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Pharmacology, Biochemistry and Behavior 84 (2006) 275–281
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Central effects of the anabolic steroid 17α methyltestosterone in female anxiety

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Received 1 February 2006; received in revised form 26 April 2006; accepted 13 May 2006
Available online 30 June 2006

Abstract

The androgen 17α-methyltestosterone (17α-meT) is one of the most commonly abused anabolic androgenic steroids (AAS). We assessed the impact of 17α-meT after bilateral infusion into the dorsomedial hypothalamus (DMH) in female anxiety. A paradoxical effect in Vogel conflict test (VCT) behavior was noted: while AAS infusion induced an increase in the latency to display the appetitive reaction of the task, it also increased the number of punished responses. No changes in elevated plus maze (EPM) behavior were noted. However, AAS infusion induced an increase in social interactions. Changes in social interactions were mimicked by muscimol infusion and counteracted by co-infusion of AAS plus the $GABA_A$ receptor ($GABA_A$-R) antagonist GABAzine. A reduction of systolic blood pressure was registered after AAS infusion in the DMH. No changes in fluid intake or locomotor behaviors were noted. We conclude that the AAS 17α-meT modulates distinct anxiety domains in females through a fast-acting mechanism.

Keywords: Anabolic steroids; Anxiety; Dorsomedial hypothalamus; Vogel conflict test; Elevated plus maze; Social interaction test; Systolic blood pressure

1. Introduction

In order to exert a regulatory role over androgen-like compounds, USA Congress enacted the Anti Drug Abuse Act of 1988 and the Anabolic Steroids Control Act of 1990, which resulted in the classification of these compounds as Schedule III substances. Anabolic androgenic steroids (AAS) were originally designed for legitimate medical use. However, these compounds are becoming drugs of abuse at an alarming rate. Unlike other drugs, AAS are popular among teenagers who seek to enhance their athletic performance, body image, and/or popularity among peers (Johnson, 1990). AAS misuse leads to a number of clinical side effects including heart disease, liver dysfunction, and infertility (Haupt and Rovere, 1984; Narducci et al., 1990). In addition, disruption of affective components of behavior is evident upon AAS misuse including aggressive bouts, mood swings, hostility, dependency, acute psychosis, manic and/or depressive episodes, and anxiety (Johnson, 1990; Pope and Katz, 1988; Franke and Berendok, 1997; Schwerin et al., 1996). These changes in effect among AAS users can have lethal consequences (Thiblin et al., 2000).

It has been reported that anxiety disorders are more frequent in women than men (for reviews, see Cameron and Hill, 1989; Genazzani et al., 1999; Pigott, 1999; Seeman, 1999). However, the majority of basic research has focused on male rodents. Although a growing body of work has shown that there are sex-specific differences in anxiety-related behaviors, there is no consensus as to the net effects of gonadal hormones in female anxiety (for a review, see Palanza, 2001). With regard to androgens, it has been shown that the testosterone metabolite 5α-androstane-3α, 17β-diol (3αDIOL) and the AAS 17a-meT is anxiolytic in the female rodent (Frye and Lacey, 2001; Barreto-Estrada et al., 2004). More recently, we have shown that systemic or central infusion of 3αDIOL has minimal or no effect in female anxiety (Jorge et al., 2005; Pérez-Acevedo et al., in

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doi:10.1016/j.pbb.2006.05.009

press). Therefore, it is of crucial importance to determine the central effects of structurally different androgens in anxiety-related behaviors among females.

We have recently proposed that chronic exposure to the AAS 17α-meT may alter risk-assessment behaviors in male mice (Rojas et al., 2006). In addition, we have also noted that chronic exposure to 17α-meT modulates social behaviors and Vogel conflict test behavior in female mice (Barreto-Estrada et al., 2004). The purpose of this study was to determine the central effects of AAS in VCT, elevated plus maze, social interactions, and systolic blood pressure in the female rat. Whereas the Vogel conflict test is a conditioned response model with a punishment component that requires learning, the elevated plus maze and the social interaction test are unconditioned response models with no punishment component that do not require learning (Panksepp, 1998; Crawley, 2000). Furthermore, we also measured the physiologic responses of systolic blood pressure associated with anxiety responses (Shekhar, 1993). Therefore, an assessment of distinct anxiety domains after AAS exposure is possible by contrasting the behavioral output in these tasks.

Although the dorsomedial hypothalamus (DMH) has been classically implicated in autonomic functions including ingestive behavior, body weight regulation, cardiovascular regulation, and response to stress (for review, see Bellinger and Bernardis, 1998), the DMH is also involved in behavioral responses associated with anxiety. In fact, a series of studies in humans (Sano et al., 1970), primates (Smith 1980), and rodents (Shekhar, 1993) have shown that the DMH plays an important role modulating anxiety-related behaviors. For instance, it has been shown that lesion of the DMH causes anxiolytic effects (Inglefield et al., 1994), exposure to anxiety tests induces c-fos expression in this region (Beckett et al., 1997), and benzodiazepine infusion into this region produces anxiolytic effects (Jardim and Guimarães, 2001). Infusion of a $GABA_A$-R antagonist into the DMH increases heart rate, respiratory rate, blood pressure, ACTH and corticosterone levels, and those effects are characterized as physiological responses associated with anxiety and stress (Keim and Shekhar, 1996; Shekhar et al. 1993; Soltis and DiMicco, 1991). In addition, increases in anxiety-related behavior were shown in elevated plus maze and social interactions after infusion of $GABA_A$-R antagonists (Shekhar, 1993; Shekhar and Katner, 1995). In contrast, $GABA_A$-R agonists produces anxiolytic effects in elevated plus maze and social interactions (Shekhar, 1993; Shekhar and Katner, 1995). Therefore, we chose to study the effects of 17α-meT in female anxiety-related behaviors through the DMH.

2. Materials and methods

2.1. Subjects

Adult (postnatal (PN) > 70) female Sprague–Dawley rats (n = 91) were purchased from Harlan Farms and housed individually with food and water available ad libitum. Rats were kept in a 12:12 h light/dark reverse cycle, in a humidity and temperature control room. All experiments were performed following the guidelines and approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico–Medical Sciences Campus and adhered to USDA, National Institutes of Health, and the American Veterinary Medical Association regulations.

2.2. Stereotaxic surgery and infusions

Rats were anesthetized with a cocktail of ketamine and xylazine (80:10 mg/kg; i.p.) and placed in the stereotaxic apparatus (Kopf Intruments). Microinjection guided cannulas were implanted bilaterally in the dorsomedial hypothalamus (DMH). The coordinates for DMH were anteroposterior (AP) −2.80; mediolateral (ML) ±0.5; and dorsoventral (DV) −6.1 according to Paxinos and Watson (1998). Seven days were allowed for recovery before behavioral testing. Microinjections were made with stainless steel injector cannula, extending 2.0 mm beyond the ventral tip of the guide, and attached to a syringe pump (Harvard Apparatus), via polyethylene tubing (0.28 mm ID). Animals were bilaterally infused with 1 μM vehicle (0.9% NaCl), pH = 7.4, 17α-methyltestosterone, 17α-methyltestosterone + GABAzine, or muscimol at a volume of 0.5 μl/side for 60 s. After infusion, injectors remained in place for an additional 60 s to allow drug diffusion. Except for the sucrose preference test, animals were placed in a given testing arena for behavioral testing 5 min after the infusion.

2.3. Behavioral testing

Behavioral testing occurred during the dark portion of the light/dark cycle. Anxiety-related behaviors were measured according to the modified Vogel conflict test (VCT), the elevated plus maze (EPM) test, and the social interaction test (SIT). Locomotor behaviors were measured with automated activity chambers. Except for the SIT, behavioral testing occurred under dim red light illumination (20 W). Several subjects were lost during behavioral testing due to cannula occlusion or appearance of neurological signs (n = 12). In all cases, the testing arena was cleaned with a mild detergent between sessions to minimize olfactory cues. The sucrose preference test (SPT) was done in the home cages at the end of behavioral testing with a group of females selected randomly. One batch of animals was tested in the following order: elevated plus maze, activity chambers, and Vogel conflict test. A second batch of animals was tested in the following order: social interaction test, sucrose preference test, and systolic blood pressure. Behavioral testing was limited to one task per day. The pharmacological studies assessing the role of $GABA_A$ receptors were conducted after the behavioral effects of 17α-meT were established in order to minimize animal use according to IACUC guidelines.

2.4. Vogel conflict test

Plexiglas testing chambers (18.42 cm length × 23.5 cm height × 22.23 cm width) were equipped with a grid floor of stainless steel bars and a drinking bottle containing filtered water (AccuScan Instruments, Columbus, OH). Animals were habituated to the testing arena for 20 min followed by 24 h of water deprivation. Individual animals were placed in the recording chambers for 20 min. Testing protocol included a "warm-up"

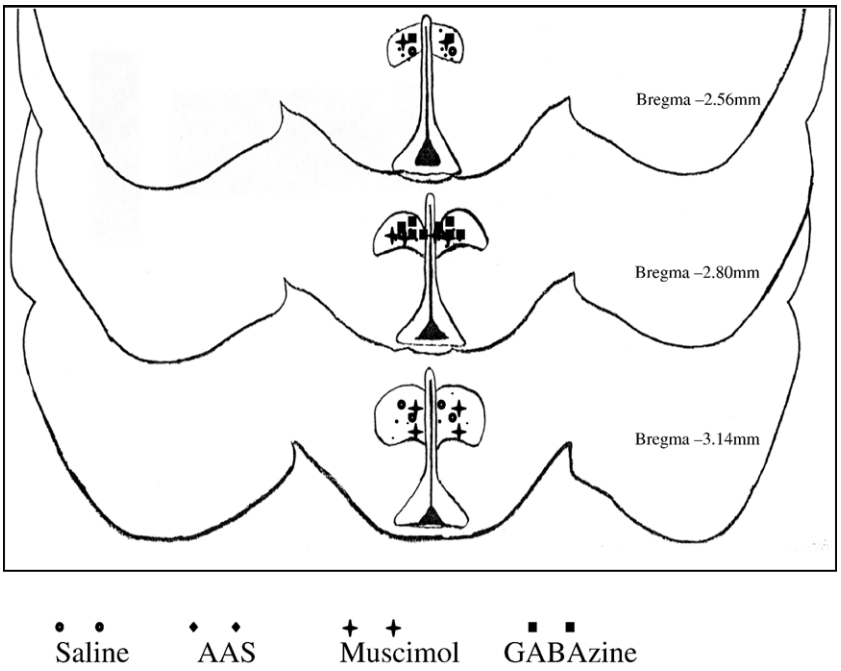

Fig. 1. Schematic representation of injector tip location within the dorsomedial hypothalamus (DMH). Representative injector tip locations within the DMH according to treatment infusions (saline, 17α-meT, musicimol, 17α-meT+GABAzine) are shown. Coordinates from bregma are shown. (Adapted from Paxinos and Watson, 1998.)

period (30 s), followed by alternated "free" and "punishment" periods (20 s each). During the punishment period, animals received a small shock to the paws every three licks (500 μA/10 ms). The following parameters were automatically recorded: number of licks, number of shocks, latency to first lick (seconds), and recovery time (seconds) after the first shock.

2.5. Elevated plus maze (EPM)

The maze consisted of two open arms and two closed arms (10×50 cm/arm) perpendicular to each other, and elevated 50 cm from the floor (AccuScan Instruments, Columbus, OH). The animal was placed in the center of the plus-maze and behavior was recorded for 5 min. The following variables were recorded automatically: total number of entries into open and closed arms and total time spent (seconds) in open and closed arms. Values are expressed as percent (%) number of entries in the open arms of maze according to number of entries into open arms/(number of entries into open arms+closed arms)×100. The same formula was used for % time in open arms.

2.6. Social interaction test (SIT)

Treatment-matched animals were paired in a novel circular testing arena (98 cm in diameter and 23 cm wall height) and were allowed to interact freely for a 5 min period in a brightly lit room. Non-aggressive physical direct contact among the pair (touching, body and anogenital sniffing, crawling over and under the partner, following, and grooming to partner) was registered as social interaction time (in seconds). Each experimental session was videotaped for further analysis.

2.7. Activity chambers

Locomotor and rearing activity were recorded by automated activity monitors for 20 min (AccuScan Instruments, Columbus,

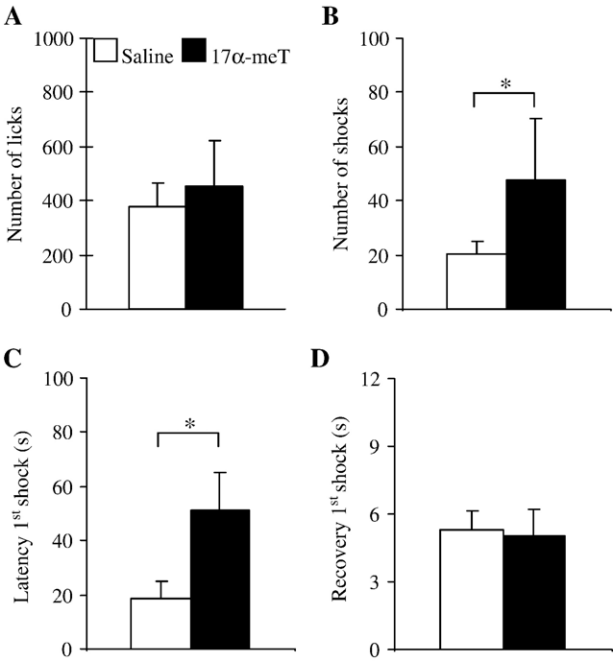

Fig. 2. Central infusion of 17α-meT modulates Vogel conflict test behavior. Infusion of 17α-meT into the DMH increases the number of shocks (panel B) and the latency to the first shock (panel C). No significant effects were noted for the number of licks (panel A) or the recovery time after the first shock (panel D). Saline (n=20), 17α-meT (n=10), *p<0.05.

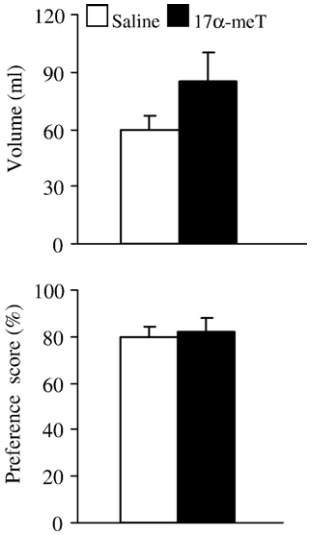

Fig. 3. DMH cannulation does not alter water consumption or sucrose preference. Prior history of saline or 17α-meT infusion into the DMH had no long-term consequences in water consumption (A) or the preference for sweetened water (B). Saline ($n=6$), 17α-meT ($n=6$).

OH). Clear acrylic chambers (42 cm length × 42 cm width × 30 cm height) consisted of 16 horizontal and vertical beams/side. Data was acquired 'on line' with line IBM/pc for further statistical analyses.

2.8. Sucrose preference test

Females were allowed to drink from two bottles: one containing filtered water and the other a 1% sucrose solution. Both bottles were available *ad libitum* for 3 days. Water consumption was registered every 24 h. Sucrose preference score was determined by: % Preference = (sucrose intake/total intake) × 100.

2.9. Systolic blood pressure

Systolic blood pressure (SBP) was determined by placing a pressure cuff on the tail. The cuff was inflated to a pressure of 250 mm Hg. SBP was then recorded near the pressure cuff with a piezoelectric sensor, which was connected to a microcomputer system (RTBP-2000, Kent Scientific, Litchfield, CT). The data were recorded and analyzed using the LabView program. With this setup, seven SBP measurements separated by 3 min intervals were taken for each animal.

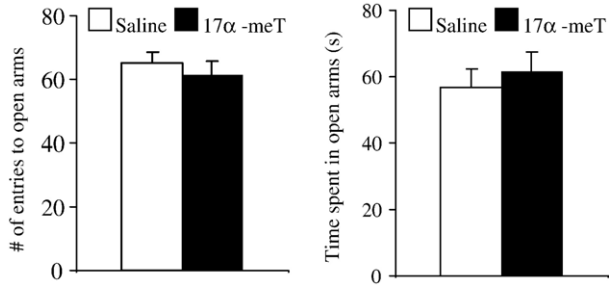

Fig. 4. Central infusion of 17α-meT does not impact elevated plus maze behavior. Acute infusion of 17α-meT had no effect in the number of entries (A) or the time spent (B) in the open arms of the elevated plus maze. Saline ($n=22$), 17α-meT ($n=20$).

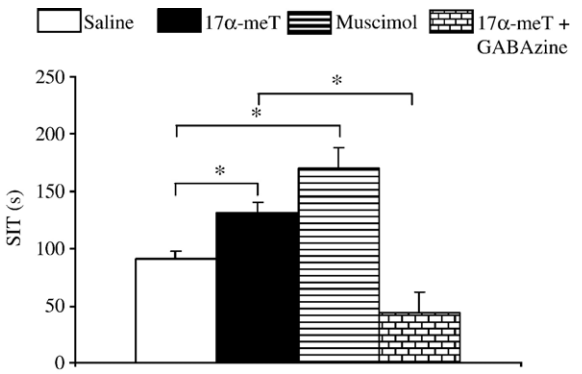

Fig. 5. Effects of 17α-meT, muscimol, or 17α-meT plus GABAzine infusion in social interactions. Infusion of 17α-meT (1 μM) or muscimol (1 iM) increases social interaction time (in sec) when compared to saline infusion. However, co-infusion of 17α-meT (1 μM) plus GABAzine (1 mM) does not increase social interactions. Saline ($n=5$ pairs), 17α-meT ($n=6$ pairs), muscimol ($n=3$ pairs), 17α-meT plus GABAzine ($n=5$ pairs), $^{*}p<0.05$.

2.10. Statistics

Data are expressed as mean ± S.E.M. Statistical analysis was performed by using one-way ANOVA followed by Student–Newman–Keuls or paired Student's *t*-test whenever appropriate. Statistical significance was established at $p<0.05$.

3. Results

Sprague–Dawley female rats received bilateral cannula targeting the dorsomedial hypothalamus (DMH). Fig. 1 shows the his2tological verification for cannula tip location corresponding to individual subjects according to treatment group. No significant changes in body weight were registered during the duration of this study (data not shown). Females were tested in the modified Vogel conflict test (VCT) after receiving a bilateral infusion of a 0.5 μl infusion/side of either saline or the anabolic androgenic steroid (AAS) 17α-methyltestosterone (17αmeT, 1 μM) in the dorsomedial hypothalamus (DMH). Four independent measures

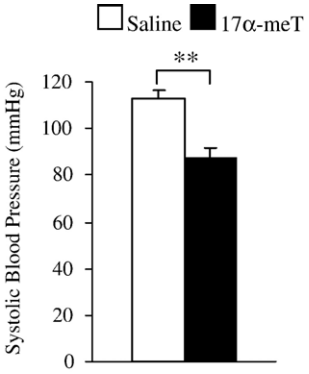

Fig. 6. Central infusion of 17α-meT infusion reduces systolic blood pressure. Infusion of 17α-meT into the DMH decrease systolic blood pressure when compared to vehicle infusion. Saline ($n=11$), 17α-meT ($n=6$), $^{**}p<0.001$.

were registered automatically in this task: number of licks, number of shocks, the latency to the first lick (seconds), and the recovery time after the first shock (seconds). Infusion of 17α-meT had no effect in the number of licks (Fig. 2A) but it produced a significant increase in the number of punished responses (Fig. 2B). Although 17α-meT-infused females took a longer time to lick from the water-containing bottle for the first time (Fig. 2C), the recovery time after receiving a shock between saline- and 17α-meT-infused females was similar (Fig. 2D).

In order to determine whether prior exposure to 17a-meT had a long-term effect in water intake or hedonia, the sucrose preference test was used over a 3-day period in animals that previously received androgen infusion for testing in other tasks. Fig. 3A shows that the total water consumption did not differ between controls and animals that previously received 17α-meT infusion into the DMH. In addition, animals that received 17α-meT infusion did not alter the preference score for 1% sucrose (Fig. 3B).

Whereas the unpredictable punishment is a key feature of the Vogel conflict test, the elevated plus maze test relies in the naturalistic conflict for a rodent to face height and open spaces. We did not detect significant changes in the number of entries or the time spent in the open arms of the elevated plus maze after 17α-meT infusion (Fig. 4).

Pairs of females were placed in an unfamiliar testing arena for a 5-min session according to treatment infusion. Analysis revealed a significant treatment effect ($F(3,18)=14.79$, $p<0.001$). Specifically, Fig. 5 shows a significant increase in social interaction time (SIT) after bilateral infusion of the AAS 17α-meT. This effect was mimicked by microinjection of the $GABA_A$ receptor agonist muscimol. Co-application 17α-meT plus the high-affinity $GABA_A$ receptor antagonist GABAzine prevented the increment in social interactions induced by 17α-meT alone.

To determine the physiological response associated to anxiety-related behaviors we measured systolic blood pressure 5 min after vehicle or 17α-meT infusion. Fig. 6 shows that the AAS 17α-meT elicited a significant decrease in systolic blood pressure compared to vehicle infusion.

Finally, females were tested in automated activity recording chambers. Fig. 7 shows that 17α-meT infusion into the DMH did not modulate the total distance traveled in the testing arena. Similarly, no difference in rearing activity was noted between saline-infused and AAS-infused females.

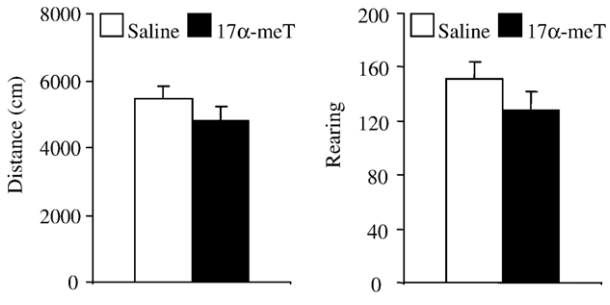

Fig. 7. Central infusion of 17α-meT infusion does not impact locomotor-related behaviors. Infusion of 17α-meT had no significant effects in the distance traveled in activity chambers (A) or rearing activity (B). Saline ($n=15$), 17α-meT ($n=20$), *$p<0.05$.

4. Discussion

We found that infusion of the AAS 17α-meT into the DMH increased the number of punished responses and the latency to display the appetitive reaction in the Vogel conflict test. This measure has been classically used as an indicator for anxiolysis (Vogel et al., 1971). In addition, it enhanced social interactions among female pairs. No changes were observed in elevated plus maze behavior. These behavioral changes did not correlate with fluid intake or locomotor behaviors. A reduction in systolic blood pressure was noted after AAS infusion. To our knowledge, this is the first study that reports the effects of central administration of an anabolic steroid in female anxiety.

The data presented here are consistent with previous reports showing that a single systemic exposure to androgens induces anxiolytic effects in rodents (Bitran et al., 1993; Bing et al., 1998; Aikey et al., 2002), albeit these studies were performed with male rodents. In the female rat, it has been shown that systemic exposure to the endogenous androgen 3αDIOL produces anxiolysis according to the elevated plus maze (Frye and Lacey, 2001), although this is not an universal finding (Jorge et al., 2005). It is interesting to notice that 17α-meT-infused females showed a longer latency to display the appetitive component of the behavior in the task (latency to the first lick). However, once the behavior was triggered, 17α-meT infused females received a greater number of shocks than saline-infused females. We have previously seen this paradoxical effect after chronic exposure to 17α-meT in mice (Barreto-Estrada et al., 2004), which suggests a change in impulse control. We have also observed changes in risk assessment after chronic AAS exposure in male mice (Rojas et al., 2006). Others have proposed that defensiveness (Johansson et al., 2000) or anticipatory anxiety (Ågren et al., 1999) is modified upon chronic exposure to AAS.

While behavioral modification was evident in Vogel conflict test and Social interaction test behaviors, no changes were detected in Elevated plus maze behavior. This task can also be used to measure risk assessment through the quantification of discrete behaviors such as stretch attended postures, head dippings, flat back approach, and rearing (Barreto-Estrada et al., 2004; Rojas et al., 2006). In this study, changes in risk assessment were not noted (data not shown). Similarly, no changes in locomotor behaviors upon acute infusion of 17αmeT into the DMH were observed. We have recently shown that systemic exposure to 17α-meT has no effect in open arm behavior or locomotor behaviors when tested in activity chambers (Barreto-Estrada et al., 2004; Rojas et al., 2006).

With regard to the AAS employed in this study, the chemical modification of AAS compounds can include substitution of a hydrogen for a methyl group at C19 along with the addition of long side chain moieties, esterification of the 17β-hydroxyl group of testosterone, or alkylation at C17. While the first two testosterone modifications render these compounds aromatizable and therefore can induce physiological actions through estrogenic metabolites via estrogen receptors, 17α-alkylated steroids cannot be converted into dihydrotestosterone or 17β-estradiol. Therefore, the behavioral effects seen here cannot be mediated through the activation of estrogen receptors in females. However, since females were tested with 24-h intervals between tasks, it is still

plausible that genomic mechanisms through androgen receptors contributed to some of the behavioral changes observed in the study.

Positive allosteric modulators of $GABA_A$-R such as benzodiazepines (Flores and Pellón, 2000; Griebel et al., 2001) and neurosteroids (Carboni et al., 1996; Czlonkowska et al., 1999) are known to induce anxiolytic responses in the Vogel conflict test (for a review, see Millan and Brocco, 2003). Similarly, enhancement of $GABA_A$-mediated currents within the DMH is associated with anxiolytic responses (Milani and Graeff, 1987; Shekhar and Katner, 1995) whereas blockade of $GABA_A$-mediated currents within the DMH is associated with anxiogenic responses (Shekhar, 1993; Shekhar and DiMicco, 1987; Shekhar et al., 1990), including the Social interaction test (Shekhar and Katner, 1995). It is important to highlight that the behavioral changes reported in this study were noted 5 min after brain infusions, suggesting that fast-acting mechanisms were at play. Given that modulation of social interaction test behavior was mimicked by muscimol infusion and prevented by co-application of 17α-meT plus the high-affinity $GABA_A$ receptor antagonist GABAzine, we propose that the behavioral modifications seen here were induced via activation of $GABA_A$ receptors within the DMH. In fact, it is well-established that enhancement of GABAergic currents within the DMH reduces blood pressure (DiMicco and Monroe, 1998; Keim and Shekhar, 1996; Shekhar et al., 2002). Therefore, the fact that infusion of 17α-meT into the DMH reduced blood pressure further supports this hypothesis. However, it will be of interest for future studies to assess the impact of GABAzine alone in the social interaction test and the effects of $GABA_A$ receptor modulators in locomotor-related behaviors within this task.

In humans, it has been argued that rather than modulating anxiety, high doses of testosterone reduces impulse control (Su et al., 1993; Lukas, 1996; Bahrke et al., 1996). It is important to consider that in the human population, AAS users have reported changes in affect ranging from anxiety to lack of inhibition or impulsivity (Daly et al., 2001, 2003; Parrott et al., 1994, Pope and Katz, 1994, Conacher and Workman, 1989, Schulte et al., 1993). This broad range of effects may reflect ontological changes in affect depending on the type and the number of AAS that are misused, the duration and the pattern of exposure, and the administered dosages. Taken together, infusion of AAS into the DMH, an important network that coordinates behavioral and physiological responses to novel and challenging environments (Inglefield et al., 1994), has a direct impact in impulse control and social interactions perhaps through a fast-acting mechanism in females.

Acknowledgements

Support provided by the NIH-Biomedical Research Infrastructure Network (BRIN) Undergraduate Research Program (RR15565) to Juan Carlos Rivera-Arce, the Louis Stroke Alliance for Minority Participation Grant HRD0114586 to Lizannette Morales-Crespo, and the Minority Biomedical Research Support-Research Initiative for Scientific Enhancement Program at the University of Puerto Rico Medical Sciences Campus Grant GM 61838 to Noelia Vargas-Pinto and Kandy T. Velázquez is acknowledged. We thank Dr. María José Crespo for providing access to the RTBP-2000 set-up. The study was also funded by National Institutes of Health (NIH) Centers of Biomedical Research Excellence Grant RR 15565, NIH-BRIN Young Investigator Award RR16470 and Grant G12RR03051 to Juan Carlos Jorge.

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