

## Anxiolytic-like Effect of Testosterone in Male Rats: GABA<sub>C</sub> Receptors Are Not Involved

\*<sup>1</sup>Ali Roohbakhsh, <sup>2</sup>Akbar Hajizadeh Moghaddam, <sup>3</sup>Karim Mahmoodi Delfan

### Abstract

#### Objective(s)

The effect of testosterone on anxiety-like behaviors has been the subject of some studies. There is evidence that testosterone modulates anxiety via GABA (gamma aminobutyric acid) and GABAergic system. The involvement of GABA<sub>C</sub> receptors in those effects of testosterone on anxiety-like behaviors of the rats was investigated in the present study.

#### Materials and Methods

A group of rats received subcutaneous injections of testosterone (5, 10 and 20 mg/kg). Two groups of rats received intracerebroventricular injections of either CACA (GABA<sub>C</sub> agonist, 0.125 µg/rat) or TPMPA (GABA<sub>C</sub> antagonist, 3 microg/rat) following administration of testosterone (5, 10 and 20 mg/kg). After the injections, the rats were submitted to the elevated plus-maze test of anxiety.

#### Results

The rats received testosterone alone, showed a decreased in anxiety-like behaviors ( $P < 0.01$ ). Administration of either CACA or TPMPA did not modify animals' behavior compared to the rats received testosterone alone.

#### Conclusion

The results of the present study showed that administration of testosterone induces anxiolytic-like behaviors in the rats and GABA<sub>C</sub> receptors possibly are not involved in the anxiolytic effect of testosterone.

**Keywords:** Anti-Anxiety Agents, GABA-C receptors, Testosterone, Infusions, Intraventricular

1-Physiology and Pharmacology Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

\* Corresponding author: Tel: +98-391-8226927; Fax: +98-391-8220072; email: aroohbakhsh@rums.ac.ir

2- Department of Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran

3- Department of Biology, Tarbiat Moallem University, Tehran, Iran

## Introduction

There are both basic and clinical reports showing that steroid hormones are involved in the modulation of anxiety. Indeed, women are 85% more likely to experience anxiety disorders than men are (1), suggesting that androgens may protect men against anxiety disorders. A wide range of studies have implicated androgens in the modulation of anxiety disorders. In animal studies, the anxiolytic-like actions of androgens either following acute (2-4) or long chronic (3-6) administration have been observed. Conversely, the anxiogenic effects of anabolic-androgenic steroids have also been documented (7, 8). Testosterone interacts with intracellular androgen receptors (9), suggesting that its anxiolytic activity could be mediated by this mechanism. Meanwhile, there is increasing evidence that certain steroids may alter neuronal excitability via interaction with cell membrane receptors (10). For example, it has been demonstrated that the reduced testosterone metabolites, androstenediol and androsterone, have little or no affinity for androgen receptors (11), but they are potent GABA<sub>A</sub> (gamma aminobutyric acid A) receptor agonists and have GABA-mediated functions (5). This finding is suggesting that testosterone possibly exerts its behavioral actions via conversion to these reduced metabolites and this may explain the mechanism by which GABA<sub>A</sub> receptor antagonists, picrotoxin and bicuculline, block the anxiolytic effect of testosterone (2). Therefore, GABAergic system may be a candidate for the mediation of many of the behavioral effects of anabolic androgenic steroids.

Gama aminobutyric acid is the main inhibitory neurotransmitter in the mammalian central nervous system. It exerts its effects through three distinct classes of membrane receptors: GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub> (12). GABA<sub>C</sub> receptors appear to be much simpler

than GABA<sub>A</sub> receptors and are more sensitive to GABA than GABA<sub>A</sub> receptors, but they have not been studied as extensively as GABA<sub>A</sub> receptors (13). They have been implicated in visual processing, regulation of sleep-waking rhythms, pain perception, memory, learning, regulation of hormones and neuroendocrine gastrointestinal secretion (14). Beside the GABA<sub>A</sub> receptors, GABA<sub>B</sub> receptors have also been considered in the modulation of some neuroendocrine effects of testosterone (15). The interaction of GABA<sub>C</sub> receptors with testosterone and its metabolites has not been studied well. There is little evidence that shows such interaction may exist. For example, along with GABA<sub>A</sub> and GABA<sub>B</sub> receptors, high density of GABA<sub>C</sub> receptors exists in male reproductive tissues and GABA<sub>C</sub> receptors facilitate rat sperm acrosome reaction (14). Moreover, testosterone can be aromatized to estradiol which has been reported as potent inhibitor of the GABA<sub>C</sub> receptors (16). On the basis of the above evidence, we evaluated the effect of testosterone on anxiety-like behaviors of the rats and the possible involvement of GABA<sub>C</sub> receptors in those effects of testosterone on anxiety-like behaviors.

## Materials and Methods

### Animals

Male Wistar rats from Pasteur institute, Iran, weighing 200-250 g were used in the present study. Animals were housed 5 per cage, in a room with a 12:12 hr light/dark cycle (lights on 07:00 hr) and controlled temperature (23±2 °C). Animals had access to food and water *ad libitum*. Each experimental group included eight animals. All experimental procedures were carried out according to a protocol approved by the local Animal Ethics Committee.

### Surgery

Rats were anesthetized intraperitoneally with ketamine hydrochloride (50 mg/kg) and xylazine

(4 mg/kg) and fixed in a stereotaxic frame. The stainless steel guide cannula (22-gauge) was implanted unilaterally in the right lateral cerebral ventricle region (coordinates: Anterior-posterior: -0.8 mm; Medial-lateral: +1.6 mm; Ventral: -3.5 mm) according to Paxinos and Watson (17). It was then fixed to the skull with acrylic dental cement.

### **Drugs**

The drugs used in the present study were testosterone propionate [Iran Hormone, Tehran, Iran], CACA (selective GABA<sub>C</sub> receptor agonist) and TPMPA (selective GABA<sub>C</sub> receptor antagonist) [Tocris, Bristol, UK]. Testosterone was dissolved in sesame oil. CACA and TPMPA were dissolved in sterile 0.9% saline.

### **Procedure**

Intracerebroventricular (i.c.v.) injections were performed by means of an internal cannula (27-gauge, Supa; Iran), terminating 1.5 mm below the tip of the guide cannula, connected by polyethylene tubing to a 2- $\mu$ l Hamilton syringe. Rats were hand-held as the experimenter inserted the injector. On each side, 2  $\mu$ l solution was infused over a 60 sec period. To allow diffusion of the solution and to reduce the possibility of reflux, the injector was left in place for an additional 60 sec.

### **Elevated plus-maze test of anxiety**

The elevated plus-maze (EPM) comprised 2 open arms (50 $\times$ 10 cm) and 2 enclosed arms (50 $\times$ 10 $\times$ 40 cm) extended from a common central platform (10 $\times$ 10 cm). The apparatus, constructed from wood, was elevated 50 cm above floor level. Testing was conducted in a quiet room between 9.00 a.m. and 13.00. At 5 days following surgery, rats were brought into the behavioral testing room and left undisturbed for at least 1 hr prior to testing. The rats were individually placed in the center of the maze facing an open arm and allowed 5

min of free exploration. After each test, the floor was cleaned with distilled water. Measures were the frequencies of total, open, and closed arm entries (arm entry= all 4 paws into an arm) and the time spent in open, closed, and central parts of the maze. The percentage of open arm entries (%OAE) and open arm time (%OAT) as the standard indices of anxiety-like behaviors were calculated (18). A significant decrease in the percentage of time in open arms and/or open arm entries was indicative of an increased level of anxiety. Total arm entries were measured as a relative pure index of locomotor activity (18).

### **Experiments**

#### *Experiment 1: The effect of testosterone on anxiety-like behaviors*

The animals received subcutaneous injections of vehicle (1 ml/kg) or one of the three doses of testosterone (5, 10 and 20 mg/kg). The test session was performed 24 hr after subcutaneous injections. %OAT, %OAE and locomotor activity were measured (Figure 1, A1, B1, C1).

#### *Experiment 2: The effects of GABA<sub>C</sub> receptor agonist and antagonist on the anxiolytic-like effect of testosterone*

Two groups of rats received subcutaneous injections of vehicle (1 ml/kg) or one of the three doses of testosterone (5, 10 and 20 mg/kg). After 24 hr and 5 min before the test, all rats received i.c.v. injections of either CACA (0.125  $\mu$ g/rat) or TPMPA (3  $\mu$ g/rat). After the second injections, the animals were exposed to the EPM test. (Figure 1, A2, B2, C2 & A3, B3, C3).

### **Verification of cannula placements**

After completion of the experimental sessions, rats received 2  $\mu$ l of methylene blue. Approximately 10 min after the injection, the animals were decapitated and their brains were removed, blocked and cut coronally through

cannula placements. Data from the rats with injection sites located outside the ventricle were not used in the analysis.

### Statistical analysis

One-way ANOVA was used for the comparison between the effects of different doses of drugs with vehicles. Two-way ANOVA was used for evaluation of interactions between drugs. Following a significant *F*-value, *post-hoc* analysis (Tukey-test) was performed for assessing specific group comparisons. Differences with  $P < 0.05$  between experimental groups at each point were considered statistically significant.

### Results

In the first experiment, testosterone at the doses of 10 and 20 mg/kg increased %OAT [Figure 1, A1;  $F_{(3,28)} = 17.4$ ,  $P < 0.001$ ]. It also increased %OAE at the dose of 10 mg/kg [Figure 1, B1;  $F_{(3,28)} = 4.63$ ,  $P < 0.01$ ]. Testosterone produced no significant change in the locomotor activity [Figure 1, C1;  $F_{(3,28)} = 1.12$ ,  $P > 0.05$ ]. This finding is suggesting an anxiolytic-like effect for testosterone.

In the second experiment (Figure 1, A2, B2, C2), the effects of CACA with testosterone on %OAT [ $F_{(3,56)} = 0.48$ ,  $P > 0.05$ ] and %OAE [ $F_{(3,56)} = 0.28$ ,  $P > 0.05$ ] were not significant when compared with the animals received testosterone alone. Moreover, i.c.v. injection of TPMPA with testosterone did not affect animal behavior when compared with the animals received testosterone alone; %OAT [ $F_{(3,56)} = 0.7$ ,  $P > 0.05$ ] and %OAE [ $F_{(3,56)} = 2.5$ ,  $P > 0.05$ ]; (Figure 1, A3, B3, C3). These findings are suggesting that GABA<sub>C</sub> receptors possibly are not involved in those effects of testosterone on anxiety-like indices.

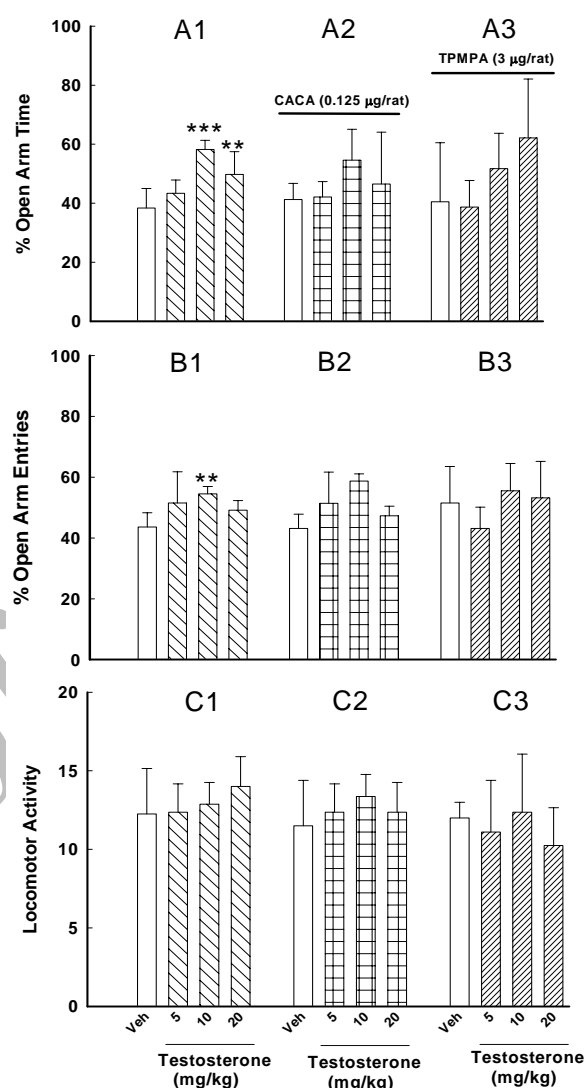


Figure 1. Effects of subcutaneous injections of testosterone alone or in the presence of CACA or TPMPA on anxiety-like behaviors. Rats received testosterone (5, 10 and 20 mg/kg, Figures A1, B1, C1) or intracerebroventricular injections of either CACA (0.125 µg/rat, Figures A2, B2, C2) or TPMPA (3 µg/rat, Figures A3, B3, C3) after subcutaneous injections of testosterone (5, 10 and 20 mg/kg). Each bar is mean±SD, n= 8. \*\*:  $P < 0.01$  and \*\*\*:  $P < 0.001$  when compared to the vehicle treated rats (1 ml/kg).

## Discussion

In the present study, administration of testosterone produced a clear anxiolytic-like action in the elevated plus-maze test. This finding is consistent with previous reports showing that a single systemic exposure to androgens induces anxiolytic-like effects in rodents (2, 3, 19). Decrease in anxiety-like behaviors and enhancement of the cognitive performance of aged male mice have also been reported following administration of androgens (20). In another study, intra-dorsomedial hypothalamus infusion of 17 $\alpha$ -methyltestosterone has shown anxiolytic-like behaviors in female rats (21). On the other hand, Edinger and Frye (22) showed that intrahippocampal infusion of flutamide, a testosterone receptor antagonist, produced anxiogenic-like behaviors in male rats. Meanwhile, female mice and rats spend less time on the open arms of the elevated plus-maze than do male mice and rats representing more anxious behaviors (23, 24). In line with these reports, an increase in vulnerability of women to anxiety disorders has been linked to lower level of endogenous testosterone compared to men. Indeed, the rise in illicit androgen use may also be related to androgens' anti-anxiety effects (25). Furthermore, it has been reported that in male rodents, removal of the primary source of endogenous androgens through gonadectomy increases anxiety-like behaviors in the open field and elevated plus-maze (4). In the same way, young hypogonadal men, with low endogenous testosterone levels, exhibit decreased performance in cognitive tasks and are more likely to be diagnosed with an anxiety or depressive disorder (26). In contrast, some studies reported that administration of androgens is anxiogenic (7, 8) or has no effect on anxiety-related behaviors in rodents (27). These inconsistent and contradictory results may be due to variation in species, sex and age of the subjects, as well as in environmental variables and the types and regimes of administered androgens.

Despite well-documented studies of the influence of testosterone on behavior, the mechanisms underlying the behavioral effects of this hormone and other steroid hormones need to be explored. Therefore, the GABAergic system has been proposed as an attractive candidate for mediating many of these effects. In 2002, Aikey *et al* (2) found that picrotoxin and bicuculline, as GABA<sub>A</sub> receptor antagonists, blocked the anxiolytic-like effect of testosterone. In a different study, Reddy and Jian (28) showed that androstenediol, a testosterone-derived metabolite, is an activator of GABA<sub>A</sub> receptors. By the way, there is evidence that anabolic androgenic steroids may also affect GABAergic system via GABA<sub>C</sub> receptors (see introduction). To test this hypothesis, we evaluated the effect of intracerebroventricular injections of a selective GABA<sub>C</sub> receptor agonist (CACA) and a selective GABA<sub>C</sub> receptor antagonist (TPMPA) on the anxiolytic action of the testosterone. Intracerebroventricular administrations were done because there is evidence that these drugs can not cross blood brain barrier (29). Recently, we reported that i.c.v. administration of CACA and TPMPA alone produced significant anxiogenic and anxiolytic-like effects in male rats respectively (30). The doses of CACA and TPMPA that we used in the present study are ineffective doses of these drugs on anxiety-like behaviors. However, to our knowledge this study is the first attempt to find an interaction between testosterone and GABA<sub>C</sub> receptors in an *in vivo* experiment, but the results of the present study failed to show such interaction. This means that GABA<sub>C</sub> receptors possibly are not mediating the anxiolytic effect of testosterone. GABA<sub>C</sub> receptors are a class of GABA receptors that have not been studied well. Especially, their possible role(s) in the brain has not evaluated well so far. However, their role in the regulation of sleep (31), memory (32) and anxiety (30) has been reported. The results of a recent study showed that estradiols, the product of aromatization of testosterone, are effective inhibitors of the GABA<sub>C</sub> receptors (16). This is

suggestive that other steroids may affect GABAergic transmission in the nervous system via GABA<sub>C</sub> receptors. This hypothesis needs to be explored in an *in vivo* study.

## Conclusion

The results of this study showed that testosterone produced a significant anxiolytic-

like effect and GABA<sub>C</sub> receptors possibly are not mediating this effect of testosterone.

## Acknowledgment

This study was part of a M.Sc. student thesis and was supported by Tarbiat Moallem University, Tehran, Iran.

## References

1. Women and anxiety disorders: implications for diagnosis and treatment. Proceedings of a conference. CNS Spectr 2004; 9:1-16.
2. Aikey JL, Nyby JG, Anmuth DM, James PJ. Testosterone rapidly reduces anxiety in male house mice (*Mus musculus*). Horm Behav 2002; 42:448-460.
3. Bing O, Heilig M, Kakoulidis P, Sundblad C, Wiklund L, Eriksson E. High doses of testosterone increase anticonflict behaviour in rat. Eur Neuropsychopharmacol 1998; 8:321-323.
4. Frye CA, Edinger KL. Testosterone's metabolism in the hippocampus may mediate its anti-anxiety effects in male rats. Pharmacol Biochem Behav 2004; 78:473-481.
5. Bitran D, Kellogg CK, Hilvers RJ. Treatment with an anabolic-androgenic steroid affects anxiety-related behavior and alters the sensitivity of cortical GABAA receptors in the rat. Horm Behav 1993; 27:568-583.
6. Fernández-Guasti A, Martínez-Mota L. Orchidectomy sensitizes male rats to the action of diazepam on burying behavior latency: role of testosterone. Pharmacol Biochem Behav 2003; 75:473-479.
7. Rocha VM, Calil CM, Ferreira R, Moura MJ, Marcondes FK. Influence of anabolic steroid on anxiety levels in sedentary male rats. Stress 2007; 10:326-331.
8. Ambar G, Chiavegatto S. Anabolic-androgenic steroid treatment induces behavioral disinhibition and down regulation of serotonin receptor messenger RNA in the prefrontal cortex and amygdala of male mice. Genes Brain Behav 2009; 8:161-173.
9. Cunningham GR, Tindall DJ, Means AR. Differences in steroid specificity for rat androgen binding protein and the cytoplasmic receptor. Steroids 1979; 33:261-276.
10. Rupprecht R. The neuropsychopharmacological potential of neuroactive steroids. J Psychiatr Res 1997; 31:297-314.
11. Roselli CE, Horton LE, Resko JA. Time-course and steroid specificity of aromatase induction in rat hypothalamus-preoptic area. Biol Reprod 1987; 37:628-633.
12. Bormann J. The 'ABC' of GABA receptors. Trends Pharmacol Sci 2000; 21:16-19.
13. Chebib M, Johnston GA. GABA-Activated ligand gated ion channels: medicinal chemistry and molecular biology. J Med Chem 2000; 43:1427-1447.
14. Li S, Zhang Y, Liu H, Yan Y, Li Y. Identification and expression of GABAC receptor in rat testis and spermatozoa. Acta Biochim Biophys Sin (Shanghai) 2008; 40:761-767.
15. Amikishieva AV. Testosterone and behavior: involvement of the hormone in psychotropic effects of baclofen. Bull Exp Biol Med 2007; 143:259-263.
16. Li W, Jin X, Covey DF, Steinbach JH. Neuroactive steroids and human recombinant rho1 GABAC receptors. J Pharmacol Exp Ther 2007; 323:236-247.
17. Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 4th ed. Academic Press: San Diego; 1998.
18. Rodgers RJ, Johnson NJT. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol Biochem Behav 1995; 52:297-303.
19. Agren G, Thiblin I, Tirassa P, Lundeberg T, Stenfors C. Behavioural anxiolytic effects of low-dose anabolic androgenic steroid treatment in rats. Physiol Behav 1999; 66:503-509.
20. Frye CA, Edinger K, Sumida K. Androgen administration to aged male mice increases anti-anxiety behavior and enhances cognitive performance. Neuropsychopharmacology 2008; 33:1049-1061.
21. Rivera-Arce JC, Morales-Crespo L, Vargas-Pinto N, Velázquez KT, Jorge JC. Central effects of the anabolic steroid 17alpha methyltestosterone in female anxiety. Pharmacol Biochem Behav 2006; 84:275-281.
22. Edinger KL, Frye CA. Intrahippocampal administration of an androgen receptor antagonist, flutamide, can increase anxiety-like behavior in intact and DHT-replaced male rats. Horm Behav 2006; 50:216-222.

23. Frick KM, Burlingame LA, Arters JA, Berger-Sweeney J. Reference memory, anxiety and estrous cyclicity in C57BL/6NIA mice are affected by age and sex. *Neuroscience* 2000; 95:293-307.
24. Johnston AL, File SE. Sex differences in animal tests of anxiety. *Physiol Behav* 1991; 49:245-250.
25. Hameed A, Brothwood T, Bouloux P. Delivery of testosterone replacement therapy. *Curr Opin Investig Drugs* 2003; 4:1213-1219.
26. Howell S, Shalet S. Testosterone deficiency and replacement. *Horm Res* 2001; 1:86-92.
27. Barreto-Estrada JL, Barreto J, Fortis-Santiago Y, Rivera-Ramos I, Fortis-Santiago A, Jorge JC. Modulation of affect after chronic exposure to the anabolic steroid 17alpha-methyltestosterone in adult mice. *Behav Neurosci* 2004; 118:1071-1079.
28. Reddy DS, Jian K. The testosterone-derived neurosteroid androstenediol is a positive allosteric modulator of GABAA receptors. *J Pharmacol Exp Ther* 2010; 334:1031-1041.
29. Chebib M, Hinton T, Schmid KL, Brinkworth D, Qian H, Matos S, *et al.* Novel, potent, and selective GABAC antagonists inhibit myopia development and facilitate learning and memory. *J Pharmacol Exp Ther* 2009; 328:448-457.
30. Roohbakhsh A, Mahmoodi Delfan K, Rostami P, Hajizadeh Moghaddam A. The Effect of Intracerebroventricular Injection of GABAC Selective Agonist and Antagonist on Anxiety-like Behaviors in Male Rats. *J Rafsanjan Unive Med Sci* 2008; 7:13-20.
31. Arnaud C, Gauthier P, Gottesmann C. Study of a GABAC receptor antagonist on sleep-waking behavior in rats. *Psychopharmacology (Berl)* 2001; 154:415-419.
32. Chebib M, Hinton T, Schmid KL, Brinkworth D, Qian H, Matos S, *et al.* Novel, potent, and selective GABAC antagonists inhibit myopia development and facilitate learning and memory. *J Pharmacol Exp Ther* 2009; 328:448-457.