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Testosterone reduces cumulative burying in female Wistar rats with minimal participation of estradiol

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ABSTRACT

Testosterone exerts anxiolytic effects, but the participation of its aromatase metabolic product estradiol is controversial. Therefore, we used the defensive burying paradigm in female Wistar rats to explore testosterone's (1.0 mg/rat, s.c.) interactions with picrotoxin (a noncompetitive γ-aminobutyric acid-A receptor [$GABA_A$] antagonist; 1.0 mg/kg, i.p.), formestane (an aromatase inhibitor; 3.0 mg/rat, s.c.), and tamoxifen (an estrogen receptor-β antagonist; 1.0 mg/kg, s.c.). Serum levels of testosterone, estradiol, and progesterone were determined in the same rats. Burying latency and locomotion did not significantly change. Systemic testosterone administration enhanced serum testosterone and estradiol levels and reduced defensive burying. This reduction in total burying was blocked by pretreatment with picrotoxin and tamoxifen, but not formestane. We conclude that testosterone produced anxiolytic-like effects in female rats that were mediated by actions at the $GABA_A$ receptor, with participation of the estradiol receptor-β, rather than estradiol aromatization.

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1. Introduction

Androgens modulate anxiety (Edinger and Frye, 2005), and testosterone produces some anxiolytic-like actions in male rats (Bing et al., 1998; Bitran et al., 1993; Gonzalez et al., 1994) and male mice (Domek et al., 1992) after either single (Aikey et al., 2002; Bing et al., 1998) or serial injections (Bitran et al., 1993; Boissy and Bouissou, 1994; Bouissou and Vandenheede, 1996; Frye and Seliga, 2001). Robust indicators of anxiety-like behavior are also observed in gonadectomized male rats in the defensive burying test (Fernández-Guasti and Martínez-Mota, 2003), open field test (Frye and Seliga, 2001), and elevated plus maze (Adler et al., 1999; Bitran et al., 1993; Edinger and Frye, 2004).

The reduced testosterone metabolites 5α-androstan-3α,17β-diol (3α-androstanediol) and 5α-androstan-3α-ol-17-one (androsterone) potentiate γ-aminobutyric acid (GABA) function in a manner similar to the reduced metabolites of progesterone (Gee et al., 1988; Turner et al., 1989). Testosterone acts on $GABA_A$ benzodiazepine receptors (Bitran et al., 1993). Picrotoxin and bicuculline (noncompetitive and competitive $GABA_A$ receptor antagonists, respectively) blocked the anxiolytic-like effects of testosterone in male house mice (*Mus musculus*) (Aikey et al., 2002). However, flutamide (an androgen receptor antagonist), but not flumazenil (a $GABA_A$ benzodiazepine receptor antagonist), reduced the anxiolytic-like effects of testosterone in male Wistar rats (Fernández-Guasti and Martínez-Mota, 2005).

Some actions of testosterone may be attributable to its conversion to estradiol, which also decreases anxiety and despair (Frye et al., 2000; Frye and Walf, 2002). Aromatase, the enzyme responsible for the conversion of testosterone to estradiol, is present in several cerebral nuclei (Roselli et al., 1998), specifically in regions where enzymatic activity is steroid hormone-dependent (Wagner and Morrell, 1997). Castration significantly reduced aromatase activity in these structures in adult rats (Roselli et al., 1998). However, systemic injection of dihydrotestosterone or 3α-androstanediol, which cannot be converted to estradiol, reduced anxiety-like behavior in the open field and elevated plus maze (Edinger and Frye, 2004, 2005). Additionally, some anti-anxiety-like effects of androgens involve the participation of estrogen receptor-β (ERβ) (Osborne et al., 2009). In mice, diarylpropionitrile, a selective estrogen receptor modulator specific to ERβ but not ERα, reduced anxiety-like behavior in the open field, elevated plus maze, elevated zero maze, and social interaction tasks (Walf et al., 2008, 2009), suggesting that the anxiolytic-like effects of testosterone are mediated by its metabolites through the ERβ receptor in male mice and rats (Frye et al., 2008).

The present study determined whether the anxiolytic-like actions of testosterone in the defensive burying test depend on its aromatization to estradiol or its actions on the $GABA_A$ receptor. We included

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female rats in this study because human females are more vulnerable than males to anxiety-related disorders (Cloitre et al., 2004; Kessler, 2003; Rapkin et al., 2006; Toufexis et al., 2006), and some clinical evidence indicates an anxiolytic action of testosterone in women (Hermans et al., 2006; Van Honk et al., 2005). The participation of the aromatization process was explored using the aromatase inhibitor formestane. We also explored the participation of ERβ using tamoxifen and the $GABA_A$ receptor using the noncompetitive antagonist picrotoxin. We also obtained serum levels of testosterone, estradiol, and progesterone using microparticle enzyme immunoassay (MEIA).

2. Materials and methods

2.1. Animals and housing

All animal procedures adhered to the general principles of laboratory animal care (National Research Council, 1985). We used 64 female Wistar rats aged 2 months, weighing 200–250 g. Animals were maintained in housing facilities in acrylic translucent boxes (45×30×30 cm), with groups of six animals per box, on a 12 h/12 h (lights on at 7:00 AM) light/dark cycle and *ad libitum* access to water and food. All experiments were conducted during the light period between 11:00 AM and 12:00 PM. We obtained authorization from the Biomedical Research Institute (UNAM, México) Ethical Committee.

2.2. Vaginal smears

Before initiating the behavioral tests, we obtained daily vaginal smears. Only those females with two continuous regular cycles (4–5 days) were included in the study. Vaginal samples were collected gently by inserting the tip of a medicine dropper into the vagina, flushing saline in and out, and placing the fluid onto microscope slides. Estrous cycle stage was determined immediately by optic microscopy (Nikon Japan Optiphot-2, 40× magnification) and classified according to Rhodes et al. (2002). Smears were classified by estimating the relative proportion of leukocytes, nucleated epithelial cells, and cornified epithelial cells as follows: diestrus (predominance of large numbers of leukocytes, some nucleated, almost no cornified cells), proestrus (primarily large, round, nucleated cells), estrus (many cornified cells), and metestrus (large numbers of leukocytes, some cornified cells, almost no nucleated cells). Because formestane (Wing et al., 1985) and tamoxifen (Misiewicz et al., 1996) induce diestrus, we selected rats in diestrus before testing in all other groups.

We selected the dose of testosterone based on previous reports that explored its anxiolytic-like effects. In castrated rats, a single dose of 1.0 mg testosterone per rat did not produce significant changes (Fernández-Guasti and Martínez-Mota, 2005). However, a single injection of 500 μg testosterone per rat (Aikey et al. 2002) produced anxiolytic-like effects in intact mice, we selected 1 mg/kg. The dose of picrotoxin was selected from a previous report (Aikey et al., 2002) in which 0.5 to 2.0 mg/kg blocked the anxiolytic-like effect of testosterone without motor changes. Tamoxifen is a nonselective, but effective, estrogen receptor antagonist that readily penetrates the blood-brain barrier (Walf and Frye, 2005b). At a dose of 1 mg/kg/day for 4 weeks, tamoxifen partially produced mammary tumor regression in rats (Zaccheo et al., 1993). We therefore used 1 mg/kg/day for 6 days. Formestane inactivates the aromatase enzyme complex through competitive irreversible binding to the enzyme (Miller, 2003). At a dose of 17.5 mg/kg twice per day for 4 days, formestane was shown to readily cross the blood-brain barrier and reduce brain aromatase activity in intact male rats (Yuan et al., 1995). We therefore used 3 mg/day for 6 days. Pretreatment with picrotoxin was administered once, and tamoxifen and formestane followed an impregnation schedule. Testosterone was administered 60 min before testing.

2.3. Experimental groups

All rats received two treatments by injection in a volume of 0.2 ml/rat. Injections contained testosterone (1.0 mg/rat, s.c.; donation from Schering Company, México), saline (0.9% NaCl, i.p.), corn oil (s.c.), picrotoxin (1.0 mg/kg, i.p.; Sigma, St. Louis, MO, USA), formestane (3.0 mg/rat, s.c.; Sigma), or tamoxifen (1.0 mg/kg, s.c.; Sigma). Picrotoxin was dissolved in saline (0.9%), and the vehicle for testosterone and tamoxifen was corn oil. We obtained a suspension of formestane using a 1:1 mixture of propylene glycol and corn oil.

Four independent groups of rats received two treatments in the following sequences: saline–oil ($n=10$), saline–testosterone ($n=10$), picrotoxin–oil ($n=10$), and picrotoxin–testosterone ($n=10$). These groups received pretreatments 90 min before the behavioral tests and treatments 60 min before the behavioral tests. Another four groups received pretreatments of six previous impregnation injections of formestane or tamoxifen: formestane–oil ($n=6$), formestane–testosterone ($n=6$), tamoxifen–oil ($n=6$), and tamoxifen–testosterone ($n=6$). In summary, pretreatments were administered according to two schedules: saline and picrotoxin were administered 90 min before testing, and formestane and tamoxifen followed an impregnation schedule for 6 days, with the final injection administered 90 min before testing. All treatments (i.e., testosterone or oil) were injected 60 min before testing.

2.4. Behavioral tests

2.4.1. Anxiety test: defensive burying behavior

For the defensive burying test, we used an acrylic box (27×17.5×15.5 cm) with a 5 cm bed of fine sawdust. From one of the walls of the box (17.7×15.5 cm), an electrode (7 cm length, 1 cm diameter) protruded horizontally 2 cm above the sawdust bed. The electrode delivered constant intensity current (0.3 mA, direct current) through an electronic stimulator (Grass Instruments S44, Quincy, MA, USA) coupled in series to a stimulus isolation unit (Grass Instruments SIU5) and a constant–current unit (Grass Instruments CCUIA). We selected 0.3 mA as the shock intensity based on its proven sensitivity to anxiolytics (Fernández-Guasti et al., 1999; Martínez-Mota et al., 2000; Picazo and Fernández-Guasti, 1995) and because higher intensities produce freezing behavior (De Boer and Koolhaas, 2003). Immediately after the placement of the animal in the box, we evaluated the latency and total time spent burying for 10 min. Once the animal received a shock, it typically moved toward the probe, sprayed urine, and pushed a pile of bedding material toward the probe with rapid alternating movements of its forepaws (Pinel and Treit, 1978). The time that elapsed between the first shock and the first attempt at burying (i.e., latency) inversely relates to the rat's reactivity. Similarly, the total time spent burying (i.e., cumulative burying) is an indicator of anxiety (Treit, 1985). Immediately after the defensive burying test, general ambulation was assessed.

2.4.2. Locomotor activity: open field test

The open field test is commonly used to exclude putative nonspecific drug actions in the defensive burying paradigm (Fernández-Guasti et al., 1992, 1999; López-Rubalcava and Fernández-Guasti, 1994). Consequently, to evaluate locomotion, we used an acrylic box (44×33 cm) with walls 20 cm high and the floor divided into 12 squares (11×11 cm each). Trained observers counted the number of squares that each rat completely crossed with all four paws in the open field box (i.e., crossings) during a 5 min videotaped test. No other behaviors, such as rearing or sniffing, were evaluated. We did not use any habituation session before the test. After each experimental session, the open field or defensive burying test boxes were carefully cleaned and deodorized with a cleaning solution (ammonia 0.5%, ethanol 15%, extran 10%, isopropyl alcohol 5%, pinol 19%, water 50.5%).

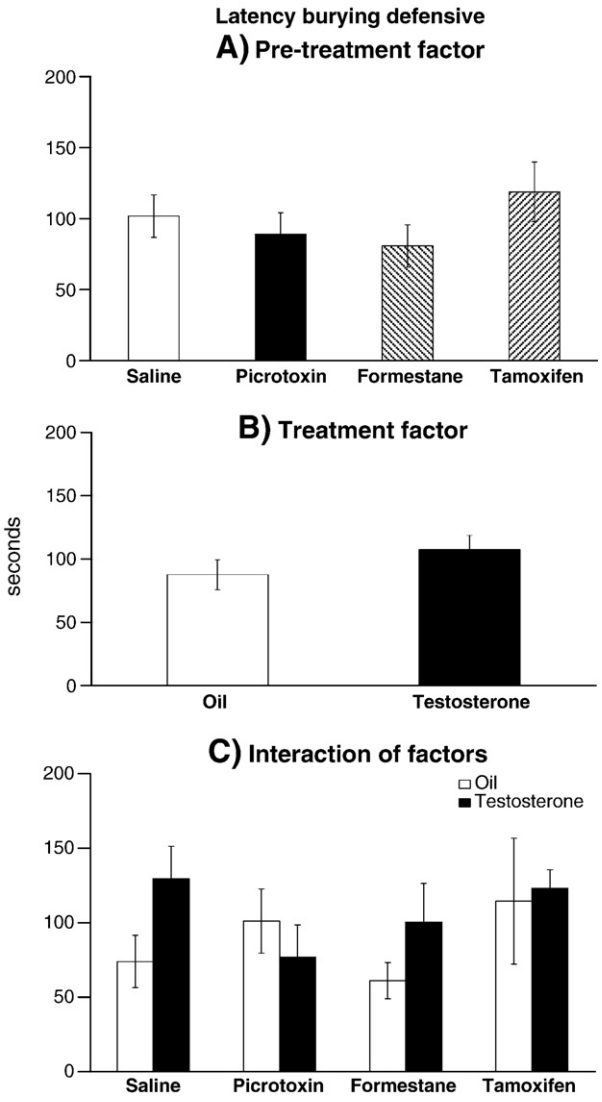

Fig. 1. Burying test. Burying latency was not significantly affected by (A) pretreatment ($F_{3,56}=0.838$, $p=0.478$) or (B) treatment ($F_{1,56}=1.407$, $p=0.240$). (C) No interaction of factors was observed ($F_{3,56}=0.21$, $p=0.64$).

2.5. Serum levels of testosterone, progesterone, and estradiol

2.5.1. Blood sampling and hormone assays

Immediately after the behavioral test, the rats underwent terminal anesthesia with pentobarbital sodium (1 ml/kg, i.p.; Anestesal®, Veterinary Use, Pfizer, Veracruz, México). A 3–4 ml blood sample was collected by intracardiac puncture into the right atrium, placed on ice in sterile Vacutainer® tubes (without anticoagulant), allowed to clot at room temperature, and centrifuged for 10 min at 3500 rpm to separate the serum. Serum was stored at −20 °C (to avoid protein denaturation) until hormone determinations were performed.

2.5.2. Hormone determination in serum

We used an automated MEIA immunoassay with an AxSYM® system (Abbott Laboratories, Abbott Park, IL, USA) in which serum samples were assayed by MEIA using commercially available kits (testosterone: 3C85-20, 1-8774; estradiol: 7A63, 34-3205/R6; progesterone: 7A64, 46-6866/R8; with a functional sensitivity of 0.05 mU/l). All samples were assayed in duplicate. The AxSYM method is a heterogeneous immunoassay in which the specimen binds to polyclonal antibodies that are linked to microparticles (Valdes and Jortani, 2002). The standard control and serum samples of investigated rats were incubated with antitestosterone-, antiestradiol-, or antiprogesterone-coated microparticles to form an antibody–antigen complex. Upon removal of unbound material, testosterone, progesterone, and estradiol–alkaline phosphatase conjugates were added and bound to available sites. After washing, 4-methyl-umbelliferyl phosphate substrate was added, and the fluorescent product was measured by the optical system, in which the intensity of the signal is inversely proportional to testosterone, progesterone, or estradiol concentrations in specimens. The sensitivity was calculated to be 2.0 U/ml, corresponding to the upper limit of the 95% confidence interval and representing the lowest measurable concentration of antigenic reactive determinant that can be distinguished from zero. However, with this procedure, the maximum detection of testosterone was fixed at a concentration 15 ng/ml.

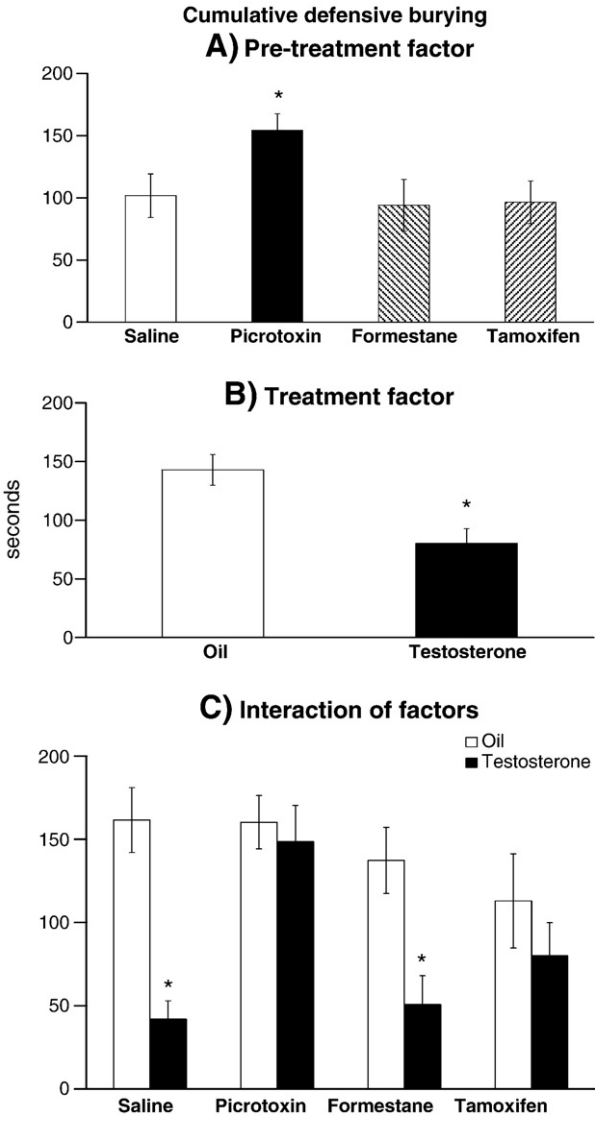

Fig. 2. Burying test. (A) Cumulative burying significantly changed in groups that received picrotoxin treatment compared with saline, formestane, and tamoxifen treatment ($F_{3,56}=4.54$, $p<0.006$; $^*p<0.05$ *vs.* control group). (B) Rats that received testosterone displayed significantly shorter cumulative burying time compared with rats that received oil ($F_{1,56}=18.26$, $p<0.0001$; $^*p<0.05$ *vs.* testosterone). (C) In the saline–testosterone and formestane–testosterone groups, cumulative burying was shorter ($^*p<0.05$) compared with the saline–oil, picrotoxin–testosterone, and tamoxifen–testosterone groups ($F_{3,56}=3.48$, $p<0.02$).

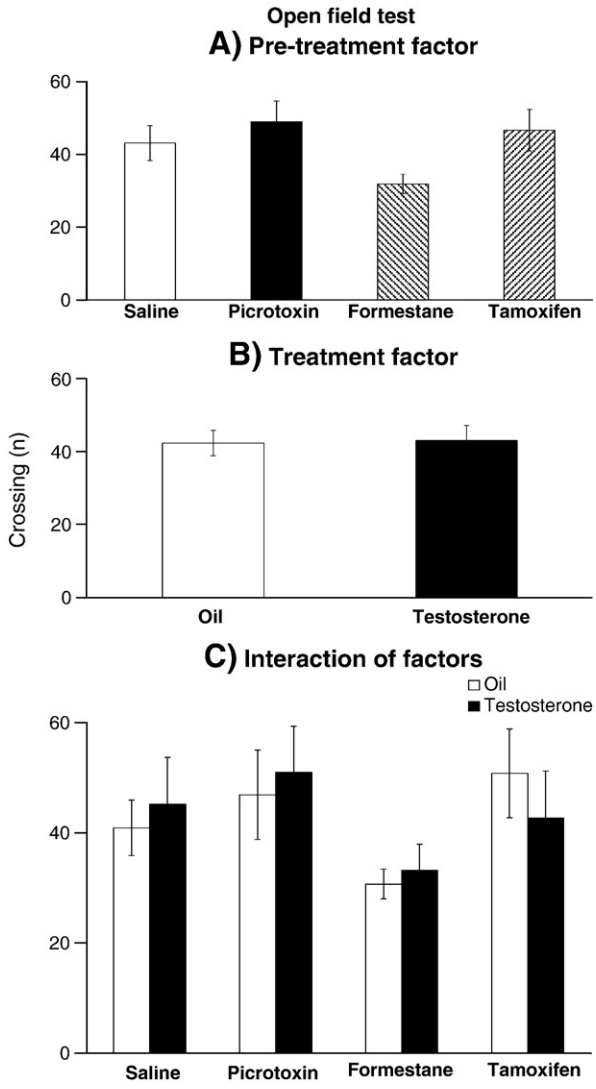

Fig. 3. Locomotor activity: open field test. Locomotor activity was not significantly affected by (A) pretreatment ($F_{3,56}=1.678$, $p=0.182$) or (B) treatment with testosterone ($F_{1,36}=0.015$, $p=0.902$). (C) No interaction of factors was observed ($F_{3,56}=0.256$, $p=0.856$).

2.6. Statistical analysis

Two-way analysis of variance (ANOVA) was used, with pretreatment (saline, picrotoxin, formestane, or tamoxifen) as one factor and treatment (oil or testosterone) as another factor. We included all groups in the ANOVA because although different impregnation procedures were used (first treatment), the second treatment was testosterone or oil. The alpha level for significance was set at $p<0.05$, in which case Student–Newman–Keuls *post hoc* test was applied. Results are expressed as mean and standard error of the mean.

3. Results

3.1. Behavioral tests

3.1.1. Burying latency

Burying latency was not significantly affected by pretreatment ($F_{3,56}=0.838$, $p=0.478$; two-way ANOVA) or treatment ($F_{1,56}=1.407$, $p=0.240$; Fig. 1A, B). No significant interaction of factors was observed ($F_{3,56}=1.395$, $p=0.253$; Fig. 1C).

3.1.2. Cumulative burying

As shown by the two-way ANOVA, the longest cumulative burying occurred in the groups that received picrotoxin ($F_{3,56}=4.54$, $p<0.006$; Fig. 2A). Rats treated with testosterone displayed a significantly ($p<0.05$) shorter time in cumulative burying compared with oil treatment ($F_{1,56}=18.26$, $p<0.001$; Fig. 2B).

Two-way ANOVA of the interaction of factors also reached significance ($F_{3,56}=3.48$, $p<0.02$), which allowed a detailed analysis of the effect of pretreatments on testosterone or oil treatments (Fig. 2C). As the *post hoc* analysis revealed, cumulative burying was shorter ($p<0.05$) in the saline–testosterone and formestane–testosterone groups compared with the saline–oil, picrotoxin–oil, picrotoxin–testosterone, formestane–oil, and tamoxifen–oil groups. In the tamoxifen–testosterone group, shorter cumulative burying was observed but did not reach statistical significance compared with the saline–oil group.

3.1.3. Open field test

In the open field test (Fig. 2A, B), square crossings were not significantly affected by pretreatment ($F_{3,56}=1.678$, $p=0.113$; two-way ANOVA) or treatment ($F_{1,56}=0.015$, $p=0.902$; two-way ANOVA). No significant interaction of factors was observed ($F_{3,56}=0.256$, $p=0.856$; Fig. 3C).

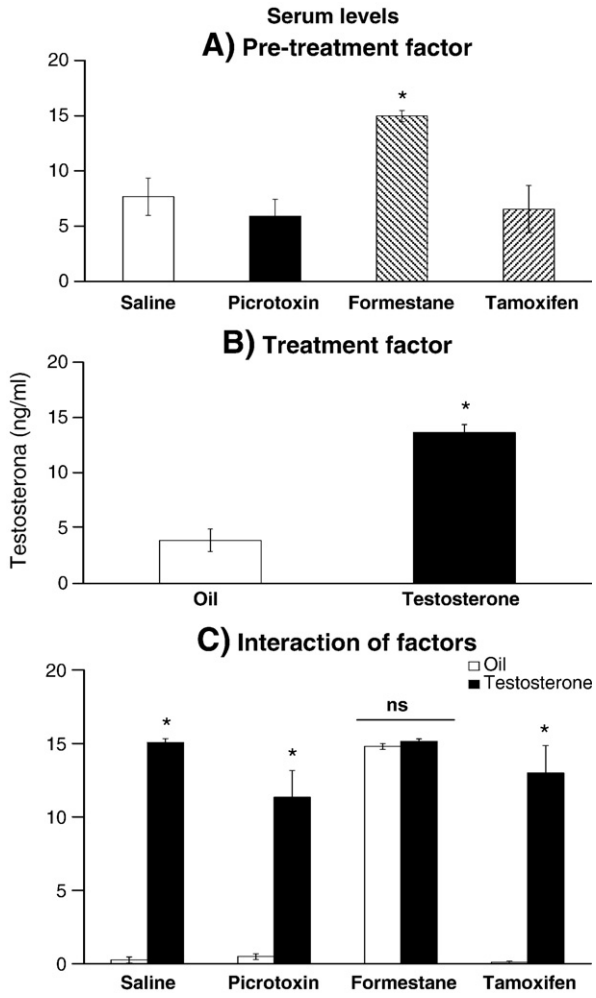

Fig. 4. Serum testosterone levels. (A) Formestane significantly increased serum testosterone levels ($F_{3,56}=31.4$, $p<0.0001$; $^*p<0.05$ *vs.* saline, picrotoxin, and tamoxifen groups). (B) Treatment with testosterone significantly increased serum testosterone levels ($F_{1,56}=192.7$, $p<0.0001$; $^*p<0.05$ *vs.* oil group). (C) All groups that received testosterone had the highest measurable serum testosterone levels compared with oil treatment ($p<0.05$), with the exception of the group that received pretreatment with formestane.

3.2. Serum levels of testosterone, estradiol, and progesterone

Two-way ANOVA revealed significant effects of pretreatment on serum testosterone levels ($F_{3,56}=31.4$, $p<0.0001$). Regardless of treatment with oil or testosterone, the group that received formestane reached the highest measurable serum testosterone levels ($p<0.05$; Fig. 4A). The treatment factor influenced serum testosterone levels, regardless of pretreatment ($F_{1,56}=192.70$, $p<0.0001$; Fig. 4B). The interaction of factors also reached significance ($F_{3,56}=19.0$, $p<0.0001$). All groups that received testosterone reached values near the highest measurable serum testosterone levels ($p<0.05$; Fig. 4C).

Two-way ANOVA revealed significant effects of pretreatment on serum estradiol levels ($F_{3,56}=10.02$, $p<0.0001$). The groups that received formestane or tamoxifen had the lowest serum estradiol levels ($p<0.05$) compared with the saline and picrotoxin groups (Fig. 5A). The treatment factor influenced serum estradiol levels regardless of pretreatment ($F_{1,56}=19.36$, $p<0.0001$; Fig. 5B). Treatment with testosterone increased serum estradiol levels in all groups ($p<0.05$). No significant interaction of factors was observed ($F_{3,56}=2.18$, $p=0.100$; Fig. 5C).

Two-way ANOVA revealed significant effects of pretreatment on serum progesterone levels ($F_{3,56}=8.09$, $p<0.0001$). Regardless of treatment with oil or testosterone, the group that received tamoxifen had the lowest serum progesterone levels ($p<0.05$) compared with the saline, picrotoxin, and formestane groups (Fig. 6A). Testosterone treatment did not affect serum progesterone levels ($F_{1,56}=1.87$, $p=0.177$; Fig. 6B). No significant interaction of factors was observed ($F_{3,56}=1.17$, $p=0.328$; Fig. 6C).

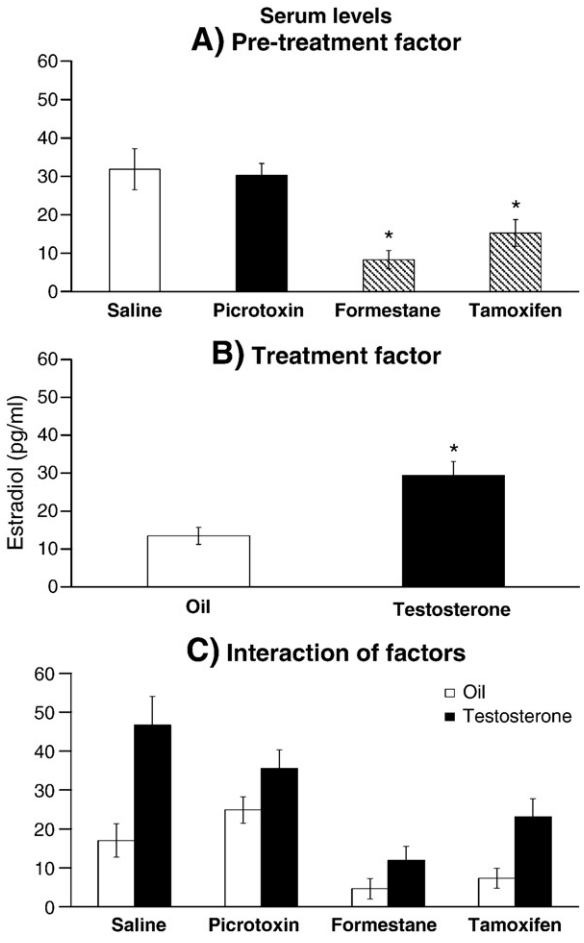

Fig. 5. Serum estradiol levels. (A) Pretreatment with formestane or tamoxifen significantly decreased serum estradiol levels ($F_{3,56}=10.02$, $p<0.0001$; *$p<0.05$ *vs.* saline and picrotoxin groups). (B) Treatment with testosterone significantly increased serum estradiol levels ($F_{1,56}=19.36$, $p<0.0001$; *$p<0.05$ *vs.* oil group). (C) No significant interaction of factors was observed ($F_{3,56}=2.18$, $p=0.100$).

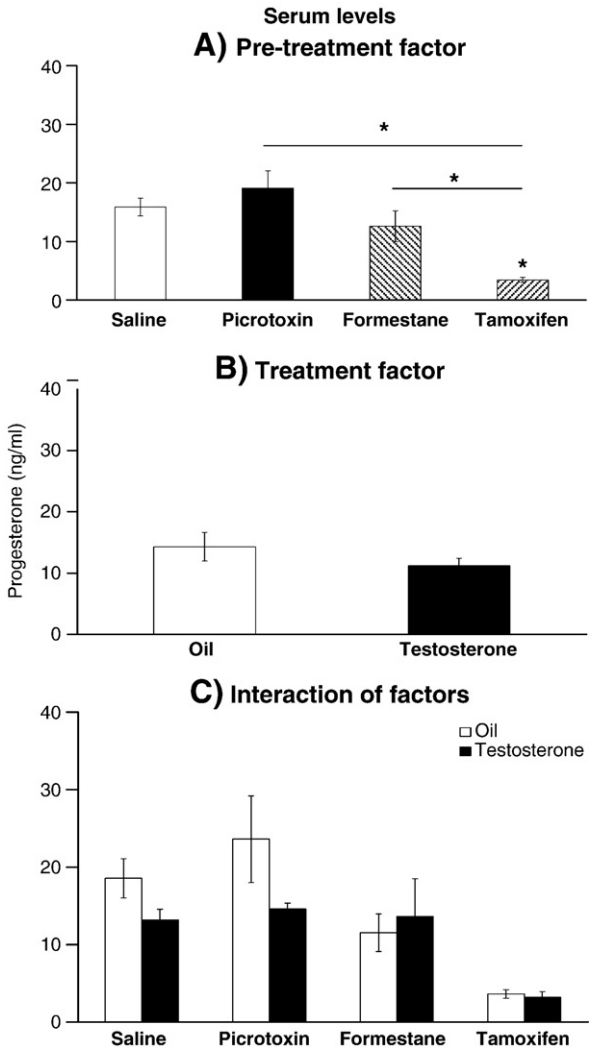

Fig. 6. Serum progesterone levels. (A) Pretreatment with tamoxifen significantly decreased serum progesterone levels ($F_{3,56}=8.09$, $p<0.0001$; *$p<0.05$ *vs.* saline, picrotoxin, and formestane groups). (B) Treatment with testosterone did not significantly modify serum progesterone levels compared with the oil group ($F_{1,56}=1.87$, $p=0.177$). (C) No significant interaction of factors was observed ($F_{3,56}=1.17$, $p=0.328$).

4. Discussion

The present study explored the participation of estradiol in the possible anxiolytic-like effects of testosterone, as well as the involvement of the $GABA_A$ receptor. Formestane did not reverse the effect of testosterone, discarding the possible participation of aromatization to estradiol. Tamoxifen reduced the effect of testosterone, and picrotoxin blocked the effects of testosterone, suggesting an anxiolytic-like effect of testosterone through $GABA_A$ receptors, with participation of ERβ.

Several studies have shown that testosterone and its metabolites reduce anxiety-like behavior in male and female rats. The anxiolytic-like effects of testosterone have been mainly studied in male rodents. Testosterone exerts anxiolytic-like effects by itself. The antiandrogen flutamide (Simard et al., 1986) was shown to block the anxiolytic-like effect of testosterone in orchidectomized male rats (Fernández-Guasti and Martínez-Mota, 2005). Repeated injections of testosterone in castrated male rats reduce anxiety-like behavior in the defensive burying test (Fernández-Guasti and Martínez-Mota, 2005), elevated plus maze (Bitran et al., 1993; Frye and Seliga, 2001), and Vogel

conflict test (Bing et al., 1998). Additionally, testosterone, dihydrotestosterone, and 3α-androstanediol (α-reduced neuroactive metabolites of testosterone) exert anxiolytic actions measured in the elevated plus maze in ovariectomized rats (Frye and Lacey, 2001) and in intact mice (Aikey et al., 2002). Consistent with these results, we found that testosterone reduced cumulative burying in the defensive burying test in non-ovariectomized female rats. This reduction of cumulative burying corresponds to an anxiolytic-like effect (Treit, 1985).

We included in our study female Wistar rats. Progesterone produces anxiolytic-like effects in the defensive burying test (Martínez-Mota et al., 2000; Picazo and Fernández-Guasti, 1995) and elevated plus maze (Bitran et al., 1991, 1993, 1995). This action is mediated by an increase in the number of $GABA_A$ receptors in rat brain (Maggi and Perez, 1984), positive endogenous modulation of $GABA_A$/benzodiazepine receptors (Majewska et al., 1986), and increased $GABA_A$ receptor sensitivity (Frye, 2001). In the present study, the $GABA_A$ receptor antagonist picrotoxin reduced the anxiolytic-like effect of testosterone in female rats, consistent with previous reports in male mice (Aikey et al., 2002). Furthermore, $GABA_A$ receptor sensitivity significantly increased in animals exposed to testosterone (Bitran et al., 1993; Gee et al., 1988; Turner et al., 1989).

In the present study, serum estradiol levels were higher in testosterone-treated groups than in vehicle groups, indicating testosterone aromatization (Patchev et al., 2004; Simpson, 2002, 2004). Tamoxifen is an antiestrogen with antagonist/agonist activity (Bellido et al., 2003). Tamoxifen exerts agonist properties on ERα and antagonistic properties on ERβ (Walf and Frye, 2005a; Watanabe et al., 1997). In fact, ERβ participates in the anxiolytic-like actions of testosterone and its 5α-reduced metabolites (Frye et al., 2008). In our study, tamoxifen reduced serum estradiol and serum progesterone content in physiological ranges (Freeman, 1988), consistent with previous reports (Donath and Nishino, 1998; Misiewicz et al., 1996). However, tamoxifen injections preceding testosterone treatment attenuated the reduction of cumulative burying produced by testosterone alone, suggesting that the androgen acts on ERβ. Administration of 5α-androstane,17β-diol-3α-diol, which has high affinity for ERβ, produced anxiolytic-like effects and enhanced cognitive performance, and androsterone, which binds to GABA/benzodiazepine receptors (Gee et al., 1988) but not ERβ, decreased anxiety-like behavior in the elevated plus maze but had no effects on cognition. Osborne et al. (2009) concluded that actions at ERβ are important for mediating androgenic effects on anxiety-like behavior and cognitive performance in male rats.

Formestane is an androgen analog that binds competitively and irreversibly to aromatase (Miller, 2003) and consequently reduces the synthesis of estrogens from testosterone (Dowsett, 1999), inclusively, in the brains of male rats (Yuan et al., 1995) by about 70% (Wing et al., 1985). However, formestane did not reverse the anxiolytic-like effects of testosterone. In groups that were not pretreated with formestane but received testosterone, serum estradiol levels increased significantly in the physiological range reported in female rats (Freeman, 1988). Serum testosterone clearly exceeded the physiological range (Ceccarelli et al., 2007) because testosterone is one of the main factors that increase plasma estradiol concentrations (Ceccarelli et al., 2006, 2007). However, after calibrating the procedures employed for measuring serum testosterone, we observed a ceiling effect.

Testosterone exerts its actions via both classical and non-classical signaling mechanisms (Walker and Cheng, 2005). One metabolite of testosterone, 5α-androstane-3α,17β-diol (3α-androstanediol), has high affinity for GABA/benzodiazepine receptors, leading to an anxiolytic action. However, 5α-androstane-3β,17β-diol (3β-androstanediol), which does not bind to GABA/benzodiazepine receptors, also decreases anxiety-like behavior (Frye et al., 2008). Consequently, a dual action of testosterone can be expected. The present study demonstrated that the anxiolytic action of testosterone was attenuated by picrotoxin via GABAergic membrane receptor blockade and by tamoxifen via ERβ nuclear receptor blockade (i.e., by reducing the influx of chloride into neurons; Aikey et al., 2002) or by decreasing the synthesis and affinity of the GABA receptor (Schumacher et al., 1989), respectively. At least one α-reduced metabolite of testosterone, 3α-androstanediol (a positive allosteric modulator of the $GABA_A$ receptor), influences GABAergic activity in both directions through modulation of mRNA expression of several $GABA_A$ receptor subunits (González-Flores et al., 2004; Zhang et al., 1999).

Commonly, studies of the anxiolytic-like effects of testosterone use male animals. The main finding of the present study was that androgens have an anxiolytic-like effect in females. This finding may be clinically relevant for women, particularly those receiving hormone-replacement therapy. A wide body of evidence indicates that some androgens possess anxiolytic activity in both humans and animals (Kessler, 2003). In women, administration of androgens decreased anxiety (Hermans et al., 2006; Sherwin, 1988). Testosterone replacement therapy resulted in significant improvement in symptomatology and quality of life in climacteric women (Sherwin and Gelfand, 1985). Furthermore, Panzer and Guay (2009) suggested that testosterone replacement therapy can be used safely in women, avoiding the risk of endometrial or breast cancer. Androgens diminish estrogen-induced breast epithelial proliferation and abolish estrogen-induced gene expression (Zhou et al., 2000), and testosterone exhibits growth-inhibitory and apoptotic effects in most breast cancer lines (Somboonporn and Davis, 2004). For this reason, we suggest that testosterone or its metabolites, in addition to traditional anxiolytic treatments, may be used to treat anxiety disorders in woman, particularly those at higher risk of breast cancer or those with a history of breast cancer.

We conclude that the reduction in cumulative burying in the present study illustrates the anxiolytic effects of testosterone or some of its reduced metabolites, rather that its aromatization to estradiol. The participation of estradiol appears to be minimal because (*i*) the main reduction of cumulative burying exerted by testosterone was observed in diestrus when serum estradiol and progesterone levels are low (Freeman, 1988), (*ii*) blocking the aromatization process of testosterone to estradiol with formestane did not modify the anxiolytic-like effect of testosterone, (*iii*) blocking the estradiol receptor with tamoxifen reduced the anxiolytic action of testosterone, and (*iv*) blocking the $GABA_A$ receptor with picrotoxin attenuated the effects of testosterone.

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