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Gender, Estrous Cycle, Ovariectomy, and Ovarian Hormones Influence the Effects of Diazepam on Avoidance Conditioning in Rats

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DIAZ-VELIZ, G., S. BUTRON, M. S. BENAVIDES, N. DUSSAUBAT AND S. MORA. *Gender, estrous cycle, ovariectomy, and ovarian hormones influence the effects of diazepam on avoidance conditioning in rats.* PHARMACOL BIOCHEM BEHAV **66**(4) 887–892, 2000.—This study examines whether the hormonal condition of the rat modifies the effects of diazepam (0.25 and 1.0 mg/kg) on avoidance conditioning and other behavioral responses. Acquisition of a conditioning avoidance response (CAR) and spontaneous motor behaviors were assessed in intact male, in intact diestrous and estrous females, and in ovariectomized (OVX) rats injected with estradiol (2 μg/rat, SC) or progesterone (5 mg/rat, SC). A higher dose (1.0 mg/kg) of diazepam significantly impaired the acquisition of CARs in diestrous, OVX, OVX + progesterone, and male rats. Conversely, both doses of diazepam significantly improved the acquisition of CAR in estrous rats and in OVX rats injected with estradiol. These effects on conditioning avoidance were not accompanied with equivalent changes in spontaneous motor behaviors. Motor activity and grooming behavior decreased in all experimental groups after administration of 1.0 mg/kg of diazepam. On the contrary, diazepam 0.25 mg/kg increased motor activity in estrous, OVX + estradiol, and OVX + progesterone rats after, whereas grooming behavior was not affected in any group. These findings suggest a physiological influence of ovarian steroid hormones in modifying the benzodiazepine effects on conditioning avoidance and motor activity. The results are discussed considering that ovarian steroids may interact with diazepam on the $GABA_A$/benzodiazapine/chloride ionophore complex, modifying the coupling between benzodiazepine sites and $GABA_A$ receptors. © 2000 Elsevier Science Inc.

$GABA_A$ receptor Benzodiazepines Diazepam Estrous cycle Avoidance conditioning Ovarian hormones

THE neuropharmacological properties of benzodiazepines (BZ) in mammals have been attributed to their ability to facilitate GABA-mediated neurotransmission. The central BZ binding site is an integral part of the macromolecular $GABA_A$ receptor complex that includes binding sites for the inhibitory neurotransmitter GABA, BZs, and a chloride channel. There are additional distinct allosteric modulatory sites that bind barbiturates, neuroactive steroids, alcohols, and intravenous and volatile anesthetics, and that exert a regulatory influence upon $GABA_A$ receptor activity (19). The BZ site is capable of modulating the affinity and/or the availability of $GABA_A$ binding sites and recognizes ligands with greatly varying chemical structures (benzodiazepines and nonbenzodiazepines) and different intrinsic efficacies (11). Full agonists, such as diazepam (DZP), act with high affinity at this site, and exhibit high intrinsic efficacy (21). At maximally effective doses, DZP produce a parallel, left-ward shift of the GABA dose–response curve for Cl^- current induction.

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There is considerable experimental evidence indicating that BZ site mechanisms participate in the modulation of learning and memory processes (4,12,18). Whereas DZP-like BZ site agonists interfere with memory processes, BZ antagonist compounds have been reported to facilitate memory performance (12). In addition, the BZ antagonist flumazenil enhances both acquisition and retention of a discriminated escape response in young mice (13), and improves age-related memory dysfunction (14).

Changes in the density of the BZ sites in brain and in peripheral organs in response to steroid hormone exposure have been reported. Sex hormones modulate the GABAergic neurotransmission by increasing the number of $GABA_A$ receptors (15,16); in addition, the synthesis of GABA is also modulated by these substances (20). Some in vivo studies have demonstrated that anxiolytic effects of DZP are evident in female mice or rats at estrus and diestrus, but not at proestrus and metestrus (1,2). Because concentrations of DZP in the brain after IP injection are not influenced by the stage of the estrous cycle, it has been suggested that the observed changes in responses to DZP reflect changes in sensitivity to this drug rather than alterations in distribution or metabolism (2).

In several studies we have reported that the estrous cycle (5), the ovariectomy, and the systemic administration of ovarian hormones (6,7) influence the acquisition of conditioned avoidance responses in rats. We found that female rats showed a significant impairment in the acquisition of a conditioned task at estrus and metestrus, with minimal changes in spontaneous motor activity, while ovariectomy induced an enhancement of the acquisition (5). Systemic administration of a single dose of estradiol benzoate (2 μg) to ovariectomized rats induce a decreased in the acquisition of conditioned task tested at 48 h after injection (6), whereas progesterone (5 mg/rat) injected 6 h prior to the test, significantly enhanced the performance exhibited at estrus and antagonized the avoidance depression induced by estradiol (7). The aim of the present study was to investigate whether the hormonal condition of the rat [gender, estrous cycle, ovariectomy, and ovarian hormones (EB or PROG)] might influence the effect induced by the systemic administration of DZP, an agonist of BZ site at the $GABA_A$/benzodiazepine/chloride ionophore complex, on the expression of avoidance conditioning and some spontaneous motor responses.

METHOD

Animals

A total of 150 female rats and 30 male Sprague–Dawley rats, weighing 180–220 g, were housed six per cage under a 12 L:12 D cycle (lights on 0800 to 2000 h), with free access to food and water.

Vaginal smears were taken daily from 60 intact female rats to determine the different stages of the estrous cycle. Only rats exhibiting three or more consistent 4-day cycles were utilized. Because a previous report (5) showed great differences in the acquisition of CARs between diestrous and estrous female rats, only these phases were considered for the pharmacological treatment. Additionally, another group of 90 female rats was bilaterally ovariectomized under light ether anesthesia. Fourteen days after surgical removal of the ovaries, ovariectomized (OVX) rats were randomly assigned to different groups that received either estradiol benzoate (EB, 2 μg/rat), progesterone (PROG, 5 mg/rat), or corn oil vehicle (0.2 ml/rat), injected subcutaneously (SC) into the dorsal region of the neck, 48 or 6 h prior to the experimental tests, respectively. Vaginal smears were taken for at least 4 days before the administration of ovarian hormones; rats were invariably found to be in diestrus, confirming the completeness of ovariectomy. The dose of EB used is considered a physiological dose able to induce effects on behavior similar to that observed at estrous, with a latency of 48 h (6). The schedule of progesterone administration used has demonstrated to influence behavioral changes in intact and OVX rats (7).

A group of 30 male Sprague–Dawley rats was also included in the study to compare their behavioral responses with those exhibited by female rats. Male and OVX rats were handled in the same manner as the intact females on several consecutive days before the experiments to exclude differences between groups in terms of handling, which may have influenced the behavior of the animals.

Drug

Diazepam (DZP, Hoffmann–LaRoche, Basel, Switzerland) was administered intraperitoneally (IP), 30 min before the behavioral testing, at doses of 0.25 or 1.0 mg/kg, in a constant volume of 1.0 ml/kg body weight, to 10 rats of each hormonal condition. DZP doses were chosen on the basis of previous results described in the literature. In fact, DZP 1 mg/kg is commonly used to induce minimal sedative and anxiolytic effects in male rats (1) and DZP 0.25 mg/kg was used considering that female rats are more sensitive to depressant drugs in some stages of their estrous cycle (10). Control rats received an equivalent volume of solvent (propylene glycol). Although separate solvent groups were tested for both doses of DZP, they were combined, because statistical analysis of the behavioral data revealed no significant differences between them. Rats were injected with DZP or solvent only once, and were tested between 1000 and 1400 h to minimize diurnal variations.

Spontaneous Motor Activity

Each rat was individually placed in a plexiglass cage (30 × 30 × 30 cm), inside a soundproof room. The floor of the cage was an activity platform (Lafayette Instrument Co., Lafayette, IN) connected to an electromechanical counter. Spontaneous motor activity was monitored during 15 min and, simultaneously, were recorded the number of times each animal reared, the number of head shakes, and the time (in seconds) spent in grooming behavior. Each animal was observed continuously via a Sony video camera connected to a VHS tape recorder. Scores were generated from live observations, while video sequences were used for later reanalysis when necessary.

Active Avoidance Conditioning

Immediately after the spontaneous motor activity test, the animals were individually placed in a two-way shuttle box (Lafayette Instrument Co.) composed of two stainless steel modular testing units, which were equipped with a 18-bar insulated shock grid floor, two 28-V DC lights and a tone generator (Mallory Sonalert 2800 Hz, Lafayette Instrument Co.). Electric shocks were provided through the grid floor by a master shock supply (Lafayette Instrument Co.). Each rat was trained over 50 trials, after a 5-min period of habituation. A trial consisted of the presentation of a tone, which, after 5 s, was combined with a 0.20-mA foot shock. The shock persisted until the animal escaped to the opposite chamber, with a maximum shock duration of 10 s. The intertone interval was 30 s. A conditioned avoidance response (CAR) was defined

as a crossing to the opposite chamber within the first 5 s (tone alone). If the rat did not escape by crossing the box during the foot shock it was considered as an escape failure (EF).

Statistical Analysis

Results were expressed as the means and standard errors. The data were analyzed by two-way analysis of variance (ANOVA), followed by post hoc Newman–Keuls's multiple comparison test. A value of $p \leq 0.05$ was considered to be statistically significant.

RESULTS

Spontaneous Motor Responses

In Table 1 are summarized the overall effects of hormonal condition and DZP administration on spontaneous motor responses. Two-way ANOVA revealed a significant effect of the hormonal condition on motor activity, $F(5, 162) = 5.17$, $p < 0.001$, number of head shakes, $F(5, 162) = 6.03$, $p < 0.001$, and grooming behavior, $F(5, 162) = 4.76$, $p < 0.001$. DZP administration significantly modify motor activity, $F(2, 162) = 64.01$, $p < 0.0001$, number of head shakes, $F(2, 162) = 12.85$, $p < 0.0001$, and grooming behavior, $F(2, 162) = 6.45$, $p < 0.001$. In motor activity and head shaking was also observed a significant interaction DZP treatment × hormonal condition, $F(10, 162) = 12.85$, $p < 0.0001$, and, $F(10, 162) = 4.31$, $p < 0.0001$, suggesting that the effects of DZP on these behaviors are dependent upon the hormonal status of the rat.

Subsequent Newman–Keuls test indicated that in saline-injected rats, motor activity and grooming behavior were significantly lower at estrus than at diestrus ($p < 0.005$), and EB administration reduced both behaviors in OVX rats ($p < 0.05$). Head-shaking behavior was lower at estrus than at diestrus ($p < 0.05$), and this behavior was increased in OVX rats injected with EB ($p < 0.005$).

Post hoc comparisons indicated that the lower dose of DZP (0.25 mg/kg) significantly increased motility in rats at estrus and in OVX rats injected with EB or PROG ($p < 0.05$ for all groups). However, this dose of DZP failed to induce significant changes on motor activity in diestrous, OVX, and male rats. The higher dose of DZP (1 mg/kg) significantly depressed motor activity in all rats treated ($p < 0.05$ for all groups). Both doses of DZP did not induce any significant change in head shaking in females at estrus and in OVX rats, whereas significantly diminished this behavior in the remaining groups. No significant interaction between DZP treatment and hormonal condition was observed on grooming behavior. The higher dose of DZP (1 mg/kg) significantly diminished the time spent in grooming behavior in all female and male rats ($p < 0.005$ in all comparisons), while the lower dose of DZP (0.25 mg/kg) was without effect in all female and male rats.

TABLE 1
EFFECTS OF DIAZEPAM ON SPONTANEOUS MOTOR ACTIVITY UNDER DIFFERENT HORMONAL CONDITIONS

Hormonal Condition	*n*	Motor Activity (Counts)	Head Shaking (No.)	Grooming (s)
Diestrus				
Solvent	10	524.4 ± 33.5	9.5 ± 1.3	235.0 ± 11.3
DZP 0.25 mg/kg	10	482.0 ± 51.4	5.9 ± 1.0*	197.4 ± 16.9
DZP 1.0 mg/kg	10	250.6 ± 43.7*	3.5 ± 0.9*	87.4 ± 14.8*
Estrus				
Solvent	10	374.5 ± 24.1†	6.5 ± 0.8†	172.5 ± 16.0†
DZP 0.25 mg/kg	10	536.9 ± 54.2*	7.6 ± 1.9	168.0 ± 23.4
DZP 1.0 mg/kg	10	284.6 ± 31.2*	8.3 ± 1.7	83.4 ± 15.8*
OVX				
Solvent	10	464.2 ± 26.5	7.4 ± 1.6	245.9 ± 24.9
DZP 0.25 mg/kg	10	461.1 ± 31.5	8.5 ± 2.1	203.1 ± 26.1
DZP 1.0 mg/kg	10	188.9 ± 25.4*	8.3 ± 2.4	90.2 ± 10.5*
OVX + EB 2 μg				
Solvent	10	368.6 ± 38.9†	14.3 ± 1.7†	187.4 ± 15.9†
DZP 0.25 mg/kg	10	494.4 ± 47.1*	8.8 ± 1.5*	230.7 ± 23.4
DZP 1.0 mg/kg	10	214.8 ± 22.3*	6.0 ± 1.6*	69.8 ± 11.3*
OVX + PROG 5 mg				
Solvent	10	454.7 ± 26.4	9.0 ± 1.4	237.2 ± 19.5
DZP 0.25 mg/kg	10	567.7 ± 44.3*	5.1 ± 0.8*	226.0 ± 28.4
DZP 1.0 mg/kg	10	343.4 ± 45.8*	2.4 ± 0.5*	59.4 ± 15.6*
Males				
Solvent	10	501.0 ± 34.6	17.9 ± 1.9	234.9 ± 17.8
DZP 0.25 mg/kg	10	527.2 ± 55.1	10.6 ± 1.8*	244.7 ± 23.4
DZP 1.0 mg/kg	10	273.8 ± 47.3*	4.8 ± 1.0*	73.1 ± 16.4*

Values are expressed as mean ± SEM. Comparisons were made by using two-way ANOVA followed by post hoc Newman–Keuls test: *$p < 0.05$ compared with its solvent group; †$p < 0.05$ comparing diestrus with estrus or OVX with OVX + EB.

Conditioned Avoidance Responses (CARs)

The results of the avoidance conditioning are showed in Fig. 1 (top panel). Two-way analysis of variance (ANOVA) revealed a significant effect of hormonal condition, $F(5, 162) = 7.42$, $p < 0.001$, and DZP administration, $F(2, 162) = 18.87$, $p < 0.001$, on the acquisition of CARs. Because the interaction between these two factors was also significant, $F(10, 162) = 12.88$, $p < 0.001$, the effect of DZP seems to be dependent upon the hormonal condition of the rat. Post hoc comparisons indicated that the avoidance conditioning in control rats was similar in diestrous and OVX rats, but this behavior was seriously impaired in estrous female rats and in OVX rats after a single injection of EB ($p < 0.001$ in both cases). The treatment with 1.0 mg/kg DZP significantly impaired the acquisition of CARs in diestrous ($p < 0.001$), OVX ($p < 0.005$), OVX + PROG ($p < 0.001$), and male rats ($p < 0.05$). However, in estrous rats and in OVX rats with EB administration, both doses of DZP significantly improved the acquisition of CARs ($p < 0.05$ for all cases).

Figure 1 (bottom panel) showed that the impairment in the acquisition of CARs was accompanied with increased escape failures. Two-way ANOVA revealed that hormonal condition, $F(5, 162) = 6.82$, $p < 0.001$ and DZP administration, $F(2, 162) = 13.47$, $p < 0.001$, exert a statistically significant effect on escape failures. The interaction between these two factors was also significant, $F(10, 162) = 10.01$, $p < 0.001$. Subsequent Newman–Keuls tests demonstrated that control estrous rats failed significantly more than diestrous ($p < 0.001$) and male rats ($p < 0.01$). Treatments with estradiol markedly increased escape failures in control OVX rats ($p < 0.001$). Higher dose of DZP increased escape failures in diestrous and male rats ($p < 0.05$ in both cases). It is interesting that both doses of DZP reversed the escape failures observed in estrous and OVX rats with estradiol administration ($p < 0.001$ in both cases), whereas in OVX rats and in OVX rats with progesterone administration, no significant effect of DZP was observed.

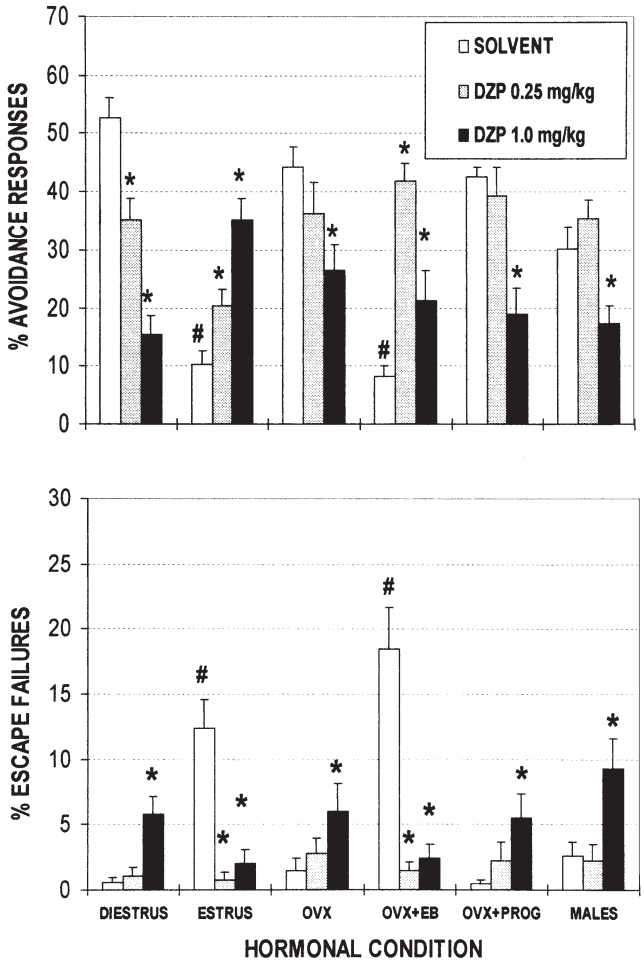

FIG. 1. Influence of hormonal condition (females at diestrus, estrus, ovariectomized = OVX, ovariectomized with estradiol administration = OVX+EB, ovariectomized with progesterone administration = OVX+PROG, and male rats) on the effects of diazepam. Top panel: acquisition of conditioned avoidance responses (CARs), and bottom panel: escape failures (EF). Each bar represents the mean ± SEM of the percentages of CARs or EF for 50 trials. Comparisons were made by using two-way ANOVA followed by post hoc Newman–Keuls test: $*p < 0.05$ compared with its solvent groups and $\#p < 0.05$ comparing Diestrus with Estrus or OVX with OVX+EB rats. The number of rats on each group was 10.

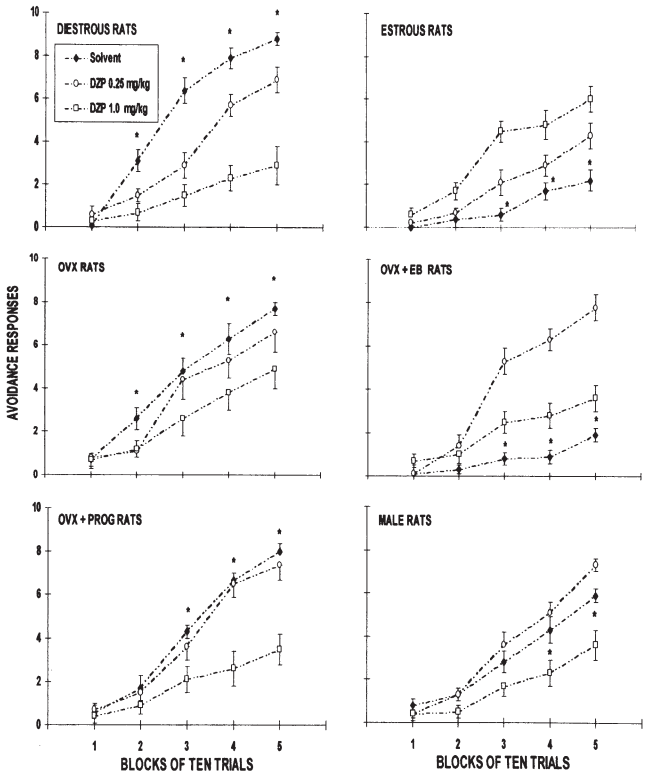

FIG. 2. Influence of hormonal condition (females at diestrus, estrus, ovariectomized = OVX, ovariectomized with estradiol administration = OVX+EB, ovariectomized with progesterone administration = OVX+PROG, and male rats) on the effect of diazepam on the acquisition of conditioned avoidance responses (CARs). Each point of the curves represents the mean ± SEM of the percentages of CARs by blocks of 10 successive trials. Comparisons were made by using two-way ANOVA followed by post hoc Newman–Keuls test: $*p < 0.05$ compared with its solvent group. The number of rats on each group was 10.

The effects of the interaction between DZP and hormonal condition on the overall rate of acquisition, expressed as the percent of CARs by blocks of 10 successive trials, are presented in Fig. 2. Females in diestrus acquired the CARs faster than females in estrus. Administration of estradiol markedly decreased acquisition rate in OVX rats, while progesterone did not modify the performance in these rats. Both doses of DZP markedly decreased acquisition rate in diestrous rats, whereas increased this behavior in estrous and OVX + EB rats. In male, OVX and OVX + PROG rats DZP 1.0 mg/kg decreased the learning rate, while DZP 0.25 mg/kg failed to induce any effect.

DISCUSSION

The results of the current study clearly demonstrates that gender, estrous cycle, ovariectomy, and ovarian hormones modify behavioral responses to DZP, suggesting that the sexual hormonal condition of the rat could trigger behavioral changes through the interaction with $GABA_A$/benzodiazepine/chloride systems in the brain. The main findings derived from this work can be summarized as follows: (a) DZP, 0.25, and 1 mg/kg, induce opposite effects on avoidance conditioning according to the hormonal condition; and (b) DZP 1 mg/kg reduces motor activity and grooming behavior, independent of the hormonal condition.

In control female rats, the acquisition of conditioned avoidance responses (CARs) was facilitated during diestrus and almost suppressed at estrus. Whereas estradiol induced a severe disruption of the response in ovariectomized rats, progesterone was not able to induce any change in the acquisition of CARs. The present experiments indicate that the same dose of DZP exerts both stimulatory and inhibitory effects on the acquisition of an avoidance task according to the hormonal condition of the rat. In fact, both doses of DZP effectively enhanced acquisition of CARs in rats at estrus and in OVX rats primed with EB, but the dose of 1.0 mg/kg im-

paired the acquisition behavior in all the other groups. As the stimulant effects of DZP on conditioning behavior were evident late after the peak plasma concentration of estradiol; that is, during estrus (5) and 48 h after a single injection of EB to OVX rats (6), estradiol might act probably modulating the GABA recognition site via genomic receptors in neural sties (15). Although it is known that conditioned avoidance performance can vary according to the foot shock intensity, the present results could not be explained by changes in pain sensitivity because no significant differences were observed between the foot shock thresholds applied to the different groups, in our experimental conditions (data not shown). On the other hand, it has been reported that concentrations of DZP in the brain after an intraperitoneally injection (0.28 mg/kg) are not influenced by the stage of the estrous cycle (2), suggesting that the observed changes in response to diazepam reflect changes in sensitivity to this drug rather than alterations in distribution or metabolism.

Two-way active avoidance is often used to assess drug effects on learning or on memory processes. In other occasions, this paradigm have been used to assess behavioral aspects that are not directly related to learning and memory per se, such as anxiety and learned helplessness (3). It is possible that DZP may alter avoidance performance by altering not only cognitive processes but also noncognitive factors necessary for avoidance performance, such as motor activity, emotionality or anxiety, motivation, and so on. The lower dose of DZP stimulated motor activity in estrous and in OVX rats injected with EB, whose exploratory responses were depressed before treatment. However, the improvement in the acquisition of CARs induced by DZP in intact female rats at estrus and in OVX + EB rats cannot be merely explained by the increase in motor activity. The higher dose of DZP depressed the motor activity in all groups of rats, although it improved the acquisition behavior in estrous and in OVX rats injected with EB. These data suggest that modulatory influences of gonadal hormones on avoidance response be not necessarily accompanied with equivalent changes in spontaneous motor activity, indicating that different mechanisms may be involved. In addition, the effects of DZP on grooming behavior were not distinctively affected by the hormonal condition of the rats. In fact, the higher dose of DZP reduced grooming behavior in all groups studied, while the lower dose was without effect in all groups.Dopamine receptors can be primarily involved in this behavior (10).

Ovarian hormone fluctuations could determine different levels of anxiety across the estrous cycle associated with changes in the avoidance performance (17). Lower conditioning performances during estrus or in OVX rats primed with estradiol, along with an increase in escape failures, may be attributed to high levels of anxiety. Then, the improvement on acquisition performance induced by DZP in these experimental groups could be explained through a higher sensitivity to the anxiolytic effects of DZP. Both doses of this drug reversed escape deficits in rats at estrus and OVX primed with EB, but DZP 1.0 mg/kg increased escape failures in the remaining groups. The latter effect, along with the impairment in conditioning performance, could be attributed to an increased sensitivity to the sedative or motor depressant effects of DZP.

Estradiol appears to be the principal ovarian steroid modulating the acquisition of an avoidance task. On the other hand, progesterone was not able to induce changes in the acquisition of CARs in OVX rats, suggesting that this ovarian hormone have a secondary role in conditioning behavior. Progesterone, in our experimental conditions, also failed to induce consistent changes in spontaneous motor behaviors. These data are in agreement with previous results obtained in our laboratory (7).

Our results suggest a modulating influence of gonadal hormones on behaviors, which are thought to reflect the activity of GABAergic neurons. Behavioral responses induced by DZP in estrous and in OVX rats injected with EB are different, and sometimes opposite, of that observed in other hormonal conditions. Although the function of central GABA systems is differentially affected by the hormonal condition of the rat, the precise mechanism of action of this effect remains unknown. Ovarian hormones have been reported to interact directly with $GABA_A$/benzodiazepine/chloride ionophore complex regulating the number of GABA receptors in selected areas of the rat brain and affecting several nonreproductive behaviors (15,16). The hormonal condition has been shown to modulate the GABA recognition site and possibly GABA-related responses in rats. This is evidenced by the decrease in cortical $GABA_A$ receptor affinity seen in females compared with other hormone groups and the gender-related difference observed in susceptibility to seizures induced by the GABA antagonist bicuculline (22). Previous findings, sometimes contradictory, seem to depend on procedures employed and, in particular, on the time elapsing between administration of ovarian steroids and testing. A short latency effect occurring less than 1 h after ovarian hormone administration implies a direct neuronal activity, but at longer latencies, hormonal effect implies a more traditional genomic mechanism. Estrogens may act directly, modifying receptor sensitivity or influencing other neurotransmitter systems. For example, there is behavioral evidence that central serotonergic, cholinergic, and dopaminergic activity varies with the hormonal condition of the rat (8–10).

Although this study does not contribute to clarify the precise mechanism of the estrogen–diazepam interaction, the demonstration of an influence of ovarian hormones on the effects of DZP could have clinical impact. These findings suggest that therapeutic effects of DZP may depend upon the sex and the endocrine cycle phase. The interaction between estrogens and $GABA_A$/benzodiazepine/chloride activity could be especially important in physiological and pathophysiological conditions where ovarian steroids levels change, for example, during pregnancy, premenstrual syndrome, or stress, and may help to explain some of the behavioral changes known to occur in these conditions.

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