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Sex differences in the burying behavior test in middle-aged rats: Effects of diazepam

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ABSTRACT

The full behavioral profile displayed during the burying behavior test was studied in middle aged (11–14 months) males, females with irregular estrous cycles, and females in persistent diestrus, with and without diazepam (0.5–2.0 mg/kg). Ambulation and motor coordination were also tested to discern behavioral changes from general motor alterations. Without diazepam treatment, middle-aged males showed longer burying behavior latencies, more prod explorations and less freezing than both groups of females. Untreated middle aged males also showed less cumulative burying and more immobility compared to females with irregular cycles. None of the parameters showed any difference between the female groups. Diazepam (0.5 and 1.0 mg/kg) increased burying behavior latency in females, but had no effect on any parameter in middle aged males. However, a higher dose (2.0 mg/kg) of diazepam increased immobility, freezing and the number of prod shocks and decreased prod explorations and groomings, but impaired motor coordination in males. In contrast with young males and females, diazepam at any dose reduced cumulative burying. Data are discussed on the bases of (1) sex and age differences in burying behavior and on (2) the anxiolytic-like action of diazepam and its side effects.

1. Introduction

Defensive burying is a rodent behavior that consists of displacing bedding material with typical alternating pushing movements of their forepaws and shoveling movements of their heads directed at localized sources of aversive stimulation, such as an electrified prod. Although controversial, sex differences in burying behavior have been suggested, since young adult males bury less than females (Fernández-Guasti and Picazo, 1990; Treit et al., 1980 vs. Maggio and Harder, 1983; Sluyter et al., 1999). In addition, other studies report that burying behavior changes along the female estrous cycle (Fernández-Guasti and Picazo, 1992; Frye et al., 2000), since females in proestrus show less cumulative burying than ovariectomized rats or females in diestrus or metestrus. The levels of burying behavior in proestrus females are reduced when the levels of progesterone and its reduced metabolite, allopregnanolone, are increased during this stage (Fernández-Guasti and Picazo, 1992; Frye et al., 2000). Age has also been explored as another variable that affects burying behavior. Treit and coworkers in 1980 described that 60 and 90 day old rats displayed more burying than 30 day old animals. In line, 21 day old males showed less burying than 80 day old animals and burying behavior began to decline by 150 days of age (López-Rubalcava et al., 1996). Thus, burying behavior levels change depending upon the sex, the endocrine stage and animal´s age.

The demonstration that anxiolytic drugs suppress burying behavior has lead to the proposal that it reflects experimental anxiety (De Boer and Koolhaas, 2003). Thus, a large body of evidence showed that diazepam importantly reduces the time animals spent burying, at doses ranging from 0.25 to 2.0 mg/kg (see De Boer and Koolhaas, 2003 for review). In addition, diazepam increased burying behavior latency, interpreted as impaired reactivity (Fernández-Guasti and Picazo, 1990). The actions of diazepam on burying behavior depended on the sex and the estrous cycle phase of females (Carey, et al., 1992). Thus, males were more sensitive than females to the anxiolytic-like effects of diazepam, evidenced by a reduction in cumulative burying; while females in proestrus were more susceptible than males and females in metestrus to an increase in burying behavior latency (Fernández-Guasti and Picazo, 1990). These data indicate that the effects of diazepam in this test vary according to the sex and the endocrine state of the female.

Unfortunately, most of these studies only scored burying behavior; therefore, the influence of sex, age and the actions of diazepam on parameters denoting competing avoidance behaviors is largely unknown. De Boer and Koolhaas (2003) recommended observing and quantifying the full defensive behavioral repertoire: defensive burying, immobility, freezing, prod exploration and shocks (avoidance/defensive behaviors); as well as grooming (self-care behavior). This ethological analysis (e.g., De Boer et al., 1990; Moser and Tait, 1983; Peacock and Wong, 1982; Treit et al., 1986; Tsuda et al., 1988) has shown that in addition to defensive prod-burying, animals also display immobility

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postures away from the prod, indicating a different behavioral expression of experimental anxiety. As defensive burying implies motor coordination, general ambulation and motor coordination must be also assessed to discern specific reductions in cumulative burying from motor alterations.

One of the most important hormonal changes related to aging is the gradual reduction in testosterone (T) in males and the relatively abrupt cessation of ovarian hormone production in females (Lamberts et al., 1997; Perheentupa and Huhtaniemi, 2009). In female rats, as in humans, there is a transition from regular to irregular cycles that eventually leads to the complete absence of ovarian cyclicity (characterized, in rats, by constant vaginal diestrus) (Lu et al., 1979). In contrast to humans, some rats show an intermediate phase of permanent estrus distinguished by constant high estradiol levels and low levels of progesterone (Lu et al., 1979; vom Saal and Finch, 1988). Thus, middle-aged rats with irregular cycles are proposed as the best animal model to study human perimenopause and those in constant diestrus (or in estropause) are estimated as the most suitable model for studying human menopause. Interestingly, a large body of clinical and experimental evidence shows that the reduction in gonadal hormones – associated or independent of aging – is related with increased anxiety (Chung-Park, 2006; Sağsöz et al., 2001; Walf and Frye, 2010). In line, recently, Walf et al. (2009) showed that middle-aged reproductively competent female rats had less anxiety-like behavior in the elevated plus maze, elevated zero maze and Vogel punished drinking task than females transitioning to reproductive senescence or reproductively senescent animals. There were no differences between these groups in the mirror maze and in the defensive burying task although reproductive-senescent rats spent more time burying the prod than the other groups. This study suggested different experimental anxiety between middle aged females with particular endocrine stages. However, it did not discern if the differences between the groups depended upon the specific estrous cycle phase.

The clinical analyses of the prevalence of anxiety disorders in the elderly have reported that women show a higher prevalence of generalized anxiety disorder than men (Krasucki et al., 1998). Additionally, considerable clinical evidence suggests that the elderly are more sensitive to the actions of benzodiazepines (Greenblatt et al., 1989, 1991; Meyer, 1982; Morgan 1990); particularly to their side effects (such as drowsiness, sedation, confusion, ataxia and falls) which are more than twice as high in patients over 70 than in patients under 40 years (Ashton, 1994; Boston Collaborative Drug Surveillance Program 1973). In aged animals, Wikinski et al. (2001) showed that aged male rats were insensitive to the anxiolytic-like actions of diazepam (putatively related to the failure of diazepam to potentiate GABA-induced $^{36}Cl^-$ flux in cortical micro-sacs) and hypersensitive to the motor side effects of this benzodiazepine. Therefore, aged humans and animals treated with benzodiazepines present a blunted anxiolytic response and increased sedative effects.

In this study we compared the behavioral repertoire of middle-aged males and females in two endocrine conditions, irregular cycles and constant diestrus, when exposed to the burying behavior test. We also assessed whether the anxiolytic-like effect of diazepam differed between middle-aged males and females in this test. Besides analyzing burying behavior, we also recorded avoidance/defensive (immobility and exploration) and self-care (grooming) behaviors, as well as ambulation and motor coordination. We hypothesize that middle-aged males and females in two endocrine conditions would differ in their behavioral response in the burying behavior test with and without diazepam treatment.

2. Materials and methods

2.1. Animals

Male and female middle-aged (10–14 months) Wistar rats were used in this study. All animals were housed in a room under controlled conditions of temperature (22 °C) and light (12/12 h light–dark cycle, lights off at 10:00 h) with *ad libitum* access to commercial rat chow and tap water. As in previous studies (Fernández-Guasti and Picazo, 1990; Jiménez-Velázquez et al., 2006; Korte et al., 1992) animals were individually housed 72 h before the burying behavior test, in cages measuring 27×16×23 cm. These series of experiments followed the general principles of laboratory animal care (NIH publication 85–23, 1985 and NOM-062-ZOO-1999) and were approved by the local ethical committee.

The stage of the estrous cycle was determined by vaginal smears taken daily, at 10:00 h, for at least 14 days before the experiment. The four estrous cycle phases were established according to vaginal-cytology, as follows: proestrus (round nucleated cells), estrus (cornified cells), metestrus (round-nucleated and cornified cells, as well as leukocytes) and diestrus (predominance of leukocytes). Females in constant diestrus and those with irregular estrous cycles were selected. The former were defined as females showing at least 10 continuous days of vaginal diestrus, while the latter were defined as females showing a given phase (usually metestrus) continuously (for 3–4 consecutive days) and an alteration in the natural sequence of the estrus cycle. Females with irregular cycles were tested in metestrus, since this phase is characterized by relatively low levels of estrogens and progestins and to perform possible comparisons with young females tested in the same phase (Fernández-Guasti and Picazo, 1990). Animals were tested for burying behavior after determining their estrous cycle phase. Males were similarly manipulated for at least 3 weeks before testing. Animals were tested only once. Diazepam (Hoffman-La Roche, Mexico City, Mexico) was dissolved in a 15% solution of propyleneglycol and injected i.p. at 0.5 or 1.0 mg/kg, 30 min before the test (Fernández-Guasti and Picazo, 1990). Only middle-aged males were treated with a higher dose of diazepam (2.0 mg/kg).

2.2. Burying behavior test

The burying behavior test was used to assess experimental anxiety (Fernández-Guasti and Picazo, 1990; Pinel and Treit, 1978). Briefly, the experiments were performed under dim red light to prevent behavioral alterations. The cage where the test was performed was of the same dimensions than those used to house the animals; but had a 7 cm long electrified probe placed in one of the cage walls, 2 cm above the bedding material (fine sawdust). When the rat touched the electrified probe with its snout or forepaws it received a 0.3 mA shock (the electric source was a constant current shocker, model 5806, LaFayette Instruments). This test lasted for 10 min and the following measures were recorded: (1) burying behavior latency: time between the first shock and the display of burying behavior; (2) cumulative burying behavior: total amount of time spent spraying the bedding material towards and on top of the shock probe; (3) immobility: crouching, lying, sitting or standing still on at least 3 ft, with the body motionless; (4) number of prod explorations: body posture oriented towards the prod in attend-like position followed by a sudden withdrawal; (5) number of prod shocks; (6) number of rearings: body raised on the hind limbs in a vertical position, (7) number of groomings: sequential motor acts in a cephalo-caudal direction, including licking the paws, washing movements over the head, fur licking and cleaning of the tail or genitals, and (8) freezing: amount of time spent completely motionless immediately after touching the prod (De Boer and Koolhaas, 2003). Independent groups of 8–14 animals were evaluated 10 min after vehicle or diazepam.

2.3. Ambulatory behavior and motor coordination tests

Ambulatory activity was tested immediately after experimental anxiety using the same animals. The activity test consisted of placing a rat on a clean Plexiglas cage measuring 43×36×19 cm, located over a

38×40 cm sensitive plate (Stoelting Co., Chicago, USA). Activity counts were recorded during a 10 minute session. A clean testing cage was used for each rat. In addition, motor coordination was tested after ambulation; using a rotarod gyrating at 11 rpm. Animals were trained to walk in the rotarod during three previous consecutive sessions; as previously described (Fernández-Guasti and López-Rubalcava, 1998). We recorded the number of falls from the rotarod during a 5 minute session.

2.4. Statistics

A two way ANOVA was performed for each parameter, with sex or endocrine condition (males, females with irregular cycles and females in constant diestrus) as one factor, diazepam treatment (0, 0.5 and 1.0 mg/kg) as another and the interaction between them, followed by post hoc Tukey tests. In addition, as data did not follow a normal distribution, one way non-parametric Kruskal–Wallis ANOVAs within each condition (sex or endocrine stage) were performed, with diazepam as factor. Finally, Kruskal–Wallis ANOVAs were carried out to compare all the parameters between the groups without diazepam treatment. Mann–Whitney U tests followed Kruskal–Wallis ANOVAs. The proportions of rats showing each behavior were compared using the Fisher F test.

3. Results

Fig. 1 shows the proportion of regular-, irregular-, persistent diestrus and constant estrus-Wistar female rats aged 10 to 14 months. At 10–11 months, most rats (77–82%) showed irregular cycles and quite few (9–12%) displayed persistent diestrus. Between the 12th and the 14th month, the percentage of females showing persistent diestrus (21–28%) increased and the proportion of females in irregular cycles (69–72%) decreased. The percentage of females with regular estrous cycles and those in constant estrus remained constant (below 10%).

Table 1 shows the proportion of animals displaying burying behavior after treatment with diazepam. In all three groups, but particularly in males and in persistent diestrus females, 1.0 mg/kg of diazepam tended to decrease the percentage of subjects showing burying behavior. The proportion of middle aged males displaying this behavior significantly decreased after treatment with 2.0 mg/kg of diazepam [only 3 out of 13 (27%) as compared with 9 out of 10 (90%) in the control saline-treated group]. As shown in Table 2, diazepam at 2.0 mg/kg increased immobility, freezing time and prod shocks; while reduced prod explorations and groomings. This dose also drastically impaired motor coordination, since it significantly increased falls from the rotarod; thus, this dose was not tested in middle-aged females.

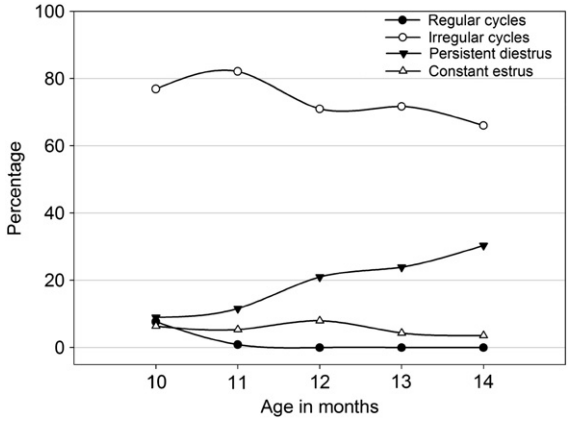

Fig. 1. Proportion of middle aged (10–14 months) Wistar female rats showing regular estrous cycles, irregular estrous cycles, persistent diestrus and permanent estrus. The total population was 90 females.

Table 1
Percentage of middle aged rats displaying burying behavior after diazepam.

	Vehicle	Diazepam 0.5 mg/kg	Diazepam 1.0 mg/kg	Diazepam 2.0 mg/kg
Irregular cycles	90%	69%	80%	–
Persistent diestrus	100%	100%	64%	–
Males	90%	83%	62%	27%**

Fisher exact probability test **$p<0.01$.

The effects of diazepam on burying behavior and on motor coordination are shown in Figs. 2 to 4. Fig. 2A, shows the latency to bury in middle-aged males, females with irregular cycles and females in persistent diestrus; with and without diazepam treatment. Only the animals that buried were considered in the analyses. The two way ANOVA revealed statistical significance for condition (sex and endocrine stage; $F_{2,69}=7.497$, $p<0.001$), but diazepam treatment had no effect ($F_{2,69}=1.093$, NS) and these factors did not interact ($F_{4,69}=2.397$, NS). Untreated males showed much longer burying behavior latencies than both groups of females (Kruskal–Wallis ANOVA, $H=7.800$, $p<0.05$). No differences were found between the female groups. The one way Kruskal Wallis ANOVA revealed that diazepam increased burying behavior latency in females with irregular cycles ($H=9.200$, $p<0.01$) and in females with persistent diestrus ($H=7.515$, $p<0.05$), but had no effect in middle-aged males ($H=2.305$, NS).

Panel B of Fig. 2 shows cumulative burying behavior. The two way ANOVA revealed statistical significance for condition (sex and endocrine stage; $F_{2,69}=4.106$, $p<0.05$), but diazepam treatment had no effect ($F_{2,69}=0.907$, NS) and these factors did not interact ($F_{4,69}=0.212$, NS). Untreated males displayed slightly less burying behavior than females, close to statistical significance (Kruskal–Wallis ANOVA: $H=4.921$, $p=0.085$). Amazingly, in contrast to adult young animals, diazepam treatment did not reduce cumulative burying in middle-aged subjects at any dose (Kruskal–Wallis ANOVAs: females with irregular cycles: $H=2.292$, NS; females in persistent diestrus: $H=1.229$, NS; and males: $H=0.566$, NS). Furthermore, cumulative burying was not reduced in those middle aged males that showed burying behavior after 2.0 mg/kg diazepam (see Table 2).

Fig. 3 shows immobility (panel A), the number of prod explorations (panel B) and shocks (panel C) in middle aged males and females with and without treatment. For the analyses of these parameters all animals were included, regardless of whether they did or did not display burying behavior. Untreated middle-aged males, as compared with females, showed slightly increased immobility almost statistically significant (Kruskal–Wallis ANOVA: $H=5.363$, $p=0.060$). The two way ANOVA showed a significant effect of condition ($F_{2,88}=3.248$, $p<0.05$), but diazepam treatment had no effect ($F_{2,88}=2.451$, NS) and these factors did not interact ($F_{4,88}=1.224$, NS). Diazepam at these doses (0.5 and 1.0 mg/kg) produced no effect on immobility (Kruskal–Wallis ANOVAs: females with irregular cycles: $H=4.936$, NS; females in permanent diestrus: $H=3.272$, NS; and males: $H=2.384$, NS).

Similar results were found for the number of prod explorations (Fig. 3B): untreated males showed more prod explorations than females (Kruskal–Wallis ANOVA: $H=7.918$, $p<0.05$). The two way ANOVA revealed an effect of condition ($F_{2,88}=3.915$, $p<0.05$); no effect of diazepam ($F_{2,88}=0.581$, NS) and no interaction between these factors ($F_{4,88}=0.905$, NS). The one way Kruskal–Wallis ANOVAs within each condition confirmed that diazepam was without effect (females with irregular cycles: $H=0.288$, NS; females in persistent diestrus: $H=1.525$, NS; and males: $H=4.446$, NS).

The number of prod shocks is shown in Fig. 3C. The number of shocks did not vary between males and females without diazepam treatment (Kruskal–Wallis ANOVA: $H=1.203$, NS). Diazepam produced a dose-dependent increase in the number of shocks received in

Table 2
Behavioral effects of 2.0 mg/kg of diazepam in middle-aged males.

	Latency to bury	Cumulative burying	Immobility	Prod-exploration	Shock	Grooming	Rearing	Freezing	Ambulatory activity	Falls
	Seconds	Seconds	Seconds	Number	Number	Number	Number	Seconds	Counts	Number
Vehicle	118 ± 69	109 ± 26	204 ± 43	4.9 ± 0.66	6.4 ± 1.1	1.5 ± 0.4	12.7 ± 2.9	0.19 ± 0.11	95 ± 14	2 ± 0.5
	n = 9	n = 9	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10
Diazepam	149 ± 30	70 ± 37	417 ± 39*	2.4 ± 0.69*	17.4 ± 2.5***	0.09 ± 0.09*	6.5 ± 1.4	0.44 ± 0.08*	90 ± 16	8 ± 2*
2.0 mg/kg	n = 3	n = 3	n = 11	n = 11	n = 11	n = 11	n = 11	n = 11	n = 11	n = 11

The table shows means ± SE. Mann–Whitney U test *p < 0.5 and ***p < 0.001. The number of rats displaying each behavior is shown below the data.

all three groups (Two way ANOVA: condition: $F_{2,88} = 2.737$, NS; diazepam treatment: $F_{2,88} = 3.374$, $p < 0.05$; and their interaction: $F_{4,88} = 0.340$, NS). The number of rearings, groomings and general ambulatory activity did not vary between untreated or diazepam-injected middle-aged males and females (data not shown).

Freezing (panel A) and the number of falls (panel B) are shown in Fig. 4. Without diazepam treatment, males showed much less freezing than females (Kruskal–Wallis ANOVA: $H = 8.795$, $p < 0.05$). Diazepam (1.0 mg/kg) only slightly reduced freezing in females with irregular cycles, but this effect was not significant (Two way ANOVA for freezing: condition: $F_{2,88} = 1.064$, NS; diazepam: $F_{2,88} = 0.196$, NS; and their interaction: $F_{4,88} = 1.876$, NS. Kruskal–Wallis ANOVAs for each condition: females with irregular cycles: $H = 4.434$, NS; females in persistent diestrus: $H = 3.158$, NS; and males: $H = 2.365$, NS).

No differences were found in the number of falls between males and females with and without diazepam treatment at the 0.5 and

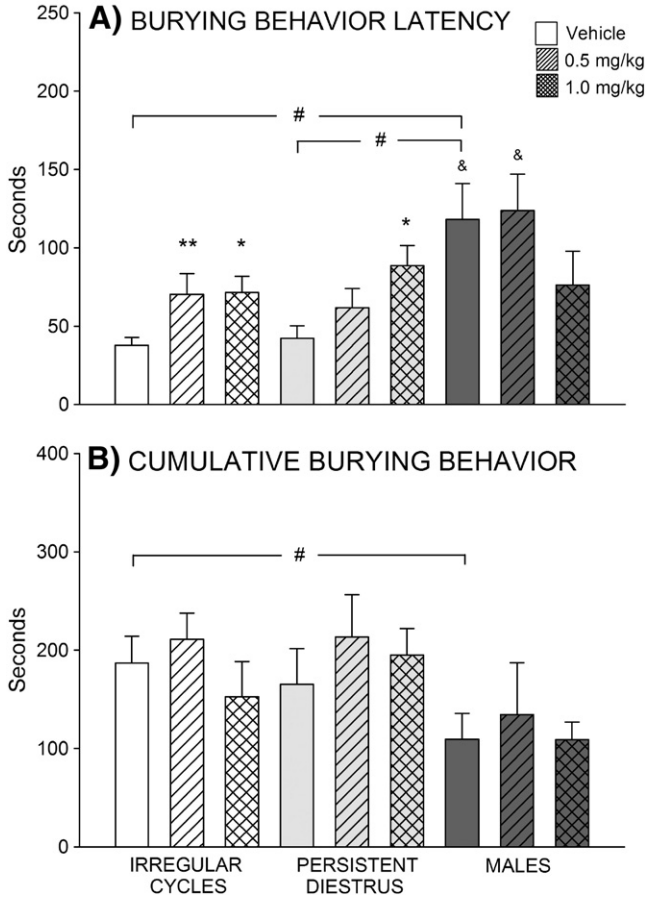

Fig. 2. Effect of diazepam on burying behavior latency (A) and cumulative burying behavior (B) in females with irregular cycles, females in persistent diestrus, and males. The figure shows means ± SE. Tukey test: &p < 0.05 males *versus* both groups of females. Mann–Whitney U-test: vehicle treated #p < 0.05 shown by bracket, and within each condition *p < 0.05, **p < 0.01 versus vehicle treated.

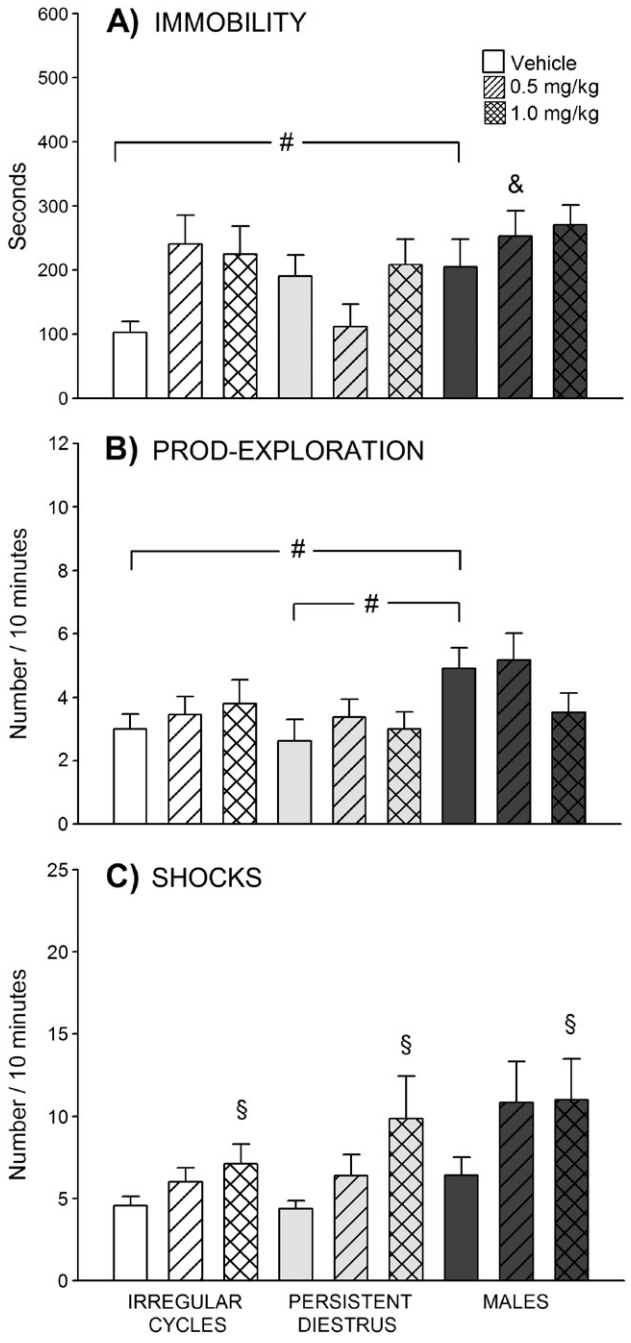

Fig. 3. Effect of diazepam on immobility (A), number of prod-explorations (B) and number of shocks (C) in females with irregular cycles, females in persistent diestrus and males. The figure shows means ± SE. Tukey test: &p < 0.05 males *versus* females in persistent diestrus; §p < 0.05 diazepam 1.0 mg/kg *versus* vehicle control. Mann–Whitney U-test: vehicle treated #p < 0.05 shown by brackets.

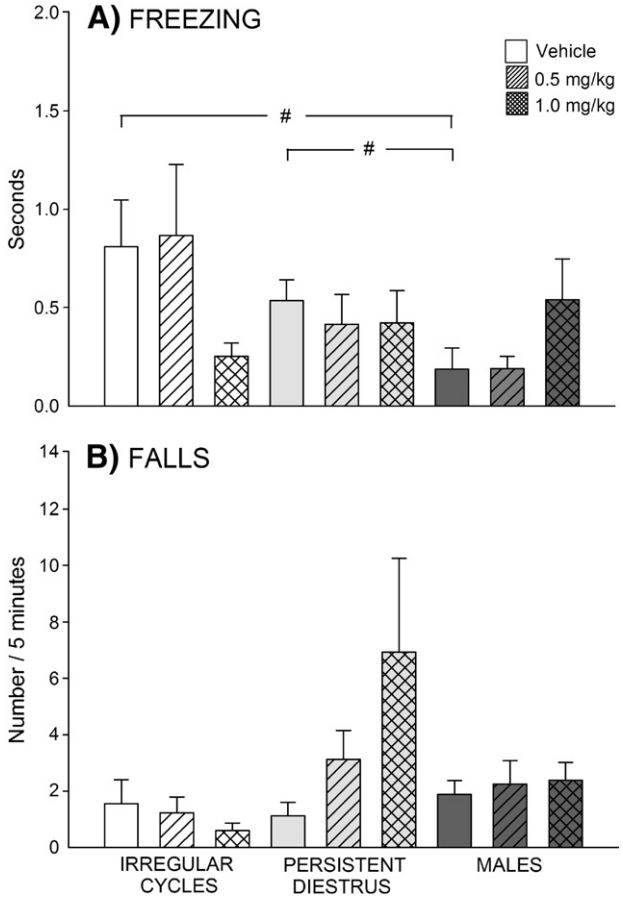

Fig. 4. Effect of diazepam on freezing (A) and number of falls (B). The figure shows means ± S.E. Mann–Whitney U-test: vehicle treated #$p<0.05$ shown by brackets.

1.0 mg/kg doses (Two way ANOVA: condition: $F_{2,88}=2.068$, NS; diazepam: $F_{2,88}=0.998$, NS; and their interaction: $F_{4,88}=1.295$, NS). The one way Kruskal–Wallis ANOVAs for each condition confirmed these findings (untreated, different conditions: $H=1.806$, NS; treated females with irregular cycles: $H=0.680$, NS; treated females in permanent diestrus: $H=2.729$, NS; and treated males: $H=0.170$, NS). However, females in persistent diestrus treated with 1.0 mg/kg diazepam showed a clear increase in the number of falls. As aforementioned, a high dose (2.0 mg/kg) of diazepam impaired motor coordination in males (Table 2).

4. Discussion

The present series of results may be summarized as follows: (a) 74% of females aged 10–14 months showed irregular cycles, 19% persistent diestrus and 5% constant estrous. (b) Without diazepam treatment, middle-aged males showed longer burying behavior latencies, more prod explorations and less freezing than both groups of females. When compared with females with irregular cycles, middle aged males also showed less cumulative burying behavior and more immobility. None of the parameters showed any difference between the female groups. (c) Diazepam (0.5 and 1.0 mg/kg) increased burying behavior latency in females with irregular cycles; while the high dose (1.0 mg/kg) had this effect and increased the number of falls in a rotarod test only in females with persistent diestrus. No other parameter was modified. These doses of diazepam had no effect on middle aged males. However, a higher dose (2.0 mg/kg) increased immobility, freezing, the number of prod shocks and of falls in a rotarod test; while decreasing the number of prod explorations and groomings. Diazepam did not reduce cumulative burying at any dose. All these results support the idea that middle aged males and females in two endocrine conditions vary in their behavioral strategy to cope with an aversive stimulus in the burying defensive test and in their response to diazepam.

In middle aged females with irregular cycles the increase in the length of the estrous cycle as well as the proportion of rats with irregular cycles and in persistent diestrus agrees with previous results (LeFevre and McClintock, 1988; Walf et al., 2011). The Wistar rats in our colony showed a much lower proportion (5%) of middle-aged females in constant estrous, compared with the 42% reported by these authors in Sprague–Dawley females. These differences could be due to the variation in the average age of onset of acyclicity and predominant postcyclic vaginal state in several strains of laboratory mouse and rat due to a variety of factors such as prenatal testosterone exposure by intrauterine position, environmental and social conditions (vom Saal and Finch, 1988).

It has been proposed that young adult females exhibit less anxiety and fearfulness than males (Gray, 1971, 1979; Johnston and File, 1991) because they show greater ambulatory and rearing activity and defecate less than males in the open-field test (Archer, 1975; Beatty and Fessler, 1976; Blizard et al., 1975; Johnston and File, 1991; Masur et al., 1980). However, this conclusion depends on the animal model used, which may possess a sex differential connotation. For example, Johnston and File (1991) reported sex differences in three animal models of anxiety. Females were less anxious than males in the elevated plus-maze, male rats were less anxious than females in the Vogel test and conclusions could be drawn in either direction in the social interaction test. In the burying behavior test, using young adults, males and females in late proestrus showed less cumulative burying than females without ovaries or females in metestrus/diestrus (Fernández-Guasti and Picazo, 1992, 1997). In middle aged rats (present results), males showed longer burying behavior latencies, reduced cumulative burying, more prod explorations and immobility than females tested in metestrus/diestrus. This behavioral profile suggests that males cope with the aversive stimulus with a different behavioral strategy than females (tested in these estrous cycle phases); i.e., they show less active behaviors (cumulative burying or prod explorations) and more passive responses (immobility).

It is well established that the reduction of testicular hormones by castration or aging increased experimental anxiety which is reversed by T or its metabolites (Bitran et al. 1993a; Edinger and Frye 2005; Fernández-Guasti and Martínez-Mota 2003; Frye and Seliga 2001; Frye et al., 2010a; Osborne et al., 2009). However, young and middle-aged males show similar latencies to bury, levels of cumulative burying (Fernández-Guasti and Picazo, 1990, 1992, 1997) and of immobility (Rohmer et al., 1990), suggesting that the decrease in testicular secretions characteristic of aging (Herrera-Perez et al., 2008) does not influence this particular responses.

Few studies have analyzed the behavioral profile of middle-aged females in anxiety tests. As aforementioned, Walf et al. (2009) reported that middle aged rats in three different reproductive statuses (competent, transitioning and senescent) responded similarly in the burying behavior test. The present results showing that middle aged females display a similar behavioral profile independently of their endocrine status agree with this observation. In addition, Walf et al. (2009) described that middle-aged reproductively competent females presented reduced anxiety-like behavior in other tests, compared with rats in reproductive senescence with very low ovarian activity. This is consistent with previous reports showing that in young adults' ovariectomy increased anxiety-like behaviors, compared to intact females or rats in proestrus (Fernández-Guasti and Picazo, 1992; Walf and Frye, 2007). In support, middle aged and young females tested in metestrus or diestrus showed similar levels of cumulative burying than ovariectomized rats (Fernández-Guasti and Picazo, 1990, 1992, 1997).Thus, the increased levels in burying in females with low ovarian hormone secretion could be underlied by the reduction in

estrogens, progesterone or its reduced metabolites as allopregnanolone reported by some authors (Bitran et al., 1993b; Genazzani et al., 2004; Walf and Frye 2005a) but not by others (Paris et al., 2011; Walf et al., 2011). Interestingly, middle-aged subjects, as young-adults ovariectomized, preserve the ability to respond to the exogenous administration of these steroids (Fernández-Guasti and Picazo, 1995; 1999; Frye et al., 2010b; Gallo and Smith, 1993; Picazo and Fernández-Guasti, 1995; Saavedra et al., 2006; Walf and Frye 2005b; 2010).

In 1990, Rohmer et al. reported the complete behavioral effect of diazepam in young adult male rats exposed to the burying behavior paradigm. They found that diazepam reduced cumulative burying, escapes from the prod and digging; increased prod exploration and did not affect locomotor activity or rearing. This pattern is consistent with the effects of anxiolytics (Jolles et al., 1979; Treit and Fundytus, 1988). In middle aged males, the highest diazepam dose (2.0 mg/kg) failed to affect cumulative burying or its latency; but it reduced grooming and the number of prod explorations, while increasing the number of shocks. The effect of diazepam on grooming and the number of shocks indicates an anxiolytic-like action; suggesting that some of the behavioral responses are shared between aged males and young adults. However, this diazepam dose also impaired motor coordination; complicating interpretations specific to experimental-anxiety (although the animals showed normal levels of burying behavior, which implies fine motor coordination). The differential effect of diazepam between young adults and middle aged rats does not depend on testicular steroids; since diazepam decreased cumulative burying in young adult castrates (Fernández-Guasti and Martínez-Mota, 2003).

Middle-aged females with irregular cycles were always tested in metestrus to analyze the anxiolytic-like effect of diazepam, because diazepam reduces cumulative burying time and increases burying behavior latency in young adult females tested in this phase (Fernández-Guasti and Picazo, 1990). Therefore, the lack of an anxiolytic-like effect of diazepam in middle-aged females was probably due to age- (rather than estrous cycle-) factors such as an absent steroid ovarian secretion (for females in persistent diestrus) or an irregular secretion of estradiol and progesterone with relatively long intervals of ovarian quiescence (for animals with irregular cycles). However, the observation that diazepam produces clear anxiolytic-like actions in ovariectomized young females (Fernández-Guasti et al., 2001) argues against the idea of an age gonadal-hormone-reduction mediated phenomenon.

In summary, diazepam did not affect any parameter reflecting experimental anxiety (active or passive responses) in middle aged females; but increased burying behavior latency indicating impaired reactivity. This last effect was the main sex difference after diazepam treatment, because not even the highest dose (2 mg/kg) of this benzodiazepine increased burying behavior latency in males. Additionally, in contrast to males, females in persistent diestrus showed a trend to an increased motor in-coordination after a dose of 1 mg/kg diazepam. From these data, it seems that middle-aged females are more vulnerable than males to the main side effects of benzodiazepines. In this line, clinical data shows that women fall more frequently than men after benzodiazepine treatment (Verhaeghe et al., 1996).

The absence of an anxiolytic-like effect of diazepam in middle aged animals agrees with a previous report (Wikinski et al., 2001) employing the dark-light transition test and with the results reported by File (1990) using chlordiazepoxide in the elevated plus maze. The hypersensitivity of middle aged animals to the motor effects of diazepam is also in line with a previous observation showing that the dose-effect curve of diazepam on locomotor activity shifts to the left with increasing age (Wikinski et al., 2001). An exacerbation of benzodiazepine-induced sedation and motor in-coordination in aged patients has also been consistently reported (Ashton, 1994; Dunbar et al., 1989; Ray et al., 1987; Sorock and Shimkin, 1988).

The age differences in the anxiolytic-like effect of diazepam could be due to pharmacokinetic changes (Klotz, 1979). Middle-aged males show a reduction in diazepam metabolism, compared to young adult animals (Fujita et al., 1990); implying that the former have higher plasma diazepam levels than the latter and failing to explain the non-anxiolytic-like response of middle-aged rats. Additionally, Fujita et al. (1990) reported that 3–12 month old males possess a much higher diazepam metabolism than females of the same age. These results may underlie the behavioral effects we found; since diazepam produced more side effects in females than in males. Although aging is associated with pharmacokinetic changes in the disposition and elimination of benzodiazepines, the sensitivity to benzodiazepines in aged subjects does not depend exclusively on plasma or brain concentrations (Barnhill et al., 1990; Castleden et al., 1977; Greenblatt and Shader, 1990; Greenblatt et al., 1980; Pomara et al., 1985; Swift et al., 1985a, 1985b); suggesting a pharmacodynamic mediation. In this line, age-dependent changes in $GABA_A$ receptor expression have been reported (Gunnersen et al., 1996; Gutierrez et al., 1996). However, the functional consequences of these changes remain controversial (Ruano et al., 1996 *vs.* Mhatre and Ticku, 1998). Wikinski et al. (2001) suggested that in the cerebral cortex the sensitivity of the $GABA_A$ receptor to benzodiazepines is reduced during aging which may account for the absent anxiolytic-like effect. Additionally, the excessive sedation, motor in-coordination and reduced reactivity produced by diazepam and chlordiazepoxide in aged rats (File, 1990; Wikinski et al., 2001, present data) may be due to changes in $GABA_A$ receptor subunits; which increase the sensitivity to benzodiazepines in other brain areas like the locus coeruleus and the hippocampus.

All these data taken together, suggest that middle-aged males use a different behavioral strategy than females of the same age to cope with an aversive stimulus. The aging-related reduction in gonadal steroids does not seem to influence the behaviors displayed during the burying behavior test. Diazepam lacked an anxiolytic-like action in middle-aged subjects (males or females), but increased behaviors denoting side-effects, such as impaired reactivity and motor coordination; a pharmacological profile that may be due to alterations in the subunits of the $GABA_A$ receptor in different brain areas.

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