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RESEARCH ARTICLE

Similar rewarding effects of testosterone in mice rated as short and long attack latency individuals

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Abstract

An attempt was made to confirm and extend the findings of an earlier study on the rewarding properties of testosterone in male mice using conditioned place preference (CPP). Previous results had only partially demonstrated such an effect because the reinforcement depended on environmental cues such as the colour of the compartment. High individual variability was evident, suggesting that basal levels of aggressiveness may modulate such effects. Animals were pre-screened for aggressive behaviour and allocated to short and long attack latency (SAL and LAL) categories. Five days later the CPP procedure started. This involved pre-conditioning tests, conditioning and post-conditioning tests. SAL and LAL animals were treated with vehicle, 1 or 2 mg/kg of testosterone. During conditioning (on alternate days), a distinctive floor was paired four times with testosterone. On the intervening days animals were paired with a different floor with vehicle. CPP was clearly observed after testosterone treatment when the colour of the compartment was controlled in both SAL and LAL animals. These results provide additional support for the idea that testosterone and its derivatives have rewarding properties, which could explain processes of dependence.

Introduction

Numerous testosterone derivatives, collectively called anabolic-androgenic steroids (AASs) are used increasingly in the general population.[1] This widespread consumption has led to an abuse syndrome,[2–5] which includes dependence reported to develop with long-term abuse in some individuals.[6] Few controlled studies have been carried out, but none have shown that all AASs abusers meet DSM-III-R criteria for dependence.[6–10]

Conditioned place preference (CPP) studies have demonstrated that testosterone has rewarding properties in gonadectomized[11] and intact[12–17] male rats. This may be related to the fact that androgen-dependent behaviours (such as sex, aggression and dominance) are natural rewards. The CPP technique, consisting of repeated pairing of a distinctive environment with the affective consequences of a drug,[18] has been used widely to assess the rewarding effects of drugs of abuse. Comparing results is frequently

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difficult due to methodological differences between studies such as number of conditioning sessions, the duration of the pre- and post-conditioning tests, the presence or absence of a control group (receiving vehicle in both compartments) and counterbalanced versus fixed assignment of animals to compartments.[19]

A previous study,[20] using peripheral injections of testosterone, suggested that low supraphysiological doses of testosterone have rewarding effects in gonadally intact male mice conditioned against their initial preference. CPP was only partially confirmed, as the effect was dependent on the environmental cues used as conditioned stimulus. CPP was observed in animals pairing testosterone/black compartment but not when pairing testosterone/white compartment. Furthermore, the great variability in the acquisition of CPP suggested possible individual differences in the rewarding capacity of testosterone.

A few studies in humans suggest that basal levels of aggressiveness may explain individual differences in the behavioural effects of abuse of these substances.[21,22] These individual differences could also mediate vulnerability to developing dependence. Data in animals generally confirm this assumption. Several studies using intact male mice suggest that basal levels of aggressiveness moderate effects of chronic and acute AAS treatment on testosterone-dependent behaviours such as aggression.[23,24] Secondly, the rewarding properties of testosterone involve the dopaminergic system,[14–17] which differs in aggressive and non-aggressive male mice.[25]

This study was carried out to confirm and extend the findings of the earlier pilot. In the present experiment, CPP was assessed in two black boxes differing in the texture of the floor to control the influence of environmental cues such as colour of the compartment. To analyse further the diversity found in the acquisition of CPP in the previous study the possibility that basal levels of aggressiveness modulate responding was investigated.

Materials and methods

Subjects

Seventy-two outbred, 42-day-old (30–32 g), OF-1 male mice purchased from Criffa Credo Laboratories (Lyon, France) were housed individually in plastic cages (20 × 10 × 13 cm) and used as experimental animals. A further 150 animals were housed in groups of five in larger cages (24.5 × 24.5 × 15 cm) and used as "standard" opponents only once, after being rendered temporarily anosmic by intranasal lavage with a 4% zinc sulphate solution a day before testing. Anosmic mice were employed as "standard" opponents because they elicit attack but never initiate such behaviour.[26]

All animals were acquired and cared for in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). They were maintained on a 12-hour light/dark cycle (lights off at 0800 hours local time) and were housed in facilities for 3 weeks prior to experimental procedure. Laboratory temperature was kept at 20 ± 1°C. Food and water were available *ad libitum*. All tests were carried out during the animal's dark cycle, starting at the 2nd hour.

Drugs

4-Androsten-17 β-ol-3-one testosterone (Sigma, Madrid, Spain) was dissolved in peanut oil (Guinama, Valencia, Spain) to obtain three testosterone doses: 0 (vehicle); 1 and 2 mg/kg. These doses are similar to those used in other studies focused on the rewarding properties of testosterone in male rats.[11–13] Injections were administered subcutaneously at a volume of 0.1 ml 30 minutes before each conditioning session. The period between the injection and the beginning of the session was based on a previous experiment.[20]

Apparatus

CPP was assessed in a standardized box (30 × 15 × 20 cm) made of aluminium and metacrilate, similar to that described by Cunningham *et al.*[29] and was purchased from PANLAB® S.L. (Barcelona, Spain). This type of box has been used to assess rewarding properties of several substances of abuse in mice.[27–29]

The walls of the box were made of black metacrilate and the floor, which was stainless steel, consisted of interchangeable halves of one of two different textures, i.e. "grid" floors composed of 3-mm rods mounted 6.4 mm apart in Plexiglas rails, and "holed" floors comprised of perforated stainless steel with 6.4-mm round holes 6.4 mm apart. The floor texture was used as a distinctive environmental stimulus to establish

place conditioning. Several studies in mice have shown that control animals spend approximately equal times on these floor types during preference tests.[29,30]

Procedure

Agonistic encounter. After 3 weeks of isolation, experimental animals were pre-screened for aggressive behaviour in an agonistic encounter. They confronted anosmic opponents and their attack latency was used to classify them into two groups: short (SAL) and long (LAL) attack latency mice. The registration of the attack behaviour was carried out using the ethological technique developed by Brain *et al.*[31] which considers "attack" category having the following constituent elements: charge, lunge, attack and chase. These encounters were carried out in a neutral area ($59 \times 29 \times 32.5$ cm) illuminated by a red light and were preceded by a minute of adaptation in which the animals were separated by a plastic partition. The test finished when the experimental animals attacked for the first time, being classified into either SAL group if they attacked before the fifth minute of the encounter or into LAL group if they attacked after the fifth minute or if the 10-minute test finished without attack. Thirty-six SAL and 36 LAL animals were selected for the CPP procedure.

Conditioned place preference. The experimental sessions were performed in a dimly illuminated room. The box was cleaned of urine and faeces after removing each animal from it. The CPP procedure started 5 days after the agonistic encounter. It required 10 days (one session per day), involving three phases: pre-conditioning test (one session); conditioning (eight sessions); and post-conditioning test (one session).

In the pre-conditioning test (day 1), the floor of the box was half grid and half holed. Subjects were situated in the middle of the box and allowed to explore the environment freely for 30 minutes to determine their initial preference for the floor texture under non-drug conditions. The preference levels for one floor texture was never larger than 60% in the pre-conditioning test. Animals were allocated to randomly one of the three treatment groups generating six experimental groups (all $N = 12$): SAL + vehicle; SAL + 1 mg/kg of testosterone; SAL + 2 mg/kg of testosterone; LAL + vehicle; LAL + 1 mg/kg of testosterone; LAL + 2 mg/kg of testosterone.

Conditioning was carried out over an 8-day period in which in every session, each mouse was injected with testosterone or vehicle (alternate days) and 30 minutes later was confined in the apparatus for 30 minutes. In each conditioning session mice had free access to the whole apparatus and floor texture was identical on both sides of the box. For half the animals within each group, the grid floor was assigned randomly as the conditioned stimulus ($n = 6$) and the holed floor as the neutral environment; for the rest ($n = 6$), the conditioned and the neutral stimuli were reversed. This procedure was in accordance with an unbiased place conditioning method.[18] The order of exposure to the drug-paired vs. neutral floor texture was counterbalanced across groups.

Animals were exposed to one conditioning session per day for a total of four vehicle pairings and four testosterone pairings in testosterone-treated groups. Control groups received vehicle pairings in all conditioning sessions. The rationale for this group was to control changes in preference for drug-paired floor due to the repeated exposure to the conditioned stimulus during conditioning phase without being paired with the drug.

CPP assessments followed the last conditioning session by 24 hours (post-conditioning test). The floor of the boxes was half grid and half holed, exactly as in the pre-conditioning test, and drug-free animals had free access to both sides for 30 minutes.

The behaviour of the animals in the pre-conditioning and post-conditioning test was video-recorded and used for the evaluation of the CPP. These videotapes were assessed using a computerized observational procedure.[31] This analysis was performed by a trained observer who was blind as to which experimental group each animal belonged. The analysis of videotapes involved assessment of the time spent on each floor texture and the latency of the first entry to each floor. An animal was judged to be inside an area when more than half of the body was on that floor.

Statistical analysis

All calculations were performed using the SPSS package. Results were considered statistically significant at $p < 0.05$.

Table 1. *Time (mean + SEM) spent (in seconds) by experimental groups on the drug-paired floor before and after conditioning*

Experimental groups		Test	
Treatment	Aggressiveness	Preconditioning	Test
Control		861 ± 44	893 ± 48
	SAL	868 ± 58	908 ± 64
	LAL	854 ± 70	878 ± 73
1 mg/kg of Testosterone		863 ± 20	1015 ± 50*
	SAL	887 ± 33	1024 ± 60
	LAL	840 ± 21	1007 ± 73
2 mg/kg of Testosterone		909 ± 27	1051 ± 29*
	SAL	892 ± 37	1007 ± 46
	LAL	926 ± 42	1095 ± 32

*Indicates a statistically significant increase in the time spent on the drug-paired floor.

Data of time spent on the drug-paired floor before and after conditioning were assessed by analysis of variance (ANOVA). The design consisted of two between-subject factors: "treatment" with three levels [0 (vehicle), 1 or 2 mg/kg of testosterone] and "aggressiveness" with two levels (SAL, LAL). The within-subject factor was "test" with two levels (pre- and post-conditioning). Following a significant *F*-value, *post-hoc* analyses (Newman–Keuls) were performed for specific group comparisons. CPP involved evaluation of differences in time spent on the drug-paired floor vs. pre-conditioning times for that floor. Preference was defined as a significant increase in the time spent in the drug-paired floor after conditioning. The same design and statistical analysis was performed for an additional dependent variable, the latency of the first entry to the drug-paired floor.

Results

Table 1 displays time spent (mean ± SEM) by experimental groups in the drug-paired compartment before and after conditioning. The repeated measures ANOVA ($2 \times 3 \times 2$) with "test" as within-subjects factor and "treatment" and "aggressiveness" as between-subjects factors revealed a significant effect of "test" [$F(1,66) = 29.11$, $p = 0.001$], and interaction "test" × "treatment"

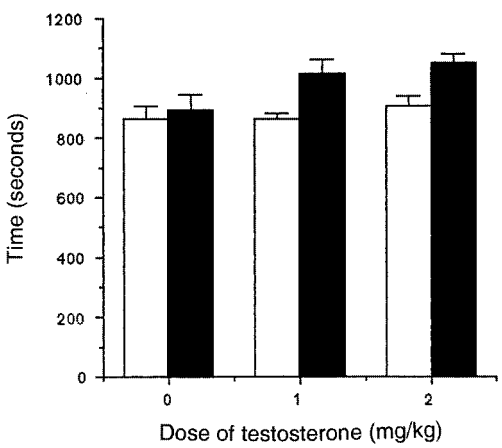

Figure 1. *Time (mean ± SEM) spent (in seconds) by treatment groups on the drug-paired floor before* □ *and after* ■ *conditioning.*

[$F(2,66) = 3.63$, $p = 0.032$]. With regard to "test" effect, all the animals spent more time on the drug-paired floor in the post-conditioning test (mean = 986.5 seconds) than in the pre-conditioning test (mean = 877.8 seconds). Moreover, the significant interaction "test" × "treatment" showed that these changes in the amount of time spent on the drug-paired floor were different depending on the treatment administered. To interpret this interaction, three one-way ANOVAs with the factor "test" were applied separately to the data from each group of treatment (Fig. 1). The effect of factor "test" was not significant for the vehicle group, whereas in both testosterone-treated groups a significant increase in the time spent on the drug-paired floor was observed (1 mg/kg testosterone [$F(1,23) = 16.77$, $p = 0.001$]; 2 mg/kg testosterone [$F(1,23) = 18.11$, $p = 0.001$]).

No effect involving "aggressiveness" was significant in this analysis, so data were reanalysed selecting extreme groups in this variable. Animals above percentile 70 ($n = 21$) and below percentile 30 ($n = 21$) in the latency of attack registered in the agonistic encounter were selected, whereas the rest of the sample was discarded. No differences in the CPP were found between these groups. No significant effects were observed with respect to the latency of first entry to the drug-paired floor.

Discussion

The present results indicate that testosterone possesses rewarding properties in intact male mice. CPP was observed after peripheral treatment with 1 and 2 mg/kg of testosterone. These results confirm the findings of an earlier study, where it was shown partially that administration of low supraphysiological doses (0.8, 1 and 1.2 mg/kg) of testosterone had rewarding properties in intact male mice. In the pilot study, CPP was assessed in an apparatus where brightness (black/white) and floor texture (rough white/fine grey plastic mesh floor) were used as discriminative stimuli to produce conditioning, whereas in the present study only floor texture (grid/holed floor) was used for this purpose. These findings give support to the relevance for the environmental cues involved in stimuli selection in CPP. It was thought that these variables could also affect CPP studies of other substances in some unknown manner.

It has recently been established that a primary rewarding mechanism is possible in the dependence on these substances, although such a phenomenon is far from confirmed.[32] Despite several differences between CPP studies,[20] all suggested that dependence on ASS could (to a certain degree) be produced by neurochemical actions of these compounds on the brain reward system in rats. Peripheral administration of different forms of testosterone produced CPP in male rats. The treatment with 1 mg/kg of testosterone produced CPP in gonadectomized male rats.[11] CPP of testosterone has also been reported in intact male rats after administration of testosterone-hydroxypropyl-β-cyclodextrin inclusion complex (0.8 and 1.2 mg/kg).[12] Moreover, intra-accumbens administration of this compound also produced CPP, showing that one target for ASS in the brain could be this area.[15] Additionally, this CPP can be blocked by the treatment with α-flupenthixol, a dopaminergic antagonist.[14,16,17] Although further investigation is needed, the present results suggest that this mechanism also occurs in male mice.

Until now, very few studies have focused on the rewarding properties of testosterone and its derivatives in animals using techniques other than CPP, used traditionally in the study of several substances of abuse. The effect of an ASS "cocktail" on the brain reward system was analysed using the rate-frequency curve shift paradigm of intracranial self-stimulation. AAS treatment did not modify the rewarding properties of brain stimulation directly but induced changes in the sensitivity of the brain reward system to the acute administration of d-amphetamine.[33]

Supraphysiological doses of testosterone showed similar rewarding properties in both groups of mice differing in their latency of attack recorded in a single encounter. This finding suggests that this variable does not moderate individual differences in the dependence on AAS. However, both groups of mice belonged to the same strain, so the absence of effects could also be due to the fact that the disparity between SAL and LAL animals was not sufficient to be able to observe differences in CPP; in addition, the behavioural difference was established in a sole encounter which could also be insufficient. Benus *et al.*[25] found a higher susceptibility to a dopamine agonist in aggressive in comparison with non-aggressive male mice, suggesting that a difference in the dopaminergic system existed

between both groups. However, they came from two different lines of wild mice selected for long- and short-attack latency that also differed on several behavioural profiles. Moreover, further information about possible changes in the rewarding properties as a consequence of different experiences through several social encounters appears necessary. In fact, differences in social and fighting experience (e.g. participating in an agonistic encounter or winning/losing a fight) should also be considered. In the present experimental protocol, screening for aggressiveness has been included and, as is well known, fighting experience affects aggressive behaviour, which may result rewarding or not depending on the outcome. Because the experimental animals in this study were confronted with non-aggressive, anosmic mice, their experience never finished in defeat; hence, the rewarding properties of testosterone would be reinforced by this experience.

In summary, future research should analyse the factors that enhance vulnerability to AAS use and lead to dependence together with the mechanisms involved. Determining these variables could improve the prevention and treatment of dependence as well as lead to a better understanding of the underlying processes.

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