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Rewarding Properties of Testosterone in Intact Male Mice: A Pilot Study

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ARNEDO, M. T., A. SALVADOR, S. MARTINEZ-SANCHIS AND E. GONZALEZ-BONO. *Rewarding properties of testosterone in intact male mice: A pilot study.* PHARMACOL BIOCHEM BEHAV **65**(2) 327–332, 2000.—The present study examined the rewarding properties of 4-androsten-17β-ol-3-one testosterone in intact male mice using the conditioned place preference (CPP) technique. In Experiment 1, the pharmacokinetics of 0.8 and 1.2 mg/kg of testosterone were studied to determine the most appropriate temporal interval to test behavior. Additionally, the locomotor activity was recorded to control a possible interfering effect on CPP. The maximum testosterone concentration was registered at 45 min of administration, and no effects on activity were found. In Experiment 2, three groups of male OF-1 mice received four pairings of the least-preferred compartment with testosterone (0.8, 1, or 1.2 mg/kg, SC) for 30 min. On alternate days the preferred compartment was paired with vehicle for 30 min. The control group received vehicle in both compartments. No significant differences between groups were found in the time spent in the drug-paired compartment. However, when separate analyses were performed in conjunction with the color of the drug-paired compartment, CPP was observed only in animals pairing testosterone/black compartment. These results suggest that rewarding properties of testosterone treatment can be observed in male mice; these effects probably being dependent on the environmental cues used as conditioned stimuli.   © 2000 Elsevier Science Inc.

Conditioned place preference   Testosterone   Intact male mice   Rewarding properties   Pharmacokinetics

ANABOLIC androgenic-steroids (AAS) are synthetic testosterone derivatives that have been increasingly used by athletes to improve their performance, and more recently by other groups to enhance their physical appearance through increases in their muscular mass (11,12). Parallel to its abuse, increasing attention has been paid to the dependence on AAS in the last years. Based on two case reports of apparent dependence (6,23), Kashkin and Kleber (13) proposed the "anabolic steroid addiction hypothesis," although without support from the scientific research at that time. Soon afterwards, several studies showed that AAS abuse can lead, in some cases, to a substance abuse disorder similar to that produced by other abused substances [for review (3)]. Despite this fact, the mechanisms involved in AAS dependence are still not clear. It has been suggested that positive effects of abuse like euphoria, sense of well-being, better performance and improvement of appearance together with the social reinforcement derived from these effects probably perpetuate the intake (4). Recently, a few studies have focused on the rewarding properties of testosterone and its derivatives using animals as experimental subjects.

Several studies have employed the conditioned place-preference (CPP) technique to test the rewarding properties of drugs or natural rewards [for review (8)]. With this method, it has been shown that peripheral (1,7,10) and intraaccumbens (19) administration of testosterone has rewarding properties in male rats. Furthermore, the place preference produced by testosterone has been inhibited by treatment with a dopaminergic antagonist (20), suggesting that the dopaminergic system is involved in the testosterone rewarding effects, as in other abused substances (2).

The main aim of this work (Experiment 2) was to throw light on testosterone's rewarding properties using the CPP in male mice on whom, to our knowledge, there are no published data addressing this issue. Before this work, an experiment (Experiment 1) was carried out to test pharmacokinetics of different doses of testosterone, taking different temporal intervals and analyzing the testosterone serum concentration

to determine the timing of drug administration in the CPP study. Moreover, it has been suggested that increases in locomotor activity produced by many rewarding drugs could inhibit CPP of these substances (8). In consequence, in Experiment 1 motor activity was registered before blood extraction to assess whether the doses of testosterone administered produce increases in activity that could interfere in the CPP process.

METHOD

Subjects

One hundred and ten male OF-1 mice (CRIFFA-CREDO, Lyon, France), aged 6–7 weeks at arrival at laboratory, were housed in groups of four in cages measuring 28 × 11 × 12, or six in bigger cages (21.5 × 21.5 × 15 cm) under a constant ambient temperature (20 ± 1°C) and a 12 h light/dark cycle automatically controlled (lights off: 1800–0600 h, in Experiment 1 and 0800–2000 h, in Experiment 2). The animals had free access to food and water. All tests were conducted and blood samples obtained during the animals' dark cycle, starting at the second hour.

Drugs

4-androsten-17β-ol-3-one testosterone (Sigma, Madrid, Spain) was dissolved in peanut oil (Guinama, Valencia, Spain) to obtain testosterone doses: 0.8, 1, and 1.2 mg/kg. These doses were selected in conjunction with studies in male rats on the rewarding properties of testosterone (1,10). Each mouse was injected subcutaneously with testosterone or vehicle (peanut oil) at a volume of 0.1 ml.

In Experiment 1 (pharmacokinetics study), groups differ in the substance and dose administered (peanut oil, 0.8 mg/kg and 1.2 mg/kg of testosterone) and in the temporal interval between the administration and the blood extraction (45, 75, and 105 min). In Experiment 2 (CPP study), animals received testosterone (0.8, 1, or 1.2 mg/kg) or vehicle 30 min prior to testing.

Apparatus

In Experiment 1, an open field consisting of a transparent glass cylinder with a floor divided into four identical segments was used to test activity.

In Experiment 2, CPP was assessed using four Plexiglas apparatus consisting of two main conditioning compartments (30 × 30 × 30 cm) connected to each other by a neutral middle compartment (10 × 12 × 30 cm). The lateral compartments offered distinct stimuli in color and floor texture. One of them was white, with the floor covered by rough white mesh (60 mm), and the other one was black with a fine gray plastic mesh floor. The animals were observed through a translucent wall of the boxes.

At the beginning of the experiment each animal was randomly assigned to one of the four boxes where all the behavioral procedure was carried out. The experimental sessions were performed in a dimly illuminated room (red bulb of 40 w). After removing each animal from the boxes, the mesh floor was changed and the boxes were cleaned of urine and feces.

Procedure

Ten days after arrival at laboratory animals were randomly assigned to experimental conditions.

In Experiment 1 (see Table 1), animals were placed into

the open field for 15 min. The first 5 min were considered as an adaptation phase, while the remaining 10 min were evaluated by taking into account the cross and rearing frequency and the number of fecal boluses. These activity tests were carried out under a red light. Five minutes afterwards, animals were sacrificed with a lethal intraperitoneal injection of Pentothal (Laboratories Abbott S.A., Madrid, Spain), and blood samples were obtained.

In Experiment 2, the CPP procedure required 10 days, involving three phases: preconditioning test (one session); conditioning (eight sessions); and postconditioning test (one session).

On day 1, the preconditioning test was carried out. Subjects were situated in the middle compartment and allowed to freely explore the three compartments for 30 min to determine the initial preference for the two larger compartments under nondrug conditions. The compartment, of the two largest, where subjects spent less time was defined as the initially least preferred compartment (ILPC). Thirty-two animals showed less preference for the white compartment, whereas only eight of them showed less preference for the black one.

Animals were allocated to four groups: vehicle (0.0 mg/kg testosterone) (*n* = 10); 0.8 mg/kg testosterone (*n* = 10); 1 mg/kg testosterone (*n* = 10) and 1.2 mg/kg testosterone (*n* = 10). Each group was formed of eight mice with less preference for the white compartment and two mice with less preference for the black one. Subjects were distributed in such a manner that there were no significant differences in the time experimental groups spent in the less preferred and preferred compartments. Conditioning was conducted over an 8-day period. At every conditioning session each mouse was injected with testosterone or vehicle (alternate days), and 30 min later was confined in the appropriate compartment for 30 min, so that it could not explore or see the other compartments of the apparatus.

The ILPC in the preconditioning test was designated as the conditioned stimulus; thus, it was paired with testosterone, whereas the preferred compartment was paired with vehicle except for a control group that received vehicle in both compartments. The rationale for this group was to control changes in preference for the drug-paired compartment due to the repeated exposure to the conditioned stimulus during conditioning phase without being paired with the drug.

In consequence, thirty-two animals associated testosterone with the white compartment while the other eight associated testosterone with the black one. One conditioning session per day and four conditioning sessions per compartment were car-

TABLE 1
EXPERIMENTAL CONDITIONS IN EXPERIMENT 1

Number of Group	Substance	Temporal Interval	Size (*n*)
1	peanut oil	45 min	6
2	peanut oil	75 min	5
3	peanut oil	105 min	6
4	0.8 mg/kg T*	45 min	6
5	0.8 mg/kg T	75 min	6
6	0.8 mg/kg T	105 min	7
7	1.2 mg/kg T	45 min	6
8	1.2 mg/kg T	75 min	7
9	1.2 mg/kg T	105 min	7

*T = testosterone.

ried out. Within each group (except the control group), half of the animals received testosterone on even-numbered days and vehicle on odd-numbered days, whereas for the other half, the pattern was reversed.

The postconditioning test followed the last conditioning session by 24 h, when the drug-free animals were again allowed to freely explore the three compartments, exactly as in the preconditioning test. The time spent in each compartment was videorecorded and used for the evaluation of the CPP. The coder was blind as to each animal's experimental group.

Two animals died during the experimental procedure, so they were removed from the data analysis.

Hormone Assay

The hormone assay, was only carried out in Experiment 1. Blood samples were withdrawn by cardiac puncture and prepared by centrifugation to separate the serum, which was frozen ($-80°C$) until assay. Testosterone analyses were carried out in the Central Research Unit, Faculty of Medicine, University of Valencia (Valencia, Spain) using Coat-A-Count Total Testosterone Kits (Diagnostic Products Corporation, Los Angeles). The sensitivity was 4 ng/dl. The intra- and interassay variation coefficients were respectively 3 and 13%. Testosterone (nmol/l) values were the mean of duplicated determinations.

Statistics

Data from Experiment 1 were analyzed by means of two-factor (treatment, time) analyses of variance (ANOVA) for the main effects and their interactions, followed by post hoc pairwise comparisons (Newman–Keuls tests).

In Experiment 2, data of time spent in the ILPC (drug-paired compartment except for the control group) before and after conditioning were assessed by ANOVA. The design consisted of one between-subject factor (treatment, with four levels: vehicle (0.0 mg/kg testosterone); 0.8 mg/kg testosterone; 1 mg/kg testosterone; and 1.2 mg/kg testosterone) and one within-subject factor (test, with two levels: pre- and postconditioning). Additionally, the same ANOVA was performed including another between-subject factor "drug-paired compartment" with two levels: black and white (conditioned to the black or white compartment, respectively).

All calculations were performed using the statistical package SPSS (18). Results were considered statistically significant if $p < 0.05$.

Ethics

The use and care of animals and the experimental protocol were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

RESULTS

Experiment 1

No significant effects due to treatment or time were found with respect to the activity indexes (cross and rearing frequency, and fecal boluses).

However, effects of the treatment, $F(2, 47) = 53.72$, $p < 0.001$, time, $F(2, 47) = 29.01$, $p < 0.001$, and their interaction, $F(4, 47) = 5.68$, $p < 0.001$, were found on testosterone concentrations. The one-factor ANOVAs revealed that testosterone-treated animals showed higher testosterone levels than vehicle treated, 45, $F(2, 15) = 26.40$, $p < 0.001$, 75, $F(2, 15) =$

12.80, $p < 0.001$, or 105, $F(2, 17) = 25.10$, $p < 0.001$, min after receiving injection. Furthermore, the hormone concentration in animals that received 0.8, $F(2, 16) = 21.14$, $p < 0.001$, or 1.2 mg/kg of testosterone, $F(2, 17) = 9.25$, $p < 0.001$, was higher 45 min after treatment than 75 or 105 min afterwards.

Experiment 2

In the first ANOVA performed, no effect was significant, but when the drug-paired compartment factor was included a significant effect of the test factor showed that in the postconditioning test animals spent more time in the ILPC than in the preconditioning tests, $F(1, 30) = 16.36$, $p < 0.001$. When this effect was analyzed only for the testosterone-treated animals it did not reach statistical significance, $F(1, 27) = 1.94$, $p < 0.175$. A main effect of drug-paired compartment factor, $F(1, 30) = 26.48$, $p < 0.001$, revealed that animals spent more time in the ILPC when it was the black one than when it was the white one.

The treatment × test interaction was also significant, $F(3, 30) = 3.36$, $p < 0.032$, indicating a different behavior in control and testosterone-treated groups. In the control group, a decrease in the time spent in the ILPC in the postconditioning test was observed, whereas in the three testosterone-treated groups increments were noticed (Fig. 1A). The significant interaction drug-paired compartment × test, $F(1, 30) = 34.78$, $p < 0.001$, showed that the test factor had a different effect, depending on the characteristics of the drug-paired compartment. When it was the black one, a significant increment in the time spent in such a compartment in the postconditioning test was noticed, $F(1, 7) = 13.33$, $p < 0.008$, whereas when it was the white one, a significant decrease was observed, $F(1, 29) = 5.25$, $p < 0.029$.

Because different results in observation of the color of the drug-paired compartment were observed, separate analyses for the animals conditioned to the black and white compartment were performed with treatment and test as the main factors. Separate analysis for the animals conditioned to the white compartment showed that only the test factor was significant, $F(1, 26) = 4.94$, $p < 0.035$, noticing a significant decrease in the time spent in the ILPC in the postconditioning test (Fig. 1B). When the effect of the test factor was analyzed for controls and treated animals separately, this factor had an effect near to the level of significance in control group, $F(1, 7) = 4.71$, $p < 0.067$, that was not found in testosterone-treated animals. The test factor was also significant for the animals conditioned to the black compartment, $F(1, 4) = 14.49$, $p < 0.019$, but in this case a significant increase in the time spent in the ILPC was observed (Fig. 1C). This augment was significant in the testosterone-treated animals, $F(1, 5) = 23.82$, $p < 0.005$, indicating that CPP was produced, whereas no significant difference in the time spent in this compartment (ILPC) before and after conditioning was observed in the control group.

Table 2 displays time spent (mean ± SEM) by control and testosterone groups (black and white subgroups) in the ILPC (drug-paired compartment for the treated groups) before and after conditioning.

DISCUSSION

The present study reveals that low supraphysiological doses of testosterone may have rewarding effects in intact male mice, depending on the environmental cues used as conditioned stimulus. CPP was observed in animals pairing the testosterone/black compartment but not in animals pairing the testosterone/white compartment. In this experiment, vi-
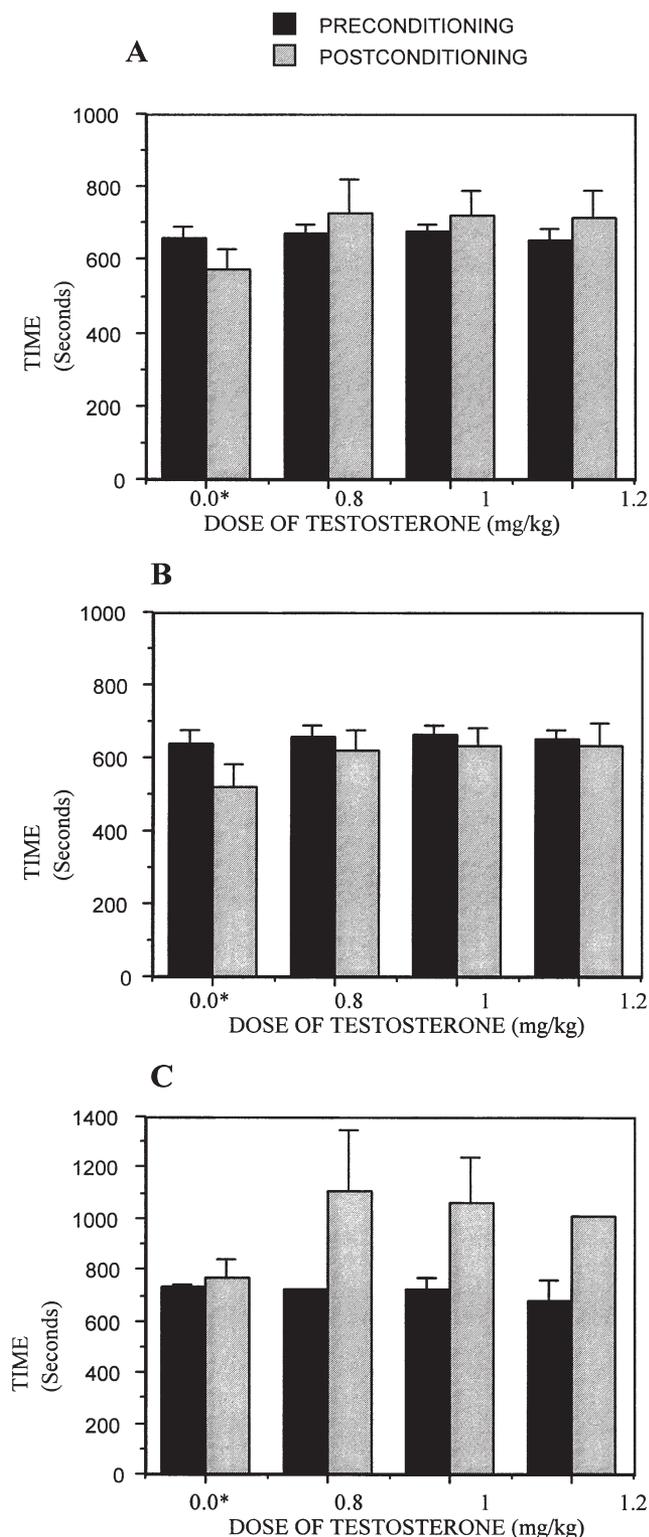
FIG. 1. Time spent (mean ± SEM) in the initially least preferred compartment before and after conditioning: (A) control and treatment groups; (B) control and treatment subgroups paired with white compartment; (C) control and treatment subgroups paired with black compartment. *0.0 mg/kg = control group.

TABLE 2
TIME (MEAN ± SEM) SPENT BY CONTROL AND TESTOSTERONE GROUPS (BLACK AND WHITE SUBGROUPS) IN THE INITIALLY LEAST-PREFERRED COMPARTMENT BEFORE AND AFTER CONDITIONING

		Test	
Groups	Subgroups	Preconditioning	Postconditioning
Control		655.900 ± 31.678	569.800 ± 59.810
	black	727.000 ± 12.000	767.500 ± 72.500
	white	638.125 ± 37.175	520.375 ± 61.778
0.8 mg/kg T		671.333 ± 24.462	726.333 ± 92.582
	black	720.500 ± 5.500	1109.500 ± 234.500
	white	657.286 ± 29.603	616.857 ± 55.558
1 mg/kg T		675.500 ± 21.178	719.200 ± 72.385
	black	723.500 ± 44.500	1061.000 ± 178.000
	white	663.500 ± 23.394	633.750 ± 45.539
1.2 mg/kg T		654.333 ± 26.947	714.667 ± 72.383
	black	680.000 ± 77.000	1007.000 ± 5.000
	white	647.000 ± 30.367	631.143 ± 61.223

sual and tactile cues, brightness, and floor texture were utilized to distinguish between alternate compartments. In CPP studies, several stimuli have been manipulated; however, little is known about stimulus selection (22), and how it can affect the experimental results. A partial CPP similar to that observed in this experiment was also found in another study where reinforcing properties of successful intermale agonistic encounters were studied using the same CPP apparatus and mouse strain (14).

Several studies using CPP have found rewarding effects of testosterone in intact (1,7,19,20) and gonadectomized (10) male rats. However, several methodological differences in the experimental procedures make it difficult to compare them, due to the fact that it is not clear how these differences could affect the results obtained. Variations in the number of conditioning sessions, strain of rat, form of the testosterone administered, and the time between injection and the placement of the animal into the box have been previously emphasized (7). Other differences such as the duration of the pre- and postconditioning tests and the inclusion in the design of a control group receiving vehicle in both compartments can also be relevant. Much more has been written about the counterbalanced vs. fixed assignment to compartments. In the former procedure, animals are randomly assigned to any compartment, whereas in the latter, the drug is paired with a given compartment, which is usually the nonpreferred one (8). Most of the studies reviewed that observed a testosterone CPP have chosen a random assignment. In our study, animals showed a clear preference for one compartment in the preconditioning test, so they were conditioned against their initial preference. This implies stricter assessment of a CPP, which could explain why the testosterone CPP was only partially found. Furthermore, statistical management of the data to assess CPP has also been very different between studies. In some of them, the time spent in the drug paired compartment before and after conditioning is compared (10), whereas in others, the comparison is made between the time spent in the drug and the vehicle-paired compartment without taking into account the initial preference (1,19,20).

Despite the procedural discrepancies described, the testosterone doses (administered peripherally) producing a significant CPP were in a dose range clearly above physiological levels. In intact male rats, testosterone CPP has been reported after 0.8, 1.2, and approximately 3.6 mg/kg of testosterone (1,7,19,20). In gonadectomized male rats, a significant CPP has been reported administering a testosterone dose of 1 mg/kg (10). Doses of $\leq$ 0.5 mg/kg did not produce a significant CPP in either intact (1,7) or gonadectomized (10) male rats. Our study suggests that some testosterone doses with rewarding properties in male rats can also have these effects in male mice.

In the testosterone-treated animals, a great variability in the place preference was observed within each group. If this fact were corroborated in similar studies, it would suggest that individual differences could exist in the rewarding capacity of testosterone. Human studies of dependence on AAS have shown that not all AAS users develop dependence (5), so this variability could be explained by other individual differences in addition to variations in the abuse patterns. Additionally, individual differences such as basal aggressiveness have proven to be an important factor that modulates other behavioral effects of testosterone (15,16).

In the nervous system, steroids show genomic actions that imply changes in protein synthesis and require several hours or even days to produce observable effects (9), and nongenomic effects that consist in rapid changes (17). Specifically, testosterone can induce rapid changes in the neuronal excitability through short-latency effects on cell membranes (21).

Using a form of testosterone that is rapidly metabolized (testosterone-hydroxypropyl-β-cyclodextrin inclusion complex), rewarding effects of testosterone are produced within the 30-min time period following injection, so it is unlikely that these rapid effects are mediated by a genomic mechanism; therefore, they must be produced by nongenomic actions (19,20). As very few works have been published with respect to the pharmacokinetics of synthetic testosterone, the chosen temporal period between the injection and the beginning of the CPP session was based on the first experiment where the highest levels of testosterone in blood were found at 45 min in comparison with the levels found 75 and 105 min after testosterone treatment. Animals were confined for 30 min in the testosterone-paired compartment 30 min following injection, so rewarding effects, when produced, occurred during the first hour following injection.

In summary, the present study suggests that testosterone administration can have rewarding effects on male mice, although, the testosterone CPP was influenced by the environmental cues used as conditioned stimuli. Thus, it could be said that dependence on testosterone and its derivatives may be a consequence of their rewarding affective properties.

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