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# A New Method for Screening Anxiolytic Drugs in Rats

TSUNEYUKI YAMAMOTO[1] AND SHOWA UEKI

*Department of Pharmacology, Faculty of Pharmaceutical Sciences*
*Kyushu University 62, Fukuoka 812, Japan*

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YAMAMOTO, T. AND S. UEKI. *A new method for screening anxiolytic drugs in rats.* PHARMACOL BIOCHEM BEHAV **26**(1) 111–117, 1987.—In order to evaluate the anxiolytic action of drugs, a simple experimental procedure using a corridor-type runway was designed. In this apparatus, five food pellets were set in a row on a plastic platform. Rats with one day food-deprivation take a food pellet and then usually return to the start box. The time required to take 5 pellets (total time) and the number of returns were recorded. Diazepam (DZP) at 1–3.2 mg/kg and zopiclone (ZOP) at 10 mg/kg caused decreases in both parameters. These effects were blocked by the benzodiazepine receptor blocker, Ro15-1788, at 10 mg/kg. However, tracazolate failed to produce any change in both parameters. Haloperidol and imipramine prolonged the total time while reducing the number of returns. In contrast to DZP and ZOP, pentetrazol, well known to possess an anxiogenic effect, prolonged the total time. These results suggest that decreases in both the total time and the number of returns produced by DZP and ZOP may be related to their anxiolytic action which is mediated by a benzodiazepine receptor. Therefore, this procedure would be a simple and selective method for detecting benzodiazepine-type anxiolytics.

Diazepam Zopiclone Tracazolate Ro15-1788 Pentetrazol Anxiolytic drugs
New behavioral method Rat

IN order to evaluate the potential of anti-anxiety drugs for clinical use, it is essential to have an animal model that is capable of measuring anxiety or fear. The behavioral effects of anxiolytic drugs have been evaluated in several well-documented animal behavior paradigms, including conflict procedures [9, 12, 29], punished crossings [1], social interactions [8,27], exploration in novel environments [17,19], antagonism of pentetrazol discrimination [25], staircase test [26] and isolation-induced fighting of mice [14].

The experimental model most widely used today is the conflict experiment using an operant apparatus. However, this model has the following disadvantages: (1) the experimental setup is costly, and (2) the required animal training is laborious and time-consuming. Furthermore, there is some doubt about the validity of comparing the conflict state provoked by physical stimulation such as foot-shock to one of human anxiety. In a test based on punishment, false positive results may also be obtained with drugs which affect the neural processes involved in pain perception of motivation even though they do not possess an anxiolytic action. Though more simple models using locomotor activity have also been proposed, the drug effect on spontaneous locomotor activity itself should be carefully distinguished from an anxiolytic effect. An increase in spontaneous activity sometimes tends to be confused with an anxiolytic action. Thus, these methods are not fully satisfactory, since it is questionable whether the observed effects can be attributed exclusively to an anxiolytic action.

In view of these considerations, the authors constructed a corridor-type runway in which food-deprived rats took food pellets as a simple and selective method for the preclinical evaluation of anxiolytic drugs.

## METHOD

### *Animals*

Male Slc:Wistar-KY rats (purchased from Shizuoka Laboratory Animals Center) weighing 180–250 g were used. The animals were housed 5 per cage in a colony room thermostatically maintained at 20±1°C under a controlled light-dark schedule (light on between 07:00 and 19:00) and allowed free access to food and water in the home cages. The animals were deprived of food for 24 hr before the behavioral test.

### *Apparatus*

The experimental apparatus (Fig. 1) was a plastic U-shaped runway having two corners with dimension of 15 cm (width) ×20 cm (height) ×185 cm (total length). The apparatus consisted of a floor with a stainless steel grid and a transparent plastic cover for the ceiling. This plastic cover was wrapped in blue cellophane to minimize external distraction. In the section containing the food pellets, a black plastic board covered the stainless steel floor.

Five food pellets (50 mg each) were set in a row on the black plastic board. The last pellet (position 5) was placed

[1]Requests for reprints should be addressed to Tsuneyuki Yamamoto.

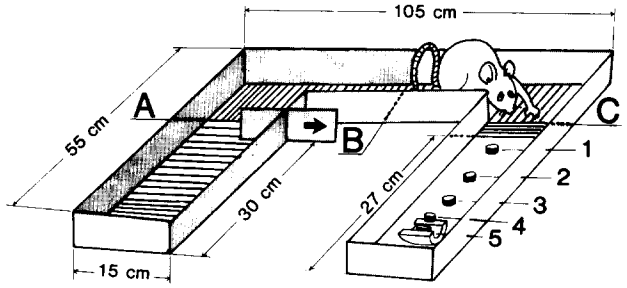


FIG. 1. Experimental design of a corridor-type runway. A broad arrow indicates a sliding door.

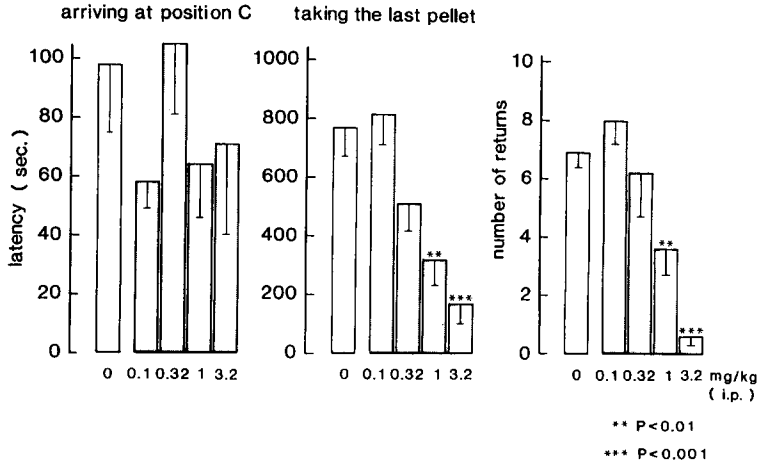


FIG. 2. The effect of diazepam. Left panel: the time required to reach position C. Middle panel: total time (the time required to take the last pellet). Right panel: number of returns. Each column with a bar represents the mean with S.E.M. by asterisks, Mann-Whitney's U-test in left and middle panels and Student's *t*-test in right panel.

within a semi-circular enclosure so that it was not visible to the animals when at position C on the runway (Fig. 1). The plastic floor provided with food pellets was illuminated with a 60 W lamp placed 65 cm above.

*Experimental Procedure*

The rats were used only once in this experiment. They were unfamiliar with the apparatus and the 50 mg food pellets before the test. A 24 hr food-deprived rat was placed in the start box (Fig. 1) for 1 min and the sliding door was opened. The rat's behavior was observed for a 20 min period. During this period, the time required for the rat to pass each of points A, B and C (Fig. 1), the time required to take each pellet and the number of returns were recorded. A "return" was registered when a rat moved back to pass at least one position (C, B and A). For example, a rat passing position C after consuming a food pellet was counted as one return. If that same rat continued to return to the start box, it was still only counted as one return. In the same way, a rat moving back position A from position B was also counted as one return. The total number of returns displayed by each rat during the 20 min test period recorded.

The time taken by a rat to eat 5 pellets completely was considered 'total time.' When a rat failed to take all the pellets within a 20 min observation period, 20 min was recorded as total time.

The amount of food intake by 24 hr-starved rats was measured individually in a test cage (25×12×30 cm). The effect of drugs on food intake was determined during a 60 min period from 30 to 90 min after drug administration.

*Drugs*

The following drugs were used in the present experiment: diazepam (Cercine injection; 5 mg/ml including benzyl alcohol 15.72 mg: Takeda), zopiclone (powder: Rhone-Poulenc), Ro15-1788 (powder: Roche), tracazolate (powder), imipramine hydrochloride (powder: CIBA-GEIGY), haloperidol (Serenace Injection: Dainippon), methamphetamine hydrochloride (powder: Dainippon) and pentetrazol sodium (powder: Sigma). Zopiclone and tracazolate were suspended in a 0.5% aqueous solution of carboxymethylcellulose (CMC), Ro15-1788 was suspended in 1% Tween 80 solution, and diazepam was diluted with a 40% polyethyleneglycol solution. Other drugs were dissolved in distilled water. All drugs were intraperitoneally (IP) administered 30 min before the runway test in a volume of 1 ml/kg. In the antagonism test,

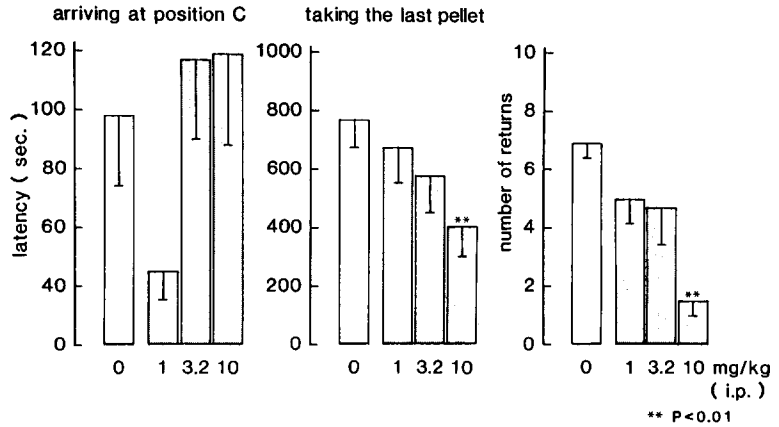


FIG. 3. The effect of zopiclone on the time required to reach position C, and to take the last pellet, and the number of returns.

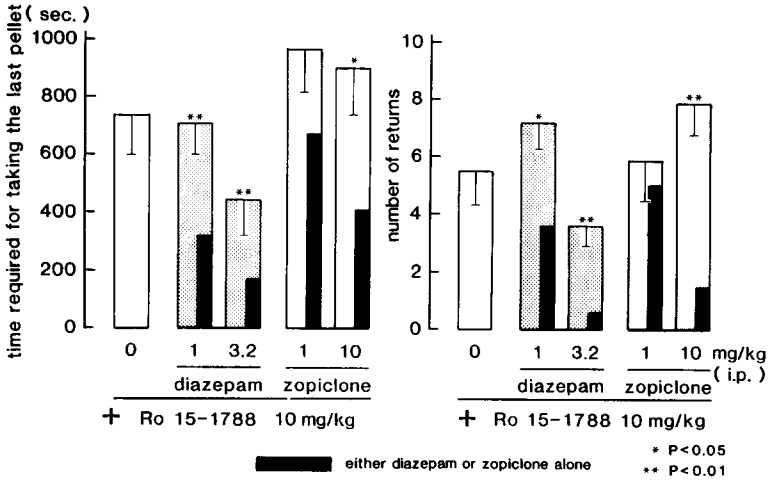


FIG. 4. The effect of benzodiazepine receptor blocker Ro15-1788 on the action of diazepam and zopiclone. Ro15-1788 was administered IP 20 min prior to the administration of either diazepam or zopiclone. A significant difference from the values for diazepam or zopiclone alone is expressed.

Ro15-1788 was administered IP 20 min prior to the administration of either diazepam or zopiclone.

*Statistical Analysis*

The statistical significance of differences was determined using two-way analysis of variance followed by Student's *t*-test and one-tailed Mann-Whitney's U-test. A probability level of 0.05 or less was considered as a significant difference.

## RESULTS

For normally fed rats (N=15) that were administered vehicle (saline or CMC), the time required to pass position A was 23.1±4.2 sec (mean±S.E.M.) and the time required to pass position C was 50.5±6.8 sec. Fourteen of 15 rats took at least one food pellet, and only 5 rats took all of the pellets within 20 min test period. The total time for taking 5 pellets was 1112.9±47.4 sec. The number of returns was 7.6±0.6 (mean±S.E.M.).

For the 24 hr-deprived rats (N=15), the times required to pass positions A and C after receiving vehicle were 34.9±7.7 sec and 97.9±23.8 sec, respectively. There was no significant difference between fed and fasted rats in the time required to pass all points up to position C. Five of 15 rats failed to take all 5 pellets. The total time (769.2±97.7 sec) in fasted rats was significantly less than that of normal, fed rats ($p<0.01$; Mann-Whitney's U-test). The number of returns for fasted rats was 6.9±0.5.

Diazepam (DZP) at 0.1 mg/kg (N=8) and 0.32 mg/kg (N=10) IP produced no significant differences in the total time and number of returns. However, at doses of 1 mg/kg (N=10) or greater, there was a significant decrease in both the total time and number of returns (Fig. 2). After administration of DZP 3.2 mg/kg (N=7), the total time was 168.1±64.6 sec ($p<0.01$; Mann-Whitney's U-test) and the

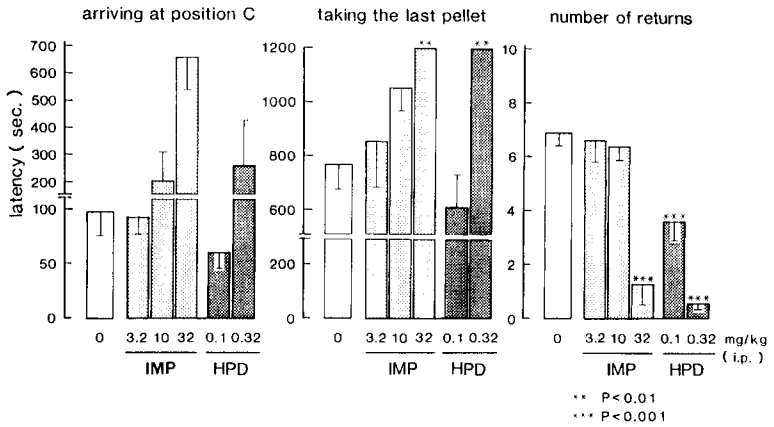


FIG. 5. The effect of imipramine (IMP) and haloperidol (HPD) on the time required to reach position C and to take the last pellet, and the number of returns.

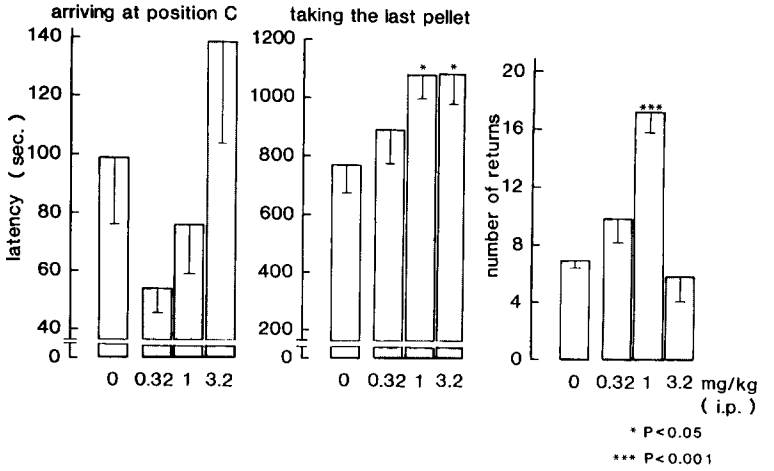


FIG. 6. The effect of methamphetamine on the time required to reach position C, and to take the last pellet, and the number of returns.

number of returns was 0.6±0.3 ($p<0.001$; Student's $t$-test). However, there was no significant difference between control and DZP-treated group in the time taken to arrive at position C.

Zopiclone (ZOP) also resulted in less total time and fewer returns in a dose-dependent manner, though doses of 1 mg/kg (N=12) and 3.2 mg/kg (N=11) did not produce a significant change (Fig. 3). At 10 mg/kg (N=13), the total time was 408.3±101.4 sec ($p<0.05$) and the number of returns was 1.5±0.5 ($p<0.001$). The time taken to pass all points up to C was not significantly affected by ZOP.

Tracazolate did not produce any significant differences in the number of returns at doses up to 10 mg/kg. At 10 mg/kg (N=9), the time required to arrive at position C was significantly higher (154.1±31.6; $p<0.05$) in comparison to that of control, and the total time was also greater with tracazolate at 10 mg/kg (1129.4±66.5; $p<0.001$), but not significantly at 1 mg/kg (1166.8±28.8; N=4) and 3.2 mg/kg (1034.5±82.8; N=4). At 10 mg/kg, 8 of 9 rats did not take all 5 pellets completely, though they took pellets at position 1–2. Even at 10 mg/kg, the rats exhibited no marked ataxia.

Figure 4 shows the antagonism of a benzodiazepine receptor blocker Ro15-1788 on the effects of the DZP and ZOP. Ro15-1788 (10 mg/kg, IP) alone was without effect (N=8). The rat given Ro15-1788 20 min prior to the injection of either DZP or ZOP did not take significantly less total time and have a smaller number of returns, compared with rats given Ro15-1788 alone (Fig. 4). The effects of DZP 1 mg/kg (N=9) and 3.2 mg/kg (N=10) were markedly blocked by Ro15-1788 in both parameters. Two out of 10 rats treated with DZP 3.2 mg/kg and Ro15-1788 failed to take all 5 pellets. On the other hand, the lower values of both total time and number of returns induced by ZOP 10 mg/kg was also significantly blocked by Ro15-1788 (N=7; Fig. 4). In this case, 4 out of 7 rats failed to take all 5 pellets.

There was no significant difference in the time taken to arrive at position C between imipramine 3.2 mg/kg (N=5) or 10 mg/kg (N=10) treated rats and control rats. However, 3 of 6 rats treated with imipramine at a high dose of 32 mg/kg failed to reach even position C. The time required for the other 3 rats to reach position C was 662.7±120.8 sec (Fig. 5). Administration of imipramine at doses of 10–32 mg/kg re-

TABLE 1

THE INFLUENCE OF DIAZEPAM, ZOPICLONE AND METHAMPHETAMINE UPON FOOD INTAKE IN 24 HR-STARVED RATS

| | | Food-Intake (g) | | | Body Weight (g) | |
|---|---|---|---|---|---|---|
| Conditioning | Drug (IP) | 30–60 min | 60–90 min | Total | Before injection | 24 hr after |
| Non-starvation | Saline | 0 | 0.5 ± 0.2* | 0.5 ± 0.2† | 218.8 ± 2.0 | 223.3 ± 1.8 |
| Starvation | Saline | 3.0 ± 0.4 | 1.7 ± 0.4 | 4.7 ± 0.7 | 208.4 ± 2.6 | 224.0 ± 2.6 |
| | Diazepam 3.2 mg/kg | 1.7 ± 0.2‡ | 1.4 ± 0.2 | 3.2 ± 0.2 | 209.1 ± 4.0 | 222.4 ± 4.5 |
| | Zopiclone 10 mg/kg | 2.1 ± 0.4 | 1.7 ± 0.2 | 3.8 ± 0.4 | 203.9 ± 2.4 | 219.0 ± 2.3 |
| | Methamphetamine 3.2 mg/kg | 0.1 ± 0.1§ | 0 | 0.1 ± 0.1§ | 208.2 ± 2.7 | 222.6 ± 2.7 |

*$p<0.05$, †$p<0.001$ (non-starvation vs. starvation; Student's $t$-test).
‡$p<0.01$, §$p<0.001$ (saline vs. drug treated group in starvation; Student's $t$-test).

sulted in significantly greater total time ($p<0.05$ and $p<0.01$, respectively). All rats injected with imipramine 32 mg/kg failed to take a food pellet. The number of returns in rats treated with imipramine at 3.2 mg/kg and 10 mg/kg was not different from that of controls, while it was decreased markedly fewer with imipramine at 32 mg/kg ($p<0.001$; Fig. 5).

Haloperidol at 0.1 mg/kg (N=7) produced no apparent effect on the total time, but resulted in significantly fewer number of returns ($p<0.001$; Fig. 5). At 0.32 mg/kg (N=8), the total time was greater ($p<0.01$) and the number of returns fewer ($p<0.001$). Only 2 of 8 rats arrived at position C. Furthermore, the rats took none of the pellets. Concurrently, locomotor activity was less.

Methamphetamine at 0.32 mg/kg (N=9) produced no effect (Fig. 6). At 1 mg/kg (N=10), however, methamphetamine significantly prolonged the total time to 1083.6±88.2 sec ($p<0.05$) and increased the number of returns to 17.2±1.3 ($p<0.001$). In this case, some rats quickly reached the food, but did not eat it. This phenomenon was observed at 1 mg/kg in 7 of 10 rats. Methamphetamine at 3.2 mg/kg resulted in greater time (1086.4±106.3; $p<0.05$) spent displaying stereotyped behavior such as head-nodding and sniffing, but did not affect the number of returns. In this case, 7 of 8 rats failed to take the pellets.

Pentetrazol at doses of 10 mg/kg (N=11) and 32 mg/kg (N=8) produced no influence on the number of returns (8.4±0.7 and 7.9±0.8, respectively). However, the total time (1103.5±90.3 sec) was greater at a dose of 32 mg/kg ($p<0.05$), and 7 of 8 rats did not take all 5 pellets. At this dose, 1 of 8 rats showed clonic convulsions.

Table 1 shows the amount of food intake over a period of 30–60 min beginning 30 min after administration of DZP 3.2 mg/kg, ZOP 10 mg/kg and methamphetamine 3.2 mg/kg. Following 24 hr of food-deprivation, food-intake during a 30 min period from 30 min to 60 min after drug administration was markedly lower in DZP- and methamphetamine-treated rats ($p<0.01$ and $p<0.001$, respectively; Student's $t$-test), but not in ZOP-treated rats. However, food-intake during a 60 min period from 30 min to 90 min after administration showed no significant difference among saline-, DZP- and ZOP-treated groups. Furthermore, there were no significant differences among the four treated groups in body weight 24 hr after injection. DZP and ZOP failed to increase the amount of food-intake in rats under our experimental condition.

## DISCUSSION

DZP caused dose-related decreases in both the total time and the number of returns, using the present procedures. ZOP, a cyclopyrolone derivative, which has a pharmacological property very similar to that of benzodiazepines [11, 23, 24, 28], also caused the same anti-conflict effect as DZP, though it has not so far been studied in a simple anxiety condition like the present method. The ambulation after finding of the first food pellet can be regarded as a food seeking behavior. After taking a food pellet, saline or CMC-treated rats generally returned to the start box or its vicinity and came back again to take another pellet. The rats given the anxiolytic DZP took 5 food pellets all at once. Furthermore, ZOP, anxiolytic effects, produced similar results.

On the other hand, pentetrazol is known to inhibit DZP-elicited discriminative stimuli [13] and to enhance conflict behavior [18]. Shearman and Lal [25] have also reported that pentetrazol has an anxiety-inducing action on the basis of studies of pentetrazol vs. saline discrimination in rats. In the present experiments, pentetrazol, in contrast to DZP and ZOP, prolonged the total time without disturbing motor coordination. In this paradigm, it is also suggested that the prolongation of total time produced by pentetrazol may be related to the anxiogenic action of this drug. From these findings, the DZP- and ZOP-induced decrease in the total time as well as in the number of returns seems to be due to alleviation of anxiety or fear in a new environment.

Usually, rats do not take unfamiliar food and water. Benzodiazepines have an alleviating effect on this neophobic response [20]. Based on this point, our method seems to be closely related to food neophobia. If such is the case, results of DZP and ZOP in the present study may be due to disinhibition of behavioral suppression caused by anxiety.

In any event, the experimental state in our procedure, in which neither long-term training nor any kind of punishment was required differently from that of the Geller type, can be regarded as a natural "conflict" situation rather than artificial. This effect of DZP and ZOP was blocked by the specific benzodiazepine antagonist Ro15-1788, which has no intrinsic activity by itself. This finding agrees with other reports [4, 7, 10] and suggests that the behavioral effects of DZP and ZOP in our experimental procedure is mediated by the benzodiazepine receptor.

Tracazolate, a so-called second-generation anxiolytic, also exhibits dose-related anticonflict activity in mice and rats without causing sedative and anticonvulsant activities [16,22]. However, the anticonflict potency of tracazolate is reported to be one-quarter to one-half that of chlordiazepoxide in the water-lick test [16]. In the Geller-Seifter conflict test in rats, tracazolate fails to exhibit significant anticonflict activity [16]. It is therefore likely that the anticonflict activity of this drug is too weak to cause a significant effect in our procedure. On the other hand, a good correlation exists within the benzodiazepines between potency as a displacer of $^3$H-benzodiazepine binding and their activity in neuropharmacological tests [15] as well as their clinical anxiolytic activity [5]. In contrast to benzodiazepine, tracazolate produces a concentration-dependent increase in $^3$H-flunitrazepam binding [16]. Non-benzodiazepine ZOP is also known to displace benzodiazepine binding [3]. Based upon these studies, it is suggested that the mechanism of anxiolytic action induced by tracazolate is different from those of DZP and ZOP. Therefore, our present method may detect only typical anxiolytics such as DZP and ZOP mediated via direct activation of benzodiazepine receptor.

Our experimental procedure resembles the staircase test [26] in that physical stress such as foot-shock is not applied, and similar doses of anxiolytic drugs produce a pharmacological response. In the water-lick experiment [21], a conflict situation can be produced as easily as in the present experiment. However, the water-lick procedure has some drawbacks; e.g., not a few animals in a non-drug state (training session) display the water-lick behavior under foot-shock.

There are some problems in simplified anxiety models as to whether the results obtained show specificity to drug effects or not, because central nervous system stimulants often produce the same behavioral patterns as do anxiolytic drugs. In the present experimental model, antidepressant imipramine and antipsychotic haloperidol prolonged the total time and decreased the number of returns because of their sedative action and occurrence of catalepsy, producing a specific behavioral pattern different from that produced by anxiolytics. Furthermore, there is a possibility that an increase in spontaneous activity sometimes tends to be confused with an anxiolytic effect in simple models using locomotor activity. However, behavioral pattern induced by methamphetamine was also different from that of anxiolytics. In addition, both DZP and ZOP tended to decrease ambulation. At least, they did not display an increase in locomotor activity. Therefore, it is suggested that the decrease of both parameters induced by DZP and ZOP was not directly based on an increase of ambulation.

On the other hand, anxiolytic drugs are well known to have appetite-increasing activity [2, 6, 30]. Therefore, there is another possibility that the results observed in the present experiment was produced by increased appetite.

In actuality, the longer the food-deprivation, the more decreased the total time and number of return in our preliminary tests. Namely, three day food-deprived rats showed significant decrease in both the total time ($104.0\pm10.8$ sec) and number of returns ($0.9\pm0.3$). However, the food intake during the test period in animals treated with DZP 3.2 mg/kg or ZOP 10 mg/kg under food deprivation for 24 hr did not increase significantly in comparison with that in the control animals, though methamphetamine 3.2 mg/kg decreased food-intake. However, Sanger *et al.* [24] reported that chlordiazepoxide and ZOP produced a dose-related increase in food intake during a 2 hr test period in starved rats. Though this discrepancy with our results cannot be explained at the present time, it may be related to the difference of test period and condition of starvation. At any rate, there is little possibility in our experiment that the decreases in the total time and number of returns was directly related to the appetite-increasing effect of anxiolytic drugs. Therefore, the time required for taking food varied according to the duration of food-deprivation and it was related to an elevation of the hunger drive, i.e., increase in motivation for taking food, which induces impaired attention or alleviation of anxiety on obtaining food under food-deprived condition.

Based on these findings, it is concluded that the experimental procedure described above represents a useful and simple method for the preclinical evaluation of anxiolytic drugs.

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