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Low-Dose Midazolam Attenuates Predatory Odor Avoidance in Rats

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DIELENBERG, R. A., J. C. ARNOLD AND I. S. McGREGOR. *Low-dose midazolam attenuates predatory odor avoidance in rats.* PHARMACOL BIOCHEM BEHAV **62**(2) 197–201, 1999.—Previous studies have shown that predatory odors are a potent anxiogenic stimulus for rodents, yet the ability of benzodiazepines to block odor-induced anxiety remains uncertain. The present study reevaluated this issue using a novel apparatus that, in contrast to previous studies, allowed rats to hide from the odor in a small wooden "hide box" placed within a larger arena. The odor stimulus used was a fabric cat collar that had been worn by a domestic cat for a period of 3 weeks. The experiment was divided into three phases on successive days: 1) habituation, where all rats were placed in the apparatus without cat odor present; 2) conditioning, where rats were presented with the cat odor in the apparatus; and 3) test, where rats previously exposed to the odor were tested for a conditioned avoidance response in the absence of the odor. Results showed that rats exposed to the cat collar displayed a robust avoidance response, spending about 70% of a 20-min session in the hide box compared to 25% in control rats. This avoidance response was completely reversed in rats given a low dose (0.375 mg/kg) of midazolam. During the test phase, rats exposed to the cat odor on the previous day showed elevated levels of hiding when returned to the test apparatus without the cat odor present. This conditioned avoidance was significantly attenuated in rats who had received midazolam (0.375 mg/kg) during cat odor exposure but not in rats given the same dose during the test. These results show that low-dose midazolam is an effective anxiolytic agent in rats during exposure to predatory odor. © 1999 Elsevier Science Inc.

Midazolam Cat odor Anxiety Predator Rat Benzodiazepine

AN increasing number of behavioral and pharmacological studies have documented the induction of anxiety-like states and defensive behaviors in rodents exposed to the odors of predators (3,8–10,14,16,20,21). Substantial interest has been expressed in the possible utility of such procedures for modeling specific anxiety states in humans and in the screening of novel anxiolytic drugs (4,7,21). One interesting proposal is that the state induced by predatory odors in rats is akin to "phobic avoidance" in humans (21). In support of this proposal, it was shown that the anxiogenic response to cat odors in rats is innate, does not habituate over repeated exposure to the odor, generalizes to previously neutral olfactory stimuli, and is relatively insensitive to the effects of benzodiazepines (19–21).

The apparently weak effect of benzodiazepines on predatory odor-induced anxiety is particularly interesting, because it is well known that human phobias, in contrast to other anxiety disorders, do not respond well to treatment with these agents (11). In one study, Blanchard's group (3) investigated the effects of diazepam on the "risk assessment" behaviors of rats exposed to a cat odor-impregnated cloth. Risk assessment behaviors include orientation to and visual scanning of a potentially threatening stimulus, together with approaches to and contact with that stimulus. It was shown that high doses of diazepam reduced risk-assessment behaviors including approaches to the cloth, although there were some difficulties of interpretation due to sedative effects of the drug.

Zangrossi and File (20) also investigated the responses of rats exposed to a cat odor-impregnated cloth, but this time in the home cage environment. They measured contact with the cloth and the time spent sheltering from the cloth under the food hopper in the cage. In contrast to the results of Blanchard (3), it was found that a low dose of chlordiazepoxide increased the time spent in contact with the cloth while not affecting the "sheltering response." It was concluded that benzodiazepines are only weakly effective in reducing cat odor-induced anxiety. Like Blanchard's study, however, sedative effects of the higher doses of the drug made exact interpretation of effects problematic.

Given the somewhat inconsistent results of these two studies, we decided to reexamine the effects of benzodiazepines

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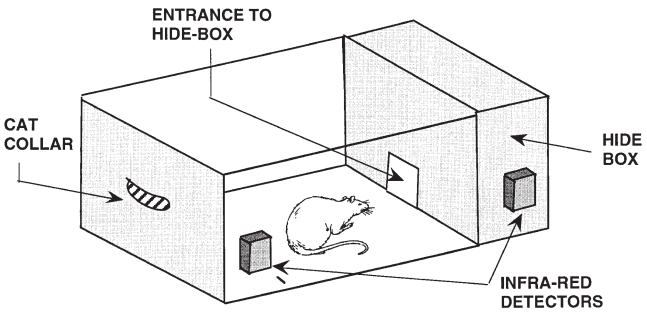

FIG. 1. The cat odor avoidance apparatus used in the study.

on responses to predatory odors using a somewhat simplified model. The guiding assumption in this new model was that if rats are given a clear option to hide from a predatory odor, then a clearer anxiolytic action of benzodiazepines may be seen. Retreat to a strategically defensible location is a primary defensive response in rodents exposed to a predator or a predatory cue (7), yet this response has rarely been measured in studies of odor-induced defense. While the "sheltering under the food hopper" response employed previously (20) may arguably qualify as such a response, it does not provide an altogether robust parallel with defensive behaviors seen in the wild.

We, therefore, constructed a novel "cat odor avoidance" apparatus that allowed rats the choice between being in an open arena in the presence of a predatory odor, or hiding in a small wooden box located at the opposite end of the apparatus to the odor source (see Fig. 1). To avoid the possible confounds introduced by the motoric effects of high benzodiazepine doses (3,20), we employed a low dose (0.375 mg/kg) of midazolam that is devoid of obvious ataxic effects and assessed its effects on odor avoidance.

It was also of interest to determine whether a conditioned avoidance response would be obtained whereby rats reexposed to the environment in which they had previously experienced cat odor show elevated hiding, even when the cat odor is no longer present (21). This would be in line of the previous finding that avoidance responses to a cat odor generalize to other stimuli in the environment in which the odor is presented (21). The effect of a low dose of midazolam on this conditioned avoidance was also investigated.

METHOD

Animals

The subjects were 60 naive male albino Wistar rats (CULAS, Sydney) aged 80 days and weighing an average of 420 g at the time of testing. They were housed in large plastic tubs in groups of seven or eight, with food and water freely available. The colony room was maintained at 24°C with lights on from 2000–0800 h. Rats were handled regularly for 2 weeks prior to the start of the experiment. All experiments were run during the dark cycle. All experimentation was approved by the University of Sydney Animal Care and Ethics Committee.

Apparatus

Testing occurred in four chambers as depicted in Fig. 1. The chambers comprised a rectangular arena with perspex walls: (60 cm (L) × 26 cm (W) × 36 cm (H)) and a metal grid floor that was raised 2 cm above a tray containing wood shavings. At one end of the chamber was a small wooden box (21 cm (L) × 24 cm (W) × 22 cm (H)) termed the "hide box." On the front wall of the hide box was a small 6 × 6 cm square hole that allowed just enough space for a rat (but not a cat) to enter the box. The apparatus was raised on legs for easy access to the underlying tray for cleaning in between trials. During testing, the room in which the chambers were located was illuminated by a 40-W red light suspended 1.5 m above the apparatus.

On the opposite wall to the hide box was an alligator clip positioned 4 cm above the metal grid floor. During testing, a piece of cat collar was attached to the clip. This cat collar had been worn by a domestic cat for a period of 3 weeks before the start of the experiment. On removal from the cat, the collar had been placed in an air-tight plastic container and was stored in a refrigerator at 4°C. The collar was cut into four equivalent pieces, with one piece being used in each of the four test chambers. Before the beginning of trials requiring exposure to cat odor, the collar was attached to the clip and left to stand for 30 min before testing. The cat collar was always handled with plastic gloves. An identical cat collar that had not been worn by a cat ("unworn cat collar") was used as a control stimulus in some conditions (see below).

Photocell detectors were located at opposite ends of the chamber (see Fig. 1), which fed their output to a Macintosh computer running "WorkbenchMac" data acquisition software (12). The placement of the photocells allowed determination within each session of 1) the amount of time (in seconds) the rats spent in close vicinity to the cat collar (hereafter called "approach time"), and 2) the amount of time spent in the hide box (hereafter called "hide time"). All sessions were of 20 min duration. Note that in any given session there were usually substantial periods when rats were not in the hide box or close enough to the cat collar to trigger the photobeam used to calculate "approach time." Thus, "hide time" plus "approach time" rarely approaches 20 min. An approximate measure of locomotor activity was also obtained in each session by counting the total number of photobeam interruptions (both "hide" and "approach" photobeams) made during a session.

Drugs

Midazolam ("Hypnovel," Roche Ltd., Sydney), a short half-life water-soluble benzodiazepine agonist, was diluted in 0.9% saline and injected SC at a dose of 0.375 mg/kg in a volume of 1 ml/kg. The midazolam dose was selected on the basis of pilot studies showing little effect of a 0.25 mg/kg dose on cat odor avoidance and signs of sedation with a higher 0.5 mg/kg dose. The 0.375 mg/kg dose is low in comparison to the doses commonly used with rats in the existing literature (5,6,18).

Procedure

The experiment consisted of three phases, each spaced 24 h apart: habituation, conditioning, and test. Four groups (n = 15

TABLE 1
GROUP TREATMENTS ACROSS PHASES

Group	Habituation	Conditioning	Test
CONTROL	sal, no collar	sal, unworn collar	sal, no collar
SAL/SAL	sal, no collar	sal, worn collar	sal, no collar
MDZ/SAL	sal, no collar	mdz, worn collar	sal, no collar
SAL/MDZ	sal, no collar	sal, worn collar	mdz, no collar

mdz = midazolam, sal = saline.

each) of rats were used and the treatments they received in each phase are shown in Table 1.

Habituation. In the first phase, all rats were given an injection of saline and 10 min later were placed in the apparatus for 20 min in the absence of any cat collar. This phase allowed rats to habituate to the injection procedure and the novel apparatus and gave an indication of baseline levels of hide and approach times in the absence of any odor stimulus.

Conditioning. In this phase, rats were injected with either saline or midazolam and 10 min later placed in the apparatus for 20 min in the presence of either a worn or unworn cat collar. Prior to the conditioning phase rats were divided into four groups such that hide times and approach times during habituation were approximately equal across groups. The CONTROL group received saline injections in the conditioning phase and were presented with an unworn (i.e., odor free) cat collar. The other three groups were all exposed to a worn cat collar during the conditioning phase. Group MDZ/SAL received midazolam prior to worn cat collar exposure while groups SAL/SAL and SAL/MDZ received saline injections.

Test. In the test phase, rats were again injected with either saline or midazolam and 10 min later placed in the apparatus in the absence of any cat collar. This phase allowed determination of any conditioned avoidance occurring as a result of the pairing of the worn cat collar with the environment during the conditioning phase. Groups CONTROL, MDZ/SAL, and SAL/SAL all received saline injections prior to the test phase, while group SAL/MDZ received a midazolam injection.

The experiment was run as two replicates involving 32 (n = 8 per group) and then 28 (n = 7 per group) subjects. The results from both replicates were essentially identical and so the data were pooled to give the total of 15 rats per group.

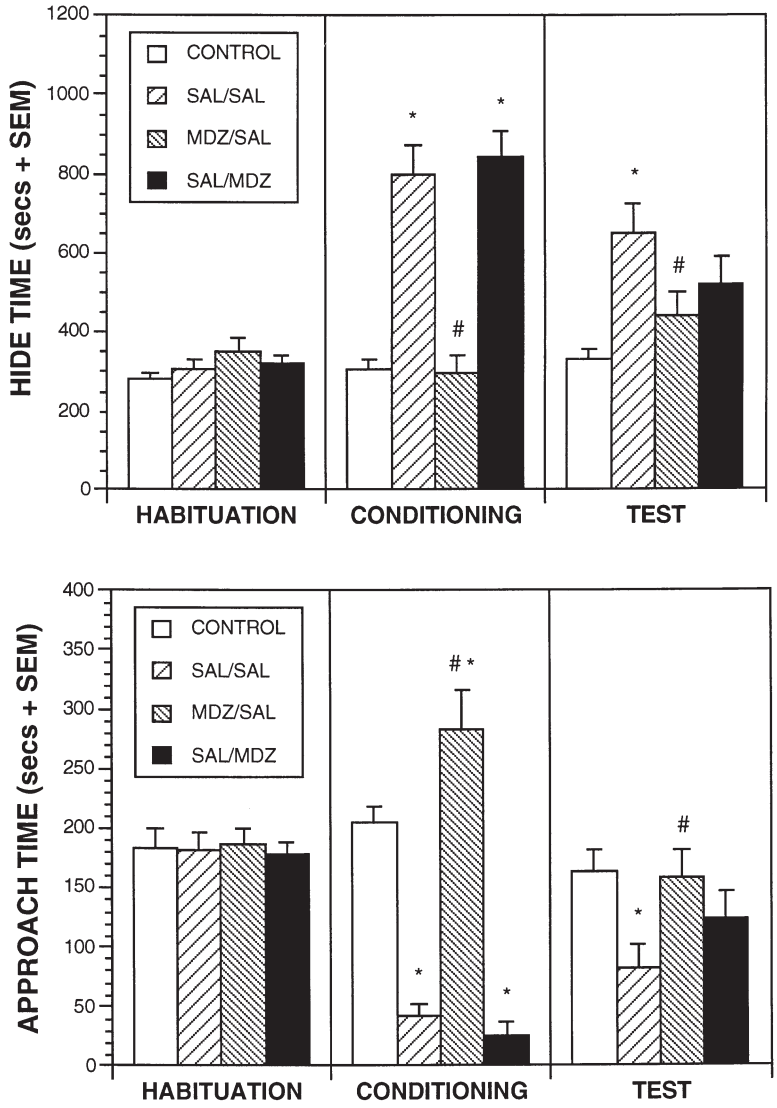

FIG. 2. Hide times (upper) and approach times (lower) for rats in the four groups used in the study. *Significantly different from group control ($p < 0.05$); #significantly different from group SAL/SAL ($p < 0.05$). Note the different scale for the ordinates of the two graphs.

Statistics

Data for "hide time" and "approach time" (in seconds) were compared across groups for each of the habituation, conditioning, and tests phases using one-way ANOVA followed by Newman–Keuls post hoc tests. A significance level of 0.05 was adopted for all tests.

RESULTS

Habituation Phase

The results from all phases are depicted in Fig. 2. Analysis of data for hide time and approach time revealed no significant group differences in the habituation phase (Fs < 1.3).

Conditioning Phase

One-way ANOVAs revealed a significant group difference in hide time, $F(3, 56) = 28.61$, $p < 0.001$, and approach time, $F(3, 56) = 42.91$, $p < 0.001$, during the conditioning phase. Post hoc tests showed that both the SAL/SAL and SAL/MDZ groups differed from group CONTROL on both hide time and approach time. This indicates that rats exposed to the worn cat collar during conditioning hid significantly more and approached the collar significantly less than rats exposed to an unworn cat collar. In addition, group MDZ/SAL differed significantly from both SAL/SAL and SAL/MDZ groups in approach time and hide time during conditioning, indicating that rats given midazolam during conditioning hid less and approached the cat collar more than rats given saline. Group MDZ/SAL also showed significantly higher approach time than group CONTROL.

The number of photobeam interruptions (both "hide" and "approach" photobeams) for each of the four groups during the conditioning phase were as follows (mean ± SEM): CONTROL 84.33 ± 4.53, SAL/SAL 34.47 ± 6.27, MDZ/SAL 62.27 ± 5.19, SAL/MDZ 26.60 ± 5.55. Such data are obviously not an unconfounded measure of activity, because the high level of hiding seen in some groups (i.e., SAL/SAL and SAL/MDZ) is likely to be linked to a lower level of activity. Nonetheless, the data illustrate that the rats in the MDZ/SAL group made a relatively high number of photobeam breaks during conditioning and were, therefore, not suffering from any profound motor impairment.

Test Phase

One-way ANOVAs showed a significant group difference in hide time, $F(3, 56) = 4.97$, $p < 0.01$) and approach time, $F(3, 56) = 3.00$, $p < 0.05$, during the test phase. Post hoc tests revealed a significant difference between groups CONTROL and SAL/SAL in both hide time and approach time during test. This indicates the presence of a conditioned avoidance response whereby rats previously exposed to a cat odor in the test environment subsequently hide more in that environment than nonexposed controls when tested in the absence of the cat odor. A significant difference was also evident between groups SAL/SAL and MDZ/SAL during test on both hide times and approach times. Thus, midazolam given during exposure to cat odor significantly attenuated the conditioned avoidance response to the environment in which the cat odor was experienced.

In the group (SAL/MDZ) given midazolam during the test phase, there was no significant difference in hide times or approach times during test relative to the group (SAL/SAL) given saline. Thus midazolam did not significantly attenuate the conditioned avoidance response established by prior exposure to the cat odor.

DISCUSSION

Although studies involving actual predators (e.g., a live cat) have sometimes measured hiding responses in rodents (2), previous studies involving only predatory odors have not employed apparatus in which a hiding option is given (4,14, 16,19–21). Thus, odors have been presented in a large open arena (14,16), a long narrow Plexiglas box (3), or in the home cage (19–20). Arguably, none of these environments have allowed a strategically defensible location analogous to the burrows, nests, or hollow logs that may be used by rodents in the wild to put a barrier between themselves and a predator. The evidence presented here that rats show a robust hiding response to a cat odor suggests that this measure may be particularly useful to assess in future studies involving predatory odors.

The present paradigm also clearly shows that a cat collar is a convenient stimulus for inducing anxiety in rodents. Previous studies have either used a cloth that has been vigorously rubbed on a cat just prior to testing (3,19–21) or the compound trimethyl thiazoline, which is a principle component of fox feces (14,16). Our preliminary experiments with the latter stimulus showed it to be an acrid compound that is readily detected and avoided by humans. In contrast, the cat collar does not present any odor cue to humans, but is clearly an effective anxiogenic stimulus for rats.

Previous studies by Hogg and File (8,9) have indicated substantial variability across rats in the anxiogenic response to cat odor. These researchers separated their rats into approximately equal numbers of "responders" and other "nonresponders" on the basis of the amount of time spent sheltering under the food hopper in the home cage in the presence of cat odor (8,9). However, in the present study 26 out of the 30 saline-treated rats exposed to cat odor during the conditioning phase increased their hide time by more than 50% relative to the habituation phase, while only 1 out of 15 rats in the nonexposed control group showed such an increase. This indicates a robust and fairly uniform hiding response to cat odor across subjects. In other unpublished work we have shown a similar high proportion of cat odor responders in both Lewis and Hooded Wistar strains of rat. Thus, previous reports of a high prevalence of "nonresponders" to cat odor may be peculiar to the Lister strain of rat used by Hogg and File (8,9) or may reflect a possible lack of sensitivity in the screening procedure used to determine "nonresponders."

A key finding of the present study is that a low dose of midazolam, which has no apparent ataxic properties, completely reversed the elevated hiding and reduced approach time seen in rats exposed to cat odor. The increase in approach times with midazolam corroborates the finding of Zangrossi and File (20) of increased approach to a cat odor-impregnated cloth following a low 5 mg/kg dose of chlordiazepoxide. However, it is inconsistent with the previous finding of reduced approaches following administration of diazepam (3). It, therefore, seems plausible that the relatively high doses of diazepam used in this previous study (2 and 4 mg/kg) may have reduced approaches due to a sedative or ataxic effect.

The finding of a clear and unambiguous reduction in hide times with midazolam challenges the assertion that cat odor induces in rats a state akin to phobic avoidance in humans that is only weakly affected by benzodiazepines (20,21). It is argued here that this earlier conclusion may have been a function of the testing procedures that involved conducting tests in

the home cage environment and that did not allow rats a clear opportunity to hide. Future studies will hopefully test other benzodiazepines such as diazepam and chlordiazepoxide in a "hiding" model so that the generality of the effect observed here with midazolam can be determined.

A second interesting finding in the present study is the presence of a conditioned avoidance response whereby rats hid more in the test environment the day after they had been exposed to cat odor in that environment. This agrees with the earlier finding that defensive responses to cat odor can generalize to previously neutral stimuli (21). Interestingly, rats in the present study that received midazolam during cat odor exposure (group MDZ/SAL), showed a greatly attenuated conditioned avoidance response during the test phase. This observation agrees with many previous findings of a reduction or abolition of aversive conditioning in rats given benzodiazepines immediately prior to conditioning (5,13,17,18). This well-documented effect is thought to reflect either an anterograde amnesia induced by the drug (15) or a failure to acquire conditioned fear due to the anxiolytic properties of the drug (18). There are studies in the literature to support both hypotheses, and unfortunately, the results of the present study do not allow a clear choice to be made between them.

The effects of midazolam in group SAL/MDZ (that received the drug only during the test phase) were suggestive of an attenuation of conditioned avoidance but were not conclusive. There was a reduction in hide time and increase in approach time in this group relative to the SAL/SAL group, but both effects failed to reach statistical significance. However, this group also did not significantly differ in hide time or approach time from the control group, and so essentially displayed an intermediate conditioned avoidance response. Some studies have suggested that benzodiazepines are ineffective in attenuating the expression of conditioned avoidance in rats that have been conditioned in the drug -free state (13). Other studies have found a clear attenuation of conditioned fear in rats given midazolam, but only at doses considerably higher than those used in the present study (6). The effect seen in the SAL/MDZ group was in the right direction to support the latter findings, but it appears that a higher dose of midazolam may be necessary to comprehensively reverse the conditioned avoidance obtained in the present paradigm.

To conclude, the present study clearly shows that a low dose of a benzodiazepine causes a complete attenuation of the hiding response elicited in rats by exposure to cat odor. This apparently "fearless" behavior induced by the drug does not necessarily mean that rats given midazolam fail to detect that the cat odor is threatening. Rather, midazolam may shift the defensive strategy of the rat from hiding to information gathering. This suggestion is akin to the conclusion of Blanchard (1) that the main effect of the benzodiazepines in prey–predator situations is to induce a shift within the defensive repertoire of the animal.

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