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Male and female C57BL/6 mice respond differently to diazepam challenge in avoidance learning tasks

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Abstract

Benzodiazepines (BZ) impair learning and memory performance of animals. The goal of this study was to examine sex differences in the effects of diazepam on learning and memory of C57BL/6 mice in avoidance paradigms. Male and female C57BL/6 mice were tested in the one-way active avoidance, step-down passive avoidance, and foot-shock pain threshold tasks, following administration of vehicle or diazepam (1 mg/kg). No substantial sex or drug effects on the threshold of the pain response to shock were found. There were no significant differences in avoidance performance between vehicle-treated male and female mice while 1 mg/kg of diazepam produced opposite effects on performance of males and females in both tasks. Diazepam-treated females learned faster in the active avoidance task and showed stronger retention in the passive avoidance task. In contrast, diazepam impaired learning of males in the active avoidance task and had no effect on their performance in the passive avoidance task. Diazepam-induced impairment in males was not due to higher sensitivity to the sedative effect of diazepam as females were more sedated than males on the first trial of the passive avoidance task. Our data showed that sedative and amnesic effects of BZs are not tightly linked. This study also suggests that cognitive effects of BZs in rodents could be sex dependent and highlight the importance of using both sexes in studies on behavioral effects of psychoactive drugs.

Keywords: C57BL/6 mice; Diazepam; Gender differences; Avoidance learning; Foot-shock pain threshold

1. Introduction

Gender differences exist in a number of physiological measures, including body size and body weight, genitalia, presence or absence of reproductive cycle, the size of certain brain areas, and differences in sexual as well as nonsexual behaviors (for review see Kelly et al., 1999; Migeon and Wisniewski, 1998). Differential performance of males and females can also be found in learning and memory tests, although results often depend on the particular task and experimental procedure employed. In both humans and rodents, males are usually better than females in spatial learning and memory (Berger-Sweeney et al., 1995; Geary et al., 2000; Mathis et al., 1994; Mishima et al., 1986; Roof, 1993). In other tasks, such as visual memory and object recognition (Barnfield, 1999; Ghi et al., 1999) and the nonspatial version of the water radial-arm maze (Hyde et al., 2000), females perform better than males. On the other hand, no gender differences have been found in measures of avoidance learning in rodents (Lamberty and Gower, 1988; Mishima et al., 1986; Parra et al., 1999).

Benzodiazepine (BZ)-induced cognitive impairments are a well-known phenomenon in humans (for review see Barbee, 1993; Curran, 1991; Lister, 1985) with the degree of impairment depending on task difficulty as well as on the particular BZ used, its dose and route of administration (for review see Curran, 1986). BZs also produce dose-dependent effects on learning and memory processes in animals (for review see Thiebot, 1985), but these effects depend on the time that the drug is administered (prelearning vs. postlearning administration), the strain of animals used, and the learning and memory tests used. For example, the BZ chlordiazepoxide impaired passive avoidance response in ddY mice when administered before training, but not when administered immediately after training or before the retention test (Nabeshima et al., 1990). On the other hand, diazepam did not impair performance of Balb/c male mice in spatial learning task (Borde and Beracochea,

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1999) and even improved performance of poorly learning STD-ddY male mice in the active avoidance paradigm (Oka et al., 1980).

The $GABA_A$/BZ receptor complex in the amygdala is a site of action for the anxiolytic effects of BZs but is also involved in the modulation of memory storage, particularly in emotional-based memory (Dickinson-Anson and McGaugh, 1997; Tomaz et al., 1991, 1993). BZ-induced impairments in avoidance learning are mainly due to stimulation of BZ/$GABA_A$ receptors in the basolateral nucleus of the amygdala (Tomaz et al., 1992), where BZRs are most densely concentrated (Niehoff and Kuhar, 1983). Gender differences in the expression of BZRs in the basolateral nucleus of the amygdala have also been described in wood mice (Canonaco et al., 1997), with females expressing a higher number of $GABA_A$ and BZR binding sites than males. This could implicate a differential effect of BZs on cognitive performance of male and female rodents. Yet, the majority of research is done exclusively on males. Individual differences, such as age or hepatal functioning, are always taken into account when studying behavioral effects of psychoactive drugs while gender, as one of the most important variables, is often not included in either preclinical or clinical studies. This is surprising not only because females consume more psychotropics than men (Cafferata et al., 1983) but also because sex differences in reactivity to psychoactive medication including BZs are well documented (for review see Yonkers et al., 1992).

The goal of the present study was to assess gender differences in behavioral effects of the BZ diazepam on avoidance behavior of male and female C57BL/6J mice. Avoidance paradigms were selected for the following reasons: (1) avoidance behavior is amygdala-dependent (LeDoux, 1993), (2) there are no gross gender differences in avoidance performance between sexes under untreated conditions (Lamberty and Gower, 1988; Mishima et al., 1986; Parra et al., 1999), (3) avoidance learning is disrupted by BZs via activation of the BZRs in the amygdala (Dickinson-Anson and McGaugh, 1997; Tomaz et al., 1992), and (4) there are sex differences in the expression of $GABA_A$/BZ receptors in the amygdala (Canonaco et al., 1997). The C57BL/6 strain of mice was used as these mice are moderate learners in avoidance tasks (Crawley et al., 1997) and, thus, both impairment and improvement could be observed. Since our experimental design included many groups, we used only one dose of diazepam. We selected 1 mg/kg of diazepam as this dose induced cognitive impairments in avoidance tasks (Decker et al., 1990).

2. Materials and methods

2.1. Subjects and housing

Adult male and female C57BL/6NCrlBR (C57BL/6) mice, from Charles River Laboratories (St. Constant, Quebec, Canada), were used in this study. Mice of the same sex were housed in groups of two to three in standard Plexiglas cages (30×15×12 cm) in a vivarium with a room temperature of 22±1 °C. They were kept on a reversed 12:12 h light–dark cycle with lights off from 9:30 a.m. to 9:30 p.m., starting 2 weeks prior to behavioral testing. Laboratory rodent chow No. 5001 (Agribrand Purina, Strathroy, Ontario, Canada) and tap water were available ad libitum.

One hundred and seventy-two mice were purchased for this experiment. Forty-nine mice ($n=12–13$) were used in the one-way active avoidance test, 83 mice ($n=9–11$) were used in the step-down passive avoidance test, and 40 mice ($n=10$) were used to assess foot-shock pain threshold. All animal protocols were approved by the Dalhousie University Animal Care committee and conformed to the Canadian Council on Animal Care guidelines. No mice died during the experiment. One C57BL/6 female mouse was excluded from the foot-shock threshold assessment for a low weight (9 g).

2.2. Behavioral testing

Behavioral testing started 2 weeks after the arrival of the animals in the laboratory. Before each behavioral procedure, animals were handled for 2 days to habituate them to handling. In all tests, mice were randomly tested with regard to sex and test condition. To reduce any lingering olfactory cues, the apparatus was cleaned with a 70% ethanol solution after each animal was tested. All tests were carried out during the dark phase of the light/dark cycle.

2.2.1. One-way active avoidance

2.2.1.1. Apparatus. The one-way active-avoidance test apparatus was a two-compartment box. The small compartment was 8 cm long and 3 cm wide at the bottom, with V-shaped sides, 10 cm high and sloping to 10 cm wide at the top. It was made of semitransparent white Plexiglas and illuminated from above with a 5-W light bulb. This compartment was connected to a large compartment through a 3-cm doorway, which had a black sliding door that allowed the doorway to be opened and closed. The large compartment was the same width and height as the small one, but was 18 cm long and made of black Plexiglas. The sidewalls and the floor were covered with two pieces of sheet metal separated by a 3-mm gap along the midline of the floor. A buzzer was located in the near proximity of the apparatus.

2.2.1.2. Procedure. On each experimental day, male and female mice were transported in their home cages to the laboratory adjoining the test room and left there undisturbed for at least 10 min. They were then weighed and administered diazepam (1 mg/kg) or vehicle in a volume of 10 ml/kg. Propyleneglycol mixed with distilled water (ratio 1:1) was used as vehicle. After injection, mice were returned to their home cages for 30 min.

Testing started 30 min after injections. Mice received 10 learning trials per day for 3 days. On the first trial, mice were placed individually into the large compartment of the apparatus with the door closed and left there for about 5 s. Once the door opened, the buzzer immediately delivered an auditory stimulus (70 dB) for 5 s followed by unscrambled shock for 15 s (0.6 mA). On the following nine trials (from the second till the tenth trial), the procedure was the same except that mice were placed in the large compartment with the door open. After each trial, the mice were removed from the apparatus and placed in a cage similar to their home cage with clean wood shavings covering the floor for 30–60 s.

The latency to escape from the large compartment was measured from the beginning of the auditory stimulus, using a stopwatch. Mice that escaped before the buzzer (on Trials 2–10) were given a latency of 0 s. All escapes that occurred before or during the auditory stimulus were scored as conditioned responses.

2.2.1.3. Statistics. Data from the active avoidance test were analyzed using a repeated two-way ANOVA (with factors of SEX and DRUG) followed by Student–Newman–Keuls multiple post hoc comparisons.

2.2.2. Step-down passive avoidance

2.2.2.1. Apparatus. It was a box made of gray Plexiglas. The grid floor had a square shape (48×48 cm) and walls were 46 cm high. There was a stainless steel tray 2.5 cm beneath the grid floor that was cleaned with alcohol between each session. The grid floor consisted of 36 parallel steel rods (0.4 cm in diameter) set 1.1 cm apart and was wired to a Grason-Stadler AC shock generator (model L4956) and a Massey-Dickinson peripheral power supply (model 11722). The shock generator was set to deliver a 0.6 mA scrambled electric shock for 2 s when the switch was turned on by an experimenter. A round Plexiglas platform (7.5 cm in diameter, 1.2 cm high) was located in the center of the floor. A Plexiglas restraining tube (7.6 cm in diameter, 18 cm high) was used for placing the subjects on the platform prior to measuring the step-down latency.

2.2.2.2. Procedure. The test paradigm consisted of an acquisition (learning) session followed by retention sessions given 1, 7, and 14 days after the acquisition session. Male and female mice were randomly assigned to one of the following experimental conditions: shocked vehicle-treated, nonshocked vehicle-treated, shocked diazepam-treated, and nonshocked diazepam-treated mice. Before the acquisition session, mice were transported to the laboratory adjoining the test room, weighed, and given either vehicle (propyleneglycol with distilled water in ratio 1:1) or diazepam (1 mg/kg) in a volume of 10 ml/kg 30 min prior to the first acquisition trial.

The acquisition session began by placing the mouse on the platform using the tube. The tube was then removed from the apparatus allowing the mouse to move off the platform. The latency for the mouse to step down onto the grid floor was recorded using a stopwatch. Once the mouse was on the steel grid floor with all four paws, an electric shock was delivered for 2 s. After each trial, the mouse was removed from the apparatus and placed in a cage with clean wood shavings for a rest period of 15–30 s. The nonshock group underwent the same procedure without receiving foot-shock. The learning criterion was reached when the mouse remained on platform for 100 s or when the mouse underwent 10 trials. Once the mouse met criterion, it was returned to its home cage. On the *retention* sessions, untreated mice were placed onto the platform using the restraining tube and the latency to step down with all four paws was measured, with a maximum cut off time of 600 s. No foot-shock was delivered during retention sessions.

2.2.2.3. Statistics. Step-down latency on the first acquisition trial and the number of trials to criterion were analyzed using a three-way ANOVA with factors of sex, drug, and group (shocked or nonshocked). Step-down latencies on retention trials in this test were analyzed using a repeated three-way ANOVA. Post hoc multiple comparisons were carried out using Student–Newman–Keuls tests.

2.2.3. Foot-shock pain threshold

2.2.3.1. Apparatus. The test arena was the same as that used for the step-down passive avoidance tests except that the round Plexiglas platform was removed for this experiment. A Plexiglas restraining tube (as described above) was used for placing the subjects, this time in the middle of the arena.

A microphone was placed 43 cm above the center of the test arena and connected to a sound level meter (Radio Shack, model 33-2050). The sound level meter was set to show sound levels between 60 and 76 dB so that it did not pick up external noise. A Panasonic color CCTV camera (model WV-CP230) was placed 150 cm in front of the test arena on a Velbon tripod (model PX-751) and connected to a Panasonic Time Lapse Video Recorder (model AG-6050) with a Panasonic Video Monitor (model TR-930C). The shock generator and the sound level meter were placed beneath the apparatus so that the video camera could record the behavior of the mice, the settings of the shock generator, the "shock on" light, and the sound meter.

2.2.3.2. Procedure. Male and female mice were randomly assigned to diazepam (1 ml/kg sc) or vehicle (propyleneglycol/distilled water) treatment. After injection, mice were returned to their home cages for 30 min.

Thirty minutes later, each mouse was removed from its home cage and placed into the start tube. The tube was

Table 1
Definitions of behaviors observed in mice in response to foot-shock

Scale	Behavior	Definition
0	No response	The mouse's behavior does not change during the 1-s shock.
1	Flinch	The mouse shudders, shakes its shoulders, lifts its feet up and down, shakes its head, straightens the tail, and points the tail straight up into the air.
2	Hop/run	The mouse takes one (hop) or more (run) rapid jumps across the bars in any direction, often hopping with the tail up and head lowered.
3	Two-paw jump	The mouse lifts two paws (front or back) from the surface of the bars, lifts the tail and shakes it while taking small hops ahead.
4	Vocalization	The mouse makes an audible squeak that moves the needle on the sound level meter.
5	Four-paw jump	The mouse lifts all four paws from the surface of the bars.
6	Freeze during shock	The mice's hind legs remain still while the front legs move towards the hind legs. Often the back is arched while the tail goes in the air.
7	Freeze during the initial 20-s interval	The hind legs of the mouse remain still although there can still be movement among the front legs, upper body, and tail. This must occur for a minimum of 10 s.

removed and 20 s later the first shock was delivered. The shocks were of 1-s duration and of the following shock intensities: 0.05, 0.08, 0.10, 0.13, 0.16, 0.20, 0.25, 0.30, 0.35, and 0.40 mA. First half of the mice were given shocks in ascending order, starting with 0.05 mA, and the second half were given shocks in descending order starting with 0.40 mA. At the end of testing, each mouse was returned to its home cage and the metal bars of the apparatus and the tray under it were cleaned with alcohol.

Behavioral response to shock of each mouse was scored from videotapes by an observer using an ordinal scale of response (Table 1). This scale and definitions of behaviors were derived from our pilot study (using three male CD-1 mice) and from Schrott and Crnic (1994). The lowest shock level to stimulate each behavior was recorded. If no response was shown to a shock level the mouse was not scored. Behaviors were scored on a check sheet by an observer during the shock procedure. That same observer then viewed the videotapes and rescored the behaviors. For reliability a blind observer then scored the videotapes and the results were correlated with those of the first observer. Since both correlation coefficients were high, scores obtained by the first observer were used for statistical analysis.

2.2.3.3. Statistics. Data were analyzed using a three-way ANOVA with factors of sex, drug, and order (ascending or descending).

3. Results

3.1. One-way active avoidance

Data for active avoidance are summarized in Fig. 1. Mice in each of the four groups showed a significant reduction in escape latency over the 3 days [$F(3,90) = 70.78$, $P < .0001$; Fig. 1A] and a significant increase in the conditioned escape responses [$F(3,90) = 81.76$, $P < .0001$; Fig. 1B]. Two-way ANOVA also revealed overall differences between sexes [$F(1,45) = 4.53$, $P < .05$] and Sex×Drug interactions [$F(1,90) = 7.36$, $P < .01$] for the escape latency and for the conditioned escape responses [sex: $F(1,90) = 4.39$, $P < .05$; Sex×Drug interaction: $F(1,90) = 5.89$, $P < .05$]. The Day×Sex×Drug interaction was significant for the conditioned responses [$F(3,90) = 4.57$, $P < .05$].

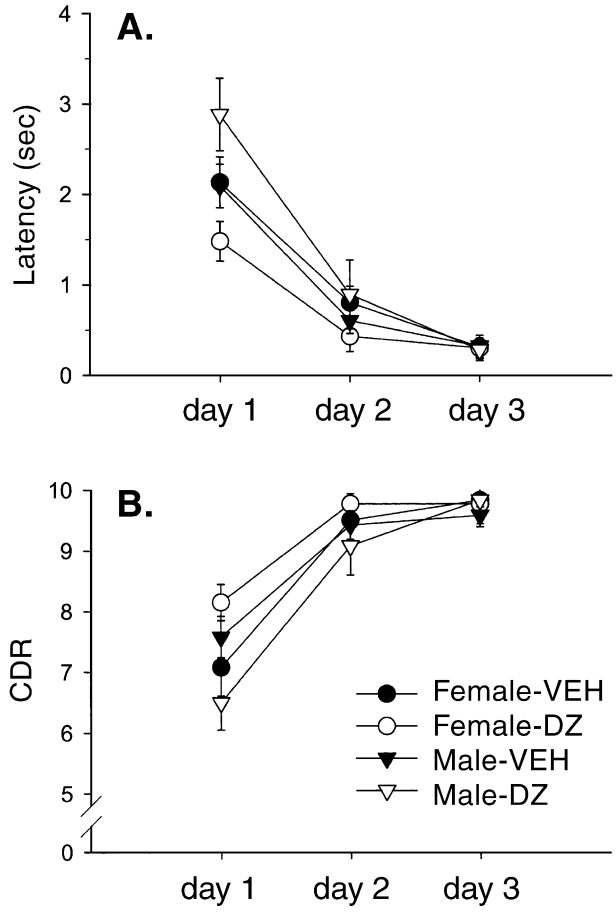

Fig. 1. Active avoidance task. Performance of male and female C57BL/6 mice treated with vehicle (full symbols) or 1 mg/kg of diazepam (empty symbols) 30 min prior to testing on three subsequent days. Upper plot (A) shows mean escape latency in seconds (± S.E.M.), lower plot (B) the mean number of conditioned responses (± S.E.M.).

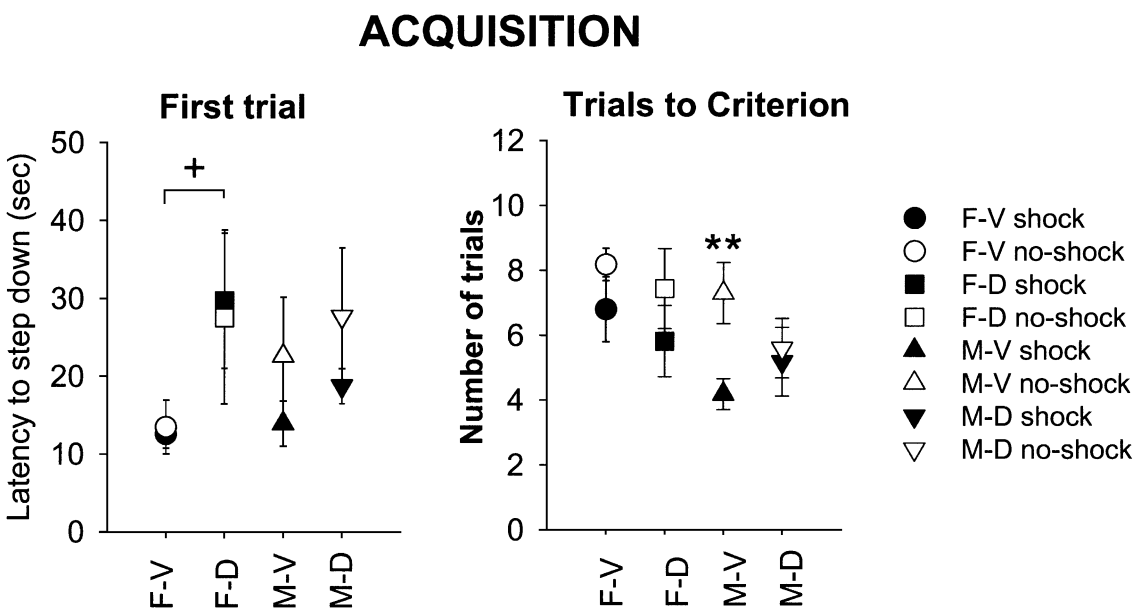

Fig. 2. Acquisition of the step-down passive avoidance task. The mean latency in seconds (±S.E.M.) to step down on the first acquisition trial (left panel) and the mean number of trials (±S.E.M.) to criterion (right panel) by shocked (full symbols) and nonshocked (empty symbols) male (M) and female (F) C57BL/6 mice. Vehicle (V) or 1 mg/kg of diazepam (D) were administered 30 min prior to the first acquisition trial. $^{+}P<.05$ between vehicle- and diazepam-treated female mice, $^{**}P<.01$ between shocked and nonshocked vehicle-treated male mice.

The significant differences between sexes and in sex–drug interactions were, however, found only on Day 1. Thus, female mice treated with diazepam showed the shortest escape latency and the highest number of conditioned escape responses while males treated with diazepam had the longest escape latency and the lowest number of conditioned escape responses. No differences were found between vehicle-treated male and female mice on Day 1. There were no significant differences between groups on Days 2 and 3 escape latency or in the conditioned escape responses.

3.2. Step-down passive avoidance

On the first acquisition trial, there was a significant effect of diazepam on the step-down latency [$F(1,75)=5.024$, $P<.05$; Fig. 2A], but only in female C57BL/6 mice (Student–Neuman–Keuls multiple post hoc comparisons: $P<.05$). Males treated with diazepam did not differ from males treated with vehicle. Three-way ANOVA revealed no significant effect of sex or condition (shocked vs. nonshocked) on the step-down latency in the first learning trial.

The number of trials to reach the learning criterion was affected by sex [$F(1,75)=5.208$, $P<.05$] and by condition [$F(1,75)=6.236$, $P<.05$]. Thus, shocked mice learned faster than nonshocked mice and males learned faster than females (Fig. 2B). Post hoc comparisons showed that a significant difference in the number of trials to criterion was found between the vehicle-treated groups of males (Fig. 2B). There was no effect of diazepam on acquisition in the step-down passive avoidance task for males or females (Fig. 2B).

Data from the retention sessions (1, 7, and 14 days posttraining) are summarized in Fig. 3. Repeated three-way ANOVA revealed a significant effect of condition over the retention sessions [$F(1,74)=15.431$, $P<.001$] with shocked groups having longer step-down latencies than their nonshocked controls on each of the retention sessions. The Time×Sex×Drug×Condition interaction also reached significance [$F(275)=3.17$, $P<.05$]. However, post hoc multiple comparisons (Student–Newman–Keuls) showed that the effect of interaction was present only on the first retention session (24 h posttraining), with diazepam-treated shocked females having longer step-down latency than their nonshocked controls. No effect of drug was found in males 24 h posttraining as well as on the second (7 days posttraining) and third (14 days posttraining) retention sessions in both sexes.

3.3. Foot-shock pain threshold

Data are shown in Table 2. All 39 mice showed a flinch response at a mean shock intensity of 0.08 mA. There was no sex, drug, or order (ascending or descending) effect. All 39 animals ran after the shock at the mean intensity of 0.09 mA. There was no sex or drug effect. There was an effect of order [$F(1,31)=23.29$, $P<.0001$] as in the ascending series the mean intensity was 0.10 mA and in the descending series the mean was 0.07 mA. Sixty-nine percent of mice jumped after the shock, with a threshold of 0.20 mA. There was no sex or drug effect but there was an effect of order [$F(1,31)=7.45$, $P<.05$] with ascending series having a higher threshold (0.25 mA) and descending

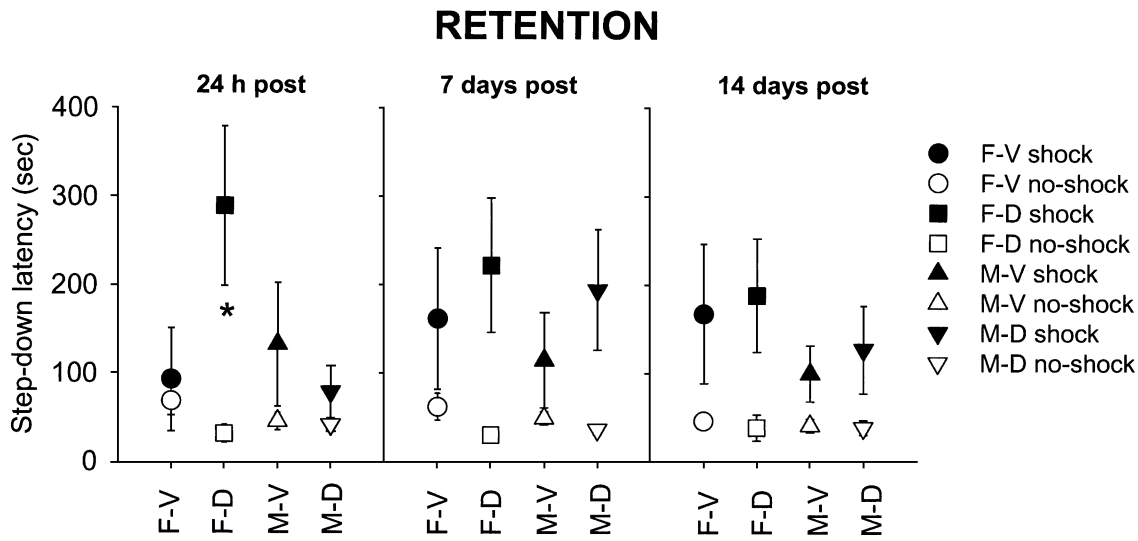

Fig. 3. Step-down latencies on retention trials in the step-down passive avoidance task. Shocked (full symbols) and nonshocked (empty symbols) male (M) and female (F) C57BL/6 mice were treated with 1 mg/kg of diazepam (D) or vehicle (V) 30 min prior to the first acquisition trial. On retention trials, all experimental groups remained untreated. Data show mean latencies ± S.E.M. There was an overall effect of shock ($P < .001$) and "time×sex×drug×condition" interaction ($P < .05$). *$P < .05$ between shocked and nonshocked diazepam-treated female mice.

series having a lower threshold (0.15 mA). Freezing during shock was shown by 62% mice. There was no effect of sex, drug, or order.

All 39 mice vocalized after shock. The mean threshold shock required to elicit a vocalization was 0.12 mA. There was a drug effect [$F(1,31) = 17.29$, $P < .001$], order effect [$F(1,31) = 61.43$, $P < .0001$], and Drug×Order effect [$F(1,31) = 6.23$, $P < .05$]. The threshold for ascending series was about 0.08 mA and for descending series was 0.15 mA, and the drug groups were less sensitive than the vehicle groups. Ninety-five percent animals froze during the 20-s initial interval. There was a drug effect [$F(1,29) = 14.85$, $P < .001$], and a trend toward sex effect ($P = .06$) and a Sex× Drug interaction ($P = .55$). Thus, the male vehicle group were more likely to freeze in the initial 20-s interval than the female vehicle group, and vehicle-treated mice were more likely to freeze than diazepam-treated mice of both sexes.

4. Discussion

Sex differences in learning and memory performance of rodents have been found in various tasks, with males performing better than females on spatial tasks (Berger-Sweeney et al., 1995; Mishima et al., 1986) and females performing better than males on visual memory tasks (Ghi et al., 1999). No sex differences were found in avoidance paradigms (Lamberty and Gower, 1988; Mishima et al., 1986; Parra et al., 1999), thus providing equal baseline performance for studying gender-dependent effects of drugs on avoidance learning and memory. Our study revealed the opposite effect of diazepam at the dose of 1 mg/kg on avoidance learning and memory in male and female C57BL/6 mice. While 1 mg/kg of diazepam improved avoidance behavior in female mice in both the active and passive avoidance paradigms, it impaired performance of male

Table 2
Mean pain threshold to elicit behavioral response in C57BL/6J mice

	Flinch	Run	Jump	Freeze-shock	Vocalization	Freeze-20 s interval
M-VEH ascending	0.082 ± 0.009	0.114 ± 0.010	0.250 ± 0.029	0.056 ± 0.006	0.068 ± 0.007	0.265 ± 0.041
M-VEH descending	0.072 ± 0.016	0.072 ± 0.010	0.153 ± 0.050	0.050 ± 0.000	0.126 ± 0.016	0.222 ± 0.063
M-DZ ascending	0.088 ± 0.018	0.114 ± 0.010	0.232 ± 0.060	0.070 ± 0.012	0.094 ± 0.010	0.122 ± 0.021
M-DZ descending	0.060 ± 0.010	0.065 ± 0.010	0.143 ± 0.054	0.050	0.202 ± 0.014	0.094 ± 0.010
F-VEH ascending	0.074 ± 0.006	0.100 ± 0.012	0.317 ± 0.017	0.060 ± 0.010	0.078 ± 0.008	0.210 ± 0.039
F-VEH descending	0.106 ± 0.037	0.076 ± 0.011	0.164 ± 0.035	0.050	0.126 ± 0.016	0.094 ± 0.010
F-DZ ascending	0.085 ± 0.005	0.085 ± 0.005	0.165 ± 0.035	0.050 ± 0.000	0.085 ± 0.005	0.130 ± 0.030
F-DZ descending	0.062 ± 0.007	0.062 ± 0.007	0.160 ± 0.028	0.050 ± 0.000	0.182 ± 0.023	0.088 ± 0.005
Statistics		c	c		b,c	b,c

Mean shock intensity (mA) ± S.E.M. that elicited behavioral response in male (M) or female (F) C57BL/6J mice treated with vehicle (V) or diazepam (D) 30 min prior to test. Within each experimental group, half of the mice were given increasing intensities of shocks (ascending) while the other half decreasing intensities of shocks (descending). Three-way ANOVA was used: a significant effect of sex (a), of drug (b), or of order (c) are depicted on the last line of the table.

C57BL/6 mice in the active avoidance task and had no effect on behavior of males in the passive avoidance task. Male and female C57BL/6 mice treated with vehicle showed substantially the same performance in both the active and passive avoidance paradigms.

In the one-way active avoidance paradigm, animals are required to run from one compartment of the apparatus to another in order to escape or avoid a foot-shock (Schulteis and Koob, 1993). We measured not only the escape latency but also the number of conditioned avoidance responses, i.e., responses made before the foot-shock was applied. Although all groups of mice learned the task over the 3 days of testing, significant differences were found between experimental groups. While no gender differences were found between vehicle-treated mice, diazepam at the dose of 1 mg/kg improved performance of females but impaired performance of males on Day 1.

In the step-down passive avoidance test, mice must "remember" that a step down from the platform leads to an unpleasant event, i.e., a foot-shock, and will therefore hesitate to repeat it in the future. The consequent increase in response latency is believed to reflect the strength of the memory trace for the aversive event (Sahgal, 1993). Diazepam (1 mg/kg) administered before training had no effect on acquisition in our step-down passive avoidance task, in a run-through passive avoidance task, (Nabeshima et al., 1990) or in an elevated T-maze (Conde et al., 1999; Decker et al., 1990). However, all of these studies found that diazepam caused cognitive impairment on retention trials in males that was not observed in our study. In contrast, diazepam prolonged the step-down latency on the 24-h posttraining retention test in shocked female C57BL/6 mice, suggesting a better memory of the aversive event in these females.

We also found that shocked vehicle-treated males, but not females, needed fewer trials to reach the learning criterion than their nonshocked controls (see Fig. 2). Thus, vehicle-treated females showed a similar number of trials to reach criterion whether or not they were shocked, which is in agreement with our previous study (Podhorna and Brown, 2002). However, behavioral observation of mice in that study showed that shocked C57BL/6 mice attempted to jump off of the platform toward the top of the arena in order to escape from the apparatus. Similar behavior was adopted by shocked C57BL/6 females in the present study suggesting that step-down latency on acquisition is confounded by jumping in this strain of mice. It is difficult to conclude whether this jumping represents a confound of anxiety or whether C57BL/6 mice have general difficulties in acquiring a passive response.

Impaired avoidance learning in males was not due to higher sensitivity of males to the sedative effect of diazepam. Diazepam-treated females, but not males, were more sedated than vehicle-treated females as they showed longer step-down latency on Trial 1 of the acquisition phase in the passive avoidance test. Similarly, Fisher and Hughes (1996) reported greater suppression of walking in female than in male rats following diazepam administration. However, the higher level of sedation in females in comparison with males did not interfere with their learning and memory performance. This suggests that the cognitive impairment effects of BZs are not inextricably linked to their sedative properties (Curran, 1986).

Anxiety interferes with learning and memory in humans and BZs generally impair cognitive performance of healthy volunteers as well as anxious subjects, without gender discrimination (Lucki and Rickels, 1988). However, improvement of cognitive functioning after BZs can be found in high-anxiety subjects (Daniels and Hewitt, 1978), presumably due to the decreased level of their anxiety. This suggests that the opposite effect of diazepam on avoidance learning of male and female C57BL/6 mice could be due to sex differences in their anxiety level or sensitivity to the anxiolytic action of BZs. Generally, C57BL/6 females are less anxious and more exploratory than males (Brown et al., 1999; Mathis et al., 1994), although gender differences are usually subtle and task specific. Gender differences in the anxiolytic action of BZs are still controversial as some studies have found higher responsiveness in females than in males (Mathis et al., 1994; Pericic et al., 1985), some have found no sex differences (Stock et al., 2000), and still others have found lower responsiveness in females than in males (Fernandez-Guasti and Picazo, 1997). Still, we cannot exclude the possibility that sex differences in levels of anxiety and exploration or in the anxiolytic action of BZs might be responsible for our results, as they were obtained in stressful situations.

In our study, we tested females randomly throughout the estrous cycle. Under such conditions, sex differences in pain sensitivity in rodents are not significant (for review see Mogil et al., 2000). Indeed, there were no differences between vehicle-treated male and female mice in either of our avoidance paradigms. However, $GABA_A$/BZ receptors play a modulators role in nonopioid analgesia (Kunchandy and Kulkarni, 1987) and thus, differences in avoidance performance between males and females could be due to gender-dependent modulation of pain sensitivity by diazepam treatment. Unfortunately, studies on the analgesic effects of BZs are controversial. For example, systemic or intrathecal administration of the BZ alprazolam did not induce analgesia in male C57BL/6 mice (Pick, 1996) and diazepam had no effect on basal nociception of male DBA/2 mice (Rodgers and Randall, 1987). In contrast, BZs clonazepam, chlordiazepoxide, and diazepam induced analgesia in albino mice of both sexes (Kunchandy and Kulkarni, 1987). However, both sexes were included within each treatment group in this study and no evaluation of gender differences was performed. Therefore, we ran a third experiment to study effects of diazepam treatment on foot-shock pain threshold in male and female C57BL/6J mice. Our results showed substantially no sex or drug effect on the threshold of the pain responses to shock, as measured by the

flinch, run, jump, and freeze during the shock. Diazepam influenced the threshold to vocalize and freeze during the initial 20-s interval. However, the threshold for these behaviors were below 0.20 mA, so that at the shock level used in avoidance paradigms all animals showed a pain response.

Very little basic or clinical research has been conducted on sex differences in the metabolism of psychoactive drugs. In general, human findings show minimal effects of gender on pharmacokinetics of BZs such as chlordiazepoxide (Greenblatt et al., 1989), diazepam (Greenblatt, et al., 1980a), alprazolam (Greenblatt and Wright, 1993), or midazolam (Thummel et al., 1996), although a greater clearance of oxazepam in men has been reported (Greenblatt et al., 1980b). Gender-dependent differences in metabolism of BZs in animals have received even less attention. Hepatic enzymatic activities are higher in male than in female rats (Reilly et al., 1990; Watanabe et al., 1997). This could lead to increased metabolism of BZs in males and could explain why males were less sedated than females, when given the same doses of BZs. However, faster metabolic rate cannot explain why males were more cognitively impaired after diazepam than females. This suggests that there is little relationship between metabolic rate and the amnestic properties of BZs. This is supported by the findings that other psychoactive drugs, such as antidepressants, exert their CNS activity even when their plasma level is low (Marcourakis et al., 1999). Thus, the pharmacokinetic and pharmacodynamic properties of psychoactive drugs are not always tightly linked.

Different levels of circulating gonadal hormones in males and females could also be responsible for the sex-dependent effect of DZ on avoidance learning. Circulating female sex hormones, mainly neurosteroid metabolites of progesterone, modulate GABAergic transmission via the $GABA_A$ receptors (Lambert et al., 1995), as do BZs. Moreover, progesterone and its naturally occurring metabolite, 3α-hydroxy-5α-dihydroprogesterone, increase binding of [^{3}H]flunitrazepam in the basolateral amygdaloid nucleus and in the CA1 layer of the hippocampus (Canonaco et al., 1989). The basolateral nucleus of the amygdala is known to play an important role in the amnesic action of BZs (Tomaz et al., 1993) and the BZ receptor in the hippocampus has been shown to be involved in avoidance learning (Ribeiro et al., 1999). In regard with these findings it is interesting that the expression of $GABA_A$ binding sites is significantly higher in females than in males in both the basolateral nucleus of the amygdala and the CA1 layer of the hippocampus (Canonaco et al., 1997). It is therefore possible that neurosteroids modulate (either positively or negatively) the function of BZRs and this in turn results in gender-dependent behavioral actions of BZs.

The amygdala is involved not only in the anxiolytic action of BZs but also in the storage of emotional memory (Tomaz et al., 1993). As mentioned above, gender differences in the expression of $GABA_A$/BZ receptors in the amygdala have been found (Canonaco et al., 1997), suggesting that behavioral action of BZs mediated via the amygdala might be dependent on sex. There is an increasing body of evidence showing that anxiolytic and sedative properties of BZs might be affected by gender (Mathis et al., 1994; Pericic et al., 1985) but the role of gender in the amnesic action of BZs was not known. Our study showed that 1 mg/kg of diazepam had negative effect on avoidance performance in male mice but not in female C57BL/6 mice. The impairment was not linked to the sedative action of BZs as female mice showed better learning performance but higher level of sedation following administration of diazepam. As there is ample evidence of gender differences in the effects of BZs on learning and memory and BZ-steroid hormone interactions in the modulation of behavior, future research should focus on the nature of these gender differences and their implications for human psychopharmacology.

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