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The Effects of Diazepam on "Fear" Reactions in Rats are Modulated by Environmental Constraints on the Rat's Defensive Repertoire

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TREIT, D., V. M. LOLORDO AND D. E. ARMSTRONG. *The effects of diazepam on "fear" reactions in rats are modulated by environmental constraints on the rat's defensive repertoire.* PHARMACOL BIOCHEM BEHAV **25**(3) 561–565, 1986.—The simultaneous effects of diazepam on three shock-induced reactions in rats were studied in order to determine the reliability of these behaviors as indices of anxiolytic drug action. Rats were injected with 1 mg/kg of diazepam or vehicle, placed in a 2-compartment chamber containing bedding material, and shocked with 1, 2, or 6 mA when they first touched a wire-wrapped prod attached to one end of the chamber. Diazepam-treated animals displayed significantly less burying behavior, but paradoxically, they also displayed more passive avoidance behavior and fewer exploratory side-transitions than vehicle-injected controls. Defensive burying behavior tended to be negatively correlated with passive avoidance behavior and positively correlated with exploratory side transitions. When the "competitive" relationship between defensive burying and passive avoidance was eliminated by testing rats in a 2-compartment chamber not containing bedding material, diazepam produced a significant suppression of passive avoidance and a significant increase in exploratory side-transitions, compared to control. Taken together, these results suggested that the validity of any single behavioral model of anxiolytic drug action might vary as a function of environmental constraints on the subjects' defensive repertoire.

Diazepam Fear reactions Animal models Cross-validation

RODENTS display a variety of stereotyped, easily quantified responses when exposed to painful, novel, or sudden stimuli [3]. These responses are most often characterized as "fear" reactions, or "defense" reactions, perhaps analogous to human anxiety reactions [1,13].

Some of these "fear" reactions appear to be selectively sensitive to suppression by anti-anxiety agents and thus might be useful as "models" for the study of anxiolytic compounds (for a recent reveiw, see [19]). For example, it has been shown that anxiolytics, in a variety of experimental settings, can reliably inhibit passive avoidance of places or responses associated with aversive stimulation (e.g., [11, 18, 25]). Under more limited conditions, it has been shown that anxiolytics can selectively increase the "exploration" of brightly illuminated places, which rodents otherwise appear to find aversive [4–7], as well as decrease "defensive burying" behavior that rodents normally direct toward well-localized souces of aversive stimulation, such as shock-prods [2, 20–23].

The pharmacological specificity of these drug effects suggests that passive avoidance, inhibited exploration, and defensive burying may be appropriate models for the study of anxiolytic agents. However, most of these data have been gathered in experimental settings that either constrain the animal's ability to display its full range of defensive behaviors, or in which its range of defensive behaviors is not systematically quantified. Thus these "anxiolytic" drug effects have not been validated in the sense of being observed concurrently in a situation where all the putative fear responses occur in the control condition. Clearly, this approach to the validation of anxiolytic models may be difficult, especially in view of the fact that the relationship between different indices of animal "fear" may be quite complex, even in simple observational settings (e.g., [17]). Nevertheless, until the correspondence between different indices of anxiolytic drug action is established, the validity of any single measure of anxiolytic drug action is questionable. Thus, the present experiments were designed to assess the correspondence of the "anxiolytic" effects of diazepam on three concurrent measures of rodent "fear" (i.e., passive avoidance, inhibited exploration, and defensive burying).

EXPERIMENT 1

The purpose of Experiment 1 was to assess the effects of 1 mg/kg of diazepam on the three indices of rodent "fear," over a range shock intensities.

METHOD

Subjects

The subjects were 64 naive, 250–450 g male hooded rats purchased from Charles River Canada (St. Constant, Quebec). These rats were individually housed in the laboratory between 7 and 14 days before the experiment, in wire-mesh cages with free access to food and water. A 12 hr light/dark cycle was in effect throughout the experiment (lights on 7:00 a.m.).

Apparatus

The test apparatus was an 80×30×40 cm Plexiglas chamber, which was divided into two 40×30×40 cm sections by a Plexiglas partition. A 7×7 cm opening in the center of the bottom of the partition enabled rats to cross freely between the two sides of the apparatus. Throughout all phases of the first experiment, the floor of the chamber was evenly covered with 5 cm of bedding material (Cat Litter, Hagen Corp., Mansfield, MS). On the center of one of the end walls of the chamber, 2 cm above the level of the bedding material, was a small hole through which a 7.0×0.5×0.5 cm wire-wrapped Plexiglas prod could be inserted. The section of the test chamber in which this wire-wrapped shock conductor was inserted was designated as the "shock" side, while the other section was the "safe" side (cf. [15]).

Procedure

Habituation. On each of four consecutive days before a test, the rats were placed in the experimental chamber in groups of 8 to 10 for 30 min. The wire-wrapped prod was not present during these habituation sessions.

Drug administration. On the fifth day of each experiment, the rats were randomly assigned to treatment conditions. Thirty min before a test, the rats in the drug condition received an intraperitoneal (IP) injection of 1 mg/kg of diazepam, whereas rats in the control condition received an IP injection of an equivalent volume of the vehicle (approximately 0.2 ml of 40% propylene glycol, 10% ethyl alcohol, and distilled water). This 1 mg/kg dose of diazepam was chosen specifically because of its apparent ability to reliably suppress each of the three indices of rodent "anxiety" without producing detectable motor inhibition [2, 4, 5, 18, 23]. All injections occurred in the rats' home colony room.

Shock administration. Immediately before the test session on day 5, the shock prod was inserted 6 cm into the 2-compartment Plexiglas test chamber. When each individually tested rat first touched the wire-wrapped prod with a forepaw it received a brief electric shock from a 1000 V power source (cf. [16]). Rats received either a 1 (n=24), 2 (n=20) or 6 mA shock (n=20), on the forepaw. Current intensity was varied using a variable resistor, and current duration was determined by the latency for the rat to withdraw its paw from the prod (typically 30–35 msec; [16,24]). The interval between the time the rat was placed into the test chamber and the time that it received shock varied somewhat, with the mean interval being 90 sec (SEM 30.6) across all conditions. However, in no case was there a significant difference between drug-treated and vehicle-treated rats in their latency to receive shock (all t values <0.08).

Behavioral measures and data analysis. Immediately following shock administration, the behavior of each animal was observed for 15 min from a separate room through one-way glass. The three "fear" reactions were recorded either on electronic counters operated through an Apple II+ computer, or on a 6 pen event recorder. Specifically, these measures were (1) the duration of the test session spent on the "safe" side of the 2-compartment chamber (i.e., "passive" avoidance, cf. [11]) (2) the duration of the rapid, alternating, forward thrusts of the forepaws by which rats direct bedding material toward or over sources of aversive stimulation (i.e., "defensive burying behavior" [16]) and (3) the frequency of transitions between the two sides of the Plexiglas chamber (i.e., "exploration," cf. [5,7]).

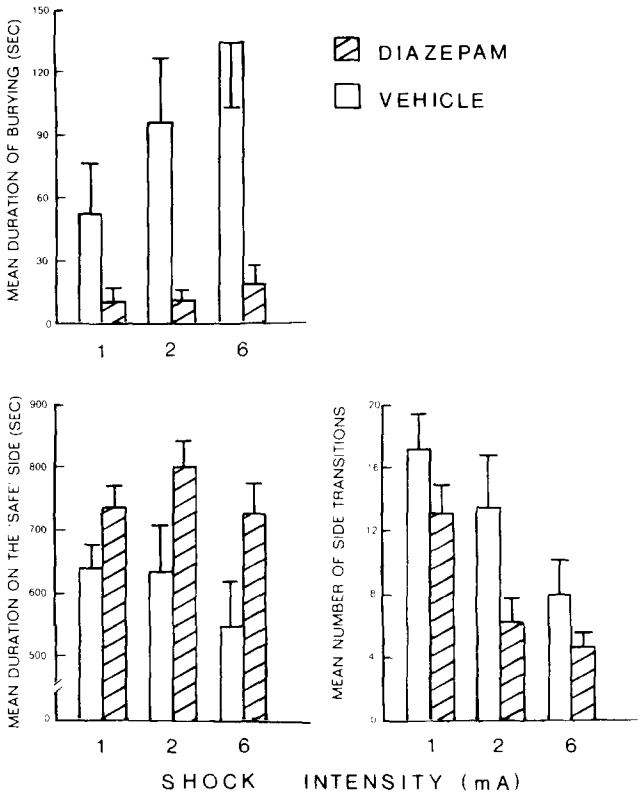

FIG. 1. Mean (SEM) duration of burying, duration on the "safe" side, and number of side transitions for rats shocked with 1, 2, or 6 mA in Experiment 1.

Since each measure was selected on the basis of a priori considerations, the reliability of the effects of 1 mg/kg diazepam on these behaviors was simply analyzed with a series of univariate ANOVAs. However, it was also obvious that there might be mutual dependencies among the basic measures that could be important for the interpretation of the univariate analyses (see introduction; [17]), and therefore correlational analyses were also conducted.

RESULTS AND DISCUSSION

As can be seen in Fig. 1, 1 mg/kg diazepam produced a significant suppression defensive burying behavior, whether the behavior was elicited by 1 mA, $F(1,22)=4.80$, $p<0.04$, 2 mA, $F(1,18)=6.21$, $p<0.02$, or 6 mA shock, $F(1,18)=10.70$, $p<0.004$. Surprisingly, however, 1 mg/kg diazepam *increased* the duration of time that animals spent on the "safe" side of the 2-compartment chamber (i.e., increased passive avoidance) at 1 mA, $F(1,22)=5.09$, $p<0.03$, 2 mA,

$F(1,18)=3.67$, $p<0.06$, and 6 mA, $F(1,18)=4.27$, $p<0.05$. Although the effect of 1 mg/kg diazepam on the frequency of side transitions was not significant at the 1 mA shock level, $F(1,22)=2.20$, $p>0.15$, there was an unexpected but significant *reduction* (cf. [5,7]) in side transitions in diazepam-treated rats at 2 mA, $F(1,18)=4.73$, $p<0.04$, and at 6 mA, $F(1,18)=4.26$, $p<0.05$.

These results are somewhat difficult to reconcile with the idea that diazepam has a uniform anti-anxiety action. In particular, the reduction in side transitions and defensive burying, and the increase in passive avoidance, seem contradictory.

The direction of the correlation coefficients computed in Experiment 1 between the different measures of rodent "fear" pointed fairly clearly toward the rationale of Experiment 2: Statistically reliable correlations were found in control animals between the duration of burying and the duration of time spent on the "safe" side (1 mA: $r=-.61$, $p<0.05$; 2 mA: $r=-.85$, $p<0.05$); between the duration of burying and the frequency of side crossings (1 mA: $r=+.70$, $p<0.05$; 2 mA: $r=+.82$, $p<0.05$); and between duration of time spent on the "safe" side and the frequency of side crossings (1 mA: $r=-.89$, $p<0.05$; 2 mA: $r=-.69$, $p<0.05$). At 6 mA, the negative correlation between the duration of burying and the duration of time spent on the "safe" side was also significant ($r=-.70$, $p<0.05$); the other correlation coefficients failed to reach significance at 6 mA, although they were directionally the same as those found at the lower shock intensities.

EXPERIMENT 2

The results of the previous experiment are difficult to reconcile with the simplistic notion that diazepam, a prototypical anxiolytic in humans, has a uniform, inhibitory effect on animal fear reactions. This is especially problematic because, when viewed in isolation, each of these animal reactions (i.e., defensive burying, passive avoidance, reduced exploration), does seem to be selectively sensitive to anxiolytic drug effects [19]. Why this does not seem to be the case when these "anxiety" reactions are viewed in concert in unclear. Both the drug and the dose used in the Experiment 1 have been repeatedly shown to produce "anti-anxiety" effects in animals without producing gross motoric side effects [12]; the intensity of the "fear"-inducing stimulus, electric shock, was varied over quite a wide range; and male rats are commonly used in many animal models of anxiolytic drug effects [19], although it has been suggested that their utility in some paradigms involving explortion may be limited [4].

One possible reason for some of these complex results, which was suggested by the correlation coefficients computed in Experiment 1, is that some fear reactions may be behaviorally incompatible, or "competitive." For example, if the negative correlation found between defensive burying and passive avoidance was an indication of response competition, this would surely complicate the observation of uniform, anxiolytic drug effects. Perhaps 1 mg/kg of diazepam, by reducing the probability of defensive burying, thereby increased the probability of other, "competing" reactions in the rat's defensive repertoire, such as passive avoidance.

In view of these observations, it seemed potentially useful to test the animals in an environment where the baseline probability of one of these reactions to aversive shock, defensive burying, could be arbitrarily set to 0, as well as in another environment, where this probability could be >0. If the paradoxical facilitative effect of 1 mg/kg diazepam on passive avoidance seen in the previous experiment was a behavioral consequence of the suppressive effect of 1 mg/kg diazepam on defensive burying, then the effects of the drug on passive avoidance should be opposite in the two environments. The present experiment was designed to assess such drug by environment interactions, and their implications for our understanding of anxiolytic drug effects.

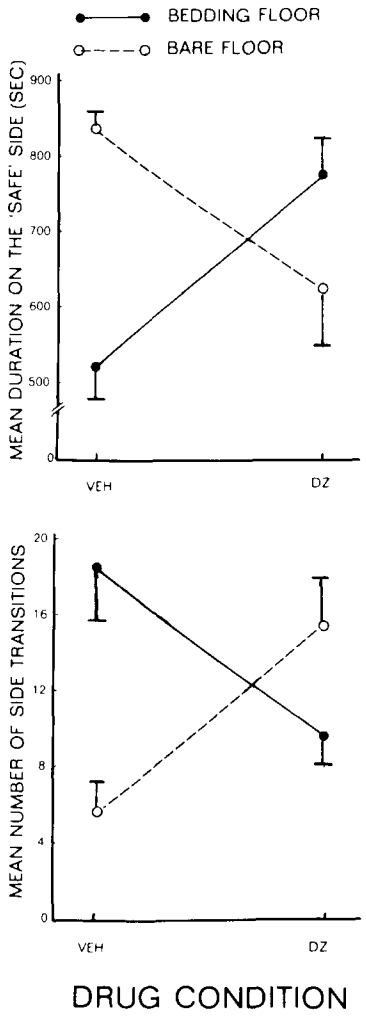

FIG. 2. Mean (SEM) duration on the "safe" side, and number of side transitions for rats shocked in the two different chambers of Experiment 2.

METHOD

The methods were similar to those used in the previous experiment. Twenty-four, randomly selected, naive rats were habituated for 4 days in the 2-compartment chamber containing bedding material, whereas another 24 naive rats were habituated in an identical chamber not containing bedding material. On the test day, a randomly selected half of each of these two groups of rats was injected with 1 mg/kg of diazepam, whereas the other half was injected with vehicle. Thus the design was a 2 by 2 factorial, with two levels of drug and two levels of test environment. Thirty min after injec-

tion, the rats were individually placed into the chamber to which they had been habituated, and when they first touched the wire-wrapped prod with a forepaw, they received a 2 mA shock. During the next 15 min, the behaviors measured in the previous experiment were recorded on electronic counters.

RESULTS AND DISCUSSION

As was the case in Experiment 1, rats tested in a chamber with bedding material and injected with 1 mg/kg diazepam (M=13.75 sec; SEM=9.90) buried the prod significantly less, $F(1,22)=8.94$, $p<0.006$, than rats injected with the vehicle (M=77.50 sec; SEM=18.85). More importantly, however, Fig. 2 shows a completely crossed interaction for the effects of drug and environment on the duration of time animals spent on the "safe" side, $F(1,44)=21.54$, $p<0.001$. A posteriori comparisons (Newman Keuls, $p=0.05$) showed, consistent with the first experiment, that in the bedding-floor chamber drugged animals spent significantly more time on the "safe" side of the chamber than controls. In the bare floor chamber, exactly the opposite was true: drugged animals displayed a reduction in passive avoidance, spending significantly *less* time on the "safe" side of that chamber. These results suggest that a clear "anti-punishment" effect can be demonstrated in the apparatus used in the present studies, but only when the animal's defensive repertoire is arbitrarily constrained.

Figure 2 also shows the number of side-transitions made by animals in each of the four conditions. As can be seen, this measure of anxiolytic drug action also seemed to depend on constraints on the animals' defensive repertoire, as only the drug by environment interaction was significant, $F(1,44)=19.38$, $p<0.001$. Subsequent pair-wise comparisons confirmed that 1 mg/kg diazepam produced a significant suppression of side crossings compared to controls in the environment with bedding material, but a significant facilitation of side crossings in the chamber without bedding material (cf. [4,5]). The latter results suggest that a diazepam-induced increase in suppressed "exploration" can be replicated in rats in the present apparatus, but only when the animal's other defensive options are selectively blocked.

Thus, in an environment which constrains some of the rat's "fear" reactions, one might characterize the effects of diazepam on side crossings and passive avoidance as anxiolytic, whereas in an environment which allows the animal to display more active, directed responses to the source of aversive stimulation, these same measures would suggest that diazepam is an anxiogenic. Moreover, these highly significant drug by environment interactions argue against the possibility that the effects of diazepam on side-crossings and passive avoidance were somehow unique to the single dose of diazepam used, or that they were simply due to general drug effects such as "motor inhibition," or "sedation" (cf. [2,23]).

GENERAL DISCUSSION

In Experiment 1, rats were shocked with 1, 2, or 6 mA when they first touched a wire-wrapped prod in a 2-compartment chamber which contained bedding material. It appeared from the initial results that a non-sedating (i.e., 1 mg/kg) dose of diazepam reduced rats' fear as indicated by a suppression of defensive burying behavior, but *increased* rats' fear as indicated by an increase in passive avoidance behavior and a decrease in exploratory, transition behavior.

The significant, negative correlations found in Experiment 1 between the amount of time that rats spent burying the prod and their duration of passive avoidance suggested that there is a "competitive" relationship between avoidance behavior and burying behavior. It seemed possible that by inhibiting defensive burying, 1 mg/kg diazepam was simultaneously enhancing the probability of passive avoidance, perhaps by default rather than by a paradoxical anxiogenic effect. In order to assess this possibility, rats in the second experiment were tested under 1 mg/kg diazepam or placebo, in an environment which did not permit the burying response, or in one in which burying was possible. Rats tested in a chamber with bedding material and injected with diazepam again showed a greater duration of passive avoidance than vehicle-injected controls, who were engaged in significantly more defensive burying behavior. However, in the chamber without bedding material, where rats could not bury, diazepam-injected animals showed significantly less passive avoidance behavior than vehicle-injected controls. Thus, environmental constraints on the rats' defensive repertoire appeared to modulate one of the "anxiolytic" effects of 1 mg/kg diazepam. Similarly, rats injected with 1 mg/kg diazepam in the chamber with bedding material showed fewer exploratory side-transitions than vehicle-injected controls, but in the chamber without bedding material 1 mg/kg diazepam produced significantly more side-transitions than vehicle-injected controls. Taken with the results of the previous experiment, these drug by environment interactions suggest that the effects of 1 mg/kg diazepam on different measures of rodent "fear" have no inherent correspondence with one another, and therefore the general validity of these models remains questionable.

In spite of the pessimistic implications of the present results for the general validity of animal models of anxiolytic drug action, there is a considerable body of behavioral and pharmacological evidence, including the results of Experiment 2, that suggests that some of these models are reliable predictors of anxiolytic drugs, albeit under limited experimental conditions (cf. [19]). Furthermore, it could be argued that these limitations on the "external" validity of the models are not particularly surprising or problematic, given the number and diversity of human anxiety disorders and the variability in humans' responses to anti-anxiety agents (cf. [8,14]). However, when the probability of one response that is supposed to index animal "anxiety" (e.g., passive avoidance, exploratory side-transitions) is either increased or decreased by an anxiolytic agent, depending upon the environmental context in which it is observed, it seems to follow that we must sacrifice the assumption that these reactions reflect animals' fear, and/or allow the possibility that anti-anxiety agents do not reduce fear in animals. In the face of strong evidence to the contrary, neither one of these alternatives seems particularly attractive, yet the data of the present investigations seem to leave us in exactly this quandry.

In addition, although there are some reports that proven anxiolytics can sometimes produce a paradoxical increase of fear in humans who are already anxious [8], these anecdotal observations do not weigh heavily against the vast body of evidence showing that anxiolytic agents such as the benzodiazepines potently and selectively inhibit human anxiety (e.g., [9, 10, 14]). Thus, many of the results of the present investigation are at odds with the notion that drug-induced suppression of the diverse reactions of animals to novel or

aversive stimuli provide unambiguous models of anxiolytic drug effects in human (cf. [19]).

Although the present studies failed to provide behavioral cross-validation of passive avoidance, reduced exploration, and defensive burying as models for the study of anxiolytic agents, some of the results might be partially rationalized in terms of a hierarchial organization of defensive reactions [3,15]. If we assume that the probability of a particular "fear" reaction in a certain setting is greater than that of other defensive reactions, then the prepotent reaction might be more likely to be affected by the anti-anxiety actions of drugs. It follows then that diazepam might selectively affect the most prominent fear reaction in a given setting rather than uniformly suppressing all manifestations of the animal's fear; in fact, anxiolytics may not reduce a fear "state" at all but rather the most probable response to this state.

This general hypothesis might be applied to some of the present data in the following way: Suppose that the most probable response of rats to prod-shock in an environment with bedding material is to bury the prod, and that the next most probable option is passive avoidance. Diazepam, by primarily suppressing defensive burying, might secondarily increase the probability of passive avoidance. In an environment without bedding material, where the burying option is totally eliminated, the most probable response might be passive avoidance, and so diazepam might preferentially suppress this response (see Experiment 2). Although this account is a posteriori, and depends on a knowledge of the actual probabilities of a rodent's "fear" reactions under various constraints, it might serve as a useful qualitative heuristic for future studies. In any case, when individual responses are used as the primary level of analysis, the present results show that anxiolytic drug effects in rats can be critically modulated by environmental constraints on the rat's defensive repertoire.

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