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Evidence That Tolerance Develops to the Anxiolytic Effect of Diazepam in Rats

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TREIT, D. *Evidence that tolerance develops to the anxiolytic effect of diazepam in rats.* PHARMACOL BIOCHEM BEHAV **22**(3) 383–387, 1985.—The development of tolerance to the anxiolytic effect of diazepam was studied using suppression of defensive burying as an animal model of anxiolytic action. Although tolerance to the suppressive effect of diazepam was not apparent after chronic administration of diazepam when the rats were tested with a low-intensity shock, anxiolytic tolerance was detected under exactly the same drug regimen when the rats were tested with somewhat higher intensity shocks: under the latter conditions, chronically treated rats buried significantly more than acutely treated rats. Furthermore, this tolerance effect did not appear to depend upon the injection environment, the control vehicle, or the strain of rat; under each of these experimental variations rats chronically treated with diazepam buried significantly more than acutely treated rats when they had received a moderately high intensity shock. These results suggested that tolerance to the anxiolytic effects of benzodiazepines may be detectable when the stimuli eliciting anxiety are relatively intense.

Tolerance Anxiolytic tolerance Benzodiazepines Defensive burying Animal models

DURING the past decade, benzodiazepines have been widely prescribed as sedative-hypnotics, muscle relaxants, anticonvulsants, and anti-anxiety (i.e., anxiolytic) agents [18]. Because of their widespread use, there has been some concern that tolerance may develop to the therapeutic effects of benzodiazepines [12, 16, 18]. Drug tolerance is commonly said to occur when the desired effects of a particular drug diminish as a function of repeated administrations, or when the dose of the drug must be increased in order to maintain its effects. Tolerance is not only an index of the long-term efficacy of a drug, it is also an index of drug safety, since it is often associated with the development of physical dependence [9]. While it is now clear that tolerance can develop to the sedative, muscle relaxant, and anticonvulsant effects of benzodiazepines [2, 3, 4, 5, 16], there is little agreement that tolerance can also develop to the anxiolytic effects of these drugs (cf. [8, 11, 12, 18, 24]).

Evidence from the clinical literature that tolerance develops to the anxiolytic effects of benzodiazepines is sparse and somewhat anecdotal [8,12]. Although there are reports that after extended treatment some patients may consume more than their prescribed dose of a benzodiazepine [12], it is difficult to determine whether this increased self-administration resulted from tolerance to general CNS depressant effects of benzodiazepines, such as euphoria or sedation, or from a specific tolerance to the anxiolytic actions of benzodiazepines. Furthermore, drug regimens for the treatment of anxiety are typically sporadic, dictated mainly by the recurrence or remission of the syndrome being treated, so that a systematic assessment of a drug's anxiolytic effect as a function of repeated exposures is often not possible in a clinical setting (cf. [18]). In view of these problems, it is not surprising that there is little convincing evidence in the clinical literature that tolerance may develop to the anxiolytic effect of benzodiazepines.

Evidence from animal studies of tolerance to the anxiolytic effect of benzodiazepines is equally sparse as well as contradictory. Using an increase in the "social interaction" of rats as an index of anxiolytic action, Vellucci and File [24] found that chlordiazepoxide lost some of its anxiolytic action after 15 administrations. This evidence of anxiolytic tolerance could have been more convincing, however, had the 'tolerant' and 'non-tolerant' animals been compared within the same set of studies; instead, this critical comparison was made between groups of animals from different studies (cf. [24]). In contrast to the results of Vellucci and File, studies that employ the 'conflict' test procedure [6] are uniform in suggesting that benzodiazepines do not lose their anxiolytic effect with repeated administration [2, 10, 11]. In fact, the 'anti-conflict' effect of benzodiazepines is usually delayed until a number of exposures to these drugs have occurred [2,11].

Recently, Treit, Pinel and Fibiger [21,22] introduced a new animal model for the study of anxiolytic agents, based on rats' species-typical propensity to bury novel [19] or noxious objects ([13, 23, 25]; for reviews, see [14,15]). Treit *et al.* [21] found that a variety of anxiolytic agents reliably suppressed this "defensive burying" response in a dose-dependent manner, with a relative potency that was similar to that found in clinical settings. Furthermore, the suppressive effect of anxiolytic agents could be distinguished from the effects of non-anxiolytic agents. Thus, the defensive burying test appeared to fulfil three pharmacological criteria of a useful animal model of anxiolytic drug action: dose-dependent sensitivity, relative potency, and selectivity (cf. [7,10]).

In view of the paucity of experimentally controlled clinical studies of tolerance to the anxiolytic effects of benzodiazepines, and the contradictory evidence thus far provided by animal studies, a systematic study of the acute and

chronic effects of diazepam on the defensive burying response seemed worthwhile.

EXPERIMENT 1

The purpose of Experiment 1 was to assess the suppressive effect of a single test injection of diazepam on the defensive burying behavior of diazepam-experienced and diazepam-naive rats.

METHOD

Subjects

Naive male Sprague-Dawley rats (Canadian Breeding Farm and Laboratories, La Prairie, Quebec) weighing between 350 and 450 g, served as subjects. They were individually housed in wire mesh cages, under a 12-hr light/dark cycle (lights on 7 a.m.), with Purina rat chow and tap water available ad lib.

Apparatus

The defensive burying test was conducted in a 40×30×40 cm Plexiglas chamber, the floor of which was evenly covered with 5 cm of bedding material (Cat Litter, Hagen Corp., Mansfield, MS). On the center of one wall 2 cm above the level of the bedding material was a small hole through which a 7×0.5×0.5-cm wire-wrapped Plexiglas prod could be inserted (cf. [20, 21, 22]).

Drug Administration

Diazepam (Roche) was dissolved in a vehicle of 40% propylene glycol, 10% ethyl alcohol, and distilled water to a concentration of 5 mg/ml. The drug was injected intraperitoneally at a dose that had been previously shown to reliably suppress defensive burying acutely without producing a response impairment (i.e., 1 mg/kg; [1, 20, 21, 22]). Control injections consisted of equal volumes of distilled water. Control and drug injections occurred on each of 10 consecutive days before the burying test, as well as 30 min before the burying test on the eleventh day. All injections were administered in the rats' colony room.

Procedure

Eighteen rats were randomly assigned to three groups (n=6). Rats in the first ("chronic") group were injected with diazepam, whereas rats in the second ("acute") and third ("control") group were injected with equivalent volumes of distilled water. On the last four days before the burying test, the rats in each of the three groups were placed in the Plexiglas test chamber in squads of six for 10 min. Then, on the test day, rats in both the chronic and acute groups were injected with diazepam (1 mg/kg), and rats in the control group were injected with water, 30 min before the test. Just before the test, the shock prod was inserted 7 cm into the test chamber through the hole in the chamber wall and fixed there. Each rat was then placed individually into the chamber; when the animal first touched the prod with a forepaw, it received a brief 1.5 mA shock from a 400 V constant current shock source.

Immediately following the shock administration, the behavior of each rat was viewed for 20 min from a separate room through one-way glass. The duration of burying behavior (i.e., rapid, alternating thrusts of the forepaws that directed litter toward or over the prod) was recorded on an electronic counter.

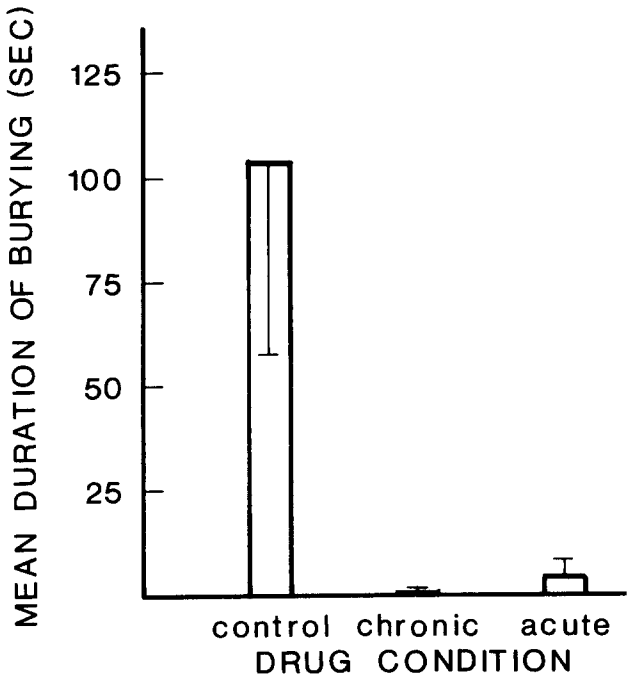

FIG. 1. Mean (±SEM) duration of burying by the control, chronic, and acutely-treated rats in Experiment 1.

RESULTS AND DISCUSSION

As can be seen in Fig. 1, diazepam had a substantial suppressive effect on defensive burying, even in rats that had previously received a number of diazepam injections. The significance of this residual suppressive effect of diazepam in the chronically-treated rats was confirmed by analysis of variance, $F(2,15)=4.92$, $p<0.02$, followed by pair-wise multiple comparisons (Newman-Keuls, $p=0.05$). These analyses showed that diazepam produced a significant suppression of burying behavior in both chronically-treated and acutely-treated rats, compared to water-treated controls, but there was no significant difference in the magnitude of this suppressive effect between the two drug-treated groups. Thus, the results of Experiment 1 suggested that diazepam maintains its anxiolytic effect, even after 10 previous injections of the drug.

EXPERIMENT 2

There is some evidence that the effect of repeated drug-exposures may interact with the intensity of the stimuli used to test drug effects. For example, File [4] found that tolerance to the protective effects of diazepam against pentylenetetrazol-induced (PTZ) seizures was not clearly evident after 45 days of diazepam pre-treatment when the subjects were challenged with 60 mg/kg of PTZ, whereas significant tolerance to the anticonvulsant effects of diazepam could be detected after the 5th pre-treatment day when the subjects were challenged with 120 mg/kg of PTZ (cf. [5]). These results suggest that the sensitivity of a test for tolerance to the therapeutic effects of benzodiazepines may increase with the magnitude or intensity of the stimulus used

to elicit the test response. Thus, it seemed possible that the failure to detect tolerance to the suppressive effect of diazepam in Experiment 1 might have been due to the relative low intensity shock received by the rats.

Accordingly, in Experiment 2, the suppressive effects of diazepam on defensive burying elicited by more intense shocks was assessed in drug-naive and drug-experienced rats. Because it had been shown in a previous experiment [21] that the suppressive effect of diazepam on defensive burying could be completely blocked by increasing the intensity of the aversive stimulus tenfold, only moderate increases in shock intensity were employed in the present experiment in order to replicate the suppressive effect of diazepam in drug-naive animals and at same time show that tolerance to this suppressive effect could occur in drug-experienced animals.

METHOD

The methods were basically the same as those used in Experiment 1, with the following exceptions. Thirty, 350–450 g naive male Sprague-Dawley rats were randomly assigned to the 3 basic treatment groups used in Experiment 1 (n=10). Rats in the "chronic" group were injected with 1 mg/kg of diazepam on each of 10 consecutive days before the burying test. Rats in the "acute" and "control" groups were injected with equivalent volumes of distilled water. After being habituated to the test chamber, rats in the chronic and acute groups were injected on the test day with 1 mg/kg of diazepam 30 min before the test session, whereas rats in the control group were injected with water. Just prior to the test, the shock prod was inserted 7 cm into the test chamber and half the rats in each of the three basic groups were randomly assigned to receive a 2 mA shock from the prod, while the other half received a 4 mA shock. Thus, the design was a completely crossed, 3 by 2 factorial, with three levels of drug exposure and two levels of shock intensity. Following shock administration, the duration of burying behavior was recorded on the electronic counter for 20 min.

RESULTS AND DISCUSSION

Figure 2 shows the duration of burying behaviour as a function of the three basic drug treatment conditions. As can be seen, although chronically treated rats buried somewhat less than control rats, they buried substantially more than did acutely treated rats, suggesting some degree of tolerance to the anxiolytic effect of diazepam. The duration data were analyzed with a 3 by 2 ANOVA which showed a significant effect of drug pre-treatment, $F(2,24)=7.56$, $p<0.01$, but no significant effect of shock, $F(1,24)=0.01$, $p>0.05$, and no significant shock by drug pre-treatment interaction, $F(2,24)=2.37$, $p>0.05$. Subsequent pair-wise comparisons of the marginal means associated with the drug treatment effect (Newman Keuls, $p=0.05$) showed that whereas there was no significant difference in the amount of burying displayed by the chronically treated and control treated rats, both of these groups buried significantly more than the acutely treated rats. These findings of significant tolerance to the suppressive effect of diazepam on defensive burying at moderately high shock levels can be contrasted to the previous failure in Experiment 1 to detect such tolerance at a lower shock level. Thus, it appears that tolerance to the anxiolytic effects of diazepam may be detected in the present model when the intensity of the aversive stimulus used to elicit defensive burying is increased (cf. [4]).

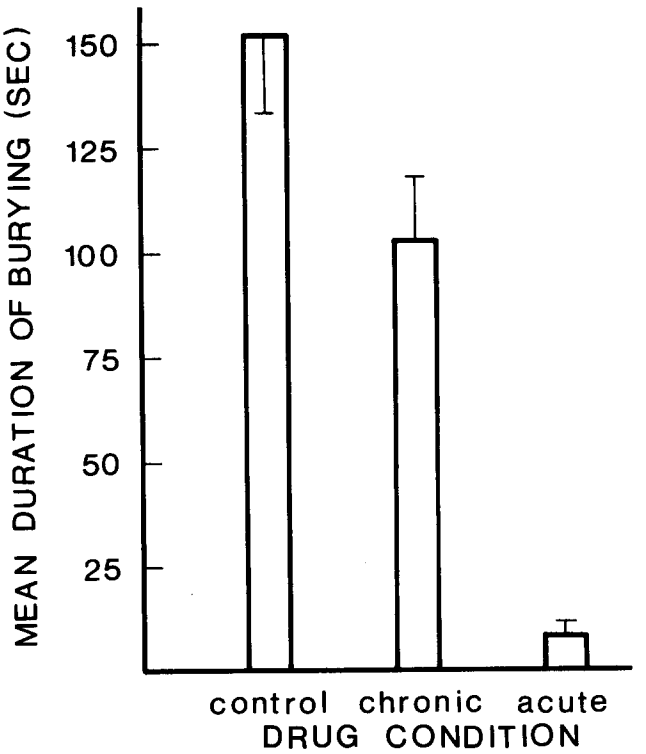

FIG. 2. Mean (±SEM) duration of burying by the control, chronic, and acutely-treated rats in Experiment 2.

EXPERIMENT 3

The major purpose of Experiment 3 was to replicate the finding that tolerance to the suppressive effect of diazepam can occur at a higher shock intensity. A secondary purpose was to examine the generality of this finding by varying the strain of rats, the control vehicle, and the injection environment.

METHOD

The methods were basically the same as those used in the previous experiment, with the following exceptions. Thirty-six naive, 350–450 g naive, male hooded rats (Canadian Breeding Farm and Laboratories, La Prairie, Quebec) served as subjects. The rats were randomly assigned to one of four treatment groups (n=9). Rats in two "chronic" groups were injected with 1 mg/kg of diazepam on each of 10 consecutive days before the burying test, whereas rats in two "acute" groups were injected with equivalent volumes of the diazepam vehicle (i.e., 40% propylene glycol, 10% ethyl alcohol, 50% distilled water). As in the previous experiment, all 10 pre-test injections occurred in the colony room, and rats were habituated for four days to the test chamber. On the test day, one of the chronic groups and one of the acute groups was administered the test injection of 1 mg/kg of diazepam in the colony room 30 min before the test (the "same environment" condition). Then the rats were individually placed into the Plexiglas test chamber and shocked once (2 mA) when they first touched the prod with a forepaw. Rats in the other chronic group and acute group were treated

TABLE 1
DURATION OF BURYING BY THE FOUR GROUPS IN EXPERIMENT 3

Drug condition:	Chronic		Acute	
Injection environment:	Same	Different	Same	Different
Mean:	82.2	91.4	26.0	32.9
S.E.M.:	(20.3)	(23.9)	(11.2)	(15.1)

in exactly the same way on the test day, except that they received their test injection of diazepam in a dimly lit, quiet room where they were kept for 30 min in a 50×50×50 cm wooden holding chamber before being taken to the behavioral test room (the "different environment" condition). Thus, the design was a 2 by 2 factorial, with two levels of prior drug experience and two levels of injection environment. Following shock, the duration of burying behavior was recorded on an electronic counter for 20 min.

RESULTS AND DISCUSSION

As can be seen in Table 1, the average duration that rats in the two "chronic" groups buried the prod was substantially greater than the average duration of the two "acute" groups, thus suggesting that some tolerance had developed to the suppressive effect of 1 mg/kg of diazepam. The effect of the pre-test injection environment, however, was only slight and well within the standard error. These results were confirmed with a two-way ANOVA which showed a significant main effect of prior drug experience, $F(1,32)=9.79$, $p<0.003$, but no significant effect of pre-test injection environment, $F(1,32)=0.192$, $p>0.5$, and no significant interaction, $F(1,32)=0.004$, $p>0.5$.

GENERAL DISCUSSION

In Experiment 1, rats given ten daily administrations of 1 mg/kg of diazepam did not become tolerant to its suppressive effect on defensive burying behavior elicited by low-level shock; the same dose of diazepam was equally effective in drug-naive and drug-experienced animals. Tolerance to the anxiolytic effect of diazepam was indicated, however, when rats were challenged with more intense aversive stimuli; rats in Experiment 2 were given exactly the same drug regimen as rats in Experiment 1, but their defensive behavior was induced by higher shock intensities. Using this procedure, tolerance to the suppressive effect of diazepam on defensive burying was detected after 10 days of diazepam treatment; the rats chronically treated with diazepam buried the shock source significantly more than rats acutely treated with diazepam. Taken together, the results of Experiments 1 and 2 suggest that diazepam may lose some of its anxiolytic action on fear-motivated behavior when the "fearful" stimulus is relatively severe, but not when it is relatively mild. The results of Experiment 3 replicated the basic tolerance effect found in Experiment 2, and indicated that this effect is not peculiar to a particular strain of rat, a particular control vehicle, or to a particular pre-test injection environment. Although the latter finding might suggest that "conditioned tolerance" does not occur to the anxiolytic effect of diazepam, this conclusion must await more systematic manipulations of the cue-properties of the injection environment (cf. [17]). In any case, the results clearly show that tolerance can develop to the suppressive effect of diazepam on defensive burying induced by higher shock levels.

Treit *et al.* [21] have previously shown that the suppressive effect of several doses of diazepam on rats' defensive burying behavior can be blocked by shocking rats with 10 mA; diazepam-treated rats under these conditions buried the prod as much as non-drugged controls. It is of some importance, therefore, to note that the shock intensities used in the present experiments were all well below 10 mA, and that a significant suppression of defensive burying did occur in rats acutely treated with diazepam. Thus, the lack of suppression observed in the chronically treated groups could not be attributed to a simple blocking effect of high intensity shock. Nevertheless, further experiments in which shock intensity and dose of diazepam are systematically varied should serve to further characterize the tolerance effects indicated by the results of the present studies.

In addition to providing further support for the view that tolerance can develop to the anxiolytic effects of benzodiazepines [24], the present results suggest that the demonstration of anxiolytic tolerance in clinical settings may be even more difficult than it was first assumed (see the introduction). The results of the present studies suggest that fear-motivation may interact with drug experience: only rats that received the relatively intense electric shocks showed significant tolerance to repeated exposures of diazepam. Thus, it is possible that tolerance to the anxiolytic effects of benzodiazepines in clinical settings might be masked in patients with moderate anxiety, and only detectable in those patients who suffer from relatively intense anxiety. According to the present studies, the demonstration of anxiolytic tolerance in clinical settings may require a careful assessment of the severity of anxiety in the patient population under study, as well as adequate experimental control over the regimen of benzodiazepine treatment. Although it is clear that comparisons between animal models and actual clinical syndromes must be made with caution, the present findings suggest that controlled clinical study may reveal that severely anxious patients can become tolerant to the anxiolytic effects of benzodiazepines.

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