Pharmacology Biochemistry & Behavior, Vol. 4, pp. 323–327.

Chronic Morphine Administration: Plasma Levels and Withdrawal Syndrome in Rats[1]

CHIARA CERLETTI, SUSAN H. KEINATH, MARCUS M. REIDENBERG AND MARTIN W. ADLER

Department of Pharmacology, Temple University Medical School
Philadelphia PA 19140 U.S.A.

(Received 12 August 1975)

CERLETTI, C., S. H. KEINATH, M. M. REIDENBERG AND M. W. ADLER. *Chronic morphine administration: plasma levels and withdrawal syndrome in rats.* PHARMAC. BIOCHEM. BEHAV. 4(3) 323–327, 1976. – Morphine, administered to Sprague-Dawley rats over a period of 65 hr either by the simultaneous implantation of two 75 mg pellets, or by a series of twice daily 20 or 30 mg/kg injections, produced dependence as indicated by the precipitation of the abstinence syndrome with the antagonist, naloxone. Plasma morphine levels, analyzed fluorometrically at various times during the treatment procedures, revealed peak concentrations that were 3 or 4 fold higher for injected animals than the maximum steady-state level established in the pellet-implanted animals. The calculated plasma concentration of the drug over time was not statistically different for the groups. It is noted that although the 2 methods of morphine administration produce a qualitatively identical dependent state, the pellet implantation technique causes greater weight loss and a higher incidence of jumping and wet-dog shakes during withdrawal.

Morphine Naloxone Precipitated abstinence syndrome Pellets Injections Plasma concentration Dependence

PHYSICAL dependence to a narcotic agent is determined by the development of a specific physical abstinence syndrome following withdrawal of the agent either by termination of narcotic administration or by treatment with a narcotic antagonist such as naloxone. In order to produce physical dependence in the experimental animal, many investigators have administered a series of injections over a period of weeks, using a schedule of increasing doses of the narcotic drug [5,13]. Recently, Frumkin [9] reported that as few as 3 once-daily injections of 10 mg/kg IP of morphine sulfate given to rats resulted in an abstinence syndrome following naloxone administration. Maruyama and Takemori [14], however, noted that doses above 20 mg/kg of morphine 3 times a day for 3 days were required to produce naloxone-precipitated jumping in mice. In discussing drug administration and dependence, Deneau and Seevers [8] stated that the daily dose of a drug should be "administered at such intervals that the organism is continuously exposed to the drug". Some investigators have used methods in which the animal is exposed to an apparently constant, low amount of the narcotic agent by continuous intraperitoneal infusion [17], or through subcutaneous implantation of a morphine reservoir [11] or one or more morphine pellets [4, 7, 18].

Establishing and quantitating the withdrawal syndrome is essential in studies of physical dependence. Body weight loss during narcotic withdrawal is considered the most reliable index of addiction by some investigators [2,11]; scoring the motor and autonomic signs appearing during abstinence can likewise provide data for assessing the severity of withdrawal. No previous studies have been reported comparing the withdrawal syndrome in animals made dependent by the injection versus the pellet method.

The primary objective in undertaking this study was to determine whether the more critical factor in producing dependence is (1) continuous or intermittent exposure of the organism to morphine or (2) the total amount of drug to which the animal is exposed. To answer this question, equivalent total areas under the plasma morphine concentration versus time curve (integral of plasma concentration over time) were produced either by twice daily injections (intermittent exposure) or by pellet implantation (continuous exposure). The precipitated abstinence syndromes were then compared.

Owing to the scarcity of published data on morphine blood levels after injections or pellet implantation in the rat, a second objective of this study was to measure drug levels in the plasma of treated animals.

[1] The work in this study was supported by USPHS Grants DA 00049 and DA 00376 from the National Institute of Drug Abuse.

METHOD

Animals and Treatment

The animals were adult male, Sprague-Dawley rats (Zivic Miller) initially weighing 330–370 g. For the duration of the experiment, the animals were housed in groups of 6 per cage (stainless steel, approximately 60 × 50 × 20 cm) at 22 ± 2°C and at a relative humidity of approximately 60%. Lights were cycled on a standard 12 hr light–dark schedule and food and water were available ad lib except during withdrawal testing.

Eighty rats were implanted subcutaneously with 2 morphine pellets in the middorsal region under light ether anaesthesia. The pellets were formulated according to the method of Gibson and Tingstad [10], each containing 75 mg of morphine base. Groups of 6 animals were decapitated at 10, 16, 20, 24, 34, 48, 58, 72, and 92 hr after implantation and the blood collected. The dose of morphine given by injection was chosen on the basis of preliminary experiments as that which would be expected to produce the same integral of plasma concentration over time as does the pellet dosage.

For the injection group, morphine sulfate (Mallinckrodt) dissolved in physiological saline was given subcutaneously twice a day (8:00 a.m. and 6:00 p.m.) in the middorsal region of 90 animals on the following dosage schedule: 10 mg/kg body weight at time 0, and 20 mg/kg at 14, 24, 38, 48 and 62 hr (Treatment A). At short time intervals (i.e. 30, 60, 120, and 180 min) after the first, second and fourth injections, groups of 6 animals each were decapitated and blood collected.

In an additional experiment, higher morphine doses were used to inject the animals, according to the following schedule: 10 mg/kg at 0 hr, 20 mg/kg at 14 hr, and 30 mg/kg at 24, 38, 48 and 62 hr (Treatment B). Groups of 6 animals each were sacrificed at 30, 60, 120, and 180 min after the fourth and the sixth injections. Blood was collected in heparinized tubes, and plasma samples obtained by centrifugation were stored in a freezer until analyzed for morphine content.

Determination of Morphine Levels

Morphine plasma levels were measured by the methods of Kupferberg *et al.* [12] and Takemori [16] with minor modifications. The plasma samples (1–3 ml), deproteinated with trichloroacetic acid (5.25% final concentration) and extracted with 10% n-butanol in chloroform, were subsequently reextracted in 1.2 ml of 0.1 N HCl. The oxidation of morphine to pseudomorphine was performed in a 1 ml acid volume with the addition of 1 ml of 0.1M sodium pyrophosphate, pH 8.5, and 0.1 ml ferri-ferrocyanide reagent composed of 57.7 mg $K_3Fe(CN)_6$ and 4.9 mg $K_4Fe(CN)_6$ per 100 ml distilled water diluted 1:10 just prior to use. The fluorometric readings were determined with a Farrand Spectrofluorometer Mark I with excitation wavelength 250 mμ (uncorrected) and emission 430 mμ (uncorrected). Fluorometric readings of serial dilutions of morphine in plasma were linear but did not pass through zero. For this reason, a standard plasma morphine curve was run with each analysis and the fluorometric readings were plotted against concentration. Morphine concentrations for unknown samples were determined by simultaneous graphing with standard samples. The sensitivity of this method was approximately 0.1 μg/ml; its precision, 97 ± 7% of the amount present. The total amount of the drug to which the animals were exposed was ascertained using a Keuffel and Esser compensating polar planimeter to measure the area under the curve.

Precipitation of Withdrawal

The abstinence syndrome was precipitated by the intraperitoneal injection of 1.0 mg/kg of naloxone hydrochloride dissolved in physiological saline. The antagonist was administered 3 hr following the final injection of morphine (65 hr after the initial injection), or 65 hr after implantation for the pellet group. Pellets were not removed prior to testing. Each animal was placed into a Plexiglas test chamber (20 × 26 × 30 cm) covered with transparent red plastic to minimize visual exposure to neighboring animals and to the experimenters. Following a 20 min acclimatization period, naloxone was administered and observations were conducted on a blind basis for a period of 30 min. Animals were weighed prior to naloxone administration and again 1 hr later.

Using modifications of previously reported methods for measuring withdrawal behavior [1, 4, 18], 2 classes of signs were distinguished: counted and checked. Counted behaviors, for which the actual number of occurrences was recorded, include jumping (all 4 paws off the cage floor), teeth chattering (number of episodes), wet-dog shakes (involving whole body), and writhing (abdominal stretching). For checked behaviors, only presence or absence during the 30 min test period was noted. These signs include chromodacryorrhea, diarrhea, eyetwitch, ptosis, rhinorrhea, and salivation. Although a number of additional signs were tabulated (e.g. exploring, grooming, chewing, digging, dyspnea), only those generally accepted as specifically indicative of morphine abstinence were used in assessing the presence and severity of withdrawal.

RESULTS

Figure 1 depicts the temporal course of morphine levels in the plasma of rats treated with the methods used to produce physical dependence. Peak values for morphine in plasma after each of the injections measured were between 3 and 5 fold higher than the maximum level attained with pellet implantation, but morphine disappeared rapidly from the plasma after each injection. From a period beginning approximately 4 hr after a morphine injection until the subsequent administration, the plasma levels of morphine fell beneath the sensitivity of the fluorometric method (0.1 μg/ml) with all 3 injection doses (10, 20, 30 mg/kg) used.

In morphine pellet-implanted rats, a maximum drug plasma level of 0.72 μg/ml is reached after 10 hr, decreasing to a plateau level averaging 0.43 μg/ml maintained between 16 and 72 hr. At 92 hr, plasma levels had dropped markedly and were not accurately measurable.

The total amount of morphine, expressed as μg/ml × hr, to which the animals were exposed after multiple injection treatments or pellet implantation was calculated by determining the area under the plasma concentration of morphine versus time curve and is shown in Table 1. The measured peak levels as well as the calculated half-life values are nearly the same for the second and the fourth injections of 20 mg/kg and for the fourth and sixth injections of 30 mg/kg; for this reason, averaging the areas under the plasma concentration time curve of measured injections

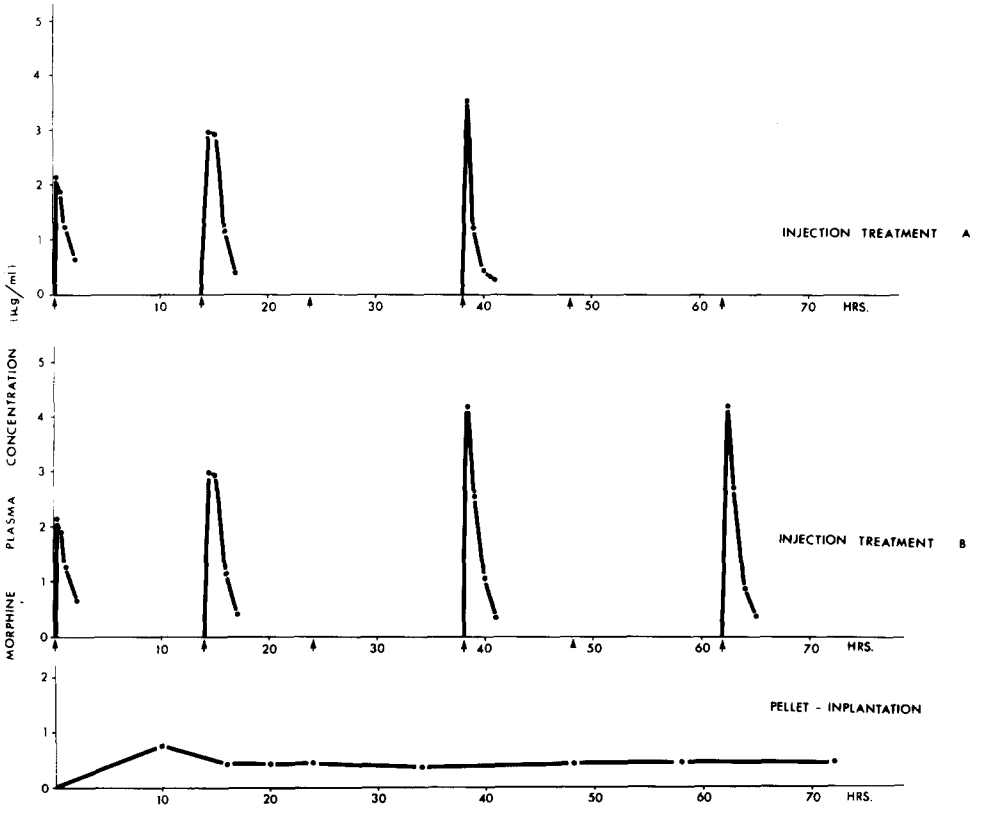

FIG. 1. Morphine plasma concentration in rats after different treatments. All graphed points represent mean values. The number of animals per mean was between 4 and 8 with an average of 6. The arrows on the graph show injection times. Injections (mg/kg) in order given are A: 10; 20; 20; 20; 20; 20, B: 10; 20; 30; 30; 30; 30.

was deemed significantly accurate for estimating the areas of unmeasured injections.

The total areas for injection groups A and B were obtained by combining the calculated and estimated values for all injections in each group. Although the shape of the curve was very different with the 2 methods of morphine administration (i.e. injections and pellet implantation), the areas under the curve representing the total amount of drug in the plasma were similar: injection A treatment (20 mg/kg final dose) resulted in a total area lower only by 9% than the pellet implantation method. To ensure that the small difference in the areas did not account for the difference in our withdrawal results, the dosage of morphine in treatment B was raised to 30 mg/kg final dose. The resulting total area under the plasma concentration time curve was almost 6% higher than that of the pellet implantation method.

In Table 2 the signs generally accepted as most characteristic of the morphine abstinence syndrome and the proportion of rats showing each sign after naloxone precipitated withdrawal are shown.

The pattern of the withdrawal syndrome, as well as the body weight loss, was not significantly different for injection treatments A and B. Consequently, the withdrawal

TABLE 1

TOTAL AREAS UNDER THE MORPHINE PLASMA VS. TIME CURVE IN THE RAT AFTER DIFFERENT TREATMENTS

Treatment	Area (μg morphine/ml x hrs)
1. Morphine Sulfate Injections (0–65 hr)	
A. Final dose 20 mg/kg	25.69*
B. Final dose 30 mg/kg	29.78*
2. Pellet Implantation (0–65 hr)	28.09

*Sum of calculated areas for measured injections and estimated areas for unmeasured injections (see Results.)

TABLE 2

PROPORTION OF RATS SHOWING VARIOUS ABSTINENCE SIGNS FOLLOWING NALOXONE PRECIPITATION OF WITHDRAWAL*

Signs	Pellet Implantation	Injection: Treatment A+B
	N=18	N=26
Jumping	0.94+	0.42
Wet-dog Shakes	0.94+	0.38
Teeth Chattering	0.89	0.61
Writhing	0.50	0.50
Chromodacryorrhea	0.28	0.08
Diarrhea	0.61	0.42
Eye twitch	0.11	0.04
Ptosis	0.94	0.85
Rhinorrhea	0.33	0.15
Salivation	0.44	0.15
Body Weight Loss (Percent±S.D.	9.40±2.04‡	4.37±3.13

*Control animals exhibited none of these signs nor body weight loss.
+Significantly different with chi-square test at $p<0.001$.
‡Significantly different with t-test at $p<0.001$.

data from the 2 experimental injection groups were pooled and compared to the withdrawal signs shown by the pellet-implanted animals.

Both methods, pellet implantation and short-term injection of low doses of morphine, result in narcotic dependence as determined by the appearance of a naloxone-precipitated abstinence syndrome.

DISCUSSION

Judging by the rate of disappearance of the drug from the plasma and the absence of fluorometrically measurable plasma levels several hr following morphine injection, it appears that only exceedingly small amounts of the drug are present in plasma for about 5 hr prior to the next injection of a series. Such a finding does not necessarily imply that there is no morphine present in other body compartments. In fact, it is important to note that our experimental animals did not evidence any overt signs of withdrawal between consecutive injections. Furthermore, nanogram amounts of morphine have been detected in the rat cerebral cortical hemisphere 24 hr after a single 10 mg/kg subcutaneous injection of ^{14}C morphine [15]. In our laboratory, it has been shown that the rate of disappearance of morphine after acute administration in rats is much slower from the brain than from the plasma (unpublished data).

Blasig *et al.* [4] estimated the amount of drug absorbed from pellets implanted subcutaneously by comparing the amount of morphine in 2 intact pellets with that remaining in the subcutaneous depot at various times after implantation. From these data they postulated an absorption pattern that attains a steady state only after several days. The present study does not support this view and actual measurements of morphine plasma levels after pellet implantation show that a constant level is attained in somewhat less than 1 day.

The plasma morphine levels measured after pellet implantation are basically in agreement with the report of Berkowitz *et al.* [3] where the same plasma concentrations were reported 10 hr after morphine pellet implantation. The minor difference in the plateau of morphine concentrations could be explained by the fact that they implanted the 2 pellets at different times.

The precipitated abstinence syndrome resulting from both treatments is qualitatively similar, as reported in an earlier study from this laboratory [6]. The more complete work reported here demonstrates that there are some quantitative differences. We have repeatedly found that the proportion of rats showing each sign is a more consistent measure of withdrawal than the actual number of occurrences of each. This observation is in agreement with Wei [9] and other investigators. The syndrome in pellet-implanted rats results in a greater incidence of jumping and wet-dog shakes and in higher body weight loss than in injected animals. It is possible that variations in treatment procedures, such as anesthesia, surgery, and handling, might account for some part of the differences observed in the incidence and magnitude of withdrawal signs. It is more likely, however, that the quantitative differences arising from the 2 methods of narcotic administration are due to either: (1) unequivalent integrals of brain morphine levels over time; or (2) different shapes of the concentration vs. time curves for morphine in the brain. At present, it is impossible to distinguish between these 2 possibilities. It seems appropriate to conclude that, at equivalent total plasma levels of morphine over a short period of time, continuous exposure to the opiate produces a greater degree of dependence than intermittent exposure.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Ellen Geller, Dr. Chen-ho Lin, and Ida Biunno for their help throughout the course of the study and to Shirley Braverman, Department of Biometrics, for her assistance with the statistical aspects. Naloxone was generously supplied by Endo Laboratories. Morphine pellets were prepared by Dr. William E. Smith of the Philadelphia College of Pharmacy and Science.

REFERENCES

1. Adler, M. W., C. Bendotti, D. Ghezzi, R. Samanin, and L. Valzelli. Dependence to morphine in differentially housed rats. *Psychopharmacologia* **41**: 15–18, 1975.
2. Akera, T. and T. M. Brody. The addiction cycle to narcotics in the rat and its relation to catecholamines. *Biochem. Pharmac.* **17**: 675–688, 1968.
3. Berkowitz, B. A., K. V. Cerreta, and S. Spector. The influence of physiologic and pharmacologic factors on the disposition of morphine as determined by radioimmunoassay. *J. Pharmac. exp. Ther.* **191**: 527–534, 1974.
4. Blasig, J., A. Herz, K. Reinhold and S. Zieglgansberger. Development of physical dependence on morphine in respect to time and dosage and quantification of the precipitated withdrawal syndrome in rats. *Psychopharmacologia* **33**: 19–38, 1973.
5. Buckett, W. R. A new test for morphine-like physical dependence (addiction liability) in rats. *Psychopharmacologia* **6**: 410–416, 1964.
6. Cerletti, C., S. H. Keinath, E. B. Geller, M. M. Reidenberg and M. W. Adler. Relationship between method of morphine administration, plasma levels and the withdrawal syndrome in rats. Proc. 37th Meeting of National Academy of Science – Nat. Res. Council Comm. on Prob. of Drug Dependence, 1975.
7. Cheney, D. L. and A. Goldstein. Tolerance to opioid narcotics: Time course and reversibility of physical dependence in mice. *Nature* **232**: 477–478, 1971.
8. Deneau, G. A. and M. H. Seevers. Pharmacological aspects of drug dependence. In: *Advances in Pharmacology*, Vol. 3, edited by S. Garattini and P. A. Shore. New York: Academic Press, 1964, p. 271.
9. Frumkin, K. Physical dependence in rats after low morphine doses. *Life Sci.* **15**: 455–462, 1974.
10. Gibson, R. D. and J. E. Tingstad. Formulation of a morphine implantation pellet suitable for tolerance-physical dependence studies on mice. *J. pharmac. Sci.* **59**: 426–427, 1970.
11. Goode, P. G. An implanted reservoir of morphine solution for rapid induction of physical dependence in rats. *Br. J. Pharmac.* **41**: 558–566, 1971.
12. Kupferberg, H., A. Burkhalter and E. L. Way. A sensitive fluorometric assay for morphine in plasma and brain. *J. Pharmac.* **145**: 247–251, 1964.
13. Martin, W. R., A. Wikler, C. G. Eades, and F. T. Pescor. Tolerance to and physical dependence on morphine in rats. *Psychopharmacologia* **4**: 246–260, 1963.
14. Maruyama, Y. and A. E. Takemori. The role of dopamine and norepinephrine in the naloxone-induced abstinence of morphine-dependent mice. *J. Pharmac. exp. Ther.* **185**: 602–608, 1973.
15. Misra, A. L., C. L. Mitchell, and L. A. Woods. Persistance of morphine in central nervous system of rats after a single injection and its bearing on tolerance. *Nature* **232**: 48–50, 1971.
16. Takemori, A. E. An ultrasensitive method for the determination of morphine and its application in experiments *in vitro* and *in vivo*. *Biochem. Pharmac.* **17**: 1627–1635, 1968.
17. Teiger, D. Induction of physical dependence on morphine, codeine, and meperidine in the rat by continuous infusion. *J. Pharmac. exp. Ther.* **190**: 408–415, 1974.
18. Way, E. L., H. H. Loh, and F. Shen. Simultaneous quantitative assessment of morphine tolerance and physical dependence. *J. Pharmac. exp. Ther.* **167**: 1–8, 1969.
19. Wei, E. Assessment of precipitated abstinence in morphine-dependent rats. *Psychopharmacologia* **28**: 35–44, 1973.