THE EFFECT OF THE ANABOLIC STEROID, 17α-METHYLTESTOSTERONE, ON INHIBITORY AVOIDANCE LEARNING AND GENERALIZED ANXIETY IN PERIADOLESCENT RATS

By

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Abstract

The cognitive effects due to exposure to the anabolic steroid, 17α-methyltestosterone (17α-meT) during adolescence, remain undetermined. An inhibitory avoidance learning task (IAT) was used to assess the effect of 17α-meT; (7.5 mg/kg) on inhibitory avoidance learning (IAL), while the elevated plus maze (EPM) was used to measure generalized anxiety. Male periadolescent rats were divided into either an acute or chronic treatment. Both acute and chronic AAS exposure produced significant impairment of inhibitory avoidance learning (IAL) in males. Female periadolescent rats showed no significant IAL alteration after acute exposure. Generalized anxiety, locomotion, and risk assessment behaviors (RABs) were not affected after AAS treatment in either males or females. Our data suggest that early AAS exposure exerts sex-specific negative cognitive effects without affecting anxiety or locomotion.

Keywords: anabolic androgenic steroids, periadolescents, inhibitory avoidance learning, cognition, emotional memory, anxiety
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAS</td>
<td>Anabolic Androgenic Steroids</td>
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<tr>
<td>AR</td>
<td>Androgen Receptor</td>
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<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<td>17α-meT</td>
<td>17 alpha-methyltestosterone</td>
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<tr>
<td>IAT</td>
<td>Inhibitory Avoidance Task</td>
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<td>EPM</td>
<td>Elevated Plus Maze</td>
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<tr>
<td>IAL</td>
<td>Inhibitory Avoidance Learning</td>
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<tr>
<td>RABs</td>
<td>Risk Assessment Behaviors</td>
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<tr>
<td>T</td>
<td>Testosterone</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>CRF</td>
<td>Corticotrophin Releasing Factor</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral Amygdala</td>
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<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
</tr>
<tr>
<td>SAP</td>
<td>Stretch Attended Posture</td>
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<tr>
<td>HD</td>
<td>Head Dipping</td>
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<tr>
<td>FBA</td>
<td>Flat Back Approach</td>
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<tr>
<td>RABs</td>
<td>Risk Assessment Behaviors</td>
</tr>
<tr>
<td>HPG</td>
<td>Hypothalamic Pitiutary Gonadal</td>
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<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
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<tr>
<td>3α-diol</td>
<td>3α-Androstanediol</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomized</td>
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<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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Problem:

Anabolic androgenic steroids (AAS) are potent exogenous androgens used acutely or chronically to increase athletic performance and appearance. The consumers of AAS vary in age and sex. Although it is well known about the psychological consequences of AAS abuse in adults, cognitive effects during adolescence remain undetermined. Findings in our laboratory have demonstrated that an acute dose of the AAS, 17α-methyltestosterone (17α-mT), is sufficient to impair emotional memory in periadolescent male rats. In contrast, no generalized anxiety or locomotion was affected. The main goal of this proposal was to extend upon this finding by addressing sex specificity of the acute dose in emotional memory and generalized anxiety, as well as to compare the acute treatment with prolonged AAS exposure.

Aims:

- **Aim I**: Determine if emotional memory and generalized anxiety differ by sex among periadolescent rats after acute exposure to a supraphysiological dose of 17α- mT (7.5 mg/kg).

  *Rationale*: Sexual differences arise during puberty, and emotional, cognitive and anxiety circuits differ by sex (for review see Graham et al., 2012).

  *Hypothesis*: An acute dose of 17α- mT will result in a sex-specific impairment of emotional learning.

- **Aim II**: Determine the effect of chronic exposure to 17α- mT in emotional memory and generalized anxiety in periadolescent male rats.
**Rationale:** Since AAS abuse varies according to interval cycles and is usually used for extended time periods (for reviews see Quaglio et al., 2009), it is imperative to study chronic AAS exposure at the behavioral level.

**Hypothesis:** A chronic two week exposure to 17α-met will result in deleterious effects of emotional memory in male periadolescent rats. In comparison to results observed under the acute treatment, the impairment after chronic doses should be exacerbated.

**Justification**

Studies report that about 2-6% of males in many western industrialized countries have used AAS at least once in their lives (Pope & Kanayama, 2012). Past evidence has shown that these percentages have gradually increased throughout the last decades (Yesalis & Bahrke, 2000). Although there are numerous reports on steroid incidence and prevalence across the world (Yesalis, 2001), considerable debate still remains regarding the safety of these synthetic compounds. Given the wide problem of steroid doping among an increasing community of adolescents (Lorang et al., 2011), it is imperative to address psyche abuse effects during early development. This study will be the first to address cognitive impairment risks due to AAS abuse using a peri-adolescent animal model. This study will contribute with new behavioral insights of androgen effects during puberty, a sensitive age to engage in androgen misuse and where most deleterious neuro-endocrine and psychiatric symptoms are observed (Kindlundh et al., 1999).

Although sexual differences arise during puberty, a large gap remains in regards to differences in female response to AAS. This study will provide further insight into sex
differences in hormonal disruption on behavioral domains such as memory and anxiety. In addition, different AAS interval treatments, or exposure regimes, will be compared, since it is well known that users expose themselves for either short or extended periods of time (for review see Quaglio et al., 2009). Therefore, our study will highlight behavioral sex-differences after AAS exposure during adolescence, and also will emphasize AAS exposure modalities as a factor to consider when evaluating cognitive and anxiety effects after steroid abuse.

Introduction

Anabolic Androgenic Steroids

Anabolic Androgenic Steroids (AAS) are synthetic derivates of the hormone Testosterone (T), chemically manipulated to remain longer in circulation by reducing the rate of absorption, inactivation and degradation. Manipulations consist in modifications of functional groups attached to T’s four rings and its nineteen carbon structure (Fig. 1). According to alterations and metabolism, AAS are divided in three main classes (Fig. 2). Those belonging to Class I compounds undergo esterification at the 17β-hydroxyl group; Class II have a substituted methyl group for a hydrogen at carbon number 19; while Class III have been alkylated at carbon 17 (Clark & Henderson, 2003).

The four common forms of administration are determined by the chemical structure of the synthetic steroid, therefore different for each class. The esterification of Class I compounds reduces the release into circulation making them good injectable AAS. Class II AAS are viable injectable compounds characterized by longer half life and
increased duration of effectiveness. Alkylation in Class III retards metabolism by the liver, resulting in orally active steroids. The other two common administration routes cream/gels and skin patches vary by class.

By having longer physiological presence, these compounds induce higher anabolic potency by accelerating the growth of muscle and bone tissue. Also, they have androgenic and virilizing effects by developing and maintaining masculine characteristics.

AAS have been clinically used since 1930s to accelerate puberty, and to stimulate appetite and bone and muscle growth. Also they have been used to counteract wasting, as seen in HIV and cancer patients (Lorang et al., 2011). Anabolic steroids use has been prohibited in sports since 1974 by the Medical Commission of the International Olympic Committee (IOC) (Fragkaki et al., 2009) and they were later added to the list of Controlled Substances in 1990. Despite its strict prohibition, recent data shows that illicit use continues, as AAS ranked as the most common banned substance detected in urine tests during 2011 at the World Anti-Doping Agency.

**AAS Abuse**

In the USA, findings report that between 1 million and 3 million people are thought to have misused AAS (National Institute on Drug Abuse, 2000). The consumption of these compounds by athletes is well documented and although strongly persecuted, in 2011 the World Anti-Doping Agency reported that 59.4% of controlled substances identified were AAS. In other associated regions of the USA, this trend seems
to be higher with misuse detected among males to be 62% and females 38% (Acevedo et al., 2011).

A changing trend to the previous main use by adults is that adolescent consumption has gradually increased throughout the last decade (Yesalis & Bahrke, 2000). This population is growing quickly with trends showing that 0.5% of 8th graders, 1.0% of 10th graders, and 1.5% of 12th graders have abused anabolic steroids at least once in the year prior to being surveyed (Johnston, 2010).

The resulting enhancement of athletic performance and body image is the primary incentive of abuse among the athletic and nonathletic community (Petersson et al., 2010). Data from large observational studies in adults suggest that the majority (88%-96%) of AAS users experience at least one minor side effect (Amsterdam et al., 2010), associated with a number of physiological and psychiatric complications. Understanding the degree of mental and behavioral complications remains a long endeavor. This results from heterogeneous findings due to the use of a variety of dosages, compounds and animal models. Therefore, to date, psychological turn outs induced by AAS consumption remain inconclusive in both adult and younger populations at risk of exposure.

Health Effects

The use of synthetic androgens results in negative physiological effects (Amsterdam et al., 2010) and continuously debated psychological alterations (Ip et al., 2012). The body’s response to supra-physiological levels of AAS is determined by the longevity and dosage regime. Increased acne problems, cardiovascular risks and reproductive infertility are some of the general established consequences of continuous
exposure to synthetic androgens (for review see Hartgens & Kuipers, 2004). Mood and behavioral alterations reported in users range from aggression and anxiety to psychiatric disorders (for reviews see Graham et al., 2008).

With the vast majority of human surveys being done in adult populations, closer attention needs to be addressed to adolescents. Anabolic steroid use during teen years results in adverse effects in proper bone growth (Irving et al., 2002) and brain development (Cunningham et al., 2012). In addition, virilizing and feminizing effects are observed in both sexes (Lumia & McGinnis, 2010). AAS have the potential to result in addiction and withdrawal effects, with additional evidence of their effect to increase the abuse of other substances such as alcohol or cigarettes for adolescent males, and marijuana in adolescent females (Irving et al., 2002). Compared to non-users, teen steroid users from both sexes report having poorer attitudes regarding health and nutrition (Irving et al., 2002). Finally, teens that use AAS show lower self-esteem and greater depressed mood than non-users (Irving et al., 2002). Since mood and behavioral changes have been reported, it is important to characterize the amount of AAS exposure necessary for adolescent psychiatric vulnerability.
Cognition and AAS

Although there is data in adult rodents, little is known about AAS potential to alter cognitive behavior during adolescence (for review see Lumia & McGinnis, 2010), where simple cognitive learning tasks are enhanced (for review see Spear & Brake, 1983).

Cognition is a broad term used to define an array of neural processes that allow for higher brain function processing. The cognitive neuroscience branch defines it as mental processes that emerge from the function of the brain (Charney & Nestler, 2009). These cognitive processes are divided into attention, problem solving, decision making and memory.

There are different classifications of memory, branching primarily into two main types in relation the task and cues involved with storage: short term and long term memory. The second is further divided into explicit or conscious memory and implicit or unconscious memory. Our study focuses on the division of explicit memory, specifically emotional or episodic memory. It is defined as the formation of memories related to unpleasant experiences (Ledoux & Muller, 1997). A model in animals to study the formation of this specific memory is through inhibitory avoidance learning (IAL), also known as passive avoidance. In this model, an undesirable stimulus results in the avoidance of an expected behavior, and the degree of avoidance is a measurement of emotional memory formation.

When addressing endogeneous testosterone effects, an acute (single injection following training; 1 mg/kg) or chronic (5 weeks through Silastic capsules) testosterone administration enhanced cognitive performance on the inhibitory avoidance task (IAT) in adult
male rats (Edinger et al., 2004; Frye et al., 2010; Frye & Seliga, 2001). Findings of AAS alteration in cognitive tasks in general show that in adult male rats, daily subcutaneous injections of a high dose (15 mg/kg) of the AAS, nandrolone decanoate, elicits impairment in spatial (Magnusson et al., 2009) and social memory (Kouvelas et al., 2008). In contrast, a single acute injection of the same steroid (4 mg) was enough to improve cognitive task (Vázquez-Pereyra et al., 1995). Studies have demonstrated that a single intracerebral administration of several doses of exogenous testosterone (10, 20, 40, 80, and 120 μg/0.5 μl) caused a dose-dependent impairment of spatial memory (Naghdi et al., 2001; 2003), as well as acquisition, consolidation and retrieval of inhibitory avoidance learning (Harooni et al., 2008). Regarding neurosteroids, pregnenolone sulfate, which is known to influence cognitive function (Flood et al., 1995), also impaired retention on the passive avoidance task in adult rats (Isaacson et al.1995; 2000). The heterogeneous findings present in regards to steroid effect in cognitions are a result of lack of consistency in experimental paradigms. Since these findings have focused only in male adults, it is imperative to undertake studies focusing on learning and memory alterations after AAS exposure during adolescence in both sexes.

**Drug Regime**

Drug regimens of AAS are characterized by long interval heterogeneous patterns with the purpose of increasing the exposure of the synthetic compound in circulation and therefore increasing the synergistic actions that augment the anabolic response (Evans, 2004). Throughout the intervals of abuse, users undertake cycles of intense consumption followed by a 4- to 6-week drug free period. Others remain intake free for months before
retaking AAS consumption (Evans, 2004). Although two recent surveys indicate that the majority (76%-96%) of AAS users self-administer injectable (intramuscular) formulations of AAS, a combination of different administration routes is also an important factor in the long interval cycle patterns.

Since users commonly consume AAS for prolonged periods, the minimum exposure required for physiological and a mood change is not established. It is important to address the degree of alteration caused by short intervals in adolescents resulting in subjects that were exposed to synthetic androgens briefly. Our study will focus on the impact of an acute exposure in cognition and anxiety, since innate developmental sensitivity of neuronal substrates to androgens, might be increasing the risk for psychological effects (Wichstrom & Pedersen, 2001).

Methods of Action

AASs are manufactured to maximize anabolic and minimize androgenic effects of the active ingredient, T, which has several signaling pathways. Endogenous T can exert its effects through direct signaling by binding to either the androgen receptor or through non genomic mechanisms. The androgen receptor is a cytoplasmic receptor that binds to the steroid after it has fused through the cellular membrane. Binding of a ligand results in a conformational change in the receptor which in turn causes dissociation of heat shock proteins, dimerization and transport from the cytosol to the cell nucleus where the androgen receptor dimer binds to androgen receptor elements, where it initiates gene transcription (Fragkaki et al., 2009).
Since AAS are derivatives of T, they mimic the same effects by targeting the same peripheral and central tissues. They can act through the androgen receptor (AR) directly as the parent compound, or after a reduction to dihydrotestosterone (DHT). AAS binding to AR is highly active, and exposure to AAS results in ARs up-regulation in muscle (Evans, 2004) and a number of brain regions (Lynch & Story, 2000).

Non genomic mechanisms involve activation of transient and quicker responses that do not involve genetic changes. A responsive cellular external element is the GABA\textsubscript{A} receptor. This pentameric chloride channel, once allosterically activated by the hormone, results in a transient ionic gradient change that induces inhibition of neuronal activity. Other non genomic targets result in second messenger signal transductions leading to diverse cellular effects such as: increases in free intracellular calcium or activation of protein kinase A (PKA), protein kinase C (PKC) or mitogen activated protein kinase (MAPK) (Li & Al-Azzawi, 2009).

An alternative mechanism of action consists of the potential of T and AAS to be aromatized to estradiol. Once metabolized to estradiol, the hormone can exert estrogenic activity through the Estrogen Receptor (ER) \(\alpha\) and \(\beta\).

Both the amygdala and hippocampus are abundant in androgen sensitive substrates, making them responsive and regulated by steroid hormones (Kerr et al., 1995; Roselli et al., 1989; Wood & Newman, 1999). They have been implicated as key cognitive sites for emotional learning and memory (Davis, 1992; Farmer & Thompson, 2012; McCaugh et al., 2002; Truitt et al., 2009). Given neuro-endocrine sensitivity changes throughout development, these limbic structures are highly responsive to
hormone levels throughout prenatal and pubertal development. The increased androgen response in these brain regions during puberty (Ahmed et al., 2008) makes them likely affected targets of supra-physiological exposure to AAS.
INTRODUCTION FIGURES
Figure 1. Representation of base testosterone structure and individual site modifications. The specific sites modified determine both the binding of the molecule to the androgen receptor or other targets, and the activity of the compound (Fragkaki et al., 2009).
Anabolic Steroids and Avoidance Learning

- 11-position: reduces binding
- 17α-OH group: favours binding
- 19-norsteroids with 7α-methyl substituents favor binding
- 3-keto group: favours binding
- 5α-steroidal framework: favours binding
- 17α-side chain and Δ4,9,11 double bonds favor binding
- 7α- small substitutions: favour binding, but large ones reduce binding
Figure 2. Main three classes of AAS according to modification of the T backbone. Those in class I undergo esterification, compounds of class II have adjoined long side chains and a methyl group instead of a hydrogen at carbon number nineteen. Class III steroids are alkylated at carbon number seventeen (Clark & Henderson, 2003).
I. Testosterone Esters

Testosterone Cypionate

II. 19-Nor-testosterone AAS

Nandrolone Decanoate

III. 17α-alkyl AAS

17α-Methyltestosterone

Stanozolol

Methandrostenolone

Oxymetholone
Methodology

General Procedures

Gonadally-intact male and female Sprague Dawley rats were purchased from Charles Rivers Labs (Wilmington, MA). Animals were received at PN-25, maintained in the vivarium for acclimation for four days after arrival, and separated and housed individually in a humidity and temperature control room on a 12:12-hour reverse light/dark cycle. Male and female rats were housed in different rooms. Food and water was available ad libitum. Animals began daily handling for a minimum of seven days prior to testing.

Periadolescent males rats were used for either experiment 1 and 3, while female rats were used for experiment 2. All behavioral tests were performed during the dark phase of the cycle at age range: PN-40 to PN-45. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico, Medical Sciences Campus.

Vaginal Lavages

We performed daily vaginal lavages to female rats to determine differences through estrus cycle. Starting at PN-31 vaginal lavage was performed for at least two estrous cycles using a small eyedropper containing 0.25 ml of 0.9% saline. The fluid obtained after the lavage was evenly distributed onto a microscope slide for further cytology analysis under low magnification. We monitored for a normal four-day cycle,
consisting of vaginal smears evaluating the presence of leukocytes, nucleated epithelial cells, or cornified epithelial cells (Fig. 3, Cooper et al., 1993).

Drug treatment

Experimental animals were exposed to 17α-met, dissolved in 0.9% saline containing 30% cyclodextrin, at a dose of 7.5 mg/kg (0.2 cc/kg, i.p.). This pharmacological dose reflects a medium to high dose range of abuse in humans on a milligram per kilogram basis (Blasberg et al., 1997; for review see Clark & Henderson 2003). In parallel, control rats received the equivalent volume of vehicle (30% cyclodextrin). Compounds were purchased from Sigma (St. Louis, MO).

In experiment 1, male periadolescent rats were exposed to an acute treatment receiving a single i.p. AAS injection during behavioral testing. In experiment 2, female periadolescent rats were exposed to a similar acute treatment. For experiment 3, male periadolescent rats received a chronic treatment that consisted of fifteen daily i.p. AAS injections prior to behavioral testing.

Inhibitory Avoidance Task (IAT)

Apparatus- To measure emotional memory we used the step-through inhibitory avoidance apparatus (AccuScan Instruments; Columbus, OH). It consisted of a two-compartment acrylic box (45 X 22 X 33 cm) with an illuminated compartment connected to a darkened one, by a movable guillotine door and a stainless steel grid floor.
General Protocol- Rats were placed in the dark compartment of the chamber during two minutes, and then transferred to the home cage for one additional minute. Thereafter, rats were placed again in the illuminated side of the chamber and the latency to enter to the dark compartment was recorded. Animals were removed from the chamber after crossing to the dark compartment. During day 2 (acquisition phase), rats in either acute or chronic experiment were injected with 17α-met or vehicle, and immediately placed in the dark compartment with the door closed. A mild electric footshock (0.3 mA; 3 sec) was delivered through the grid floor. The retention test was carried out 24 hours later, where crossover latency to enter the dark compartment measured the index of IAL. The maximum entry latency allowed in the retention test was 300 seconds.

In experiment 1, males were divided into controls (n=19) and AAS (n=20) prior to testing, with habituation at PN-41, learning phase at PN-42 and retention at PN-43. In experiment 2, females were tested for emotional memory while they were in diestrus (low hormone levels) or proestrus (high hormone levels). They were divided in cohort I (Control, n=20; AAS, n=20) and cohort II (Control, n=10; AAS, n=10) respectively. Behavioral testing was not homogeneous due to dependence on the rats’ estrous cycle. Specifically, the age interval for habituation was from PN-41 to 43, for acquisition between PN-42 to 44, and for retention between PN-43 to PN-45.

In experiment 3, male peri-adolescent controls (n=6) and AAS (n=6), received fifteen daily injections of cyclodextrin vehicle or the steroid prior to behavioral testing. They did not receive injection during habituation at PN-41, were re-exposed to drug or vehicle during acquisition phase at PN-42 and then measured for retention at PN-43.
Hot Plate Test (HPT)

General Protocol- In order to measure nociceptive changes due to treatment exposure, a hot plate surface at a constant temperature of 55°C was used to measure any induced antinociceptive responses. Animals were placed with all four paws on top of the surface surrounded by a beaker of high temperature resistance. The time between placement and response of either: hind-paw lick, hind paw flick or jump was recorded. Animals were not exposed to heat longer than 60 seconds. Each animal was tested only once, at the end of day 3 of the IAT.

Elevated Plus Maze (EPM)

Apparatus- The Elevated Plus Maze (AccuScan; Columbus, OH) has been validated in rodents to measure generalized anxiety (Pellow et al., 1985).

General Protocol- Rats were injected with AAS or vehicle before placement in the EPM for an interval of 5 minutes. An increase in time spent or number of entries to the open arms reflected anti-anxiety behavior.

Risk assessment behaviors (RABs) such as stretch attended posture (SAP), flat back approach (FBA), and head dipping (HD), as well as grooming (non-emotional) and freezing (fear) were monitored over the EPM. The biological function of RABs, acts and postures, is to inform behavioral strategies in potentially dangerous situations (for review see Carobrez & Bertoglio, 2005). Therefore these parameters are more accurate observations of anxiety-related behaviors than conventional measures obtained from the EPM alone (Rodgers and Cole, 1993).
For experiment 1, male periadolescent rats at PN-43 were divided into controls (n=10) and AAS (n=10) to later undergo EPM behavioral testing. In experiment 2, female rats of both control (n=10) and AAS (n=10) groups were tested for generalized anxiety while they were in diestrus during PN-43 to PN-45. In experiment 3, male controls (n=5) and AAS (n=5) underwent testing at PN-43. This paradigm was used in animals that did not undergo prior testing in the IAT, in order to avoid confounding effects due to the foot-shock. Data from animals that fell from the maze was discarded.

**Statistical Analysis**

Data are presented as mean ± standard error of the mean (SEM). Two-way Repeated Measures Analysis of Variance followed by Tukey test post-hoc analysis was employed for comparison of IAT results of Experiments 1 and 2, while Two-way Analysis of Variance was employed to analyze their EPM and RABs. For Experiment 3, a Student’s t-test was used to analyze the IAT, EPM and RAB’s. Statistical significance was established at p ≤ 0.05.
METHODOLOGY FIGURES
Figure 3. Vaginal lavage classification of female rat estrous cycle. Schematic diagram of vaginal lavage microscopic examination for identification of cell topology throughout the female rat estrous cycle (modified from McLean et al., 2012). During Proestrus nucleated epithelial cells are dominant, followed by the Estrus stage where cornified squamous epithelial cells are a majority. During Metestrus there is a combination of both cornified squamous epithelial cells and leukocytes. Finally during the last stage, Diestrus, leukocytes are the major cells visible.
Results

Experiment 1 & 2: Male and Female Acute Treatment with 17α-meT

Inhibitory Avoidance Task

Male peri-adolescent rats exposed to an acute dose of 17α-meT showed an impairment of inhibitory avoidance learning. Specifically, there was a significant difference in the crossover latency during day 3 (Fig. 4A), where AAS decreased this latency. Particularly, crossover latencies were $165.8 \pm 47.98$ for controls and $35.18 \pm 29.45$ for AAS treated animals. For males, post-hoc analysis revealed the main effect of treatment during day 3 (Fig. 4; $F (1, 78) = 5.34, p < 0.001$). In contrast to males, females showed no significant effect on the IAL after 17α-meT, with (Control: $28.09 \pm 12.27$; AAS: $4.82 \pm 6.81$) (Fig. 4B). Since no difference was observed between females in diestrus (cohort I) or proestrus (cohort II) (data not shown), the presented data represents both cohorts.

When comparing both day of testing and sex, a two-way ANOVA repeated measures indicated both a significant effect of testing day (Day 1 vs. Day 3; $F (1, 77) = 25.14, p < 0.001$), as well as sex (males vs. females; $F (1, 77) = 15.06, p < 0.001$). In addition, there was a significant Testing Day x Sex interaction ($F (1, 77) = 14.80, p < 0.001$).

A sexual dimorphism on the crossover latency on Day 3 was observed. There was a significant difference between control males and females with value for males: $165.80 \pm 47.98$ and females: $28.09 \pm 12.27$. The post-hoc analysis values were $F (1, 77) = 14.77, p < 0.001$.
When comparing crossover results of only day 3, after animals were exposed to 17\(\alpha\)-
meT, a two-way ANOVA showed a significant main effect of both sex (\(F (1, 78) =18.94, p<0.001\))
and treatment (\(F (1, 78) =15.88, p<0.001\)), with a statistically significant Sex x Treatment interaction (\(F (1, 78) =7.73, p=0.007\)).

**Elevated Plus Maze**

Periadolescent rats were tested in the EPM to determine if 17\(\alpha\)-meT affects
generalized anxiety. Acute injection of 17\(\alpha\)-meT did not cause any significant changes on
the time spent in the open arms in either males (Fig. 5A; Control 83.0 ± 19.45; AAS 129.7
±13.79) or females (Fig. 5B; Control 107.6± 13.53; AAS 110.4 ± 10.84). Likewise, the
number of entries to the open arms (Fig. 5C, Males: Control 5.0 ± 0.85, AAS 4.3 ± 0.45;
Fig. 5D, Females: Control 6.8 ± 0.53, AAS 7.0 ± 0.74) or the locomotion, as measured by
total number of arm entries (Fig. 5E, Males: Control 10.4 ± 1.31, AAS 8.6 ± 0.62; Fig. 5F,
Females: Control 13.1 ± 0.87, AAS 12.6 ± 0.65) were not altered by AAS treatment.

No effect was observed in the RAB’s in either males or females (Table 1) after acute
exposure to 17\(\alpha\)-meT, still significant sex-differences were found in HD (males 8.8 ± 0.77;
females 4.4 ± 0.25; \(F(1,13)=15.309, p=0.002\)), and SAP (males 1.8± 0.36; females 0.4 ±
0.25; \(F(1,13)=6.635, p=0.023\)). Non-emotional measures and fear response such as
grooming and freezing, respectively, were not affected by treatment or sex (data not shown).
Experiment 3: Male Chronic 17α-meTTreatment

Inhibitory Avoidance Task

Similar to the acute treatment, a significant impairment of emotional memory was evident after a chronic exposure to 17α-meT (7.5 mg/kg). When comparing crossover latencies on day 3, periadolescent male rats treated chronically with AAS showed a significant decrease as compared with control animals (Fig. 6; Control = 10.03 ± 1.59; AAS = 5.21 ± 0.44, p = 0.016.)

Elevated Plus Maze

A fifteen day exposure of 17α-meT did not cause any significant changes on the time spent in the open arms in males (Fig. 7A; Control 165.0 ±24.96; AAS 155.4 ± 29.12). Likewise, the number of entries to the open arms (Fig. 7B, Control 10.4 ± 2.15, AAS 11.2 ± 3.62) or the locomotion, as measured by total number of arm entries (Fig. 7C, Control 23.2± 2.74, AAS 25.6 ± 5.85) were not altered by AAS treatment. No effects were observed in the RAB’s (Table 2) after a chronic exposure to 17α-meT. HD (Control 13.2 ± 2.63; AAS 11±1.04), rearing (Control 1.6 ± 0.04; AAS 0.4 ± 0.25), SAP (Control 3.2 ± 0.53 ; AAS 1.4 ± 0.50) and FBA (Control 1.6 ± 0.67; ASS 0.6 ± 0.24).
RESULT FIGURES
Figure 4. **Effect of acute 17α-met on the IAT in peri-adolescent rats.** (A) Control male rats showed a significant decrease in the crossover latency (sec) during retention (Day 3), (B) while in females, the crossover latency during retention (Day 3) was not affected by AAS. **Two-Way ANOVA RM; p< 0.001.** Males: Control n=19, AAS n=20; Females: Control n=30, AAS n=30. Error bars represent SEM.
Figure 5. Effect of acute 17α-methyltestosterone (17α-meT) on the elevated plus maze (EPM) in peri-adolescent rats. Acute exposure to AAS did not affect the time spent in the open arms (A, B), the open arms entries (C, D), or the total number of entries (E, F) on the EPM in either male (A, C, E) or female (B, D, F) rats. Control n=10, AAS n=10. Error bars represent SEM.
Table 1. Effect of acute 17α-meT on risk assessment behaviors (RABs) on the EPM in peri-adolescent rats.

<table>
<thead>
<tr>
<th>RABs</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>AAS</td>
</tr>
<tr>
<td>HD</td>
<td>8.8 ± 0.77</td>
<td>10.1 ± 0.50</td>
</tr>
<tr>
<td>FBA</td>
<td>0.8 ± 0.29</td>
<td>1.0 ± 0.33</td>
</tr>
<tr>
<td>SAP</td>
<td>1.8 ± 0.35</td>
<td>2.3 ± 0.30</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. Flat back approach (FBA), stretched attend posture (SAP), head dipping (HD). Control n=10, AAS n=10. Two-way ANOVA, *p<0.05, **p<0.005; control females different from control males.
**Figure 6. Effect of chronic 17α-met on the IAT in peri-adolescent rats.** During retention day (Day 3), chronically AAS-treated male rats showed a significant decreased in the crossover latency.

* t-test; p< 0.05. Males: Control n=6, AAS n=6. Error bars represent SEM.
Figure 7. Effect of chronic 17α-methyltestosterone (17α-MET) on the EPM in male peri-adolescent rats. Chronic exposure to AAS did not affect the time spent in the open arms (A), the open arm entries (B), or the total number of entries (C) in male peri-adolescent rats. Control n=5, AAS n=5. Error bars represent SEM.
Table 2. Effect of chronic 17α-meT on risk assessment behaviors (RABs) on the EPM in peri-adolescent rats.

<table>
<thead>
<tr>
<th>RABs</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>HD</td>
<td>13.2 ± 2.63</td>
</tr>
<tr>
<td>FBA</td>
<td>1.6 ± 0.04</td>
</tr>
<tr>
<td>SAP</td>
<td>3.2 ± 0.53</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. Flat back approach (FBA), stretched attend posture (SAP), head dipping (HD). Control n=5, AAS n=5.
Discussion

Inhibitory Avoidance Learning

The present study is the first one addressing negative cognitive effects after acute and chronic exposure of AAS in periadolescent rats. Our results showed that an acute treatment of 17α-meT (7.5 mg/kg), when tested on the IAT, produced cognitive impairment in male but not female peri-adolescent rats. Injection of 17α-meT interfered with avoidance learning, as evidenced by the significant reduction of the crossover latency on day three. This suggests that a single injection of AAS is sufficient to impair emotional memory as measured by an inhibitory avoidance learning task. Also, the impairment in males was also evident after a chronic exposure to the steroid. When comparing sexes within control groups, a sexual dimorphism previously cited (Heinsbroek et al., 1988) was observed, with male rats having longer crossover latencies than females and showing stronger acute stress outcome (Andreano & Cahill, 2009).

AAS Memory Impairment

In adult male rats, previous reports showed that intra-amygdalar and intra-hippocampal injections of testosterone produced impairment on spatial memory (Naghdi et al., 2003), with peak effects at 120 μg/0.5 μl and 80 μg/0.5 μl, respectively. Additionally, intra-hippocampal injections of testosterone produced impairment on IAL acquisition (1 and 80 μg/0.5 μl/side), consolidation (20 μg/0.5 μl/side), and retrieval (20 and 40 μg/0.5 μl/side) (Harooni et al., 2008). Moreover, anabolic steroids compromise learning and memory. Particularly, daily subcutaneous injections of a high dose (15 mg/kg) of nandrolone decanoate, a Class II AAS and a 19-nortestosterone derivative, elicit impairment in spatial
(Magnusson et al., 2009) and social memory (Kouvelas et al., 2008). Conversely, a single intramuscular injection of a lower dose of nandrolone (4 mg) improved short and long-term memory (Vázquez-Pereyra et al., 1995). As a matter of fact, from the four nandrolone doses tested (1, 2, 4 and 6 mg), only the 4 mg was effective, suggesting that the beneficial effects of steroids on learning and memory may require optimum level of androgens. Regarding neurosteroids, pregnenolone sulfate, which is known to influence cognitive function (Flood et al., 1995), also impaired retention on the passive avoidance task in adult rats (Isaacson et al., 1995; 2000). From these studies in adults, it is suggested that the diverse spectrum of AAS effects on cognitive performance depends on an optimal level of testosterone (Muller et al., 2005), where low doses enhance, and high doses impair cognition. In this respect, endogenous androgen circulating levels have been shown to be important for the appropriate memory formation, since castration during periadolescence showed reduced contextual fear memory (McDermott et al., 2012). Therefore it is possible that the impairment of the IAL we observed after a pharmacological dose of androgen is the result of feedback inhibition of the HPG axis, a response that might be comparable with diminished androgen levels after castration.

**AAS Memory Enhancement**

However, the fact that some studies showed opposite results about steroid-enhanced memory (Schneider-Rivas et al., 2007) and resistance to extinction (Rivas-Arancibia &Vázquez-Pereyra, 1994) might be due to other factors such as foot shock intensity and the cognitive demands of each task. For example, we showed memory impairment using a 0.3
mA foot shock in a 3-day duration IAT, while Schneider-Rivas and colleagues (2007) reported that testosterone enanthate (20 mg) improved long term memory and delay extinction of the conditioned response in a one trial passive avoidance task using a foot shock intensity of 3 mA; an intensity ten times higher than the one used in our study. Nonetheless, in studies where low intensity (0.25 mA) foot shock and an enhancement of IAL were evident, this might be because of the lower androgen dose (testosterone, DHT or 3α-diol: 1 mg/kg) rather than to the intensity of the foot shock (Edinger et al., 2004). Similarly, studies in which androgens were micro-infused directly to the brain followed by impaired cognition after a high intensity foot shock (Harooni et al., 2008), highlight the possible correlation between high androgen levels and disruption of cognitive processes.

**Sex specific Memory Impairment**

Concerning females, previous studies reported that adult female rats injected with 500 μg T propionate (class I AAS) or 500 μg DHT for two consecutive days have no effect on memory in adult rats on the spatial Morris water maze task (Frick et al., 2004), while chronic exposure to an AAS cocktail with equal combination of three steroids (testosterone cypionate, nandrolone decanoate, methandrostenolone; 7.5 mg/kg) for 4 weeks in female adolescent mice did not affect learned fear (Costine et al., 2010). Correspondingly, our study showed no differences on the IAL in AAS-treated periadolescent females. Since the exact timing of adolescence is a matter of dispute in laboratory animals, and females reach puberty before males (for review see Spear, 2000), it is possible that sex-specific effects on IAL might be due to differences in the surge of gonadal hormones during development.
This raises the possibility that the majority of females may have been in a post-pubertal stage, while males might be pre-pubertal. Nevertheless, other studies have demonstrated that androgen- treated females enhance their cognitive performance on the IAT and Y maze (Frye & Lacey, 2001). Differences in type of androgens (natural versus synthetic), their metabolites, developmental stages (adults versus periadolescents) and/or the hormonal manipulation (ovariectomized or intact) might account for differences in Frye’s studies and ours, respectively.

Even though we used intact females, no differences were detected between females in diestrus or proestrus, ruling out the possibility of endogenous hormonal status having a role on IAL. This result contrasts from previous findings indicating that learning in adult female rodents differ through the estrus cycle (Gupta et al., 2001; Milad et al., 2009). In fact, differences in learning and greater variability among female rats, as opposed to male rats, are usually associated with changes in hormonal status across the cycle. For instance, contextual fear conditioning in female rats fluctuates across the estrous cycle, with less freezing during proestrus compared to estrus (Markus & Zecevic, 1997; Milad et al., 2009). In a study to test if ovarian steroids play a role in fear conditioning, sham-operated (intact) female rats extinguished fear faster than males and also than ovariectomized (OVX) females (Gupta et al., 2001).
Generalized Anxiety

Interestingly, we found that acute and chronic AAS impaired acquisition of IAL without having effects on anxiety. Our results are consistent with Oberlander and Henderson (2012), who demonstrated that a single acute injection of an AAS cocktail (7.5 mg/kg) did not affect anxiety in adolescent females. In addition, results in other rodents have also shown a lack of effect on generalized anxiety after a 15 day chronic exposure to 17α- meT at the same 7.5 mg/kg dose used in this study (Rojas-Ortiz et al., 2006).

The sexual dimorphism between control males and females in RABs supports literature findings that there are sex differences in rat behavior during this task (Naslund et al., 2013). For female rats to have lower HD and SAP suggests that a higher stressful response inhibits behavior that allows to analyze the danger of an environmental context. In accordance, studies have reported higher baseline plasma levels of the stress hormone cortisol in female rats compared to males (Weinstock et al., 1998).

Nociception

It is noteworthy that in our protocol, 17α-meT did not elicit pain nociception as tested in the hot-plate test (data not shown). Consistently with this result, recent studies have shown that subcutaneous injections of testosterone, DHT, or stanozolol (5mg/kg; acute or chronic) did not alter pain nociception or induce chronic inflammatory pain in adult intact male rats (Tsutsui et al., 2011). However, we cannot rule out the possibility that 17α-meT elicited some kind of impulsivity effect, given that testosterone (5 mg/kg) displayed increased punished responses in the Vogel Conflict Test (Bing et al., 1998), although this
measure is more associated with anxiolytic processes (Millan & Brocco, 2003) than to impulsivity.

*Possible Neural Alteration induced by 17α-meT*

As 17α-meT holds the potential to modulate memory circuitries for emotional memory formation, many studies have focused on the neural mechanisms that mediate learning and memory, in which the amygdala and hippocampus have been identified as key structures for these processes (for review see Phelps, 2004). The amygdala and hippocampus are parts of the limbic system, which subserve tasks related to memory and emotional regulation (Okada et al., 2011). These regions are abundant in androgen sensitive substrates, making them responsive and regulated by steroid hormones (Roselli et al., 1989). The increased androgen response in these brain regions during puberty (Ahmed et al., 2008) could explain why an acute exposure to a single steroid is enough to induce cognitive impairment in acquisition.

The amygdala is involved in mediating influences of emotional arousal and stress on learning and memory. Specifically, the basolateral nucleus of the amygdala (BLA) is greatly implicated in the modulation of memories of meaningful events (for review see LeDoux, 2000). Evidence indicates that memory formation on the IAT may be mediated by the BLA (Liang, 1999; Tomaz et al., 1992; Roozendaal et al., 2009; Sajdyk et al., 2004). In fact, intra-BLA injections of different drugs and neurotransmitter agents modulate memory in this task (McGaugh et al., 2002). In this line of evidence, and as discussed before, intra-
amygdalar and intra-hypocampal testosterone injections impaired spatial memory and interfered with the IAL (Naghdí et al., 2003; Harooni et al., 2008).

However, it is established that the BLA does not work alone in the modulation of memory, and performs its role via potent interactions with other cortical and limbic structures such as the hippocampus (McIntyre et al., 2003). A previous study showed a cooperative interaction between the BLA and the ventral tegmental area (VTA) on IAL (Nazari-Serenjeh & Rezayof, 2013). It has also been found that outburst projections from the BLA lead to IAL neuroplastic responses in hippocampal pyramidal neurons (Farmer & Thompson, 2012). Thus, the reciprocal relationship between the BLA and the VTA and hippocampus in memory consolidation of IAL may be dependent on cooperative interactions between the glutamatergic and GABAergic systems via NMDA and GABA-A receptors.

**Hypothetical GABA modulation by 17meT**

Although androgens and its synthetic compounds are known to act primarily through the AR, non-genomic pathways are rapid and transient alternative responses. The inhibitory gabaergic system, which plays an important role in learning and memory (Erhlic et al., 2009) has shown to be modulated by 17α-meT (Jones et al., 2006; Penatti et al., 2011; Yang et al., 2002, 2005). In fact, it has been found that extinction memory (for review see Barad et al., 2006) and IAL (Khajehpour et al., 2011) are modulated through GABA-A receptors in the BLA. Particularly, intra-BLA microinjection of muscimol, a GABA-A receptor agonist,
impaired dexamethasone-induced memory; while bicuculline, a competitive GABA-A receptor antagonist, increased memory retrieval using an IAT (Khajehpour et al., 2011).

Since we suggest a sex-specific effect of the hormonal milieu on IAL in periadolescent rats, it is plausible to suggest a sexually-dimorphic GABAergic transmission in which males have higher GABA-A receptor B$_{\text{max}}$ (maximal number of binding sites) than females (Jüptner & Hiemke, 1990), although Stefanova (1998) showed that females have more GABA-expressing neurons in the BLA. Other point of regulation to attain sex-specific effects on behavior is at the GABA receptor subunit level. This was evidenced by the increase in GABA-A receptor $\alpha2$ subunit mRNA after 17$\alpha$-meT (7.5 mg/kg) treatment in the hypothalamus of adolescent females, but not male mice. This differential expression of the receptor subunit was accompanied by a decrease in spontaneous inhibitory synaptic current frequency in this brain region (Penatti et al., 2005). Still, limbic areas such as the amygdala and hippocampus have greater gabaergic response to steroids than the hypothalamus (Wilson & Biscardi, 1997). As a matter of fact, in males, neuroactive steroids produced greater GABA-activated chloride influx in the amygdala as compared to the hypothalamus; a trend not seen in females (Wilson & Biscardi, 1997).

Memory consolidation of inhibitory avoidance learning (IAL) may be dependent on cooperative interactions between the GABAergic system and neuropeptide Y substrates. NPY Y1 has shown to be implicated in the mediation of fear and anxiety behaviors (for review see Kask et al., 2002; Gutman et al., 2008; Verma et al., 2012), where it is needed for synaptic plasticity that leads to emotional memory (Sajdyck et al., 2004; 2006). Findings have shown that both positive and negative chronic modulation of the GABA$_A$ receptor induces transgene expression changes of NPYY1 in the medial amygdala (Oberto et al.,
Regarding steroids, changes in cerebro-cortical neuroactive steroids in the medial and central amygdala also caused NPY Y1 transgene expression alterations (Eva et al., 2006).

Taken together, it is suggested that pharmacological doses of steroids has the potential to allosterically modulate GABA-\(A\) receptors that might result in alteration of GABA-induced currents and the subsequent modulation of learning and memory through androgen-sensitive brain regions. Within the amygdala GABA has shown to coexist and have a close relationship with neuropeptide Y (NPY) Y1 receptors (Oberto et al., 2001). This interaction strongly suggests a circuit model in the regulation of anxious behavior and neuronal excitability (Eva et al., 2006). Future electrophysiological and molecular studies will warrant the understanding of the mechanism of action of 17\(\alpha\)-meT through the GABAergic system in avoidance learning.

**Conclusion**

The findings in this thesis are the first to suggest that a single dose is sufficient to induce changes in normal memory throughout adolescence in rats. Further studies need to address if the same effect is observed in humans. If so, this study should be addressed as an imperative flag for prevention, since adolescence is a crucial period of development and a population at risk of acquiring and consuming AAS. Strategies to reduce overall AAS risk in population should target a younger audience, and by our findings also have a sex specific intervention.
Bibliography:


