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Testosterone treatment induces behavioral disinhibition in adult male rats

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Abstract

The importance of testosterone for impulsive-like behavior is unclear. Here we studied the effect of testosterone administration during 6 and 14 days (separate experiments) with one, three and five testosterone-filled silastic capsules implanted subcutaneously on shock-induced behavioral inhibition and on flunitrazepam-induced disinhibition in a modified Vogel's drinking conflict model in rats. Alleviation of shock-induced behavioral inhibition has been suggested to reflect impulsive-like behavior and/or anxiolysis. Treatment with the highest testosterone dose used for 6 (Experiment 1) and 14 (Experiment 3) days increased the number of shocks accepted. Testosterone treatment affected serum levels of testosterone and accessory sex organ weights. Flunitrazepam induced behavioral disinhibition in both testosterone-treated (for 14 days) and sham-treated rats. Moreover, testosterone treatment for 14 days resulted in enhanced GABA-induced $^{36}Cl^-$ uptake into synaptoneurosomes as compared to controls. In conclusion, testosterone produces behavioral disinhibition and may enhance brain $GABA_A$ receptor function.

Keywords: Conflict behavior; Flunitrazepam; HPG axis; Impulsivity; Testosterone; GABA; Shock-induced behavioral inhibition; Rat; $GABA_A$ receptor; Alcohol

1. Introduction

Earlier reports have suggested a link between androgens and impulsive behavior in man. Thus, abuse of anabolic–androgenic steroids (AAS) has been reported to induce impulsive and aggressive behavior in correlative studies (Conacher and Workman, 1989; Galligani et al., 1996; Pope and Katz, 1988, 1990, 1994). Moreover, use of AAS together with drugs that lower inhibited behavior, e.g., GABAergic drugs, has been suggested to impair impulse control more than either AAS or GABAergic drugs do alone (Kindlundh et al., 1999).

Further support for a link between androgens and impulsive behavior was provided by a report demonstrating that women with bulimia nervosa, a disorder possibly involving poor impulse control, display significantly higher serum concentrations of testosterone as compared to age-matched controls (Sundblad et al., 1994). In another study, bulimic symptoms were reduced after treatment with the testosterone antagonist flutamide (Bergman and Eriksson, 1996).

However, other investigators have not found a correlation between testosterone levels in cerebrospinal fluid (CSF) and impulsive behavior, neither in man (Virkkunen and Linnoila, 1993; Virkkunen et al., 1994) nor nonhuman primates (Higley et al., 1996). It is instead suggested that testosterone levels in CSF correlate to aggressive behavior both in man and in nonhuman primates (Virkkunen and Linnoila, 1993; Virkkunen et al., 1994; Higley et al., 1996).

Regarding research in rats, a single high dose of testosterone has been reported to induce disinhibited behavior in the elevated plus-maze (Bitran et al., 1993) and in a modified Vogel's conflict model (Bing et al., 1998) in intact adult male rats. Furthermore, shock-induced behavioral inhibition in group-housed adult male rats is enhanced by gonadectomy and testosterone substitution prevents this effect (Svensson et al., 2000a). However, in single-housed adult male rats, gonadectomy did not affect this behavior (Svensson et al., 2000b).

Disinhibited behavior in the Vogel's conflict model, which involves shock-induced behavioral inhibition, and

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in the elevated plus-maze has commonly been interpreted to reflect anxiolytic-like effects. However, disinhibition in these models could just as well reflect impulsive behavior, at least after certain pharmacological manipulations, e.g., serotonin (5-HT) depletion (cf. Soubrié, 1986; Söderpalm and Svensson, 1999). Thus, lesioning of the ascending 5-HT pathways in the brain by means of the selective 5-HT neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) induces impulsive-like behavior in the (1) delay of reward (Wogar et al., 1993), (2) DRL (Wogar et al., 1992) and (3) autoshaping (Harrison et al., 1997) paradigms. These three models have frequently been suggested to reflect impulsive behavior, even if they also may reflect reinforcer sensitivity or habituated behavior (Evenden, 1998). In addition, treatment with positive modulators of the $GABA_A$ receptor (e.g., benzodiazepines) produces impulsive-like behavior in the delay of reward paradigm (Thiébot et al., 1985), DRL paradigm (Jackson et al., 1995) and behavioral disinhibition in a modified Vogel's conflict test (reviewed in Söderpalm, 1990). Thus, both 5-HT depletion and GABAergic stimulation produce behavioral disinhibition in the modified Vogel's test and impulsive-like behavior in the paradigms mentioned here.

Hence, the abovementioned findings indicate that testosterone may be involved in mechanisms underlying behavioral disinhibition in rat. However, in the rat, just as in man and primates, conflicting data have been reported. Thus, testosterone treatment, possibly yielding supraphysiological levels, failed to produce behavioral effects in adult male rats in two tests measuring different aspects of impulsive-like behavior; a paced fixed consecutive number schedule and a delayed reinforcement procedure (Evenden, 1998). Furthermore, testosterone treatment did not affect the behavior of castrated adult male rats in the elevated plus-maze (Gonzalez et al., 1994). Importantly, in this study, the testosterone treatment probably did not produce supraphysiological serum levels of testosterone.

Taken together, conflicting data have been presented regarding the effect of testosterone treatment on impulsive-like behavior in rats. However, in none of these studies was the effect of testosterone on accessory sex organ weights reported, and in some studies the serum concentrations of testosterone have not been presented. Furthermore, few reports have examined dose–response effects of testosterone treatment on impulsive-like behavior.

The potential association between testosterone and impulsive-like behavior may involve both serotonergic and GABAergic mechanisms. Thus, testosterone in supraphysiological levels decreases brain 5-HT tissue levels (Martinez-Conde et al., 1985), whereas gonadectomy increases 5-HT levels (Engel et al., 1979). Moreover, the reduced testosterone metabolites androsterone and 3α-androstanediol are positive modulators of the $GABA_A$ receptor (Bitran et al., 1996). Androsterone and 3α-androstanediol induce behavioral disinhibition in the elevated plus-maze (Aikey et al., 2002). Interestingly, both serotonergic and GABAergic mechanisms have been implicated in the regulation of behavioral disinhibition (Söderpalm and Svensson, 1999).

Thus, in this study we further explored the possible effect of testosterone on shock-induced behavioral inhibition. We studied the effect of three different testosterone doses administrated during 6 and 14 days (separate experiments) on shock-induced behavioral inhibition in group-housed (intact) adult male Sprague–Dawley rats. In addition, serum levels of testosterone and sex accessory organ weights were determined in these animals.

To study the potential involvement of GABAergic mechanisms and to experimentally address the suggestion that AAS (including testosterone) together with positive $GABA_A$ receptor modulators impair impulse control more than either AAS or GABAergic drugs do alone, the effects of flunitrazepam and ethanol on shock-induced behavioral inhibition were studied in testosterone- and sham-treated rats. Importantly, in man this effect of combined use may be obvious even after a long period of AAS use (cf. Kindlundh et al., 1999); hence a 14-day treatment period was used in these experiments. In addition, GABA-induced $^{36}Cl^-$ uptake into synaptoneurosomes from sham- or testosterone-treated animals was determined after pretreatment for 14 days.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (B&K, Universal, Sollentuna, Sweden) weighing 270–350 g were used. A total of 198 rats were used in this study. The animals were kept under controlled light–dark conditions (light on at 5:00 a.m. and off at 7:00 p.m.) and at constant temperature (25 °C) and humidity (65%). Four rats were housed in each cage (size: 59 × 38.5 × 20 cm). A 7-day adaptation period to the animal maintenance facilities of the department was allowed before the start of the experiments. The animals had free access to standard laboratory chow and water when not participating in the modified Vogel's drinking conflict test, drinking motivation or shock threshold experiments. All animal procedures were approved by the Ethics Committee for Animal Experiments, Göteborg, Sweden.

2.2. Capsule implantation

The rats were anaesthetized with ketamine 50 mg/ml and xylazine 20 mg/ml in a mixture of 2:1, which was injected in a volume of 2 ml/kg intraperitoneally. The rats were randomly allocated to different treatments (see Experimental designs). One, three or five silastic capsules containing testosterone or one empty capsule were implanted subcutaneously (sc) in the flank. All capsules were incubated in 0.9% NaCl for 1 day before use, then the capsules were washed in 70% ethanol for 30 min and thereafter in saline 30 min before implantation. All the rats weighed at least 275

g when operated on. The same rats that were housed together before operation were also housed together after operation. The rats were treated with testosterone-filled capsules or empty capsules for 6 or 14 days before being used in the behavioral test described in the next section. Rats were used only once in the behavioral test.

2.3. Shock-induced behavioral inhibition

A modified Vogel's drinking conflict model was used. On the first day of the experiment, the animals were adapted for 10 min to a Plexiglas box (inner dimensions $30 \times 24 \times 20$ cm) enclosed in a soundproof cage and equipped with a grid floor of stainless steel bars and a drinking bottle containing a 5.5% (wt/vol) glucose solution. After a 24-h period of water deprivation, the animals were adapted to the same test chamber for a further 10 min. During this period, the animals had free access to the glucose solution. After a further 24 h of water deprivation, the animals were returned to the Plexiglas box. Thus, the rats were given 10 min of fluid access in a 48-h period. When approaching the drinking spout (usually within 20 s), the animal was allowed to drink for 30 s, after which the first electric shock (0.16 mA for 2 s) was administered between the spout of the drinking bottle and the grid floor. Upon every further attempt to drink, an electric shock was administered. The number of shocks accepted during a 10-min session was recorded. All experiments were carried out between 10:00 a.m. and 4:00 p.m. The animals were used only once in this test.

2.4. Shock threshold and drinking motivation tests

The same rats were used for the shock threshold, drinking motivation and modified Vogel's test. Vogel's test was performed first. To conform with Vogel's conflict test the animals were treated identically in the shock threshold test including water deprivation for 48 h. Each rat was placed in the Plexiglas box previously described. The shock threshold was determined stepwise by manually increasing the current delivered through the grid floor (0.05, 0.06, 0.08, 0.10, 0.13, 0.16, 0.20, 0.25, 0.3, 0.4, 0.5 and 0.6 mA) until the rat showed an escape reaction to the electrical stimulus (jump, jerk or similar) as judged by an assistant that was blind to the treatment and the shock level applied. There was a 15-s shock-free interval between each step. The current amplitude threshold was recorded. Immediately after the shock threshold had been determined (see above) each rat was placed in its individual home cage and this was supplied with a drinking bottle containing 50 ml of a 5.5% (wt/vol) glucose solution. The total amount of liquid (grams) consumed during 10 min was recorded for every rat.

2.5. Preparation of synaptoneurosomes

Synaptoneurosomes were prepared essentially according to the method of Hollingsworth et al. (1985). Rats were decapitated and their brains rapidly taken out and placed in ice-cold preparation buffer (PB: NaCl 118.5 mM, KCl 4.7 mM, $MgSO_4$ 1.18 mM, $CaCl_2$ 2.5 mM, HEPES [2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid] 20 mM and Tris–Base 9 mM). The cerebral cortices and hippocampi were dissected from white matter, and the dura and pia mater were removed. The cortices and hippocampi from two animals were pooled and placed in ice-cold PB. The tissue was homogenized in 7 ml of PB in a glass–Teflon homogenizer. The homogenate was gravity filtered through two layers of nylon filter (160 μM) in a Sweenex filter holder. The resultant material was filtered once more over a 10-μm Millipore filter. The filtrate was centrifuged at $1000 \times g$ for 15 min and the supernatant was decanted. The pellet was resuspended in 2 ml of PB in a glass–Teflon homogenizer, and was thereafter diluted with 30 ml of PB and centrifuged once more at $1000 \times g$ for 15 min. The supernatant was again decanted and the remaining pellet weighed and diluted with PB to yield a final protein concentration of approximately 10 mg/ml. The resulting material after a similar cortical preparation has been described in detail and is considered to be mainly made up of sealed neuronal membranes, often arranged in a "snowman"-like fashion ("synaptoneurosomes"), with a limited number of other "cells" (a "cell-free" preparation) (Schwartz et al., 1984; Luu et al., 1987). In the present study we added hippocampus to the preparation, since this brain region shows a high density of $GABA_A$/benzodiazepine receptors, and since this procedure increases the tissue yield from each animal.

2.6. $^{36}Cl^-$ uptake into synaptoneurosomes

Assay tubes containing 300 μl of PB (with pH 7.4 at 30 grades) were prewarmed in a water bath (30 grades) before addition of 100 μl of the synaptoneurosomal suspension. The suspension was left to incubate for 20 min before the addition of 50 μl GABA (or PB) together with 0.5 μCi of $^{36}Cl^-$. The mixture was rapidly vortexed and the flux of $^{36}Cl^-$ terminated 5 s later by the addition of 5 ml ice-cold PB containing 100 μM picrotoxin. The mixture was then immediately vacuum-filtered (Schleicher & Schuell filters, GF31, 24 mm) and the tube and the filter was rinsed twice more with 5 ml of the picrotoxin-containing buffer. The filters were placed into scintillation vials and 4.5 ml of scintillation fluid (Beckmann Ready-Safe) were added. Radioactivity was counted overnight (DPM) using conventional liquid scintillation techniques. Data are expressed as percent stimulation of baseline $^{36}Cl^-$ accumulation (5 s in the absence of GABA).

2.7. Serum concentration of testosterone

The serum concentration of testosterone in trunk blood was estimated by using a ^{125}I testosterone DA kit (ICN, 07-189102). This method has been used earlier to measure testosterone in adult male rats (Doherty et al., 1990).

2.8. Experimental designs

2.8.1. Experiments 1 and 2

Shock-induced behavioral inhibition was estimated in rats treated with one, three or five testosterone-filled silastic capsules implanted sc in the flank for 6 (Experiment 1) or 14 days (Experiment 2). Experiments 1 and 2 were separate experiments, and thus each rat was used only once in the behavioral test. As controls, rats treated with one empty capsule implanted in an identical manner were used. Thus, four different treatment groups including the control group were obtained. One rat from each treatment group was housed in fours in each cage. After the shock-induced behavioral inhibition test, shock threshold and drinking motivation were determined for each rat. Furthermore, serum testosterone concentrations and absolute and relative weights of the prostate gland, testes and epididymides were determined.

2.8.2. Experiment 3

Although the behavioral effect of 14 days of testosterone administration was not statistically significant in Experiment 2, this treatment was used to further study the interaction between testosterone and the $GABA_A$ receptor. The effects of flunitrazepam (0.25, 0.5 and 1.0 mg/kg; 2 ml/kg ip 30 min before the test) and ethanol (ethanol concentration: 15% wt/vol in doses of 0.75, 5 and 1.0 g/kg; 6.67 ml/kg ip, 10 min before the test) on shock-induced behavioral inhibition were determined in sham-treated and testosterone-treated rats (five testosterone-filled capsules implanted sc as in Experiments 1 and 2). Controls received the corresponding vehicles (see Drugs). The rats were tested in the conflict task 14 days after implantation. As controls, rats treated with one empty capsule implanted in an identical manner were used. Two sham-treated (one empty capsule sc) and two testosterone-treated (five testosterone-filled capsules sc) were housed together. Each rat was only used once in the behavioral test. After the shock-induced behavioral inhibition test, shock threshold and drinking motivation were determined for each rat after behavioral relevant doses of flunitrazepam and ethanol.

2.8.3. Experiment 4

The amount of GABA-induced $^{36}Cl^-$ uptake was measured in corticohippocampal synaptoneurosomes prepared from sham-treated (one empty capsule sc) and testosterone-treated rats (five testosterone-filled capsules sc). Two sham-treated (one empty capsule sc) and two testosterone-treated (five testosterone-filled capsules sc) were housed together. The rats were decapitated 14 days after the implantation of the capsules. As described in the section Preparation of synaptoneurosomes, the brain tissue from two rats were pooled together. Hence, the amount of GABA-induced $^{36}Cl^-$ uptake was compared between synaptoneurosomes prepared from two pooled testosterone-treated and two pooled sham-treated animals.

2.9. Drugs

Ketamine hydrochloride (Parke-Davies, Barcelona, Spain) and xylazine hydrochloride (Bayer, Leverkusen, Germany) were used for anaesthesia. Silastic capsules (length 50 mm., inner diameter 1.57 mm, SIKEMA) were filled with crystalline testosterone (4-androsten-17β-ol-3-one, Sigma, St. Louis, MO, USA) as described by Damassa et al. (1977). Flunitrazepam (courtesy of Hoffmann-LaRoche) was dissolved in 0.5% Tween 80, gently warmed and diluted with 0.9% NaCl. Ethanol (Svensk Sprit) was diluted with 0.9% NaCl. γ-Amino-*n*-butyric acid (GABA; Sigma) and picrotoxin (Sigma) were dissolved in assay buffer. Picrotoxin was light protected throughout the experiment.

2.10. Statistics

The effect of different doses of flunitrazepam and ethanol on the number of shocks accepted in the modified Vogel's test in sham- and testosterone-treated rats was analyzed by two-way analysis of variance (ANOVA; Factor 1: sham- or testosterone treatment; Factor 2: flunitrazepam or ethanol dose). One-way ANOVA followed by Fischer's PLSD (Davies, 1949; Winer, 1971) implemented for comparisons between groups with different numbers of rats (Abacus Concepts, 1992) was used for evaluations of differences in the modified Vogel's test. The shock threshold data were analyzed using Kruskal–Wallis ANOVA followed by Mann–Whitney *U* test. A P value $<.05$ was considered to be statistically significant. When appropriate, multiple comparisons were corrected for using a sequentially rejective Bonferroni procedure.

3. Results

3.1. Shock-induced behavioral inhibition

Control rats accepted approximately 10 shocks/10 min in absolute terms. After 6 days of testosterone administration, rats treated with five testosterone capsules accepted statistically significantly more shocks than control rats (Experiment 1). A tendency to accept a lower number of shocks was observed in rats treated with only one testosterone capsule after 6 days of testosterone treatment (Fig. 1A). After 14 days of testosterone administration (pooled from two independent experiments; all data from Experiment 2), in another set of animals, none of the testosterone doses statistically significantly altered shock-induced behavioral inhibition. A tendency to accept more shocks was, however, noted in the testosterone-treated rats, as compared to controls (Fig. 1B).

Administration of 0.5 mg/kg flunitrazepam increased the number of shocks accepted both in sham-treated ($P<.05$) and in testosterone-treated ($P<.05$) rats (Fig. 2; Experiment 3; F values are given in the figure legend). Overall, the testosterone-treated rats accepted more shocks as compared

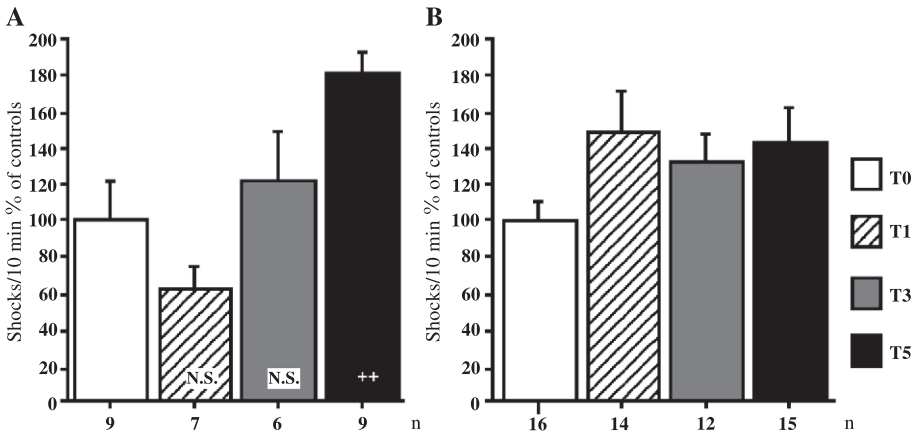

Fig. 1. Effects of testosterone treatment (with one, three or five testosterone-filled capsules) for 6 (A) or 14 (B) days on shock-induced behavioral inhibition (expressed in % of sham-treated controls). Shown are the means ± S.E.M. T0: one empty silastic capsule sc; T1, T3, T5: one, three or five testosterone-filled silastic capsule(s) sc. Statistics: One way ANOVA followed by Fischer's PLSD. The multiple comparisons were corrected for using a sequentially rejective Bonferroni procedure. (A) $F(3,27) = 8.51$, $P < .001$, $n = 31$. ++, $P < .01$, N.S., $P > .05$. (B) $F(3,53) = 1.49$, $P = .229$, $n = 57$.

to sham-treated animals when different doses of flunitrazepam were applied [0–1 mg/kg; $F(1,68) = 4.6$, $P < .05$]. Flunitrazepam produced dose-related (biphasic) behavioral disinhibition [$F(3,68) = 9.3$, $P < .0001$]. There was no significant Treatment × Drug dose (flunitrazepam) interaction [$F(3,68) = 0.6$, $P = .64$].

Ethanol failed to alter the behavior in this model [$F(2,36) = 2.4$, $P = .11$]. Testosterone-treated rats accepted more shocks as compared to sham-treated animals when 0–1.0 g/kg of ethanol was given [$F(1,36) = 5.2$, $P = .03$]. There was no Treatment × Ethanol dose interaction [$F(2,36) = 0.05$, $P = .95$].

3.2. Shock threshold and drinking motivation tests

Neither shock threshold nor drinking motivation was statistically significantly altered in rats treated with any of the testosterone doses during 6 or 14 days, as compared to control rats (Table 1A and B; *F*, *H* and *P* values are given in the table legend). Furthermore, neither flunitrazepam (evaluated after behavioral relevant doses) nor ethanol statistically significantly affected shock threshold or drinking motivation in sham-treated or testosterone-treated rats (for 14 days; Table 1C, see table legend).

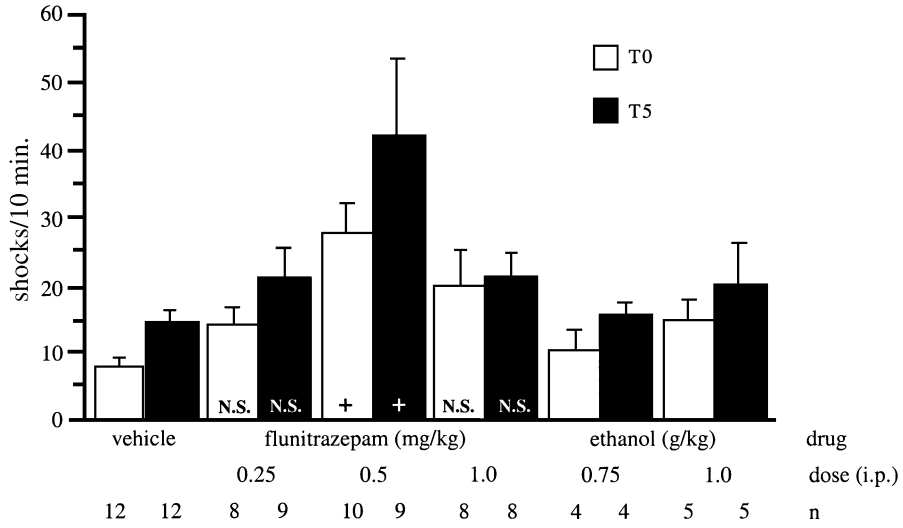

Fig. 2. Effects of flunitrazepam and ethanol on shock-induced behavioral inhibition in sham-treated rats (T0: one empty capsule implanted sc for 14 days) and in rats treated with testosterone for 14 days (T5: five testosterone-filled capsules implanted sc). Shown are the means ± S.E.M. Statistics: Two-way ANOVA (Factor 1: sham or testosterone treatment; Factor 2: flunitrazepam/ethanol dose; see text for *F* and *P* values). One-way ANOVA followed by Fischer's PLSD was used for evaluations of differences between individual groups (*F* and *P* values are below). The multiple comparisons were corrected for using a sequentially rejective Bonferroni procedure. T0, flunitrazepam: $F(3,34) = 6.82$, $P = .001$. T5, flunitrazepam: $F(3,34) = 4.18$, $P = .013$. +, $P < .05$, N.S.:$P > .05$.

Table 1
Lack of effect of testosterone treatment (with one, three or five testosterone-filled capsules) for 6 days (A) or 14 days (B) with or without flunitrazepam or ethanol treatment (C) on the shock threshold and on drinking motivation (the amount of 5.5% glucose solution consumed during 10 min after a 48-h period of water deprivation)

Treatment	Shock threshold (mA) (*n*)	Liquid consumption (g)
A		
T0	0.19 ± 0.03 (9)	15.89 ± 1.01 (9)
T1	0.17 ± 0.02 (8)	14.88 ± 1.26 (8)
T3	0.19 ± 0.03 (6)	15.50 ± 0.56 (6)
T5	0.14 ± 0.03 (9)	16.11 ± 1.53 (9)
B		
T0	0.09 ± 0.01 (8)	19.14 ± 1.40 (7)
T1	0.10 ± 0.01 (7)	17.44 ± 0.48 (7)
T3	0.12 ± 0.02 (6)	16.64 ± 0.96 (5)
T5	0.11 ± 0.01 (8)	17.59 ± 1.02 (6)
C		
T0 + vehicle	0.12 ± 0.02 (7)	17.57 ± 0.72 (7)
T5 + vehicle	0.13 ± 0.01 (7)	16.83 ± 1.68 (6)
T0 + flunitrazepam 0.5 mg/kg	0.14 ± 0.01 (7)	21.67 ± 1.78 (6)
T5 + flunitrazepam 0.5 mg/kg	0.16 ± 0.02 (7)	20.57 ± 0.97 (7)
T0 + flunitrazepam 1.0 mg/kg	0.13 ± 0.01 (7)	16.67 ± 4.34 (6)
T5 + flunitrazepam 1.0 mg/kg	0.15 ± 0.02 (7)	17.83 ± 1.99 (6)
T0 + ethanol 1.0 g/kg	0.12 ± 0.01 (7)	18.50 ± 0.89 (6)
T5 + ethanol 1.0 g/kg	0.13 ± 0.01 (7)	16.67 ± 1.43 (6)

Shown are the means ± S.E.M. of five to nine observations. T0: one empty silastic capsule sc; T1, T3, T5: one, three or five testosterone-filled silastic capsule(s) sc Statistics: Kruskal–Wallis ANOVA was used for evaluation of shock threshold data and one way ANOVA was used for drinking motivation data. Shock threshold data: (A) $H = 3.17$, $P = .366$. (B) $H = 1.77$, $P = .622$. (C) $H = 4.21$, $P = .755$. Drinking motivation data: (A) $F(3,28) = 0.20$, $P = .894$. (B) $F(3,22) = 0.98$, $P = .421$. (C) T0 + T5: $F(7,42) = 0.92$, $P = .504$; T0: $F(3,21) = 0.86$, $P = .479$; T5: $F(3,21) = 1.50$, $P = .245$. Thus, there were no statistically significant differences in shock threshold or drinking motivation between the treatment groups.

3.3. GABA-induced $^{36}Cl^-$ uptake into synaptoneurosomes

Rats treated with five testosterone capsules during 14 days displayed enhanced GABA-induced $^{36}Cl^-$ uptake into synaptoneurosomes prepared from these rats as compared to that observed in synaptoneurosomes prepared from sham-treated animals (Fig. 3; Experiment 4).

3.4. Serum testosterone levels

Testosterone treatment during 6 and 14 days resulted in increased serum concentrations of testosterone as compared to control treatment after all doses examined. Furthermore, rats treated with three or five testosterone capsules displayed significantly higher serum levels of testosterone as the rats treated with only one capsule (Table 2A and B; *F* and *P* values are given in the table legend).

3.5. Epididymides, testes, prostate and body weights

3.5.1. Six days of testosterone administration (Table 2A)

In rats treated with three testosterone capsules, the relative and absolute (data not shown) epididymides weights were significantly higher as compared to control rats. Furthermore, absolute testes weights were statistically significantly lower in rats treated with one testosterone capsule as compared to sham-treated rats (data not shown). A tendency for a similar effect was obtained regarding relative testes weights. Furthermore, all doses of testosterone produced higher absolute (data not shown) and relative prostate weights as compared to controls (see table legend for *F* and *P* values).

3.5.2. Fourteen days of testosterone administration (Table 2B)

The relative epididymides weights were significantly higher in rats treated with three or five testosterone capsules as compared to sham-treated rats. The absolute weights of the epididymides in these rats also tended to be higher as compared to in controls (data not shown). Rats treated with one or three testosterone capsules displayed significantly lower absolute testes weights (data not shown) and rats treated with one testosterone capsule displayed significantly lower relative testes weights as compared to control rats.

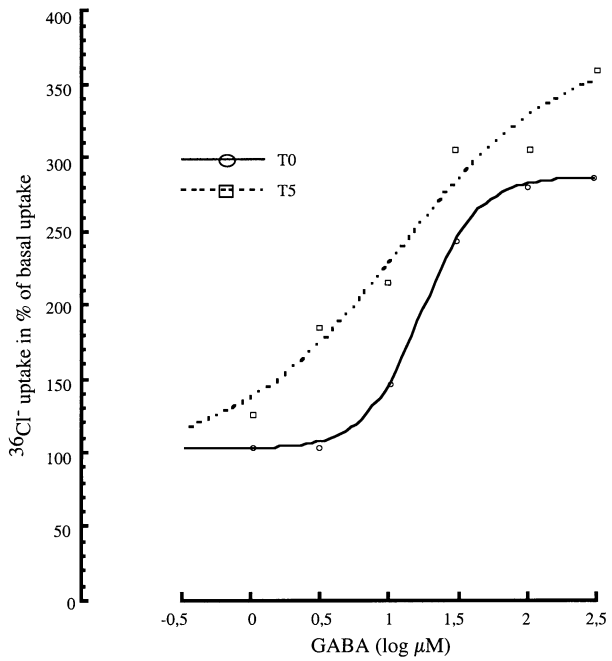

Fig. 3. Effects of testosterone treatment for 14 days (T5: five testosterone-filled capsules implanted sc) on GABA-induced (1, 3, 10, 30, 100 and 300 μM) $^{36}Cl^-$ uptake into corticohippocampal synaptoneurosomes as compared to sham-treated rats (T0: one empty capsule sc). Shown are the means of one representative experiment performed in triplicates. The experiment was repeated three times with similar results (GABA-induced $^{36}Cl^-$ uptake was higher in testosterone-treated as compared to sham-treated rats in all four experiments).

Table 2
Effect of testosterone treatment (with one, three or five testosterone-filled capsules) for 6 days (A) or 14 days (B) on serum concentrations of testosterone (S-testosterone) and relative accessory sex organ weights

	Treatment			
	T0 (*n*)	T1 (*n*)	T3 (*n*)	T5 (*n*)
A				
S-testosterone (nmol/l)	7.21 ± 2.68 (9)	13.09 ± 1.16^{c} (8)	37.67 ± 2.32^{c,f} (6)	66,87 ± 4.49^{c,f,h} (8)
Body weight (g)	329.3 ± 6.1 (9)	320.0 ± 7.0 (8)	325.3 ± 5.6 (6)	315.3 ± 6.4 (9)
Relative epididymides weights (mg/100g)	90.0 ± 3.3 (9)	86.9 ± 2.7^{ns} (8)	95.7 ± 4.8^{a} (5)	91.9 ± 3.6^{ns} (8)
Relative testes weights (mg/100g)	929.6 ± 29.3 (9)	844.7 ± 28.0 (8)	896.3 ± 26.2 (6)	916.8 ± 28.9 (9)
Relative prostate weight (mg/100g)	219.6 ± 20.4 (8)	297.3 ± 21.8^{a} (7)	371.0 ± 27.3^{a} (4)	372.0 ± 16.6^{a} (8)
B				
S-testosterone (nmol/l)	8.17 ± 1.98 (8)	12.48 ± 1.59^{b} (5)	44.67 ± 13.72^{b,e} (3)	66,50 ± 2.22^{b,e} (4)
Body weight (g)	398.5 ± 8.6 (8)	388.0 ± 3.1^{ns} (7)	366.0 ± 4.6^{a} (6)	361.3 ± 10.8^{a,g} (7)
Relative epididymides weights (mg/100g)	77.3 ± 2.0 (8)	85.1 ± 2.7^{ns} (7)	94.2 ± 3.8^{a} (6)	94.8 ± 4.2^{a} (7)
Relative testes weights (mg/100g)	789.2 ± 27.5 (8)	655.9 ± 24.1^{a} (7)	759.4 ± 22.0^{ns,g} (6)	794.8 ± 36.2^{ns,d} (7)
Relative prostate weights (mg/100g)	183.7 ± 4.2 (8)	238.5 ± 9.2^{a} (7)	279.4 ± 13.1^{a} (6)	292.9 ± 26.9^{a} (7)

Shown are the means ± S.E.M. and *n* values. T0: one empty silastic capsule sc; T1, T3, T5: one, three or five testosterone-filled silastic capsule(s) sc. Statistics: One-way ANOVA followed by Fischer's PLSD. The multiple comparisons were corrected for using a sequentially rejective Bonferroni procedure. S-testosterone: (A) $F(3,27) = 86.87$, $P < .001$. (B) $F(3,16) = 40.49$, $P < .001$. Body weight: (A) $F(3,28) = 0.98$, $P = .415$. (B) $F(3,24) = 5.42$, $P = .005$. Relative epididymides weight: (A) $F(3,26) = 3.18$, $P = .041$. (B) $F(3,24) = 7.14$, $P = .001$. Relative testes weight: (A) $F(3,28) = 1.75$, $P = .180$. (B) $F(3,24) = 5.22$, $P = .006$. Relative prostate weight: (A) $F(3,23) = 12.67$, $P < .001$. (B) $F(3,24) = 10.70$, $P < .001$.
Comparisons to T0: $^{a}P < .05$, $^{b}P < .01$, $^{c}P < .001$, ^{ns}nonsignificant ($P > .05$). Comparisons to T1: $^{d}P < .05$, $^{e}P < .01$, $^{f}P < .001$, ^{g}nonsignificant ($P > .05$). Comparisons to T3: $^{h}P < .001$.

Furthermore, relative testes weights, but not absolute testes weights (data not shown), were higher in rats treated with five testosterone capsules as compared to rats treated with one capsule filled with testosterone. All doses of testosterone increased relative prostate weights and the two highest doses increased absolute prostate weights (data not shown) as compared to sham-treated rats. The body weights were statistically significantly lower in rats treated with three and five testosterone capsules, but not one testosterone capsule, as compared to controls (*F* and *P* values are given in the table legend).

4. Discussion

In the present study, a high dose of testosterone treatment for 6 days to intact group-housed adult male rats produced disinhibitory behavior in a modified Vogel's drinking conflict model involving shock-induced behavioral inhibition. In the experiment where flunitrazepam was used, testosterone-treated rats (for 14 days) accepted more shocks as compared to sham-treated animals (see the following paragraphs). However, no statistically significant behavioral effects were obtained after 14 days of testosterone administration per se with any of the doses used. There could be several explanations for these different results regarding the behavioral effect of testosterone treatment in Experiments 2 and 3. The most plausible explanations may be that (1) more rats were used in Experiment 3, which increased the ''statistical power'' and/or (2) flunitrazepam and testosterone produced additive effects on disinhibition and sedation. In control experiments, testosterone treatment did not alter shock threshold or drinking motivation, after either 6 or 14 days of treatment. Thus, it is not likely that the effect of testosterone administration on shock-induced behavioral inhibition was secondary to unspecific effects on shock threshold and/or drinking motivation. Taken together, our interpretation of the present results is that overall, testosterone treatment for both 6 and 14 days produced behavioral disinhibition.

Treatment with any of the testosterone doses used for 6 or 14 days statistically significantly elevated the serum levels of testosterone as compared to controls. To further confirm an adequate exposure to testosterone in this study, the effect of testosterone treatment on the weights of accessory sex organs was evaluated. Testosterone treatment induced changes in testes weights, prostate weights and epididymides weights. These changes confirm that testosterone was peripherally active (O'Shaughnessy and Sheffield, 1990; Podesta et al., 1975; Orgebin-Crist et al., 1983; McLachlan et al., 1994). Thus, the testosterone treatment here applied resulted in adequate exposure to the hormone.

Data have been presented supporting our findings that testosterone treatment promotes behavioral disinhibition. In the Vogel's conflict model and in the elevated plus-maze behavioral disinhibition may be interpreted as impulsive-like and/or anxiolytic-like behavior (see Introduction). In a previous study it was found that testosterone treatment, both chronic (with five testosterone-filled silastic capsules implanted sc for 8 weeks) and ''acute'' (5 mg/kg sc 24 h before testing) in group-housed rats, produced disinhibited behavior in a modified Vogel's conflict model (Bing et al., 1998).

In contrast to our findings, it has earlier been reported that testosterone treatment (0.5 and 5.0 mg/kg sc three times per week) for 6 weeks in intact adult male Sprague–Dawley

rats does not affect impulsive-like behavior in two tests measuring different aspects of impulsive-like behavior; a paced fixed consecutive number schedule and a delayed reinforcement procedure. The last testosterone injection before testing was administered 1 h before the tests (Evenden, 1998). However, in this study, in contrast to the present study, serum levels of testosterone or weights of accessory sex organs were not evaluated. Hence, it was not demonstrated that the testosterone treatment produced an adequate exposure to the hormone.

In another study the elevated plus-maze was used to study the behavioral effect of testosterone. Treatment with one sc implanted testosterone propionate filled capsule (only one dose was used) induced disinhibited behavior in intact isolated adult male Long–Evans rats after 6, but not 14, days of treatment (Bitran et al., 1993). The treatment resulted in both elevated serum concentrations of testosterone and enhanced ventral prostate weights. In the same study, it was found that the concentration of GABA that produced 50% of the maximal Cl^- influx in cortical synaptoneurosomes was significantly decreased after 6, but not 14, days of testosterone propionate treatment. The authors argued that the finding that 14 days of testosterone propionate treatment did not alter the sensitivity of the $GABA_A$ receptor in the method used may be due to tolerance.

In the present study, however, we found that testosterone treatment during 14 days induced behavioral disinhibition in the modified Vogel's test. As noted above, the rats were single-housed in the study by Bitran et al. (1993). We have earlier found that the housing condition may be of importance when studying the relation between testosterone and behavior in the modified Vogel's test. Gonadectomy enhanced shock-induced behavioral inhibition in group-housed rats and decreased diazepam-induced sedation and muscle relaxation (Svensson et al., 2000a). In single-housed rats, gonadectomy did not affect shock-induced behavioral inhibition or GABA-induced $^{36}Cl^-$ uptake into synaptoneurosomes when determined 14 days after operation (Svensson et al., 2000b). Thus, it is plausible that the lack of effect of testosterone treatment for 14 days on conflict behavior and GABA-induced $^{36}Cl^-$ uptake into synaptoneurosomes reported by Bitran et al., 1993 (see earlier) is due to single housing of the rats. Another possibility may be that testosterone treatment affects the behavior differently in the elevated plus-maze as compared to Vogel's test. This appears, however, less likely, since different pharmacological manipulations generally affect the behavioral outcome (behavioral disinhibition/inhibition) in the two models in a similar way (reviewed in Söderpalm, 1990).

Testosterone treatment has been reported to induce dominance in rats (Albert et al., 1986) and subordination is associated with lower plasma testosterone and reduced testes weights (Blanchard et al., 1993). It has been consistently reported that social defeat in rats enhances behavioral inhibition in the elevated plus-maze (reviewed in Blanchard et al., 2001). Thus, it may be speculated that the testosterone-treated rats in our study have become dominant and the sham-treated rats subordinate and that this phenomenon at least partly explains our findings. Arguing against this interpretation is, however, the fact that testosterone treatment induces behavioral disinhibition also in *single*-housed rats (Bitran et al., 1993).

The $GABA_A$ receptor has been implicated in the mechanisms regulating behavioral disinhibition in animals, e.g., in the shock-induced behavioral inhibition test (e.g., Vogel et al., 1971; Söderpalm and Svensson, 1999; Svensson et al., 2000a). From a clinical perspective, intake of AAS together with drugs that are positive modulators of the $GABA_A$ receptor appear to impair impulse control more than intake of AAS alone, also after a longer period of AAS intake (see Introduction). This important relationship between potential disinhibitory effects of subchronic androgen exposure and positive modulators of the $GABA_A$ receptor, e.g., benzodiazepines, needs further studies. Therefore, we studied the behavioral effect of flunitrazepam and ethanol on shock-induced behavioral inhibition in rats that had been treated with testosterone for 14 days. Both flunitrazepam (e.g., Braestrup and Squires, 1978) and ethanol (e.g., Suzdak et al., 1986) are positive modulators of the $GABA_A$ receptor.

Flunitrazepam exhibited biphasic inversely U-shaped dose–response relationships with regard to the number of shock accepted, i.e., in a low dose behavioral disinhibition was induced in both treatment groups, whereas after the highest dose the number of shocks tended to decrease in both treatment groups, but most evident in the testosterone-treated rats. This effect of the highest dose may reflect flunitrazepam-induced sedation (gross observation). Thus, it may be plausible that flunitrazepam induced sedation at a lower dose in testosterone- than in sham-treated rats. However, there was no significant Treatment × Drug dose (flunitrazepam) interaction, suggesting that the effects of testosterone and flunitrazepam are independent of each other. It is plausible that the appearance of the biphasic dose–response curve had been similar for testosterone- and sham-treated rats if a higher dose of flunitrazepam had been applied in the latter group.

Ethanol, however, did not statistically significantly affect shock-induced behavioral inhibition, neither in testosterone-treated nor in sham-treated animals after any of the two doses used (0.75 and 1.0 g/kg ip). This negative finding is in contrast with earlier findings demonstrating that ethanol induced disinhibited behavior in the present model in similar doses (Engel and Liljequist, 1983). This discrepancy may be due to differences in the time when the rats were tested in the conflict task after ethanol injection. Accordingly, here the rats were behaviorally tested 10 min, and in the referred study 50 min, after ethanol administration. It should also be noted that ethanol affects many other receptors than $GABA_A$ receptors in the brain, e.g., nicotinic acetylcholine receptors and $5\text{-}HT_3$ receptors (cf. Ericson, 2000), which could also influence the results obtained.

To further explore potential GABAergic mechanisms in testosterone-treated rats we studied the functional activity of $GABA_A$ receptors in vitro. Interestingly, testosterone-treated animals displayed an enhanced GABA-induced $^{36}Cl^-$ uptake into synaptoneurosomes as compared to sham-treated animals.

Taken together, our findings demonstrate the following: (1) Biologically significant exposure to testosterone promotes behavioral disinhibition. (2) Flunitrazepam induces behavioral disinhibition in both sham- and testosterone-treated rats. (3) These effects are independent of each other. (4) Testosterone treatment for 14 days results in an increased GABA-induced $^{36}Cl^-$ uptake into synaptoneurosomes, as compared to sham-treated animals.

All these findings support an involvement of GABAergic mechanisms in testosterone-induced behavioral alterations after subchronic exposure. However, it is also plausible that the behavioral effects of 14 days of testosterone may be independent from these on GABA-induced $^{36}Cl^-$ uptake.

The mechanisms underlying these changes can only be speculated upon. Testosterone treatment may effect behavior by nongenomic and/or genomic mechanisms. Nongenomic mechanisms may involve testosterone metabolites, such as androsterone and 3α-androstanediol, which induce behavioral disinhibition in the elevated plus-maze and are positive modulators of the $GABA_A$ receptor (Bitran et al., 1996). Also genomic mechanisms could be involved, e.g., in the regulation of brain 5-HT systems, since castration increases brain 5-HT levels with a time lag of a few days (Engel et al., 1979). Conversely, testosterone propionate given every 7 days to male rats produces a decrease in diencephalic 5-HT levels (Martinez-Conde et al., 1985). Increased and decreased brain 5-HT activity produces behavioral inhibition and disinhibition, respectively (cf. Soubrié, 1986). Finally, since serotonergic and GABAergic mechanisms appear to be intimately connected in the regulation of behavioral disinhibition (Söderpalm and Svensson, 1999), testosterone-induced effects (nongenomic and/or genomic) on both systems (serotonergic and GABAergic) could be involved.

In conclusion, our data provide experimental support for the notion that abuse of anabolic steroids may impair impulse control. The data also suggest that individuals with increased androgen activity (e.g., AAS abusers) are more susceptible to potential $GABA_A$ receptor mediated behavioral disinhibition, e.g., after intake of the benzodiazepine flunitrazepam.

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