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Genetic Differences in the Development of Physical Dependence upon Diazepam in Lewis and Fischer 344 Inbred Rat Strains

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SUZUKI, T., M.-S. LU., H. MOTEGI, T. YOSHII AND M. MISAWA. *Genetic differences in the development of physical dependence upon diazepam in Lewis and Fischer 344 inbred rat strains.* PHARMACOL BIOCHEM BEHAV 43(2) 387–393, 1992. – The purpose of the present study was to investigate physical dependence upon diazepam systematically in two inbred strains of rats, Lewis (LEW) and Fischer 344 (F344). Rats were chronically fed food containing diazepam on an escalating drug dosage schedule, from 1 and 2 to 12 mg/g of food, over a period of 30 days. During treatment, the growth curve in LEW and F344 rats was suppressed compared with the respective controls. Motor incoordination was evaluated by a rotarod performance test. The ranking of the motor incoordination during the final concentration of diazepam was as follows: F344 > LEW. After substitution of normal food for the diazepam-admixed food, various signs of diazepam withdrawal occurred 16–120 h later. These signs included vocalization, irritability, muscle rigidity, ear-twitching, Straub's tail, piloerection, fascicular twitch, tremor, convulsion, and death. The incidences of vocalization, ear-twitching, piloerection, and tremor in F344 were significantly higher than those in LEW rats. Furthermore, two of six F344 rats showed spontaneous convulsions and one rat died of convulsions. Overall withdrawal scores were significantly greater in F344 (16.0) than in LEW (6.3) rats. These results suggest that diazepam withdrawal severity is strongly influenced by genetic factors, and F344 rats are highly susceptible to dependence upon benzodiazepines.

Diazepam Physical dependence Lewis rat Fischer 344 rat Genetic difference Drug-admixed food method

THERE have been several genetic studies of susceptibility to benzodiazepines. Gallaher et al. (10) demonstrated that mice could be selectively bred to separate into two distinct lines, diazepam resistant (DR) and diazepam sensitive (DS), using diazepam-induced rotarod impairment. Furthermore, the Maudsley reactive (MR) and nonreactive (MNR) rat strains were selectively bred by Broadhurst for differences in open-field defecation (2). Rats of the MNR strain exhibited a significantly greater anticonflict effect following diazepam or alprazolam treatment than did rats of the MR strain (3). Thus, the results of a number of studies suggest that genetic variables affect behavioral responses to benzodiazepines (3,5,8,10,19).

Genetic differences in physical dependence upon benzodiazepines have not been reported because induction of physical dependence upon benzodiazepines in rodents is difficult. We developed a drug-admixed food (DAF) method for assessing physical dependence upon drugs in rats (28). Others have utilized dietary administration of diazepam to demonstrate development of dependence in rats (9,12). As a result, rats, like dogs and monkeys, can be shown to be suitable experimental animals for studying early stages of dependence liability, and the administration of DAF is a useful method for inducing physical dependence upon opioids (21,29) and barbiturates (31). However, the incidence of severe physical dependence manifested by withdrawal convulsions has not been shown with short-acting barbiturates, for example, pentobarbital, or benzodiazepines (22,30) in rats. Therefore, we developed an escalating dose schedule for inducing physical dependence on short-acting barbiturates (20), nonbarbiturates, and benzodiazepines in rats. With this procedure, animals became severely dependent upon pentobarbital (27) and methaqualone (24,25) and subsequent withdrawal convulsions could be detected.

Recently, we found that ethanol serves as a strong positive reinforcer for Lewis (LEW) rats but a weak positive reinforcer for Fischer 344 (F344) rats (23). Furthermore, we reported that F344 rats treated with pentobarbital-admixed food showed mild withdrawal signs in comparison with pentobarbital-treated LEW rats (26) and that withdrawal signs from

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chronic ethanol or barbital were significantly greater in F344 than in LEW rats (28).

The main purpose of the present study was to demonstrate a method for producing severe physical dependence upon diazepam characterized by spontaneous convulsions during withdrawal in rats. In addition, the study was designed to compare physical dependence upon diazepam in F344 and LEW rats to study the genetic aspects of the phenomena.

METHOD

Animals

Male Fischer 344 (F344/DuCrj) and Lewis (LEW/Crj) rats aged 6 weeks (Charles River Japan, Inc., Atugi, Japan) at the beginning of experiment were used in groups of four to six. All rats were housed individually. Powdered food (CA-1; Clea Japan, Inc., Tokyo) and tapwater were supplied ad lib. The animal room was illuminated daily from 8:30 a.m.–8:30 p.m. and maintained at 21 ± 1°C.

Drug Treatment

Diazepam was mixed with powdered food in a mortar (33). Each rat was allowed to eat the diazepam-admixed food and drink tapwater ad lib. The concentration of diazepam in the food was gradually increased over the course of days according to the dose schedule (20) shown in Table 1. When rats were treated with one dose of diazepam, there was one food container in a cage. When rats were treated two doses (e.g., 1 and 2 mg/g food), there were two food containers in a cage, one with each dose. Rats were given simultaneous access to two concentrations of diazepam to avoid or minimize toxicity. Body weight and food consumption were measured daily at 4:00 p.m. Daily diazepam intake was calculated as follows:

$$\text{diazepam intake (mg/kg/day)} = \frac{\text{food intake (g/day)} \times \text{concentration (mg/g food)}}{\text{body weight (kg)}}.$$

Measurement of Motor Incoordination

Motor incoordination in diazepam-treated rats was measured with a 5-min test on a rotarod performance apparatus (9 cm diameter, 5.3 rpm; Natsume Seisakusho Co., Tokyo, Japan). Each rat was trained to run on a rotarod until it could remain there for 5 min without falling before the beginning of the diazepam treatment. The rotarod performance test was carried out at intervals of 2–3 days during diazepam treatment.

TABLE 1

SCHEDULE OF PROGRESSIVELY INCREASED DOSES FOR DEVELOPMENT OF PHYSICAL DEPENDENCE ON DIAZEPAM IN RATS

Diazepam Concentration of Food (mg/g food)	Duration (days)
1 and 2	5
2 and 4	5
4 and 6	3
6	3
8	3
10	5
12	6

Withdrawal

Withdrawal was conducted by substituting normal food for diazepam-admixed food on the last day of the treatment at 4:00 p.m. Body weight and food intake were measured and withdrawal signs were observed at various times after termination of drug. To quantify the intensity of physical dependence upon diazepam, withdrawal signs (Table 2) were scored on the following 5-point scale: no abnormality (score 0), mild (score 1), intermediate (score 2), severe (score 3), and very severe (score 4). The criteria of these signs are as follows.

Vocalization: vocalization in response to the tactile stimuli.
Irritability: become wild in response to the tactile or auditory stimuli and/or restlessness.
Ear-twitching: shaking or trembling of ear.
Fascicular twitch: shaking or trembling of nape.
Aggression: destructive or attacking behavior toward observers in response to the tactile or auditory stimuli.
Tremor: shaking or trembling of the whole body.
Convulsion: handling-induced clonic convulsion.
Spontaneous convulsion: spontaneous clonic convulsion.

Blood Diazepam Assay

One day before termination of diazepam treatment, a 200-μl blood sample was obtained from the tail of each rat. Diazepam levels in serum were determined by high-pressure liquid chromatography according to the method of Kabra et al. (14).

Statistical Analysis

Analysis for the incidence of each withdrawal sign was performed by the χ^2 (2 × 2) test. Statistical comparisons for body weight, food intake, diazepam intake, rotarod performance, and withdrawal scores were done by means of analysis of variance (ANOVA) for repeated measures. All other analyses were carried out using Student's *t*-test.

RESULTS

Growth Curve and Diazepam Intake During Treatment

Relative to naive LEW and F344 rats, body weight of diazepam-treated F344 rats was significantly suppressed at a diazepam concentration of 8 mg/g food, $F(1, 24) = 48.2$, $p < 0.01$, whereas body weight of diazepam-treated LEW rats was significantly suppressed at a diazepam concentration of 10 mg/g of food, $F(1, 40) = 150.3$, $p < 0.01$. Weight gain in both diazepam-treated rat strains was significantly suppressed thereafter in comparison with respective control: for LEW, $F(1, 48) = 282.0$, $p < 0.01$, and for F344, $F(1, 48) = 152.8$, $p < 0.01$ (Fig. 1). Changes in food intake of diazepam-treated LEW and F344 rats were in parallel with the changes in body weight: for LEW, $F(1, 48) = 26.9$, $p < 0.01$, and for F344, $F(1, 48) = 264.1$, $p < 0.01$ (Fig. 1).

As shown in Fig. 2, daily diazepam intake rose from 70–950 mg/kg/day. The mean diazepam intakes during feeding with the final diazepam concentration (12 mg/g food) were 773.3 and 833.9 mg/kg/day in F344 and LEW rats, respectively. There were no significant strain differences in diazepam intake during the final concentration.

Serum Diazepam Levels

Diazepam concentrations 1 day before termination of diazepam treatment were 252.9 ± 20.8 ng/ml in LEW rats and

TABLE 2

MAXIMAL BEHAVIORAL CHANGES DURING DIAZEPAM WITHDRAWAL IN LEW AND F344 RATS

	Rats Exhibiting Withdrawal Signs/Total Number of Animals			
	LEW		F344	
Withdrawal Score	Control	Diazepam	Control	Diazepam
Weight loss				
5–10% (1)	0/4	6/6	0/4	6/6
10–15% (2)	0/4	3/6	0/4	6/6*
Vocalization (2)	0/4	1/6	0/4	5/6*
Irritability (2)	0/4	3/6	0/4	3/6
Muscle rigidity (2)	0/4	6/6	0/4	6/6
Ear-twitching (2)	0/4	2/6	0/4	6/6*
Piloerection (2)	0/4	0/6	0/4	6/6†
Straub's tail (2)	0/4	0/6	0/4	2/6
Fascicular twitch (3)	0/4	0/6	0/4	2/6
Nosebleed (3)	0/4	1/6	0/4	1/6
Aggression (3)	0/4	0/6	0/4	0/6
Tremor (3)	0/4	0/6	0/4	3/6*
Convulsion (3)	0/4	0/6	0/4	2/6
Spontaneous convulsion (4)	0/4	0/6	0/4	2/6
Death (4)	0/4	0/6	0/4	1/6
Withdrawal scores	0	6.3 ± 0.6	0	16.0 ± 3.3†

The χ^2 (2 × 2) test was used for statistical evaluation for each withdrawal sign. Student's t-test was used for withdrawal scores. Convulsion was elicited by handling.
*$p < 0.05$, †$p < 0.01$ vs. LEW.

242.3 ± 19.7 ng/ml in F344 rats. No strain difference was found.

Motor Incoordination

Figure 3 presents changes in rotarod performance as a function of increases in diazepam concentration. The rotarod performance in LEW and F344 rats was moderately affected at a drug concentration of 2 and 4 mg/g, and the impairment in both strains of rats was gradually increased thereafter. The inhibition of rotarod performance at 8 mg/g food in LEW rats (84.6%) was significantly greater than that in F344 rats (23.0%), $F(1, 20) = 18.6$, $p < 0.01$. At the final concentration (12 mg/g food), the inhibition was greater in F344 rats (86.7%) than in LEW rats (72.3%), although the effect was not statistically different.

Withdrawal Signs

After substituting normal powdered food for diazepam-admixed food, several signs of diazepam withdrawal were observed 16–120 h later. These signs included vocalization, irritability, muscle rigidity, ear-twitching, Straub's tail, piloerection, fascicular twitch, tremor, convulsion, and death (Table 2). The incidences of vocalization, ear-twitching, piloerection, and tremor in F344 rats were significantly higher than those in LEW rats. Furthermore, two of six F344 rats showed spontaneous convulsions and one rat died of convulsions. Withdrawal scores were significantly greater in F344 than in LEW rats. The time course of withdrawal scores in LEW and F344 rats is shown in Fig. 4. Withdrawal scores in F344 rats were significantly greater than those in LEW rats, $F(1, 141) = 46.1$, $p < 0.01$. As shown in Fig. 5, body weights

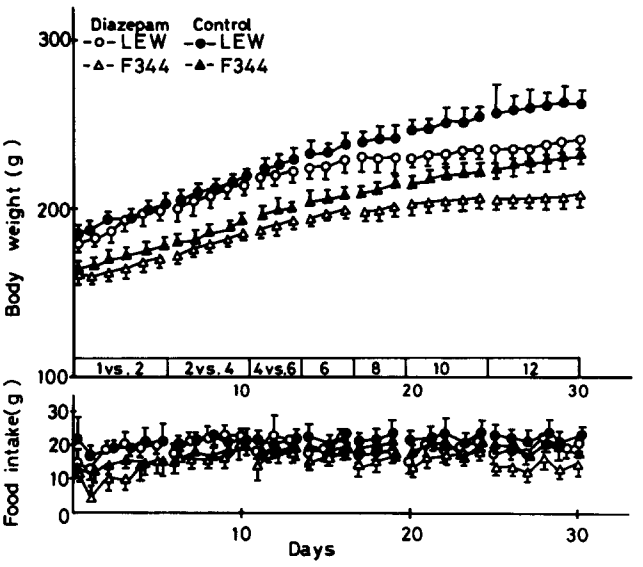

FIG. 1. Changes in mean body weight and daily food intake during diazepam-admixed food treatment on a schedule of gradually increasing dosages in two inbred strains of male, LEW and F344 rats. Each point is the mean with SEM of four rats for control and six rats for diazepam treatment. The numerals in the column show concentrations of diazepam-admixed food (mg/g food).

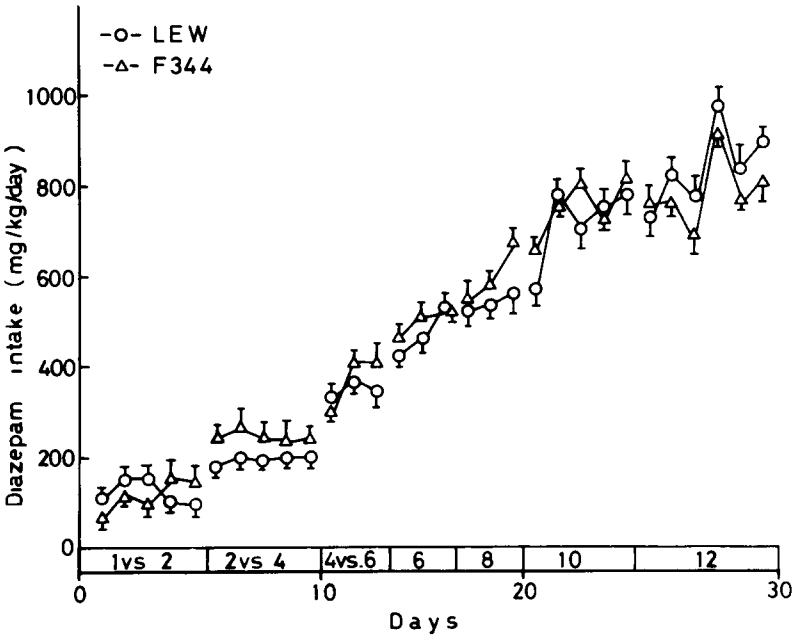

FIG. 2. Changes in mean daily food intake during chronic diazepam on a schedule of gradually increasing dosages in LEW and F344 rats. Each point is the mean with SEM of six animals. The numerals in the column show concentrations of diazepam in food (mg/g).

of rats that were treated with diazepam decreased after diazepam withdrawal. The maximum weight loss after withdrawal in LEW and F344 rats was 8.7% after 48 h and 11.8% after 72 h, respectively. Weight in all groups fluctuated on a diurnal basis. Withdrawal disrupted this rhythm until day 3 in F344 rats, whereas LEW rats began to exhibit normal rhythm by day 2.

DISCUSSION

Several investigators have observed withdrawal effects after chronic administration of benzodiazepines in primates. Yanagita and Takahashi (32) found withdrawal characterized by a variety of signs, such as convulsions and delirium, following chronic administration of diazepam and chlordiazepoxide. Lukas and Griffiths (15) observed overt signs of withdrawal, such as nose rubbing, yawning and tremor, in baboons following 45 days of intragastric administration of diazepam. On the other hand, most primary dependence studies of benzodiazepines in rodents have examined withdrawal induced by drug discontinuation after chronic postoperative administration of diazepam or chlordiazepoxide, although dependence upon benzodiazepines has also been demonstrated when the drugs were placed in the animals' food ration (9,33). Withdrawal signs reliably observed after treatment with various doses of diazepam were weight loss, increased locomotor activity, muscle rigidity, tremor and decreased eating (16,18, 22,30,33,34). Furthermore, Gallaher et al. (9) reported that two of nine mice treated with diazepam-admixed food pellets for 53 days exhibited convulsions after withdrawal. In other rodent studies, animals have not shown spontaneous withdrawal convulsions. In the present study, we developed an escalating dose schedule to produce physical dependence upon benzodiazepines in rats. Drug concentrations in drug-admixed food were rapidly increased until animals showed moderate to severe CNS depression, with motor incoordination, and then this condition was maintained for at least 10 days. With this procedure, animals became severely dependent upon diazepam and withdrawal convulsions could be induced in F344 rats. Thus, diazepam physical dependence in F344 rats was produced more rapidly, and to a greater degree, than has been achieved by a daily injection method despite the shorter administration period (34). These results indicate that F344 rats may be useful for studies of dependence on benzodiazepines.

There are several pharmacogenetic studies on benzodiazepines (3,4,8,10,19). Animal behavior studies with benzodiazepines have been hampered by large variations in drug effect to the extent that individual animals are sometimes pretested to remove "nonresponders" (6,7). File et al. (8) reported strain differences in the anticonvulsant activity of diazepam. Taken together, these data suggest that genetic factors contribute to the differences observed among individual animals. Furthermore, Gallaher et al. (10) demonstrated that genetic factors also contribute to benzodiazepine sensitivity on the rotarod test and that mice can be selectively bred to separate these factors into two distinct lines, DR and DS. On the other hand, Belknap et al. (1) showed that ethanol withdrawal-seizure-prone (WSP) mice displayed more severe withdrawal handling-induced convulsion than withdrawal-seizure-resistant (WSR) mice after chronic treatment with diazepam, and Crabbe et al. (4) showed parallel differences after acute treatment with diazepam. However, in these studies flumazenil was used for withdrawal. Utilizing spontaneous withdrawal, no pharmacogenetic study has yet been reported for physical dependence upon benzodiazepines. In the present study, we found that physical dependence upon diazepam in F344 rats

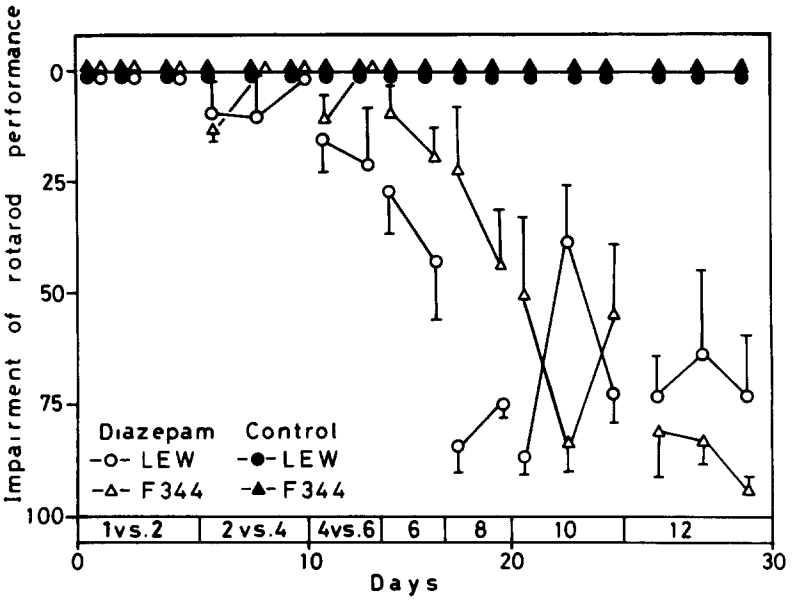

FIG. 3. Impairment in rotarod performance during chronic diazepam treatment in LEW and F344 rats. Each point is mean with SEM of four rats for control and six rats for diazepam treatment. The numerals in the column show concentrations of diazepam in food (mg/g).

is more severe than in LEW rats, suggesting that strain differences in physical dependence upon diazepam may be strongly influenced by genetic factors. We observed similar large strain differences in physical dependence upon ethanol and barbital in LEW and F344 rats (28). However, we also reported that the severity of signs of withdrawal from pentobarbital is greater in LEW rats than in F344 rats (26). The order of severity of withdrawal signs for diazepam, barbital, and ethanol was reversed in F344 and LEW rats as compared with the order for pentobarbital. Harris and Allan (13) demonstrated that the site of action of ethanol on the chloride channel is closely related to the sites of action of benzodiazepines and phenobarbital but is distinct from the sites of action of pentobarbital and muscimol. Therefore, the site of action of diazepam, barbital, and ethanol may differ from the site of action of pentobarbital.

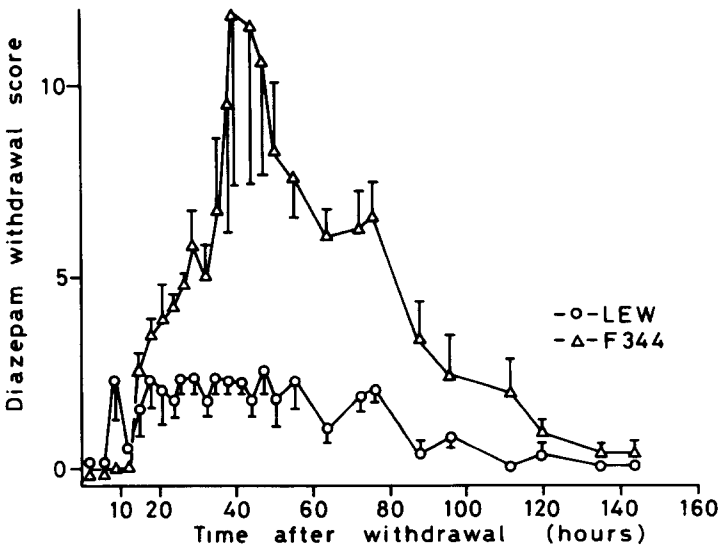

FIG. 4. Time course for changes in withdrawal score after termination of diazepam treatment in LEW and F344 rats. Each point is the mean with SEM of six animals.

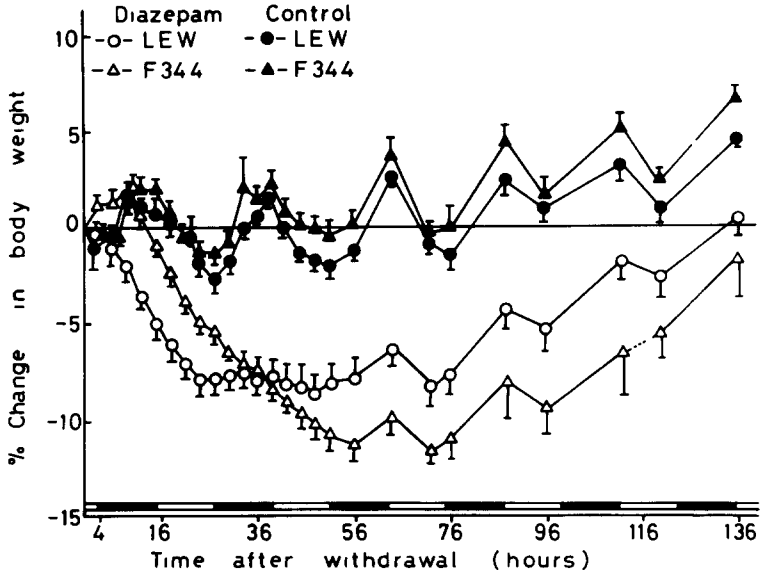

FIG. 5. Time course of changes in body weight (%) after termination of diazepam treatment in LEW and F344 rats. Each point is the mean with SEM of four rats for control and six rats for diazepam treatment. Closed and open columns represent dark and light periods, respectively. The broken line indicates the rat died during the study.

The intensity of physical dependence upon diazepam was expected to be influenced by brain and/or blood diazepam levels. In the present study, there was no significant difference in blood diazepam levels between diazepam-dependent LEW and F344 rats. Therefore, strain differences in physical dependence upon diazepam may not result from difference in blood diazepam levels. On the other hand, the correlation between severity of withdrawal and extent of chronic CNS depression is consistent with previous studies in which the degree of depression was manipulated by using different ethanol, barbiturate, or benzodiazepine doses or giving the drug for different durations (11,17,18,27). Therefore, the intensity of physical dependence upon diazepam was influenced by development of tolerance. In the present study, tolerance to the impairment of rotarod performance induced by diazepam tended to be greater in LEW than in F344 rats (Fig. 3). From our results, it is concluded that the strain differences in physical dependence upon diazepam may have resulted from differences in diazepam tolerance development and, in addition, CNS sensitivity to diazepam.

In conclusion, our findings suggest that diazepam withdrawal severity is strongly influenced by genetic factors, and F344 rats are particularly sensitive to dependence upon benzodiazepines.

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