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The Actions of Diazepam and Serotonergic Anxiolytics Vary According to the Gender and the Estrous Cycle Phase

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FERNÁNDEZ-GUASTI, A. AND O. PICAZO. *The actions of diazepam and serotonergic anxiolytics vary according to the gender and the estrous cycle phase.* PHARMACOL BIOCHEM BEHAV **37**(1) 77–81, 1990.—The anxiolytic effect of diazepam (0.5, 1.0 and 2.0 mg/kg), buspirone (2.5 and 5.0 mg/kg), indorenate (2.5 and 5.0 mg/kg) and ipsapirone (5.0 and 10.0 mg/kg) was evaluated in male and female rats during the proestrus and metestrus phases. The burying behavior test was used to measure the anxiety levels. In this test, increases in the behavior latency are interpreted as prolonged reactivity, while reductions in the burying behavior are considered to reflect anxiolytic states. Diazepam increases in burying behavior latency were consistently higher than those observed after serotonergic anxiolytics. Buspirone, at no dose tested, affected the burying behavior latency, while indorenate and ipsapirone had only minor effects. Male individuals were more sensitive than females to the actions of diazepam on burying behavior. The serotonergic anxiolytics produce similar responses in both sexes. Metestrus females were much less sensitive to the action of all anxiolytics on burying behavior latency than proestrus females. Proestrus females were highly sensitive to the actions of diazepam on burying latency as compared both with males and metestrus females. Data show that a larger gender and within females variation occurs after treatment with diazepam as compared with the serotonergic anxiolytics. The results are discussed considering the relationships between ovarian hormones and the GABA-benzodiazepinic and serotonergic systems.

Burying behavior Diazepam Gender differences Serotonergic anxiolytics Estrous cycle phase differences

SEVERAL lines of evidence suggest that the GABAergic [cf. (15)] and the serotonergic (9, 10, 16) transmissions importantly participate in the regulation of anxiety. Recently, some anxiolytic compounds acting on the serotonergic system (on 5-HT_{1A} receptors) have been proposed: buspirone (4, 13, 33), ipsapirone (5, 11, 12, 28, 29) and indorenate (5,6).

Gender differences in the neurotransmitter systems involved in the control of anxiety have been established. For example, it has been shown that the serotonin (5-HT) turnover rate is larger in females than in males (25), and that the female threshold to display the serotonergic syndrome is lower when compared with males (1). The changes in these neurotransmissions are, however, not gender restricted, but also occur along the various phases of the estrous cycle (2) probably determined by steroid hormones (3,17).

Recently, we reported on the changes in anxiety along the various phases of the estrous cycle (7). In this experiment, low anxiety levels during the proestrus phase, presumably related to the presence of steroid hormones, were found. No differences in the anxiety levels between the metestrus and the diestrus phases and ovariectomized females were observed.

On the basis of the gender and within-females differences in the neurotransmitters involved in the control of anxiety, the purpose of the present study was to analyze the effect of the antianxiety agents, diazepam, buspirone, ipsapirone and indorenate on males and females in different phases of the estrous cycle.

The conditioned defensive burying behavior has been considered as a selective paradigm for testing antianxiety agents [cf. (31)]. The main advantages of this test are: (a) selectivity, only antianxiety agents produce clear responses; (b) sensitivity, relatively low doses of anxiolytic compounds are highly effective; and (c) relative potency, a relationship between the anxiolytic action in this animal model and in clinics has been established (30).

METHOD

General

Male (250 g body weight) and female (200 g body weight)

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Wistar rats were used in this study. All animals were individually housed in a room under reversed and controlled (12-hr light:12-hr dark, lights on at 2200 hr) light:dark conditions. All animals had ad lib access to water and commercial rat chow. After two weeks of adaptation to the light:dark conditions the estrous cycle was determined by daily vaginal smears. Four estrous phases were recognized depending upon the vaginal cellular population: proestrus, oestrus, metestrus and diestrus. Only those females with at least two regular estrous cycles were selected for participation in this study. On the basis of a previous report (7) showing large differences in the anxiety levels between proestrus and metestrus females, only these phases were considered for the pharmacological treatments. In order to analyze whether the treatments affected the estrous cyclicity the vaginal inspection was continued for four days. All male rats were manipulated in the same manner as the females during at least two weeks before the experiments.

Anxiety Test

The anxiety tests were performed immediately after the determination of the estrous cycle phase. The anxiety levels were measured in the defensive burying behavior paradigm. As previously described (5,30), the paradigm consisted of a cage measuring 27 × 16 × 23 cm with a prod placed two cm above the bedding material through which the animal received an electric shock of 0.3 mA. The source of the shock was a constant current shocker (LaFayette Instruments Co., model 5806). Immediately after the placement of the animal in the test cage its behavior was observed for 10 min. Once the animal received a shock it displayed a stereotyped behavior characterized by pushing the bedding material (fine sawdust) ahead with rapid and alternative movements of its forepaws. The parameters registered in this test were: (a) burying behavior latency, time in minutes from the first shock to the burying behavior display and (b) cumulative burying behavior, cumulative time (in minutes) that the animal spends burying the aversive stimulus (prod) in a 10-min test. The data were statistically analyzed using the Kruskal-Wallis analysis of variance followed by the Mann-Whitney U-test (26).

The burying behavior latency has been considered to reflect the animals' reactivity, while the burying behavior itself has been considered as an expression of the anxiety levels. Benzodiazepine anxiolytics cause an increase in burying behavior latency accompanied by a decrease in burying behavior (21,30).

Drugs

The following drugs were used in this experiment: diazepam (La Roche, México City, México), ipsapirone (Miles Pharmaceutical Division, West Haven, CT), indorenate (Department of Pharmacology, CINVESTAV, México City, México) and buspirone (Mead Johnson, México City, México). All drugs but diazepam were dissolved in physiological saline. Diazepam was dissolved in propylene glycol 40%. All drugs were injected IP in a volume of 2.0 ml/kg. The latencies and the doses used for each drug were as follows: diazepam (0, 0.5, 1.0 and 2.0 mg/kg, −30 min), ipsapirone (0, 5 and 10 mg/kg, −30 min); indorenate (0, 2.5 and 5.0 mg/kg, −90 min); and buspirone (0, 2.5 and 5.0 mg/kg, −20 min). Doses and latencies were chosen on the basis of previous data showing maximum effects of these drugs under this schedule.

RESULTS

The results of these experiments are shown in Figs. 1–4. Figure 1 shows the effect of various doses of diazepam on the burying behavior latency (A) and on the cumulative burying behavior (B)

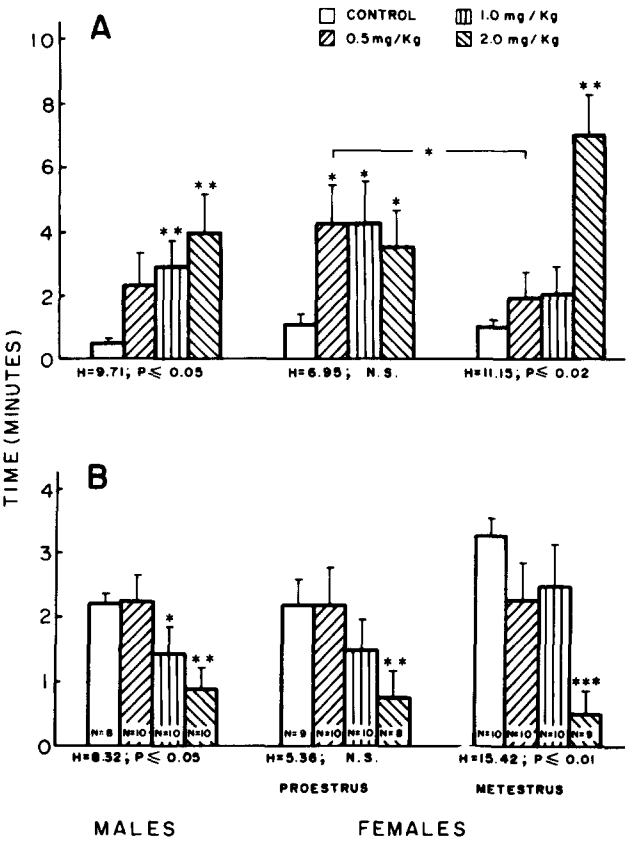

FIG. 1. Effect of diazepam (0, 0.5, 1.0 and 2.0 mg/kg) on burying behavior latency (A) and cumulative burying behavior (B) of male and female (proestrus or metestrus) rats. The figure shows mean ± S.E. Values under columns represent the result of the Kruskal-Wallis analysis of variance with the respective probability (for all cases in these experiments gl = 3). Asterisks over columns show comparisons between the experimental (drug-treated) and the control (vehicle-treated) groups. Other comparisons are shown by brackets. Mann-Whitney U-test, $*p<0.05$; $**p<0.02$; $***p<0.01$.

of male and female (proestrus and metestrus) rats. The burying behavior latencies showed no differences within the vehicle-treated animals (Fig. 1A). In males, a clear dose-dependent increase in latency was found. By contrast, females tested in proestrus showed a marked increase in burying behavior latency already at the lower dose used (0.5 mg/kg). No further latency increases with higher doses were seen. Conversely, metestrus females did not show changes in burying behavior latency at the low and middle diazepam doses used (0.5 and 1.0 mg/kg). However, a large increase was found after administering the highest dose (2.0 mg/kg). Within-phase comparisons showed that diazepam at 0.5 mg/kg produced a different effect depending upon the estrous cycle phase. Thus, while proestrus females were drastically affected by this dose, metestrus females were completely unaltered.

The diazepam actions on the cumulative burying behavior are shown in Fig. 1B. As previously reported (7), differences in the anxiety levels between males and females and between females in different stages of the estrous cycle were observed. Common to all experiments presented, consistently high anxiety levels were shown by females tested during the metestrus phase. A similar dose-response decrease in the cumulative burying behavior after

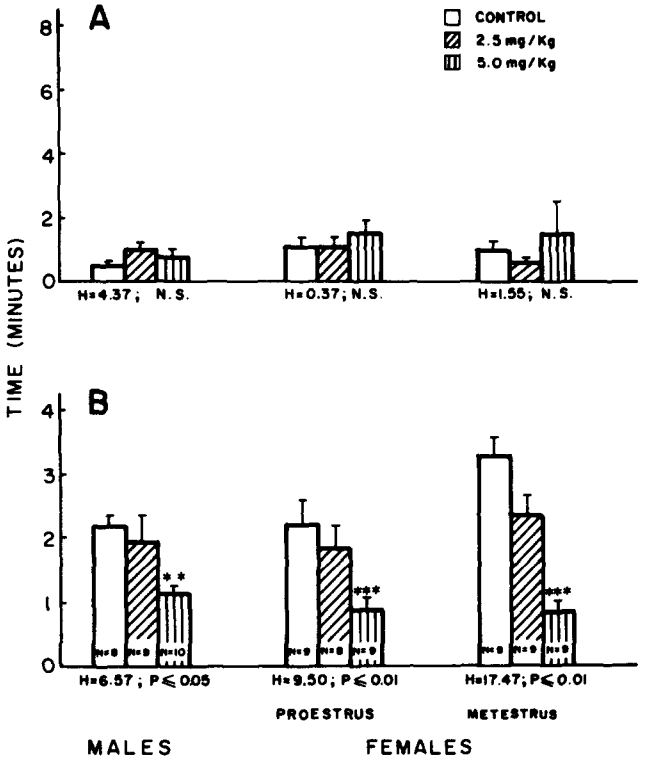

FIG. 2. Effects of buspirone (0, 2.5 and 5.0 mg/kg) on burying behavior latency (A) and cumulative burying behavior (B) on male and female rats. The figure shows mean ± S.E. Values under columns represent the result of the Kruskal-Wallis analysis of variance with the respective probability (for all cases in these experiments gl = 2). Asterisks over columns show comparisons between the experimental (drug-treated) and the control (saline-treated) groups. Mann-Whitney U-test, $^{**}p<0.02$; $^{***}p<0.01$.

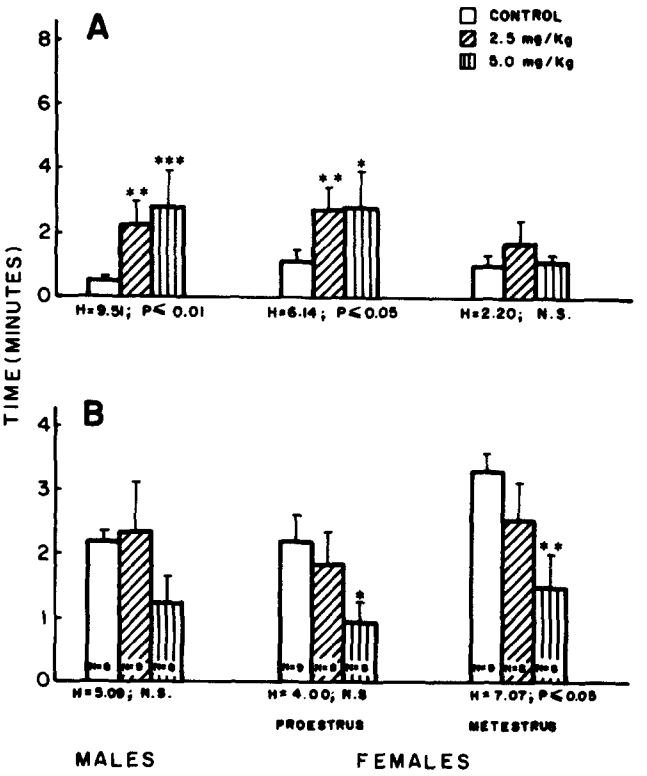

FIG. 3. Effects of indorenate (0, 2.5 and 5.0 mg/kg) on burying behavior latency (A) and cumulative burying behavior (B) on male and female rats. The figure shows mean ± S.E. Values under columns represent the results of the Kruskal-Wallis analysis of variance with the respective probability (for all cases in these experiments gl = 2). Asterisks over columns show comparisons between the experimental (drug-treated) and the control (saline-treated) groups. Mann-Whitney U-test, $^{*}p<0.05$; $^{**}p<0.02$; $^{***}p<0.01$.

diazepam injection was observed for males and females (Fig. 1B).

Figure 2A and B shows the effect of buspirone on the burying behavior latency and on the cumulative burying behavior, respectively. It is clear from Fig. 2A that none of the buspirone doses used (2.5 or 5.0 mg/kg) affected the burying behavior latency neither in males nor in proestrus or metestrus females. However, a clear dose-response inhibitory effect of this drug on the cumulative burying behavior was observed. This action was similar independently of the animal sex or estrous cycle stage (Fig. 2).

The effects of the putative anxiolytic drugs, indorenate and ipsapirone are shown in Figs. 3 and 4, respectively. Figure 3A shows the effect of indorenate on burying behavior latency. The administration of indorenate 2.5 or 5.0 mg/kg resulted in a dose-dependent increase in burying behavior latency in males and proestrus females. This action was not observed in metestrus females. Common to all animals a dose-dependent decrease in the cumulative burying behavior was observed after indorenate injection (Fig. 3B). The effects of ipsapirone on burying behavior latency are shown in Fig. 4A. Clearly, the low dose of ipsapirone (5 mg/kg) produced an increase in this parameter in males, while female rats (independently of the estrous cycle phase) were not affected. A high dose of this compound (10 mg/kg) produced similar increases in all animals tested. Figure 4B shows the actions of ipsapirone on the cumulative burying behavior. A clear dose-response decrease in cumulative burying behavior was observed in all animals, although metestrus females seemed more sensitive since lower doses (5 mg/kg) were required to significantly decrease the burying behavior.

It was also clear that the administration of these drugs, at the dosages used, do not interfere with the estrous cyclicity as evaluated by vaginal smears (data not shown).

DISCUSSION

The results from the present experiments can be analyzed from three different points of view: (a) pharmacological, general differences between the benzodiazepinic and the serotonergic anxiolytics; (b) generic, variations in the drug effects according to the animal gender; and (c) within females differences, changes in the drug actions according to the female endocrine cycle phase.

According to the pharmacological variations it was observed that diazepam effects on burying behavior latency were consistently higher than those observed after the serotonergic anxiolytics; conversely, the decrease in cumulative burying behavior was similar for all compounds used. In relation to the gender the main difference was that male individuals were more sensitive than females (considering both proestrus and metestrus) to the inhibitory actions of diazepam on burying behavior and of ipsapirone on burying latency. The different responses observed between females in the various phases of the estrous cycle were that proestrus females are highly sensitive to the increase in burying behavior latency induced by diazepam as compared with metestrus females or males.

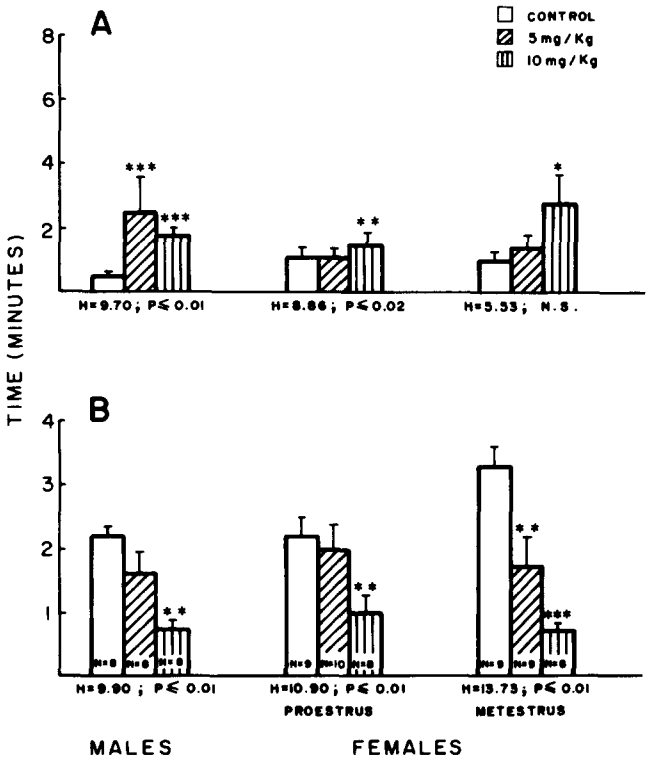

FIG. 4. Effects of ipsapirone (0, 5 and 10 mg/kg) on burying behavior latency (A) and cumulative burying behavior (B) on male and female rats. The figure shows mean ± S.E. Values under columns represent the results of the Kruskal-Wallis analysis of variance with the respective probability (for all cases in these experiments gl = 2). Asterisks over columns show comparisons between the experimental (drug-treated) and the control (saline-treated) groups. Mann-Whitney U-test, $*p<0.05$; $**p<0.02$; $***p<0.01$.

The decrease in reactivity, shared by various benzodiazepine anxiolytics, has been considered as one of the main side effects of these drugs (14). Additionally, it has been proposed that the serotonergic anxiolytic, buspirone, effectively reduces anxiety without modifying reactivity (13). In the burying behavior paradigm, it has been considered that increases in the burying behavior latency are related to prolonged reactivity, while decreases of burying behavior time are interpreted as a reduction in the anxiety levels (21,30). According to this interpretation, present findings show that, in general, all serotonergic drugs used, by contrast with diazepam, have much less effect than the benzodiazepines on the animal reactivity to aversive stimuli. Indeed, it has been observed that ipsapirone (28,29), and particularly buspirone (13,33), do not affect the reactivity tested in animal paradigms or in clinics. Additionally, on the basis of the action of these drugs on the cumulative burying behavior, it could be concluded that all these pharmaca exert nearly the same anxiolytic action. It should be noted, however, that the serotonergic drugs were less potent than diazepam in producing the anxiolytic action since the inhibitory actions of diazepam were observed between 0.5 and 2.0 mg/kg and the effects of the serotonergic drugs were seen in a dose range varying between 2.5 and 10 mg/kg.

It has been proposed that females are more sensitive than males to the convulsant action of GABAergic antagonists (20). From these data it is possible to suggest that the GABAergic transmission is higher in males than in females. The present results showing that males are more sensitive to the action of diazepam (i.e., lower doses of diazepam are required to produce similar effects in males as compared with females), are in line with the idea that in males a higher GABAergic tone is present. Needless to mention, further studies are required to investigate this proposition.

During proestrous the peak of ovarian steroid hormones, estrogen and progesterone, occurs (8). It has been demonstrated that the exogenous administration of these hormones result in an increase in the GABAergic transmission (18, 19, 32) particularly reflected as an increase in the number of ^{3}H-muscimol binding sites (17). Although controversial, it is generally believed that the benzodiazepine receptor is coupled to the GABA-A receptor selectively labeled by muscimol or bicuculline (15). Furthermore, behavioral data have revealed that during proestrous (7) or after the exogenous administration of estrogen and progesterone (23,24), low anxiety levels are observed. Indeed, the anxiolytic actions of progesterone have been interpreted on the basis of the actions of this progestin on the GABAergic system (27). Present data showing that proestrus females are more sensitive than males, and particularly more than females in metestrus, to the actions of diazepam suggest that in this phase an increase in baseline GABAergic activity is present probably due to the peak of steroid hormones. Further experiments designed to test this idea should be undertaken.

The metestrous phase is endocrinologically characterized by an absence of ovarian steroid hormones (8). During this phase the anxiety levels are similar to those shown by ovariectomized females (7). By contrast with proestrous females (vide supra), rather high doses of diazepam were required to modify the reactivity of animals tested in this phase. Interestingly, none of the serotonergic anxiolytics was effective in modifying the burying behavior latency of these females. These data would indicate that females in metestrus respond differently to the action of anxiolytics on burying behavior latency suggesting that the reactivity of females during this phase is not affected by the anxiolytic compounds. The reason for the insensitivity of this group of females could be based on the lack of steroid hormones present during this phase, however, further studies aimed to study this possibility should be made.

It is worth noting that most of the differences between the sexes and the estrous cycle phases occur between the groups treated with diazepam. This finding suggests that diazepam's therapeutic actions may depend upon the sex or the endocrine cycle phase. This observation, however, needs to be confirmed. It has been shown that drastic differences (40–50%) in the number of serotonin receptors occur along the various phases of the estrous cycle (2) and that such differences depend upon the actions of steroid hormones (mainly estradiol) (3). Additionally, on the basis of the binding studies it is suggested that the serotonin receptors affected belong to the 5-HT_{1A} subtype (3), where most likely the serotonergic anxiolytics act (4, 6, 11, 28). Surprisingly, minor gender and within females differences were found after the administration of the 5-HT_{1A} anxiolytics used. It is worth mentioning that the actions of steroids on serotonergic neurons have been mainly demonstrated in neurons belonging to the ventromedial hypothalamus (2,3). Thus, a possible explanation for the lack of sex or endocrine phase variability after serotonergic anxiolytics could be based on the fact that different brain serotonergic areas, insensitive to the action of steroid hormones, are involved in the control of anxiety. This idea, however, needs further exploration.

Recently, buspirone has been suggested as effective in the treatment of various symptoms that characterize the premenstrual syndrome (22). According to present experiments, the therapeutic actions of the serotonergic anxiolytics may have at least two advantages over treatment with benzodiazepines: lack of effects upon reactivity and similar actions independent of sex and endo-

crine cycle phase. Further clinical studies should be undertaken to explore these hypotheses.

Finally, the present study points out the necessity of analyzing preclinically the role of anxiolytic drugs in both sexes and in various phases of the female endocrine cycle.

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