changes in the maximal binding and the Km of specific binding sites for (^{3}H)-dihydromorphine and for (^{3}H)-naloxone *in vitro* using chronically morphine-treated animals.

The primary aims of this investigation were to further characterize the properties of specific dihydromorphine and naloxone binding sites *in vitro* and to demonstrate that these specific binding sites are consistent with what is known about the pharmacologic receptor. Studies also were performed to determine if the development of tolerance and physical dependence during chronic morphine treatment is associated with changes in the concentration or affinity of specific binding sites for dihydromorphine and naloxone.

MATERIALS AND METHODS

A. Materials

Dihydromorphine (7, 8-^{3}H labelled) was purchased from New England Nuclear, Boston, Mass. This compound was supplied with a specific radioactivity of 46 Curies/m mole and radiochemical purity of 98.5%. Naloxone (^{3}H, randomly labelled) was also purchased from New England Nuclear. This compound was obtained with a specific radioactivity of 23.6 Curies/m mole and a radiochemical purity of 98.5%. These two compounds were used without further purification. (^{3}H)-dihydromorphine was supplied as an ethanol solution (1 mCi; 6.4 µg/ml) and (^{3}H)-naloxone was supplied as a methanol solution (1 mCi; 1.39 µg/ml). Both compounds were stored in the refrigerator or in the cold room at 0-5°C. Fresh solutions of both compounds at the appropriate concentration were prepared for each experiment by diluting the supplied solution with ice-cold buffer solution, pH 7.4 (usually more than a 200 times dilution) in the cold room.

Morphine sulfate USP, was purchased from Mallinckrodt Chemical Works (St. Louis, Mo.). Levorphanol tartrate and dextrorphan tartrate were obtained from Hoffman-LaRoche Inc. (Nutley, N.J.). Naloxone HCl was purchased from Endo Laboratories Inc. (Garden City, N.Y.). Dihydromorphine HCl, d,l-methadone HCl were generous gift of Dr. J. H. Woods,

(Department of Pharmacology, University of Michigan, Ann Arbor). Morphine base pellets were supplied by Dr. E. L. Way, (Department of Pharmacology, School of Medicine, University of California, San Francisco). 6-Hydroxydopamine HBr was purchased from Sigma Chemical Company (St. Louis, Mo.). 5,7-Dihydroxytryptamine creatinine sulfate was a generous gift of Dr. A. A. Manian, (NIMH, Rockville, Md.). Other chemicals were analytical reagent grade.

B. Tissue preparation

Male, Sprague-Dawley rats weighing 200 to 300 grams were used. The animals were decapitated and the brains were rapidly excised. Meninges and choroid plexuses were removed with a dissecting forceps. The brain-stem, that is the brain without cerebral cortex and cerebellum, was separated as will be described later, weighed and homogenized in 19 volumes of 50 mM Tris-HCl buffer (pH 7.4) using a motor-driven Teflon-pestle homogenizer (Potter type: A. H. Thomas Company, Philadelphia, Pa; type A, BB or B; clearance 0.10-0.18 mm at 25°C). Pestle was driven with a Tri-R motor at approximately 900 rpm and the tissue was homogenized with 5 passes in 30 seconds.

When regional studies of the specific binding of dihydromorphine or naloxone were performed, brains were dissected as essentially described by Nybäck and Sedvall [1969]. First, the cerebellum was removed with a forceps. Then, with the aid of the central fissure, an incision to the

corpus callosum was made with a small spatula. The cerebral cortices were separated from subcortical structures using the lateral ventricles as a guide. The caudate nuclei (striatum) were carefully separated from the cortex using the exposed upper surface of caudate nuclei (external capsule) as a guide and then separated from the rest of diencephalic structures by cutting through the internal capsule. The diencephalon (thalamus-hypothalamus) and mesencephalon (mid-brain) were separated by cutting through the anterior border of the anterior colliculi. Pons and medulla oblongata were separated from midbrain by cutting through the posterior border of the posterior colliculi. The pons and medulla oblongata were combined. The striatum and midbrain were pooled from four rats and cerebral cortex, thalamus-hypothalamus, pons-medulla oblongata and cerebellum were pooled from two rats.

The homogenate was centrifuged in a Beckman L3-50 ultracentrifuge using a type 40 rotor at 100,000 x g (40,000 rpm) for 25 minutes at 1°C. The supernatants were discarded and the pellets were rehomogenized in Tris-HCl buffer and recentrifuged as described above. Pellets were finally homogenized in Tris-HCl buffer as described above and then diluted with 9 volumes of Tris-HCl buffer, i.e., to the 200 volumes of the original tissue. The homogenate of the particulate fraction of indicated brain regions were assayed immediately for (^{3}H)-dihydromorphine or (^{3}H)-naloxone binding as described later.

All preparative procedures were performed in a cold room in which the temperature is kept below 5°C.

In some experiments, tissue preparations were prepared and incubated with (^{3}H)-dihydromorphine or (^{3}H)-naloxone in artificial cerebrospinal fluid (CSF) or in simulated intracellular fluid (ICF). The artificial cerebrospinal fluid contained 125 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 25 mM sodium bicarbonate. The simulated intracellular fluid contained 22 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$ and 25 mM sodium bicarbonate [Davson, 1967]. Salts were dissolved in double-distilled water and the solution was bubbled with 95% O_2-5% CO_2 gas for 60 minutes. The solutions were kept in the refrigerator or cold room and bubbled with 95% O_2-5% CO_2 gas for 20 minutes prior to use. Both CSF and ICF solutions prepared as described above had a pH of 7.4.

Protein concentrations of homogenates were assayed by the method of Lowry et al. [1951]. One ml aliquot of the homogenate was taken at the beginning and the end of each experiment and diluted with 3 ml of double-distilled water. Two 0.3 ml samples of the diluted homogenates were stored in the freezer until assayed. Bovine serum albumin (crystallized and lyophilized, Sigma Chemical Company, St. Louis, Mo.) was used as the protein standard. It was found that Tris-HCl buffer increased the background absorbance whereas CSF and ICF had no such effect. Therefore, when the homogenate was prepared in 50 mM Tris-HCl buffer, bovine serum albumin was dissolved and diluted in 12.5 mM Tris-HCl buffer.

Frozen samples of tissue homogenates were thawed to room temperature prior to assay.

C. Binding assays

Tissue preparations (0.4 mg of protein in a final volume of 2 ml) were incubated with or without 10 μM levorphanol, unless otherwise indicated, at 35°C for 5 minutes in the presence of 50 mM Tris-HCl buffer (pH 7.4). Subsequently, (^{3}H)-dihydromorphine or (^{3}H)-naloxone was added to the reaction mixture and incubated for an additional 15-minute period at 35°C. Each experiment was performed in triplicate. Bound drug was collected on Millipore filters (type AA, pore size, 0.8 μm) and washed immediately with 18 ml of ice-cold Tris-HCl buffer (pH 7.4). Each filter was dissolved in 1.0 ml of ethyleneglycol monomethylether and assayed for radioactivity in a liquid scintillation counter [Beckman, L-100]. Counting efficiency was monitored with the external standard channel-ratio which was calibrated with internal standards. For (^{3}H)-labelled compounds, counting efficiency was approximately 32%. The amount of tissue-bound drug assayed in the presence of 10 μM levorphanol (non-saturable binding) was subtracted from that assayed in the absence of levorphanol (total binding) to calculate the saturable, stereospecific binding. Km value and maximal specific binding were determined using double-reciprocal [see Webb, 1963] or Scatchard plots [Scatchard, 1949]. Ki value for various opiate agonists and antagonists on specific (^{3}H)-dihydromorphine binding were determined using Dixon plots

[see Webb, 1963].

D. Chronic morphine treatments of rats

Rats weighing about 200 grams were rendered tolerant to and physically dependent on morphine by subcutaneous injections of morphine sulfate solution at 9 a.m. and again at 5 p.m. The initial dose of 10 mg morphine sulfate per Kg of body weight per injection was increased every other day over a period of 12 days to a final dose of 100 mg morphine sulfate per Kg of body weight per injection. Control animals were given a comparable volume of isotonic saline solution subcutaneously. Compared to control animals, the growth rate of morphine treated animals was greatly retarded. At the beginning of treatment, morphine induced catalepsy, rigidity of the muscles of body and limbs and constipation. After 5-6 days of chronic treatment, the catalepsy become less apparent and hyperactivity and gnawing became prominent. When morphine injections were terminated after the chronic treatment, severe diarrhea and acute body weight losses were observed and the animals were hypersensitive to handling and to external stimuli. These signs would indicate that the animals had become physically dependent on morphine following chronic morphine treatement [Akera and Brody, 1968; Nozaki et al., 1974].

Rats treated with morphine as described above had a high mortality rate (1-2 out of 6 rats) when the dose of morphine sulfate was increased from 10 to 20 or from 20 to

40 mg/Kg/injection. Therefore a log scale doses of morphine sulfate starting from 10 mg/Kg/injection to 100 mg/Kg/injection was adopted in later experiments. No rat died during this dosage schedule and tolerance to 100 mg morphine sulfate can be achieved in a shorter time period.

In a series of experiments (results are shown in Figure 19 and Table 5) rats were injected with morphine solution (morphine base dissolved in 0.01 N HCl) subcutaneously at 8-hour intervals (6 a.m., 2 p.m. and 10 p.m.). The initial dose of 1.67 mg/Kg/injection was increased to 10 mg/Kg/injection over a period of 16 days and this dose was maintained for at least 6 days prior to the sacrifice. Control animals received comparable volumes of 0.01 N HCl solution.

In another series of experiments (results are shown in Figure 18) rats were rendered tolerant to and physically dependent on morphine by implanting two 75 mg morphine base pellets subcutaneously 36 hours apart as described by Way et al. [1969]. These investigators and Cicero and Meyer [1973] have demonstrated that rats treated by this procedure developed morphine tolerance and physical dependence.

Animals were sacrificed at about 9 a.m. in the morning 16 hours after the last injection or 36 hours after the second morphine base pellet implantation unless otherwise indicated.

E. 6-Hydroxydopamine and 5,7-dihydroxytryptamine treatment of rats

6-Hydroxydopamine HBr and 5,7-dihydroxytryptamine creatinine sulfate solutions were freshly prepared in isotonic saline solution containing ascorbic acid (1 mg/ml). Ascorbic acid was used to prevent the oxidation of these two drugs in the aqueous solution. Rats were anesthetized with sodium pentobarbital (50 mg/Kg, i.p.). Ten µl of a solution containing 250 µg of 6-hydroxydopamine HBr was then injected into the left lateral ventricle of the brain over a period of one minute by the method essentially described by Noble et al. [1967]. Briefly, rat was placed in a stereotaxic apparatus and a mid-sagittal incision was made from eyes to the ears and the bregma was exposed. A small hole, made with a dental drill, was placed 1.5-2.0 mm lateral to the crossing of the sagittal and coronal sutures. A 23-gauge needle, connected to a 100 µl Hamilton syringe with a polyethylene tube, was lowered 3.5-4.0 mm into the brain through the hole and 10 µl of drug solution or saline was injected. Seven days later, the same dose of 6-hydroxydopamine HBr was injected into the right lateral ventricle. Control animals were similarly treated with 10 µl injections of isotonic saline solution containing ascorbic acid (1 mg/ml). By the same method other groups of rats were treated with a single dose of 75 µg of 5,7-dihydroxytryptamine creatinine sulfate. The drug solution was injected into the left lateral ventricle.

All animals were sacrificed 14 to 21 days after the first drug treatment. The dose of both drugs chosen in the present studies has been shown to produce prominent damage of either catecholaminergic or 5-hydroxytryptaminergic nerve terminal structures [Uretsky and Iversen, 1970; Baumgarten and Lachenmayer, 1972].

F. Statistical analysis

Statistical evaluations of the data were performed by Student's t-test. The level of significance was selected as a probability of less than 5%.

EXPERIMENTS AND RESULTS

A. Characterization of specific (^{3}H)-dihydromorphine binding to brain-stem particulate fraction assayed in 50 mM Tris-HCl buffer (pH 7.4)

Since levorphanol and dextrorphan have been used to differentiate stereospecific and non-specific binding [Goldstein et al., 1971; Pert and Snyder, 1973a, 1973b], the effects of these agents on (^{3}H)-dihydromorphine binding were investigated.

The binding of (^{3}H)-dihydromorphine to brain-stem particulate fraction in the absence of levorphanol or dextrorphan (total binding) was 0.24 ± 0.01 pmole/mg protein (mean ± standard error of 5 experiments) with 6 nM (^{3}H)-dihydromorphine present in the incubation mixture. Both levorphanol and dextrorphan inhibited (^{3}H)-dihydromorphine binding (Figure 1). Levorphanol significantly inhibited (^{3}H)-dihydromorphine binding when its concentration was higher than 5×10^{-10}M and inhibited 50% of the total dihydromorphine binding (ID_{50}) at 3×10^{-9}M. On the other hand, dextrorphan did not inhibit dihydromorphine binding significantly until its concentration was higher than 5×10^{-6}M and its ID_{50} was 9×10^{-6}M. However, both levorphanol and dextrorphan produced a similar maximal inhibition: approximately 75% of total (^{3}H)-dihydromorphine

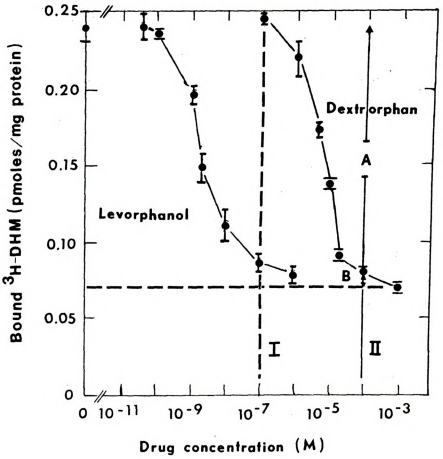

Figure 1. Levorphanol and dextrorphan inhibition of dihydromorphine binding assayed with 6 nM (^{3}H)-dihydromorphine(^{3}H-DHM) in 50 mM Tris-HCl buffer(pH 7.4).

Brain-stem particulate fraction was incubated for 5 minutes with indicated concentrations of either levorphanol or dextrorphan. Subsequently, 6 nM (^{3}H)-dihydromorphine was added to the incubation mixture and the binding was assayed. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

binding (Figure 1, horizontal broken line). Thus, although the magnitude of maximal inhibition obtained were similar with both compounds, dextrorphan was markedly less potent than levorphanol, indicating that the affinity of dextrorphan for the binding sites was approximately three orders of magnitude lower than that of levorphanol. Under this experimental condition, the difference in (^{3}H)-dihydromorphine binding assayed in the presence of 10^{-7}M dextrorphan and in the presence of 10^{-7}M levorphanol would represent the saturable binding which is relatively stereospecific (Figure 1, line I). This observation is consistent with the data shown by Pert and Snyder [1973a]. The use of higher concentrations of dextrorphan and levorphanol, for example 10^{-4}M as employed by Goldstein et al. [1971], would artificially separate the saturable binding into two components: saturable, non-stereospecific binding (Figure 1, line II A) and saturable, stereospecific binding (Figure 1, line II B).

In addition to the difference in potency of inhibiting the (^{3}H)-dihydromorphine binding, levorphanol and dextrorphan also appeared to inhibit the dihydromorphine binding differently. While levorphanol had a sigmoid inhibition curve with a dose range of more than 5 log units, dextrorphan had a steeper inhibition curve with a dose range of 3 log units. A steeper asymmetric curve suggests that dextrorphan might inhibit dihydromorphine binding irreversibly. Therefore, in several preliminary experiments, tissue preparations were incubated with (^{3}H)-dihydromorphine first and then

levorphanol or dextrorphan was added to the incubation mixture and incubated for an additional 15-minute period. Results similar to that depicted in Figure 1 were obtained. Thus, both (^{3}H)-dihydromorphine binding and dextrorphan binding was reversible. Preliminary studies also indicated that specific dihydromorphine binding was greatly reduced when homogenates stored in a freezer overnight were used for the assay.

In the presence of 20 nM (^{3}H)-dihydromorphine both total and non-saturable binding were significantly higher (Figure 2). The concentrations of levorphanol and dextrorphan required to inhibit the saturable binding were also higher. Approximately 10 μM levorphanol was required to inhibit the saturable binding (Figure 2, A+B). Dextrorphan remained approximately three orders of magnitude less effective than levorphanol. Ten μM dextrorphan thus inhibited only portions of the saturable binding (Figure 2, A). At 10^{-4}M, the inhibitory effect of both drugs appeared to be resulted from nonspecific changes in membrane properties. Therefore, the difference in (^{3}H)-dihydromorphine binding assayed in the absence and presence of 10 μM levorphanol was used as an estimate of the saturable, stereospecific binding in the following studies. The inhibition curve of levorphanol became more shallow with a dose range of more than 6 log units whereas the inhibition curve of dextrorphan was steeper with a dose range of 2 log units. The saturable, relatively stereospecific component was approximately 50%

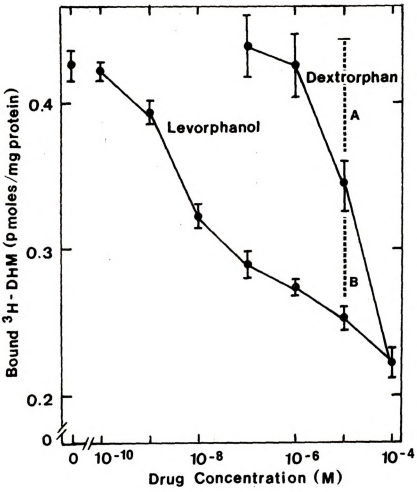

Figure 2. Levorphanol and dextrorphan inhibition of dihydromorphine binding assayed with 20 nM (^{3}H)-dihydromorphine in 50 mM Tris-HCl buffer(pH 7.4).

Brain-stem particulate fraction was incubated for 5 minutes with indicated concentrations of either levorphanol or dextrorphan. Subsequently, 20 nM (^{3}H)-dihydromorphine was added to the incubation mixture and the binding was assayed. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

of the total binding assayed with 20 nM (^{3}H)-dihydromorphine compared to approximately 75% of the total binding assayed with 6 nM (^{3}H)-dihydromorphine.

The binding of (^{3}H)-dihydromorphine increased with higher (^{3}H)-dihydromorphine concentrations in the medium (Figure 3). Binding in the presence of 10 μM levorphanol was proportional to the (^{3}H)-dihydromorphine concentration, indicating that this fraction is non-saturable. When this fraction was subtracted from the (^{3}H)-dihydromorphine binding in the absence of levorphanol (total binding) to calculate the levorphanol-inhibitable binding, a typical absorption isotherm curve was observed (Figure 3, broken line). Figure 4 shows a Scatchard plot of the data (saturable, stereospecific component) shown in Figure 3. The regression line was calculated by the non-weighted, least squares method. In this type of plot, the slope of the regression line indicates the Km value whereas the intercept of the regression line at the ordinate represents the maximal binding. In this study, a Scatchard plot of data from each independent experiment was performed in order to calculate the apparent Km and maximal binding. Then the mean and the standard error of the apparent Km and maximal binding was calculated. The apparent Km value of the saturable, stereospecific binding site for (^{3}H)-dihydromorphine was 7.9 ± 1.2 nM and the maximal binding was 0.25 ± 0.01 pmole/mg protein.

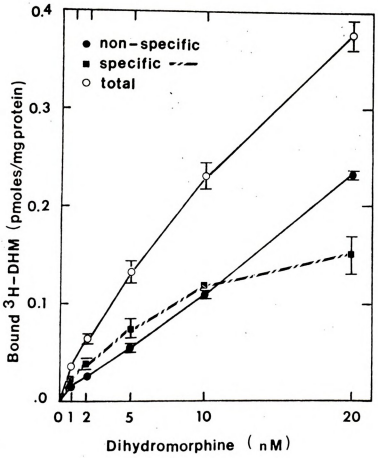

Figure 3. Binding of (^{3}H)-dihydromorphine(^{3}H-DHM) in the absence and presence of 10 μM levorphanol assayed in 50 mM Tris-HCl buffer(pH 7.4).

Brain-stem particulate fraction was incubated for 5 minutes with or without 10 μM levorphanol. Subsequently, various concentrations of (^{3}H)-dihydromorphine were added to the incubation mixture and the binding was assayed. Substracting the non-specific binding (binding assayed in the presence of 10 μM levorphanol) from the total binding(binding assayed in the absence of 10 μM levorphanol) gives the specific binding. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

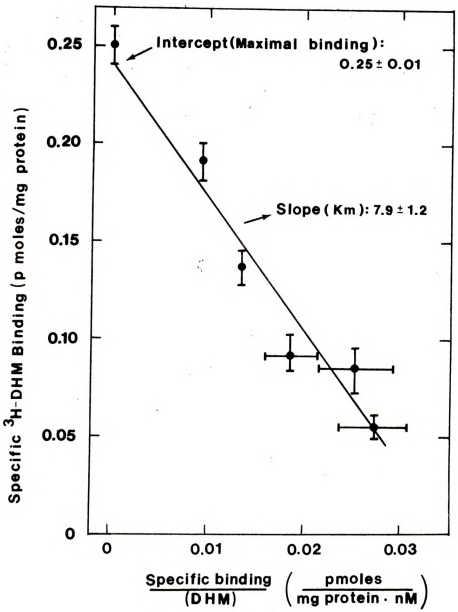

Figure 4. Scatchard plot of the specific (^{3}H)-dihydromorphine binding to brain-stem particulate fraction assayed in 50 mM Tris-HCl buffer(pH 7.4).

Brain-stem particulate fraction was incubated for 5 minutes with or without 10 μM levorphanol. Subsequently, various concentrations of (^{3}H)-dihydromorphine were added to the incubation mixture and the binding was assayed. The saturable, stereospecific (^{3}H)-dihydromorphine binding was plotted in this figure. Each point represents the mean of 5 experiments. Vertical and horizontal lines indicate standard errors. The regression line is calculated by the least squares method fitted to the means.

B. Relative potency of opiate analogs to inhibit specific (^{3}H)-dihydromorphine binding to brain-stem particulate fraction assayed in 50 mM Tris-HCl buffer (pH 7.4)

Since the consequences of the drug-receptor interaction cannot be observed with in vitro studies, the pharmacologic significance of the specific binding can only be explored from a comparison of the characteristics of the in vitro receptor binding with the known characteristics of pharmacologic receptor. Thus the affinity of several opiate analogs for the specific (^{3}H)-dihydromorphine binding sites were studied and compared to their analgesic potency for each drug.

In previous studies [Pert and Snyder, 1973a, 1973b; Simon et al., 1973; Wong and Horng, 1973], the concentrations of various opiate analogs to inhibit 50% of the specific binding of a given concentration of a radiolabelled opiate analog (ID_{50}) have been determined and used to assess the affinity of these opiate analogs for the specific binding sites. As discussed earlier in Introduction, Section A-3, the specific binding of a certain compound observed at one concentration is not only determined by the affinity of specific binding site for that particular compound but also by its maximal binding. Therefore, determination of the inhibition constant, Ki, is a more appropriate assessment of the affinity of opiate analogs for the specific binding site. Ki values of several opiate analogs for specific dihydromorphine binding were determined graphically

from Dixon plots. Brain-stem particulate fraction, prepared in 50 mM Tris-HCl buffer, pH 7.4, were incubated with various concentrations of opiate analogs for 5 minutes. Subsequently, the specific binding was assayed using 0.6 and 6 nM (^{3}H)-dihydromorphine. Figure 5 shows a Dixon plot of specific dihydromorphine binding inhibited by dextrorphan. The regression line was calculated by the non-weighted, least squares method fitted to the data obtained from 4 independent experiments. The concentration range of dextrorphan shown in Figure 5 correspond to that producing a linear inhibition of specific (^{3}H)-dihydromorphine binding. Table 1 shows the results of such studies for several opiate analogs. Levorphanol had the highest affinity for the specific (^{3}H)-dihydromorphine binding site. Naloxone, morphine and d, l-methadone had moderate affinities. Dextrorphan and codeine had markedly lower affinities and thebain had the lowest affinity. Compared to ID_{50} studies, the ranking order of affinity for these opiate analogs is similar and is generally correlated with their analgesic potency as determined by the systemic injection of these drugs. However, the Ki values are 3 to 4 times lower than the ID_{50} values of corresponding compounds for specific (^{3}H)-naloxone binding reported earlier [Pert and Snyder, 1973a], 40 to 100 times lower than the ID_{50} values of corresponding compounds for specific (^{3}H)-etorphine binding [Simon et al., 1973] and 2 to 4 times lower than the ID_{50} values of corresponding compounds for specific

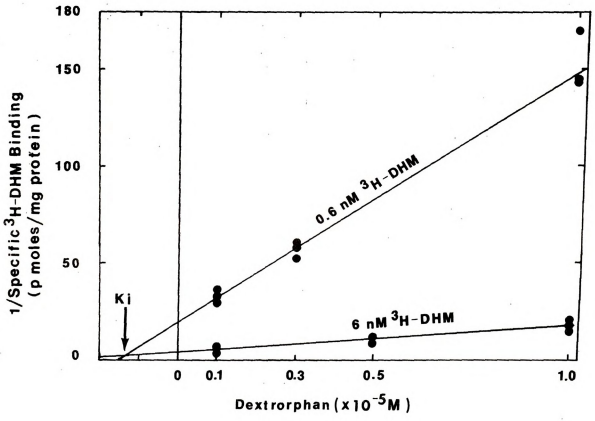

Figure 5. Dixon plot: Dextrorphan inhibition of specific (^{3}H)-dihydromorphine binding assayed in 50 mM Tris-HCl buffer(pH 7.4).

Brain-stem particulate fraction was incubated with various concentrations of dextrorphan for 5 minutes. Subsequently, the specific dihydromorphine binding was assayed with 0.6 and 6 nM (^{3}H)-dihydromorphine. Each point represents a result of an independent experiment performed in triplicate. The regression line is calculated by the least squares method fitted to the data from 4 experiments.

Table 1. Inhibition constant(Ki) of several opiate analogs for spfcific (^{3}H)-dihydromorphine binding.

Opiate Analogs	Ki (nM)
Levorphanol tartrate	0.52
Naloxone hydrochloride	1.74
Morphine sulfate	2.22
d,l-Methadone hydrochloride	2.25
Dextrorphan tartrate	1300
Codeine phosphate	2020
Thebaine hydrochloride	2520

Brain-stem particulate fraction was incubated with various concentrations of opiate analogs for 5 minutes. Subsequently, the specific dihydromorphine binding was assayed with 0.6 and 6 nM (^{3}H)-dihydromorphine. Ki values were determined from Dixon plots. One of the Dixon plots is shown in Figure 5.

(^{3}H)-dihydromorphine binding [Wong and Horng, 1973]. The ID_{50} of opiate analogs determined in the present studies are shown in Table 2. It is clearly demonstrated that the ID_{50} values increased as the concentration of (^{3}H)-dihydromorphine increased. The ID_{50} values for 6 nM (^{3}H)-dihydromorphine are similar to those determined by Pert and Snyder [1973a] using 5 nM (^{3}H)-naloxone. Since etorphine is much more potent than dihydromorphine [Blane et al., 1967] and thus supposedly has a higher affinity for the specific binding sites, the ID_{50} values of other opiate analogs for the specific (^{3}H)-etorphine binding are expected to be higher than the ID_{50} values for the specific binding of (^{3}H)-dihydromorphine and (^{3}H)-naloxone. However, the difference was about 100 times lower than that expected. Simon et al. [1973] argued that the affinity for receptors is only one of several factors responsible for the enormous potency of etorphine.

As pointed out earlier, dextrorphan and levorphanol appeared to inhibit specific (^{3}H)-dihydromorphine binding by different mechanisms. This speculation, however, was not substantiated by the Ki determination of dextrorphan. A Dixon plot of the specific dihydromorphine binding inhibited by dextrorphan (Figure 5) indicated that the two inhibitory regression lines did not intercept on the abscissa. That is, dextrorphan did not appear to exhibit non-competitive inhibition on specific dihydromorphine binding as speculated. All opiate analogs tested in the present studies, therefore,

Table 2. ID_{50} of several opiate analogs for specific binding of (^{3}H)-dihydromorphine, (^{3}H)-naloxone(^{3}H-NLX) and (^{3}H)-etorphine.

Opiate Analogs	ID_{50} (nM)				
	^{3}H-DHM 0.6 nM	^{3}H-DHM 6 nM	^{3}H-DHM(1) 2 nM	^{3}H-NLX(2) 5 nM	^{3}H-Etorphine(3) 3 nM
Levorphanol tartrate	0.75	3	2	2	20
Naloxone hydrochloride	1.5	10	3.3	10	30
Morphine sulfate	1.5	10	1.5	6	200
d,l-Methadone hydrochloride	2.5	12			
Dextrorphan tartrate	1300	7500	4000	8000	80000
Codeine phosphate	1400	9000		20000	
Thebaine hydrochloride	3500	10000			

(1) Wong and Horng(1973). (2) Pert and Snyder(1973a). (3) Simon et al.(1973).

Specific dihydromorphine binding was assayed with 0.6 and 6 nM (^{3}H)-dihydromorphine. The percent decrease of specific dinydromorphine binding was determined for 5 or more concentrations of each opiate analog. The ID_{50} values(the concentration of drug that reduced specific dihydromorphine binding by 50%) were determined from probit-log plots of the data. Values represent the mean of 4 determinations. ID_{50} values determined by previous studies are also listed in this table for comparison.

exhibited competitive inhibition of the specific binding of (^{3}H)-dihydromorphine.

Effects of several non-opiate drugs on the specific binding of 3.33 nM (^{3}H)-dihydromorphine have also been studied. Apomorphine, dopamine, chlorpromazine, xylazing [Bayer 1470] and N-methylnicotinamide did not affect specific (^{3}H)-dihydromorphine binding at concentrations up to 10^{-5}M. SKF-525A inhibited approximately 81% of specific (^{3}H)-dihydromorphine binding at 10^{-5}M (Table 3). Such an inhibition, however, was greatly reduced at 10^{-6}M. Thus, it appeared that the inhibition of (^{3}H)-dihydromorphine binding by SKF-525A is not a competitive type. Since 10^{-5}M SKF-525A has been shown to alter the properties of biological membrane, the effect of SKF-525A on (^{3}H)-dihydromorphine binding could be nonspecific. Apomorphine is a relatively specific stimulator of dopaminergic receptors at low concentrations [Ernst, 1967] while chlorpromazine is a blocker of dopaminergic receptors [Carlsson and Lindqvist, 1963]. It has been suggested that central dopaminergic receptors are important in morphine analgesia and tolerance development [Vander Wende and Spoerlein, 1972, 1973]. Xylazine (Bayer 1470) is a potent, non-narcotic analgesic [Kroneberg et al., 1967]. N-methylnicotinamide and SKF-525A have been shown to inhibit the active transport of morphine from the systemic circulation into the CSF [Wang and Takemori, 1972]. The present data would indicate that the stereospecific, saturable binding of dihydromorphine is neither a nonspecific

Table 3. Inhibitory effect of several non-opiate drugs on specific (^{3}H)-dihydromorphine binding assayed in 50 mM Tris-HCl buffer(pH 7.4).

Drugs	% Inhibition of Specific Binding at 10^{-5}M, at 10^{-6} M		
Apomorphine	8.8		n=2
Dopamine	-		n=2
Chlorpromazine	-		n=2
Xylazine(Bayer 1470)	-		n=2
N-Methylnicotinamide	-		n=6
SKF-525A	80.9	10.5	n=4

Brain-stem particulate fraction was incubated for 5 minutes with various concentrations of drugs. Subsequently, the specific dihydromorphine binding was assayed with 3.33 nM (^{3}H)-dihydromorphine. Inhibition was calculated and expressed in this table as percent. Short horizontal bars indicate no significant inhibition was observed at concentrations up to 10^{-5}M.

binding phenomenon nor a transport phenomenon.

C. Specific (^{3}H)-dihydromorphine binding to particulate fraction obtained from various brain regions assayed in 50 mM Tris-HCl buffer (pH 7.4)

Goldstein et al. [1971] have shown that there were no regional variations of specific (^{14}C)-levorphanol binding in the mouse brain. On the other hand, other investigators [Pert and Snyder, 1973a; Kuhar et al., 1973; Hiller et al., 1973] have demonstrated regional differences of specific binding of (^{3}H)-naloxone, (^{3}H)-dihydromorphine and (^{3}H)-etorphine. It should be pointed out that in all these studies specific binding was assayed with only a low concentration of radiolabelled compound. Since the magnitude of binding observed with a given concentration of compound is determined by the maximal binding and the affinity, one cannot ascertain whether the regional differences in specific binding observed in the previous studies are due to the differences in the concentration of specific binding sites, differences in affinity of specific binding sites for the opiate analogs or both. Therefore, these two kinetic parameters were determined using Scatchard or double-reciprocal plots of the data in order to delineate whether specific opiate binding site distribute differently in various regions and to determine whether characteristics of specific opiate binding site are different in various brain regions.

The particulate fraction obtained from striatum had

the highest specific (^{3}H)-dihydromorphine binding (Figure 6), followed by those obtained from midbrain, cerebral cortex, thalamus-hypothalamus and pons-medulla, in decreasing order. Cerebellum exhibited only minimal specific dihydromorphine binding. There was more than a 20-fold differences in specific dihydromorphine binding between the striatum and cerebellum. The nonspecific binding assayed with 10 nM (^{3}H)-dihydromorphine was quite similar in various brain regions, ranging from 0.101 pmole/mg protein in the cerebellum to 0.135 pmole/mg protein in the pons-medulla. The specific binding was only about 10% of the total binding in the cerebellum whereas it was about 57% in the striatum.

Figure 7 shows Scatchard plots of specific dihydromorphine binding to particulate fraction obtained from 5 brain regions. Scatchard plot of the specific dihydromorphine binding to particulate fraction obtained from cerebral cortex appeared to have two regression lines with different slopes. Thus, the data suggest that there are two types of specific binding sites for dihydromorphine in the cerebral cortex. Because of the limitation of the specific radioactivity of (^{3}H)-dihydromorphine, the specific dihydromorphine binding assayed with low concentrations of (^{3}H)-dihydromorphine tends to show large variability. Therefore, in the present studies, the apparent Km value and the maximal binding were determined from a regression line calculated by the least squares method fitted to all data in each experiment. Scatchard plots of the specific dihydromorphine

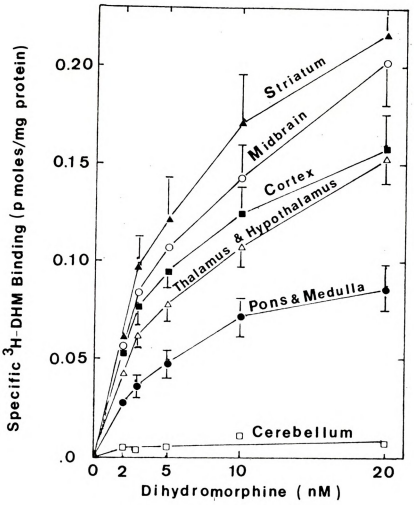

Figure 6. Specific (^{3}H)-dihydromorphine binding to particulate fraction obtained from various brain regions assayed in 50 mM Tris-HCl buffer(pH 7.4).

Particulate fraction was incubated for 5 minutes with or without 10 μM levorphanol. Subsequently, various concentrations of (^{3}H)-dihydromorphine were added to the incubation mixture and the binding was assayed. Values shown are differences in (^{3}H)-dihydromorphine binding assayed in tne absence and presence of 10 μM levorphanol(saturable, stereospecific binding). Brain tissues were pooled from 2 or 4 rats for each preparation. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

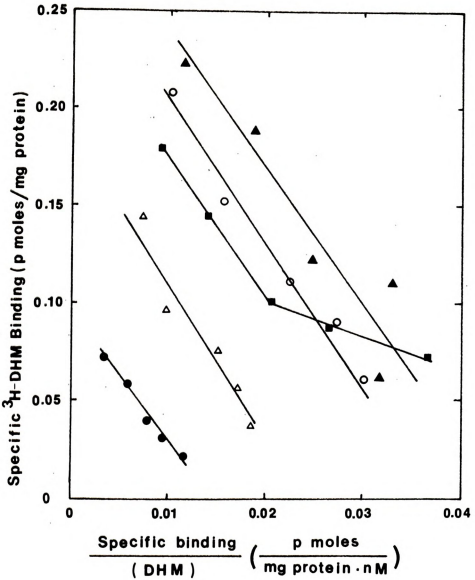

Figure 7. Scatchard plots of specific (^{3}H)-dihydromorphine binding to particulate fraction obtained from various brain regions assayed in 50 mM Tris-HCl buffer(pH 7.4).

(■—■):Cerebral cortex; (△—△):Thalamus-Hypothalamus; (▲—▲):Striatum; (○—○):Midbrain; (●—●):Pons-Medulla. Eacf point represents the mean of triplicate determinations. Regression lines are calculated by the least squares method fitted to the means in each experiment.

binding to other brain regions appeared to have only one regression line and thus suggests that there is only one major type of specific binding site for dihydromorphine in these brain regions.

Table 4 shows the maximal binding and apparent Km for dihydromorphine calculated from Scatchard plots of the data as shown in Figure 7. The maximal binding for dihydromorphine was significantly higher in the striatum, midbrain and thalamus-hypothalamus than in cerebral cortex and pons-medulla. The affinity, which can be expressed as the reciprocal of the apparent Km value, was similar in all brain regions studied except the cerebral cortex where the affinity for dihydromorpine was significantly higher than for other brain regions. The apparent Km and maximal binding for dihydromorphine in the cerebellum could not be determined because the specific dihydromorphine binding in this region was only minimal under these experimental conditions.

D. Specific (^{3}H)-naloxone binding to particulate fraction obtained from various brain regions assayed in 50 mM Tris-HCl buffer (pH 7.4)

Pert and Snyder [1973a] have demonstrated that the corpus striatum had the highest specific naloxone binding followed by midbrain, cerebral cortex and brain-stem. In their study, the cerebellum had no detectable specific naloxone binding. These investigators, however, employed only one concentration of (^{3}H)-naloxone(5 nM). Moreover,

Table 4. Maximal binding and apparent Km for specific (^{3}H)-dihydromorphine and (^{3}H)-naloxone binding to particulate fraction obtained from various brain regions assayed in 50 mM Tris-HCl buffer(pH 7.4).

Brain Regions	Maximal Binding (pmoles/mg protein)		Apparent Km* (nM)	
	DHM	NLX	DHM	NLX
Cerebral cortex	0.17±0.02	0.49±0.03+	3.71±0.32	24.7±2.0
Striatum	0.31±0.03	0.46±0.06	7.65±1.83	14.4±2.5
Thalamus-Hypothalamus	0.24±0.03	0.60±0.06+	11.01±1.11	31.0±7.9
Midbrain	0.28±0.02	0.40±0.03+	9.63±2.66	12.4±1.6
Pons-Medulla	0.13±0.02	0.54±0.14+	8.48±1.10	36.9±11.7

+Significantly different from corresponding values for dihydromorphine ($p < 0.05$).

*Apparent affinity for dihydromorphine is greater than that for naloxone in 50 mM Tris-HCl buffer but smaller in CSF and ICF(see Table 5).

Maximal binding and apparent Km were determined from Scatchard plot of the data in each experiment. The mean and standard error of 5 experiments were then calculated.

there is evidence suggesting that naloxone may bind to another site in addition to the morphine binding site (see Introduction, Section A-1). Therefore, regional differences for specific (^{3}H)-naloxone binding were also studied and compared with that for dihydromorphine. Specific naloxone binding was defined as the difference in binding observed in the absence and presence of 10 μM levorphanol.

The particulate fraction obtained from the striatum had the highest specific (^{3}H)-naloxone binding (Figure 8), followed by midbrain, thalamus-hypothalamus, cerebral cortex, pons-medulla and cerebellum. Thus, a somewhat similar pattern of regional differences in specific binding were observed with both dihydromorphine and naloxone. The regional differences in specific (^{3}H)-naloxone binding, however, were rather small; there was only a two-fold difference in specific naloxone binding between striatum and cerebellum. This was primarily due to the higher specific naloxone binding in cerebellum. This particular result is not in agreement with that reported by Pert and Snyder [1973a].

Figure 9 shows several Scatchard plots of data shown in Figure 8. In contrast to the specific dihydromorphine binding, the specific (^{3}H)-naloxone binding to particulate fraction obtained from cerebral cortex, striatum, thalamus-hypothalamus and midbrain appeared to have two regression lines. Only the specific binding of naloxone to the pons-medulla and cerebellum appeared to have a single regression

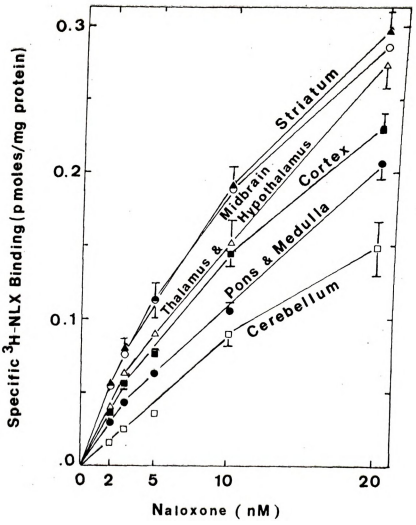

Figure 8. Specific (^{3}H)-naloxone(^{3}H-NLX) binding to particulate fraction obtained from various brain regions assayed in 50 mM Tris-HCl buffer(pH 7.4).

Particulate fraction was incubated for 5 minutes with or without 10 μM levorphanol. Susequently, various concentrations of (^{3}H)-naloxone were added to the incubation mixture and the binding was assayed. Values shown are differences in (^{3}H)-naloxone binding assayed in the absence and presence of 10 μM levorphanol(saturable, stereospecific binding). Brain tissues were pooled from 2 or 4 rats for each preparation. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

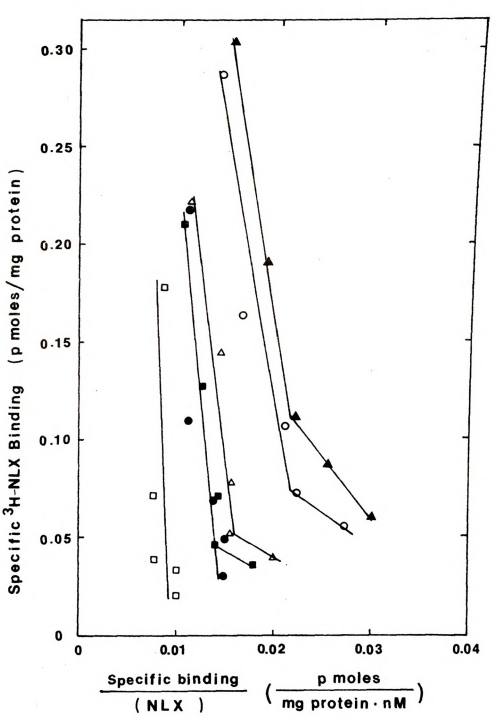

Figure 9. Scatchard plots of specific (^{3}H)-naloxone binding to particulate fraction obtained from various brain regions assayed in 50 mM Tris-HCl buffer(pH 7.4).

(■——■):Cerebral cortex; (△——△):Thalamus-Hypothalamus; (▲——▲):Striatum; (○——○):Midbrain; (●——●):Pons-Medulla; (□——□):Cerebellum. Each point represents the mean of triplicate determinations. Regression lines are calculated by the least squares method fitted to the means in each experiment.

line. Thus, it appears that there are two types of specific binding sites for naloxone in brain regions such as the striatum, thalamus-hypothalamus, midbrain and cerebral cortex. However, as pointed out earlier, the specific binding assayed with low concentrations of (^{3}H)-naloxone tends to show large variability. Therefore, more specific binding studies, particularly in the lower concentration range, are necessary in order to substantiate the two specific binding sites for naloxone. In the present studies, the apparent Km values and maximal binding were determined from a regression line calculated by the least squares methods fitted to all data in each experiment. The Scatchard plot of the specific naloxone binding to cerebellar particulate fraction appeared to indicate that there is only one type of specific binding site for naloxone. However, the slope is steep and the specific naloxone binding assayed with the low concentration of (^{3}H)-naloxone is quite small, and neither the Scatchard plot nor the double-reciprocal plot could provide an unequivocal answer. Nevertheless, the present studies would suggest that the specific binding sites in cerebellum could bind naloxone but not dihydromorphine.

Maximal binding and the apparent Km value for specific naloxone binding to various other brain regions are also shown in Table 4. In contrast to dihydromorphine binding, regional differences in the concentration of naloxone binding sites (maximal binding) were rather small. It was

relatively higher in thalamus-hypothalamus and lower in midbrain. Apparent affinities, on the other hand, were different among various brain regions.

The apparent Km for naloxone was significantly higher than that for dihydromorphine in each brain region, i.e., the apparent affinity for naloxone was lower than that for dihydromorphine in 50 mM Tris-HCl buffer. The maximal binding for naloxone was higher than that for dihydromorphine in each brain region. Since naloxone, a competitive antagonist for dihydromorphine binding, should bind to dihydromorphine binding sites, these data appear to substantiate the findings revealed in Scatchard plots that naloxone has two or more types of specific binding sites, only one of which is available to dihydromorphine.

E. Comparison of specific (^{3}H)-dihydromorphine and (^{3}H)-naloxone binding assayed in 50 mM Tris-HCl buffer, cerebrospinal fluid (CSF) and simulated intracellular fluid (ICF)

The finding that naloxone had a lower affinity than dihydromorphine for specific binding sites is not compatible with the pharmacologic potency of these two drugs. Moreover, most of the opiate receptor binding studies were performed using relatively simple buffer solution, 50 mM Tris-HCl buffer, as the incubation medium. It has been shown that sodium decreases specific (^{3}H)-etorphine and other opiate agonists binding [Simon et al., 1973; Pert et al., 1973] but it enhances specific (^{3}H)-naloxone and opiate agonist-

antagonist binding [Pert et al., 1973]. Potassium, on the other hand, decreases binding of both agonists and antagonists [Pert et al., 1973]. Calcium and magnesium also inhibit specific (^{3}H)-naloxone binding at 5 mM but has no effect at physiological concentrations of these ions [Pert and Snyder, 1973a, 1973b]. Since it appears reasonable to assume that the environment surrounding opiate receptors is either extracellular fluid (cerebrospinal fluid) or intracellular fluid which contains various concentrations of sodium, potassium and calcium, it was of interest to study the binding of dihydromorphine and naloxone in simulated cerebrospinal fluid (CSF) and intracellular fluid (ICF) and compare it to the binding assayed in 50 mM Tris-HCl buffer.

Total (^{3}H)-dihydromorphine binding was greatly reduced in CSF and ICF. The specific dihydromorphine binding in CSF or in ICF was approximately 50% of that in Tris-HCl buffer (Figure 10). Scatchard plots of these data indicate that the maximal binding is 0.25 ± 0.01, 0.15 ± 0.01 and 0.17 ± 0.01 pmoles/mg protein in Tris-HCl buffer, CSF and ICF, respectively (Table 5). Apparent Km values, however, were significantly higher in CSF and in ICF than in Tris-HCl buffer. Thus, the reduced specific dihydromorphine binding assayed in CSF or ICF is due both to reduced maximal binding capacity and reduced affinity of binding sites for dihydromorphine.

In contrast to (^{3}H)-dihydromorphine binding, total

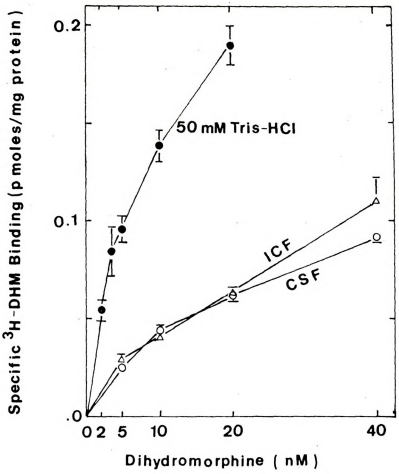

Figure 10. Comparison of specific (^{3}H)-dihydromorphine binding assayed in 50 mM Tris-HCl buffer, artificial cerebrospinal fluid(CSF) and simulated intracellular fluid(ICF).

(^{3}H)-Dihydromorphine binding to brain-stem particulate fraction was assayed in 50 mM Tris-HCl buffer(pH 7.4), CSF or ICF. Values shown are the differences in dihydromorphine binding assayed in the absence and presence of 10 μM levorphanol. Each point represents the mean of 4 experiments. Vertical lines indicate standard errors.

Table 5. Maximal binding and apparent Km for specific (^{3}H)-dihydromorphine and (^{3}H)-naloxone binding to brain-stem particulate fraction assayed in different media.

Incubation Media	Maximal Binding (pmoles/mg protein)		Apparent Km (nM)	
	DHM	NLX	DHM	NLX
50 mM Tris-HCl buffer	0.25±0.01	0.57±0.10 $^{+}$	7.9±1.2	24.0±6.6 $^{+}$
Cerebrospinal fluid (CSF)	0.15±0.01*	0.29±0.01^{*+}	27.8±4.1*	6.5±0.5^{*+}
Intracellular fluid (ICF)	0.17±0.01*	0.29±0.04^{*+}	30.0±4.5*	10.2±2.1 $^{+}$

* Significantly different from corresponding values in 50 mM Tris-HCl buffer ($p < 0.05$).

$^{+}$ Significantly different from corresponding values for dihydromorphine ($p < 0.05$).

Maximal binding and apparent Km were determined from Scatchard plot of the data in each experiment. The mean and standard error of 4 experiments were then calculated.

(^{3}H)-naloxone binding was increased in CSF and in ICF (Figure 11). This increase in total naloxone binding was primarily due to an increase in the nonspecific binding (binding in the presence of 10 μM levorphanol). Specific naloxone binding was also slightly higher in CSF at low naloxone concentrations (Figure 12). The specific naloxone binding sites, however, saturated at lower concentrations in both CSF and ICF as compared to those in Tris-HCl buffer. Thus, maximal binding was significantly lower in CSF and ICF than in Tris-HCl buffer, wereas affinities for naloxone were significantly higher in CSF and ICF as indicated by lower apparent Km values in these media than in Tris-HCl buffer (Table 5). Thus, it would appear that the maximal binding for both dihydromorphine and naloxone was reduced in CSF or in ICF compared to that in Tris-HCl buffer, whereas the apparent affinity for dihydromorphine was decreased and that for naloxone was increased. In any media, however, the maximal binding for naloxone was increased. In any media, however, the maximal binding for naloxone was significantly greater than that for dihydromorphine. In CSF or ICF, but not in Tris-HCl buffer, naloxone had a significantly higher apparent affinity than did dihydromorphine indicating that the binding observed in CSF or ICF may be closer to the pharmacologic properties of these compounds. Thus, while some experiments, such as the comparison of the binding site population, may be performed using a simple buffer solution, in vitro studies may be pharmacologically more relavant if

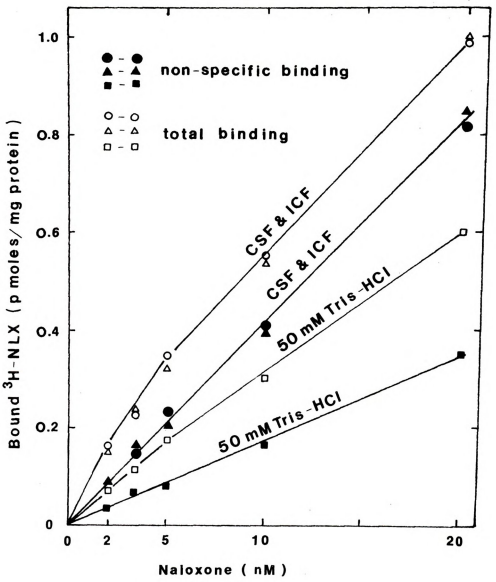

Figure 11. Binding of (^{3}H)-naloxone (^{3}H-NLX) in the absence and presence of 10 μM levorphanol assayed in 50 mM Tris-HCl buffer, CSF and ICF.

Brain-stem particulate fraction was incubated for 5 minutes with or without 10 μM levorphanol. Subsequently, various concentrations of (^{3}H)-naloxone were added to the incubation mixture and the binding was assayed. Binding assayed in the absence of 10 μM levorphanol is total binding. Binding assayed in the presence of 10 μM levorphanol is non-specific binding. Each point represents the mean of triplicate determinations. Since binding assayed in CSF was similar to binding assayed in ICF, they are represented by a single line.

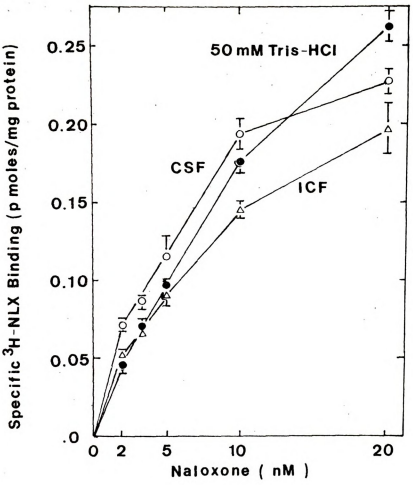

Figure 12. Comparison of specific (^{3}H)-naloxone binding assayed in 50 mM Tris-HCl buffer, CSF and ICF.

(^{3}H)-Naloxone binding to brain-stem particulate fraction was assayed in different media. Values shown are the differences in binding assayed in the absence and presence of 10 μM levorphanol. Each point represents the mean of 4 experiments. Vertical lines indicate standard errors.

performed using CSF or ICF.

F. Effect of dihydromorphine and naloxone on (^{3}H)-naloxone binding to particulate fraction obtained from cerebellum and striatum assayed in CSF

In earlier studies presented here, kinetic analyses have indicated that naloxone binds to dihydromorphine binding site and to another type of binding site that has a different affinity for naloxone and is not available to dihydromorphine. If this is true, then dihydromorphine may inhibit naloxone binding poorly in the cerebellum, a region where dihydromorphine binding sites are minimal compared to the naloxone binding sites. In striatum, however, a significant portion of naloxone should be bound to binding sites that are capable of binding both dihydromorphine and naloxone, and therefore a significant portion of naloxone binding should be inhibited by dihydromorphine. Thus, the effects of non-labelled dihydromorphine and naloxone on (^{3}H)-naloxone binding were compared in both the cerebellum and striatum using CSF as an incubation medium. In cerebellum, non-labelled dihydromorphine failed to inhibit total (^{3}H)-naloxone binding significantly at all concentrations tested whereas non-labelled naloxone inhibited total (^{3}H)-naloxone binding (Figure 13). At 10^{-5}M, non-labelled naloxone inhibited approximately 10% of the total (^{3}H)-naloxone binding, that is, approximately 10% of the total naloxone binding is saturable. The remaining portion, approximately 90% of the total naloxone binding,

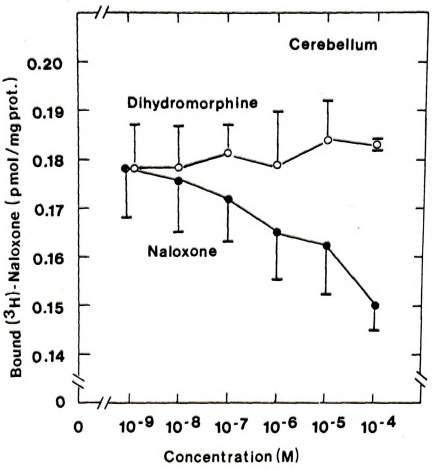

Figure 13. Effect of dihydromorphine and naloxone on (^{3}H)-naloxone binding to cerebellum particulate fraction assayed in CSF.

Particulate fraction obtained from cerebellum was incubated for 5 minutes with indicated conentrations of either dihydromorphine or naloxone. Subsequently, 3.33 nM (^{3}H)-naloxone was added to the incubation mixture and the binding was assayed. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors. Note: (^{3}H)-naloxone binding in the absence of inhibitor represents the total binding, which includes non-specific binding.

therefore, is non-saturable binding in cerebellum. Since non-labelled dihydromorphine did not inhibit the total (^{3}H)-naloxone binding at 10^{-5}M, or even with concentrations as high as 10^{-4}M, these data would suggest that the specific binding sites for dihydromorphine is negligible in the cerebellum. In striatum, both non-labelled naloxone and dihydromorphine inhibited total (^{3}H)-naloxone binding (Figure 14). Naloxone appeared to have a higher affinity than dihydromorphine for specific binding sites in striatum when assays were performed using CSF. Naloxone also tended to inhibit a greater portion of the total (^{3}H)-naloxone binding than that inhibited by dihydromorphine but the difference was not statistically significant. These data clearly indicate that naloxone binds to at least two distinct specific binding sites, one of which is not available to dihydromorphine.

G. Effect of 5,7-dihydroxytryptamine pretreatment of rats in vivo on the specific (^{3}H)-dihydromorphine binding assayed in CSF in vitro

The specific (^{3}H)-naloxone binding has been shown to be restricted to neuronal tissue [Pert and Snyder, 1973a]. These authors have concluded that regional differences in acetylcholine concentration within the brain parallel the observed regional differences in specific naloxone binding. In a subsequent study [Kuhar et al., 1973], electrolytic lesions resulting in the destruction of cholinergic,

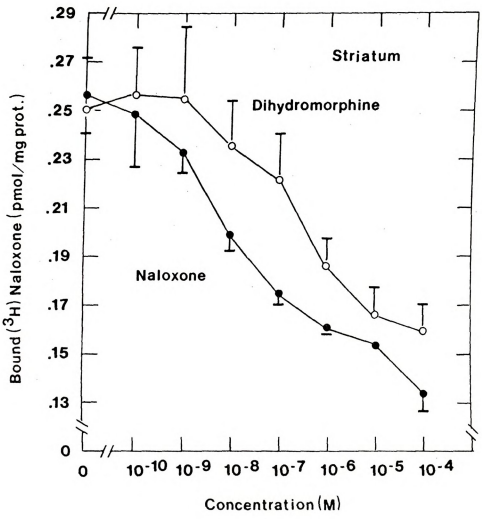

Figure 14. Effect of dihydromorphine and naloxone on (^{3}H)-naloxone binding to striatum particulate fraction assayed in CSF.

Particulate fraction obtained from striatum was used in these studies. The binding of 3.33 nM (^{3}H)-naloxone was assayed in the absence and presence of either dihydromorphine or naloxone. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

noradrenergic or 5-hydroxytryptaminergic pathways failed to affect specific (^{3}H)-dihydromorphine binding in regions where the lesioned pathways terminate. However, the failure of electrolytic lesions to affect specific dihydromorphine binding could be due to an incomplete destruction of the particular nerve pathways. Dilution by non-lesioned cells may mask the alterations caused by the electrolytic lesions. Chemical destruction induced by drugs such as 5,7-dihydroxytryptamine or 6-hydroxydopamine may be a better method, since these agents selectively destroy the terminals associated with the particular neurotransmitter and this destruction is not limited to a focal region. The main sites of morphine analgesia have been shown to be associated with periventricular structures (see Introduction, Section A-2). Therefore, lesions of 5-hydroxytryptaminergic or catecholaminergic nerve endings in brain were induced by injecting 5,7-dihydroxytryptamine or 6-hydroxydopamine solution into the lateral ventricles of rats and the specific binding of (^{3}H)-dihydromorphine and (^{3}H)-naloxone was monitored in several brain regions.

5,6-Dihydroxytryptamine, administered intraventricularly, has been shown to produce prominent, long-lasting degenerative damage to 5-hydroxytryptaminergic neurons in the rat brain [Baumgarten et al., 1972; Daly et al., 1973]. Recently, an isomer, 5,7-dihydroxytryptamine has been shown to produce similar toxic damage to central tryptaminergic neurons [Baumgarten and Lachenmayer, 1972]. Since

5,7-dihydroxytryptamine is claimed to have lower toxicity [Baumgarten et al., 1973], this compound was used. 5,7-Dihydroxytryptamine creatinine sulfate, 75 µg in 10 µl normal saline solution containing ascorbic acid (1 mg/ml), was injected into the lateral ventricle of rats by lowering the needle stereotaxically through a small hole made 1.5-2.0 mm lateral to the bregma and 3.5-4.0 mm down into the brain tissue. Rats became hypersensitive in first two days but behaved quite normally thereafter. The body weight of treated animals was generally slightly less than that of control animals. The brains of treated animals appeared normal. The behavioral response in treated animals were apparently smaller than that previously reported. For example, no incidence of convulsion was observed following 5,7-dihydroxytryptamine injection. This may be due to the pretreatment of animals with sodium pentobarbital. Sodium pentobarbital has been used to treat convulsions developed in rats treated with high doses of 5,7-dihydroxytryptamine [Baumgarten and Lachenmayer, 1972]. All of the 24 rats survived following the treatment in the present studies. Methoxyflurane and ether have been used to anesthetize rats in preliminary studies and previous studies cited above. However, anesthesia induced by these two anesthetics was generally not deep enough for the surgery and animals were very frequently overdosed and be killed by these two anesthetics. In the present studies, 5,7-dihydroxytryptamine was administered in a smaller volume (10 µl) and

injected slowly over a period of 1 minute, therefore, may produce less non-specific damage [Rech, 1968] compared to most of previous studies in which 20 µl or more of drug solution was injected rapidly into the lateral ventricle.

Two weeks after the injection, rats were decapitated. At this time treated animals appeared to have no spinal reflexes. Particulate fraction of diencephalon and midbrain-low brain-stem regions were prepared in CSF and assayed immediately for specific (^{3}H)-dihydromorphine binding. Since the forebrain region has been examined previously [Kuhar et al., 1973], the diencephalon, which is rich in 5-hydroxytryptaminergic nerve terminals, and midbrain-low brain-stem, which is rich in cell bodies and axons of 5-hydroxytryptaminergic neurons [Ungerstedt, 1971a], were used in the present studies. In both brain regions studied, 5,7-dihydroxytryptamine pretreatment of rats did not significantly alter the total or specific (^{3}H)-dihydromorphine binding (Figure 15). A Scatchard plot was found to be inadequate for analyzing these data, particularly the specific (^{3}H)-dihydromorphine binding to particulate fraction obtained from midbrain-low brain-stem. Therefore, a double-reciprocal plot was used to determine the maximal binding and the apparent Km for specific (^{3}H)-dihydromorphine binding (Figure 16). Table 6 shows that there are no remarkable changes occurred in these two kinetic parameters of specific binding sites for dihydromorphine in either brain region after 5,7-dihydroxytryptamine treatment.

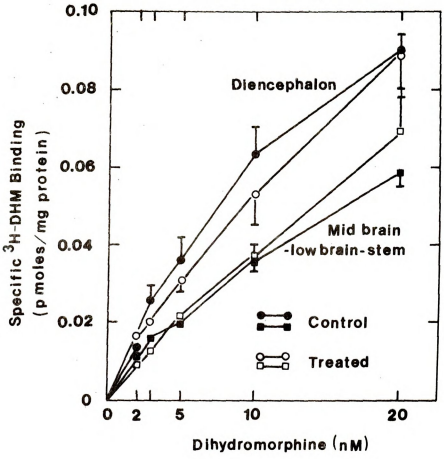

Figure 15. Effect of 5,7-dihydroxytryptamine pretreatment of rats on specific (^{3}H)-dihydromorphine binding to particulate fraction obtained from diencephalon and midbrain-low brain-stem assayed in CSF.

Rats were treated with an intraventricular injection of 75 µg 5,7-dihydroxytryptamine creatinine sulfate(dissolved in 10 µl isotonic saline solution containing ascorbic acid; 1 mg/ml). Control animals were similarly injected with the same volume of vehicle. Animals were sacrificed 2 weeks later. Particulate fraction of indicated brain regions was prepared in CSF and assayed for specific (^{3}H)-dihydromorphine binding. Each point represents the mean of 6 experiments. Vertical lines indicate standard errors.

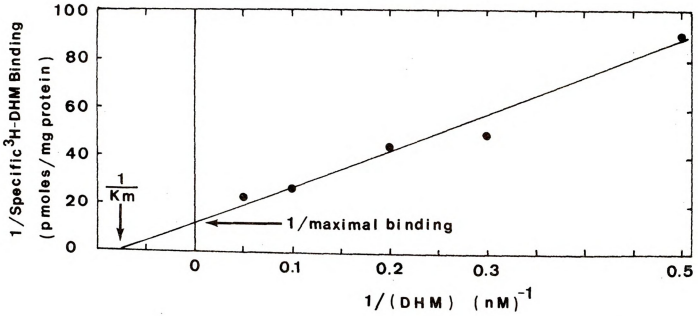

Figure 16. Double-reciprocal plot of specific (^{3}H)-dihydromorphine binding to midbrain-low brain-stem particulate fraction obtained from control rats assayed in CSF.

Each point represents the mean of triplicate determinations. The regression line is calculated by the least squares method fitted to the means.

Table 6. Maximal binding and apparent Km for specific (^{3}H)-dihydromorphine binding to particulate fraction obtained from control and 5,7-dihydroxytryptamine(5,7-DHT)-treated rats assayed in CSF.

Brain Regions	Maximal Binding (pmoles/mg protein)		Apparent Km (nM)	
	Control	5,7-DHT treated	Control	5,7-DHT treated
Diencephalon	0.26±0.03	0.21±0.02	32.2±2.5	28.5±2.2
Midbrain-low brain-stem	0.10±0.02	0.13±0.04	16.6±3.2	23.7±6.5

Maximal binding and apparent Km were determined from double-reciprocal plot of the data in each experiment. The mean and standard error of 6 experiments were then calculated.

H. Effect of 6-hydroxydopamine pretreatment of rats in vivo on the specific (^{3}H)-naloxone binding assayed in CSF

6-Hydroxydopamine, given intraventricularly, has been shown to produce a long lasting damage to catecholaminergic neurons [Uretsky and Iversen, 1969]. Therefore, this drug was used to produce degeneration of central catecholaminergic neurons.

Rats were treated with two intraventricular injections of 250 µg of 6-hydroxydopamine HBr given 7 days apart. Rats treated with 6-hydroxydopamine became hypersensitive in the first few days but behaved normally thereafter. One week after the last injection, these rats appeared normal although their body weights were slightly less than those of control animals. There was apparent damage to periventricular structures of brains of 6-hydroxydopamine treated rats at the time of sacrifice.

Rats were decapitated 7 days after the second 6-hydroxydopamine injection, and particulate fraction of cerebral cortex and brain-stem were prepared in CSF and assayed immediately for (^{3}H)-naloxone binding. In both brain regions studied, pretreatment with 6-hydroxydopamine did not alter the total or specific (^{3}H)-naloxone binding significantly (Figure 17). The maximal binding and the apparent Km value for naloxone as calculated from Scatchard plots also indicated no significant difference (Table 7).

It should be noted that, in this study, the specific (^{3}H)-naloxone binding was defined as the difference in the

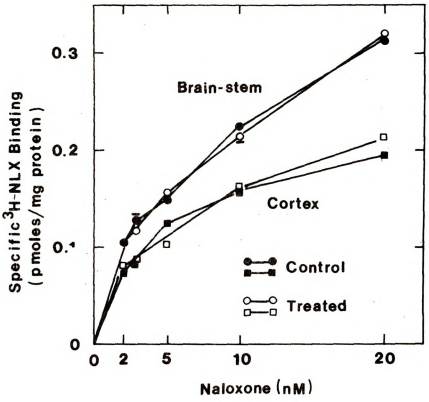

Figure 17. Effect of 6-hydroxydopamine pretreatment of rats on specific (^{3}H)-naloxone binding to particulate fraction obtained from brain-stem and cerebral cortex assayed in CSF.

Rats were treated with two intraventricular injections of 250 µg of 6-hydroxydopamine HBr(dissolved in 10 µl isotonic saline solution containing ascorbic acid; 1 mg/ml) given 7 days apart. Control animals were similarly injected twice with the same volume of vehicle. Particulate fraction of indicated brain regions was prepared in CSF and incubated with or without 10 µM non-labelled naloxone for 5 minutes. Subsequently, various concentrations of (^{3}H)-naloxone were added to the incubation mixture and the binding was assayed. Values shown are differences in (^{3}H)-naloxone binding assayed in the absence and presence of 10 µM non-labelled naloxone (saturable, stereospecific binding). Each point represents the mean of 6 experiments. Vertical lines indicate standard errors.

Table 7. Maximal binding and apparent Km for specific (^{3}H)-naloxone binding to particulate fraction obtained from control and 6-hydroxydopamine(6-OHDA)-treated rats assayed in CSF.

Brain Regions	Maximal Binding (pmoles/mg protein)		Apparent Km (nM)	
	Control	6-OHDA treated	Control	6-OHDA treated
Brain-stem	0.35±0.02	0.35±0.04	5.33±0.47	4.23±0.19
Cerebral cortex	0.22±0.01	0.22±0.01	5.44±0.47	3.95±0.22

Maximal binding and apparent Km were determined from Scatchard plot of the data in each experiment. The mean and standard error of 6 experiments were then calculated.

(^{3}H)-naloxone binding observed in the absence and in the presence of 10 μM non-labelled naloxone. A previous study present here has shown that the affinity of the specific binding sites for naloxone was increased whereas that for dihydromorphine was decreased in CSF and ICF (see Table 5). Pert et al. [1973] also demonstrated that sodium enhanced the specific binding of naloxone but reduced the specific binding of dihydromorphine and levorphanol. Therefore, non-labelled naloxone, rather than levorphanol, is a more appropriate opiate analog for the inhibition of the saturable, stereospecific component of (^{3}H)-naloxone binding assayed in CSF. Compared to an earlier experiment in which 10 μM levorphanol was used to define the specific binding, the specific (^{3}H)-naloxone binding observed in this study was slightly higher, the maximal binding was increased and affinity was also increased as indicated by the lower apparent Km value.

A similar study was also performed using the striatum obtained from 6-hydroxydopamine treated mice. Twelve male albino mice were injected with 16 μg of 6-hydroxydopamine HBr into the left striatum.[2] It has been shown that after the unilateral injection of 6-hydroxydopamine into the striatum, mice exhibited a marked ipsilateral reduction in forebrain dopamine concentration (reduced to 17% of the opposite non-lesioned side) and turned preferentially toward

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[2]Work performed collaboratively with Dr. John E. Thornburg.

the side of lesion. Apomorphine, in doses too low to produce stimulation of spontaneous motor activity, caused contralateral turning [Von Voigtlander and Moore, 1973]. All mice exhibited marked contralateral turning after apomorphine injection. Mice were decapitated and the striatum were dissected. Left striata were pooled and used as 6-hydroxydopamine-treated tissue whereas right striata (not directly injected with 6-hydroxydopamine) were pooled and used as the control tissue.

Particulate fraction of treated and control striata were prepared with 50 mM Tris-HCl buffer and immediately assayed for (^{3}H)-dihydromorphine binding as described previously. Neither total nor specific (^{3}H)-dihydromorphine binding to particulate fraction obtained from 6-hydroxydopamine-treated and control striata were significantly different. The maximal binding and the apparent Km values calculated from double-reciprocal plots also indicated no significant difference. The maximal binding was 0.13 pmoles/mg protein and the apparent Km was 3.47 nM for both 6-hydroxydopamine-treated and control striatum.

These data would suggest that the specific binding sites for dihydromorphine and naloxone are not specifically associated with central monoaminergic nerve terminal elements.

I. Effect of chronic morphine treatment on (^{3}H)-dihydromorphine binding to brain-stem particulate fraction assayed in 50 mM Tris-HCl buffer (pH 7.4)

Effects of levorphanol and dextrorphan on (^{3}H)-dihydromorphine binding were first studied. Rats were rendered tolerant to and dependent on morphine by implanting two 75 mg morphine base pellets 36 hours apart. Animals were sacrificed 36 hours after the implantation of the second pellet. In withdrawn animals, morphine base pellets were removed 36 hours after the implantation of the second pellet and animals were sacrificed 24 hours later. Withdrawn animals were hypersensitive, had severe diarrhea and lost weight sharply for 24 hours. About 48 hours after withdrawal, animals started to regain their body weight gradually and returned to their normal body weight 4-5 days following withdrawal. Particulate fraction of brain-stem were prepared in 50 mM Tris-HCl buffer and assayed for dihydromorphine binding immediately by incubating the preparation with 6 nM (^{3}H)-dihydromorphine.

Levorphanol and dextrorphan appeared to be similarly effective in inhibiting (^{3}H)-dihydromorphine binding to brain-stem particulate fraction obtained from control, tolerant and withdrawn animals (Figure 18). Dextrorphan remained to be about three orders of magnitude less potent than levorphanol. Both the total and the nonspecific (^{3}H)-dihydromorphine binding to the three preparations were not significantly different and thus the specific binding

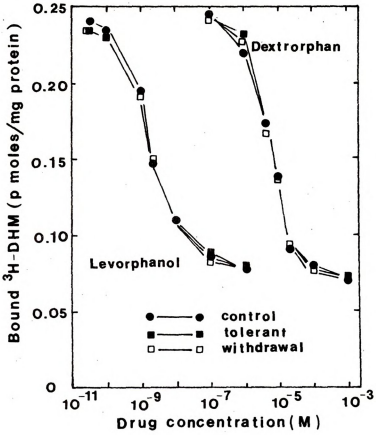

Figure 18. Effect of chronic morphine treatment on (^{3}H)-dihydromorphine binding to brain-stem particulate fraction assayed in 50 mM Tris-HCl buffer(pH 7.4).

Rats were implanted with two 75 mg morphine base pellets 36 hours apart. Tolerant animals were sacrificed 36 hours after the implantation of the second pellet. In withdrawn animals, morphine base pellets were removed 36 hours after the implantation of the second pellet and these animals were sacrificed 24 hours later. Control animals had sham operation. Particulate fraction of brain-stem was prepared in 50 mM Tris-HCl buffer(pH 7.4) and assayed for dihydromorphine binding with 6 nM (^{3}H)-dihydromorphine. Each point represents the mean of 5 experiments.

component was not altered after chronic morphine treatment or 24 hours after the withdrawal under these experimental conditions.

Since the specific binding was assayed with 6 nM (^{3}H)-dihydromorphine, a concentration of dihydromorphine close to its Km value, the magnitude of specific dihydromorphine binding is dependent on two factors, i.e., the affinity and the maximal binding capacity of specific binding sites for (^{3}H)-dihydromorphine. This study indicated that the apparent affinity for dihydromorphine, levorphanol and dextrorphan was not altered by chronic morphine treatment or during morphine abstinence. Alternatively, the apparent affinity for these three drugs was changed by the same magnitude in the same direction. The concentration of (^{3}H)-dihydromorphine was well below saturable levels, therefore, the alteration of the maximal binding may not be great enough to be detected by this assay.

In further studies, specific dihydromorphine binding to brain-stem preparation obtained from control, chronically morphine-treated and morphine-withdrawn rats was assayed with various concentrations of (^{3}H)-dihydromorphine and the maximal binding and the apparent Km values were determined separately in order to detect possible changes of these kinetic parameters induced by chronic morphine treatment or subsequent morphine withdrawal. Rats were rendered tolerant to and dependent on morphine by the subcutaneous injections of morphine solution (morphine base dissolved in

0.01 N HCl) at 8-hour intervals. The initial dose of 1.67 mg/Kg/injection was increased to 10 mg/Kg/injection over a period of 16 days and this dose was maintained for at least 6 days. Control animals received comparable volumes of 0.01 N HCl solution. Withdrawal syndrome such as hypersensitivity, severe diarrhea and weight loss appeared within 24 hours after termination of morphine injections in animals tolerant to high dose of morphine. These syndromes indicated that morphine-treated rats were physically dependent to morphine.

Chronic morphine treatment and subsequent morphine withdrawal failed to alter total and specific (^{3}H)-dihydromorphine binding significantly (Figure 19). The maximal binding and the apparent Km value for (^{3}H)-dihydromorphine, determined by a double-reciprocal plot of data in each independent experiment (Figure 20), also indicated no significant changes occurred after chronic morphine treatment or during morphine withdrawal (Table 8). During the withdrawal period, there was an apparent increase in the Km value for dihydromorphine indicating that the apparent affinity for dihydromorphine may be decreased at the peak of the withdrawal syndrome (35 hours). The apparent Km value returned toward control levels as withdrawal syndrome subsided (7 days).

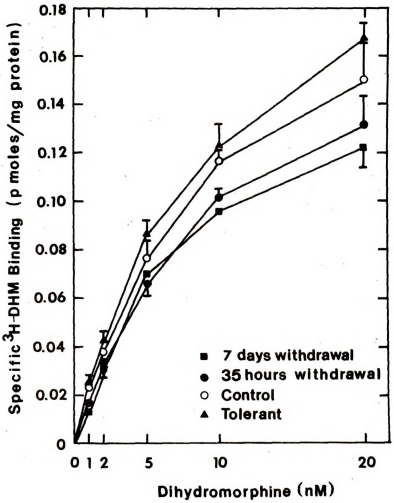

Figure 19. Effect of chronic morphine treatment and subsequent morphine withdrawal on specific (^{3}H)-dihydromorphine binding to brain-stem particulate fraction assayed in 50 mM Tris-HCl buffer (pH 7.4).

Rats were injected with morphine solution(morphine base dissolved in 0.01 N HCl solution) subcutaneously at 8-hour intervals. The initial dose of 1.67 mg/Kg/injection was increased to 10 mg/Kg/injection over a period of 16 days and this dose was maintained for at least 6 days prior to the sacrifice. Control animals received comparable volumes of 0.01 N HCl solution. Particulate fraction of brain-stem obtained from control, chronically morphine-treated and subsequently morphine-withdrawn rats was prepared in 50 mM Tris-HCl buffer(pH 7.4) and assayed for specific (^{3}H)-dihydromorphine binding. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

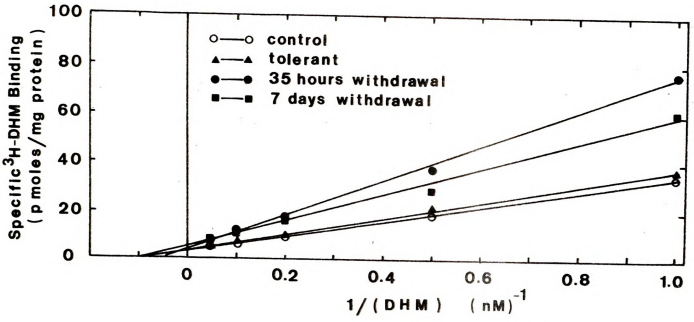

Figure 20. Double-reciprocal plots of specific (^{3}H)-dihydromorphine binding to brain-stem particulate fraction obtained from control, chronically morphine-treated and subsequently morphine-withdrawn rats assayed in 50 mM Tris-HCl buffer (pH 7.4).

Each point represents the mean of triplicate determinations. Regression lines were calculated by the least squares method fitted to the means in each experiment.

Table 8. Maximal binding and apparent Km for specific (^{3}H)-dihydromorphine and (^{3}H)-naloxone binding to brain-stem particulate fraction obtained from control, chronically morphine-treated and subsequently morphine-withdrawn rats assayed in 50 mM Tris-HCl buffer(pH 7.4).

Treatment	Maximal Binding (pmoles/mg protein)		Apparent Km (nM.)	
	DHM	NLX	DHM	NLX
Control	0.22±0.02	0.55±0.08	9.9±2.2	35.3±8.3
Tolerant	0.21±0.01	0.41±0.01	7.6±0.9	22.1±2.2
35 hours withdrawal	0.29±0.05		17.8±4.0*	
7 days withdrawal	0.19±0.03		9.7±2.7	

* Significantly different from corresponding value for preparation obtained from tolerant rats($p < 0.05$).

Maximal binding and apparent Km were determined from double-reciprocal plot of the data in each experiment. The mean and standard error of 5 experiments were then calculated.

J. Effect of chronic morphine treatment on specific (^{3}H)-naloxone binding to brain-stem particulate fraction assayed in 50 mM Tris-HCl buffer (pH 7.4)

Since the dose of naloxone required to precipitate the withdrawal syndrome become progressively smaller as the animals become more tolerant to morphine [Way et al., 1969], it was of interest to determine whether chronic morphine treatment would increase the affinity of specific binding sites for naloxone. Rats were rendered tolerant to and dependent on morphine by the subcutaneous injections of morphine sulfate solution twice per day. The initial dose of 10 mg/Kg/injection was increased to 100 mg/Kg/injection over a period of 12 days. Control animals received comparable volumes of isotonic saline solution.

Specific naloxone binding to brain-stem particulate fraction obtained from morphine tolerant and dependent rats was not significantly different from that of control preparations at all (^{3}H)-naloxone concentrations studied (Figure 21). The maximal binding and the apparent Km for (^{3}H)-naloxone, calculated from double-reciprocal plots, also indicated that no remarkable changes occurred after chronic morphine treatment (Table 8).

K. Effect of chronic morphine treatment on the specific (^{3}H)-dihydromorphine and (^{3}H)-naloxone binding to brain-stem particulate fraction assayed in CSF

It is well known that tolerance develops to morphine and to various natural and synthetic opiate analgesics after

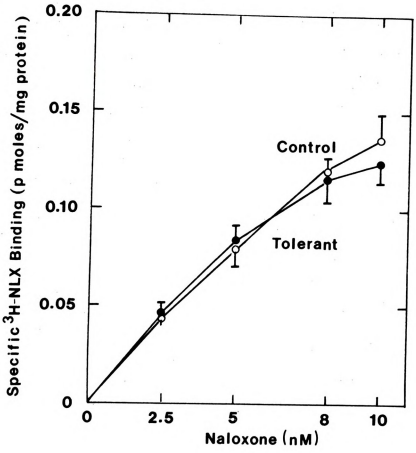

Figure 21. Effect of chronic morphine treatment on specific (^{3}H)-naloxone binding to brain-stem particulate fraction assayed in 50 mM Tris-HCl buffer(pH 7.4).

Rats were injected with morphine sulfate solution, twice per day, starting with 10 mg/Kg/injection. The dose was increased to 100 mg/Kg/injection over a period of 12 days. Control animals received comparable volumes of isotonic saline solution. Specific (^{3}H)-naloxone binding to brain-stem particulate fraction obtained from control and chronically morphine-treated rats was assayed in 50 mM Tris-HCl buffer(pH 7.4). Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

repeated doses of these drugs. At the same time as animals become more tolerant to morphine, the dose of naloxone required to precipitate the withdrawal syndrome becomes progressively less [Way et al., 1969]. It was of interest to note that sodium reduced the specific binding of several opiate agonists whereas the specific binding of naloxone and opiate agonist-antagonist were enhanced [Pert et al., 1973]. Thus changes in properties of opiate receptors after chronic morphine treatment may be related to the change in the sodium-sensitivity of the binding site. Therefore, the effect of chronic morphine treatment on the specific binding of (^{3}H)-dihydromorphine and (^{3}H)-naloxone were reinvestigated using CSF, which contains a high concentration of sodium, rather than using Tris-HCl buffer.

Under these experimental conditions, total and non-specific (^{3}H)-dihydromorphine binding were greatly reduced as observed in the earlier study. Chronic morphine treatment did not alter the total and the specific (^{3}H)-dihydromorphine binding significantly although the specific binding to preparations obtained from tolerant animals was generally slightly higher than that of control preparations (Figure 22). The maximal binding for dihydromorphine of brain-stem particulate fraction obtained from control and chronically morphine-treated animals, calculated from double-reciprocal plot of the data in each independent experiment, was 0.19 ± 0.01 and 0.16 ± 0.02 pmoles/mg protein (mean ± standard of 5 experiments) respectively. The apparent Km values for

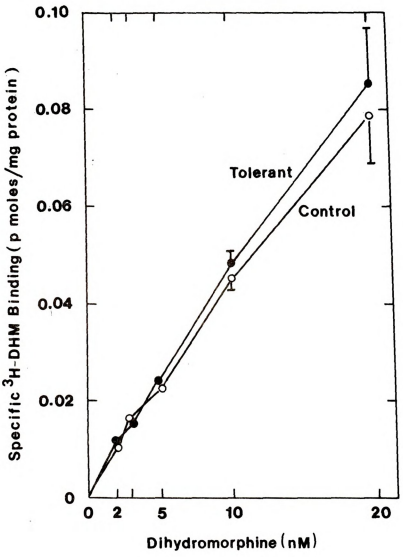

Figure 22. Effect of chronic morphine treatment on specific (^{3}H)-dihydromorphine binding to brain-stem particulate fraction assayed in CSF.

Brain-stem particulate fraction obtained from control and chronically morphine-treated rats was prepared in CSF and then assayed for specific (^{3}H)-dihydromorphine binding. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

(^{3}H)-dihydromorphine binding in control and tolerant preparations were 33.5 ± 4.2 and 27.2 ± 3.0 nM, (mean ± standard of 5 experiments) respectively. The maximal binding and apparent affinity for (^{3}H)-dihydromorphine in preparations obtained from tolerant animals were not significantly different from those of control preparations.

Both the total and the nonspecific (^{3}H)-naloxone binding were increased in CSF as observed in previous study. The specific (^{3}H)-naloxone binding, defined as the difference between the (^{3}H)-naloxone binding observed in the absence and in the presence of 10 μM non-labelled naloxone, was not significantly altered after chronic morphine treatment (Figure 23). In this experiment, the maximal binding and the apparent Km for (^{3}H)-naloxone were determined from Scatchard plots of data in each independent experiment and the mean and the standard error were calculated. The maximal binding for naloxone to brain-stem particulate fraction of control and chronically morphine-treated animals was 0.36 ± 0.02 and 0.38 ± 0.02 pmoles/mg protein, respectively, and they were not statistically different. The apparent Km for naloxone was 5.5 ± 0.47 and 6.4 ± 0.51 nM in control and chronically morphine-treated animals, respectively. These were not statistically different.

The present data thus indicate that the maximal binding and the apparent affinity for (^{3}H)-dihydromorphine and (^{3}H)-naloxone of brain-stem particulate fraction are not altered by chronic morphine treatment.

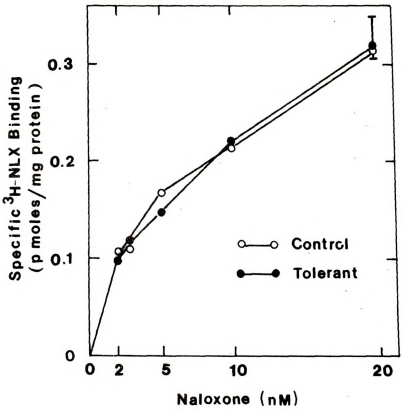

Figure 23. Effect of chronic morphine treatment on specific (^{3}H)-naloxone binding to brain-stem particulate fraction assayed in CSF.

Brain-stem particulate fraction obtained from control and chronically morphine-treated rats was prepared in CSF and then assayed for specific (^{3}H)-naloxone binding. Each point represents the mean of 5 experiments. Vertical lines indicate standard errors.

DISCUSSION

A. Specific binding and uptake(transport) of opiate analogs

The present studies and several previous reports demonstrated that it is possible to observe the specific binding of several opiate analogs to brain homogenates or particulate fraction obtained from brain homogenates. However, it should be noted that tissue binding and active transport of drugs have many features in common: saturability, chemical specificity and competition among structurally related analogs. This can be expected since the initial phase of an active transport mechanism is the binding of the drug to a carrier of the transport system and thus it resembles the binding of a drug to a specific receptor site. Since the existence of active transport of opiate analgesics has been reported [see Hug, 1971; Wang and Takemori, 1972; Vasko and Hug, 1973], it is important to differentiate specific binding from an active transport mechanism which accumulates opiate analogs into synaptosomes or other organelles.

One of the generally recognized features of active transport is an energy requirement. It was shown that specific naloxone binding was temperature dependent with a maximal binding at 35°C and a Q_{10} (change in the reaction rate for each 10°C change in temperature) value of 1.5 when measured between 25°C and 35°C and 1.3 when measured between

15°C and 25°C after 15 minute incubation. At 4°C, binding was reduced to 25% of the values at 35°C [Pert and Snyder, 1973a, 1973b]. The specific naloxone binding could reach a steady state within 15 minutes at 35°C and 25°C [Pert and Snyder, 1973a; Pasternack and Snyder, 1974]. Generally speaking Q_{10} value should be discussed in relation to the rate of reacion. Therefore, the Q_{10} value could be small at the steady state, as reported for specific naloxone binding, if both binding rate and dissociation rate were affected to the same degree by a temperature change. The Q_{10} value for specific naloxone binding may be large if a shorter incubation period is employed or at lower temperatures because the binding may not reach the steady state under these conditions. Thus, the reported small Q_{10} values *per se* may not support the conclusion that the observed uptake is not an active transport process.

The specific (^{3}H)-etorphine binding was not affected by high concentrations of sodium azide or sodium fluoride [Simon *et al.*, 1973]. Thus, the specific etorphine binding is not dependent upon energy from oxidative metabolism or glycolysis. Specific (^{3}H)-dihydromorphine binding was not affected by 10^{-5}M ouabain [Wong and Horng, 1973]. The same concentration of ouabain has been shown to inhibit approximately 60% of the active accumulation of 5-hydroxytryptamine and norepinephrine by synaptosomes [Tissari *et al.*, 1969]. Wong and Horng [1973] have further reported that valinomycin and monensin, which can function as mobile carriers for

monovalent cations in biological membranes in general, had no effect on the binding of (^{3}H)-dihydromorphine. Therefore, the specific (^{3}H)-dihydromorphine binding is not coupled with sodium transport.

One of the features which clearly distinguishes active transport from binding is the storage of the transported drug. For instance, the uptake of norepinephrine by synaptosomes in Krebs bicarbonate buffer was linear for 50 minutes and the amine was concentrated well above 200 times the concentration in the incubation media [Colburn et al., 1967]. The specific binding of (^{3}H)-naloxone, (^{3}H)-etorphine and (^{3}H)-dihydromorphine to brain homogenates was linear for only 1 to 3 minutes and a steady state was reached in 10 minutes [Pert and Snyder, 1973b; Simon et al., 1973; Wong and Horng, 1973]. On the other hand, when brain slices were used, (^{3}H)-etorphine, at low concentrations, was accumulated into slices and a steady state was not reached until 30 minutes later [Huang and Takemori, 1974]. However, it was also found that (^{3}H)-etorphine accumulation into brain slices was not affected by metabolic inhibitors such as dinitrophenol, fluoride, azide or iodoacetamide. Thus, this accumulation phenomenon may be just a redistribution of (^{3}H)-etorphine between the incubation medium and the brain slice which has a high lipid content.

Organic basic compounds such as N-methylnicotinamide and SKF-525A have been shown to inhibit the active transport of morphine from the systemic circulation into the

CSF [Wang and Takemori, 1972]. However, the present studies have shown that N-methylnicotinamide did not affect specific (^{3}H)-dihydromorphine binding (see Table 3). SKF-525A did inhibit the specific binding of 3.33 nM (^{3}H)-dihydromorphine at concentrations higher than 10^{-6}M. However, the inhibition of specific dihydromorphine binding by SKF-525A appears not to be a competitive type and may be resulted from nonspecific changes in membrane properties. (^{3}H)-Etorphine accumulation into brain slices also was not affected by N-methylnicotinamide and hexamethonium [Huang and Takemori, 1974]. These data would suggest that in brain tissue there is no active transport mechanism for opiate analogs in contrast to that demonstrated in choroid plexus. The active transport of opiates by cerebral cortical slices demonstrated by Hug and his associates [see Hug, 1971; Vasko and Hug, 1973] may not be involved in the present binding studies because active transport was demonstrated only with high concentrations of opiates. Moreover, it has been shown that at concentrations above 15 nM, the accumulation of (^{3}H)-etorphine into brain slices appeared to be nonsaturable [Huang and Takemori, 1974]. Therefore, the overall evidence appears to indicate that the specific binding of opiate analogs to brain homogenates assayed *in vitro* is not a transport phenomenon.

B. Components of the binding of opiate analogs

Goldstein *et al.* [1971] first demonstrated that approximately 2% of the (^{14}C)-levorphanol binding to mouse brain

homogenates (assayed with 1.95 μM (^{14}C)-levorphanol) was saturable and stereospecific binding, 53% of the binding was saturable, nonspecific binding and 46% of the binding was non-saturable, nonspecific binding. The saturable, stereospecific binding was defined as the difference in the (^{14}C)-levorphanol binding observed in the presence of 100 times excess of dextrorphan, a pharmacologically inactive enantiomer of levorphanol, and in the presence of 100 times excess of non-labelled levorphanol. The saturable, non-specific binding was defined as the difference in the (^{14}C)-levorphanol binding observed in the absence and in the presence of 100 times excess of dextrorphan. Recently, Pert and Snyder [1973] have shown that approximately 70% of the (^{3}H)-naloxone binding (assayed with 5 nM (^{3}H)-naloxone) was saturable and stereospecific.

The present studies clearly demonstrated that when dihydromorphine binding was studied with low concentrations of (^{3}H)-dihydromorphine, the bulk of the binding is saturable (Figure 1; Vertical line I). This is in agreement with the finding of Pert and Snyder [1973a] for naloxone binding. Since the affinity for dextrorphan and levorphanol differed by more than three orders of magnitude, the saturable binding may be called stereospecific. As the concentration of (^{3}H)-dihydromorphine increased, the non-saturable binding increased proportionally whereas the saturable binding was close to the maximal binding which is achieved at approximately 80 nM. Thus, the proportion of this component

decreased as the concentration of (^{3}H)-dihydromorphine was increased. These findings are therefore also in agreement with the finding of Goldstein et al. [1971]. In the presence of high concentrations of levorphanol and dextrorphan, the saturable binding may be further separated into saturable, non-stereospecific and saturable, stereospecific components (Figure 1 and Figure 2, A and B, respectively) as postulated by Goldstein et al. [1971]. However, this separation appears to be due to the use of a concentration of dextrorphan that inhibits the saturable binding only partially and is also due to the relative stereospecificity of the binding sites and hence may be artifactual. Therefore, the binding of opiate analogs to brain tissue in vitro can be separated into two components; one is saturable, relatively stereospecific and the other is non-saturable, non-stereospecific.

C. Affinity and specificity of the specific binding sites for opiate analogs

According to Ki values of several opiate analogs for the specific (^{3}H)-dihydromorphine binding, the affinity for levorphanol was approximately 4 times higher than that for morphine. Naloxone, morphine and d,l-methadone had similar affinities. Dextrorphan, codeine and thebaine had much lower affinities. These data and previous studies of ID_{50}'s of several opiate analogs [Pert and Snyder, 1973a, 1973b; Simon et al., 1973; Wong and Horng, 1973] all indicate that, in general, the affinity of opiate analogs parallel

their analgesic potency as determined by the systemic injection of these drugs. The low potency of codeine *in vitro* may be due to the lack of metabolic activation under these experimental conditions. It has been proposed previously that the demethylation of codeine is required for its pharmacologic activity [Johannesson and Schou, 1963].

In the present studies, the affinity of levorphanol was approximately 2000 times higher than that of dextrorphan. Based on ID_{50}'s, the affinity of levorphanol is about 4000 times higher than that of dextrorphan. The affinity of *l*-levallorphan was 5000 times higher than that of its *d*-enantiomer [Pert and Snyder, 1973a; Simon *et al.*, 1973]. The affinity of *l*-methadone was 10 to 50 times higher than that of *d*-methadone [Pert and Snyder, 1973a; Wong and Horng, 1973]. These data thus clearly demonstrate that the specific binding sites for etorphine, dihydromorphine and naloxone are relatively stereospecific. The less impressive difference in stereospecificity between *l*-methadone and *d*-methadone may be due to that the methadone molecule has greater conformational mobility than the other opiate analogs [Portoghese, 1966].

A serious problem in comparative studies of analgesic potency of various opiate analgesics *in vivo* and *in vitro* is that the assessment of *in vivo* potency are not based on drug concentrations at receptor sites. It is well known that the blood-brain barrier impedes or prevents the

penetration of drugs with low lipid solubility into the brain [see Schanker, 1962]. Thus, the pharmacokinetic differences will probably be of considerable magnitude for the chemically heterogeneous opiates and related synthetic analgesics. For instance, heptane/water partition coefficients range from less than 0.0001 for the more hydrophilic morphine and normorphine to 100 for the lipophilic methadone and fentanyl [von Cube et al., 1970]. One would expect methadone and fentanyl to cross the blood-brain-barrier more easily than morphine and normorphine. Therefore, it may not be appropriate to estimate the relative analgesic potency of various opiate analgesics from a comparison of the dosages which produce equal analgesic effects when administered systemically.

The comparative analgesic potency of various opiate analogs according to their brain concentrations has been reported by Herz and Teschemacher [1971]. In these studies, morphine has been shown to be 8 times more potent than levorphanol and 30 times more potent than d,l-methadone. Thus, the relative affinities for various opiate analogs, estimated from their Ki or ID_{50} values, do not correlate well with their analgesic potencies as estimated from their brain concentrations [Terenius, 1974; Takemori, 1974]. However, some cautions must be taken before making this conclusion. Herz and Teschemacher [1971] have concluded that drugs with lower lipid solubilities reach their maximal activity much later when the drug is administered

intraventricularly. With intravenous application, the differences are much less pronounced. It has been shown that intraventricularly applied morphine produces its effects very slowly and does not reach its maximal effect until 1.5 hours later, while fentanyl, a highly lipid soluble opiate analgesic, produced its maximal effect within minutes. No more than 10% of intraventricularly applied fentanyl and etorphine were found in brain 15 minutes after the application, while 40-60% of morphine and dihydromorphine were found at that time. More than 10% of morphine and dihydromorphine were present in the CSF even after one hour. Autoradiographic studies have shown that various opiate analgesics had different distribution patterns. Morphine and dihydromorphine preferentially diffused into gray matter, whereas fentanyl exhibited a pronounced preference for white matter. Whereas morphine and dihydromorphine continue to penetrate further into the brain, fentanyl does not. Fentanyl can scarcely be detected in a small zone at the periventricular wall 120 minutes after intraventricular injection [see Herz and Teschemacher, 1971]. Thus a depot of morphine and dihydromorphine is maintained in the ventricular system for a good length of time, which insures a steep concentration gradient within the brain tissue whereas the concentration of fentanyl and etorphine in ventricular system falls at a rapid rate. In the presence of a steep concentration gradient, the concentration of morphine or dihydromorphine at receptor sites, which is

presumably located a few mm from the ventricular wall, increases with time. The concentration of fentanyl or etorphine at receptor sites, on the other hand, reaches a peak earlier but then declines rapidly.

In studies in which the comparative analgesic potency of various analgesics was estimated from the drug concentration in brain tissue [Herz and Teschemacher, 1971; Terenius, 1974], neither the time of onset nor the grade of analgesia was taken into consideration. Therefore, more careful studies should be performed in order to estimate the true potency of various opiate analogs.

The present studies show that the affinity of specific binding sites for naloxone was higher whereas that for dihydromorphine was lower in CSF and ICF compared to those in 50 mM Tris-HCl buffer. Therefore, if affinities for various opiate analogs are monitored in CSF or ICF, the absolute value of Km or Ki would be different. The ranking order of affinity for opiate agonists in CSF or ICF may be similar to that in 50 mM Tris-HCl buffer. However, the absolute affinity for opiate antagonists or agonist-antagonists such as naloxone, nalorphine, levallorphan, cyclazocine and pentazocine would be higher in CSF or ICF and the ranking order may be affected by the incubation media. Since it is reasonable to assume that the opiate receptors are exposed either to extracellular fluid or intracellular fluid, the affinities of opiate agonists or antagonists assayed in CSF or ICF may be more relevant to the study of

opiate receptors. Thus, studies of affinity and specificity of the specific binding sites for opiate analogs need to be carefully reevaluated.

D. Regional distribution of the specific binding sites for dihydromorphine and naloxone in rat brain

The present data confirm and extend the marked regional differences in (^{3}H)-dihydromorphine and (^{3}H)-etorphine binding reported earlier [Kuhar et al., 1973; Hiller et al., 1973]. The differences in dihydromorphine binding observed in various brain regions appear to be primarily due to the difference in the concentration of binding sites. Binding sites for dihydromorphine from various brain regions would appear to have a similar apparent Km value except for those from cerebral cortex, which has a significantly higher apparent affinity. This would suggest a similarity in binding sites in different brain areas but does not rule out the possibility that multiple binding sites exist. It is possible, for example, that dihydromorphine bound to low affinity binding sites may be lost during the washing of the Millipore filters if the complex of dihydromorphine with such a low affinity binding sites has a high dissociation rate.

Kuhar et al. [1973] and Hiller et al. [1973] have demonstrated that the specific binding of (^{3}H)-dihydromorphine and (^{3}H)-etorphine were high in the limbic cortex such as amygdala, temporal lobe, parahippocampal gyrus and

cingulate gyrus but low in the hippocampus and very low in cortical white matter areas. The specific binding in these studies was assayed with 1 or 3 nM of tritium-labelled compounds. Present data show that cerebral cortex has a high affinity specific (^{3}H)-dihydromorphine binding site and thus would suggest that the high specific (^{3}H)-dihydromorphine or (^{3}H)-etorphine binding observed in the previous two studies may be due to the high affinity of specific binding sites in those brain regions. The maximal binding for (^{3}H)-dihydromorphine was relatively low in the whole cerebral cortex. Whether the specific agonist binding sites are mainly located in certain cerebrocortical regions such as those structures categorized as the limbic cortex remains to be elucidated. A Scatchard plot of the specific (^{3}H)-dihydromorphine binding to particulate fraction of rat cerebral cortex (Figure 7) indicates that there are probably two types of specific binding sites with different affinities for dihydromorphine. This observation is compatible with the finding that whereas several cortical limbic structures have high specific binding, other cortical limbic structures such as the hippocampus and cortical white matter have low specific binding when assayed at one low concentration of (^{3}H)-dihydromorphine or (^{3}H)-etorphine.

The binding sites for naloxone distribute differentially in various brain regions not only in quantity but also in quality(apparent Km values). Differences in affinities indicate the presence of different binding sites for the

antagonist. Since naloxone is capable of displacing dihydromorphine from its binding site, it appears that naloxone interacts with dihydromorphine binding sites and with other type of binding sites which have different affinities and are not available to dihydromorphine. This hypothesis is supported by the finding that in each brain region tested, maximal binding, i.e., the concentration of binding sites accessible under the particular experimental condition, is greater for naloxone than for dihydromorphine. Similarly, greater values for maximal naloxone binding than that for dihydromorphine binding were observed in artificial cerebrospinal fluid or in simulated intracellular fluid. Finally, experiments shown in Figure 13 and 14 clearly indicate that a part of the (^{3}H)-naloxone binding cannot be displaced by dihydromorphine, although it can be displaced by non-labelled naloxone. The naloxone binding that cannot be displaced by dihydromorphine was the primary fraction of (^{3}H)-naloxone binding in the cerebellum but a relatively small portion in striatum. These data are consistent with results shown in Figure 6 and 8, which indicate that the levels of dihydromorphine binding are only small fractions of the naloxone binding in the cerebellum but a substantial portion in the striatum. Thus, it would appear that the observed regional differences in the apparent Km values for naloxone binding represent the presence of at least two different populations of binding sites with different Km values, and that the observed Km value depends upon the

relative abundance of each type of binding site.

The present data also clearly indicate that naloxone but not dihydromorphine binds to cerebellar tissue. This is in contrast with a previous report by Pert and Snyder [1973a] who claim that cerebellar tissue does not contain naloxone binding sites. The discrepancy appears to depend on differences in the concentrations of (^{3}H)-naloxone used and those of levorphanol employed to dilute specific naloxone binding. The binding of (^{3}H)-naloxone to cerebellar tissue was indeed small at low (^{3}H)-naloxone concentrations and may be regarded as insignificant if higher concentrations are not examined (Figure 8). Additionally, (^{3}H)-naloxone binding to cerebellar tissue is primarily due to a site which is not available to dihydromorphine. Inhibition of (^{3}H)-naloxone binding to such a site may require a higher concentration of levorphanol, such as the 10 μM concentration used in the present study, rather than that used by Pert and Snyder (0.1 μM). Thus, the successful demonstration, in the present study, of a naloxone binding site which is not available to dihydromorphine may partly depend on the use of higher concentrations of levorphanol. With high concentrations, dextrorphan was capable of inhibiting the specific opiate binding indicating that the stereospecificity of the binding site is only relative.

In CSF or ICF, the affinity for dihydromorphine was decreased while the affinity for naloxone was increased as compared to those in Tris-HCl buffer. Moreover, the present

data indicate that naloxone has more binding sites than dihydromorphine. Therefore, when specific (^{3}H)-naloxone binding is to be studied in CSF or ICF, non-labelled naloxone, rather than levorphanol, should be used to define the saturable, specific naloxone binding. Results of regional studies of specific naloxone binding would not be affected by this change in design because these studies were performed in 50 mM Tris-HCl buffer and levorphanol appears to have a higher affinity than naloxone for agonist sites in 50 mM Tris-HCl buffer (Table 1).

Previous studies have shown that the main sites of morphine analgesia in rats are periventricular structures of the third ventricle (see Introduction, Section A-2), whereas the primary sites of naloxone action appears to be located in the medial thalamus and medial areas of diencephalic-mesencephalic junctures. However, Martin [1967] has suggested that the receptors responsible for the analgesic, respiratory depressant and psychotomimetic effects could be stereochemically quite similar since all of these pharmacologic effects could be antagonized by naloxone in non-toxic doses. Thus, it is not possible to correlate a specific pharmacologic receptor, namely the analgesic receptor with the results of present regional studies. Studies with more delicately dissected brain regions may yield a relevant answer to this question. On the other hand, Martin's suggestion gives the support for the attempts to correlate the affinity of the specific binding site for various opiate

analogs with their analgesic potency in order to demonstrate the pharmacologic significance of the specific binding site observed in vitro.

E. The specific binding sites for opiate analogs and the central monoaminergic preterminal axons and nerve endings

The central nervous system has been categorized biochemically as containing several types of neuronal systems according to the neurotransmitter utilized for nerve transmission. If the specific binding sites for opiate analogs were associated with certain presynaptic terminals, such an association may be demonstrated by destroying those presynaptic terminals and monitoring the change in the specific binding of opiate analogs. This would be an important finding since the functional role of the binding macromolecules is not known.

The present studies show that specific dihydromorphine and naloxone binding to tissue preparations obtained from rats treated with 5,7-dihydroxytryptamine creatinine sulfate and 6-hydroxydopamine HBr were not significantly different from the specific binding of dihydromorphine and naloxone in control preparations. This would suggest that the specific binding sites for dihydromorphine and naloxone are not a unique component of preterminal axons or nerve endings of central monoaminergic neurons.

Ungerstedt [1971b] concluded that intraventricularly injected 6-hydroxydopamine produces a two stage effect with

a large dose (200 µg/20 µl). The first stage involved an area limited to a periventricular zone, being about 2.0 mm wide. The second stage involved the noradrenergic axons in the lateral hypothalamus, tegmentum, pons and medulla oblongata and the dopaminergic cell bodies in the substantia nigra and the ascending dopaminergic axons in the hypothalamus. Noradrenergic terminals in the cerebral and cerebellar cortices and dopaminergic terminals in the caudate nucleus were also affected. Therefore, the failure of 6-hydroxydopamine to alter opiate binding in the present studies is not due to the route of administration which may limit the drug to the periventricular structures.

Richards [1971] has pointed out that structurally-damaged axon profiles, after two intraventricular injections of 200 µg 6-hydroxydopamine, were not more than 1-3% of the total number of nerve terminals observed in survey micrographs. The present experimental method cannot detect such a small change if the specific opiate binding is not unique to catecholaminergic preterminal axons and nerve endings.

In previous studies, the specific binding of opiate analogs has been proposed to be primarily associated with synaptosomal membranes (see Introduction, Section A-3). In a subsequent detailed study, Pert et al. [1974] demonstrated that the specific binding of 1 nM (^{3}H)-dihydromorphine was primarily found in synaptosomal fractions. Within synaptosomal fractions, the specific dihydromorphine binding was

highly restricted to the membrane fraction. Since Kuhar et al. [1973] have failed to correlate the specific dihydromorphine binding with central noradrenergic, 5-hydroxytryptaminergic or cholinergic nerve terminal elements, Pert et al. [1974] proposed that specific dihydromorphine binding might be associated with post-synaptic thickening which could persist after electrolytic lesions of axons in previous studies [Kuhar et al., 1973]. Alternatively, the specific binding sites for opiate analogs may be associated with other pathways which are not yet clearly understood; such as the GABA and histamine pathways or other pathways which are not demonstrated yet. It is also possible that the specific binding sites are not specifically associated with nerve terminal elements of any particular nerve pathway.

F. Specific binding of dihydromorphine and naloxone in vitro and hypotheses of narcotic tolerance and physical dependence

Tolerance to and physical dependence on opiate analgesics may develop as a result of quantitative changes in macromolecules (proteins) associated with the binding of opiates (receptors) or with the expression of drug-receptor interaction. In attempts to explain the development of narcotic tolerance and physical dependence, it has been postulated that chronic morphine treatment induces changes in the concentration of opiate receptors [Collier,

1965; Martin, 1968; Jaffe and Sharpless, 1968], changes in amounts of opiate sensitive enzymes that synthesize central neurotransmitters [Goldstein and Goldstein, 1961; Shuster, 1961], the synthesis of new proteins which could affect the morphine-receptor binding (see Introduction, Section B-5; Cochin and Kornetsky, 1964] or the synthesis of new proteins which affect the neurotransmitter-receptor interactions involved in pharmacologic manifestations of opiate effects [Smith, 1971]. Narcotic tolerance and physical dependence may also develop as a result of qualitative changes in opiate sensitive brain proteins if chronic morphine treatment alters the affinity of the existing receptors for opiate analogs.

However, the present studies indicate that neither the maximal binding nor the apparent Km of the specific binding sites for dihydromorphine and naloxone were significantly altered after chronic morphine treatment of rats. A significant decrease in the apparent affinity of specific binding sites for dihydromorphine was observed during the morphine withdrawal in rats chronically treated with morphine. This phenomenon, however, does not appear to correlate with the development of tolerance since no significant change in the apparent affinity of specific dihydromorphine binding sites was observed shortly after the termination of morphine injections. It appears that this phenomenon is a result rather than the cause of morphine withdrawal. These data thus suggest that morphine tolerance and physical dependence

is not the result of alteration in the number or the affinity of the specific receptor sites in the rat brain. The finding that there is no specific binding of levorphanol and naloxone in the soluble supernatant [Goldstein et al., 1971; Pert and Snyder, 1973] would rule out the possibility that opiate analogs could specifically interact with certain soluble enzymes which may be important in neurotransmitter synthesis. The possibility that chronic morphine treatment could induce the synthesis of new soluble proteins which may regulate the opiate-receptor or neurotransmitter-receptor interaction remains to be elucidated.

Klee and Streaty [1974] argued that if narcotic tolerance and dependence were simply the result of an increased number or a decreased affinity of receptor sites much large doses of naloxone or nalorphine, not decreased doses as documented, should be required to precipitate withdrawal syndrome. Since the present studies clearly indicate that naloxone has at least two types of specific binding sites, the assumption which states that naloxone binds only to the morphine receptors is too simple.

It should be pointed out, however, that rat brain regions not including the cerebral cortex and cerebellum were utilized in the present studies. Relatively large regions such as cerebral cortex, brain without the cerebellum or the whole brain were used in other studies [Terenius, 1973; Klee and Streaty, 1974; Hitzemann et al., 1974]. Thus, it is not possible to rule out the possibility that

changes in properties of the specific opiate binding site occurs only in a very small brain region; for instance, in the hypothalamus or medulla oblongata, and therefore would not be detected in the present studies.

Recently, Ahtee [1974] has shown that 2 hours after methadone injection (10 mg/Kg), the striatal homovanillic acid (HVA) concentration of rats receiving methadone for 8 weeks was increased to about the same degree as in control (saline) rats receiving the same dose of methadone as an acute single injection. However, 19 hours after the last injection of methadone the striatal HVA concentration of rats receiving methadone for 8 weeks was decreased to 55% of that of untreated control rats. Since it is generally accepted that the distribution and metabolism of opiates and synthetic analgesics is not significantly altered after chronic treatment of these drugs (see Introduction, Section B-3), Ahtee's observations may be interpreted as indicating that the affinity of methadone receptors on dopaminergic neurons which innervate striatal neurons is decreased. Neither the concentration nor the efficacy of receptors are altered after chronic methadone treatment because HVA increased to about the same degree in control rats 2 hours after the last injection and only decreased thereafter. If dopaminergic neurons are not the primary neurons affected by methadone, the same interpretation would apply to the unknown primary sites of the action of methadone. Thus this study would substantiate the hypothesis that the

affinity of opiate receptors was altered after chronic treatment by opiate analogs. It also suggests that the use of brain preparations obtained from a small, particular brain region may yield positive data.

Alternatively, the effectiveness of the system with which opiate-receptor interaction is translated into the primary pharmacologic response or the function of a system which regulates such processes may be greatly altered in the tolerant state. A successful reversal of morphine tolerance by medial thalamic lesions in the rat [Teitelbaum et al., 1974] appears to support the latter hypothesis. If chronic morphine treatment causes a proliferation of an inhibitory pathway which modulates the expression of the drug-receptor interaction, then lesions of such an inhibitory pathway could restore the sensitivity to opiate analgesics. However, this study was performed using the EEG response at cortical and subcortical recording sites as the criterion for the development of morphine tolerance. A further study of morphine analgesia in animals with or without medial thalamic lesions might substantiate this hypothesis.

Since the functions of the central nervous system are mediated through neurotransmitters released from nerve terminals, if the events which follow the opiate-receptor interaction are altered in the tolerant state, then one would predict that neurotransmitter released from the neuron which possesses specific opiate receptors is either greatly

increased or decreased. As discussed in the Introduction, Section B-6, no such phenomenon could be detected. The evidence, thus, appears to encourage further studies of the properties of the specific binding sites for various opiate analogs. The use of preparations obtained from delicately dissected small brain regions or purified preparations as described by Lowney et al. [1974] and Loh et al. [1974] may be helpful in such studies.

SUMMARY AND CONCLUSION

In the present studies, the binding in vitro of an opiate agonist, (^{3}H)-dihydromorphine was studied using particulate fraction obtained from rat brain homogenates and compared with that of an opiate antagonist, (^{3}H)-naloxone. The significant observations and conclusive remarks of the present investigations are as follows:

A. Levorphanol as well as its stereo-enantiomer, dextrorphan inhibited (^{3}H)-dihydromorphine binding. Although the magnitude of maximal inhibition was similar with both compounds, dextrorphan was approximately three orders of magnitude less potent than levorphanol. The binding of (^{3}H)-dihydromorphine in the presence of 10 μM levorphanol was proportional to the (^{3}H)-dihydromorphine concentration in the medium, indicating that this fraction is non-saturable. When this fraction was subtracted from the (^{3}H)-dihydromorphine binding observed in the absence of levorphanol (total binding), the levorphanol-inhibitable binding followed a typical absorption isotherm curve. Thus, the binding of (^{3}H)-dihydromorphine may be separated into two components: one saturable and stereospecific and the other non-saturable. The use of dextrorphan results in an artifactual separation of the saturable component. The apparent Km value of the saturable, stereospecific binding sites for dihydromorphine

in brain-stem was 7.9 ± 1.2 nM. The maximal specific binding was 0.25 ± 0.01 pmoles/mg protein. Specific (^{3}H)-naloxone binding assayed under same conditions also appeared to have two components. The apparent Km value of saturable, stereospecific binding sites for naloxone in brain-stem was 24.0 ± 6.6 nM and the maximal specific binding was 0.57 ± 0.10 pmoles/mg protein.

B. Based on Ki values estimated from Dixon plots of specific (^{3}H)-dihydromorphine binding in the presence of several non-labelled opiate analogs, levorphanol had the highest affinity for the specific dihydromorphine binding site, followed by naloxone, morphine and d,l-medhadone. Dextrorphan had an affinity 2000 times lower than that of levorphanol. Codeine and thebaine had the lowest affinities. Apomorphine, dopamine, chlorpromazine, xylazine [Bayer 1470] and N-methylnicotinamide did not affect specific (^{3}H)-dihydromorphine binding at concentrations up to 10^{-5}M. SKF-525A inhibited specific dihydromorphine binding. Such inhibition, however, appeared to depend on the irreversible alteration of membranes and hence to be nonspecific to opiate binding sites. Thus, the specific dihydromorphine binding site appeared to be specific for other active opiate analogs.

C. There were marked regional differences in the distribution of specific (^{3}H)-dihydromorphine binding in the brain. These were primarily due to differences in the concentration of the binding sites within various brain

regions. It appeared that specific binding sites in various brain regions had similar affinities for dihydromorphine, except for those binding sites in cerebral cortex which had high affinity. In contrast, specific binding sites for naloxone in various brain regions had different affinities for anloxone. It appeared that naloxone has at least two types of binding sites, one of which is not available to dihydromorphine. This is based on observations that (1) the total concentration of specific binding sites for naloxone was greater than that for dihydromorphine in each brain region studied irrespective of the assay medium used and (2) unlabelled dihydromorphine inhibited the specific (^{3}H)-naloxone binding in striatum but failed to alter it significantly in cerebellum whereas unlabelled naloxone reduced (^{3}H)-naloxone binding significantly in both brain regions. The difference in total binding sites for naloxone and dihydromorphine was relatively small in striatum but large in cerebellum, indicating that the binding sites in cerebellum are predominantly naloxone-specific whereas those in striatum are capable of binding both naloxone and dihydromorphine.

D. In CSF as well as in ICF, the apparent affinity of specific binding sites for dihydromorphine was decreased while that for naloxone was increased as compared to those in 50 mM Tris-HCl buffer. The maximal specific binding for dihydromorphine and naloxone were both decreased in CSF and ICF. The apparent affinity for naloxone was significantly

higher than that for dihydromorphine in CSF and ICF in contrast to those observed in Tris-HCl buffer. This is consistent with the pharmacologic properties of these two drugs. Thus, while some experiments, such as the comparison of the binding site populations, may be performed using a simple buffer solution, in vitro studies may be pharmacologically more relevant if performed using CSF and ICF. When specific (^{3}H)-naloxone binding is to be studied in CSF or ICF, non-labelled naloxone, rather than levorphanol, should be used to define the saturable, specific naloxone binding because naloxone appeared to bind to other sites in addition to agonist binding sites and the affinity of naloxone binding sites for naloxone in CSF and ICF is higher than that for opiate agonists.

E. Two weeks after an intraventricular injection of 75 µg of 5,7-dihydroxytryptamine creatinine sulfate, specific (^{3}H)-dihydromorphine binding to preparations obtained from diencephalon and midbrain-low brain-stem of these treated animals was not significantly different from the specific binding to comparable control preparations. Similarly, pretreatment of rats with two intraventricular injections of 250 µg of 6-hydroxydopamine HBr also failed to alter significantly the specific (^{3}H)-naloxone binding to particulate fraction obtained from cerebral cortex and brain-stem. These data appear to indicate that the specific binding sites for dihydromorphine and naloxone are not a unique component of preterminal axons and nerve endings of

central monoaminergic neurons.

F. Chronic morphine treatment of rats or subsequent withdrawal failed to alter the concentration of either dihydromorphine or naloxone specific binding sites in the brain-stem when binding was assayed in 50 mM Tris-HCl buffer as well as in CSF. Chronic morphine treatment also failed to alter the affinity of these specific binding sites. During withdrawal from morphine there was a tendency toward a reduced affinity of specific binding sites for dihydromorphine, which returned toward control level upon the dissipation of the withdrawal syndrome.

G. It was concluded that the specific binding sites for dihydromorphine and naloxone could be demonstrated using low concentrations of radiolabelled compounds. These binding sites appear to be saturable, stereospecific, specific to active opiate analogs and closely related to the pharmacologic receptors. Naloxone appears to have at least two types of specific binding sites, one of which is not available to dihydromorphine. It appears that these specific binding sites are not associated with central monoaminergic preterminal axons and nerve terminals which have been emphasized to play an important role in pharmacologic actions of opiate analgesics and the development of narcotic tolerance and dependence. Chronic morphine treatment does not alter the concentration and affinity of specific binding sites for dihydromorphine and naloxone in brain-stem particulate fraction. Since a relatively large brain region was

utilized in the present studies, it cannot be ruled out that changes in the properties of the specific opiate binding sites may occur only in certain small brain region(s).

H. The present data failed to positively support one of many hypotheses which have been proposed in attempts to explain the development of narcotic tolerance and physical dependence. Several hypotheses, however, are inconsistent with the present data and may be ruled out.

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