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## THE ANABOLIC STEROIDS TESTOSTERONE PROPIONATE AND NANDROLONE, BUT NOT 17 $\alpha$ -METHYLTESTOSTERONE, INDUCE CONDITIONED PLACE PREFERENCE IN ADULT MICE

Jeffrey Parrilla-Carrero<sup>1</sup>, Orialis Figueroa<sup>2</sup>, Alejandro Lugo<sup>3</sup>, Rebecca García-Sosa<sup>4</sup>, Paul Brito-Vargas<sup>1</sup>, Beatriz Cruz<sup>5</sup>, Melanis Rivera<sup>6</sup>, and Jennifer L. Barreto-Estrada<sup>7</sup>

<sup>1</sup> Department of Science and Technology, AGMUS, Universidad del Este (UNE), Carolina, PR 00984

<sup>2</sup> Department of Biology, University of Puerto Rico, Humacao Campus, PR 00791

<sup>3</sup> Department of Biology, Interamerican University, Metropolitan Campus, San Juan, PR 00919

<sup>4</sup> Department of Biology, University of Puerto Rico, Río Piedras Campus, San Juan, PR 00936

<sup>5</sup> Department of Biology, University of Puerto Rico, Mayagüez Campus, PR 00681

<sup>6</sup> Department of Social Sciences, University of Puerto Rico, Cayey Campus, PR 00736

<sup>7</sup> School of Medicine, Department of Anatomy and Neurobiology, Medical Sciences Campus, University of Puerto Rico, San Juan, PR 00936

### Abstract

Anabolic androgenic steroids (AAS) are often misused by adolescents and athletes. Their effects vary according to chemical structure and metabolism, route of administration, and AAS regimen. In this study, adult C57Bl/6 male mice were systemically exposed to testosterone propionate (TP), nandrolone or 17 $\alpha$ -methyltestosterone (17 $\alpha$ -meT), type I, type II and type III AAS, respectively, in order to determine the hedonic or aversive properties of each drug. For this purpose, the conditioned place preference (CPP) test was employed at three different AAS doses (0.075, 0.75 and 7.5 mg/kg). Other behavioral domains monitored were light-dark transitions (side changes) and general activity. TP shifted place preference at all doses tested, and nandrolone shifted place preference at 0.75 and 7.5mg/kg, but not at 0.075 mg/kg, the lower dose tested. Conversely, mice receiving 17 $\alpha$ -meT did not show alteration in the preference score. The lower dose of nandrolone did modify exploratory based-anxiety showing a decrease in light-dark transitions if compared to vehicle-treated animals, while mice treated with TP or 17 $\alpha$ -meT were not affected. Our data suggest that when studying hedonic and rewarding properties of synthetic androgens, distinction has to be made based on type of AAS and metabolism.

### Keywords

conditioned place preference; anabolic androgenic steroids; reward; mice

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Corresponding author: Dr. Jennifer L. Barreto Estrada, PHONE: 787-758-2525, ext. 7013 TELEFAX: 787-767-0788, E-mail: E-mail: jbarreto@rcm.upr.edu.

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## 1. INTRODUCTION

The synthetic derivatives of testosterone, known as anabolic androgenic steroids (AAS), are considered drugs of abuse and are misused in supraphysiological doses by an increasing number of athletes and adolescents (Bahrke et al., 1998; Hartgens & Kuipers, 2004). It has been argued that motivation for AAS misuse includes improvement of physical appearance, muscular strength, and/or peer approval (Schwerin et al., 1996). A growing body of work in rodents shows that androgen compounds have hedonic (Frye et al., 2001; Arnedo et al., 2002; Packard et al., 1997) and reinforcing effects (Johnson & Wood, 2001; Wood et al., 2004; Frye 2007; Frye et al., 2007). These effects have been shown with a variety of experimental paradigms including conditioned place preference (CPP) (Frye et al., 2001; Arnedo et al., 2002), oral self-administration (Johnson & Wood, 2001), jugular self-administration (Jorge et al., 2005), and intracerebral self-administration (Rosellini et al., 2001; Wood et al., 2004). However, rewarding properties of AAS have received relatively little attention.

There are approximately 60 different AAS compounds that can be classified in three classes (for review see Clark & Henderson, 2003) based upon their chemical structure and metabolism. Class I AAS are injectable compounds derived from esterification of the 17 $\beta$ -hydroxyl group of testosterone, which can be aromatized to estrogens, reduced to dihydrotestosterone (DHT), or hydrolyzed into testosterone. Class II AAS are comprised of injectable 19-nortestosterone derivatives, which have a longer half-life and less androgenic activity than Class I AAS. Similar to class I, Class II AAS can be aromatized to estrogens or reduced to DHT. The alkylation at carbon 17 distinguishes Class III AAS; this modification retards the metabolism of this class of AAS by the liver, making them orally active (Kuhn, 2002), and with restricted potential for aromatization (Andersen et al., 2006; Mor et al., 2001; Zhang et al., 2007). Class II and III are probably the most abused by adolescents and athletes (for review see Clark & Henderson, 2003; Pope & Brower, 2000). In this study we sought to investigate and compare rewarding effects of testosterone propionate (TP), nandrolone and 17 $\alpha$ -methyltestosterone (17 $\alpha$ -meT), representatives of type I, type II and type III AAS, respectively. Since differences in chemical structure and metabolism are present between these three drugs, we hypothesized that a differential behavioral response will be found using CPP, a paradigm that examines positive hedonic and reinforcement effects of drugs (Reid et al., 1989; Tzschentke, 1998). Specifically, TP has been modified adding a 3 carbon (3C) ester; nandrolone (19-nortestosterone) is very similar to testosterone, but a carbon (C19) is missing; and 17 $\alpha$ -meT has a methyl group at carbon 17 (C17). Differences in metabolism between the three AAS are also present, being one of the most significant, the ability to be aromatized to estrogen by the aromatase enzyme. Anxiety-related behaviors and general activity were monitored as well, to better understand specific patterns of behavioral alteration. Our data suggest that different classes of anabolic steroids elicit differential modulation in hedonia and reward that could reflect the complex AAS metabolism, and consequently, their interaction with cellular substrates.

## 2. METHODS

### 2.1 Subjects

Adult (PN-90) C57B1/6J-NHsd mice male mice were purchased from Charles River and housed individually in the Animal Resources Center of the Medical Sciences Campus of the University of Puerto Rico (MSC-UPR) with food and water available *ad libitum*. They were kept on a 12:12 hour light/dark cycle. The temperature and relative humidity in the housing facility is 64 °F and 30%, respectively. The experiments were performed during the dark portion of the cycle, following the guidelines and approval of the Institutional Animal Care and Use Committee (IACUC) of the MSC-UPR and in accordance with USDA, National Institutes of Health, and American Veterinary Medical Association regulations.

## 2.2 Androgen administration

Control groups received intraperitoneal (i.p.) injections of vehicle, which consisted of 0.9% NaCl and 30% cyclodextrin (0.02cc/10g of body weight). Experimental groups received i.p. injections of TP, nandrolone or 17 $\alpha$ -meT at doses of 0.075, 0.75 and 7.5mg/kg (0.02cc/10g of body weight). TP, nandrolone (19-nortestosterone) and 17 $\alpha$ -meT were purchased from Sigma Chemical Co. (St. Louis, MO). As 1 mg/kg is sufficient to restore endocrine function and reproductive behaviors in rodents (Clark & Barber, 1994), the dosage used in this experiment reflects a low, intermediate and high dose, respectively.

## 2.3 Behavioral assessment

### 2.3.1 Behavioral paradigm 1

**Conditioned place preference (CPP):** This paradigm has been used typically to measure hedonic properties of drugs of abuse as well as natural reinforcers (Packard et al., 1997; Tzschentke, 1998). Acrylic chambers (42cm long  $\times$  30cm high  $\times$  42cm high) were used. A black acrylic box (40.6 cm long  $\times$  20.3 cm high  $\times$  12.7 cm wide) with an entry opening (10.2 cm  $\times$  6.4 cm) was placed alongside the activity chamber. Chambers were cleaned between animals with a mild detergent to minimize olfactory cues between sessions. Sessions were videotaped for subsequent analysis and interpretation.

CPP was performed with the lights 'on' and lasted 13 days (days 0–12), Fig. 1. On day 0, animals were habituated to the recording chambers without the black acrylic box for 20 min. On day 1 (baseline), individual animals were allowed to move freely between the light and the dark compartments of the chamber for 15 min. The time spent (in sec) in a given compartment was recorded. The preference score (PS) for a given compartment was calculated as follows:  $PS = (\% \text{ time in the non-preferred side} / \% \text{ time in the non-preferred} + \text{preferred side}) \times 100$ . Mice that demonstrated strong preference for a given compartment ( $> 90\%$ ) during the baseline were excluded from the analysis. Animals were randomly assigned to control or experimental groups. Experimental groups received either intraperitoneal (i.p.) injections of a given AAS (TP, nandrolone or 17 $\alpha$ -meT) and were restricted to the non-preferred (NP) side of the chamber, or they received i.p. vehicle injections and were restricted to the preferred side (P). Control groups received daily vehicle injections (i.p.) and were restricted to a given compartment (P vs. NP) of the chamber for 30 min. AAS versus vehicle i.p. injections in the experimental groups occurred on alternate days for a total of 5 injections of vehicle and 5 injections of a given AAS in the experimental group. On day 13 (test day), injection-free animals were allowed to move freely between the light and the dark compartments of the chambers, and the PS was recalculated.

### 2.3.2 Behavioral paradigm 2

**Light-dark transitions (side changes):** To measure exploratory-based anxiety, the number of transitions between the dark and illuminated side of the chamber were measured during the baseline and test day (30 min each). This behavior is optimized for mice, titrating the tendency of mice to explore a novel environment versus the aversive properties of a brightly lit open field (Crawley, 2000). Classic anxiolytics can be detected using this paradigm (Bourin and Hascoët, 2003). Anxiogenic animals will display reduced transitions between the dark and light chambers (Zhang et al., 2007).

### 2.3.3 Behavioral paradigm 3

**General activity:** Jumping and climbing activities were scored on baseline and test day during the CPP. The door between the dark and light chambers has a thin rim above its aperture and animals showed a tendency to climb over it. Thus, we scored jumping when animals attained

vertical locomotion (jumping) without making contact with the door rim, and climbing was scored when at least two animal's paws contacted the door rim.

## 2.4 Neuroendocrine effects

Gonadal weight- After the CPP on day 13 (test day), animals were sacrificed to analyze testicular weight between vehicle and AAS-treated animals.

## 2.5 Statistical analysis

Data are presented as mean  $\pm$  standard error of the mean (S.E.M.). One-way analysis of variance followed by Tukey Test post hoc analysis was employed. Statistical significance was established at  $p \leq 0.05$

# 3. RESULTS

## 3.1 Behavioral paradigm 1: CPP

We tested the effect of three different classes of AAS compounds, TP, nandrolone and  $17\alpha$ -meT in the CPP task after five alternating days of systemic exposure. No statistically significant differences were found in the preference score between baseline and the test day. Indeed, none of the tested behaviors showed significant differences between baseline and test day (data not shown). Thus, we proceeded to analyze the vehicle and androgen-treated animals during the test day. TP at all doses tested shifted the place preference in the CPP paradigm,  $F(3, 36) =$ ,  $p < 0.05$ . Post hoc analysis showed significant differences between the vehicle-treated group and each of the three doses (Fig. 2A). In particular, the preference score for control animals was  $(53.38 \pm 4.38)$ . This value was shifted to  $(66.80 \pm 3.23)$ ,  $(66.49 \pm 2.26)$ , and  $(66.56 \pm 2.85)$  for animals treated with TP at 0.075, 0.75, and 7.5 mg/kg, respectively, ( $p < 0.05$ ).

A statistically significant difference was observed in the preference score between nandrolone and vehicle treated animals,  $F(3, 33) = 4.79$ ,  $p < 0.01$ . Post hoc analysis showed significant differences between groups of animals treated with the higher doses of nandrolone (Fig. 2B) when compared with the vehicle group. Vehicle-treated animals exhibited a preference score of  $28.84 \pm 3.62$ , whereas those treated with nandrolone at 0.75 and 7.5 mg/kg showed significant increases in the preference score to  $41.47 \pm 1.46$  ( $p < 0.01$ ) and  $39.66 \pm 2.14$  ( $p < 0.05$ ), respectively. Preference score was not affected in animals treated with nandrolone at 0.075 mg/kg. Thus, animals treated with nandrolone at higher doses displayed an increase in the preference for the non-preferred side of the chamber. In contrast, no statistical differences were found between  $17\alpha$ -meT and vehicle groups,  $F(3, 28) = 0.209$ ,  $p = 0.889$ , during the same experimental conditions in the CPP (Fig. 2C). Specifically, vehicle treated animals showed a preference score of  $32.86 \pm 4.54$  which is not statistically different from  $17\alpha$ -meT preference scores at 0.075 mg/kg ( $34.34 \pm 3.96$ ), 0.75 mg/kg ( $37.91 \pm 5.23$ ) and 7.5 mg/kg ( $34.90 \pm 5.23$ ).

## 3.2 Behavioral paradigm 2: light-dark transitions

Figure 3 shows the effect of AAS in exploratory-based anxiety as measured by light-dark transitions (side changes). A statistical difference in the frequency of transitions between nandrolone and vehicle treated animals was observed,  $F(3, 17) = 3.91$ ,  $p < 0.05$ . Post hoc analysis revealed that mice receiving nandrolone 0.075 mg/kg showed a significant reduction in exploration (Fig. 3B) between the light and dark compartments (vehicle:  $32 \pm 2.52$ ; nandrolone 0.075 mg/kg:  $19.60 \pm 1.33$ ),  $p < 0.05$ . No differences were found at higher doses of nandrolone. On the other hand, TP (Fig. 3A) nor  $17\alpha$ -meT (Fig. 3C) produced differences in the frequency of light-dark transitions.

### 3.3 Behavioral paradigm 3: general activity

General activity as assessed by jumping/climbing activity was only affected by TP at 0.75 mg/kg. However, we have found that TP at this dose did not affect locomotor activity as assessed by the elevated plus maze (data not shown).

### 3.4. Neuroendocrine effects

Finally, the selected dose levels, the duration, and the pattern of exposure to TP, nandrolone and  $17\alpha$ -meT did not show any effect in gonadal weight as assessed by the analysis of testicular weight between control and AAS-treated animals (data not shown).

## 4. Discussion

We have found that TP and nandrolone, but not  $17\alpha$ -meT, produces a dose-dependent shift in place preference after 5 systemic AAS injections. Only the higher doses of 0.75 and 7.5 mg/kg shifted preference to the non-preferred compartment of the chamber in nandrolone-treated animals, while TP was effective at all doses tested. These effects in CPP cannot be attributed to changes in general activity of the animals. The lack of alteration of general locomotor activity after TP and nandrolone treatment was previously reported. In particular, this study demonstrated that several nandrolone doses ranging from 3.75 to 30 mg/kg given to male mice had no effect in locomotion (Salvador et al., 1999). In our study, although TP (0.75 mg/kg) displayed an increase in jumping/climbing (vertical activity), we have found no effect when animals were tested in the elevated plus maze (data not shown).

The rewarding effect of naturally-occurring androgens (testosterone) according to the CPP task has been previously demonstrated in male mice (Arnedo et al., 2002) and male rats (Frye et al., 2001). In addition, it has been shown that male rodents self-administer naturally-occurring androgens (Frye et al., 2007; Johnson and Wood, 2001; Rosellini et al., 2001; Wood et al., 2004). However, limited studies have been done to assess the rewarding properties of AAS. Whenever present, these studies preclude the effects of anabolic steroids in determining the modulatory effect in the rewarding properties of other drugs of abuse (C  lerier et al., 2003; 2006). Now, we present for the first time a study that contrast the rewarding effects of three different types of synthetic androgens that differ in chemical structure and metabolism using the conditioned place preference test.

No studies have been found in the literature showing the effect of nandrolone or  $17\alpha$ -meT in CPP, although Gans and Erskine (2003) demonstrated that neonatal TP treatment did not disrupt or enhance the CPP induced by paced mating. The differential modulation we observed in the CPP ie., TP and nandrolone vs.  $17\alpha$ -meT might reflect a distinct ability for each class of AAS to act in estrogenic receptors. It is well known that unlike TP and nandrolone,  $17\alpha$ -meT decrease the aromatase mRNA expression, or inhibit its activity acting as a competitive aromatase inhibitor (Mor et al., 2001; Zhang et al., 2007). Furthermore, a decrease in endogenous E2 levels have been reported after exposure to  $17\alpha$ -meT (Andersen et al., 2006). On the contrary, aromatase has been shown to be upregulated in a study using nandrolone as the AAS source (Roselli, 1998; Takahashi et al., 2007; 2008), while a decrease was observed after exposure to 17-alpha alkylated compounds (Roselli, 1998). Thus, the product metabolism of aromatase activity ie., estrogen, seems to be a key candidate for the differential modulation observed in the CPP. Estradiol subcutaneous injections induced CPP in ovariectomized rats (Frye and Rhodes, 2006) and may involve actions at estrogen receptors in the nucleus accumbens (Walf et al., 2007). Moreover, DiMeo and Wood (2006) have shown that estradiol is reinforcing in male hamster. Similar differential modulation has been previously shown when studying other behavioral paradigms. For instance, differences in male aggression between  $17\alpha$ -meT, testosterone propionate, stanozolol and nandrolone decanoate may reflect

differences in the abilities to act at androgen versus estrogenic receptors (for review see Clark and Henderson, 2003; McGinnis et al., 2002).

AAS compounds can target a variety of neurochemical substrates in the brain including the GABAergic, dopaminergic and peptidergic systems (for review see Clark and Henderson, 2003). It is well known from previous work that steroids interact with the benzodiazepine/GABA receptor complex (Bitran, 1993; Majewska, 1992; Gee et al., 1988) and that AAS can modulate several subunits of the GABA<sub>A</sub> receptor (Jones et al., 2006; Yang et al., 2002; 2005). Moreover, it is now clear that the testosterone metabolite 3 $\alpha$ -androstane-3 $\alpha$ , 17 $\beta$ -diol (3 $\alpha$ -diol) acts through this GABAergic complex, and is one of the most potent modulators of this system (for review see Frye, 2007). 3 $\alpha$ -diol is converted from dihydrotestosterone (DHT) by the action of the 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD). The fact that TP and nandrolone can be metabolized to 3 $\alpha$ -diol and produced a shift in place preference might be in agreement with Frye (2007) who had demonstrated that this metabolite elicited the most rewarding effects of testosterone. This contrast with the knowledge that 17 $\alpha$ -alkylated compounds can not be reduced to DHT nor converted to 3 $\alpha$ -diol (for review see Clark and Henderson, 2003; Sundaram et al., 1995). Correspondingly, 17 $\alpha$ -meT proved to be a stronger inhibitor of 5 $\alpha$ -reductase, the enzyme responsible for the irreversible conversion of testosterone to DHT (Lo et al., 2007). Therefore, if the cellular concentration of DHT is depleted by 17 $\alpha$ -alkylated compounds, a decrease in the rewarding metabolite 3 $\alpha$ -diol could be observed.

A connection between AAS, the mesocorticolimbic reward system and the central dopaminergic activity have been study for many years. Thus, dopamine and its metabolites have been studied in mesolimbic structures such as the nucleus accumbens and the striatum. A study where comparison of the dopaminergic metabolism between class I (TP), class II (nandrolone) and class III (methandrostenolone) was assessed in the striatum, found no differences in dopamine (DA) metabolites between AAS treatment, although all of the AAS tested increase their levels if compare to control animals (Thiblin et al., 1999). Based in this study, a differential modulation of DA levels or its metabolites by different classes of AAS, could not be a plausible explanation for the differences we observed in CPP.

Several domains of anxiety have been typically shown to be affected by AAS in animal models (Barreto-Estrada et al., 2004; Bitran et al., 1993; Kouvelas et al., 2008; Rocha et al., 2007). In this study we observed a decrease in exploratory-based anxiety as assessed by light-dark transitions but only when animals were exposed to the lower dose (0.075 mg/kg) of nandrolone. This result can be interpreted as an increase in anxiety during exploratory behavior at this dose level. Nandrolone (5 mg/kg) has been shown to increase anxiety levels in rats when tested in the elevated plus maze (EPM), a paradigm that measure generalized anxiety (Rocha et al., 2007). The fact that we do not observed an anxiogenic response at a similar dose (7.5 mg/kg) might be due to our short-term steroid exposure (5 alternate days), if compared with the long-term administration of twice a week for six weeks (Rocha et al. 2007) and/or drug desensitization at higher doses. Interestingly, a more recent study that also administered nandrolone for 6 weeks, showed an anxiolytic effect at a dose of 15 mg/kg (Kouvelas et al., 2008), a two-fold increase in the nandrolone concentration if compared with the study of Rocha and colleagues (2007). These results argue in favor of nandrolone producing anxiogenic versus anxiolytic effects depending of the pharmacological dose and regime of administration. In our study it is important to highlight the dissociation between hedonic properties and anxiety. It seems that low levels of nandrolone are required to attain changes in anxiety status, while higher doses (0.75 and 7.5 mg/kg) elicit shifts in place preference.

Other behavioral studies (Barreto-Estrada et al., 2004) have shown no significant effects in the light-dark transitions or in locomotor activity after two weeks of systemic exposure to 17 $\alpha$ -meT (7.5 mg/kg). In this previous study, 17 $\alpha$ -meT decreased gonadal weight. However, in the

present study five alternate injections of AAS were not enough to produce similar effects. These results interestingly suggest that it might takes longer to produce endocrine changes than to produce changes in hedonia and reward.

It is difficult to assess whether the improvement of physical appearance, the increase in muscular strength and performance, hedonia, and/or reinforcing effects is underlying the misuse of androgen compounds in the human population. However, anecdotal information from AAS users points towards an association between the misuse of anabolic steroids and mood disorders, depressive symptoms, euphoria (Bahrke et al., 1998; Perry et al., 1990; Wood, 2004) and positive (pleasure) emotions (Brower et al., 1991). Nevertheless, a growing body of experimental evidence with animal models clearly shows that androgen compounds possess rewarding and reinforcing properties. In conclusion our results suggest that: 1) not all AAS elicit hedonic effects; 2) the AAS-induced hedonic effects might be dose-dependent, 3) doses of AAS not producing hedonia might alter other behavioral domains such as anxiety-related behaviors and 4) the interaction of AAS and their metabolites with cellular substrates, more than the chemical structure, might ultimately predict their behavioral effects. Further basic and translational studies will be required to unravel the hedonic and rewarding properties of anabolic steroids in humans.

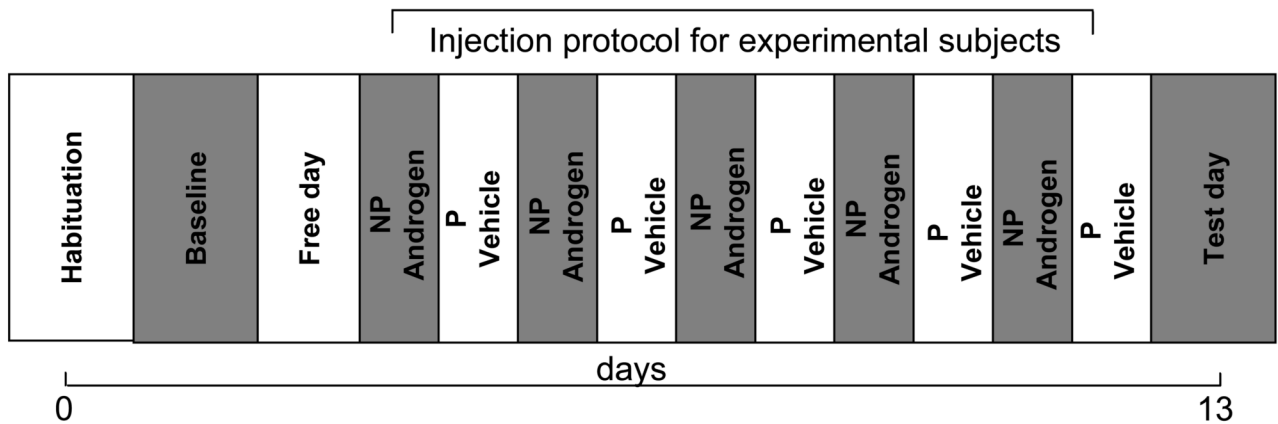
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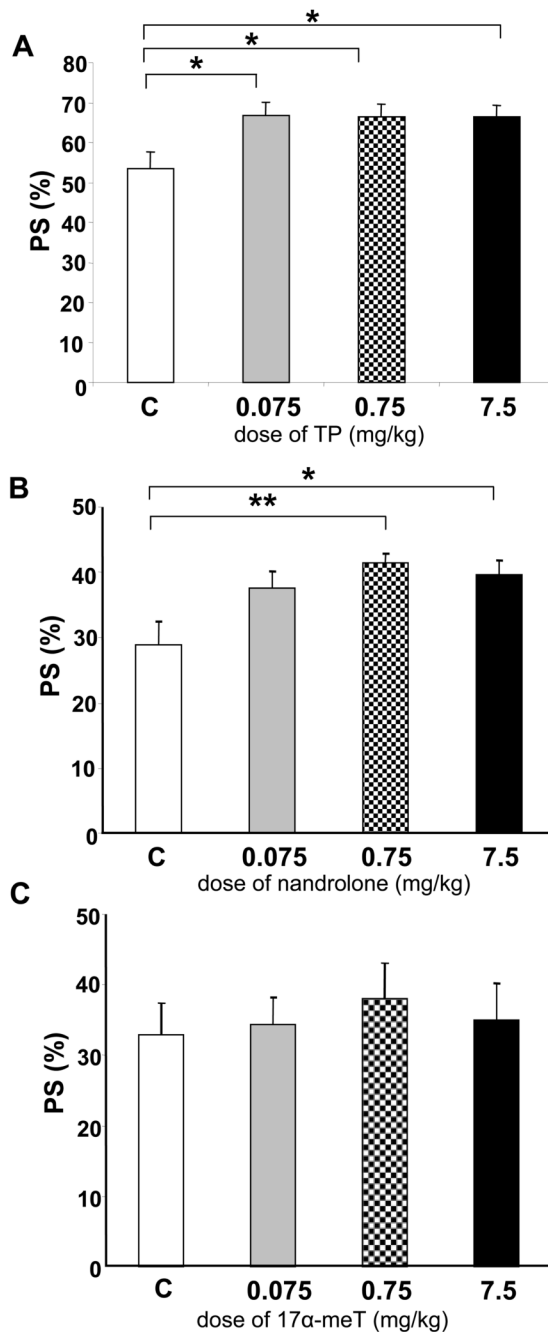


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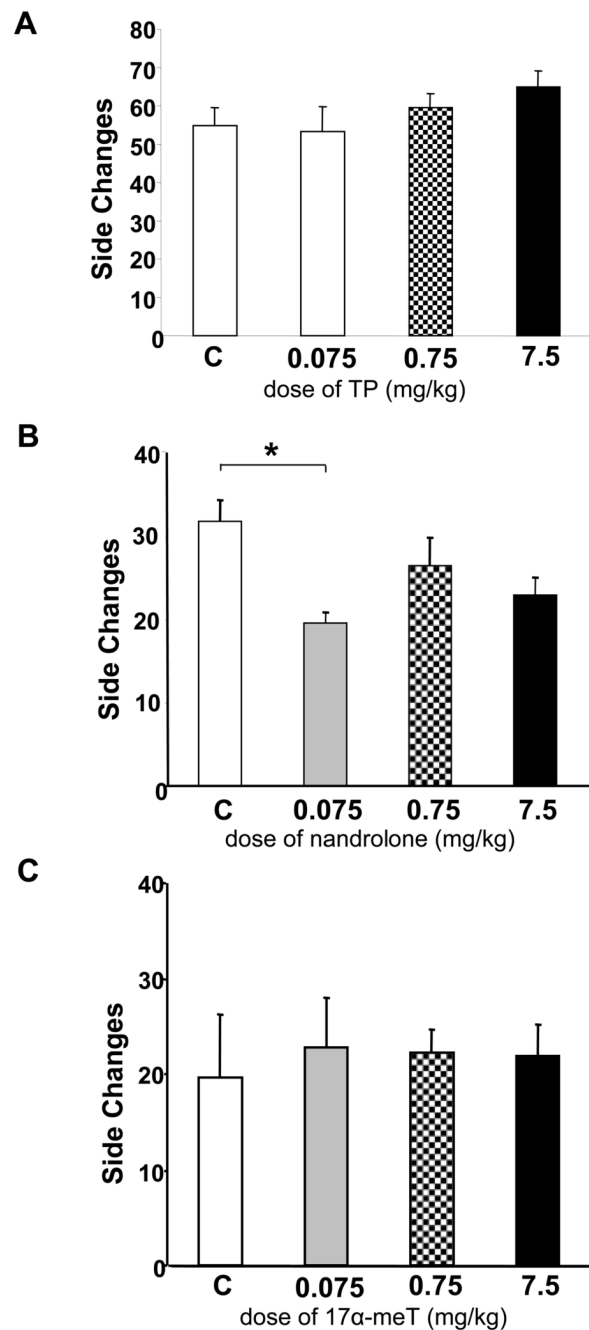


**Figure 1. Experimental design and injection protocol for the conditioned place preference (CPP) in AAS treated animals**

CPP was performed for 13 days (days 0–12). On day 0 animals were habituated to the recording chambers for 20 min. On day 1 (baseline), animals were allowed to move freely between the dark and light compartments of the chamber for 15 min. Preference score for a given compartment was immediately calculated. After a free day (day 2), alternate injections (i.p.) of androgen or vehicle were started (days 3–12) in the non-preferred (NP) and preferred side (PS) of the chamber, respectively. For each injection, animals spent 30 min in the chamber. On day 13 (test day), injection free animals were allowed to move freely between the light and dark compartments, and the preference score was recalculated. Control animals received daily injections of vehicle and were restricted to a given compartment of the chamber for 30 min each.



**Figure 2. Effect of TP, nandrolone and 17 $\alpha$ -meT on conditioned place preference (CPP) test**  
 (A) Animals receiving TP at all doses tested showed a significant increase in the preference score for a given compartment of the testing arena. (B) Similarly, nandrolone at 0.75 and 7.5 mg/kg showed a significant increase in the preference score. (C) The AAS 17 $\alpha$ -meT did not modify the place preference at any of the dose levels tested. All bars represent the data of the test day. For TP, vehicle: n= 10; 0.075 mg/kg: n= 8; 0.75 mg/kg: n= 10; 7.5 mg/kg: n= 9. \*  $p \leq 0.05$  vs. vehicle. For nandrolone, vehicle: n= 9; 0.075 mg/kg: n= 8; 0.75 mg/kg: n= 9; 7.5 mg/kg: n= 8, \* $p \leq 0.05$  vs. vehicle, \*\* $p \leq 0.01$  vs. vehicle. For 17 $\alpha$ -meT, vehicle: n= 7; 0.075 mg/kg: n= 6; 0.75 mg/kg: n= 8; 7.5 mg/kg: n= 8. Error bars represent standard error of the mean.



**Figure 3. Effect of TP, nandrolone and 17 $\alpha$ -meT on light-dark transitions**

Animals receiving nandrolone at 0.075 mg/kg throughout the CPP task (B) showed a reduction in the frequency of light-dark transitions (side changes). Animals treated with higher doses were not affected. TP (A) or 17 $\alpha$ -meT (C) do not produced changes in light-dark transitions at any of the dose levels tested. All bars represent the data of the test day. For TP, vehicle: n= 10; 0.075 mg/kg: n= 8; 0.75 mg/kg: n= 10; 7.5 mg/kg: n= 9. For nandrolone, vehicle: n= 3; 0.075 mg/kg: n= 5; 0.75 mg/kg: n= 5; 7.5 mg/kg: n= 5 \*p < 0.05 vs. vehicle. For 17 $\alpha$ -meT, vehicle: n= 5; 0.075 mg/kg: n= 6; 0.75 mg/kg: n= 12; 7.5 mg/kg: n= 8. Error bars represent standard error of the mean.