



# The effects of diazepam on the elevated T-maze are dependent on the estrous cycle of rats

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## Abstract

In order to determine the modulation of anxiolytic and panicolytic-like effects of diazepam by the hormonal cycle of female rats, male and female rats – the latter divided per estrous cycle phase (estrus, diestrus, metaestrus and proestrus) – were tested in the elevated T-maze, a behavioral model of panic and anxiety. Diazepam (0.5, 1.0 and 2.0 mg/kg) or saline solution was injected in individual animals that were submitted to one session in the elevated T-maze 25 min after drug/saline administration. The test consisted of three avoidance trials and one escape trial, separated by a 30 s interval, during which the animals were isolated in individual cages. The avoidance trials began with the animal being placed at the end of the maze's enclosed arm. The time necessary for the animal to leave the central square was considered as the response's latency. The trials that exceeded 300 s were considered as failures. Results demonstrate a decrease in the effects of diazepam in inhibitory avoidance (anxiety) trials in females in diestrus and proestrus, but no relation of gender or estrous cycle on diazepam effects on escape trials (fear). The results support the hypothesis that down-regulation of GABA<sub>A</sub> receptors by activation of nuclear estrogen receptors and induction of PKC-mediated GABA<sub>A</sub> receptor phosphorylation by activation of surface estrogen receptors in raphe neurons underlie the modulation of diazepam sensitivity by estrogen. **Keywords:** diazepam, estrous cycle, estrogen, anxiety, elevated T-maze.

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## Introduction

Anxiety is an emotional state that emerges under situations in which risk is probable, but not certain. This state is associated with behaviors linked to defense reactions, that is, a set of species-specific behavioral strategies which were selected in evolutionary history to increase the possibilities of survival in dangerous situations as well as the autonomic activation that accompanies these behaviors in danger situations, such as cautious investigation (Blanchard & Blanchard, 1988; Fanselow & Lester, 1988).

In humans, anxiety is manifested by clinical alterations with variable severity distributed along the anxiety spectrum, and is usually treated with benzodiazepines, a class of substances that act on the GABA<sub>A</sub> receptor complex by allosteric modulation.

GABA is a neurotransmitter of mostly inhibitory action that is present in about 30% of the synapses in the central nervous system (Olsen, 2002). The GABA<sub>A</sub> receptor complex prevails on mammalian nervous systems; it receives, besides the endogenous GABA neurotransmitter, benzodiazepines, barbiturates and other anxiolytic drugs, hormones and anticonvulsants (Berezhnoy, Gravielle, & Farb, 2007; Schmitt, Luddens, & Hiemke, 2000). Most ligands which modulate GABA<sub>A</sub>-associated chloride currents seem to act through allosteric modulation of the opening of a chloride channel, altering the probability that this ligand-gated channel is in an "open" state (Berezhnoy et al., 2007; Costa, 1998; Olsen, 2002). The actions of GABA at the many GABA<sub>A</sub> receptor subtypes can also be agonized or allosterically modulated by a variety of progesterone metabolites (Puia et al., 1990). Neurosteroids given in high doses can gate chloride channels associated with GABA<sub>A</sub> receptors even in the absence of GABA (Puia, Ducic, Vicini, & Costa, 1993), an effect that has been

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observed with barbiturates, but not benzodiazepines (Costa, 1998). Progesterone derivatives positively modulate GABA action on GABA<sub>A</sub> receptors (Puia et al., 1993); pregnelone sulfate (a progesterone derivative) negatively modulates chloride currents gated by GABA only in high concentrations (Puia et al., 1993). Interestingly, this neurosteroid is most efficient in its GABA-potentiating action in recombinant receptors including the  $\gamma_1$  subunit – a subunit whose mRNA is expressed predominantly in native GABA<sub>A</sub> receptors located in glial cells (McKernan & Whiting, 1996).

Besides the direct action of progesterone metabolites on GABA<sub>A</sub> receptors, estrogen has also been implicated in the regulation of GABA<sub>A</sub> receptor activity (Bitran & Dowd, 1995; Guilinello, Gong, Li, & Smith, 2001; Hamon et al., 1983; Herbison, 1997; Herbison & Fénelon, 1995; Zhu & Vicini, 1997), despite the lack of evidence to suggest that this steroid is a direct allosteric modulator of the GABA<sub>A</sub> receptor. A major contributing factor in the wide array of behaviors which are mediated by estrogen and progesterone is the existence of two different estrogen receptors in the brain: Estrogen Receptor- $\alpha$  (ER- $\alpha$ ) and Estrogen Receptor- $\beta$  (ER- $\beta$ ). In rodent brains, the first are expressed strongly in hypothalamic nuclei which are closely related to reproductive physiology, as well as in the pons and medulla (Österlund, Kuiper, Gustafsson, & Hurd, 1998; Shughrue & Merchenthaler, 2001). ER- $\beta$ , however, is expressed widely in the forebrain (neocortex as well as limbic forebrain cell nuclei) and raphe (Österlund, Gustafsson, Keller, & Hurd, 2000; Österlund et al., 1998; Shughrue & Merchenthaler, 2001; Silveira, Zangrossi, Viana, Silveira, & Graeff, 2001). Consistent with this distribution, for example, mice which were knocked out of either ER- $\alpha$  or ER- $\beta$  demonstrated differences in exploratory behavior and activity levels: while ER- $\alpha$  KO mice presented differences in spontaneous wheel running, ER- $\beta$  KO mice had an anxious phenotype (Krezel, Dupont, Krust, Chambon, & Chapman, 2001; Ogawa, Chan, Gustafsson, Korach, & Pfaff, 2003), with decreased exploration of the center of an open-field (OF) and decreased exploration of the open arms of an elevated plus-maze.

The estrous cycle of rodents lasts for about four to five days, and has four phases, denominated *estrus*, *metaestrus*, *diestrus* and *proestrus*. Estrus corresponds to ovulation, and progesterone is found at its peak level during this phase. Metaestrus corresponds to the phase between cycles, and hormone levels are restored to the “baseline” level in the vagina. At the end of the metaestrus phase, the diestrus phase begins, a period where estrogen’s action takes place. Proestrus corresponds to the peak on the action of estrogens.

Many studies explored the different phases of the estrous cycle in relation to fear and anxiety-like behavior, focusing mainly on estrus (the phase

in which progesterone is highest) and proestrus (the phase in which estrogen is highest) (for a review, cf. Morgan, Schulking, & Pfaff, 2004). John Archer (1975) observed that females in estrus not only have an increase in behaviors involving gross motor activity, but also are more reactive to fearful stimuli. In terms of conditioned fear, females in proestrus acquire eye-blink responses faster than females in other stages (Shors, Lewczyk, Pacynski, Mathew, & Pickett, 1998), exhibit facilitated acquisition of two-way avoidance (Sfikakis, Spyraiki, Sitaras, & Varonos, 1978) and less freezing to context in a Pavlovian fear conditioning paradigm (Markus & Zecevic, 1997). Cycling female rats in proestrus or estrus show less activity in the open-field than females in other stages (Díaz-Véliz, Benavides, Butrón, Dussabaut, & Mora, 1999), which the authors interpreted as a measure of increased fear. Another study (Frye, Petralia, & Rhodes, 2000) reported that female rats in proestrus and estrus were more active in the periphery of the OF (i.e., increased thigmotaxis) but not in the center, which is a less controversial measure of anxiety (Treit & Fundytus, 1989). These differences were associated with increased hippocampal content of progesterone and 3 $\alpha$ ,5  $\alpha$ -THP. The latter results are partially contradictory with observations by Rhodes and Frye (2001) that the inhibition of progesterone metabolism in the hippocampus during estrus had pro-exploratory and antinociceptive effects, also producing an anxiogenic profile. To complicate matters further, in the elevated plus-maze (EPM), females in proestrus present decreased anxiety (Frye et al., 2000; Gouveia & Morato, 2002; Mora, Dussabaut, & Díaz-Véliz, 1996; Nomikos & Spyraiki, 1988). Morgan and colleagues (2004) argued that these differences could be accounted for by different dependencies on ER- $\alpha$  or ER- $\beta$  receptors, as well as the different neuroanatomical patterns of expression for both receptors. In addition, one could argue that the modulation exerted by estrogen and progesterone is widespread in different systems which mediate anxiety, such as GABA (Bitran & Dowd, 1995; Hamon et al., 1983; Herbison, 1997; Herbison & Fénelon, 1995; Maggi & Perez, 1984), serotonin (Fernández-Guasti, Martínez-Mota, Estrada-Camarenas, Contreras, & Lórez-Rubalcava, 1999; Le Saux & Di Paolo, 2005; Maswood, Stewart, & Uphouse, 1995; Maswood, Truitt, Hotema, Caldarola-Pastuszka, & Uphouse, 1999; Österlund, Hallin, & Hurd, 2000; Robichaud & Debonnel, 2005; Rubinow, Schmidt, & Roca, 1998), oxytocin (Dellovade, Zhu, & Pfaff, 1999; Koksma et al., 2003), and protein kinase C $\epsilon$  (Fancsik, Linn, & Tasker, 2000; Hodge et al., 2002).

The elevated T-maze is an anxiety and memory animal model developed from the classic elevated plus-maze by the elimination of one of the maze’s

enclosed arms. In relation to other animal models, the elevated T-maze allows the differentiation between the behavioral elements that compose generalized anxiety, fear, and learned anxiety, based on the animal's natural aversion to open and elevated spaces, thus enabling clearer discriminations between the anxiolytic and panicolytic effects of diverse drugs (Bueno, Zangrossi, & Viana, 2007; Graeff, Viana, & Tomaz, 1993; Zangrossi & Graeff, 1996): while inhibitory avoidance of the open arm is supposed to represent learned fear, the escape response from the open arm would represent innate fear. Anxiolytic-like effects are observed in the impairment of inhibitory avoidance, while impairment of escape responses is associated with panicolytic-like effects (Graeff et al., 1993; Zangrossi & Graeff, 1996). Diazepam selectively impairs inhibitory avoidance in the elevated T-maze, leaving escape responses unchanged (Graeff, Netto, & Zangrossi, 1998; Graeff et al., 1993; Viana, Tomaz, & Graeff, 1994; Zangrossi & Graeff, 1996). In a previous work (Gouveia, dos Santos, Felisbino, Afonseca, Antunes, & Morato, 2004), we demonstrated that the estrous cycle modulates different parameters of conditioned and unconditioned fear in this model, with females in proestrus and estrus showing impairment in the acquisition of inhibitory avoidance but no alterations in escape, while females in diestrus showed increased baseline anxiety. In the present work, we assessed the effects of the estrous cycle on benzodiazepine sensitivity of rats using the elevated T-maze model.

## Methods

### Subjects

Thirty-two male rats and 128 female rats (*Rattus norvegicus*, Wistar, UNESP, Botucatu) were used in the experiment. Animals were distributed in groups ( $n = 8$ ) based on sex, drug dose and (in the case of females) estrous cycle phase. Subjects were kept in collective cages (five per cage) in the same room with *ad libitum* water and food, as well as controlled light/dark cycle (12L:12D, lights on at 0700) and temperature ( $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ).

### Apparatus

The elevated T-maze consisted of two opposed open arms (50x10 cm) and one enclosed arm (50x10x40 cm), perpendicular to the other two. The maze's arms bind to a 10x10 cm central area. The apparatus was elevated 50 cm above the ground. The whole maze was made of wood and the surfaces of its arms were covered with formica.

### Drug

Diazepam (0.5, 1.0 and 2.0 mg/kg, Roche Products Ltd., Brazil) was dissolved in saline solution (0.9%) as served as control solution. Diazepam and control solution were injected intraperitoneally at a total volume of 4 ml/kg.

### Procedure

Initially, females had their estrous cycle recorded with daily collection of vaginal material for a period that varied between 5-6 days. Females with an irregular cycle were discarded. After verification, the animals were divided in groups based on sex, and females were divided in subgroups according to the phase of their estrous cycle (estrus, diestrus, metaestrus or proestrus). Further subdivision was made as animals were divided according to drug dosage (saline; 0.5; 1.0; and 2.0 mg/kg), resulting in nested groups with eight animals each. Each experimental subject underwent a single test session that began 25 minutes after the intraperitoneal injection of diazepam.

The test consisted of three avoidance trials and one escape trial separated by 30 s intervals in which the animals were isolated in individual cages. The avoidance trials began with the animal being placed at the end of the maze's closed arm. The time necessary for the animal to leave the central square was considered as the response latency. Trials that exceeded 300 s were considered as failures. After the inhibitory avoidance test, an escape test was executed, where the animal was placed at the end of the right open arm and the latency time to cross the central square was recorded. Thus, latencies to leave the compartment in which the animal was first placed were the outcome assessed in both inhibitory avoidance and escape trials.

The maze was cleaned after each session to avoid interference of the odor of a previously tested animal on the behavior of the animal observed on the following session.

The experiments were performed in compliance with the recommendations of the SBNeC (Brazilian Society for Neuroscience and Behavior), which are based on NIH's Guide for Care and Use of Laboratory Animals. Procedures and animal housing conditions were approved by the UNESP Research Ethics Committee.

### Statistical analysis

Data are presented as means  $\pm$  S.E.M. The results of the inhibitory avoidance trials were analyzed with a two-way repeated measure analysis of variance (between-subjects factors: dose and hormonal state; within-subjects factor: trial). Data from the escape trials were analyzed with a two-way analysis of variance (between-subjects factors: dose and hormonal state). Tukey *post-hoc* tests were performed whenever appropriate, and statistically significant *p*-values were set as  $\leq .05$ .

## Results

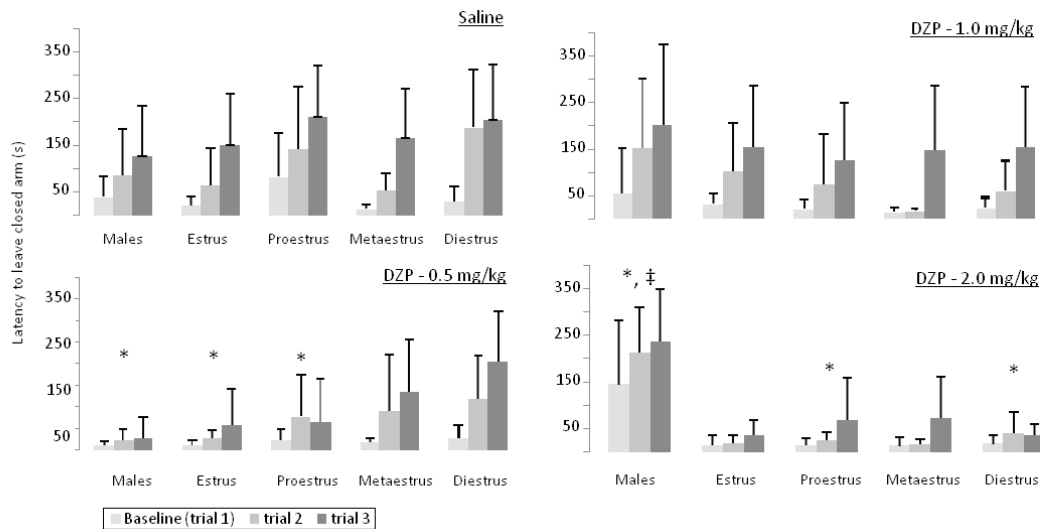
Figure 1 presents latency times to leave the closed arm, a measure of inhibitory avoidance, across trials. As expected, latency times increased monotonically as trials occurred, irrespective of estrous cycle phase or drug dose. However, this relationship was broken in

diestrus females taking the 2.0 mg/kg dose.

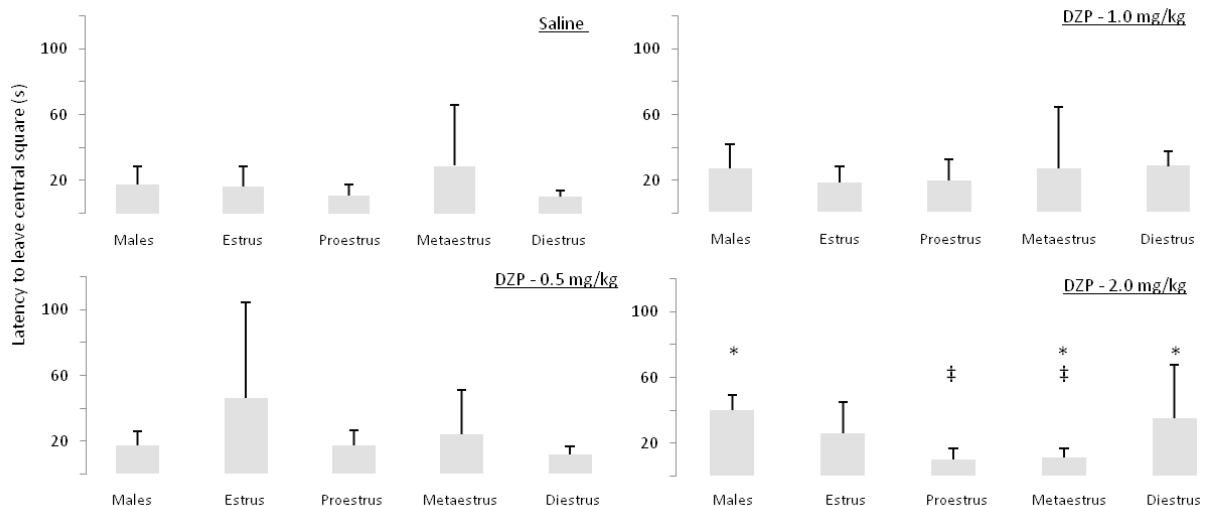
Since the data presented statistically significant sphericity ( $W = .876, p < .005$ ), all subsequent analyses assumed sphericity. Within-subjects main effects of trial number ( $F_{[2,280]} = 92.067, p < .005$ ) and dose within trial ( $F_{[6,280]} = 4.313, p < .005$ ) were found, but hormonal state did not exert significant effects on this variable ( $F_{[8,280]} = 1.667, p = .106$ ). No interaction between dose and hormonal state was found within trial number ( $F_{[24,280]} = 1.276, p = .179$ ).

Diazepam doses ( $F_{[3,140]} = 3.842, p = .011$ ) and hormonal status ( $F_{[4,140]} = 3.363, p = .012$ ) produced main between-subject effects on the mean latency times, and an interaction between both factors ( $F_{[12,140]} = 4.514, p < .005$ ) was also observed. Post-hoc tests discriminated the underlying differences; the results of these analyses can be found in Figure 1.

Figure 2 presents the effects of diazepam and gender/hormonal status on the latency to exit the central square. No main effects of diazepam dose ( $F_{[3,140]} = 1.666, p = .34$ )



**Figure 1.** Estrous cycle modulates inhibitory avoidance acquisition in the elevated T-maze and affects diazepam sensitivity in that task. The charts represent mean ( $\pm$  S.E.M.) latencies to leave the closed arm of the elevated T-maze as a function of gender and estrous cycle phase, as well as diazepam dose. Asterisks (\*) represent differences within a given dose, while the double dagger symbol (‡) represents differences against controls (0.9% saline).



**Figure 2.** Estrous cycle does not have an effect in escape trials in the elevated T-maze, and diazepam effects are detectable only at higher doses, suggesting motor impairment. The charts represent mean ( $\pm$  S.E.M.) latencies to leave the central square of the elevated T-maze as a function of gender and estrous cycle phase, as well as diazepam dose. Asterisks (\*) represent differences within a given dose, while the double dagger symbol (‡) represents differences against controls (0.9% saline).

or hormonal status ( $F_{[4,140]} = .223$ ,  $p = .2230$  were found; however, a statistically significant interaction between both factors was observed ( $F_{[12, 138]} = 1.857$ ,  $p = .045$ ).

## Discussion

The present article tested the influence of estrous cycle phase on the anxiolytic and panicolytic-like effects of diazepam, using the elevated T-maze, a rodent anxiety model. The results indicate a modulatory action of gender and hormonal status on the effects of diazepam in the elevated T-maze, evident in anxiety-like (inhibitory avoidance) but not fear-like behaviors (escape), in the diestrus and proestrus phases, correspondent to an increase in the circulating levels of estrogen present in female rats' organisms. These results are consistent with available evidence regarding increased anxiety and estrogen levels (Bitran & Dowd, 1995; Díaz-Véliz, Alarcón, Espinoza, Dussabaut, & Mora, 1997; Díaz-Véliz, Soto, Dussabaut, & Mora, 1989; Gouveia et al., 2004; Gouveia & Morato, 2002). Females in diestrus and proestrus also presented reduced latency times in inhibitory avoidance trials in relation to males and females in metaestrus and diestrus, suggesting an anxiolytic effect of progesterone also consistent with the literature (Frye et al., 2000; Frye & Walf, 2002; Frye, Walf, Rhodes, & Harney, 2004; Guilinello et al., 2001; Rhodes & Frye, 2001; Smith et al., 1998). The significant increases in latency times on males' inhibitory avoidance trials are proportional to the dose of diazepam, suggesting not a neuromodulatory phenomenon, but a sedative and ataxic effect of this benzodiazepine.

The differential result in anxiety - but not panic - is consistent with the observation that estrous cycle modulates responses to anxiogenic stimuli in the elevated T-maze, but not to panicogenic stimuli (Gouveia et al., 2004). It also indicates that the site of action is not restricted to the ventromedial hypothalamic nucleus, dorsal periaqueductal gray area or medial nucleus of the amygdala, since facilitation of GABA activity in these regions produces effects on panic measures in the elevated T-maze (Bueno, Zangrossi, Nogueira, Soares, & Viana, 2005; Bueno et al., 2007; Herdade, Strauss, Zangrossi, & Viana, 2006). Exposure to the inhibitory avoidance task of this model increases Fos-like immunoreactivity in the medial amygdaloid nucleus, anterior hypothalamic nucleus and raphe nuclei, while the escape task increases Fos-like immunoreactivity in the basolateral amygdaloid nucleus and in the dorsal periaqueductal gray area (Silveira et al., 2001). Accordingly, the modulation of diazepam sensitivity by estrogen, being anxiety-related, is likely to have happened at the level of the raphe nuclei, a set of structures presenting surface and nuclear estrogen and progesterin receptors (Alves, Weiland, Hayashi, & McEwen, 1998). Estrogen and progesterone modulate

the firing of raphe neurons (Robichaud & Debonnel, 2004, 2005), and serotonergic neurons in the raphe have been associated with the modulation of anxiety-like behavior (Graeff, 2002; Graeff, Viana, & Mora, 1997). Interestingly, the immunoreactivity of estrogen and progesterin receptors in the raphe does not coincide with tryptophan hydrolase immunoreactivity, suggesting that the targets of steroid modulation of raphe serotonergic activity are not serotonergic neurons (Alves et al., 1998).

Such differences between males and females in diestrus and proestrus may be related to a modulatory action of estrogen over the GABA<sub>A</sub> receptor complex and the benzodiazepine subunit. This action would be manifested by a diminished efficacy of diazepam during diestrus and proestrus - precisely the phases of the estrous cycle that present increased estrogen activity.

One possible mechanism involves the activation of membrane-associated estrogen receptors, whose response is mediated by the second messenger protein kinase C (Kelly & Levin, 2001; Kelly & Wagner, 1999; Le Saux & Di Paolo, 2005). PKC would phosphorylate GABA<sub>A</sub> receptors, decreasing the efficacy of benzodiazepines. This hypothesis is strengthened by the observation that phosphorylation of these receptors through the action of PKC determines sensitivity to neurosteroids (Fancsik et al., 2000), together with evidence that mice knocked out for the  $\epsilon$  isoform of PKC are more sensitive to the modulation of chloride uptake by allopregnanolone, alphaxalone, and pregnanolone (Hodge et al., 2002) and data on the behavioral and neurochemical effects of alcohol and benzodiazepines (Choi, Wang, Dadgar, Chang, & Messing, 2002; Hodge et al., 1999; Olive, Mehmert, Messing, & Hodge, 2000; Olive, Mehmert, Nannini, Camarini, Messing, & Hodge, 2001).

Another possibility is that activation of nuclear  $\beta$  estrogen receptors in raphe neurons could down-regulate GABA<sub>A</sub> receptor density, an effect that was observed in the preoptic area and in the bed nucleus of the stria terminalis of female rats (Herbison & Fénelon, 1995). The relative contribution of effects mediated through genomic modulation of GABA<sub>A</sub> receptors and effects mediated through receptor phosphorylation to the effects of estrogen on behavior is yet undetermined, and it should be considered that these two mechanisms may be operating in association (Brandon, Uren, Kittler, Wang, Olsen, & Parker, 1999; Kumar, Sieghart, & Morrow, 2002; Moss & Smart, 1996). The association between the effects of diazepam and progesterone levels is indicated by the effectiveness of the drug (i.e., diminished latency times) in the estrus phase, when this hormone is found at its peak level, and is probably due to the direct action of progesterone and its metabolites on allosteric sites of the GABA<sub>A</sub> receptor. Diazepam seems also to be effective in the metaestrus phase, where no hormonal activity is found in the female rats' organisms.

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