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DISPOSITION OF BRAIN MORPHINE AND CORRELATION WITH THE PHARMACOLOGIC EFFECTS

by

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B.S., National Taiwan University, 1968
M.S., National Taiwan University, 1970

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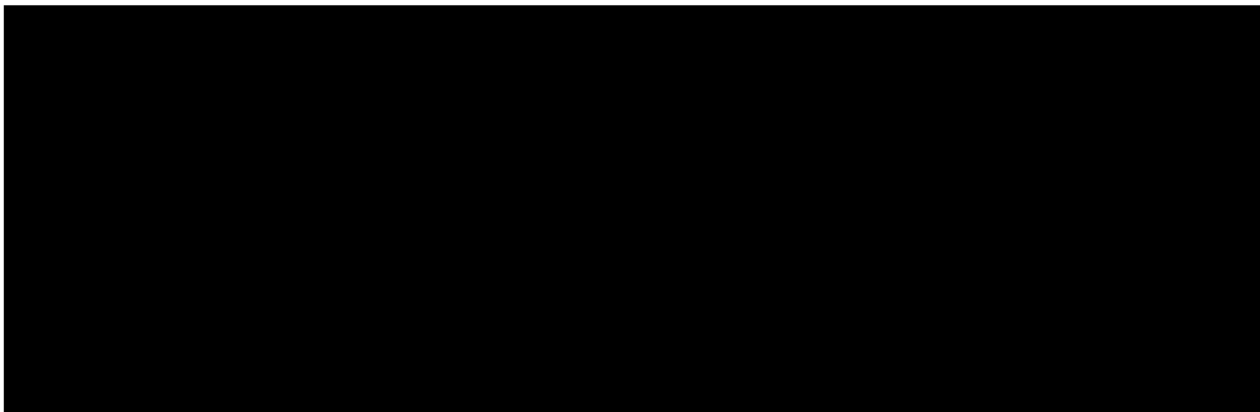

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ABSTRACT

DISPOSITION OF BRAIN MORPHINE AND CORRELATION WITH THE PHARMACOLOGIC EFFECTS

by

Jeanne Wuan-Hsiang Shen

The primary goal of this study was to investigate the disposition of morphine in the central nervous system of both naive and morphine-tolerant/dependent mice and to study the effect of naloxone on the disposition of brain morphine.

Tissue morphine was extracted with 10% n-butanol in chloroform and purified with 0.01 N H_2SO_4. Morphine content in the acid extract was analyzed by radioimmunoassay using ^{3}H-morphine or ^{3}H-dihydromorphine as the radiolabeled ligand and morphine antiserum from the rabbit (supplied by Dr. Don H. Catlin). The antiserum binds to morphine with high specificity so that the concentrations required to displace 50% ^{3}H-dihydromorphine binding were 10 picomole/ml for morphine, 1.2×10^4 picomole/ml for morphine 3-glucuronide and 2.5×10^3 picomole/ml for N-desmethyl morphine. The mean assay sensitivity is 1.3 ng/ml for serum morphine and 4.2 ng/gm for brain morphine. Naloxone neither bound to the antiserum nor changed the shape of the standard curve when the latter was prepared from brains of naloxone-treated mice. Combing the double extraction procedure with radioimmunoassay, therefore, provides a highly sensitive and specific method for the analysis of tissue morphine.

The disposition studies reveal that at peak morphine levels, the amount of morphine in the mouse brain is only 0.09% and 0.04% of the total administered intravenous and intraperitoneal dose respectively. A half-life of 36 minutes was obtained in naive mice after one single

injection. After implantation of a morphine pellet for 3 days, the decay of brain morphine after pellet removal is 2.5 times longer.

There is good correlation between serum and brain morphine concentrations and the dose of morphine administered. Good correlation also exists between brain morphine levels and the analgesic effect of morphine either in terms of degree of analgesia or in terms of time course of analgesia. However, the absolute brain morphine levels required to produce analgesia among different strains of mice vary considerably.

Naloxone displaces brain morphine in morphine-dependent animals. In mice rendered morphine tolerant/dependent by implantation with one morphine pellet for 3 days, a brain morphine level of 65 ng/gm was obtained at 4 hours after the pellet was removed. When naloxone HCl (10 mg/Kg, i.p.) was administered, a reduction of more than 80% of brain morphine occurred in 5 minutes and lasted for 30 minutes. Full recovery from the effect of this dose of naloxone occurs in 60 minutes. The effect of naloxone is dose-dependent. For a given dose of naloxone, the reduction of brain morphine is directly related to the animal's degree of physical dependence. With increasing physical dependence, there is a parallel increase in the efficacy of naloxone to displace brain morphine and to precipitate withdrawal jumping, suggesting that as the degree of tolerance and physical dependence increases, the opiate receptor decreases its sensitivity to morphine (tolerance) and this is accompanied by an increased sensitivity to naloxone to displace brain morphine and to precipitate withdrawal (physical dependence). Our findings provide strong evidence compatible with the hypothesis that antagonists displace morphine from its receptor sites and thereby

precipitate the withdrawal syndrome. In post-addict mice withdrawn from morphine for a period of 2 to 30 days, naloxone also reduces brain morphine levels at 2 hours after priming the mice with a dose of morphine sulfate (30 mg/Kg, i. p.). On the other hand, naloxone fails to change brain morphine levels in post-placebo mice primed with morphine. Since the post-addict mice were originally unresponsive to naloxone alone, the results indicate that the primary requirement for naloxone to precipitate withdrawal syndrome is the presence of morphine in the brain which is displaceable.

The disposition of morphine in discrete brain regions was studied in rats. The decline of tissue morphine after morphine pellet removal is significantly slower in the hypothalamus and thalamus than in other brain regions. In morphine-dependent rats, the most extensive reduction of tissue morphine by naloxone also occurs in these two brain areas. The results suggest that the hypothalamus and thalamus are important sites for studying the opiate receptors.

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INTRODUCTION

The diverse pharmacological property of narcotic analgesics has long been a fascinating and challenging area of interest to many investigators. However, despite extensive research in this field, the mechanism by which these drugs elicit their effects remains unknown. Nonetheless, it is generally believed that narcotic analgesics elicit their pharmacological effects in the central nervous system by interacting with specific receptor sites as evidenced by the studies on structure-activity relationship (Beckett and Casy, 1954), potency differences in enantiomers (Portoghese, 1965, 1966), and specific antagonism by structurally related antagonists.

Among several approaches aimed at elucidating the site and mechanism of action of narcotic analgesics, efforts have been made to correlate the biological disposition of these drugs in the central nervous system with their pharmacological effects. Extensive review on general accounts of the disposition and metabolism of narcotic analgesics were given by Way and Adler (1960, 1962). More recent data pertinent to distribuion within the central nervous system was summarized by Mulé (1971). From the references listed in these review articles, it is obvious that relatively large differences exist with respect to the ability to concentrate the drug in various organs and tissues. This is particularly true in the central nervous system; for example, in rats, peak morphine levels in brain were reported to occur from 14 minutes to 4 hours after the injection of doses ranging from 2 to 780 mg/Kg (Woods, 1954; Miller and Elliott, 1955; Szerb and McCurdy, 1956; Adler _et al._, 1957; Johannesson and Milthers, 1963; Johannesson and Woods, 1964). Part of the variation may be due to age, sex and strain of the animal as well as dose and route of

drug administration. However, some of the difficulty was related to specificity, sensitivity and reliability of the method utilized to quantitate morphine (for review on methodology, see Way and Adler, 1962; Taylor, 1971; Rubin, 1973). The relatively short biological half-life of morphine and the fact that the drug is used in small doses usually results in tissue levels beneath the sensitivity of most analytical methods. Consequently, the early investigators were forced to employ high doses of morphine and/or sampling at short post-injection intervals to bring morphine concentrations within the sensitivity range for analysis. As a result, early attempts to correlate brain levels of morphine with pharmacological action did not always yield data that were in complete accord. Thus, good correlation between analgetic activity and concentration of morphine in rat brain was observed by Miller and Elliott (1955) but not by Johannesson and Schou (1963). In guinea pigs, no relationship between the duration of morphine action and its brain levels was found to exist (Mulé *et al.*, 1968). However, as the methodology for both pain assessment and morphine analysis improved, it became clear that the antinociceptive effect of morphine correlates well with the concentrations of the drug in the central nervous system of mice (Paalzow and Paalzow, 1971; Patrick *et al.*, 1975) and rats (Hipps *et al.*, 1976), and indeed, the increased susceptibility of the neonatal rat to morphine over that of the adult animal was shown to be dependent upon the increased brain permeability to morphine in the young animal (Way, 1967).

While there appears to be no doubt that morphine exerts a concentration-related effect on the central nervous system, distribution studies revealed little information with respect to the site and mech-

anism of the action. No selective localization of labeled opiates was found in many regions of the central nervous system (Mulé and Woods, 1965), although the gray matter seemed to contain a higher concentration of morphine than white matter. It is interesting to note that the overt response to morphine in the cat is stimulation in contrast to depression in other species such as dogs, guinea pigs and rats. A comparison of distribution of morphine in the central nervous system of the cat with that of the dog did not reveal any clue which could account for the difference in the response to the drug between the two species (Chernov and Woods, 1965). Development of tolerance and physical dependence is a well known consequence of frequent, repeated administration of morphine and various natural and synthetic opiate analgesics. Attempts to correlate any alteration in the disposition of morphine with the development of tolerance and physical dependence have been unsuccessful. No difference was found in the brain concentration of morphine between tolerant and nontolerant monkeys (Mellett and Woods, 1956), rabbits (Siminoff and Saunders, 1958), guinea pigs (Mulé *et al.*, 1968), and rats (Szerb and and McCurdy, 1956; Johannesson and Woods, 1964). In tolerant dogs, it was observed that concentrations of ^{14}C-morphine in the central nervous system were significantly lower than that of nontolerant dogs at each time interval after a single injection of morphine. However, the difference did not appear to be of sufficient magnitude to account for tolerance development (Mulé and Woods, 1962).

While *in vivo* disposition studies did not succeed in providing significant information regarding the site and mechanism of morphine action, the stereotaxic placement technique and the *in vitro* binding assay makes it possible to delineate to some extent the anatomic localization of the

receptor. With the stereotaxic technique, the responsiveness of various brain areas toward agonists or antagonists were determined after the microinjection of the drug by means of permanently implanted cannulas at these localized areas. The data has indicated that the main site of antinociceptive effect of morphine resides in the ventricular gray matter of the following anatomic regions : 1) the third ventricle at a distance of 1-2 mm from the ventricular space in rabbits (Tsou and Jang, 1964) ; 2) the rostral hypothalamus in rats (Foster et al., 1967); 3) surrounding the aqueduct and structures on the floor of the fourth ventricle including medial part of the hypothalamus, subthalamus, and mesencephalon in rabbits (Herz et al., 1970, Albus, et al., 1970); 4) periaqueductal in the rhesus monkey (Pert and Yaksh, 1974), and 5) medial thalamic area (Wei et al., 1973). On the other hand, Buxbaum et al. (1970) demonstrated that dose-response relationship for analgesia could be obtained in rats if microinjections of morphine were made into anterior thalamic nuclei. Although the method has the advantage of avoiding the blood-brain barrier and permits precise quantities of the drug to be delivered into the intended site, it has been found that depending on the site and dose, intracerebral morphine may produce pharmacological effects significantly different from those by systemic injections. Thus, in rats, 10 ug of morphine in the posterior hypothalamus resulted in significant analgesia while the same dose of morphine injected into the medial septum, the caudate, or the periaqueductal gray matter yielded hyperalgesia (Jacquet and Lajtha, 1973, 1974).

With the same technique, it has been shown that repeated intraventricular injections of morphine produced tolerance and physical dependence in rats (Watanabe, 1971), rabbits (Herz and Teschmacher, 1973),

and monkeys (Eidelberg and Barstow, 1971). In morphine-dependent animals, intraventricular injection of narcotic antagonist precipitates withdrawal signs comparable to that produced by systemic injection. The fourth ventricle was found to be a sensitive site following injection of the antagonist to elicit a severe withdrawal syndrome in morphine-dependent rabbits (Herz et al., 1972). Using a stereotaxic approach, Wei et al. (1972, 1973) introduced naloxone crystals into various parts of the brain in morphine-dependent rats through chronically placed cannulae and found withdrawal signs were most frequently observed when naloxone was placed in the medial thalamus and medial areas of the diencephalic-mesencephalic junctions.

For the in vitro binding assay, the background dates back to 1953 when Sung and Way, using d- and l-methadone, applied the principles of stereospecificity in an attempt to identify opiate receptors. However, after intraperitoneal injection, no selective distribution of either one of the stereoisomeric pairs was found in nontolerant or tolerant rats. Ingoglia and Dole (1970) also studied the localization of ^{14}C-labeled d- and l-methadone after intraventricular injection into rat brain. Higher radioactivity was found in the ipsilateral lateral ventricle and the hypothalamus but there was no significant difference in the accumulation of the two isomers in the hypothalamus or in other brain regions they studied. The failure of these two studies might be attributed to the low specific activity of the radiolabeled isomers and the lack of sensitivity of the analytic procedure in detecting each isomer in specific areas.

Following the suggestion of Goldstein et al. (1971) that stereospecific binding can be measured as the difference between the binding of radiolabeled opiate (levorphanol) in the presence of excess, unlabeled active

(levorphanol) and inactive (dextrophan) enantiomers, three groups of investigators have independently described stereospecific binding of opiate agonists and antagonists to brain tissue *in vitro* (Simon *et al.*, 1973; Terenius, 1973; Pert and Snyder, 1973a). The chemical and pharmacological characteristics of opiate binding to brain receptors have been summarized by Goldstein (1974). While Goldstein *et al* (1971) could not demonstrate any difference in the capacity for specific ^{14}C-levorphanol binding among major mouse brain regions studied, Pert and Snyder (1973a) on the other hand, reported that specific ^{3}H-naloxone binding in mouse brain homogenate was high in striatum and low in midbrain, cortex and brain stem. Further study with monkey and human brains indicated that specific ^{3}H-dihydromorphine binding was highest in the limbic system, thalamus and hypothalamus, followed by the extrapyramidal, midbrain and cerebral cortical white areas, and no detectable specific binding was found in the cerebellum, lower brain stem or thoracic spinal cord (Kuhar *et al.*, 1973). Similar findings were noted by Hiller *et al.* (1973) in human brains, but there were some differences. Specific binding of ^{3}H-etorphine to human brain structures was found to be the highest in the limbic system, moderate in the caudate nucleus, putamen, hypothalamus and periaqueductal gray matter, low in the hippocampus, globus pallidus, colliculi, substantia nigra, area postrema and cerebellar cortex, and very low in the cerebral white matter, dentate nucleus of cerebellum, pineal gland and pituitary gland. Whether the observed variation is due to the use of different radiolabeled drugs or to true differences in the anatomical localization of the opiate receptors in the brain of mouse, monkey and human remains to be determined.

In the field of narcotic research, narcotic antagonists play an important role. In addition to their therapeutic and diagnostic uses, they provide powerful tools for studying the mechanism and site of action of morphine, for assisting in the understanding of the fundamental processes involved in tolerance and physical dependence, and for delineating the chemical structure of the narcotic receptor. The history of the development of narcotic antagonists, their diverse chemical structures, and their pharmacology has been the subject of several reviews (Woods, 1956; Wikler, 1958; Foldes, et al., 1964a; Martin, 1967; Braude et al., 1973; Kosterlitz et al., 1973).

In vivo, the effect of narcotic antagonist on the disposition of morphine has been studied in dogs. In nontolerant dogs (Mulé et al., 1962) morphine levels in the central nervous system were either increased or unchanged by the administration of nalorphine. In the tolerant dog (Mulé, 1965), a different pattern emerged. A statistically significant decrease (42-56%) in the central nervous system concentration of morphine occurred after nalorphine administration at 65 minutes. Nalorphine also caused a reduction of 5-73% in morphine levels of the heart, lung, liver, and kidney in these dogs. Thus, it is obvious that the effect obtained with nalorphine in the tolerant dog was quite different from that in the nontolerant dog. It was also found that morphine given concomitantly with nalorphine increased the brain concentration of nalorphine (Johannesson, 1963).

Despite the relative lack of in vivo evidence that displacement of morphine by its antagonist occurs at the specific receptor sites, the hypothesis that morphine and its antagonist interact competitively at a common receptor site was inferred from the in vivo study of dose-

antagonism relationship (Cox and Weinstock, 1964; Grumbach and Chernov, 1965; Blane *et al.*, 1967) and *in vitro* experiments (Kosterlitz and Watt, 1968). Furthermore, in morphine tolerant/dependent mice, the competitive interaction between agonist and antagonist at a common receptor site was suggested from the report by Tuluney and Takemori (1974a, b) who calculated PA_2 values and affinity constants for naloxone antagonism of morphine analgesia. In the same study, it was found that the efficacy of the antagonistic effect of naloxone was increased by narcotic pretreatment. Thus, the hypothesis was proposed that with acute or continuous morphine administration, and the ensuing tolerance/physical dependence development, a conformational change of the opiate receptor occurs which renders it increasingly sensitive to naloxone. Indeed, Way *et al.* (1969) have discovered that with increasing physical dependence, less naloxone was required to precipitate withdrawal signs.

The primary goal of this study was to investigate the disposition of morphine in the central nervous system of both naive and morphine-tolerant/dependent mice and to study the effect of naloxone on the central nervous system disposition of morphine. Since the *in vitro* receptor binding studies have indicated that the amount of opiate bound to the specific receptor sites is very small compared to the total amount present in the brain (Simon *et al.*, 1973). Furthermore, the percentage of the specific binding has been shown to be dependent on the concentration of the labeled agonist or antagonist used in the study, i.e., low concentration tends to reduce the nonspecific, nonsaturable binding and to affect the high affinity specific binding to a lesser extent (Lee *et al.*, 1973). With low concentration ($4x10^{-9}$ M), Pert and Snyder (1973a) were able to demonstrate that more than 70% of ^{3}H-naloxone binding is saturable,

stereospecific and can be displaced by other opiate agonists or antagonists. Therefore, for the *in vivo* study, in order to define a receptor-associated disposition, it is important to investigate the naloxone-morphine interaction at times when the ratio of specific to nonspecific binding of morphine in the brain is at its maximum, i.e., when brain morphine level is low. To achieve this, the main requirement is a sensitive method for morphine analysis. The recent development of a radioimmunoassay for morphine analysis (Spector and Parker, 1970) appears to overcome to a significant extent the problem of assay sensitivity. The specificity of the assay has been improved (Catlin *et al.*, 1976). In the present study, tissue morphine was extracted (Kupferberg *et al.*, 1964) and analyzed by radioimmunoassay with the hope that the extreme sensitivity of the method could facilitate the detection *in vivo* of any change in the selective brain disposition of the drug. In addition, the interaction between morphine and naloxone was extensively investigated with the rationale that it might not only provide the information of the effect of narcotic antagonist on the brain disposition of morphine but also assist in localizing the specific opiate binding sites in the animal's brain. On the assumption that narcotic antagonist competes effectively for the specific but not for the nonspecific binding components in the brain, naloxone would reduce brain morphine concentration as a consequence of displacing morphine from the specific binding sites.

METHODS

Animal. Male ICR white mice (Simonsen Laboratories, Gilroy, California), weighing 22 to 28 gm were used in most of the experiments. Variations in the analgesic effect of morphine on strains of mice were studied with three additional strains of similar sex and weight ; Swiss-Webster also from Simonsen, brown C3H and black C57 mice (Jackson Laboratories, Bar Harbor, Maine).

The animals were allowed a period of two to three days of acclimation before use. Mice were housed six per cage and kept under automatically controlled temperature (72-74 ^{o}F), humidity (45-55%), and lighting (on at 7 am and off at 7 pm). They were given free access to tap water and Berkeley Maintence Diet (Feedstuffs Processing Company, San Francisco, California).

Additional experiments were performed using Sprague-Dawley male rats weighing 150 to 200 gm (Simonsen, Gilroy, California) under the same environmental conditions as mice. Rats were housed two per cage and given tap water and the same diet. In all experiments, animals were used only once.

Analgetic test. The analgetic test used was a modification of the tail-flick procedure described by D'Amour and Smith (1941). Radiant heat from a high intensity lamp was focussed onto the mouse's tail blackened with marking ink. The mean response time from the initial application of heat to the tail-flick endpoint was 1.2 ± 0.1 seconds. The antinociceptive effect of morphine was quantitated as previously described (Way, Loh and Shen, 1969) by using prolongation of the tail-flick response time as a quantal response to varying doses of morphine. A reaction time of 5 or more seconds after the administration of morphine sulfate was considered to be a positive analgetic response. The percentage of the

animals showing positive analgetic response was plotted against the log dose of morphine sulfate on log-probability graph paper and the median analgetic dose (AD50) and its 95% confidence limits were determined by the method of Litchfield and Wilcoxon (1949).

The tail-flick response, an easily recognized endpoint, allowed for rapid assessment of morphine antinociception in comparison to the other methods. The utility of the tail-flick test has been demonstrated in the past. It has yielded data on narcotic potency and addiction liability that correlate well with that observed in the clinic for most of the narcotic analgesics. Although the tail-flick assay has been reported to be based on a nociceptive spinal reflex, studies of the tail-flick response have disclosed that it is modulated by supraspinal influences. It was noted that although morphine inhibited the tail-flick response in spinal animals, the effect produced in spinal and intact animals was quantitatively different. Morphine lost about 70 to 85% of its analgetic potency as a result of spinal sectioning. It was then concluded that morphine augmented supraspinal inhibitory influences on the tail-flick response which were then removed upon sectioning of the cord (Irwin *et al.*, 1951; Dewey *et al.*, 1969). Further evidence of a central inhibitory influence of morphine on spinal sensory transmission has been reported by many investigators (Fujita *et al.*, 1954; Hagbarth and Kerr, 1954; Takagi *et al.*, 1955; Urabe *et al.*, 1965; Satoh and Takagi, 1971). Thus it appears that tail-flick response is not solely mediated by a simple reflex, but is also modulated by supraspinal mechanisms.

Induction and assessment of physical dependence. Physical dependence on morphine was produced and assessed according to the methods described by Way *et al.* (1969). Animals were rendered physically dependent

on morphine by implantation with one specially formulated compressed tablet containing 75 mg of the free base of morphine. The tablet was implanted in the subcutaneous tissue in the small region of the back for varying fixed intervals. The control animals were implanted with a dummy tablet (placebo) containing the tablet fillers without morphine.

After implantation for certain fixed time periods, the pellet was removed and after four hours, abstinence was precipitated with an intraperitoneal injection of the narcotic antagonist, naloxone HCl (Endo Laboratories) dissolved in physiological saline. The doses of naloxone given are in terms of the salt. The number of mice jumping off a laboratory stool after a given dose of naloxone was determined over a 15 minute period.

Extraction of brain morphine. Animals were sacrificed by decapitation, brains were removed, wiped of adhering blood with soft tissue paper, rinsed in ice-cold saline, rewiped and weighed.

Brain morphine was extracted by the method of Kupferberg *et al.*. (1964). Brain tissue was homogenized with a polytron in 2 ml of saturated sodium bicarbonate and 10 ml of 10% n-butanol in chloroform. The mixture was centrifuged for 10 minutes at 28,000 x g and the top aqueous phase removed by aspiration. After pushing aside the tissue flap, a 8 ml aliquot was pipetted into a clean 15 ml polyethylene stoppered-centrifuge tube containing 1.2 ml of 0.01 N H_2SO_4. The mixture was shaken for 30 seconds and centrifuged at 2,500 rpm for 10 minutes, after which, 0.1 ml of the upper acid phase was transferred to a 10x75 mm reagent tube for radioimmunoassay.

Radioimmunoassay for morphine.

A. Components of the assay system.

Tritiated morphine : [6-^{3}H (N)]morphine with a specific activity of 12.5-20 Ci/mmole was purchased from New England Nuclear and diluted to 0.25-0.4 µCi/ml in de-ionized water for each assay.

Tritiated dihydromorphine : Occasionally, [7.8-^{3}H (N)] dihydromorphine which was also purchased from New England Nuclear, with a specific activity of 46-51.9 Ci/mmole was employed as radiolabeled ligand. It was diluted to 4.6-5.2 µCi/ml in de-ionized water for each assay.

Phosphate buffer saline solution : The stock solution with pH between 7.2 and 7.4 and ionic strength 0.202 was prepared with 0.004 M NaH_2PO_4 (0.55 gm of $NaH_2PO_4.H_2O$), 0.016 M K_2HPO_4 (2.79 gm) and 0.15 M NaCl (8.77 gm) in 1000 ml of de-ionized water. The working solution with ionic strength of 0.168 was freshly prepared by diluting the stock solution 5 to 6 in de-ionized water.

Normal rabbit serum was purchased from Grand Island Biological Company and stored at below 4 ^{o}C.

Antiserum used throughout the investigation was prepared in the rabbit and was a gift from Dr. Don H. Catlin. An 1:10 dilution in phosphate buffer saline solution was prepared and stored at -20 ^{o}C. Further dilutions were made freshly in normal rabbit serum diluted 1 to 10 with phosphate buffer saline solution.

A saturated solution of ammonium sulfate was prepared in de-ionized water at 4 ^{o}C and stored at the same temperature. The solution was filtered with #1 filter paper immediately before use.

Scintillation counting fluid : To 2000 ml of toluene (spectranalyzed grade, Fisher Scientific Company), 12 gm of PPO (2,5-diphenyloxazole) and 150 mg of POPOP (1,4-bis-2-[5-phenyloxazolyl]-benzene) were added.

Both PPO and POPOP were purchased from Sigma Chemical Company.

NCS tissue solubilizer : The surface-active organic base was purchased from Amersham/Searle Coporation.

Morphine standards : For the master standard solution of morphine sulfate (USP, Mallinckrodt), a 2 µmole/ml stock solution in 0.01 N HCl was prepared and stored at 4 °C. For each analysis of the unknown samples, a standard curve was prepared in the same batch of animal and assayed along with the tissue samples. To quantitate morphine in serum, the stock solution was serially diluted in pooled serum obtained from mice to give 10, 50, 100 and 500 picomoles of morphine per ml.

Internal standards for brain morphine assay were prepared by making serial dilutions of the master stock solution with phosphate buffer saline. Twenty ul of each dilution was then added to the n-butanol/chloroform brain homogenate. The internal standards were extracted and assayed along with the tissue samples of morphine-treated animals.

Direct standards for brian morphine assay were prepared by serially diluting the master stock solution in the acid extract obtained after double extraction of brain homogenate.

To study the disposition of morphine in the naive animal, morphine standards were prepared with naive animals. For the study of morphine disposition in morphine-dependent animals, morphine standards were prepared with animals which had placebo pellet implanted and removed for the same period of time as the corresponding morphine pellet-treated animals.

B. Assay procedure.

The assay procedure was a minor modification of the method described by Catlin *et al.* (1973). Four-tenths of a ml of the diluted antiserum which had an optimal titer for the assay was pipetted into 10x75 mm reagent tube

(for antiserum dilution, see p. 13). Each reagent tube contained 0.1 ml of ^{3}H-morphine (0.025-0.04 μCi, 2 picomoles) or 0.02 ml of ^{3}H-dihydromorphine (0.092-0.104 μCi) and 0.1 ml of specimen (serum or brain extract) containing either varying concentrations of the morphine standard or an unknown quantity of morphine to be determined.

After vortexing and allowing to incubate for two hours at room temperature (22 ± 2 ^{o}C), the sample was thoroughly mixed with 0.6 ml of saturated ammonium sulfate solution. After 30 minutes of incubation at 4 ^{o}C to precipitate serum globulin, the tubes were centrifuged at 4000 x g for 15 minutes in a Sorval RC-3 refrigerated centrifuge. The supernatant was aspirated and the precipitate was washed twice with 0.6 ml of a solution containing equal volume of saturated ammonium sulfate and phosphate buffer saline. The washed precipitate was then dissolved in 0.8 ml of NCS tissue solubilizer at room temperature, allowing 1 hour to completely dissolve the precipitate.

The content of the tube was then poured into a liquid scintillation vial containing 9 ml of toluene scintillation fluid. The tube was rinsed once with 1 ml of toluene scintillation fluid and the rinse was combined with the original sample. The radioactivity was then counted in a Parkard Tri-Cab liquid scintillation spectrometer.

The optimal concentration of the antiserum for assay was determined by measuring the ability of antiserum at various dilutions to bind diluted ^{3}H-morphine or ^{3}H-dihydromorphine in the absence of unlabeled morphine. Usually, the dilution chosen was that which gave a ratio of bound/free radiolabeled antigen of 1:1, i.e., the dilution required to bind 50% of the radiolabeled antigen in the absence of unlabeled antigen (Berson and Yalow, 1964). For analysis, 0.1 ml of ^{3}H-morphine or 0.02 ml of ^{3}H-

dihydromorphine and 0.1 ml of blank specimen was added to 0.4 ml of diluted antiserum.

C. <u>Logit transformation of morphine concentration-binding curve.</u>

Abbreviations;

T*: total radiolabeled ligand (^{3}H-morphine or ^{3}H-dihydromorphine) added to each tube, dpm/tube.

M : amount of unlabeled morphine present in each tube.

Ms: amount of morphine standard in each tube.

Mu: amount of unknown morphine in each tube.

Bo*: amount of labeled ligand bound to antibody in the absence of M, after correction for nonspecific binding, dpm/tube.

B*: amount of labeled ligand bound to antibody in the presence of M, after correction for nonspecific binding, dpm/tube.

F*: labeled ligand not bound to antibody, i.e., amount of free labeled ligand, dpm/tube.

N*: nonspecific or background binding of labeled ligand, i.e., amount of labeled ligand bound to serum proteins in the absence of antibody, dpm/tube.

Y : B*/Bo* x 100

X : log (M)

The logit transformation for a percentage over the range of 0 to 100% was defined by the expression: $\log_e [Y/(1-Y)]$ (Finney, 1963).

With a limited amount of antibody, an increase in M is inversely related to B*/F* according to the law of mass action. To describe this relationship graphically, a number of different transformation of the parameters have been employed. Figure 1 illustrates four such transformations of the data presented in Table 1 obtained from the first determination. The form

of concentration-binding curve given by B*/F* versus M, a natural extension of the Scatchard plot, yields a segment except over a very narrow range (Figure 1-a). Although the B*/F* ratio arises in the derivation of the Scatchard plot, it is often more convenient to plot B*/Bo* as the response variable versus M (Figure 1-b). This standardizes the scale (from 0 to 100%) and obviates the need to calculate B*/F*. The plot of B*/Bo* versus X (log M) results in a sigmoidal curve, which can be approximated by a linear segment for at least a tenfold range of concentrations, thereby, facilitating concentration interpolation (Figure 1-c). However, this form of the concentration-binding curve is still distinctly nonlinear. When the logit transformation is applied to the response variable Y (B*/Bo*) (Figure 1-d), linearization of the sigmoidal curve just described is achieved at least for Y from 10 to 90% (logit = -2.2 to +2.2) (Finney, 1963) with a relationship between Y and X as logit Y = a + bX. This permits accurate estimation of the slope and intercept either by means of linear regression analysis or graphically.

D. Calculation and statistical quality control.

The daily operation for calculation includes the following :

1. Correction of counting efficiency for each tube, i.e., transfer of cpm in each tube into dpm.
2. Calculation of B*/Bo* for each standard or unknown.
3. The corresponding logit value for each B*/Bo* was determined and recorded.
4. With logit Y and log Ms as response variables, the programmed unweighted linear regression analysis was performed on a Tektronix 21 calculator.

5. From the equation, the linear standard curve was drawn on graph paper (No. 217-L; TEAM, Tamworth, N.H.) which has both logit and per cent scales on the vertical axis and $\log_{10}$ scales on the horizontal axis.
6. The quantity of morphine in each unknown tube was estimated from the standard curve.

For statistical quality control, several variables describing the assay such as Bo*/T*, N*/T*, slope of concentration-binding curve and variability of the assay were arbitarily selected and recorded for each assay. Data of tissue morphine levels were analyzed by t-test and the criteria for significance was $p < 0.05$.

RESULTS

Radioimmunoassay for morphine.

A. Screening of antisera for titer.

Figure 2 illustrates the profiles of titration curves for morphine antisera produced by two laboratories (Catlin's and Roche's).

In the presence of a negligible amount of radiolabeled antigen, the sensitivity of radioimmunoassay is greater with higher dilutions of the antiserum. However, a wider range of unlabeled antigen concentration is covered when a more concentrated antiserum is used (Potts et al. 1967). Furthermore, since each antiserum contains multiple or heterogenous antibodies reacting with various equilibrium constants, the potential sensitivity of the assay will depend on those antibodies that react with the highest energy and that are present in a suitable concentration to be utilizable (Berson and Yalow, 1964). However, the use of a too highly concentrated antiserum will decrease the precision if highly concentrated antibodies with low affinity begin to bind a significant fraction of radiolabeled antigen (Yalow and Berson, 1970).

For morphine assay, we used the titer which gave a Bo^*/F^* ratio between 1.0 and 0.75 so that a decrease of 50% in the initial Bo^*/F^* value resulted in a decrease of Bo^*/T^* from 50% to 33% when initial $Bo^*/F^* = 1.0$, and from 43% to 27% when initial $Bo^*/F^* = 0.75$. The optimal final concentration in each tube for the assay, therefore, would be between 1:400 and 1:500 with antisera produced by Dr. Catlin's Laboratory, and between 1:1400 and 1:1500 with antisera produced by the Roche Laboratory. The antisera produced by Dr. Catlin's Laboratory was used throughout the investigation.

B. Concentration-binding curve for morphine standard.

The curve in Figure 3 represents the average of 13 direct assay

for known quantity of morphine added to the acid extract obtained from double extraction of mouse brains. ^{3}H-morphine and rabbit serum #77-8/29/73 (Catlin's) were employed as radiolabeled ligand and antiserum respectively. Figure 4 represents the concentration-binding curve for internal morphine standards added to the n-butanol/chloroform mouse brain homogenate. After correcting for the aliquot loss (8/10 x 1/1.2), the recovery was calculated to be 101 $\pm$ 3.13%.

C. Assessment of the reliability of the assay.

1. Precision: Precision is defined as the extent to which a given set of measurements of the same sample agrees with the mean, i.e., the amount of variation in the estimation of unlabeled antigen (Midgley et al., 1969). Precision can be estimated by calculating the index of precision (λ) (Armitage, 1971) which is defined as the ratio between the standard deviation (SD) of logit Y for each Y value and the slope (b) of the regression line between each value of logit Y and the corresponding X. A summary of the index of precision for 13 direct morphine assays is listed in Table 2.

2. Sensitivity: Sensitivity of the assay was defined as the smallest quantity of unlabeled antigen which gives a response significantly different from zero-concentration level, i.e., the amount of unlabeled antigen in the tube with which the value of Y is significantly (95% confidence limits) different from 100. According to this definition, the calculated lower limit of B*/Bo* with corresponding concentration for the 13 direct assay is summarized in Table 3. The estimated sensitivity in terms of ng morphine per gm mouse brain (mean brain weight = 450 mg) is also listed in the same Table.

3. Specificity: Defined as the extent of freedom from interference

by any substance other than the one intended to be measured, specificity of the assay is affected by immunologic cross-reactivity, presence of unwanted radiolabeled substances and differences in the composition of incubation medium. The specificity of morphine antiserum produced by Dr. Catlin's laboratory was tested for compounds which were likely to be present in the tissue specimen in our experiments and the results are summarized in Table 4. Approximately, 10 picomole/ml of morphine was required to displace 50% of ^{3}H-dihydromorphine, while it took 1.2×10^{4} picomole/ml of morphine-3-glucuronide and 2.5×10^{3} picomole/ml of N-desmethyl morphine to effect the same amount of displacement. Naloxone virtually did not bind to the antiserum. Furthermore, to eliminate the possibility that naloxone might interact with constituents of brain tissue, and thereby indirectly interfere with morphine binding to the antiserum, the standard curve prepared with brains of placebo-implanted, naloxone (10 mg/Kg, i. p.)-treated mice was compared to that prepared with placebo-implanted, saline treated mice. No difference was found in the two standard curves (Table 5). In addition, we also tested the possible binding ability of met-enkephalin and found that with concentrations ranging from 4×10^{3} to 2×10^{5} picomole /ml, met-enkephalin (Peninsula Laboratories) did not show any appreciable displacement of ^{3}H-morphine binding to the antiserum.

4. Accuracy: Defined as the extent to which a given measurement of a substance agrees with the exact amount of the substance which is present, accuracy of radioimmunoassay can be evaluated by comparing its results to those obtained with different methods. We compared radioimmunoassay and fluorometric assay by measuring morphine concentration in identical brains of mice which had received morphine sulfate

(30 mg/Kg, i.p.) 2 hours prior to sacrifice. As shown in Table 6, both methods yielded similar results.

5. Reproducibility: The quality control for 13 direct morphine assay is illustrated in Table 2.

Bo*/T*: The Bo*/T* ratio is one of the most important parameters describing the performance of an assay. It may be low for a variety of reasons such as high nonspecific binding, low binding affinity, wrong titer of antibody, incomplete separation of bound from free, or failure to reach equilibrium.

N*/T*: Nonspecific or background binding may be affected by the conditions of incubation, e.g., time, temperature and pH , and by the efficiency of the separation technique for bound and free. A value of N*/T* greater than 5% is indicative of trouble in the assay system.

Slope: The slope of the concentration-binding curve should be constant when the assay is under control.

Within-assay variability and between-assay variability is summarized in Table 7.

Disposition of morphine.

A. Brain levels of morphine in mice after i.v. injection.

When 15 mg/Kg of morphine sulfate was administered to mice through the tail vein, morphine appeared in brain very rapidly, reaching a level of 620 ng/gm in 15 minutes (Figure 5). At the peak level of 730 ng/gm, approximately 0.09% of the total administered morphine was calculated to be present in the brain.

The decay process for morphine in the brain appears to have two components, one with a fast rate with a half-life of 36 minutes and a slower one with a half-life of approximately 90 minutes. By 8 hours

after the injection, more than 97% of morphine in the brain had disappeared.

B. <u>Dose-related changes in brain and serum morphine content.</u>

Figure 6 shows that as the dose of morphine sulfate was increased from 0 to 20 mg/Kg, i.p., there was a linear increase in both serum and brain concentration of morphine when morphine was determined at 30 minutes after the injection. A comparison of morphine levels in serum and brain showed that the relative distribution of morphine between the two was ranging from 1.4 to 1.8. As shown in Table 8 relative uptake of morphine by the brain did not exceed 0.05% of the total morphine administered when brain morphine levels were determined at 30 minutes after the injection.

C. <u>Brain and serum morphine concentration after pellet implantation.</u>

Morphine was rapidly absorbed from the pellet after subcutaneous implantation in mice which received morphine chronically by pellet implantation. As shown in Figure 7, within 30 minutes, a level of 0.13 ug/gm was noted. A maximum brain level of about 1.10 µg/gm was obtained between 6 and 12 hours, but this level was sustained for less than one day. The brain morphine level started to decline after 12 hours, so that at 48 and 72 hours after pellet implantation, morphine content in mouse brain was 64 and 33% respectively of the peak level. At 6 and 7 days after pellet implantation, the level was only 12% of the peak morphine level.

When the morphine pellet was removed, both brain and serum content of morphine declined rapidly. As shown in Figure 8, when the pellet was removed after 72 hours of implantation, the time course of morphine disappearance indicates that the half-life of morphine in the

brain was 1.6 hours and in the serum 3 hours.

D. Regional brain distribution of morphine.

The difference in time course of tissue morphine disposition in various brain regions was investigated in rats. After one pellet implantation for 3 days, morphine concentration in various brain areas did not differ much, the range being 75 ng/gm (neostriatum) to 122 ng/gm (hypothalamus). After pellet removal, however, there was considerable difference in the rate of decline of morphine (Figure 9). Morphine levels of 18.1, 63.5, 99.5, 38.3, 47.0, 47.6 and 40.1 ng/gm were measured in the cortex, thalamus, hypothalamus, neostriatum, midbrain, brain stem and cerebellum respectively in rats with morphine pellet removed, compared to those of 114, 91.2, 122, 75.2, 100, 97.4 and 114 in rats with morphine pellet remaining.

Relationship between brain morphine levels and analgesic activity in mice.

The analgesic activity of morphine was compared with its concentration in the brain. ICR mice were administered varying doses of morphine and analgesia was quantified by the tail-flick test 30 minutes later. Mouse brains were removed for morphine analysis immediately after the analgetic test. As depicted in Figure 10, a linear concentration-effect relationship ($r = 0.996$) was observed for brain morphine levels between 50 and 200 ng/gm, with a level of 145 ng of morphine per gm of tissue corresponding to 50% animals showing inhibition of tail-flick response. Figure 10 also indicates that analgesia was not elicitable in mice with a brain morphine level lower than 37.5 ng/gm. On the other hand, maximal analgesic activity was achieved at a brain morphine level of approximately 250 ng/gm.

The time course of the analgesic effect of morphine was also found to correlate with brain morphine levels. At various time intervals after a fixed dose of morphine was administered to mice, inhibition of the tail-flick response in groups of 8 mice was recorded, and the animal was sacrificed immediately after the analgetic test. As shown in Figure 11, brain morphine levels of 441, 123 and 40 ng/gm were obtained at 1, 2 and 4 hours respectively after the i.v. injection of 15 mg/gm of morphine sulfate. At these time intervals, the positive analgesic response to morphine was found in 100, 37.5 and 0% mice respectively. This concentration-effect relationship was compatible with that noted in the previous experiment.

Although analgesic activity of morphine was quantitatively related to its concentration in the brain, the level of morphine needed to produce analgesia varied among different strains of mice. Table 9 shows that among 4 strains of mice examined, response to the analgesic effect of morphine was found to be most sensitive in C57 mice, followed by C3H, Swiss and ICR as indicated by their AD50's of 1.35, 1.60, 3.00 and 4.30 mg/Kg respectively. However, brain morphine levels determined at 30 minutes after i.p. injection of 4 mg/Kg of morphine sulfate did not reveal any significant difference among the 4 strains of mice. A mean brain morphine level of 78.4, 100, 84 and 94.4 ng/gm was obtained for C57, C3H, Swiss and ICR mice respectively. Thus the difference in the sensitivity to the analgesic effect of morphine among these 4 strains of mice could not be attributed to any difference in the disposition of brain morphine.

<u>Effect of naloxone on the disposition of morphine.</u>

A. <u>Effect of naloxone on brain morphine levels in naive animals.</u>

Effect of naloxone on morphine content in whole brain of naive animals was studied in mice and the resultsare summarized in Table 10. Naloxone HCl (1 mg/Kg, i.p.) given to naive mice at 20 minutes after the the i.p. injection of 1 mg/Kg of morphine sulfate, failed to change brain morphine level when animals were sacrificed 10 minutes after the administration of naloxone. The morphine level was 16.6 ng/gm for naloxone-treated mice as compared to 17.5 ng/gm for saline-treated mice. Similarily, mice receiving 10 mg/Kg of morphine sulfate followed 20 minutes later by either 1 or 10 mg/Kg of naloxone HCl had brain morphine levels of 153 and 184 ng/gm, respectively, at 30 minutes after morphine. Neither of these levels was significantly different from that (178 ng/gm) of mice which received the same dose of morphine followed by saline. However, a test for analgesia in mice prior to sacrifice indicated that antagonism of the analgesic effect of morphine was affected by both doses of naloxone. Analgesia produced by 10 mg/Kg of morphine sulfate was antagonized 50% by 1 mg/Kg of naloxone HCl and was completely blocked by 10 mg/Kg of naloxone HCl.

Since we had found previously that brain morphine level reached its peak at around 30 minutes after the injection, the possibility that the selective change in brain morphine level as might be affected by naloxone, could be masked by excessive amount of morphine in brain tissue needed to be excluded. Therefore, brain morphine was also determined at 60 minutes, during the decline phase of brain morphine. Again, a difference in brain morphine levels between saline and naloxone treated animals was not demonstrable. At this time point, the brain morphine level produced by 6 mg/Kg of morphine sulfate was 83.3 ng/gm in mice treated with saline 10 minutes before sacrifice and 88.0 ng/gm

in mice treated with naloxone HCl (10 mg/Kg, i. p.).

However, in an additional study in discrete brain regions of naive rats, it was possible to demonstrate a reduction in brain morphine level by naloxone. The morphine levels were found to be lower in the hypothalamus and thalamus of rats treated with morphine together with naloxone when compared to those of rats treated with morphine together with saline. As shown in Table 11, 30 minutes after morphine sulfate (15 mg/Kg, i.p.) plus saline, morphine concentrations in rat brain regions varied considerably. On the basis of ng morphine per gm wet tissue, the values were: 175 in cortex, 400 in brain stem and cerebellum, about 550 in midbrain and thalamus and 1189 in the hypothalamus. In rats treated with the same dose of morphine together with naloxone HCl (6 mg/Kg, i. p.), reduction in tissue morphine content was calculated to be 56% in the hypothalamus, and 44% in the thalamus. Other brain areas, namely, cortex, midbrain, brain stem and cerebellum did not show significant differences in tissue contents of morphine with the two treatments.

B. Displacement of brain morphine by naloxone in morphine-dependent animals.

Although naloxone failed to change brain morphine content in naive mice, a rapid and extensive displacement of morphine from brain tissue was affected by naloxone when it was administered to morphine-dependent animals.

1. Time course. As shown in Figure 12, 4 hours after pellet removal, a mean brain morphine level of 65.4 ng/gm was obtained in mice which had a morphine pellet implanted for 3 days prior to removal. When these morphine-dependent mice received saline at this time point, the brain

morphine level was found to be 47.1 ng/gm after 2 hours and further declined to 21.0 ng/gm in 5 hours. However, if naloxone HCl (10 mg/Kg, i.p.) was administered to a similar group of dependent mice, the brain content of morphine was rapidly and dramatically reduced. In 5, 10, 15 and 30 minutes after the injection of naloxone, brain morphine levels were found to be 7.90, 10.5, 9.50 and 8.17 ng/gm, i.e., reduction of more than 80% of brain morphine was affected by this dose of naloxone. Full recovery from the effect of this dose of naloxone occurred within 60 minutes as evident by the return to a morphine level of 44.7 ng/gm, comparable to that of mice which received saline. However, following a second injection of the same dose of naloxone, brain morphine level was again reduced to 8.88 ng/gm.

2. Dose-response relationship. In the determination of brain morphine levels, variability was one of the major problems encountered in animals which became dependent on morphine as a consequence of morphine pellet implantation and subsequent removal. At 3 days after pellet implantaion, the standard deviation of mean morphine level was 20% of the mean. At 4 hours after the pellet was removed, it was 37% of the mean. Since brain morphine levels after pellet implantation and/or removal may be influenced by various factors such as the composition of the pellet, sites of implantation, technique of implantaion and/or removal, in addition to biological variations among individual animals, such variability is not unexpected. The main biologic variables among individuals can be related to differences in the disposition of morphine, the degree of physical dependence development and responsiveness to naloxone. These consideration make it rather difficult to obtain a good correlation between the intensity of the effect

and the dose of naloxone.

Table 12 summarizes brain morphine levels of mice treated with saline or different doses of naloxone. Four hours after the morphine pellet was removed, saline or naloxone was administered to mice. Fifteen minutes later, brain morphine level of 64.4 ng/gm (with a range between 32.8 and 103 ng/gm) was found in saline-treated mice and 24.7 (4.13-65.2), 20.5(6.38-55.8), 19.0(3.10-85.9) and 7.64 (4.17-8.95) ng/gm was found respectively in mice received 0.1, 1.0, 10 and 50 mg/kg of naloxone HCl. Although by non-paired "t" test, the brain morphine level in each of four naloxone-treated groups was significantly different from that of its corresponding saline-treated control, no significant difference was found between two dosage groups.

A better correlation between the effect and doses of naloxone was obtained, if in each group, the per cent animals which had brain morphine levels significantly (95% confidence limits) lower than that of the saline-treated control was chosen as the response variable. As shown in Figure 13, the number of mice which had brain morphine levels significantly lower than that of the saline-treated mice was found in 29, 64, 82 and 100% mice, respectively, with 0.1, 1.0, 10 and 50 mg/Kg dosage groups.

3. Regional distribution of morphine in rat brain. Rats were rendered dependent on morphine by implantation with one morphine pellet for 3 days. Four hours after the pellet was removed, the animals were divided randomly into two groups; one was injected with saline and the other with naloxone HCl (10 mg/Kg, i.p.). The animals were sacrificed 15 minutes later and their brains removed and dissected into 7 regions for morphine analysis. The results are summarized in

Table 13. In saline-treated rats, tissue morphine concentration was found to be highest in the hypothalamus, 99.5 ng/gm, followed by the thalamus, 63.0 ng/gm. Relatively similar concentrations were found in the brain stem, 47.6 ng/gm, midbrain, 47.0 ng/gm, cerebellum, 40.1 ng/gm, and neostriatum, 38.3 ng/gm. The lowest morphine concentration was found in the cerebral cortex, 18.1 ng/gm.

Comparison between morphine-treated groups given naloxone or saline indicated that in the hypothalamus and thalamus, significant reduction of tissue morphine level was affected by naloxone. In naloxone-treated rats, the hypothalamus contained 42.7 and thalamus 24.8 ng/gm of morphine, i.e., tissue morphine content was decreased by 57% in the hypothalamus and 61% in the thalamus by naloxone. On the other hand, in other brain areas studied, namely, the brain stem, midbrain, cerebellum, neostriatum and cortex, no significant difference was observed between naloxone-treated and saline-treated mice.

4. <u>Relationship between degree of physical dependence and the ability of naloxone to displace brain morphine</u>. Mice were randomly divided into 3 groups and implantated with a morphine pellet for 1, 2 or 3 days. Four hours after the pellet removal, each group was further divided into 2 subgroups, and one subgroup received physiological saline and the other naloxone HCl (0.1 mg/Kg, i.p.). Immediately after the injection, the jumping incidence during a 15 minute observation period was recorded and the mice were then sacrificed.

As shown in Figure 14, the degree of physical dependence on morphine was greatly increased with an increasing duration of pellet implantation. The incidence of withdrawal-jumping precipitated by

0.1 mg/Kg of naloxone HCl increased from 13% on day 1 to 75% on day 2. It was further increased when the period of pellet implantation was extended. As indicated by a further increase from 75% on day 2 to 100% incidence of precipitated withdrawal-jumping on day 3.

In each group, comparison of brain morphine levels between the two subgroups revealed that naloxone-treated mice had less morphine in the brain. In the 1-day group, the brain level of morphine was 87.2 ng/gm in the naloxone-treated subgroup, compared to that of 105 ng/gm in the saline-treated subgroup. In the 2-day group, morphine content in the brain was 50.0 ng/gm in the naloxone-treated mice and 78.3 ng/gm in the saline-treated mice ($p<0.05$). In the 3-day group, the concentration of brain morphine in the naloxone-treated mice was 28.4 ng/gm, compared to that of the saline-treated mice, 72.5 ng/gm ($p<0.025$). In other words, in the naloxone-treated subgroups, the brain morphine content was reduced by 17, 36 and 61% respectively in the 1 day, 2 day, and 3 day groups when compared to that of the saline-treated subgroups.

Thus, it appears that the displacement of brain morphine by naloxone in morphine-dependent mice is related directly to the degree of physical dependence of the animal, i.e., the greatest reduction in brain morphine levels is found in the group of mice which also exhibits the highest incidence of precipitated withdrawal-jumping to naloxone.

5. Displacement of brain morphine in post-addict mice. Mice were rendered physically dependent on morphine by pellet implantation for 3 days and the pellet was then removed. A control group of mice was implantated with a placebo pellet. Two, 12, or 30 days after the pellet was removed, the mice were primed with an i.p. injection of morphine sulfate (30 mg/Kg) and then randomly divided into two subgroups. After

120 or 150 minutes, one subgroup received saline and the other naloxone HCl (10 mg/Kg, i.p.). The animals were sacrificed 15 minutes later and their brains removed for morphine assay. The results are summarized in Table 14.

After the priming dose of morphine, an injection of naloxone resulted in a marked fall in brain morphine levels in morphine-dependent mice withdrawn from morphine for 2, 12 or 30 days. In the 2-day morphine withdrawn mice, a brain morphine level of 205 and 169 ng/gm was obtained at 120 and 150 minutes, respectively, after priming with 30 mg/Kg of morphine sulfate followed by saline. However, if the priming dose of morphine was followed by naloxone, the corresponding brain morphine level was 138 and 119 ng/gm, i.e., 67 and 70% that of the saline-treated control. Similarly, in the 12-day morphine withdrawn mice, the brain morphine level after naloxone was 60% that of the saline-treated mice. Furthermore, in mice withdrawn from morphine for a period of 30 days, a marked reduction in brain morphine level was still effected by naloxone after priming with morphine. The brain morphine level in these mice was 58% that of the saline-treated mice. On the other hand, in the placebo withdrawn mice primed with morphine, naloxone failed to lower the brain morphine level significantly; brain morphine level after naloxone or saline treatment was almost identical.

Table 1

RAW DATA OF A RADIOIMMUNOASSAY FOR MORPHINE STANDARD

(B_0^* = 9735 ± 103) RUN IN DUPLICATE

MS (PMOLE/ML)	X (LOG MS)	B^*	F^*	B^*/F^*	B^*/B_0^*	LOGIT(B^*/B_0^*)
5	0.7	8277	28854	0.29	0.85	1.74
		8517	28614	0.30	0.87	1.90
10	1.0	7323	29808	0.25	0.75	1.10
		7404	29727	0.25	0.76	1.15
25	1.4	4509	32622	0.14	0.46	-0.15
		5286	31845	0.17	0.54	0.15
50	1.7	3321	33810	0.10	0.34	-0.66
		2988	34143	0.09	0.31	-0.79
100	2.0	1788	35343	0.05	0.18	-1.52
		2052	35079	0.06	0.21	-1.33
1000	3.0	273	36858	0.01	0.03	-3.54
		339	36792	0.01	0.04	-3.30

Morphine standards were prepared in phosphate buffer saline solution. 3H-dihydromorphine and rabbit serum #77-7/2/73 (Catlin) were used as radiolabeled ligand and morphine antiserum respectively.
Bo*, amount of labeled ligand bound to antiserum in the absence of morphine standard, dpm; Ms, amount of unlabeled morphine standard, pmole/ml; X, log Ms; B*, amount of labeled ligand bound to antiserum in the presence of morphine standard, dpm; F*, labeled ligand not bound to antiserum, dpm.

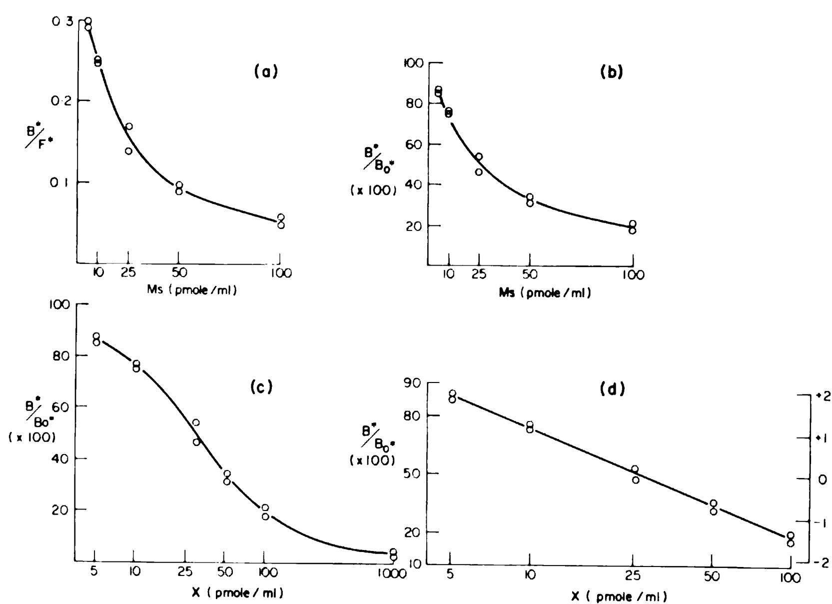

Figure 1. Four graphic methods of analyzing radioimmunoassay data (Table 1). 1-a, B*/F* vs M; 1-b, B*/Bo* x 100 vs M; 1-c, B*/Bo* x 100 vs X; 1-d, logit (B*/BO* x 100) vs X.

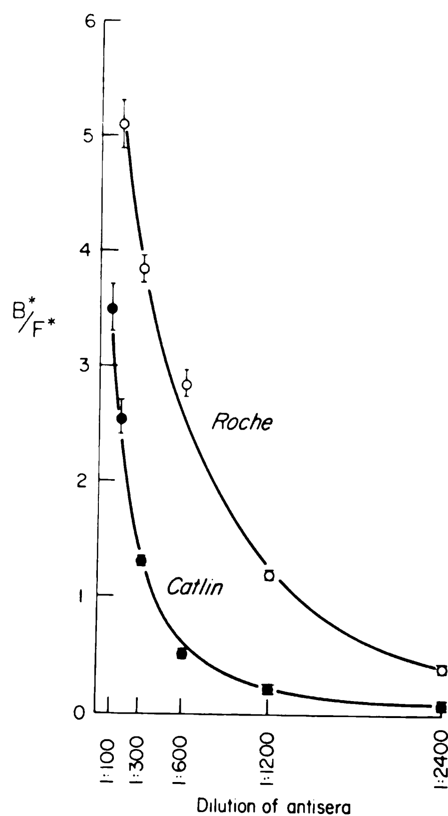

Figure 2. Effect of antiserum dilution on the degree of antibody binding to 3H-morphine. Each tube contained 0.4 ml of serial dilutions of antiserum supplied by Dr. Catlin (●—●), #77-8/29/73 or Roche (O—O), 0.1 ml of 3H-morphine (0.025-0.040 uCi) and 0.1 ml of acid extract of naive mouse brains. Each point represents the mean ± S.E. of 3 measurements.

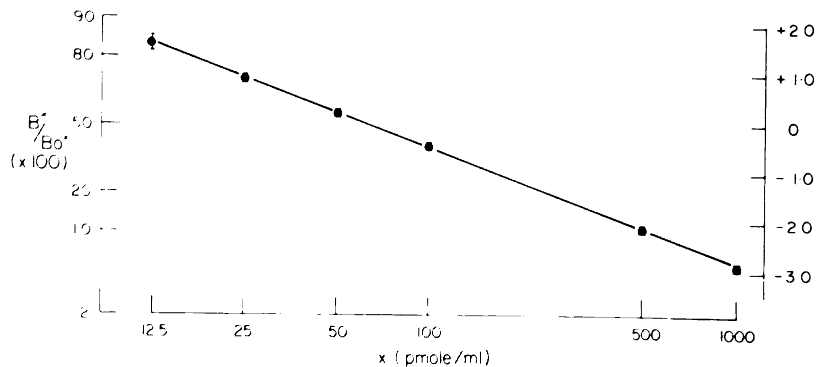

Figure 3. Concentration-binding curve of radioimmunoassay for direct morphine standards. Morphine standards were prepared by serially diluting the master stock solution of morphine sulfate in the acid extract obtained after double extraction of brian homogenate. In each assay, 3H-morphine and rabbit serum #77-8/29/73 (Catlin) were used as radiolabeled ligand and morphine antiserum respectively. Each concentration was run in duplicate in each assay. Each point represents the mean ± S.E. of 13 assays.

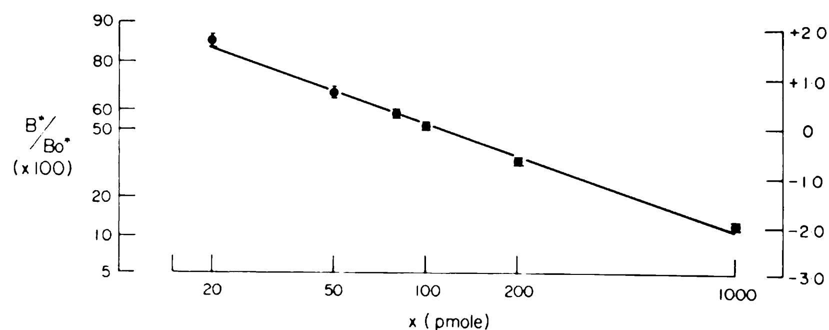

Figure 4. Concentration-binding curve of radioimmunoassay for internal morphine standards. Twenty ul of morphine standards was added to the n-butanol/chloroform mouse brain homogenate. 3H-morphine and rabbit serum #77-8/29/73 were used as radiolabeled ligand and morphine antiserum respectively. Each concentration was run in duplicate in each assay. Each point represents the mean ± S.E. of 7 assays.

Table 2

SUMMARY OF THE SLOPE, CORRELATION COEFFICIENT, INDEX OF PRECISION, BO*/T* AND N*/T* X 100 FOR 13 DIRECT ASSAY

ASSAY NUMBER	SLOPE (b)	CORRELATION COEFFICIENT(r)	INDEX OF PRECISION(λ)	BO*/T*	N*/T* X 100
1	-2.06	-0.996	0.165	0.35	0.49
2	-2.19	-0.999	0.045	0.48	0.90
3	-3.44	-0.983	0.224	0.49	0.80
4	-2.40	-0.998	0.070	0.44	0.88
5	-2.21	-0.996	0.090	0.46	1.07
6	-2.18	-0.995	0.117	0.49	1.32
7	-2.79	-0.991	0.152	0.44	0.64
8	-2.15	-0.995	0.118	0.34	0.75
9	-2.23	-0.997	0.127	0.65	0.89
10	-2.26	-0.998	0.113	0.41	0.64
11	-2.46	-1.000	0.046	0.58	0.69
12	-2.43	-0.998	0.151	0.41	0.65
13	-2.33	-0.999	0.097	0.48	0.80
$\bar{X}$	-2.39	-0.996	0.117	0.46	0.81
S.E.	0.10	0.001	0.014	0.02	0.06

3H-morphine and rabbit serum #77-8/29/73 (Catlin) were used as radiolabeled ligand and morphine antiserum respectively. Morphine standards were prepared in the acid yieled from the double extraction of mouse brains.

Table 3

SUMMARY OF THE SENSITIVITY OF THE METHOD FOR 13 DIRECT ASSAY

ASSAY#	B*/B0 X100	PMOLE/ML	NG/GM BRAIN
1	94	2.7	2.6
2	99	1.0	1.0
3	95	7.8	7.4
4	97	4.0	3.7
5	99	1.0	1.0
6	93	5.0	4.8
7	93	7.5	7.1
8	99	1.0	1.0
9	84	10	9.5
10	82	10	9.5
11	92	5.0	4.8
12	99	1.2	1.1
13	98	1.0	1.0
$\bar{X}$	94	4.4	4.2
S.E.	1.6	0.9	0.9

3H-morphine and rabbit serum #77-8/29/73 were used as radiolabeled ligand and morphine antiserum respectively. Direct morphine standards were prepared in the acid extract obtained after double extraction of the mouse brains.

Table 4

SPECIFICITY OF MORPHINE ANTISERUM #77-7/2/73 WITH RESPONSE VARIABLES TABULATED AS B*/B0* X 100

CONCENTRATION (PMOLE/ML)	MORPHINE	MORPHINE 3-GLUCURONIDE	N-DESMETHYL MORPHINE	NALOXONE
1	90 ± 2.6			
2.5	82 ± 1.5			
10	52 ± 1.0			
25	27 ± 1.4			
50	14 ± 1.0			
100	8 ± 0.5	94 ± 3.9	96 ± 1.7	100 ± 1.0
10^3		80 ± 1.6	68 ± 0.6	94 ± 1.5
10^4		56 ± 0.3	24 ± 1.0	98 + 1.9
5 X 10^4		29 ± 1.0		
10^5		22 ± 0.0	7 ± 0.0	80 ± 2.5
SLOPE	-2.42	-1.59	-1.59	-----
CORRELATION COEFFICIENT	-0.997	-0.935	-0.951	-----
50% INTERCEPT (PMOLE/ML)	10	1.2 X 10^4	2.5 X 10^3	-----

Direct standards of morphine, morphine 3-glucuronide, N-desmethyl morphine or naloxone were added to phosphate buffer saline sloution and 100 ul aliquot was assayed in replicate. 3H-dihydromorphine and rabbit serum #77-7/2/73 (Catlin) were used as radiolabeled ligand and morphine antiserum respectively. The results are the mean ± S.E..

Table 5

CONCENTRATION-BINDING CURVES OF RADIOIMMUNOASSAY FOR INTERNAL MORPHINE STANDARD PREPARED WITH BRAINS OF SALINE-TREATED AND NALOXONE-TREATED MICE

MS (PMOLE)	$B^*/B0^*$ X 100	
	SALINE	NALOXONE
20	89 ± 1.4	88 ± 0.5
100	59 ± 0.0	55 ± 1.0
200	45 ± 0.5	49 ± 0.5
1000	20 ± 0.0	22 ± 0.5

Mice implanted with one placebo pellet for 3 days and then removed for 4 hours were randomly divided into two groups; one challenged with saline and the other naloxone HCl (10 mg/Kg, i.p.). Mice were sacrificed 15 minutes later. The brain was removed and extracted with n-butanol/chloroform. Twenty µl of the master stock of morphine standard was added to the brain homogenate. After the purification with 0.01 N sulfuric acid, 0.1 ml of the acid extract was analyzed by radioimmunoassay. 3H-morphine and rabbit serum #77-7/2/73 were used as radiolabeled ligand and moprhine antiserum respectively. Each concentration was run in duplicate and the result was the mean ± S.E..

Table 6

COMPARISON OF RADIOIMMUNOASSAY AND FLUOROMETRIC ASSAY FOR THE ANALYSIS OF MORPHINE IN THE BRAIN

MOUSE	MORPHINE (NG/GM) FLUOROMETRIC ASSAY	RADIOIMMUNOASSAY
1	226	228
2	234	228
3	201	217
4	151	181
5	272	266
6	292	287
$\bar{X} \pm$ S.D.	229 ± 50.4	235 ± 37.4

Mice challenged with 30 mg/Kg of morphine sulfate (i.p.) were sacrificed 2 hours later. Morphine level in the identical brain was analyzed by both radioimmunoassay and fluorometric assay. For radioimmunoassay, 3H-morphine and rabbit serum #77-7/2/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with internal morphine standards.

Table 7

WITHIN-ASSAY VARIABILITY

CONCENTRATION (PMOLE/ML)	$\bar{X}$	SD	%CV(SD/$\bar{X}$)
12.5	85	1.60	1.88
25	73	1.70	2.33
50	55	2.47	4.49
100	37	1.60	4.20
500	9.5	0.50	5.26
1000	6.3	1.00	15.9

BETWEEN-ASSAY VARIABILITY

CONCENTRATION (PMOLE/ML)	$\bar{X}$	SD	%CV(SD/$\bar{X}$)
12.5	85	3.70	4.30
25	73	4.00	5.70
50	55	5.00	9.05
100	37	4.50	12.2
500	9.5	1.70	18.2
1000	6.3	1.50	24.3

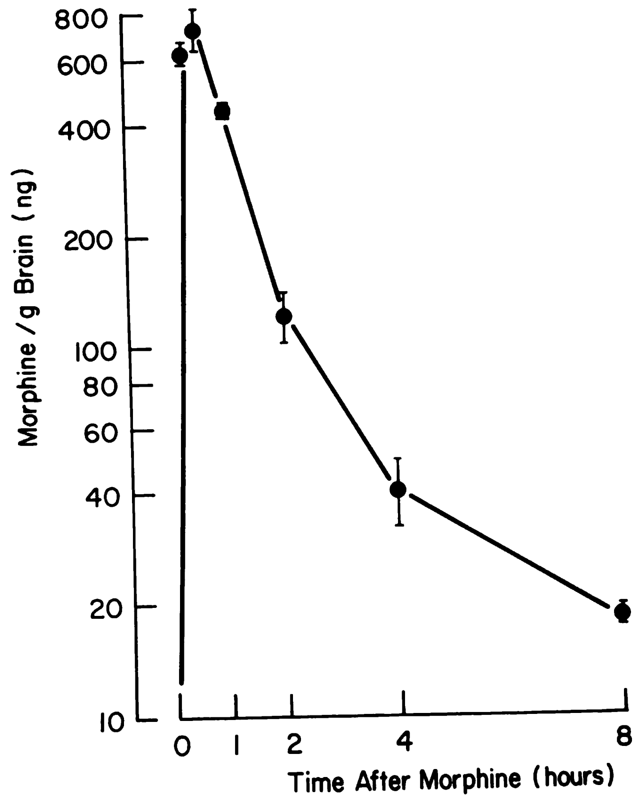

Figure 5. Brain morphine levels in mice at various intervals after one injection of morphine sulfate (15 mg/Kg, i.v.). 3H-dihydromorphine and rabbit serum #77-7/2/73 were used as radiolabeled ligand and morphine antiserum respectively for radioimmunoassay. The standard curve was prepared with internal morphine standards. Each point represents the mean ± S.E. of 5 individual mouse brain.

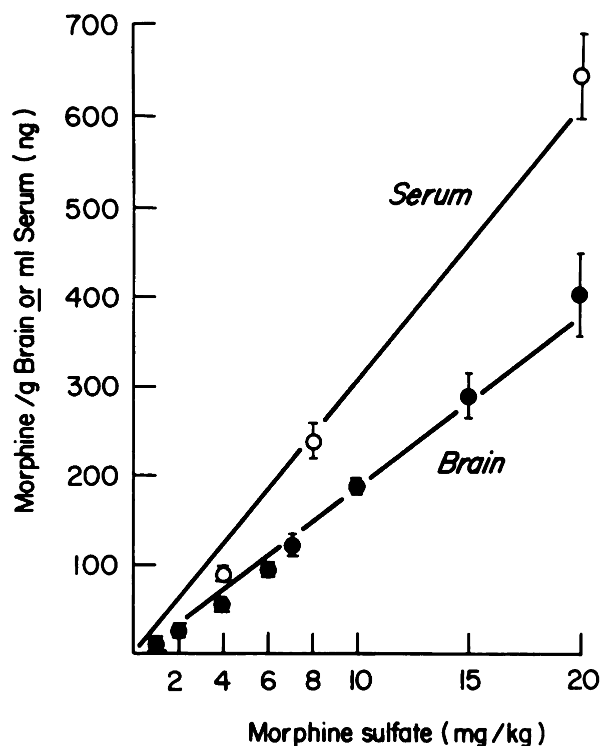

Figure 6. Effect of increasing dosage of morphine sulfate on the serum (O —O) and brain (● — ●) concentraitons of morphine at 30 minutes after the i.p. injections. For serum, each point represents the mean ± S.E. of 4 seperate determination on serum pooled from 3 animals, and for brain each point represents the mean ± S.E. obtained from 5 to 12 mice. For radioimmunoassay, 3H-dihydromorphine and rabbit serum #77-7/2/73 were used as radiolabeled ligand and morphine antiserum respectively and the standard curve was prepared with internal morphine standards.

Table 8

UPTAKE OF MORPHINE BY MOUSE BRAIN AT 30 MINUTES AFTER I.P. INJECTION

MORPHINE SULFATE (MG/KG)	BODY WEIGHT (GM)	BRAIN WEIGHT (GM)	BRAIN MORPHINE (NG/GM)	$\frac{\text{BRAIN MORPHINE}}{\text{TOTAL DOSE}}$ X100
1	24.0 ± 0.2	0.46 ± 0.01	11.2 ± 1.48	0.03
2	25.1 ± 0.3	0.45 ± 0.01	29.4 ± 6.09	0.04
4	25.0 ± 0.6	0.45 ± 0.01	55.8 ± 2.93	0.03
6	23.8 ± 0.2	0.44 ± 0.02	97.1 ± 2.28	0.04
7	25.2 ± 0.6	0.45 ± 0.01	128 ± 10.4	0.04
10	24.3 ± 0.3	0.44 ± 0.01	192 ± 12.3	0.05
15	23.7 ± 0.3	0.44 ± 0.01	291 ± 25.8	0.05

Mice were given morphine sulfate at doses indicated 30 minutes prior to sacrifice. For radioimmunoassay, 3H-dihydromorphine and rabbit serum #77-7/2/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with the internal morphine standard. Each number is the mean ± S.E. of 6 animals.

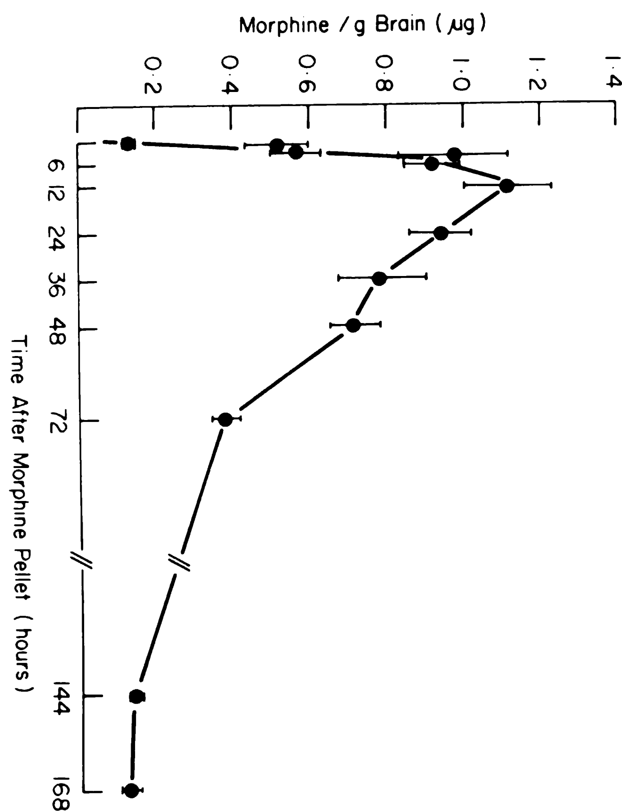

Figure 7. Brain morphine levels in mice at varying times after one morphine pellet implantation (75 mg base, s.c.). For morphine radioimmunoassay, 3H-morphine and rabbit serum #77-7/2/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with the internal morphine standard. Each point represents the mean ± S.E. of n=6.

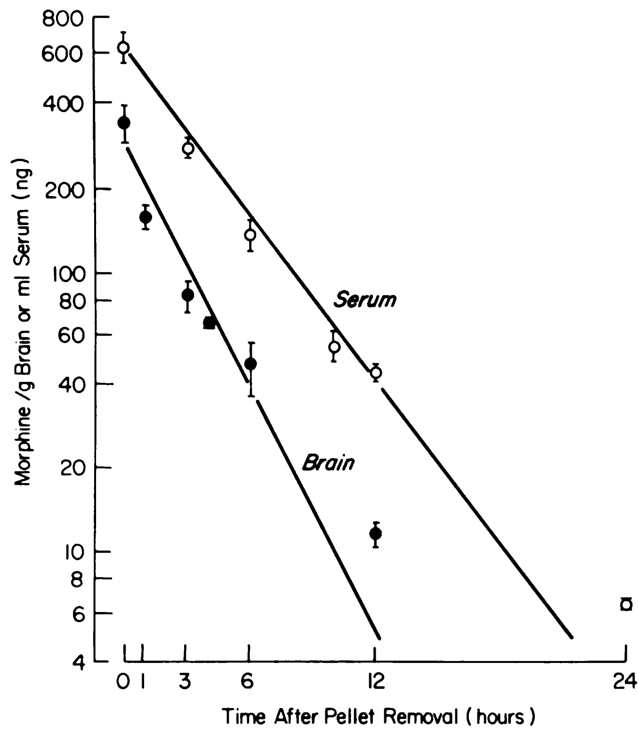

Figure 8. Rate of decay of serum (O—O) and brain (●—●) morphine levels in mice after the removal of a morphine pellet implanted for 72 hours. For morphine radioimmunoassay, 3H-morphine and rabbit serum #77-7/2/73 (Catlin) were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with internal morphine standards. Each point of serum levels represents the mean ± S.E. of 5 seperate determination on serum pooled from 3 animals, and each point of brain levels represents the mean ± S.E. of 6 individual mouse brain sample.

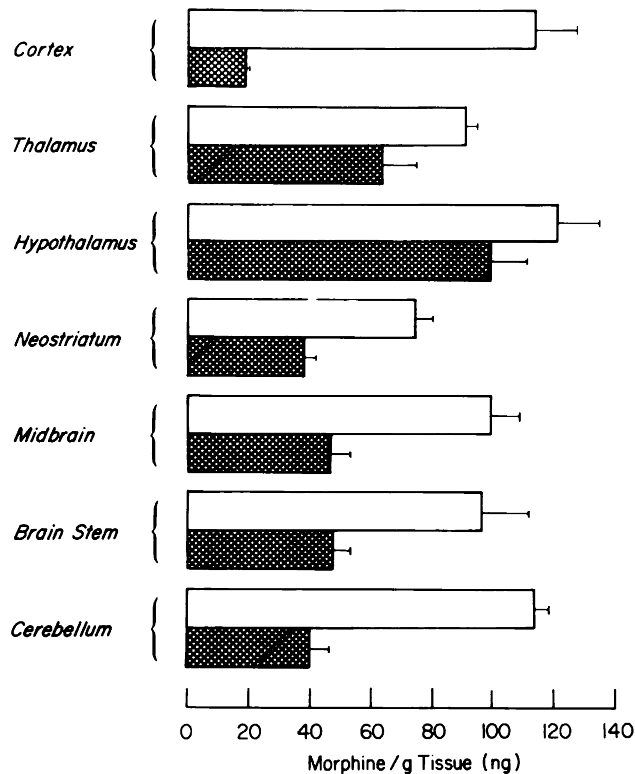

Figure 9. Comparison of morphine concentration in various brain regions of rats with and without morphine pellet removal. Mice were implanted with one morphine pellet for 72 hours and then randomly divided into 2 groups; one with morphine pellet removed and the other with morphine pellet remained. The animal was sacrificed at 4 hours later. Ratched columns are tissue morphine levels of rats at 4 hours after morphine pellet removal and empty columns are tissue morphine levels of rats without pellet removal. For morphine radioimmunoassay, 3H-morphine and rabbit serum #77-8/29/73 (Catlin) were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with direct morphine standards.

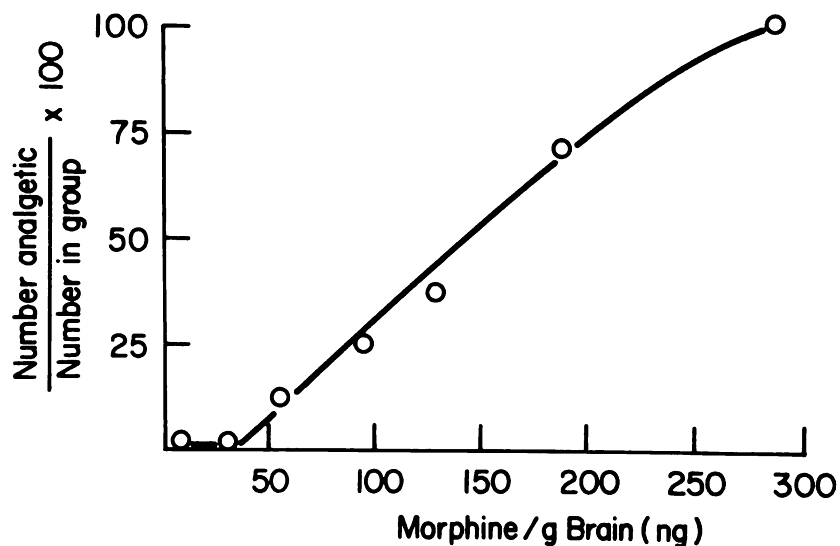

Figure 10. Relationship between brain morphine levels and analgesia. Groups of 8 mice were injected intraperitoneally with various doses of morphine sulfate and the tail-flick response was measured 30 minutes after the injection. Mice with a tail-flick reaction time of 5 or more seconds were considered to be analgetic. Each animal was sacrificed immediately after the analgetic test. For morphine radioimmunoassay, 3H-dihydromorphine and rabbit serum #77-7/2/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with internal morphine standard.

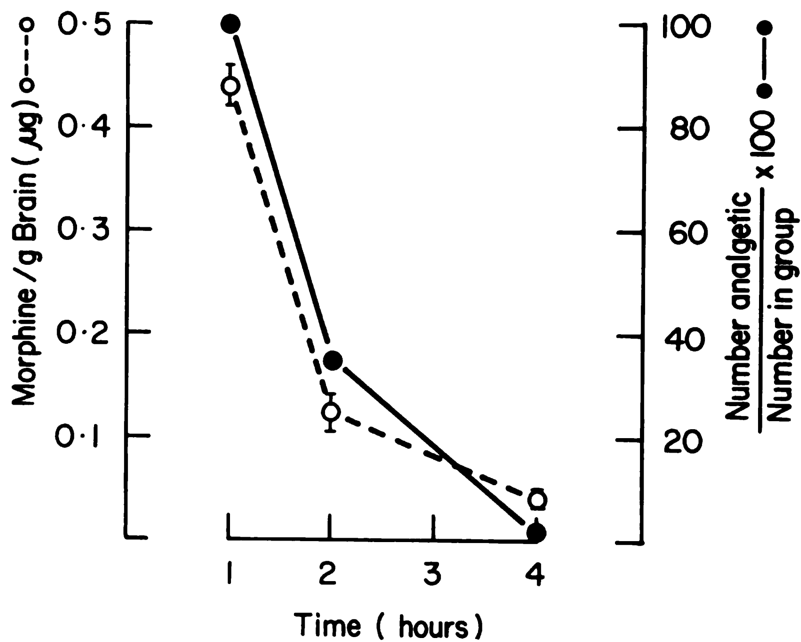

Figure 11. Correlation of brain morphine levels (O---O) with analgesia (●—●) in mice. Mice were injected with morphine sulfate (15 mg/Kg, i.v.) and were tested for analgesia at various time intervals indicated. They were sacrificed immediately after the analgetic test and the brain was removed for morphine analysis. For morphine radioimmunoassay, 3H-dihydromorphine and rabbit serum #77-7/2/73 (Catlin) were used as radiolabled ligand and morphine antiserum respectively. The standard curve was prepared with internal morphine standards. Eight mice were used in each analgetic test. Each brain morphine level represents the mean ± S.E. of 6 mouse brain samples.

Table 9

BRAIN MOPRHINE LEVELS AND ANALGESIA
IN 4 STRAINS OF MICE

MOUSE STRAIN	MORPHINE AD50 (95% CONFIDENCE LIMITS)	BRAIN MOPRHINE(NG/GM) 30 MIN. AFTER 4 MG/KG
SWISS	3.00 (2.70 - 3.30)	84 ± 12.0
C3H	1.60 (1.47 - 1.74)	100 ± 10.0
C57	1.35 (0.98 - 1.86)	78.4 ± 9.40
ICR	4.30 (3.94 - 4.68)	94.4 ± 7.92

The inhibition of the tail-flick response by morphine was tested at 30 minutes after the i.p. injection of various doses of morphine sulfate. The group of mice which had received 4 mg/kg of morphine sulfate was sacrificed immediately after the analgetic test and the brain was removed for morphine analysis. For morphine radioimmunoassay, 3H-dihydromorphine and rabbit serum #77-7/2/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with internal moprhine standards. Morphine levels were expressed as the mean ± S.E. of n=5.

Table 10

EFFECT OF NALOXONE ON BRAIN MORPHINE LEVELS
IN NAIVE MICE

DOSE(MG/KG) MORPHINE SULFATE	NALOXONE HCl	MINUTES AFTER MORPHINE	BRAIN MORPHINE (NG/GM)
1	0	30	17.5 ± 1.14
1	1	30	16.6 ± 0.69
10	0	30	178 ± 14.9
10	1	30	153 ± 14.8
10	10	30	184 ± 4.93
6	0	60	83.3 ± 6.60
6	10	60	88.0 ± 8.51

Mice were injected (i.p.) with various doses of morphine sulfate for the time intervals indicated. They were randomly divided into two groups; one was given i.p. injection of saline and the other naloxone with doses indicated at 10 minutes before sacrifice. For morphine radioimmunoassay, 3H-morphine and rabbit serum #77-8/29/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with direct morphine standards. Brain morphine levels were expressed as the mean ± S.E. (n=6).

Table 11

EFFECT OF NALOXONE ON REGIONAL BRAIN DISTRIBUTION

OF MOPRHINE IN NAIVE RATS

BRAIN REGIONS	BRAIN AREA WEIGHT(MG) SALINE	NALOXONE	BRAIN MORPHINE(MG/GM) SALINE	NALOXONE
CORTEX	781 ± 27.1	805 ± 27.2	175 ± 20.0	147 ± 5.85
THALAMUS	115 ± 7.51	110 ± 4.17	569 ± 41.9	319 ± 40.8*
HYPOTHALAMUS	49.0 ± 4.30	53.0 ± 3.55	1189 ± 81.3	527 ± 61.9*
MIDBRAIN	116 ± 9.90	129 ± 4.44	543 ± 49.3	524 ± 82.0
BRAIN STEM	179 ± 5.88	167 ± 4.11	383 ± 32.0	215 ± 40.2
CEREBELLUM	245 ± 5.53	260 ± 5.97	411 ± 15.3	347 ± 42.2

Rats were randomly divided into two groups; one was injected with morphine sulfate (15 mg/Kg, i.p.) plus saline and the other with morphine sulfate (15 mg/Kg, i.p.) plus naloxone HCl (6 mg/Kg, i.p.). The rat was sacrificed at 30 minutes after the injection. For morphine radioimmunoassay, 3H-morphine and rabbit serum #77-8/29/73 were used as radiolabeled ligand and morphine antiserum respectively. Direct morphine standards were used to prepare the standard curve. Each number represents the mean ± S.E. of 6 tissue samples.
* $p<0.05$.

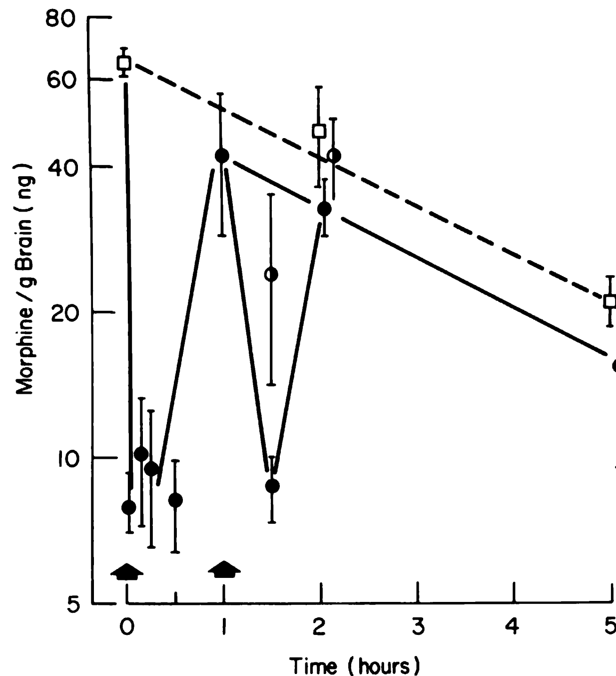

Figure 12. Time course of the displacement of brain morphine by naloxone in morphine-dependent mice. Mice were implanted with one morphine pellet for 3 days before the pellet was removed. Fours hours after the morphine pellet removal they were randomly divided into 4 groups; one was injected with saline (□--□) and the other three with naloxone HCl (10 mg/KG, i.p.)(●—●). However,one of the three naloxone-treated groups was given another dose of naloxone (10 mg/Kg, i.p.) at one hour after the first dose and onother group was given saline at one hour after the first dose of naloxone. For brain morphine analysis, 3H-morphine and antiserum #77-7/2/73 (Catlin) were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with internal morphine standards. Each morphine level is the mean ± S.E. of n=8.

Table 12

RELATIONSHIP BETWEEN DOSE OF NALOXONE AND BRAIN LEVELS OF MORPHINE IN MORPHINE-DEPENDENT MICE

NALOXONE HCl (MG/KG)	BRAIN MORPHINE (NG/GM)	% DECREASE
0	64.4 ± 5.98	-----
0.1	24.7 ± 4.37	62
1.0	20.5 ± 5.76	68
10	19.0 ± 6.93	70
50	7.64 ± 1.74	88

Mice were implanted with one morphine pellet for 3 days and then removed for 4 hours. They were randomly divided into 5 groups; one was injected with saline and the other four were injected with naloxone HCl with doses indicated for each group. They were sacrificed 15 minutes later. For morphine radioimmunoassay, 3H-morphine and rabbit serum #77-7/2/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with internal morphine standards. Each brain morphine level is the mean ± S.E.(n=6).

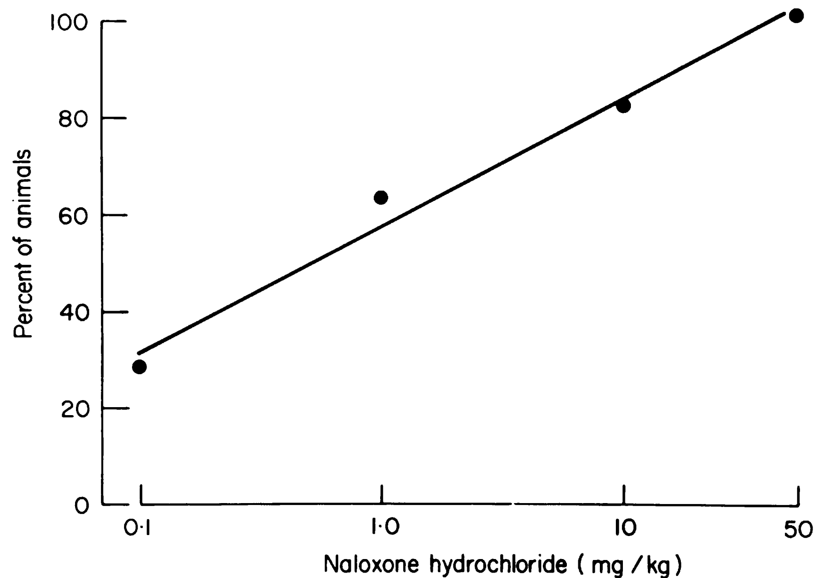

Figure 13. Dose-response relationship of the displacement effect of naloxone on brain morphine in morphine-dependent mice. The treatment of the animal was that of Table 12. Positive response to naloxone was defined in naloxone-treated mice which had brain morphine level significantly (95% confidence limits) less than that of the saline-treated mice.

Table 13

EFFECT OF NALOXONE ON MORPHINE CONTENT IN DISCRETE BRAIN REGIONS OF MORPHINE-DEPENDENT RATS

BRAIN REGIONS	BRAIN AREA WEIGHT(MG)		BRAIN MORPHINE(NG/GM)	
	SALINE	NALOXONE	SALINE	NALOXONE
CORTEX	740 ± 11.4	728 ± 15.4	18.1 ± 1.40	17.0 ± 1.15
THALAMUS	100 ± 2.24	108 ± 1.22	63.5 ± 17.4	24.8 ± 1.49*
HYPOTHALAMUS	49.3 ± 1.36	53.0 ± 0.49	99.5 ± 12.3	42.7 ± 9.20*
NEOSTRIATUM	128 ± 2.00	130 ± 1.96	38.3 ± 4.40	47.2 ± 7.65
MIDBRAIN	150 ± 8.37	139 ± 7.35	47.0 ± 7.37	61.5 ± 5.40
BRAIN STEM	166 ± 4.00	166 ± 7.48	47.6 ± 6.87	53.4 ± 11.8
CEREBELLUM	247 ± 5.10	258 ± 3.74	40.1 ± 6.91	38.4 ± 3.26

Rats were implanted with one morphine pellet for 3 days before the pellet was removed. Four hours after morphine pellet removal, they were randomly divided into two groups; one was injected with saline and the other naloxone HCl (10 mg/Kg, i.p.). The animal was sacrificed 15 minutes later. For tissue morphine radioimmunoassay, 3H-morphine and rabbit serum #77-8/29/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with the direct morphine standards. Each number represents the mean ± S.E. (n=5).
*$p<0.05$.

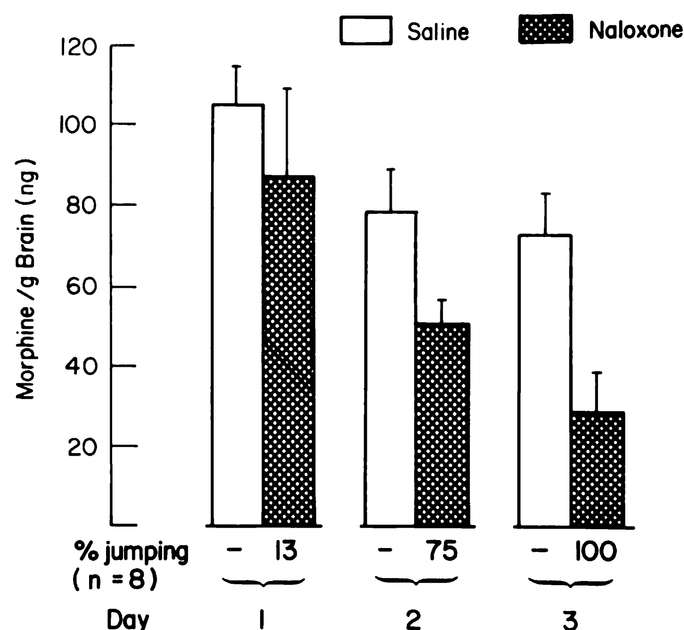

Figure 14. Relationship between the displacement of brain morphine by naloxone and the degree of physical dependence in mice. Three groups of mice were implanted with one morphine pellet for 1, 2, and 3 days respectively before the pellet was removed. Four hours after morphine pellet removal, each group was further randomly divided into 2 subgroups; one was injected with saline and the other naloxone HCl (10 mg/Kg, i.p.). The jumping incidence was recorded for 15 minutes in naloxone-treated subgroup of 8 mice. The animal was sacrificed at the end of 15 minutes. Open columns are brain morphine levels in saline-treated subgroup and ratched columns are those in naloxone-treated subgroup. They are the mean ± S.E. of 6 mice. For morphine radioimmunoassay, 3H-morphine and rabbit serum #77-8/29/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with direct morphine standards.

Table 14

DISPLACEMENT OF BRAIN MORPHINE IN POST-ADDICT MICE
BY NALOXONE AFTER PRIMING WITH MORPHINE

PELLET	DAYS AFTER PELLET REMOVAL	TIME OF MORPHINE PRIMING	BRAIN MORPHINE (NG/GM)	
			SALINE	NALOXONE
PLACEBO	2	-120 MIN	249 ± 27.0	249 ± 21.1
		-150 MIN	170 ± 13.1	177 ± 22.7
MORPHINE	2	-120 MIN	205 ± 19.0	138 ± 16.7*
		-150 MIN	169 ± 9.07	119 ± 13.2*
PLACEBO	12	-150 MIN	186 ± 19.0	171 ± 11.8
MORPHINE	12	-120 MIN	214 ± 12.4	129 ± 23.7*
PLACEBO	30	-150 MIN	158 ± 11.4	176 ± 15.9
MORPHINE	30	-120 MIN	205 ± 21.8	118 ± 14.5*

Male Swiss-Webster mice were implanted with either placebo or morphine pellet for 3 days before the pellet was removed. The pellet was removed for a period of time as indicated in the table. A priming dose of morphine sulfate (30 mg/Kg, i.p.) was then given which was followed in 120 or 150 minutes by saline or naloxone HCl (10 mg/Kg, i.p.). The animal was sacrificed 15 minutes later. For radioimmunoassay for morphine, 3H-morphine and rabbit serum #77-8/29/73 were used as radiolabeled ligand and morphine antiserum respectively. The standard curve was prepared with direct morphine standards. Each brain morphine level is the mean ± S.E. of 6 mice.
*Brain morphine levels significantly lower than that of the corresponding saline-treated mice ($p<0.05$).

DISCUSSION

The mouse has been used extensively as an animal model to study the behavioral and neuropharmacological effects of acute and chronic morphine administration because analgesia, tolerance and physical dependence have all been demonstrated in this animal model. However, due to the small body size of the animal and the limitations in sensitivity of the methods for morphine analysis, little information is available for this species regarding its disposition, particularly in the central nervous system.

The recent development of a radioimmunoassay for morphine appears to overcome to a significant extent the problem of assay sensitivity. However, one of the major problems encountered in radioimmunoassay is the possible interference by several structurally related compounds (Spector and Parker, 1970; Berkowitz *et al.*, 1974), such as the two biotransformation products of morphine, i.e., morphine 3-glucuronide and N-desmethyl morphine. It was also of importance for this particular study to rule out any interference by naloxone, the antagonist we administered to the animal. Using the antiserum provided by Dr. Catlin we were able to determine extracted tissue morphine in each individual mouse brain with a lower limit ranging from 1 to 10 ng per gm of tissue. Furthermore, this batch of antiserum seemed able to differentiate morphine from its metabolites and antagonist with high specificity. Only 10 picomole/ml of morphine was required to displace 50% of ^{3}H-dihydromorphine; however, it took 1.2×10^{4} picomole/ml of morphine 3-glucuronide and 2.5×10^{3} picomole/ml of N-desmethyl morphine to effect the same amount of displacement. Naloxone neither bound to the antiserum, nor indirectly changed the shape of the standard curve when the latter was prepared from the brains of naloxone-treated

mice. The specificity of the method was further improved by the double extraction procedure prior to the assay. Therefore, for brain morphine determinations, combining the double extraction with radio-immunoassay provides a method which is both sensitive and highly specific.

Having provided some evidence for the accuracy, sensitivity, specificity and reproducibility of the assay, we then examined the disposition of morphine in mouse brain. As in rats (Way, 1967, Oldendorf et al., 1972), brain uptake of morphine in mice is largely impeded by the presence of a blood-brain barrier. Therefore, although morphine rapidly appeared in mouse brain after either a single bolus dose or pellet implantation, only a very small fraction of the administered drug gained access to the central nervous system. At peak morphine level, the amount of morphine present in the mouse brain was calculated to be only 0.09% of the total administered intravenous dose. This figure was further reduced by 50% with intraperitoneal administration. A comparison of the serum and brain concentrations revealed that relative distribution of morphine between the two was ranging from 1.4 to 1.8. A higher serum/brain ratio has been reported in rats by other investigators (Dahlstrom and Paalzow, 1975; Hahn et al., 1976). Since in our studies, we did not eliminate the residual blood trapped in the brain, it would be expected that the true serum/brain ratio was higher than what we had found. Other non-CNS tissues e.g., kidney, liver, lung and diaphragm have been reported to attain morphine levels higher than those found in serum (Hahn et al., 1976) at 30 minutes after the subcutaneous injections of various doses of morphine. Once the morphine level reached its peak

in the brain in 15 minutes or less after a single intravenous dose, it rapidly disappeared over the next 4 hours. The half-life of the decay was about 36 minutes. Loh _et al_. (1969) reported a longer half-life of 50 minutes and Paalzow and Paalzow (1971) found a somewhat shorter half-life of 28.1 minutes. Since this decay represents the distribution of the drug to other tissues, the short half-life of morphine in the brain provides further evidence that tissues other than brain can concentrate morphine more. It is generally believed that morphine is eliminated from brain tissue predominantly by simple diffusion process. However, in rabbits, it has been demonstrated that morphine is actively removed from cerebrospinal fluid (Asghar and Way, 1970). Autoradiographs of the rat brain showed that radioactive morphine concentrates in the choroid plexus and the ventricles (Miller and Elliott, 1955). In addition, N-desmethylation of morphine in the central nervous system has been suggested (Elison and Elliott 1963; Milthers, 1962), although the data are not convincing.

With morphine pellet implantation for 3 days, the decay of brain morphine after removal of the pellet was much slower. The half-life was 2.5 times that after a single injection. In studies on the distribution pattern of morphine in discrete areas of the rat brain after a single small dose, the highest concentration of morphine was found in the hypothalamus at 30 (Hahn _et al_., 1976) and 60 minutes (Clouet and Williams, 1973) after the injection of the drug. This finding was explained by the fact that hypothalamus is rich in cell body and therefore, gray matter, has a high blood flow rate and that part of the tissue lacks a blood-brain barrier. However, the time

course of morphine in the hypothalamus, as in other brain regions, appeared to show almost the same kinetic behavior as that of the whole brain. In our study with rat brain, it is interesting to note that although morphine concentration in various brain areas did not differ much at 3 days after one morphine pellet implantation, there was considerable difference in the rate of decline of morphine among seven brain regions at 4 hours after the pellet removal. The rate of tissue morphine elimination was found to be fastest in the cortex; 84% of morphine was removed within this period of time. This was followed by the cerebellum, midbrain, brain stem and neostriatum with the fraction of morphine removed ranging from 49 to 65%. The thalamus and hypothalamus had significantly slower rates of morphine elimination, i.e., at the end of 4 hours, the amount of morphine retained in teach was found to be 70 and 82% respectively. Taking into consideration that the hypothalamus has a highly efficient blood supply and both the thalamus and hypothalamus are in the vicinity of the ventricular system, the opposite result, i.e., faster morphine elimination from these regions would be expected. One of the explanations for slower decay of morphine from these regions would be the presence of high affinity and/or capacity of the binding sites for morphine in these tissues. Indeed, it has been reported that these two brain regions contain relatively high concentration of opiate receptors (Hiller et al., 1973; Kuhar et al., 1973).

In the study of the effect of naloxone on the disposition of brain morphine, our results clearly demonstrated that during the course of antagonist induced precipitated withdrawal in morphine-dependent mice, a dramatic fall in brain morphine level occurs shortly after the

administration of naloxone. These results are supported by the studies of Mulé (1965) who reported that the administration of morphine to tolerant dogs followed 35 minutes later by nalorphine resulted in a decrease in central nervous system content of morphine at 65 minutes. Our results further demonstrated that the time course and duration of the decline in brain morphine level coincide closely with the onset, peak and duration of the acute withdrawal signs that are precipitated by naloxone. In establishing the dose-response relationship, although there was a considerable variability in brain morphine levels after pellet implantation and removal, it was possible to show that a good correlation exists between the dose of naloxone and the per cent of animals exhibiting morphine levels significantly lower than those of salinetreated mice.

With morphine-dependent rats, studies in the regional brain distribution of morphine revealed that the reduction of tissue morphine occurs mainly in the hypothalamus and thalamus. Since these two regions only account for 10% of the total brain weight, the more than 70% reduction in mouse brain morphine as effected by 10 mg/Kg of naloxone HCl suggests that displacement of tissue morphine by naloxone might also occur in other areas of mouse brain besides these two regions. It is reasonable to assume that under the same conditions, some discrepancy exists between these two species regarding the effect of naloxone. First, the degree of physical dependence resulted from one morphine pellet implantation for 3 days, and the severity of precipitated withdrawal produced by the same dose of naloxone could be different between these two species. On the other hand, it could be possible that receptor density and/or distribution in regional brain tissues are different between the two species. Unfortunately, due to the limitations in methodology, the regional brain

concentraiton of morphine in the mouse could not be accurately determined. Therefore, for the time being, we have no experimental evidence to rule out or rule in the above-mentioned possibilities.

Way and his associates (1969) have shown that in mice the intensity of physical dependence at any interval after morphine pellet implantation can be quantified by the estimation of the ED50 of naloxone to precipitate the withdrawl jumping. An inverse relationship was found to exist between the ED50 of naloxone and the duration of pellet implantation from 0 to 72 hours. In this investigation with morphine-dependent mice, we have presented quantitative data which indicates the existence of a direct relationship between the degree of physical dependence on morphine and the sensitivity in response to a given dose of naloxone. With increasing duration of morphine pellet implantation from 1 to 3 days, an increase in physical dependence was evidenced by an increase in jumping incidence precipitated by a given dose of naloxone. Correspondingly, the reduction in brain morphine concentration as effected by this dose of naloxone became greater. Thus, a parallel increase in the efficacy of naloxone to precipitate withdrawal jumping and to displace brain morphine occurred with increasing physical dependence.

While our results suggest that an alteration in the sensitivity of narcotic binding sites to naloxone, Takemori and coworkers applying the concept of PA_2 (Tulunay and Takemori, 1974a, b) demonstrated an increase in the ability of naloxone to antagonize narcotic analgesia in mice after prior exposure to morphine. They proposed that the affinity of the opiate receptor for naloxone is increased by morphine pretreatment.

Despite the evidence _in vitro_ which suggests that the saturable stereospecific bindings of various opiate agonists and antagonists to homogenate of brain tissues are related to opiate receptors, attempts to relate changes in the binding associated with the development of tolerance/physical dependence have not yielded data that are significant (Pert and Snyder, 1973b; Lee _et al._, 1973; Hitzeman _et al._, 1974; Klee and Stready, 1974). However, with rat brain stem slices, Davis _et al._ (1975) reported that chronic morphine treatment decreased the affinity of the opiate receptor binding for morphine.

Although Wikler _et al._ reported as early as 1953 that nalorphine could precipitate the abstinence syndrome in morphine-dependent subjects, the experimental basis for antagonist precipitated withdrawal could not be demonstrated. Our present findings provide strong evidence compatible with the hypothesis that antagonist displaces morphine from its receptor sites and thereby precipitates the withdrawal syndrome. The time course, dose-dependence, brain regional senensitivity and correlation with degree of physical dependence, strongly indicate that the reduction in brain morphine concentration is the consequence of direct interaction between morphine and naloxone. Although the relative purity of the antagonist activity of naloxone (Foldes _et al_ ., 1964b; Jasinski and Martin, 1967; McClane and Martin, 1967) already rules out to some extent the possibility that the reduction in brain morphine level might be secondary to the physiological alteration effected by naloxone.

Recently, it was discovered (Brase _et al._, 1976) that in post-addict mice unresponsive to naloxone, a single injection of morphine resulted in a rapid and marked sensitization of the animal to naloxone. It was

of interest, therefore, to ascertain if displacement of brain moprhine could also be effected by naloxone in post-addict mice primed with morphine and, if so, whether the displacement would be greater for post-addict mice than that for non-addict mice primed with same amount of morphine followed by the same dose of naloxone. Our results clearly demonstrate that although alteration in brain level of morphine by naloxone is not detectable in post-placebo mice, after the priming dose of morphine in post-addict mice, naloxone caused a reduction in the brain level of morphine.

Based on these observations, it appears that to precipitate the withdrawal syndrome in morphine-dependent animals, the primary requirement is the presence of morphine in the brain which is "displaceable" by naloxone. The fraction of the "displaceable" brain morphine for a given dose of naloxone is directly related to the sensitivity of the opiate receptor toward agonist or antagonist, which in turn is determined by the dose frequency and duration of morphine administration. Hence, as the degree of tolerance and physical dependence increases, the opiate receptor appears to decrease in sensitivity to morphine (tolerance) and this is accompanied by an increased sensitivity to naloxone to displace brain morphine and consequently to precipitate withdrawal (physical dependence).

Although the displacement of brain morphine by naloxone is readily demonstrated in morphine-dependent animal, we were not able to detect such displacement in naive mice. Since the antagonism of the analgesic effect of morphine by naloxone can easily be demonstrated, it is very likely that displacement of brain morphine as a consequence of naloxone-morphine interaction also occurs in the brain of naive mice. However,

as mentioned before, since the amount of morphine displaced by a given dose of naloxone is determined by the animal's degree of physical dependence on morphine, a lesser amount of morphine is expected to be displaced by a given dose of naloxone in naive animals. However, since the receptor's sensitivity toward morphine is at its maximum in the naive animal, a slight reduction in the availability of morphine at the receptor site may manifest a significant antagonism of analgesia. But this reduction in brain morphine level is likely beyond the sensitivity of our procedure for morphine determination. On the other hand, since the pharmacological parameters we quantitated were different in naive and morphine-dependent animals, i.e., analgesia in the former and precipitated withdrawal-jumping in the latter, it could be argued that different receptors were involved in the interaction between agonist and antagonist to manifest the antagonism of analgesia in naive animals and precipitated withdrawal-jumping in morphine-dependent animals. Nonetheless, with radiolabeled agonists, it has been shown that nalorphine and levallorphan reduced brain concentration of levorphanol in the dog (Wuepper _et al._, 1967), cyprenorphine lowered brain concentration of etorphine in the rat (Dobbs, 1968) and naloxone, diprenorphine and l-cyclorphan reduced _in vivo_ ^{3}H-etorphine binding in the synaptic membrane of the rat brain (Mulé _et al._, 1975).

BIBIOGRAPHY

Adler, T. K., Elliott, H. W. and George, R.: Some factors affecting the biological disposition of small doses of morphine in rats. J. Pharmacol. Exp. Ther. 120 : 475-487, 1957.

Albus, K., Schott, M. and Herz, A.: Interaction between morphine and morphine antagonists after systemic and intraventricular application. Eur. J. Pharmacol. 12 : 53-64, 1970.

Armitage, P.: Statistical Methods in Medical Research, p. 446. Wiley, New York, 1971.

Asghar, K. and Way, E. L.: Active removal of morphine from the cerebral ventricles. J. Pharmacol. Exp. Ther. 175 : 75-83, 1970.

Beckett, A. H. and Casy, A. F.: Synthetic analgesics: Stereochemical consideration. J. Pharm. Pharmacol. 6 : 986-1001, 1954.

Berkowitz, B. A., Cerreta, K. V. and Spector, S.: The influence of physiologic and pharmacologic factors on the disposition of morphine as determined by radioimmunoassay. J. Pharmacol. Exp. Ther. 191 : 527-534, 1974.

Berson, S. A., and Yalow, R. S.,: Immunoassay of protein hormones. In The Hormones Physiology, Chemistry and Applications, edited by Pincuss, G., Thimann, K. V. and Astwood, E. B., vol. IV, pp. 557-630. Academic Press, New York-London, 1964.

Blane, G. F., Boura, A. L. A., Fitzgerald, A. E. and Lister, R. E.: Actions of etorphine hydrochloride (M99): a potent morphine-like agent. Brit. J. Pharmacol. 30 : 11-22, 1967.

Blumberg, H., Dayton, H. B., George, M. and Rapaport, D. N.: N-allylnoroxymorphone: A potent narcotic antagonist. Fed. Proc. 20 : 311, 1961.

Blumberg, H., Dayton, H. B. anf Wolf, P. S.: Counteraction of narcotic antagonist analgesics by the narcotic antagonist naloxone. Proc. Soc. Exp. Biol. Med. 123 : 755-758, 1966

Brase, D. A., Iwamoto, E. T., Loh, H. H. and Way, E. L.: Reinitiation of sensitivity to naloxone by a single narcotic injection in post-addict mice. J. Pharmacol. Exp. Ther. 197 : 317-325, 1976.

Braude, M. C., Harris, L. S., May, E. L., Smith, J. P. and Villarreal, J. E.: Narcotic antagonists. Adv. Biochem. Psychopharmacol. 8 : 9-494, 1974.

Buxbaum, D. M., Yarborough, G. G. and Carter, M. E.: Dose-dependent behavioral and analgesic effects produced by microinjection of morphine sulfate (MS) into anterior thalamic nuclei. Pharmacologist 12 : 211, 1970.

Catlin, D., Cleeland, R. and Grumberg, E.: A sensitive, rapid radio-immunoassay for morphine and immunologically related substances in urine and serum. Clin. Chem. 19 : 216-220, 1973.

Chernov, H.I. and Woods, L.A.: Central nervous system distribution and metabolism of 14 C-morphine during morphine-induced feline mania. J. Pharmacol. Exp. Ther. 149 : 146-155, 1965.

Clouet, D.H. and Williams, N.: Localization in brain particulate fractions of narcotic analgesic drugs administered intracisternally to rats. Biochem, Pharmacol. 22 : 1283-1293, 1973.

Cox, B.M. and Weinstock, M.: Quantitative studies of the antagonism by nalorphine of some of the actions of morphine-like analgesic drugs. Brit. J. Pharmacol. 22 : 289-300, 1964.

Dahlstrom, B.E. and Paalzow, L.K.: Pharmacokinetics of morphine in plasma and discrete areas of the rat brain. J. Pharmacokinet. Biopharm. 3 : 293-302, 1975.

D'Amour, F.E. and Smith, D.L.: A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72 : 74-79, 1974.

Davis, M.E., Akera, T. and Brody, T.M.: Saturable binding of morphine to rat brain-stem slices and the effect of chronic morphine treatment. Res. Commun. Chem, Pathol. Pharmacol. 12: 409-418, 1975.

Dewey, W.L., Snyder, J.W., Harris, L.D. and Howes, J.F.: The effect of narcotics and narcotic antagonists on the tail-flick response in spinal mice. J. Pharm. Pharmacol. 21 : 548-549, 1969.

Dobbs, H.E.: Effect of cyprenorphine (M285), a morphine antagonist, on the distribution and excrection of etorphine (M99), a potent morphine-like drug. J. Pharmacol. Exp. Ther. 160 : 407-414, 1968.

Eidelberg E. and Barstow, C.A.: Morphine tolerance and dependence induced by intraventricular injection. Science 174 : 74-76, 1971.

Elison, C. and Elliott, H.M.: N- and O-demethylation of some narcotic analgesics by brain slices from male and female Long-Evans rats. Biochem. Pharmacol. 12: 1363-1366, 1963.

Finney, D.J.: Statistical Method in Biological Assay. Charles Griffin and Company, Ltd, London, 1974.

Foldes, F.F., Lunn, J.N., Moore, J. and Brown, I.M.: N-allylnoroxy-morphone : A new potent narcotic antagonist. Am. J. Med. Sci. 245 : 23-30, 1963.

Foldes, F.F., Swerdlow, M. and Siker, E.S.: Narcotics and Narcotic Antagonists, pp. 3-9, 10-26, 37-112. Charles C. Thomas, Springfield, 1964a.

Foldes, F.F., Duncalf, D., Davidson, G.M., Yun, D.S. and Schapira, M.: Comparison of the respiratory and circulatory effects of narcotic antagonists. Anesthesiology 25 : 95-96, 1964b.

Foster, R.S., Jenden, D.J. and Lomax, P.: A comparison of the pharmacologic effects of morphine and N-methyl morphine. J. Pharmacol. Exp. Ther. 157 : 185-195, 1967.

Fujita, S. Yasuhara, M, Yamamoto, S. and Ogiu, K.: Studies on sites of action of analgesics. The effect of analgesics on afferent pathway of pain. Jap. J. Pharmacol. 4 : 41-51, 1954.

Goldstein, A. Lowney, L.I. and Pal, B.K.: Stereospecific and non-specific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain. Proc. Natl. Acad. Sci. USA 68 : 1742-1747, 1971.

Goldstein, A.: Opiate receptors. Life Sci. 14 : 615-623, 1974.

Grumbach, L. and Chernov, H.I.: The analgesic effect of opiate-opiate antagonist combination in the rats. J. Pharmacol. Exp. Ther. 149 : 385-396, 1965.

Hagbarth, K.E. and Kerr, D.I.B.: Central influences on spinal afferent conduction. J. Neurpphysiol. 17 : 295-307, 1954.

Hahn, E.F., Norton, B.I. and Fishman, J.: Dose related changes in tissue morphine concentration. Res. Commun. Chem. Path. Pharmacol. 13 : 569-577, 1976.

Herz, A., Albus, K., Metys, J., Schubut, P. and Teschemacher, H.J.: On the central sites for the antinociceptive action of morphine and fentanyl. Neuropharmacology 9 : 539-551, 1970.

Herz, A. Teschemacher, H.J., Albus, K., Zieglgansberger, S.: Morphine abstinence syndrome in rabbits precipitated by injection of morphine antagonists into the ventricular system and restricted parts of it. Psychopharmacologia 26 :219-235, 1972.

Herz, A. and Teschemacher, H.J.: Development of tolerance to the antinociceptive effect of morphine after intraventricular injection. Experientia 29 : 64-65, 1973.

Hiller, J.M., Pearson, J. and Simon, E.J.: Distribution of stereospecific binding of potent narcotic analgesic etorphine in human brain: Predominance in the limic system. Res. Commun. Chem. Pathol. Pharmacol. 6 : 1052-1062, 1973.

Hipps, P.P., Eveland, M.R., Meyer, E.R., Sherman, W.R. and Cicero, T.J.: Mass fragmentography of morphine : Relationship between brain levels and analgesic activity. J. Pharmacol. Exp. Ther. 196 : 642-648, 1976.

Hitzemann, R.J., Hitzemann, B.A. and Loh, H.H.: Binding of 3H-naloxone in the mouse brain: Effect of ions and tolerance development. Life Sci. 14 : 2393-2404, 1974.

Ingoglia, N.A. and Dole, V.P.: Localization of d- and l- methodone after intraventricular injection into rat brains. J. Pharmacol. Exp. Ther. 175 : 84-87, 1970.

Irwin, S., Houde, R.W., Bennett, D.R., Hendershot, L.C. and Seevers, M.H.: The effects of morphine, methadone and meperidine on some reflex responses of spinal animals to nociceptive stimulation. J. Pharmacoo. Exp. Ther. 101 : 132-143, 1951.

Jacquet, Y.F. and Lajtha, A.: Morphine action at central nervous system sites in rats: Analgesia or hyperalgesia depending on site and dose. Science 182 : 490-492, 1973.

Jacquet, Y.F. and Lajtha, A.: Paradoxical effects after microinjection of morphine in the periaqueductal gray matter in the rat. Science 185 : 1055-1057, 1974.

Jasinski, D.R. and Martin, W.R.: The human pharmacology and abuse potential of N-allylnoroxymorphone (naloxone). J. Pharmcol. Exp. Ther. 157 : 420-426, 1967.

Johannesson, T. and Schou, J.: Analgesic activity and brain concentration of morphine in tolerant and non-tolerant rats given morphine alone or with neostigmine. Acta Pharmacol. Toxicol. (Kbh) 20 : 213-221, 1963.

Johannesson, T. and Milthers, K.: The lethal action of morphine and nalorphine given jointly to morphine tolerant and non-tolerant rats. Acta Pharmacol. Toxicol. (Kbh) 20 : 80-89, 1963.

Johannesson, T.: Effect of morphine on tolerant to nalorphine and its metabolism. Acta Pharmacol. Toxicol. (Kbh) 20 : 281-285, 1963.

Johannesson, T. and Woods, L.A.: Analgesic action and brain and plasma levels of morphine and codeine in morphine tolerant, codeine tolerant and non-tolerant rats. Acta Pharmacol. Toxicol. (Kbh) 21 : 381-396, 1964.

Klee, W.A. and Streaty, R.A.: Narcotic receptor sites in morphine-dependent rats. Nature 248 : 61-63, 1974.

Kosterlitz, H.W. and Watt, A.J.: Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). Brit. J. Pharmacol. 33 : 266-276, 1968.

Kosterlitz, H.W., Collier, H.O.J. and Villarrel, J.E.: Agonist and Antagonist Actions of Narcotic Analgesic Drugs. University Park Press, Baltimore, 1973.

Kuhar, M.J., Pert, C.B. and Snyder, S.H.: Regional distribution of opiate receptor binding in monkey and human brain. Nature 245 : 447-450, 1973.

Kupferberg, H., Burkhalter, A. and Way, E.L.: A sensitive fluorometric assay for morphine in plasma and brain. J. Pharmacol. Exp. Ther. 145 : 247-251, 1964.

Lee, C.Y., Akera, T., Stolman, S. and Brody, T.M.: Saturable binding of dihydromorphine and naloxone to rat brian tissue in vitro. J. Pharmacol. Exp. Ther. 194 : 583-592, 1975. 45

Litchfield, J.T. and Wilcoxon, F.: A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96 : 99-113, 1949.

Loh, H.H., Shen, F.H. and Way, E.L.: Inhibition of morphine tolerance and physical dependence development and brain serotin synthesis by cycloheximide. Biochem. Pharmacol. 18 : 2711-2721, 1969.

Martin, W.R.: Opiod antagonist. Pharmacol. Rev. 19 : 463-521, 1967.

McClane, T.K. and Martin, W.R.: Effects of morphine, nalorphine, cyclazocine and naloxone on the flexor reflex. Int. J. Neuropharmacol. 6 : 89-97, 1967.

Mellett, L.B. and Woods, L.A.: The distribution and fate of morphine in the non-tolerant and tolerant monkey. J. Pharmacol. Exp. Ther. 116 : 77-83, 1956.

Midgley, A.R., Jr., Niswender, G.D. and Regar, R.W.: Principles for the assessment of the reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity). Acta Endocrinol. (Kbh) 63, (Suppl) 142 : 163-184, 1969.

Miller, J.W. and Elliott, H.W.: Rat tissue levels of corbon-14 labeled analgesics as related to pharmacological activity. J. Pharm. Exp. Ther. 113 : 283-291, 1955.

Milthers, K.: N-dealkylation of morphine and nalorphine in the brain of living rats. Nature 195 : 607, 1962.

Mulé, S.J. and Woods, L.A.: Distribution of N-C14 -methyl labeled morphine: I. In central nervous system of nontolerant and tolerant dogs. J. Pharmacol. Exp. Ther. 136 : 232-241, 1962.

Mulé, S.J., Woods, L.A. and Mellet, L.B.: Distribution of N-C14-methyl labeled morphine: II. Effect of nalorphine in the central nervous system of nontolerant dogs and observations on metabolism. J. Pharmacol. Exp. Ther. 136 : 242-249, 1962.

Mulé, S.J.: Distribution of N-C14 -methyl labeled morphine: III. Effect of nalorphine in the central nervous system and other tissues of tolerant dogs. J. Pharmacol. Exp. Ther. 148 : 393-398, 1965.

Mulé, S.J. Clements, T.H., Layson, R.C. and Haertzen, C.A.: analgesia in guinea pigs: a measure of tolerance development. Arch. Int. Pharmacodyn. Ther. 173 : 201-212, 1968.

Mulé, S.J., Casella, G. and Clouet, D.H.: The specificity of binding of the narcotic agonist etorphine in synaptic membranes of rat brain in vivo. Psychopharmacologia 44 : 125-129, 1975.

Mulé, S.J.: Physiological disposition of narcotic agonists and antagonists. In Narcotic Drugs Biochemical Pharmacology, edited by Clouet, D.H., pp. 99-121. Plenum Press, New York-London, 1971.

Oldendorf, W.H., Hyman, S., Braun, L. and Oldendorf, S.Z.: Blood-brain barrier: Penetration of morphine, codeine, heroin, and methadone after carotid injection. Science 178 : 984-986, 1972.

Paalzow, L. and Paalzow, G.: Blood and brain concentration of morphine and its relation to the analgesic activity in mice. Acta Pharma. Suec. 8 : 329-336, 1971.

Patrick, G.A., Dewey, W.L., Spaulding, T.C. and Harris, L.S.: Relationship of brain morphine levels to analgesic activity in acutely treated mice and rats and in pellet-implanted mice. J. Pharmacol. Exp. Ther. 193 : 876, 883, 1975.

Pert. A. and Yaksch, T.: Sites of morphine induced analgesia in the primate brain: Relation to pain pathways. Brian res. 80 : 135-140, 1974.

Pert, C.B. and Snyder, S.H.: Opiate receptor: Demonstration in nervous tissue. Scinece 179 : 1011-1014, 1973a.

Pert, C.B. and Snyder, S.H.: Properties of opiate-receptor binding in the rat brain. Proc. Natl. Acad. Sci. USA 70 : 2243-2247, 1973b.

Portoghese, P.S.: A new concept on the mode of interaction of narcotic analgesics with receptors. J. Med. Chem. 8 : 609-616, 1965.

Portoghese, P.S.: Stereochemical factors and recptor interactions associated with narcotic analgesics. J. Pharm. Sci. 55 : 865-887, 1966.

Potts, J.T., Jr., Sherwood, L.M., O'Riordan, J.L.H. and Aurbach, G.D.: Radioimmunoassay of polypeptide hormones. Adv. Intern. Med. 13 : 183-240, 1967.

Rodbard, D., Rayford, P.L., Cooper, J.A. and Ross. G.T.: Statistical quality control of radioimmunoassay. J. Clin. Endocrinol. Metab. 28 : 1412-1418, 1968.

Rodbard, D., Rayford, P.L. and Ross, G.T.: Statistical quality control of radioimmunoassay. In Statistics in Endocrinology, edited by McArthur, J. and Colton, T., pp. 411-429. MIT Press, Cambridge, 1970.

Rubin, M.: Requirements and problems in urinalysis for abuse drugs, the role of gas chromatography. Am. J. Med. Techonol. 39 : 205-210, 1973.

Siminoff, R. and Saunders, P.R.: Concentration of free and conjugated morphine in brain and other tissue of tolerant and nontolerant rabbits. J. Pharmacol. Exp. Ther. 124 : 252-254, 1958.

Satoh, M. and Takagi, H.: Enhancement by morphine of the central descending inhibitory influence on spinal sensory transmission. Eur. J. Pharmacol. 14 : 60-65, 1971.

Simon, E.J., Hiller, J.M. and Edelman, I.: Stereospecific binding of the potent narcotic analgesic 3(H)-etorphine to rat-brain homogenate. Pro. Natl. Acad. Sci. USA 70 : 1947-1949, 1973.

Spector, S. and Parker, C.W.: Morphine radioimmunoassay. Science 168 : 1347-1348, 1970.

Sung, C.Y., Way, E.L. and Scott, K.G.: Studies on the relationship of metabolic fate and hormonal effects of D, L- Methadone to the development of drug tolerance. J. Pharmacol. Exp. Ther. 107 : 12-23, 1953.

Szerb, J.C. and McCurdy, D.H.: Concentration of morphine in blood and brain after intravenous injection of morphine in non-tolerant, tolerant and neostigmine-treated rats. J. Pharmacol. Exp. Ther. 118 : 446-450, 1956.

Takagi, H., Matsumura, M., Yanai, A. and Ogiu, K.: The effect of analgesics on the spinal reflex activity of the cat. Jap. J. Pharmacol. 4 : 176-187, 1955.

Taylor, J.F. : Methods of chemical analysis. In Narcotic Drugs Biochemical Pharmacology, edited by Clouet, D.H., pp. 99-121. Plenum Press, New York-London, 1971.

Terenius, L.: Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. Acta Pharmacol. Toxicol. (Kbh) 32 : 317-320, 1973.

Tsou, K. and Jang, C.S.: Studies on the site of analgesic action of morphine by intracerebral micro-injection. Sci. Sinica 7 : 1099-1109, 1964.

Tulunay, F.C. and Takemori, A.E.: The increased efficacy of narcotic antagonists induced by various narcotic analgesics. J. Pharmacol. Exp. Ther. 190 : 395-400, 1974a.

Tulunay, F.C. and Takemori, A.E.: Further studies on the alteration of analgesic receptor-antagonist interaction induced by morphine. J. Pharmacol. Exp. Ther. 190 : 401-407, 1974b.

Urabe, M., Tsubokawa, T., Sakurai, H. and Seki, M.: Relation of brain stem activity to the evoked potential in the spinal cord following the single volley of the splanchnic nerve. Folia Psychiat. Neurol. Japan. 19 : 49, 1965.

Watanabe, H.: The development of tolerance to and physical dependence on morphine following intraventricular injection in the rat. Jap. J. Pharmacol. 21 : 383-381, 1971.

Way. E.L. and Adler, T.K.: The pharmacologic implication of the fate of morphine and its surrogates. Pharmacol. Rev. 12 : 383-445, 1960.

Way, E.L. and Adler, T.K.: The biological disposition of morphine and its surrogates. Bull. World Health Organ. 25 : 227-262, 1962.

Way, E.L.: Brain uptake of morphine: Pharmacologic implications. Fed. Proc. 26 : 1115-1118, 1967.

Way, E.L., Loh, H.H. and Shen, F.H.: Simultaneous quantitative assessment of morphine tolerance and physical dependence . J. Pharmacol. Exp.Ther. 167 : 1-8, 1969.

Wei, E., Loh, H.H. and Way, E.L.: Neuroanatomical correlates of morphine dependence. Science 177 : 616-617, 1972.

Wei, E., Loh., H.H. and Way, E.L. : Brain sites of precipitated abstinence in morphine-dependent rats. J. Pharmacol. Exp. Ther. 185 : 108-115, 1973.

Wei, E., Collins, P. and Way, E.L.: The central site of analgesia in rats. Proc. West Pharmacol. Soc. 16 : 8-13, 1973

Wikler, A., Fraser, H.F. and Isbell, H.: N-allylnormorphine: Effects of single doses and precipitation of acute "abstinence syndrome" during addiction to morphine, methadone or heroin in man (post-addicts). J. Pharmacol. Exp. Ther. 109 : 8-20, 1953.

Wikler, A.: Opiates and Opiate Antagonists. A Review of Their Mechanisms and Action in Relation to Clinical Problems. Public Health Monograph No. 52, U.S. Government Printing Office, Washington D.C., 1958.

Woods, L.A.: Distribution and fate of morphine in non-tolerant and tolerant dogs and rats. J. Pharmacol. Exp. Ther. 112 : 158-175, 1954.

Woods, L.A.: The pharmacology of nalorphine (N-allylnormorphine). Pharmacol. Rev. 8 : 175-198, 1956.

Wuepper, K.D., Yeh, S.Y. and Woods, L.A.: Effect of nalorphine and levallorphan on brain concentrations of levorphanol in the dogs. Proc. Soc. Exp. Biol. Med. 124 : 1146-1150, 1967.

Yalow, R.S. and Berson, S.A. : Radioimmunoassay. In Statistics in Endocrinology, edited by McArthur, J.W. and Colton, T., pp 327-344, MIT Press, Cambridge, 1970.

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