STUDIES ON PHYSICAL DEPENDENCE INDUCIBLE
BY HOURS EXPOSURE OF MICE TO MORPHINE

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Received for publication May 29, 1972

Long exposure to morphine or its surrogates to humans inevitably results in an intoxicated state which is characterized by two types of morbid phenomena, tolerance and dependence. This state is fully or partially reproducible in some species of laboratory mammals and forms a basis to explore the mechanisms underlying those symptoms and also to screen morphine-like untoward properties of newly developed analgesics in laboratory level.

Recently, Maggiolo and Huidobro (1, 2) have defined abstinence syndrome in morphine-pellet implanted mice. Later, using jumping response as a criterion, Way et al. (3) attempted to quantify the degree of physical dependence, thus adding this animal species to the list of animal models usable in this research field. Further, the utilization of mice seems to have been confirmed in more recent experiments by Cheney and Goldstein (4) and Saelens et al. (5).

In previous papers (6, 7), it was reported that continuous infusion of morphine could develop tolerance in mice as early as a few hours and that concomitant development of physical dependence was detected by the precipitation of abstinence signs with naloxone. To the present, various techniques have been developed in order to morphinize the laboratory animals and, according to the conditions employed, obtained tolerance or dependence differed in degree one from another, however, it is not yet known whether or not their properties also differ. Thus, using various techniques we investigated how rapidly in terms of hour(s) the animal could become sensitive to naloxone, namely physically dependent, and in what aspects the rapidly inducible type of dependence would differ from that after long exposure to morphine.

MATERIALS AND METHODS

Animals used were dd strain male mice weighing 19 to 21 g. Generally, one group of mice included of 5 animals. The drug was dissolved in saline and the dose indicated in terms of salts. Naloxone was given s.c. at the dose level of 10 mg/kg throughout.

Estimation of analgesia and detection of tolerance

The time course and extent of analgesia produced by the drug were followed, as previously described (6). In some experiments, development of tolerance was detected by testing the effect of 10 mg/kg of morphine, a standard dose, which was s.c. given after
analgesia induced by scheduled morphine treatment had almost subsided.

Continuous infusion of morphine or other analgesics

The drug solution or saline alone was infused according to the method previously reported (6). The rate and route of infusion are summarized in Table 1 and duration was 1, 3, 5, 7 or 10 hr. Naloxone was given 30 min following termination of infusion. In some cases, more concentrated solution (30 mg/ml) of morphine was infused at a rate of 112 mg/kg for 1 hr and later, at varying intervals (0.5, 1, 1.5, 2, 2.5 or 3 hr), naloxone was given.

| Drugs         | Route | Concentration of drug solution (mg/ml) | Infusion rate* (mg/kg/hr) |
|---------------|-------|----------------------------------------|--------------------------|
| Morphine HCl  | s.c.  | 10                                     | 35                       |
|               | i.v.  | 10                                     | 35                       |
| Pethidine HCl | s.c.  | 10                                     | 35                       |
|               | i.v.  | 10                                     | 35                       |
| Methadone HCl | s.c.  | 1.5                                    | 52                       |
|               | i.v.  | 4.0                                    | 14                       |
| Pentazocine HCl | s.c. | 25                                     | 87                       |
|               | i.v.  | 35                                     | 122                      |

* Speed of infusion was a constant 0.07 ml/hr.

A single dose of morphine

The animal was given a single dose of 100 mg/kg of morphine i.v. or s.c. The control group were administered saline. At regulated hours (1, 2, 3, 4 or 6 hr), naloxone was injected.

Five hourly repeated injections of morphine

A fixed dose of morphine was s.c. injected hourly for a total of five times. The dosage for a injection was 5, 10 or 20 mg/kg. One hr after the final injection, the animal was given naloxone. In another series of experiments, the analgesic effect of those injected hourly with morphine (10 mg/kg) was partially or completely inhibited by giving nalorphine. Starting 30 min prior to the first hourly dose of morphine, a total of ten injections of nalorphine (dosage 1.0 or 2.0 mg/kg) were given at 30 min intervals. Naloxone was administered as described above.

Ten days treatment with morphine

The method consisted of injecting morphine twice daily at 9 A.M. and 6 P.M. for 9 days. Starting with 10 mg/kg on the 1st day and 20 mg/kg on the 2nd day, the dose was then increased in a daily increment of 20 mg/kg for the next 4 days. A daily dose thus reached 100 mg/kg on the 6th day and thereafter remained constant for the following 3 days. At 9 A.M. on the 10th day, the animal was given 100 mg/kg of morphine as the final dose, and then natural withdrawal was allowed to start or naloxone was administered after 3 hr.
Observation of withdrawal signs and suppression by readministered morphine

Following completion of prescribed treatment, five mice as a group were placed on a circular platform 30 cm in diameter and 45 cm in height. When withdrawal was precipitated with naloxone, observation was usually continued for 2 hr. In the case of natural withdrawal after 10 days treatment, a 20 min observation period started 60 min after the final morphine dose and was repeated totally ten times with a 40 min intermission between each observation. The severity of each behavioral sign was graded as follows depending on either the intensity or the frequency or both; −: no appearance, +: slight, ++: moderate and +++: severe. Suppressive effect of morphine toward developed abstinence signs was tested as follows. When withdrawal was induced by naloxone, 100 mg/kg of morphine was s.c. given just after jumping response started to appear. In the case of natural withdrawal of the 10 day morphinized mice, morphine was readministered 4.75 hr after the final scheduled dose, usually when abstinence signs were reaching the peak in severity. On the other hand, naturally induced signs in short term morphinized mice were weak in intensity and dissipated quickly. No jumping response occurred. Therefore, as soon as any recognizable sign emerged, morphine was given to the one group and saline to the other. The effect was evaluated by comparing the behavior of both groups.

RESULTS

1. Continuous infusion of morphine and naloxone induced withdrawal

During infusion and 30 min after termination thereof, the behavior listed in Table 2 was observed. The Straub tail was specific to the morphine infused group and the group infused with morphine for 10 hr exhibited ataxia. Other tabled behavior appeared commonly in the morphine as well as the saline infused group.

Following the treatment with naloxone, the behavior of the morphine infused group drastically changed in mice which had been given the infusion for only 1 hr and typical withdrawal signs as shown in Table 3 precipitated. The saline infused group remained inert to the treatment. In s.c. infused groups, as reported previously (7), jumping induced by naloxone generally increased in frequency with increasing time of infusion, whereas in the

| Behavior        | Morphine | Methadone | Pethidine | Pentazocine |
|-----------------|----------|-----------|-----------|-------------|
| **During infusion** |          |           |           |             |
| Straub tail      | ++       | ++        | +++       | –           |
| Biting of cage   | +        | +         | +         | +           |
| Urination        | +        | ++        | ++        | +           |
| Defecation       | +        | +         | +         | +           |
| Squeaking        | –        | –         | –         | +           |
| **After infusion** |          |           |           |             |
| Straub tail      | +•       | +         | +         | +           |
| Restlessness     | +        | +         | +         | +           |
| Hypermotility    | ++•      | +         | ++        | +           |
| Hyperreactivity  | +        | +         | +         | +           |
| Ataxia           | –        | –         | –         | ++          |
i.v. treated such a trend was not constantly observed and falling was the more prevailing syndrome (Fig. 1).

During infusion, the mice under a fasting condition continued to lose body weight slowly in a time-dependent manner. Naloxone, unexceptionally caused abrupt weight loss in morphine infused mice but there was no linear correlation between the extent of weight loss and the duration of infusion (Fig. 2). Such an abrupt weight loss did not occur in the saline infused control groups.

**Fig. 1.** Appearance rate of jumping response in mice infused with morphine for various hours and challenged with naloxone.
- • - • : i.v. infused groups, ○ - - ○: s.c. infused groups.

**Fig. 2.** Loss of body weight during morphine infusion and naloxone induced withdrawal.
- • - • : i.v. infused groups, ○ - - ○: s.c. infused group.
The arrow indicates administration time of naloxone.
2. Continuous infusion of dl-methadone, pethidine or pentazocine and naloxone induced withdrawal

During and after infusion of these drugs, mice showed behavior similar to that observed in the morphine infused group (Table 2). In addition, the pentazocine infused groups were characterized by the appearance of squeaking and severe ataxia if the infusion lasted longer than 3 hr. Administration of naloxone induced withdrawal signs including jumping (Table 3) and abrupt weight loss. The appearance rates of jumping response in drug infused groups are shown in Figs. 3-5.

3. One hour infusion of morphine and naloxone induced withdrawal

Fig. 6 shows the time course of analgesia induced by one hr infusion of morphine at the rate of 112 mg/kg/hr. A high level of analgesia persisted for about 3 hr and then gradually disappeared. Ten mg/kg of morphine given 4 hr after infusion induced less analgesic effect than it did in the saline infused controls, showing development of acute tolerance. In addition to the behavior listed in Table 2, some mice exhibited lenticular opacity.

In s.c. infused groups, the appearance rate of jumping plus falling* induced

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**TABLE 3. Naloxone precipitated withdrawal signs in drug infused mice.**

| Signs        | Morphine | Methadone | Pethidine | Pentazocine |
|--------------|----------|-----------|-----------|-------------|
| Restlessness | ++       | ++        | ++        | ++          |
| Urination    | ++       | +         | ++        | ++          |
| Defecation   | ++       | +         | ++        | +           |
| Hyperreactivity | ++   | +         | +         | +           |
| Rearing      | ++       | +         | ++        | +           |
| Sniffing     | ++       | +         | +         | +           |
| Peeping below | ++      | ++        | ++        | +           |

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**FIG. 3. Appearance rate of jumping response in mice infused with methadone for various hours and challenged with naloxone.**

- - - - : i.v. infused groups, - - - : s.c. infused groups.

* During the course of this study, it was observed that almost all cases of falling resulted from an unsuccessful attempt to jump off the edge of the platform, so thereafter the appearance rate of jumping plus falling was employed so as to include the times in which the animal did not jump but fell.
Fig. 4. Appearance rate of jumping response in mice infused with pethidine for various hours and challenged with naloxone.
- - - : i.v. infused groups,  o---o : s.c. infused groups.

Fig. 5. Appearance rate of jumping response in mice infused with pentazocine for various hours and challenged with naloxone.
- - - : i.v. infused groups,  o---o : s.c. infused groups.

by naloxone reached the peak 1.5 hr after infusion and later tended to decrease (Fig. 7). In contrast, any mouse in the i.v. infused groups did not exhibit jumping response. Other withdrawal signs were observed to appear equally in both groups.

4. A single dose of morphine and naloxone induced withdrawal

The time course of analgesia resulting from a 100 mg/kg of administered morphine is illustrated in Fig. 8. The test dose given at time 6 evidenced development of acute tolerance. When naloxone induced withdrawal started at varying times after the morphine injection, the rate of jumping plus falling reached the peak 3 hr after i.v. injection or 1 hr after s.c. injection of morphine (Fig. 9). Concerning other signs, there was no difference between the two groups.
5. **Five hourly injections of morphine and naloxone induced withdrawal**

By repeating hourly injections of morphine at three dose levels, graded degrees of analgesia were maintained for 5 hr as shown in Fig. 10. Naloxone, which was given 1 hr after each final dose of morphine, elicited typical withdrawal signs listed in Table 3 but jumping response was counted only in the groups which had been given 10 mg/kg or more of morphine at one time. In the groups for which 10 mg/kg was the hourly injected dose, extension of the interval between the last dose and naloxone treatment to more than 2 hr
FIG. 8. Time course of analgesia induced by a single dose of morphine and development of acute tolerance. 100 mg/kg of morphine was given i.v. (•—•) or s.c. (○—○) at time 0. The arrow indicates the time when the test dose of morphine was given.

FIG. 9. Appearance rate of jumping plus falling in mice treated with a single dose of morphine and various times later challenged with naloxone. 100 mg/kg of morphine was given i.v. (•—•) or s.c. (○—○) at time 0.

resulted in considerable alleviation of withdrawal syndrome, showing that once developed dependence would dissipate quickly.

6. **Five hourly injections of morphine in the presence of nalorphine and the withdrawal signs induced by naloxone**

In the experiments shown in Fig. 11, mice were exposed to morphine for 5 hr as described above but in the presence of nalorphine. To the group in which no overt effect of morphine was allowed to appear, naloxone failed to elicit any withdrawal sign. When morphine analgesia was only partially suppressed, however, withdrawal signs including jumping were precipitated.
FIG. 10. Analgesia and naloxone precipitated jumping in mice given five hourly injections of morphine. The arrow indicates the injection time of morphine. ○ ○ : 20 mg/kg, × × : 10 mg/kg, • • : 5 mg/kg. Naloxone was given at time 5. The fraction in parentheses shows No. of mice which jumped per No. of mice tested.

FIG. 11. Analgesia and naloxone precipitated jumping in mice given five hourly injections of morphine in the presence of nalorphine. The solid lined arrow indicates the injection time of morphine and the broken one that of nalorphine. ○ ○ : 5 × 10 mg/kg of morphine, × × : 5 × 10 mg/kg of morphine and 10 × 1.0 mg/kg of nalorphine, • • : 5 × 10 mg/kg of morphine and 10 × 2.0 mg/kg ofnalorphine. Naloxone was given at time 5. The fraction in parentheses shows No. of mice which jumped per No. of mice tested.
7. Ten days treatment with morphine and the withdrawal signs, natural or induced by naloxone

Immediately after the last injection on the 10th day morning, various behavioral effects of morphine, such as Straub tail and hypermotility, appeared, but within 2 hr practically disappeared. Approx. 3 hr later, withdrawal signs began to emerge and became maximal in severity in 5 to 6 hr. When withdrawal was induced in advance by giving naloxone 3 hr after the last morphine dose, vigorous abstinence signs were sharply elicited within minutes but, in this case, waned quickly after continuation for only approx. 1 hr.

8. Suppression by readministered morphine of abstinence syndrome

Regardless of the morphinization method used, the naturally induced abstinence syndrome was completely suppressed by readministered morphine. On the other hand, in the case of naloxone induced withdrawal, readministered morphine failed to be completely effective either in suppressing or in masking the abstinence signs (Table 4).

**Table 4. Comparison of abstinence syndrome observed in groups of mice morphinized under various conditions.**

| Methods of morphinization | Natural withdrawal | Naloxone induced withdrawal |
|---------------------------|--------------------|----------------------------|
|                           | Abstinence signs   | Masking by morphine*       | Abstinence signs | Masking by morphine* |
| Ten days treatment        | +++ (4/5)**        | complete                   | +++ (4/5)       | partial               |
| Seven hours infusion      | + (0/5)            | complete                   | ++ (3/5)        | partial               |
| Five hourly injections    | + (0/5)            | complete                   | +++ (4/5)       | partial               |
| A single injection        | + (0/5)            | complete                   | +++ (4/5)       | partial               |

* The dose of morphine was 100 mg/kg.

** Within each column, the relative intensity of abstinence syndrome was expressed as compared to that exhibited by the ten days morphinized group which was taken as ++++. No. of mice which jumped per No. of mice tested are shown in parentheses.

**DISCUSSION**

The obtained results suggest that exposure of mice to morphine in terms of hour(s) could develop physical dependence to a detectable degree. All morphinization methods used here proved capable of producing an intoxicated state identifiable as physical dependence. Ten mg/kg of naloxone which followed the morphine treatment precipitated abnormal behavior; jumping, falling, biting, restlessness, backward locomotion, etc. Behavior was comparable to that defined by Chilean investigators (1, 2) as abstinence signs in morphine-pellet implanted mice could not be distinguished from those which we observed to appear on natural withdrawal of 10 days morphinized mice.

Without naloxone treatment, however, rapidly morphinized mice seldom exhibited potent withdrawal signs. Way et al. (3) reported that jumping response did not appear when the morphine pellet had been removed 12 hr or earlier after implantation, but they also
described that the response could be elicited with naloxone as early as 3 hr after implanting morphine and that afterward the \( ED_{50} \) of naloxone necessary to precipitate the response continued to decrease progressively. Though the \( ED_{50} \) of naloxone was not estimated herein and haloxone was extensively used at a fixed and large dose level, it was constantly observed that the longer exposure to morphine resulted in a higher rate of jumping and falling responses. In addition, evidence was obtained to demonstrate that dependence produced by short-term morphinization would disappear more quickly than it did in those mice. Therefore, a weak response of shortly morphinized mice toward natural withdrawal under the 10 day treat may be explained as follows; the duration of exposure to morphine is not long enough to render mice highly dependent, but the event(s) which, on further continued exposure, could increase to potential dependence, had indeed been initiated. An alternate possibility however cannot be excluded. Apart from a rapidly inducible type of dependence, there is another type of dependence which is inducible only after a much longer exposure to morphine.

Another discrepancy observed was that the naloxone induced syndrome in shortly morphinized mice could not be masked completely by readministrating 100 mg/kg of morphine, while the same dose completely suppressed naturally induced signs in the 10 day morphinized mice. This is however, considered to be due to administration of naloxone in a relatively large dose, 10 mg/kg, which is potent enough to nullify the effects of 100 mg/kg of morphine. In fact, naloxone induced syndrome in 10 days treated mice was not completely suppressed by the dose of morphine.

In this experiment, other analgesics were compared with morphine as to the dependence liability. It was concluded that methadone and pethidine are potent and comparable to morphine dependence liability while pentazocine had a rather weak potency. The results seem to be in good agreement with those obtained by other investigators experimentally (5, 8, 9) and clinically (9).

In order to morphinize mice rapidly, three methods were tested here. Firstly, the continuous infusion method was employed since morphine could be administered at a constant rate for a desired period. Insofar as it is carried out using our methods, confinement of the animal under restricted conditions would induce unwanted stress, however, the results obtained using this method, allowed us to assume that detectable dependence might be inducible if analgesia above a certain level was maintained more than 1 hr. Thus a single injection method was tested at various dose levels. An unreasonably high and rather toxic dosage was found to be required in order to produce a detectable degree of dependence. Those problems, being identical to the above could be solved by using the five hourly injection method in which needless stress is avoided and the dose given at one time is not unreasonably high. Thus, in order to test dependence liability of the drug and to economize on time, five hourly injections and evaluation of naloxone induced behavior would be the method of choice. In addition, the single suppression test using 10 days morphinized mice has proved to be useful for the same purpose details of which are to be published elsewhere.
SUMMARY

The rapidity with which the state identifiable as physical dependence could be induced in mice in terms of hour(s) was investigated as well as the aspects in which such an rapidly inducible type of dependence would differ from that obtained after a much longer exposure.

After many hours exposure to morphine by continuous infusion, a single dose or five hourly repeated doses, the animal responded to injection of naloxone in a specific manner, exhibiting a number of behavioral signs; jumping, falling, biting, restlessness, backward locomotion, etc. These signs were comparable to those identified as abstinence syndrome in this animal species and could not be distinguished in variety. In some cases the intensity also was indistinguishable from that observed to appear on natural withdrawal of the 10 day morphinized mice.

Though the possibility cannot be denied that the type of physical dependence may depend on the methods used to morphinize the animal, the obtained results appear to suggest that at least a type of physical dependence can be produced in mice as early as a few hours if during that period a certain level of analgesia is maintained.

Acknowledgement: The authors wish to thank Sankyo Pharmaceutical Co., Ltd. for provision of naloxone and pentazocine.

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