Psychopharmacology (1999) 147:174–181

ORIGINAL INVESTIGATION

Iain S. McGregor · Robert A. Dielenberg

Differential anxiolytic efficacy of a benzodiazepine on first versus second exposure to a predatory odor in rats

Received: 14 February 1999 / Final version: 26 June 1999

Abstract *Rationale and objectives:* Rodents tested in the elevated plus maze model of anxiety only show an anxiolytic response to benzodiazepines on their first exposure to the maze. The present study investigated whether a similar phenomenon occurs with benzodiazepines in a different model of anxiety that involves exposing rats to the odor of a predator. *Methods:* Testing took place in a rectangular arena containing a cat odor-exuding collar at one end and a small "hide box" at the opposite end. Rats were initially familiarized with the odor-free apparatus for 20 min and then placed back in the apparatus 24 and 48 h later in the presence of cat odor. *Results:* Vehicle-treated rats displayed marked avoidance of the cat odor on both first and second exposures, spending most of the session in the hide box and very little time near the odor source. In contrast, rats given a low dose of midazolam (0.375 mg/kg) during first exposure spent considerable time in close proximity to the odor source and much less time in the hide box. Rats given midazolam (0.375 mg/kg) on their second exposure to cat odor displayed no such anxiolytic effect of the drug. Rats given midazolam (0.375 mg/kg) on both exposures showed a potent anxiolytic effect of the drug on each occasion. This pattern of results was replicated with a higher dose of midazolam (0.75 mg/kg). A further experiment showed that rats previously exposed to cat odor showed high levels of hiding in the test environment 24 h later even when the cat odor was no longer present. This conditioned fear was blocked by midazolam (0.75 mg/kg) suggesting that the ineffectiveness of midazolam on second exposure to cat odor is not due to a failure of the drug to affect conditioned fear. *Conclusions:* The ineffectiveness of midazolam in odor-experienced rats parallels the results obtained with benzodiazepines in the elevated plus maze. Such results may help illuminate the comparative lack of efficacy of benzodiazepines in treating certain types of anxiety disorders in humans.

Key words Cat odor · Predator · Midazolam · Anxiety · Phobia · Learning

I.S. McGregor (✉) · R.A. Dielenberg
Department of Psychology, University of Sydney,
Sydney, NSW 2006, Australia
e-mail: iain@psych.usyd.edu.au, Fax: +61-2-9351-2603

Introduction

Previous decades have seen the widespread and often indiscriminate use of benzodiazepine drugs for the treatment of anxiety and insomnia. In more recent years, the use of these drugs has come under far greater critical scrutiny (Mant and McManus 1994; Norman et al. 1997). Recognition of the interrelated problems of tolerance, dependence and withdrawal resulting from long term benzodiazepine use (Lader and Morton 1991) has caused increasing restraint in the prescription of benzodiazepines in clinical practice (Mant and McManus 1994). In addition, the realization that serotonergic compounds such as fluoxetine and buspirone can offer a superior treatment to benzodiazepines for anxiety disorders has seen benzodiazepines replaced as the first choice treatment for most, if not all, of these disorders (Norman et al. 1997).

At the preclinical level, research has also shown that the anxiolytic efficacy of benzodiazepines may be rather limited. In one of the most commonly used animal models of anxiety, the elevated plus maze, benzodiazepines are largely ineffective in reducing anxiety in rodents that have had prior experience of the maze (Lister 1987; File 1990, 1993; File et al. 1990, 1993, 1998; Rodgers et al. 1992; Gonzalez and File 1997). On first exposure, benzodiazepines reliably increase the amount of time spent on the open arms of the maze indicating decreased anxiety (Pellow et al. 1985; Lister 1987; File 1990). However, when rats or mice are tested for a second time on the maze ("trial 2"), this anxiolytic effect is much reduced or absent (Lister 1987; File 1990; Rodgers et al. 1992; Gonzalez and File 1997).

Explanations of this "trial 2" effect have centered on the idea that the type of anxiety experienced during sec-

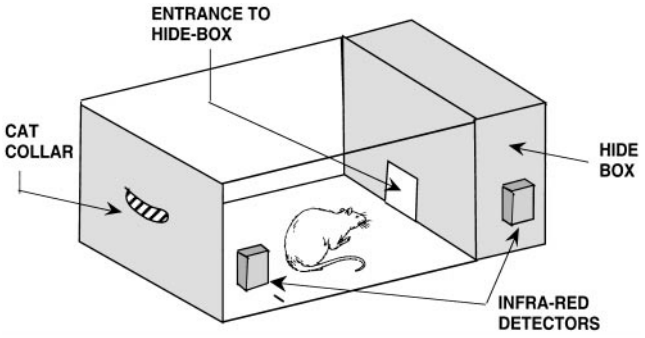

Fig. 1 Schematic of the apparatus used in the study

ond exposure to the maze is somehow different to that experienced on first exposure (File 1993; File et al. 1993). Specifically, it has been thought that anxiety on first exposure may reflect a combination of neophobia and generalized anxiety while anxiety on second exposure may be something akin to that experienced by humans with specific phobias (File 1993). Since specific phobias are insensitive to benzodiazepine treatment (Marks 1987; Norman et al. 1997), this proposal has reasonable face validity.

In the past decade, many laboratories have documented profound behavioral, endocrine and neurochemical changes in rodent species exposed to predatory odors (Blanchard et al. 1990; Vernet-Maury et al. 1992; Zangrossi and File 1992a, 1992b; Kavaliers et al. 1994; Perrot-Sinal et al. 1996; Kemble and Bolwahnn 1997). In recent work, we have developed a novel methodology for assessing the behavioral response of rats to cat odors in the laboratory (Dielenberg and McGregor 1999; Dielenberg et al. 1999). The apparatus used consists of a rectangular arena with a small wooden box (termed the "hide box") at one end and a piece of fabric collar that had been worn by a cat at the opposite end (see Fig. 1). In a typical test session, rats initially approach and sniff the collar, but then retreat rapidly and spend much of the remaining time in the hide box. From this behavior, it is inferred that the worn cat collar possesses properties that are strongly anxiogenic to the rat.

We have shown that a low dose of the short acting benzodiazepine midazolam greatly reduces the hiding response to cat odor and increases approaches towards the odor source, suggesting an anxiolytic effect of the drug (Dielenberg and McGregor 1999; Dielenberg et al. 1999). These findings are of interest, since they run counter to previous claims that cat odor causes "phobic avoidance" in rats that is benzodiazepine insensitive (Zangrossi and File 1992b). They are also at odds with suggestions that benzodiazepines reduce risk assessment behavior in rats exposed to predatory odors (Blanchard et al. 1990). Rather, the increased approach to the collar stimulus caused by midazolam in our apparatus indicates increased risk assessment. The discrepancies between our findings and these previous studies may be attributable to our use of relatively low non-ataxic doses of benzodiazepines and also from our use of a behavioral model where the hiding response to cat odor is specifically measured as an index of anxiety (Dielenberg and McGregor 1999; Dielenberg et al. 1999).

In the present experiment, we examined whether benzodiazepines retain their anxiolytic efficacy across repeated exposures to cat odor. Accordingly, rats were tested with midazolam on their first or second exposure to cat odor. It was predicted that a benzodiazepine would exert a strong anxiolytic effect on first exposure to the odor, in line with our previous results (Dielenberg et al. 1999). On the basis of the present literature, no hypothesis could be put forward as to whether such an effect would occur on second exposure. In experiment 1, a low dose (0.375 mg/kg) of midazolam was tested which has proven efficacy in reducing cat odor induced anxiety on first exposure to the odor (Dielenberg et al. 1999). In experiment 2, a higher dose (0.75 mg/kg) of midazolam was employed which is on the borderline of producing motoric impairment (Drugan et al. 1996; Austin et al. 1999).

Our previous research has shown that rats previously exposed to cat odor in a specific environment show subsequent high levels of hiding when returned to that environment 24 h later, even when the cat odor is no longer present (Dielenberg et al. 1999). The presence of this conditioned fear may be one important difference between the anxiety experienced on first versus second exposure to cat odor and might conceivably explain any difference in the anxiolytic efficacy of benzodiazepines across two consecutive exposures. It was also then of interest to determine whether benzodiazepines affect the conditioned fear resulting from prior odor exposure. This issue was addressed in experiment 3.

Materials and methods

Subjects

The subjects were 104 experimentally naive male albino inbred Wistar rats (CULAS, Sydney) aged 60–90 days and weighing an average of 400 g at the time of testing. The rats were housed in large plastic tubs in groups of eight with food and water freely available. The colony room was maintained at 22 °C on a reverse light-dark cycle with lights on from 2000 to 0800 hours. All experiments were run during the dark cycle. Rats were handled on two occasions prior to the start of the experiment. The experiments were designed to use the minimum number of subjects required for reliable statistical effects. All experiments were approved by the University of Sydney Animal Care and Ethics Committee.

Apparatus

Testing occurred in four chambers as described previously (Dielenberg and McGregor 1999; Dielenberg et al. 1999) and as shown 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 ap-

paratus 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 wool acrylic cat collar ("Tinkle Bell" safety stretch cat collar, model CC 800, manufactured in Taiwan) was attached to the clip. The collar had been worn by a domestic cat for a period of at least 3 weeks before the start of the experiment. On removal from the cat, the collar was placed in an air-tight plastic container and was stored in a freezer at –12°C. The collar was cut into four equivalent pieces [dimensions 50 mm (L)×13 mm (W)5 ×mm (D)], 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 "warmed up" by placing it on top of a computer monitor for approximately 2 min. The cat collar was always handled with latex gloves. Three different collars were used for the three experiments reported in this study. Each collar was effective in producing robust hiding behavior in rats, although it appeared that the collar used in experiment 3 was particularly potent relative to those used in experiments 1 and 2.

Photocell detectors were located at opposite ends of the test chamber approximately 7 cm from the end walls. These detectors fed their output to a Macintosh computer running "Workbench-Mac" data acquisition software (McGregor 1996). The position of the photocells allowed determination within each session of (1) the amount of time (in s) the rats spent in close vicinity (approximately 7 cm or less) 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 equals 20 min (or 1200 s).

Drugs

Midazolam ("Hypnovel", Roche Ltd, Sydney, Australia), 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 or 0.75 mg/kg in a volume of 1 ml/kg. The 0.375 mg/kg midazolam dose used in experiment 1 was selected on the basis of our previous work showing that this low dose is very effective in reversing the response to cat odor while having minimal sedative effects (Dielenberg and McGregor 1999 Dielenberg et al. 1999). In experiments 2 and 3 a higher dose of midazolam was used (0.75 mg/kg). While a 0.5 mg/kg dose of midazolam produces no ataxia in the rotarod test in rats, a 1 mg/kg dose has a substantial ataxic effect (Drugan et al. 1996; Austin et al. 1999). The 0.75 mg/kg dose is therefore on the threshold of impairing motor co-ordination.

Procedure

Experiment 1

Experiment 1 involved 32 rats that were split into four groups of eight, and were used to assess the effects of a low dose of midazolam (0.375 mg/kg) on anxiety during first and second exposure to cat odor. The groups were named according to the drug treatments given on first and second exposures to cat odor, respectively, namely SAL-SAL, SAL-MDZ, MDZ-SAL and MDZ-MDZ. The rats were tested across 3 consecutive days as follows.

Familiarization. In this 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 be familiarized with 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.

Exposure day 1. On the day after familiarization, the rats were injected with either saline or midazolam (depending upon group allocation) and 10 min later placed in the apparatus for 20 min in the presence of the cat collar. Of the four groups, groups SAL-SAL and SAL-MDZ received saline injection on this day, while groups MDZ-SAL and MDZ-MDZ received midazolam.

Exposure day 2. The procedure was identical to that of Exposure day 1 expect that groups SAL-SAL and MDZ-SAL received saline injections on this day while groups SAL-MDZ and MDZ-MDZ received midazolam.

It could be argued that a demonstration of anxiety-induced by the cat odor would require the presence of a control group that is not exposed to odor. This control was not included in any of the present experiments because our previous work has clearly shown that rats exposed to a collar that has not been worn by a cat show very stable low hide times and high approach times across consecutive test sessions (Dielenberg and McGregor 1999; Dielenberg et al. 1999). We have also found that the hiding response in our model is relatively specific to cat odor, since rats do not show elevated hiding to novel odors such as rat urine, flea repellent or peppermint (Dielenberg and McGregor, unpublished data). Similarly, File and colleagues have found that odor of disinfectant does not produce the same avoidance behavior seen in rats exposed to cat odor (Zangrossi and File 1992a).

Experiment 2

Experiment 2 investigated the effects of a higher dose of midazolam (0.75 mg/kg) on anxiety during first and second exposures to cat odor. This involved 32 rats that were split into four groups of eight and allocated to the same conditions as described in experiment 1. The only difference was that the rats given midazolam received a higher dose (0.75 mg/kg) of the drug.

Experiment 3

Experiment 3 was designed to determine whether an absence of anxiolytic efficacy of midazolam on second exposure to cat odor was due to a failure of the drug to affect conditioned fear resulting from the first odor exposure. The study involved 40 rats that were split into four groups of ten and named according to the drug treatments received on Exposure days 1 and 2 and whether a cat collar was present in the apparatus on Exposure day 2. The groups were SAL-SAL, SAL-MDZ, SAL-SAL(nc) and SAL-MDZ(nc), where (nc) denotes "no collar'. The rats were handled exactly as described for experiments 1 and 2 and were tested across 3 consecutive days as follows.

Familiarization. This was identical to experiments 1 and 2.

Exposure day 1. On the day after familiarization, all rats from all groups were injected with saline and 10 min later placed in the apparatus for 20 min in the presence of the cat collar.

Exposure day 2. Groups SAL-SAL and SAL-MDZ were given saline and midazolam (0.75 mg/kg), respectively, and exposed to cat odor. These groups were therefore equivalent to groups SAL-SAL and SAL-MDZ in experiment 2. Groups SAL-SAL(nc) and SAL-MDZ(nc) were placed in the testing apparatus without the cat collar present in order to test for conditioned fear arising from prior odor exposure. Group SAL-MDZ(nc) was given midazolam (0.75 mg/kg) during this test for while group SAL-SAL(nc) was injected with saline. Two measures were taken to avoid rats in the "no-collar" conditions being exposed to any lingering cat odors from previous trials. Firstly, the test boxes were thoroughly washed with dilute ethanol solution prior to the "no-collar" tests. Secondly, the SAL-SAL(nc) and SAL-MDZ(nc) rats were all tested prior to the SAL-SAL and SAL-MDZ rats so as to minimize further the risk of contamination between conditions.

Statistics

Data for hide time and approach time (in seconds) were compared across groups for each of the familiarization, exposure and tests phases using one-way ANOVA followed, where appropriate, by Newman-Keuls post hoc tests. To test directly for differences in the efficacy of midazolam on first versus second odor exposures, planned contrasts (one-way ANOVA) compared approach and hide times in groups MDZ/MDZ and MDZ/SAL on Exposure day 1 with those of group SAL/MDZ on Exposure day 2. An α level of 0.05 was adopted for all tests.

Results

Experiment 1

Familiarization phase

The results from all phases for experiment 1 are depicted in Fig. 2. Analysis of data for hide time and approach time revealed no significant group differences in the familiarization phase (F<1).

Exposure day 1

One-way ANOVA on the data for hide time on Exposure day 1 revealed a significant group effect [F(3,28)=6.10, P<0.01]. Post hoc tests revealed that groups that received midazolam on this day (groups MDZ-SAL and MDZ-MDZ) showed significantly lower hide times than either of the groups that received saline (groups SAL-SAL and SAL-MDZ).

A similar pattern was evident with approach times, with a significant overall group effect [F(3,28)=12.43, P<0.001], and with post hoc tests showing that both groups MDZ-SAL and MDZ-MDZ had significantly higher approach times than groups SAL-SAL and SAL-MDZ.

Exposure day 2

One-way ANOVA on the data for hide time on Exposure day 2 revealed a significant group effect [F(3,28)=4.88, P<0.01]. Post hoc tests showed that groups MDZ-MDZ had significantly lower hide times than each of the three other groups. No other between group comparisons approached significance.

A similar pattern was evident with approach times, with a significant overall group effect [F(3,28)=4.97, P<0.01]. Again, post hoc tests showed that group MDZ-MDZ had significant higher approach times than the three other groups. No other between-group comparisons were significant.

Direct comparison of hide times for group SAL/MDZ on Exposure day 2 versus those for groups MDZ/SAL and MDZ/MDZ on Exposure day 1 indicated significantly higher hide times in group SAL-MDZ [F(2,21)=6.99, P<0.05]. This contrast was also significant for approach times, indicating lower approach times in group SAL-MDZ on Exposure day 2 than the other two groups on Exposure day 1 [F(2,21)=16.76, P<0.001]. Overall, this indicates a significantly greater anxiolytic effect of midazolam on first relative to second exposure to cat odor.

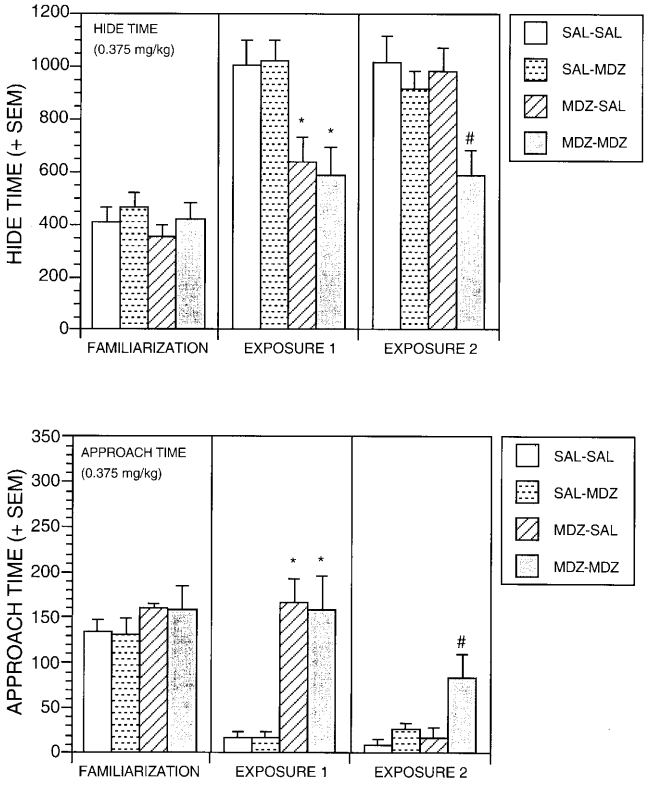

Fig. 2 Hide times (*upper*) and approach times (*lower*) on the familiarization day, Exposure day 1 and Exposure day 2 in rats from the four groups in experiment 1. Data presented are mean+SEM. Abbreviations: *SAL* saline, *MDZ* midazolam. *Significantly different from groups SAL/SAL and SAL/MDZ (Newman-Keuls post hoc tests, P<0.05). #Significantly different from groups SAL/SAL, SAL/MDZ and MDZ/SAL

Experiment 2

Familiarization phase

The results from all phases for experiment 2 are depicted in Fig. 3. Analysis of data for hide time and approach time revealed no significant group differences in the familiarization phase (F<1.4).

Exposure day 1

One-way ANOVA on the data for hide time on Exposure day 1 revealed a significant group effect [F(3,28)=14.28, P<0.001]. Post hoc tests showed that the two groups that received midazolam on this day (groups MDZ-SAL and MDZ-MDZ) showed significantly lower hide times than

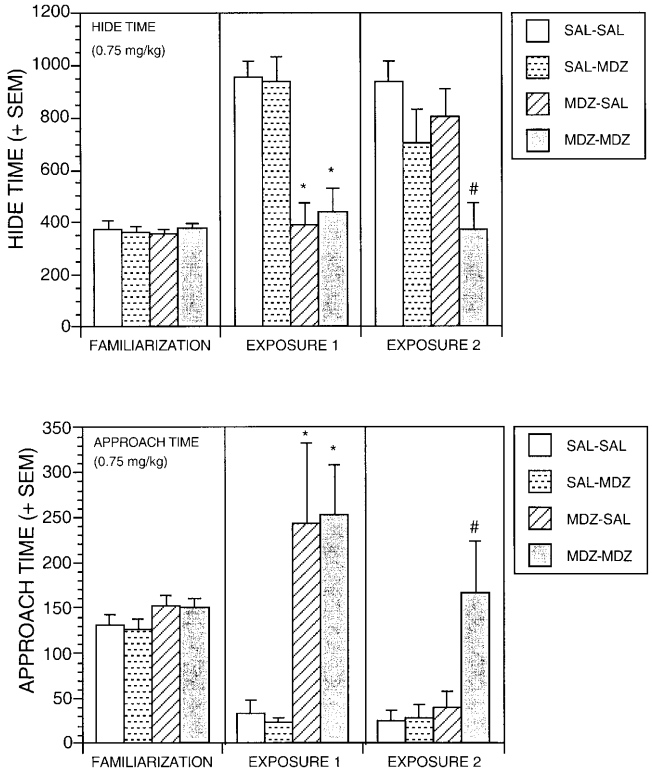

Fig. 3 Hide times (*upper*) and approach times (*lower*) on the familiarization day, Exposure day 1 and Exposure day 2 in rats from the four groups in experiment 2. Data presented are mean+SEM. Abbreviations: *SAL* saline, *MDZ* midazolam. *Significantly different from groups SAL/SAL and SAL/MDZ (Newman-Keuls post hoc tests, $P<0.05$). #Significantly different from groups SAL/SAL, SAL/MDZ and MDZ/SAL

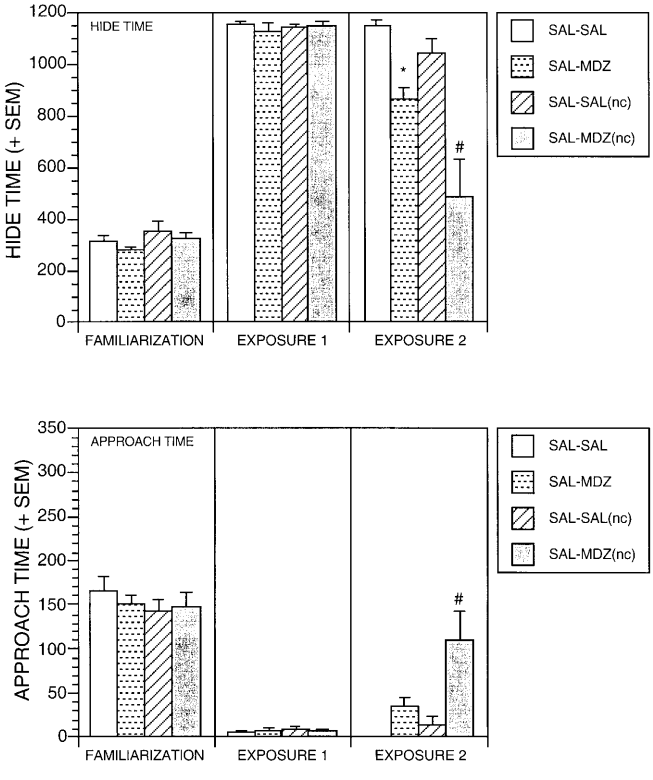

Fig. 4 Hide times (*upper*) and approach times (*lower*) on the familiarization day, Exposure day 1 and Exposure day 2 in rats from the four groups in experiment 3. Data presented are mean+SEM. Abbreviations: *SAL* saline, *MDZ* midazolam, *nc* no collar. *Significantly different from group SAL/SAL only (Newman-Keuls post hoc tests, $P<0.05$). #Significantly different from groups SAL/SAL, SAL/MDZ and SAL/SAL(nc)

the groups that received saline (groups SAL-SAL and SAL-MDZ).

A similar pattern was evident with approach times, with a significant overall group effect [$F(3,28)=5.58$, $P<0.01$], and with post hoc tests showing that both groups MDZ-SAL and MDZ-MDZ had significantly higher approach times than groups SAL-SAL and SAL-MDZ.

Exposure day 2

One-way ANOVA on the data for hide time on Exposure day 2 revealed a significant group effect [$F(3,28)=5.15$, $P<0.01$]. Post hoc tests showed that group MDZ-MDZ had significantly lower hide times than each of the three other groups. No other between group comparisons approached significance.

A similar pattern was evident with approach times, with a significant overall group effect [$F(3,28)=4.78$, $P<0.01$]. Again, post hoc tests showed that group MDZ-MDZ had significant higher approach times than the three other groups. No other between-group comparisons were significant.

Direct comparison of hide times for group SAL/MDZ on Exposure day 2 with those for groups MDZ/SAL and MDZ/MDZ on Exposure day 1 indicated significantly higher hide times in group SAL-MDZ than in the other two groups [$F(2,21)=5.54$, $P<0.05$]. The same contrast was significant for approach times [$F(2,21)=8.32$, $P<0.01$]. This again indicates a significantly greater anxiolytic effect of midazolam on first versus second exposure to cat odor.

Experiment 3

Familiarization phase

The results from all phases for experiment 3 are depicted in Fig. 4. Analysis of data for hide time and approach time revealed no significant group differences in the familiarization phase ($F<1.3$).

Exposure day 1

One-way ANOVA on the data on Exposure day 1 revealed no significant overall difference in hide time or

approach time between groups ($F<1$). This indicates an equivalent anxiogenic effect of the cat odor in all four groups.

Exposure day 2

One-way ANOVA on the data for hide time on Exposure day 2 revealed a significant group effect [$F(3,36)=12.91$, $P<0.001$]. Post hoc tests indicated that groups SAL-MDZ had significantly lower hide times than group SAL-SAL, indicating an anxiolytic effect of the drug on second exposure to cat odor. Group SAL-MDZ(nc), which received midazolam and no cat collar, showed significantly less hiding than any of the other three groups. The significantly lower hide times in group SAL-MDZ(nc) relative to group SAL-SAL(nc) indicates that midazolam counteracts the conditioned fear arising from prior cat odor exposure.

An overall significant group effect was also evident with respect to approach times [$F(3,36)=7.68$, $P<0.001$]. Post hoc tests showed that groups SAL-SAL and SAL-MDZ did not differ from each other in approach times. Group SAL-MDZ(nc) displayed significantly higher approach times than any of the other three groups. The higher approach times in group SAL-MDZ(nc) relative to group SAL-SAL (nc) further indicates that midazolam counteracts the conditioned fear arising from previous cat odor exposure.

Discussion

When given immediately prior to first exposure to cat odor, midazolam had a pronounced anxiolytic action. The low (0.375 mg/kg) dose of midazolam returned approach times to the baseline levels seen during the familiarization phase and also caused a significant decrease in hide times. The higher (0.75 mg/kg) dose of midazolam brought hide times down to baseline levels and increased approach times to a level that was even greater than baseline. These results confirm and extend our earlier findings (Dielenberg and McGregor 1999; Dielenberg et al. 1999) of an anxiolytic action of midazolam in rats given a single exposure to cat odor.

This clear anxiolytic effect of midazolam on first exposure to cat odor stands in contrast to the comparative lack of efficacy of the drug when given on second exposure to the odor. The low (0.375 mg/kg) dose failed to reduce hide times or increase approach times in rats that had experienced a single 20 min exposure to cat odor 24 h previously. The higher (0.75 mg/kg) dose had marginal efficacy in odor-experienced rats, causing a modest reduction in hide times that reached statistical significance in experiment 3. However, this effect on hide times was still significantly weaker than that obtained with the same dose of midazolam on first exposure to cat odor. In addition, the 0.75 mg/kg dose of midazolam had only very slight and non-significant effects on approach times when given on second exposure, unlike its striking effects on approach times during first exposure.

This lack of efficacy of midazolam does not appear to be due to greater anxiety being present on second exposure to cat odor than on first exposure. If this were the case then control rats would have shown greater hide times and lower approach times on the second compared to the first exposure. However, hide times and approach times in the SAL-SAL groups in each of the three experiments remained constant across the two exposures. Indeed, our recent work has shown that when rats are given repeated daily 20-min exposures to cat odor, the hiding response eventually decreases to baseline levels after five to ten sessions (Dielenberg and McGregor 1999).

The data from experiment 3 rule out another possible explanation of the weak midazolam efficacy during second exposure, namely that benzodiazepines fail to reduce the conditioned fear arising from prior exposure to cat odor. The presence of such conditioned fear was established in our previous work (Dielenberg et al. 1999) and was replicated here in Experiment 3 with the high hide times and low approach times seen in group SAL-SAL(nc) on Exposure day 2. The "conditioned fear" theory is plausible since some previous studies involving passive avoidance paradigms have suggested that benzodiazepines are ineffective in attenuating the expression of conditioned avoidance in rats that have been conditioned in the drug free state (Nabeshima et al. 1990). Such results suggest that benzodiazepines might also fail to affect the hiding response of rats that have previously experienced cat odor in the test environment. However, the present results do not support this account. Midazolam clearly reduced hiding and increased approach times in rats exposed to the now odor-free environment in which cat odor had previously been experienced. In demonstrating this, the present study rules out a role for conditioned fear as an explanation of the differential benzodiazepine effect across exposures.

In addressing the "trial 2" effect seen in the elevated plus maze, File and colleagues have suggested that the anxiety experienced on trial 2 may be qualitatively distinct to that experienced on trial 1 (File 1993; File et al. 1993). Thus on trial 1, a state of generalised anxiety and/or neophobia may be present that can be effectively treated by benzodiazepines. However, during trial 1, this generalized anxiety may be replaced by a "phobic" anxiety state arising from the fear of heights and open spaces that are present in the plus maze. This "phobic" anxiety state, which predominates on subsequent exposures to the maze, is not amenable to treatment with benzodiazepines. This account might also be a plausible explanation for the present results, namely that prior exposure to cat odor causes a long lasting change in the nature of the unconditioned fear experienced on subsequent exposures to the odor. Whether this means that the fear experienced becomes "phobic" in nature is open to speculation. One argument against the "phobic" account is the relatively fast habituation of the hiding response to cat odor that

we have recently reported in rats given repeated exposure to the odor (Dielenberg et al. 1999).

It is important to note that the MDZ-MDZ groups in experiments 1 and 2 showed a strong anxiolytic response to midazolam on both first and second exposures to cat odor. Thus midazolam can be effective on second exposure so long as it is also given on first exposure. This indicates that the anxiolytic effect of the benzodiazepine on first exposure to cat odor protects against the neural changes resulting from cat odor exposure that lead to a subsequent decreased sensitivity to the anxiolytic efficacy of benzodiazepines. These results agree with those from the elevated plus maze, showing that an anxiolytic effect of benzodiazepines may be seen on both trial 1 and trial 2, provided that rats receive the drug on both occasions and sessions are of sufficiently long duration (File et al. 1993).

Recent studies offer some insight into the neural changes that result from a single plus maze experience that lead to subsequent loss of sensitivity to benzodiazepines. Maze-experienced rats show altered sensitivity of benzodiazepine receptors in the dorsal raphe nucleus which renders them uniquely sensitive to an anxiolytic action of the benzodiazepine antagonist flumazenil injected into this region (Gonzalez and File 1997). Maze-experienced, but not maze-naive, rats are also sensitive to an anxiolytic action of the 5-HT_{1A} agonist 8-OH-DPAT injected into the dorsal raphe nucleus. Other evidence supports a role for the amygdala, since rats with lesions of the basolateral amygdala displayed an anxiolytic action of benzodiazepines on both first and second exposures to the plus maze (File et al. 1998). Finally, the dorsal hippocampus is also implicated since 8-OH-DPAT was found to have anxiogenic effects when injected into this structure in plus maze experienced, but not plus-maze naive, rats (File et al. 1996). Obviously, it is hoped that future work will uncover whether alterations in serotonergic and benzodiazepine receptor sensitivity are also involved in the shifting response characteristics of rats given repeated exposure to cat odor. This may also give insight into whether the anxiety states experienced on trial 1 and trial 2 on the plus maze are similar or even identical to those experience on first and second exposure to cat odor.

Finally, it is interesting to debate the implications of the present results for treatment of human anxiety disorders. One implication appears to be that if a strong fear of a biologically important stimulus has already developed then benzodiazepines will be of little use in attenuating this fear. The situation where benzodiazepines may be useful, however, is in producing "fearless" behavior when an anxiogenic stimulus is encountered for the first time and in preventing a change in the fear state that renders it insensitive to the anxiolytic effects of benzodiazepines.

Acknowledgements This research was supported by Australian Research Council grants to Iain S. McGregor. Robert A. Dielenberg is the recipient of an Australian Postgraduate Award from the University of Sydney. We are very grateful to Justin Harris and Fred Westbrook for their insightful comments on this work.

References

Austin M, Myles V, Brown PL, Mammola B, Drugan RC (1999) FG 7142- and restraint-induced alterations in the ataxic effects of alcohol and midazolam in rats are time dependent. Pharmacol Biochem Behav 62:45–51

Blanchard RJ, Blanchard DC, Weiss SM, Meyer S (1990) The effects of ethanol and diazepam on reactions to predatory odors. Pharmacol Biochem Behav 35:775–780

Dielenberg RA, McGregor IS (1999) Habituation of the hiding response to predatory odor in rats (*Rattus Norvegicus*). J Comp Psychol (in press)

Dielenberg RA, Arnold JC, McGregor IS (1999) Low dose midazolam attenuates predatory odor avoidance in rats. Pharmacol Biochem Behav 62:197–201

Drugan RC, Coyle TS, Healy DJ, Chen S (1996) Stress controllability influences the ataxic properties of both ethanol and midazolam in the rat. Behav Neurosci 110:360–367

File SE (1990) One-trial tolerance to the anxiolytic effects of chlordiazepoxide in the plus-maze. Psychopharmacology 100: 281–282

File SE (1993) The interplay of learning and anxiety in the elevated plus-maze. Behav Brain Res 58:199–202

File SE, Mabbutt PS, Hitchcott PK (1990) Characterisation of the phenomenon of "one-trial tolerance" to the anxiolytic effect of chlordiazepoxide in the elevated plus-maze. Psychopharmacology 102:98–101

File SE, Zangrossi H, Viana M, Graeff FG (1993) Trial 2 in the elevated plus-maze – a different form of fear. Psychopharmacology 111:491–494

File SE, Gonzalez LE, Andrews N (1996) Comparative study of pre- and postsynaptic 5-HT_{1A} receptor modulation of anxiety in two ethological animal tests. J Neurosci 16:4810–4815

File SE, Gonzalez LE, Gallant R (1998) Role of the basolateral nucleus of the amygdala in the formation of a phobia. Neuropsychopharmacology19:397–405

Gonzalez LE, File SE (1997) A five minute experience in the elevated plus-maze alters the state of the benzodiazepine receptor in the dorsal raphe nucleus. J Neurosci 17:1505–1511

Kavaliers M, Wiebe JP, Galea LAM (1994) Reduction of predator odor-induced anxiety in mice by the neurosteroid 3α-hydroxy-4-pregnen-20-one (3αHP). Brain Res 645:325–329

Kemble ED, Bolwahnn BL (1997) Immediate and long-term effects of novel odors on risk assessment in mice. Physiol Behav 61:543–549

Lader M, Morton S (1991) Benzodiazepine problems. Br J Addict 86:823–828

Lister RG (1987) The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology 92:180–185

Mant A, McManus P (1994) Benzodiazepine use – continuing to fall. Med J Aust 161:454

Marks IM (1987) Fears, phobias and rituals. Oxford University Press, New York

McGregor IS (1996) Using Strawberry Tree WorkbenchMac and Workbench PC software for data acquisition and control in the animal learning laboratory. Behav Res Meth Instr Comput 28: 38–48

Nabeshima T, Tohyama K, Ichihara K, Kameyama T (1990) Effects of benzodiazepines on passive avoidance response and latent learning in mice: relationship to benzodiazepine receptors and the cholinergic neuronal system. J Pharmacol Exp Ther 255:789–794

Norman TR, Ellen SR, Burrows GD (1997) Benzodiazepines in anxiety disorders: managing therapeutics and dependence. Med J Aust 167:490–495

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Meth 14:149–167

Perrot-Sinal TS, Heale VR, Ossenkopp KP, Kavaliers M (1996) Sexually dimorphic aspects of spontaneous activity in meadow voles (*Microtus pennsylvanicus*): effects of exposure to fox odor. Behav Neurosci 110:1126–1132

Rodgers RJ, Lee C, Shepherd JK (1992) Effects of diazepam on behavioural and antinociceptive responses to the elevated plus-maze in male mice depend upon treatment regimen and prior maze experience. Psychopharmacology 106:102–110

Vernet-Maury E, Constant B, Chanel J (1992) Repellent effect of trimethyl thiazoline in the wild rat *Rattus Norvegicus*. In: Doty RL, Muller-Schwarze D (eds) Chemical signals in vertebrates VI. Plenum Press, New York

Zangrossi H Jr, File SE (1992a) Behavioral consequences in animal tests of anxiety and exploration of exposure to cat odor. Brain Res Bull 29:381–388

Zangrossi H Jr, File SE (1992b) Chlordiazepoxide reduces the generalised anxiety, but not the direct responses, of rats exposed to cat odor. Pharmacol Biochem Behav 43:1195–1200