PHYSICAL DEPENDENCE ON MORPHINE, PHENOBARBITAL AND DIAZEPAM IN RATS BY DRUG-ADMIXED FOOD INGESTION

Saizo YANAURA, Eijiro TAGASHIRA and Tsutomu SUZUKI
Department of Pharmacology, Hoshi College of Pharmacy, Shinagawa-ku, Tokyo, Japan
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Abstract—To produce physical dependence on morphine, phenobarbital and diazepam in rats, these drugs were mixed with the powder form of rat food in concentrations of 0.5 mg/g, 1 mg/g and 2 mg/g of food. One group of rats (the lower dose group) was continuously exposed for 1 week to two morphine-admixed foods with morphine to food ratios of 0.5 mg/g and 1 mg/g in a cage. The other group (the higher dose group) could choose between two morphine-admixed foods with morphine to food ratios of 1 mg/g and 2 mg/g. After 1 week, morphine-admixed foods were replaced with morphine free food for 2 days. Both groups of rats showed greatly reduced body weight and food intake after the first 24-48 hr withdrawal. The body weight decrease was greater for rats in the higher dose group. Control groups of morphine dependent rats were kept on the morphine added food diets and showed the same body weight increase as well as normal control rats during the course of these experiments. Physical dependence on phenobarbital and diazepam was produced using the same dosage schedules as with morphine. Both the lower and higher dose groups showed significant decrease in body weight due to withdrawal after 1 week of drug-food exposure. Levallorphan (0.5, 1, 3 and 5 mg/kg, s.c.) administered to morphine dependent rats had dose-dependent effects on the intensity of abstinence symptoms (e.g., diarrhea, piloerection and wet shakes phenomena), maximal decrease in body weight and duration of decreased body weight. Cross-physical dependence between phenobarbital and diazepam was demonstrated by this method.

Animals can be made drug dependent quickly and economically for utilization in studies on drug dependence and screening tests of drug dependence. The growth curve of body weight and total daily food intake should not be allowed to become depressed during the drug administration period for detection of drug dependence liability using decrease in body weight as indicator of abstinence symptoms. Hosoya (1) first reported physical dependence liability of morphine in rats using body weight decrease by withdrawal as indicator of abstinence symptoms. In that case, morphine was injected subcutaneously twice daily for 5 weeks using a gradually increasing dosage schedule. The acquisition of morphine type drug dependence by means of other than the daily injection method in mice (2) and rats (3-6) has been reported. Nichols et al. (7, 8), Davis and Nichols (9) and Kumar et al. (10) reported morphine dependence development and preference formation in rats with morphine dissolved in water, either forcibly or ad libitum supplied. Goode (3) developed a method of subcutaneous reservoir of morphine type drugs. Rapid induction of physical dependence on morphine by both direct morphine administration and by drug substitution for morphine in morphine dependent rats (cross-physical dependence test) as well as by administration of an opiate antagonist to morphine dependent rats was confirmed by this...
same author. Recently, Risner and Khavari (11) reported morphine type drug dependence in rats by administering drug-adulterated food for 5–10 days. In their report, rats exhibited decreased body weight and decreased total daily food intake during the administration period. Crossland and Leonard (12) and Essig (13) reported that rats (which had previously been reported as dying during the administration period) developed physical dependence on sodium barbital if the fluid intake was restricted to solutions containing gradually increasing concentrations of the drug. Essig (13) stated that previously unsuccessful attempts to induce physical dependence of rats on barbiturates were based on the single daily dose method of drug administration and that the continuous availability of sodium barbital and its more frequent intake favors the development of physical dependence. Kamano and Arp (14), Harris et al. (15) and Götestam (16) reported dependence on chlordiazepoxide and medazepam in rats without using the daily injection method. In a previous paper (17) we reported that rats developed physical dependence on phenobarbital, diazepam and chlordiazepoxide by the daily injection method, but it takes longer to obtain dependent rats using this method. Phenobarbital and diazepam have poor solubility in water. Furthermore, our experiments with chronic toxicity showed that daily water intake varied with the individual animal and it was no easier measuring the daily drug intake by this method than by the food admixed method. In the present study, we used drug-admixed food to produce physical dependence on morphine, phenobarbital and diazepam by continuous exposure of the animals to these agents.

MATERIALS AND METHODS

Each group of rats included 6 male and 6 female Sprague-Dawley JCL strain rats, 5–7 weeks of age. Rats were housed individually in cages (21 cm × 25 cm × 15 cm) with 2 food containers equi-distant from the water bottle (Fig. 1). The position of the food containers was changed daily and food and water was provided ad libitum. Body weight and total food intake were measured daily between 10:00 a.m.-12:00 noon. Phenobarbital, diazepam and morphine were used in this experiment. Each drug was admixed with rat food (CA-1 powder, CLEA Japan Inc., Tokyo) at drug/food ratios of 0.5 mg/g, 1 mg/g and 2 mg/g. Preliminary studies indicated normal body weight increase and food intake in animals fed these ratios. High drug concentration depressed body weight increase and food intake due to either to the bad taste or to toxicity of the drug. Actual daily drug intake was calculated in mg/kg of body weight/day and mg/day and the higher dose level preference

Fig. 1. Apparatus for induction of drug dependence in rats. Rats were housed individually in a cage (21 cm × 25 cm × 15 cm) with 2 food containers and were continuously exposed to the drug-admixed powder food in different drug/food ratios, e.g., 0.5 mg/g vs. 1 mg/g, or 1 mg/g vs. 2 mg/g. The water bottle was set equi-distant from each of containers.
DEPENDENCE INDUCTION IN RATS

The rats were allowed to choose between the lower and higher dose level diet for 1 to 3 weeks. Non-drug added food was then provided for 48 hr to observe abstinence symptoms. When levallorphan (0.5, 1, 3 and 5 mg/kg, s.c.) was administered to morphine dependent rats being fed morphine-admixed foods, abstinence symptoms, body weight and food intake changes and preference were observed at 2 hr intervals during the first 24-48 hr. Morphine dependent rats unchallenged by levallorphan served as the control. Measurements of both the control and experimental animals were made concurrently at 2 hr intervals during the first 24-48 hr of levallorphan injection.

Physically dependent rats for the phenobarbital-diazepam cross-physical dependence test were produced by ad libitum ingestion of phenobarbital-admixed foods (1 mg/kg and 2 mg/kg) for 2-3 weeks followed by substitution of diazepam-admixed food (1 mg/g and 2 mg/g) for 24-48 hr. Body weight changes and food intake were measured at 2 hr intervals from 6:00 p.m. to 6:00 a.m.

RESULTS

Fig. 2 shows the mean body weight and food intake changes during morphine-admixed food ingestion at ratios of 0.5 mg/g and 1 mg/g for the lower dose group, and 1 mg/g and 2 mg/g for the higher dose group. After 1 week administration, non-morphine added food was provided (withdrawal phase). The average maximal body weight decrease for the lower dose group was 11.6±2.4% for males and 8.3±2.2% for females and was observed on the second day of withdrawal. Other physiological symptoms were reduced food intake and water intake during the first 24-48 hr. The faeces were moist and occasionally unformed. The food intake decrease was 67.6-70.5% in both sexes in parallel with body weight loss. In the higher dose group the average maximal body weight decrease was 12.8±1.6% for males and 8.0±1.4% for females and the food intake decrease was 91.7± for males and 60% for females. Behavioral symptoms of withdrawal such as 'wet dog' shakes and occasional aggression on handling were observed 24 hr after withdrawal. During the 1 week of administration, the mean total daily morphine intake was 40-70 mg/kg/day in the lower dose group and 80-120 mg/kg/day in the higher dose group. Almost all rats preferred the lower level of morphine in admixed food (0.5 mg/g for the lower dose group and 1 mg/g for the higher dose group). This was due mainly to the taste of the drug in the food. When morphine-admixed food was re-administered after the 48 hr withdrawal, body weight and food intake recovered to nearly pre-withdrawal level during the first 24 hr. During the 3 weeks administration period, significant growth curve and total daily food intake reductions were not observed in the experimental animals compared to the untreated control group of rats. Individual variations in decreased

(preference rate) was calculated as follows:

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\text{Preference rate (\%)} = \frac{B}{A + B} \times 100
\]

\[
A + B = \text{Total daily food intake}
\]
FIG. 2. Changes in mean body weight, total daily food intake and daily morphine intake of four groups of rats (n=6). Rats ingested 2 different doses of morphine-admixed foods (0.5 mg/g vs. 1 mg/g and 1 mg/g vs. 2 mg/g) for 1 week. Then the drug-admixed food was replaced by drug-free food for 2 days, while the control morphine dependent rats were continuously fed morphine-admixed food. Figs. A and C show mean body weight, total food intake and daily morphine intake of the lower dose groups of male and female rats, respectively. Figs. B and D show mean body weight, total food intake and daily morphine intake of the higher dose groups of male and female rats, respectively. After 2 days withdrawal, the normal food was replaced by the same dose of morphine-admixed foods as previously given. - - - - , control (non-treated) groups of rats. - - - - , control morphine dependent rats. - - - - - - - - groups of morphine withdrawal for 2 days.

Body weight and food intake due to withdrawal were minor, and almost all rats (n=6) showed parallel decreases.

In levallorphan (0.5, 1, 3 and 5 mg/kg, s.c.) administration to morphine dependent rats fed morphine-admixed foods, abstinence symptoms (e.g., piloerection, diarrhea, muscle rigidity and 'wet dog' shakes phenomena) and sudden loss of body weight appeared after 15–30 min, and the maximal decrease in body weight was observed during the 2 to 4 hr period after injection. Larger doses of levallorphan tended to cause greater decreases in body weight and longer duration of abstinence symptoms, but the time of onset of abstinence symptoms and maximal decrease in body weight was almost the same as

FIG. 3. Time course changes in mean body weight (calculated as percentage of the pre-precipitation level) during the first 30 hr after injection of levallorphan (0.5, 1, 3 and 5 mg/kg, s.c.) to 4 groups of morphine dependent rats (n=6). The 4 groups of experimental rats could choose between 2 different morphine-admixed food (0.5 mg/g and 1 mg/g) for 17 days prior to injection. Levallorphan was injected at 2:00 p.m.
for the lower doses. Maximal body weight decrease, moreover, was shown to be dose-dependent between 0.5 to 5 mg/kg of body weight (Fig. 3). There were some differences between precipitation caused by levallorphan and natural withdrawal. Abstinence symptoms were observed to occur more rapidly and severely in the former, but the maximal decrease in body weight was more intense in the latter. Un-challenged morphine dependent control rats showed less than 1% body weight decrease after 24 hr.

Fig. 4 shows that phenobarbital was ingested with the food at ratios of 0.5 mg/g and 1 mg/g (the lower dose group), and 1 mg/g and 2 mg/g (the higher dose group). Each rat was individually caged for 1 week, then the admixed food was removed for 48 hr to observe abstinence symptoms. Maximal body weight and food intake decrease were observed during the second day of withdrawal, and body weight decrease was 6.1±1.8% for males and 2.5±0.8% for females in the lower dose group, and 10.4±3.9% for males and 6.9±2.3% for females in the higher dose group. During administration, the mean total daily drug intake was 120-130 mg/kg/day in the lower dose group and 170-200 mg/kg/day in the higher dose group. No decrease in body weight or food intake was observed in any of the rats during phenobarbital administration. No preference for the higher pheno-
barbital level (1 mg/g in the lower dose group and 2 mg/g in the higher dose group) was observed. During the first 1–2 days administration of phenobarbital, behavioral changes such as sedations, staggering gait and loss of righting reflex appeared in some rats. These behaviors were tolerated following repeated intake of phenobarbital. When phenobarbital-admixed food was re-administered after the 48 hr withdrawal period, body weight and food intake recovered almost to the pre-withdrawal level after the first 24 hr, but not to the level of dependent control rats which did not undergo withdrawal. Individual variations in body weight and food intake decrease during withdrawal were minor and all rats showed the same withdrawal response. Fig. 5 shows the results of 48 hr diazepam substitution for phenobarbital in phenobarbital dependent rats (cross-physical dependence test). When phenobarbital-admixed foods (1 mg/g and 2 mg/g) were replaced by diazepam-admixed foods (1 mg/g and 2 mg/g), loss of body weight and food intake decrease apparent during phenobarbital withdrawal were completely suppressed by ingestion of 35 mg/day of diazepam (nearly the same as the total daily phenobarbital ingestion). Diazepam caused almost the same changes in body weight as phenobarbital in phenobarbital dependent rats, as shown in Fig. 5. The increase in body weight after the first 12–24 hr of diazepam substitution was greater than for phenobarbital dependent rats continually fed phenobarbital.

![Fig. 5](image)

**Fig. 5.** Time course changes in mean body weight at 2 hr intervals during the first 24 hr of diazepam substitution for phenobarbital in phenobarbital dependent rats. Control phenobarbital dependent rats were continuously fed phenobarbital-admixed food. The 3 groups of experimental rats were fed 2 phenobarbital-admixed foods (1 mg/g vs. 2 mg/g) for 2 weeks and one group of rats was fed diazepam (1 mg/g vs. 2 mg/g) instead of phenobarbital for 24 hr. – – –, control rats. – – –, diazepam dependent control rats. – – –, diazepam substitution in phenobarbital dependent rats. – – –, natural withdrawal of phenobarbital.

Fig. 6 shows the development of physical dependence on diazepam for both the lower and the higher dose groups after 1 week ingestion of diazepam-admixed foods (0.5 mg/g and 1 mg/g, and 1 mg/g and 2 mg/g). During the 1 week administration period, the mean total daily drug intake was 60–90 mg/kg/day for the lower dose group and 110–150 mg/kg/day for the higher dose group for both sexes. A no growth curve and food intake reduction were observed. After 1 week of diazepam administration, the maximal body weight
Fig. 6. Changes in mean body weight, total food intake and total daily diazepam intake of 4 groups of rats (n = 6). Rats could choose between 2 different doses of diazepam-admixed foods (0.5 mg/g vs. 1 mg/g and 1 mg/g vs. 2 mg/g) for 1 week, then the drug-admixed food was replaced by drug-free food for 2 days. The control diazepam dependent rats were fed diazepam-admixed foods continuously. Figs. A and C show mean body weight, total daily food intake and total daily diazepam intake of the lower dose groups of male and female rats, respectively. Figs. B and D show mean body weight, total daily food intake and total daily diazepam intake of the higher dose groups of male and female rats, respectively. After 2 days withdrawal, the drug-free food was replaced by the same dose diazepam-admixed food as before.

and food intake decrease during withdrawal was observed after the first 24 hr and was 3.6±1.9% for males and 2.6±1.1% for females in the lower dose group and 7.9±1.2% for males and 8.0±1.9% for females in the higher dose group. The decrease in food intake paralleled the loss in body weight except for female rats in the lower dose group and was about 50% that of the pre-withdrawal level. Rats preferred the lower level of diazepam admixed in food. This was attributed to the bad taste imparted by the drug. When diazepam-admixed foods were re-ingested after a 48 hr withdrawal period, body weight and food intake nearly recovered to the pre-withdrawal level after 24 hr. Individual variations in body weight and food intake decrease due to diazepam withdrawal were larger than those caused by morphine withdrawal. Phenobarbital-admixed food (1 mg/g and 2 mg/g) replacement of diazepam-admixed food (1 mg/g and 2 mg/g) for 48 hr in diazepam dependent rats, caused no significant withdrawal signs during the substitution period. There was no loss of body weight or food intake when diazepam was replaced in the diet by 30-40 mg/day of phenobarbital. This was approximately the total daily diazepam intake prior to the substitution. As shown in Fig. 7, phenobarbital had a similar effect to diazepam on the body weight of diazepam dependent rats.
DISCUSSION

To study drug dependence and develop routine screening for dependence liability in rats, we applied the drug-admixed food ingestion method to easily and quickly cause morphine, phenobarbital and diazepam dependence in rats. Recently, small animals have been used for dependence studies of morphine (18, 19) and barbiturates (20, 21) because of simpler handling and less cost. Disadvantages of other procedures to produce drug dependent rats include surgical stress (3–6) and solvents necessary for water insoluble drugs (12, 13). The drug-admixed food ingestion method is a simple, inexpensive and effective way to obtain the same degree of morphine dependence as the Goode or Essig procedures which also induce physical dependence on morphine or barbiturates in rats without depressing the growth curve and total daily food intake during 7 days of morphine administration.

When levallorphan (0.5, 1, 3 and 5 mg/kg, s.c.) was injected into morphine dependent rats, abstinence symptoms were observed and the decrease in body weight indicated a dose-dependent relationship in this dosage range as reported by others (6). Goldberg et al. (22) reported that small doses of nalorphine (30–300 mg/kg) and naloxone (3–10 mg/kg) markedly increased the rate of i.v. morphine self-administration in morphine dependent rhesus mon-
keys. In the present experiment, preference for a higher food level due to levallorphan injection was not observed. This was probably due either to the shorter period of morphine administration or anorexia produced by precipitation of withdrawal. However, during forced morphine administration of two food dose levels (0.5 mg/g vs. 1 mg/g) for 3 weeks, rats gradually preferred the higher level of morphine-admixed food (1 mg/g). This was probably due to the positive reinforcing properties of morphine and spontaneous intake of effective concentration of morphine/food (Fig. 8). When rats were allowed free access to two normal foods in a cage for 2 months, the daily food intake from each container was approximately the same even when the container position was changed every day. Nichols et al. (7, 8), Davis and Nichols (9) and Kumar et al. (10) have reported that morphine dependent rats prefer morphine solution (0.5 mg/ml of water) to plain water in the two bottle choice method and that unpremedicated rats also drank more morphine solution than water. These experiments suggest that morphine has both positive and negative reinforcing properties in rats.

Recently, Risner and Khavari (11) reported that morphine dependence in rats could be induced in 5 days by providing morphine-adulterated food (4 mg/g of food) and sucrose-morphine (1 mg/ml). In their report, the growth curve and food intake were apparently suppressed after morphine-adulterated food and sucrose-morphine were administered. This was probably due to excessive daily morphine intake or the bad taste of food imparted by the high morphine concentration. Their data suggest that it is important to select sufficient and non-toxic concentrations of the drug to acquire drug dependent rats through either drug-admixed food ingestion or supplying drug-dissolved liquid. In the screening test of drug dependence, drugs which are non-effective when given orally and/or have too repulsive a taste of drug-admixed food (or water) to maintain a normal level of food intake (or water intake), dependence liability by these ingestion methods could not be detected. In the morphine-admixed food ingestion method, morphine dependence in rats could be induced by 1 week of drug administration and the decrease in body weight during withdrawal was gradually intensified during the 6 weeks of experiment. This is attributed to the preference for the higher level of morphine-admixed food as tolerance developed.

Crossland and Leonard (12) first demonstrated physical dependence formation on sodium barbital in rats. In their procedure, rats were forced to increase their sodium barbital intake from 50 to 280–400 mg/kg/day over 4 to 5 weeks. Essig (13) also reported in detail that after gradually increasing the drug concentration abstinence symptoms of barbital were evident. He suggested that continuous availability of a drug and the resulting frequent intake favors the development of physical dependence. In our present experiment, the drug concentration in admixed food was not increased during the administration period. However, phenobarbital and diazepam dependence in rats was more rapid and with a greater degree of physical dependence than that by the daily injection method despite the shorter administration period. Since the rats had ad libitum access to drug-admixed foods, we assume that having two different drug-admixed food concentrations in a
cage contributes to maintain dependent states in rats without the appearance of abstinence symptoms during the administration period. Phenobarbital and diazepam treated rats showed no significant preference for the higher drug level in food and tended to a gradual decrease in the total daily drug intake (mg/kg/day) during the 3 week administration period. This suggests that the drug concentration in food should forcibly increase to non-toxic doses for the acquisition of rats made dependent on phenobarbital or diazepam, and for long term maintenance of an adequate dependent state.

The cross-physical dependence test between phenobarbital and diazepam was administered by the drug-admixed food ingestion method. Nozaki and Hosoya (23) and Nurimoto (24) previously reported on substitution of morphine type drugs and barbiturate-alcohol type drugs for morphine in morphine dependent rats and the effect of these two drug types on the body weight of morphine dependent rats measured at 2 hr intervals during the first 8 hr after substitution. Substitution of diazepam for phenobarbital or phenobarbital for diazepam, in our present study, began at 6:00 p.m. and calculations of body weight and food intake were done at 2 hr intervals during the 24 hr immediately following substitution. Figs. 5 and 7 show the results of the cross-physical dependence test. The maximal increase in body weight was observed from 8:00-10:00 a.m. in phenobarbital and diazepam dependent rats as well as in the dependent control rats. In the case of complete substitution, the body weight change curve showed the same pattern as that for dependent control rats and the maximal body weight increase was also observed between 8:00-10:00 a.m. with nearly the same weight gain as for control dependent rats. However, during withdrawal of phenobarbital and diazepam, body weight change curves for 24 hr showed quite a different pattern from that for dependent control rats. This data suggests that time course changes in body weight during the 24 hr immediately following substitution are also a useful screening method for drug dependence liability in rats.

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