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Estrus variation in anticonflict effects of midazolam microinjected into septal nuclei in female Wistar rats

Miguel Molina-Hernández*, N.P. Téllez-Alcántara

Laboratorio de Conducta, Instituto de Investigaciones Psicológicas, Universidad Veracruzana, PO Box 361, Jalapa, CP 91000 Veracruz, Mexico

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Abstract

Effects of midazolam intraperitoneally (3.0 mg/kg) administered, or locally applied into lateral septal nuclei (10 μg/μl), or into the medial septum (10 μg/μl) were assessed in Wistar rats during late proestrus or metestrus–diestrus in a conflict-operant task. A reduction in conflict behavior was found in control rats during late proestrus ($P < .05$), when compared to metestrus–diestrus. Systemic injections of midazolam ($P < .05$) or midazolam infusions into lateral septal nuclei ($P < .05$) also reduced conflict behavior only during late proestrus, whereas midazolam infusions into the medial septum produced neither of these anticonflict effects in any estrous phase. In conclusion, an endocrine-related variation in anticonflict effects of midazolam microinjected into lateral septal nuclei was displayed by female rats.

Keywords: Conflict behavior; Estrous cycle; Lateral septum; Midazolam

1. Introduction

The septal area has been implicated in the modulation of emotions, especially fear and anxiety (Thomas et al., 1991). In addition, stimulation of lateral septal nuclei reduced fear-like behavior during aversive states (Yadin et al., 1993). These effects are similar to the one seen with peripheral (Yadin et al., 1993) and lateral septal infusions (Pesold and Treit, 1994) of benzodiazepine drugs (BZDs). Conversely, stimulation of nicotinic receptors in lateral septal nuclei (Ouagazzal et al., 1999) and septal lesions (Yadin et al., 1993) produced fear-like behavior, and impaired acquisition of behaviors that depend on fear-like reduction (Gray and McNaughton, 1983). Aforementioned findings suggest the participation of the lateral septum in fear-like behavior and in anxiolytic actions of BZDs (Menard and Treit, 1999).

A reduction in fear-like levels during late proestrus (Fernández-Guasti and Picazo, 1992) may be attributed to the production of high levels of progesterone during late proestrus (Freeman, 1988). In fact, progesterone (Rodriguez et al., 1986) and some of its main metabolites reduce fear-like behavior in rats (Fernández-Guasti and Picazo, 1995), probably acting at the GABAergic system (Smith et al., 1987). Anxiolytic effects of drugs also vary in relation to estrous cycle phases (Mora et al., 1996). These behavioral changes could be attributed to an interaction between neurosteroids and BZDs, since neurosteroids enhance benzodiazepine binding (Kroboth and McAuley, 1997).

In a new variation on the standard Geller and Vogel's conflict tests, rats may choose between receiving an immediate punished reinforcer or a delayed nonpunished reinforcer. This experimental task is sensitive to anxiolytic actions of diazepam (Hascoët et al., 1994) and serotonergic agonists (Hascoët and Bourin, 1997), which increase the number of immediate punished reinforcers without affecting nonpunished responding (Hascoët and Bourin, 1997). Behavior in this task depends on the endocrine state, since rats are more sensitive to anxiolytic drugs during the proestrus phase when compared to the metestrus phase (Molina et al., in press). However, there are no reports related to effects of BZDs locally applied into lateral septal nuclei on conflict responding during different stages of the estrous cycle. Therefore, in the present study, female rats trained in the aforementioned conflict-operant task were evaluated during

* Corresponding author. Tel.: +28-12-86-83; fax: +28-12-86-83.
E-mail address: mimoli@bugs.invest.uv.mx (M. Molina-Hernández).

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late proestrus and during metestrus–diestrus after receiving intralateral septal infusions of midazolam. Considering that the participation of the medial septum in fear-like behavior has been suggested (Martins et al., 1988; Menard and Treit, 1996), another group of rats were tested in the conflict-operant task, after receiving midazolam infusions into the medial septal nucleus.

2. Methods

2.1. Animals

Adult female Wistar rats (250–300 g; $n=167$) were used. Rats were individually lodged in housing facilities (room temperature: 20–22°C; 12 light/12 dark cycle; lights on at 06:00 h). Access to food was restricted: approximately 12 h before a training or a testing session, food was removed, and training in the experimental chamber began. After finishing each experimental session, rats had free access to food again (Hurwitz and Davis, 1983). Water was continuously available. Two weeks before training, estrous phases were determined by daily microscopic examination of vaginal smears. Proestrus was identified by the predominance of nucleated epithelial cells. Estrus was identified by the presence of dense sheets of cornified epithelial cells. Metestrus was identified by the presence of scattered, nucleated, or cornified epithelial cells, and leukocytes. Diestrus was identified by the presence of leukocytes (Freeman, 1988). Only rats showing two consecutive regular estrous cycles (4–5 days) were included in the study. All experiments were conducted under strict principles of animal care (National Institutes of Health, 1996).

2.2. Drugs

Midazolam maleate (Roche, USA) was diluted in saline (0.9%) and administered intraperitoneally (3.0 mg/kg) or infused into lateral septal nuclei or into medial septal area.

2.3. Experimental design

Inclusion of rats to any experimental group (10 rats each group) was counterbalanced. On the basis of a previous report showing large behavioral differences between late proestrus and metestrus–diestrus in the conflict-operant task (Molina et al., in press), only these estrous phases were considered. Rats were tested in the conflict task during late proestrus or metestrus–diestrus after receiving midazolam infusions into lateral septal area or into the medial septal area. Control rats received vehicle. Rats receiving an intraperitoneal injection of midazolam were used as positive control groups. The number of immediate reinforcers (i.e., punished responses) and the number of unpunished responses were assessed.

2.4. Behavioral tests

2.4.1. Apparatus

Rats were trained in an experimental chamber (height: 33.0 cm, length: 30.0 cm, width: 25.0 cm; Coulbourn Apparatus, USA), placed in a ventilated, sound-attenuated cubicle. One wall of the experimental chamber contained a recess in which a dispenser delivered a reinforcer (0.10 ml of condensed milk). Two apertures located 5 cm above and 2.5 cm on either side of the recess allowed the placement of a motor-driven retractable lever on each side. The experimental chamber was supplied with four lights (3 W, 24 V each): one situated above each lever, one inside the dispenser, and one in the middle of the ceiling (house light). During punishment periods, a shock generator (Grass S48) delivered electric foot-shocks (0.4 mA, 45 ms). The experimental chamber was wiped clean after each session. Software (Coulbourn Instruments) and a computer accomplished control of light stimuli, the delivery of reinforcers, and counted the number of responses.

2.4.2. Training procedure

Rats were trained as previously described (Hascoët et al., 1994). Briefly, all rats were trained to press any of two levers continuously present in the chamber. At the beginning, a fixed ratio of 1 was used, i.e., rats received a reinforcer after one lever pressing. After that, the operant conditioning task raised progressively over a 15-day period from the fixed ratio of 1 to a fixed ratio 8, i.e., rats received one reinforcer after eight lever presses. Thereafter, rats underwent the final conflict training procedure.

Final conflict training sessions were organized in five successive periods totaling 17 min, alternating between nonpunished and punished periods. Nonpunished periods (duration: 3 min) were periods 1, 3, and 5. Punished periods (duration: 4 min) were periods 2 and 4. Each session began with a nonpunished period. During nonpunished periods, only the right lever was inserted, and a reinforcer was presented in a fixed ratio of 8. When nonpunished periods stopped, punished periods were ensued. Punished periods were signaled by illumination of the house light, and the insertion of the left lever. Each press of the left lever was nonpunished, and delivered the reinforcer in a fixed ratio of 8. Each pressing of the right lever was now reinforced according to a fixed ratio of 1, and associated with an electric foot shock. Thus, during punished periods, rats were presented with a choice of responding, i.e., if the response is followed by punishment, then, rats have the opportunity to avoid shocks by active behavior, such as pressing the nonpunished-associated lever, a clearer picture of choice and conflict results. Each daily session consisted of five successive periods alternating between nonpunished and punished periods. Punished responses were assessed, and considering that it is quite often the case that anxiolytic drugs, such as BZDs, will modify unpunished responding at anxiolytic doses, unpunished responses were also counted.

When rats displayed stable baselines of responding (about 4 weeks), stereotaxic surgery was performed.

2.4.3. Stereotaxic surgery

Rats were anaesthetized (20 mg/kg of sodium pentobarbital, intraperitoneally, plus 60 mg/kg of ketamine hydrochloride, intramuscularly) profoundly. They were placed in an stereotaxic device (Stoelting Instrument), and bilaterally implanted with guide cannulae (26-gauge stainless steel; angled 4°) positioned 1.0 mm above the middle of lateral septal nuclei (0.3 mm anterior to bregma; 2.60 mm lateral to bregma; 3.5 mm ventral to dura). Another group of rats were implanted with a guide cannula positioned 1.0 mm above the middle of the medial septal nucleus (0.70 mm anterior to bregma; 0.40 mm lateral to bregma; 4.90 mm ventral to dura; angled 4°); dummy cannulae were inserted into the guide cannulae except during infusions periods. Continuous infiltration of lidocaine (2%) was done on surgical wounds, and in pressure points of the stereotaxic device. After 2 weeks of recuperation, all rats were submitted again to the conflict task. Once rats displayed stable baselines of responding (about 2 weeks), drug testing began. Twenty minutes after receiving systemic injections of vehicle or midazolam, or 3 min after receiving midazolam infusions into lateral septal nuclei, or into medial septal area, rats were tested in the final conflict task. Finally, all rats were sacrificed with an overdose of sodium pentobarbital, and were perfused intracardially with saline (0.9%) followed by formalin (10%). Concentrated thionine (0.01 μl) stain was infused to mark the location of cannula tips. Brains were extracted and placed in formalin (10%) for a week. Lastly, frozen sections (50 μl; cresyl violet) were obtained to

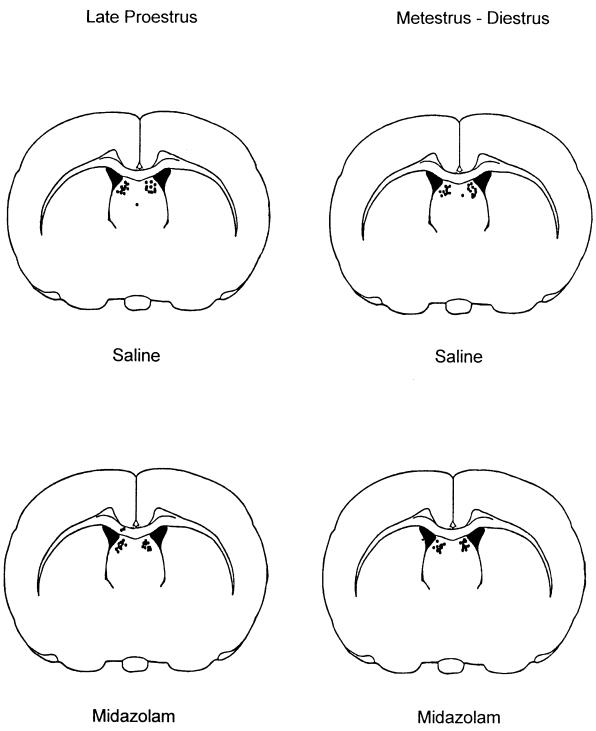

Fig. 1. Frontal sections of rat's brain showing histological reconstruction of injection sites into lateral septal nuclei of subjects tested in the operant-conflict test. Black dots indicate the location of tips of needle injections; adapted from Paxinos and Watson (1982).

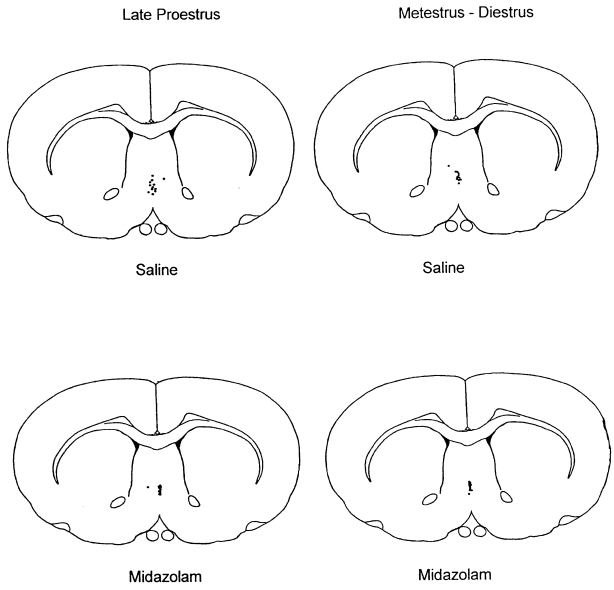

Fig. 2. Frontal sections of rat's brain showing histological reconstruction of injection sites into medial septal nucleus of subjects tested in the operant-conflict test. Black dots indicate the location of tips of needle injections; adapted from Paxinos and Watson (1982).

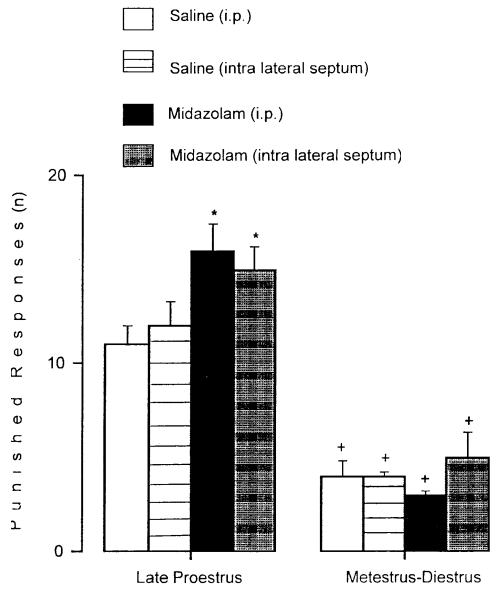

Fig. 3. Immediate punished responses (mean ± S.E.M.) were higher in control rats tested during late proestrus than in rats tested during metestrus–diestrus. Systemic midazolam (3.0 mg/kg) or midazolam infused into lateral septum nuclei increased punished responses only during late proestrus. $^{+}$Metestrus–diestrus significantly different from late proestrus for that treatment condition; $P < .05$. *Statistical comparisons between groups receiving midazolam against vehicle group; $P < .05$.

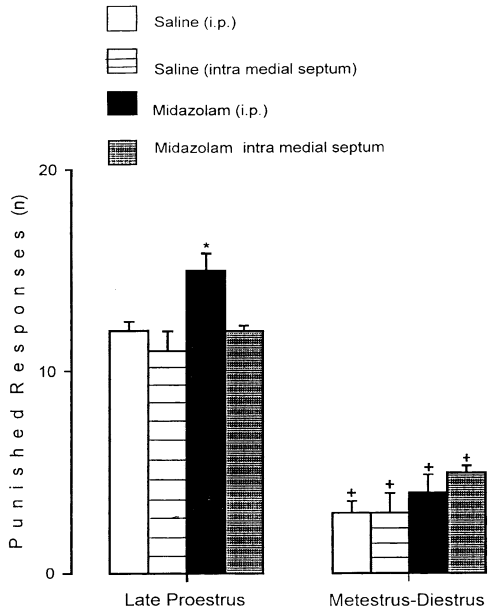

Fig. 4. Immediate punished responses (mean ± S.E.M.) were higher in control rats tested during late proestrus than in rats tested during metestrus–diestrus. Systemic injection of midazolam (3.0 mg/kg) increased punished responses only during late proestrus. Midazolam applied into medial septum nucleus was without anticonflict effects in any estrous phase. $^{+}$Metestrus–diestrus significantly different from late proestrus for that treatment condition; $P<.05$. *Statistical comparisons between groups receiving midazolam against vehicle group; $P<.05$.

confirm the correct implantation of guide cannulae into septal nuclei. Animals were excluded from statistical analysis if needle tip marks missed target areas. Lesion sites were determined by a judge blinded to data.

2.4.4. Infusion procedure

Rats received an infusion of midazolam maleate (0.5 μl/side; 10 μg/μl) or saline (0.9%) infused at a rate of 1.0 μl/min, through a 33-gauge stainless steel internal cannula lowered 1.0 mm below the tip of the guide cannula. The internal cannula was connected via polyethylene tubing to a 50-μl constant rate Hamilton microsyringe.

2.4.5. Statistical analysis

Data were analyzed by three-way analysis of variance (ANOVA), with main effects of midazolam/vehicle, proestrous/metestrous–diestrous phases, and IP/medial septum/lateral septum as three factors. When significant changes were found, analyses of covariance (ANCOVA) were conducted in order to determine the independence of changes. The significance shown in text, figures, and tables are those that remain after ANCOVA. Comparisons between individual groups were then made with Tukey's test. Results were expressed as mean ± S.E.M. Only if $P \leq .05$, differences were considered statistically significant.

3. Results

3.1. Histology

Examples of cannula track placements located into lateral septal nuclei (Fig. 1) and into the medial septal area (Fig. 2) for all rats tested in the conflict-operant test are shown. The majority of cannula placements fell within lateral septal nuclei and within the medial septum. Subjects in which injections sites were located outside of the lateral septum area ($n=4$) or the medial septum ($n=3$) were discarded from statistical analysis.

3.2. Midazolam infusions into lateral septal area during punished periods

Behavior in the conflict-operant task varied according to estrous cycle phases and treatments (three-way ANOVA: estrous phases: $F(1,144)=59.68$, $P<.0001$; treatments: $F(3,144)=7.99$, $P<.003$; Estrous phases × Treatments interaction: $F(3,144)=7.80$, $P<.001$; septal nuclei interaction: n.s.). Rats receiving saline intraperitoneally ($P<.05$) or into lateral septal nuclei ($P<.05$) obtained more immediate punished reinforcers during late proestrus than rats tested during metestrus–diestrus. In addition, midazolam elicited a significant increase in punished responding during conflict periods. Compared to control groups, systemic injections of midazolam ($P<.05$) or midazolam infusions into lateral septal nuclei ($P<.05$) increased the number of immediate punished reinforcers, only in rats tested during late proestrus (Fig. 3).

3.3. Midazolam infusions into medial septum nucleus during punished periods

Rats receiving saline intraperitoneally ($P<.05$) or into medial septum ($P<.05$) obtained more immediate punished reinforcers during late proestrus than rats tested during metestrus–diestrus. Midazolam systematically applied significantly increased the number of immediate punished

Table 1
Effects of midazolam systematically applied or infused into lateral septum nuclei during nonconflict periods

Group	Saline (intraperitoneal)	Saline intralateral septum	Midazolam (intraperitoneal)	Midazolam intralateral septum
Proestrus	350.9 ± 35.0	374.1 ± 32.5	380.2 ± 24.0	385.2 ± 31.0
Metestrus–diestrus	385.2 ± 42.5	380.3 ± 33.6	365.9 ± 45.5	375.2 ± 44.2

Table 2
Effects of midazolam systematically applied or infused into medial septum nuclei during nonconflict periods

Group	Saline (intraperitoneal)	Saline intramedial septum	Midazolam (intraperitoneal)	Midazolam intramedial septum
Proestrus	374.3 ± 28.2	360.5 ± 28.9	376.2 ± 32.5	360.2 ± 28.5
Metestrus–diestrus	380.5 ± 56.4	385.6 ± 25.8	374.9 ± 36.0	379.6 ± 45.1

reinforcers only during late proestrus ($P < .05$). Midazolam administered locally into the medial septum did not modify immediate punished behavior in any estrous phase (Fig. 4).

3.4. Midazolam infusions into the lateral septal area during unpunished periods

Table 1 shows that the total number of nonpunished lever presses did not vary significantly in any estrous phase (three-way ANOVA: estrous phases: $F(1,144) = 1.25$, n.s.; treatments: $F(3,144) = 0.54$, n.s.; Estrous phases × Treat-Treatments interaction: $F(3,144) = 2.00$, n.s.; septal nuclei interaction: n.s.). Midazolam did not elicit a significant modification in unpunished responding.

3.5. Midazolam infusions into medial septal area during unpunished periods

Table 2 shows that in control rats, the number of lever presses during nonpunished periods was similar between rats tested during late proestrus and rats tested during metestrus–diestrus. Systemic injection of midazolam or midazolam infusions into medial septal nucleus did not elicit a significant decrease in unpunished responding.

4. Discussion

Data showed an interaction between the endocrine state and midazolam infused into septal nuclei on fear-like responding in a conflict test. Results showed that: (1) control rats displayed a reduction in fear-like levels only during late proestrus; (2) a moderate anxiolytic-like effect of systemic midazolam occurred only during late proestrus; and (3) midazolam reduced fear-like behavior during late proestrus only when infused into the lateral septal area. Latter results replicated an earlier study (Pesold and Treit, 1996) of the anatomical specificity of anxiolytic effects of midazolam infusions into lateral septal nuclei, and showed an interaction between anxiolytic effects and the hormonal state of rats. Stimulation of the lateral septal area reduces fear-like behavior; similar results were produced by peripheral administration of BZDs (Yadin and Thomas, 1981). Lateral septal cells may act reducing fear-like behavior probably by modification of their firing rates when rats confront aversive stimuli, which has been observed during peripheral infusions of BZDs (Yadin and Thomas, 1981), and in rats tested during proestrus–estrus (Contreras et al., 2000). However, some contradictory results about the role of the lateral septal area in the inhibition of fear-like or in the mediation of the anxiolytic effects of BZDs (Melia and Davis, 1991) have been obtained. Moreover, septal lesions reduce fear-like behavior (Pesold and Treit, 1992; Treit and Pesold, 1990; Treit et al., 1993). Data obtained in present study are consistent with the anxiolytic-like role proposed to the lateral septal area, since rats receiving midazolam into lateral septal nuclei displayed anticonflict actions only during late proestrus, suggesting an important role of the lateral septum in mediating fear-like behavior of rats (Menard and Treit, 1999), whereas medial septum do not (Pesold and Treit, 1996). Considering that midazolam and other BZDs work fine in male rats, and comparing with previous studies (Hascoët et al., 1994) in which control male rats obtained a similar number of punished reinforcers than females tested during metestrus–diestrus in present study, we consider metestrus–diestrus to be more like male rats during punished periods. We also consider late proestrus to be a natural anxiolytic-like state, since anticonflict effects of midazolam and late proestrus were found in female rats during punished periods.

Control rats tested during late proestrus accepted more immediate punished reinforcers than rats tested during metestrus–diestrus. These results suggest that, during late proestrus, a possible reduction in fear-like levels may occur (Molina et al., in press). Late proestrus is characterized by high-circulating levels of progesterone (Freeman, 1988), and anxiolytic-like actions of progesterone have been proposed since 1940 and confirmed by several authors, either by exogenous hormone administration (Picazo and Fernández-Guasti, 1995) or by selecting endocrine phases characterized by high levels of this steroid, i.e., late proestrus (Bitran and Dowd, 1996). Consistent with this hypothesis, the exogenous administration of progestins results in an anxiolytic-like action (Bitran et al., 1995). Moreover, anxiolytic-like effects of progesterone are mediated by the neurosteroid allopregnanolone at brain GABA-A receptors (Bitran and Dowd, 1996). Progesterone and its main metabolites are potent positive allosteric modulators of GABA-A receptors. The activity of GABA-A receptors can be enhanced by a variety of structurally diverse agents that act allosterically via distinct binding sites at the receptor complex, i.e., BZDs (Costa and Guidotti, 1996) and neurosteroids (Lambert et al., 1995). BZDs (Costa and Guidotti, 1996) and neurosteroids (Twyman and MacDonald, 1992) can increase the opening frequency of Cl^- channels elicited by GABA.

During late proestrus, systemic injections of midazolam or midazolam infused into lateral septal nuclei increased the number of immediate punishment responses, suggesting that during late proestrus, rats could be more sensitive to actions of BZDs (Bitran and Dowd, 1996; Molina et al., in press). This finding could be explained by an interaction between steroid hormones, most likely neurosteroids and midazolam, since effects of compounds acting at the GABA–benzodiazepine complex vary according to estrous cycle phases (Fernández-Guasti and Picazo, 1990), and anxiolytic-like actions of BZDs are influenced by the endocrine stage (Fernández-Guasti and Picazo, 1997). Considering that progesterone (Steimer et al., 1997) and GABAergic (Gallagher et al., 1995) receptors exist in the lateral septal area, then, neurosteroids may participate in anticonflict actions of midazolam probably interacting at GABA-A receptors in brain (Bitran and Dowd, 1996).

Some operant tasks are sensitive to changes in gonadal hormones (Rodriguez et al., 1984), but the experimental task used in this study represents some advantages. In this task, during the unpunished periods, rats have to press a lever on a fixed ratio of 8 to obtain a reinforcer. After 3 min of testing, there is a change in lighting, and pressing of this lever changes from a fixed ratio of 8 to a fixed ratio of 1, but is now followed by an electric foot shock (punished periods). At the same time, another lever (nonpunished) is introduced in which reinforcers can be obtained on a fixed ratio of 8. Then, rats have the opportunity to choose between receiving an immediate (punished) or a delayed (nonpunished) reinforcement. During punished periods, control rats stop pressing the punished lever, and start pressing the unpunished lever. In contrast, rats receiving anxiolytic drugs continue pressing the punished lever. Pressing of this lever, which gives the reinforcer on a fixed ratio of 1 in combination with an electric foot shock, may be due to several causes: perseveration, differences in hunger, differential sensitivity to foot shocks (Leer et al., 1988), or diminished fear-like levels. Validation of our data was obtained by the marked increase in punished responses observed during late proestrus and after receiving midazolam (systemic or into lateral septal nuclei), and that responding during nonpunished periods (where only one lever is presented) was not affected by drug treatments or estrous phases, suggesting that food consumption and motor abilities were not factors in the increased rate of punished responding.

Metestrus and diestrus are characterized by an almost complete absence of ovarian steroid hormones, and they are associated with high levels of fear-like behavior and a low response to BZDs (Bitran and Dowd, 1996). Accordingly, in present study, rats receiving saline and tested during metestrus–diestrus accepted a low number of immediate punished reinforcers, and midazolam did not modify punished responding. These results support the hypothesis that a low sensitivity of the GABAergic pathway is associated with an absence of gonadal hormones (Rupprecht et al., 1996). In other study using the conflict task, only a high dose of diazepam increased the number of punished reinforcers during metestrus–diestrus (Molina et al., in press). However, in present study, only one dose of midazolam was used, allowing a very limited conclusion, and drawing conclusions of an absolute nature from a single dose of midazolam is unfounded. Thus, additional studies should be undertaken to further characterize the site-specific and endocrine phase-specific anticonflict effects of midazolam. In addition, a better test of the hypothesis that actions of systematically administered midazolam are derived from actions in the lateral septal area, and not the medial septum would come from experiments where the benzodiazepine antagonist Ro15-1788 could be administered locally into these regions to antagonize effects of midazolam (Pesold and Treit, 1996).

In conclusion, present study provides new evidence for the importance of lateral septal nuclei and the endocrine state in female rats, since midazolam infused into lateral septal nuclei produced anticonflict actions only during late proestrus. Conversely, midazolam applied directly into the medial septum did not modify anticonflict behavior.

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