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Testosterone as a discriminative stimulus in male rats

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ABSTRACT

Testosterone and other anabolic–androgenic steroids (AAS) are reinforcing in animals, as determined by conditioned place preference or self-administration. Most drugs of abuse produce subjective effects on mood and perception that initiate and maintain drug taking. Whether AAS have similar effects is not known. Food-restricted male Sprague–Dawley rats (n = 9) were tested for their ability to discriminate an injection of testosterone from the β-cyclodextrin vehicle using a standard two-lever operant paradigm. In drug discrimination, animals use the subjective effects of drug or vehicle to select the appropriate lever to obtain food pellets under an FR10 schedule of reinforcement. All rats demonstrated vigorous responding for food (1415.1 ± 76.1 responses/20 min) with 94.9% of responses on the active lever. For the first 30 days, rats received 1 mg/kg testosterone sc 30 min before testing. On Day 14, one rat achieved the discrimination criteria of 9/10 consecutive days with >90% responses on the active lever and ≤5 responses on the inactive lever before the first reinforcement. Subsequently, rats were tested with testosterone at different doses (2, 7.5, 15 mg/kg at 30 min before testing) and times (2 mg/kg at 30 or 60 min before testing), each for 20 days. One additional rat demonstrated successful discrimination at Day 54 with 2 mg/kg testosterone 60 min before testing. The remaining 7 rats failed to discriminate testosterone within 110 days. When analyzed according to less-stringent standards, 4 additional rats met criteria for testosterone discrimination. However, continued performance was not stable. Thus, testosterone was unable to consistently support drug discrimination. We conclude that testosterone does not produce rapid interoceptive effects (NIH DA12843 to RIW).

1. Introduction

Anabolic–androgenic steroids (AAS) are steroid molecules derived from testosterone (reviewed in Wood, 2008). Among their many physiologic and behavioral effects, AAS improve athletic ability, build muscle, and enhance competitive performance (ACSM, 2006). Not surprisingly, AAS are subject to abuse by athletes and others. Since 1991, AAS have been classified by the Drug Enforcement Agency as Schedule III controlled substances, and it has been estimated that up to 1 million Americans may have used AAS (Johnston et al., 2006; McCabe et al., 2007). Recent evidence suggests that dependence may develop in susceptible individuals with a long-term history of illicit AAS use (Kanyama et al., 2009). Nonetheless, the potential for AAS dependence remains understudied.

AAS have unique features that distinguish them from other commonly-abused substances. Athletes do not use steroids to "get high". Instead, most human users seek the anabolic (muscle-building) effects of AAS, rather than a subjective sense of pleasure and reward (Kanyama et al., 2010). Anabolic effects of AAS develop slowly over weeks or months. The classic view of steroid action is that steroids act as transcription factors within cells, and follow a relatively slow time-course with a delayed onset and persistent action (Rommets, 2004). This model is consistent with testosterone effects on libido and sexual behavior (Hull et al., 2006). The slow time-course of steroid action would argue against AAS producing a state of acute intoxication characteristic of most drugs of abuse.

On the other hand, it is now recognized that steroids may have rapid effects on brain and behavior that are not mediated through classic genomic receptors (Mermelstein and Micevych, 2008; Vasudevan and Pfaff, 2008). These non-genomic effects of steroids occur within seconds to minutes, and can be transduced in brain regions largely devoid of classic steroid receptors. Through their non-genomic actions, steroids have potential to produce subjective effects on mood and perception that serve to initiate and maintain drug taking, thereby leading to dependence.

Studies in rats and mice have demonstrated conditioned place preference (CPP) when testosterone was paired with a distinct environment for 30 min following systemic or intracerebral injection (Alexander et al., 1994; Arnedo et al., 2000, 2002; Frye et al., 2002; Packard et al., 1997; Schroeder and Packard, 2000). This time period is consistent with a non-genomic effect of testosterone. Conversely, a single study of former opiate abusers receiving testosterone injections concluded that AAS "are devoid of the usual pharmacologic effects that are associated with abuse" (Fingerhood et al., 1997). However, subjects were tested only

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with single doses of 50–200 mg testosterone, far below those advocated on body-building websites (over 2 g/week: http://www.steroid.com/advancecycle2.php). Furthermore, the subjects had no previous exposure to AAS. Thus, additional studies to test the discriminative stimulus effects of androgens are warranted. The present study used a standard 2-lever choice test for food reward (Solinas et al., 2006) to determine if AAS possess salient pharmacologic properties. With this approach, rats learn to use the subjective effects of drug vs vehicle injection to select the appropriate lever to receive a food reward. We evaluated the discriminative stimulus effects of testosterone in male rats across a range of doses comparable to those used by human AAS users. Drug discrimination has been demonstrated for a wide range of drugs of abuse (Colpaert, 1999). Whether AAS have similar effects has not previously been determined.

2. Materials and methods

2.1. Animals

Young adult male Sprague–Dawley rats (Charles River Laboratories) were 51–54 days of age, and weighed 204.9 ± 1.9 g BW at the start of the study. Rats were individually housed under a reversed 14L:10D photoperiod and stable ambient temperature (24 °C). All rats remained gonad-intact to more closely approximate AAS use in humans. Daily testing was conducted under dim illumination during the first 4 h of the dark phase when activity peaks. Water was available at all times, except when rats were in the operant chamber. To facilitate operant responding, rats were weighed daily and received a measured amount of food sufficient to maintain a slow rate of growth (ca. 1–2 g/day). Veterinary and daily care were supplied by the USC Vivaria. Experimental procedures were approved by USC's Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (DHEW Publication 80-23, revised 1985, Office of Sciences and Health Reports, DRR/NIH, Bethesda, MD 20205).

2.2. Drugs

To prepare androgen solutions for injection, testosterone (Steraloids, Newport, RI) was dissolved in an aqueous 13% cyclodextrin (RBI, Natick, MA) vehicle. Vehicle injections consisted of the 13% cyclodextrin solution alone. Male rats and hamsters self-administer testosterone iv or icv, but do not self-administer the cyclodextrin vehicle (Sato et al., 2010; Wood et al., 2004). Solutions were prepared weekly.

2.3. Experimental design

The study followed established methods for drug discrimination (Solinas et al., 2006). Training and testing were conducted in operant conditioning chambers (Med Associates, St. Albans, VT) connected to a PC running WMPC software (Med Associates). Each operant chamber was equipped with a house light, food pellet dispenser, and 2 levers each with stimulus light, and was enclosed in a sound-attenuating cubicle with a fan for ventilation. Levers were located 6 cm above the cage floor, flanking the pellet trough.

2.4. Training

Initially, rats were trained to press both levers for 45 mg food pellets (Bioserv, Frenchtown, NJ) on a fixed-ratio 1 (FR-1) schedule of reinforcement during daily 20-min sessions 5×/week, with a stimulus light to indicate the correct lever. Response rates were gradually increased to FR-10. A response on the incorrect lever caused the ratio on the correct lever to reset. Rats achieved a stable rate of responding at FR-10 (>200 responses/20 min for 5 sessions) after an average of 31.4 ± 0.9 sessions.

On subsequent days, each rat received an injection sc of 1 mg/kg testosterone or vehicle 30 min before being introduced into the operant chamber. Injections varied in a semi-random order, with no more than 2 consecutive injections of testosterone or vehicle. Reinforced responses on the two levers (for testosterone or vehicle) were balanced to account for side preferences. Exposure to 1 mg/kg testosterone for 30 min induces CPP in rats (Alexander et al., 1994; Schroeder and Packard, 2000); 30 min of testosterone exposure at 1 mg/kg is sufficient to acquire LiCl-induced conditioned taste aversion (De Beun et al., 1992).

For each rat, injection of testosterone was consistently paired with illumination of one stimulus light and reinforced responses on the underlying lever; vehicle injection was paired with the opposite light/lever combination. The specific light and lever paired with testosterone or vehicle was balanced to account for side preferences. With continued daily training (16.6 ± 0.9 sessions), both stimulus lights were illuminated to eliminate the use of this discriminative cue. This forces rats to use interoceptive cues specific to drug or vehicle injection to select the correct lever to receive reinforcement.

2.5. Testing

Testing for discrimination of testosterone took place over the next 110 days (see Fig. 1). For the first 30 days of testing, rats received 1 mg/kg testosterone 30 min before introduction into the operant chamber. Subsequently, the dose of testosterone was increased to 2 mg/kg at 30 and 60 min prior to testing, each for 20 days. Thereafter, rats received testosterone (7.5 and 15 mg/kg) 30 min before testing, each for 20 days.

We evaluated discrimination of testosterone according to different criteria for drug discrimination. A common, if strict, definition of successful discrimination is 9 of 10 consecutive sessions with >90% of total responses on the correct lever, and ≤5 responses on the incorrect lever prior to obtaining the first food reinforcer (Solinas et al., 2006). In this regard, the initial lever selection is considered a stronger index of discrimination, since rats will often continue to respond on the correct lever once they receive a food reward (Solinas et al., 2006). We also evaluated testosterone discrimination by less-

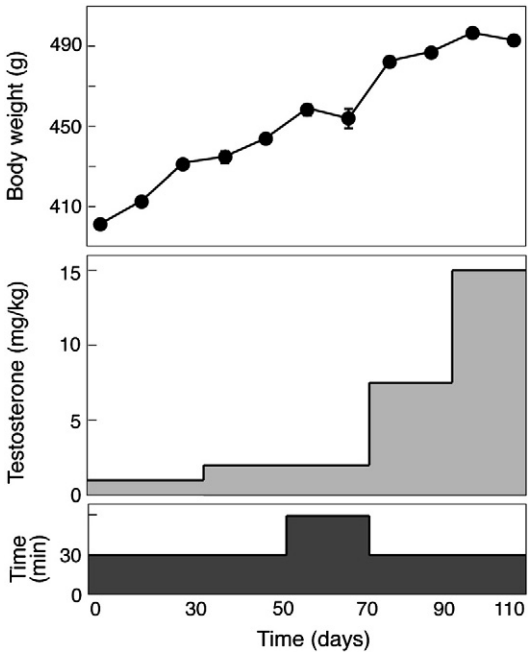

Fig. 1. Experimental design and body weight (mean ± SEM) in male rats (n = 9) during testing for discrimination of testosterone vs vehicle. To facilitate operant responding for food, rats were limited to a slow rate of growth throughout the study.

stringent standards (>90% of total responses on the correct lever, with <10 responses on the incorrect lever before the first reinforcer for 8 of 10 or 9 of 12 consecutive days), according to Overton et al. (1986).

3. Results

Rats responded vigorously for food on an FR-10 schedule following injection of testosterone or vehicle. Fig. 2 illustrates the percent of operant responses on the lever paired with testosterone following injection of testosterone or vehicle. When tested 30 min after testosterone injection across a range of testosterone doses (1–15 mg/kg), rats made 92.6 ± 2.0% of all operant responses on the testosterone lever. Conversely, rats made only 2.8 ± 0.7% of operant responses on the testosterone lever after vehicle injection. This difference was highly significant ($p<0.05$). However, by repeated measures ANOVA, there was no effect of testosterone dose and no interaction of drug and dose.

In response to 2.0 mg/kg testosterone or vehicle, there was no effect of injection time (30 or 60 min) on percent responses on the testosterone lever. Rats made 94.3 ± 4.0% responses on the testosterone-paired lever 30 min after a testosterone injection, and 97.2 ± 0.7% responses when injected 60 min before testing. By contrast, 30 or 60 min after an injection of vehicle, responses on the testosterone-paired lever averaged 2.0 ± 0.6% and 1.6 ± 0.4%, respectively. Although there was a significant effect of drug (testosterone vs vehicle, $p<0.05$), there was no effect of time (30 vs 60 min) and no drug × time interaction.

Fig. 3 presents the total number of operant responses per 20-minute session after injection of testosterone or vehicle. When tested 30 min after vehicle injection, rats averaged 1611.0 ± 83.6 responses/20 min (1.3 ± 0.1 responses/s). Across all doses of testosterone, rats made fewer responses after testosterone injection (1312.3 ± 130.5 responses/20 min), but there was no significant effect of drug ($p=0.07$ by repeated measures ANOVA). Nonetheless, there was a significant effect of dose on total responses, with maximal rates of responding at the

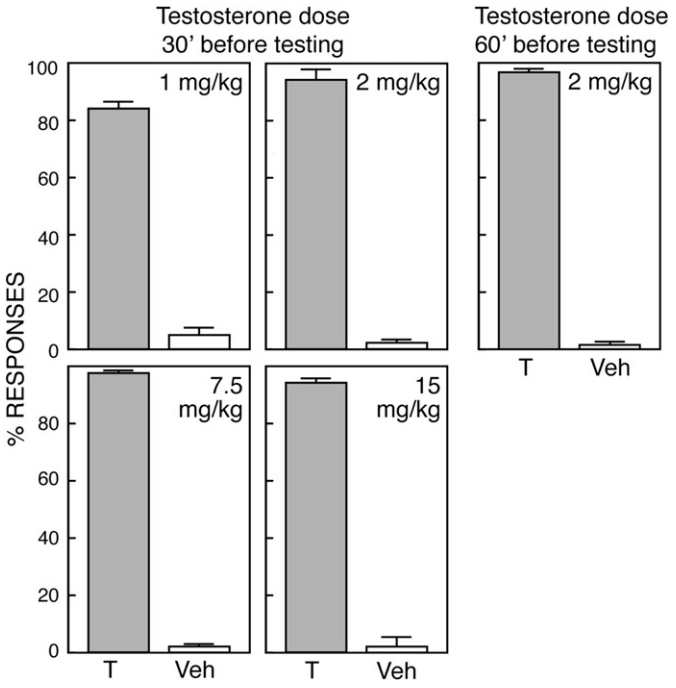

Fig. 2. Percent of total operant responses on the testosterone-paired lever following injection of testosterone (T: gray bars) or vehicle (Veh: white bars) in male rats tested for discrimination of testosterone vs vehicle to obtain a food reward. Left: Percent responses at different doses of testosterone (1–15 mg/kg) administered 30 min before testing. Right: Percent responses to 2 mg/kg testosterone administered 60 min before testing.

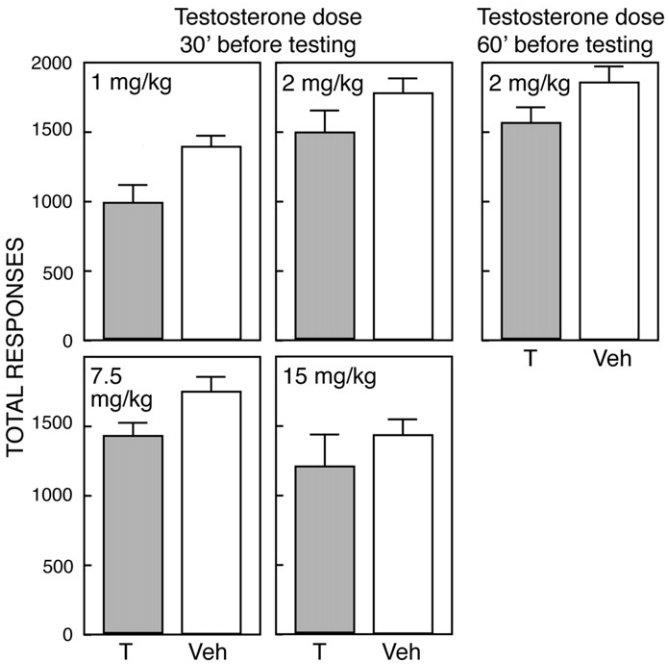

Fig. 3. Total operant responses on both levers following injection of testosterone (T: gray bars) or vehicle (Veh: white bars) in male rats tested for discrimination of testosterone vs vehicle to obtain a food reward. Left: Total responses at different doses of testosterone (1–15 mg/kg) administered 30 min before testing. Right: Total responses to 2 mg/kg testosterone administered 60 min before testing.

2 mg/kg dose (1799.0 ± 116.2 responses/20 min after vehicle, 1531.4 ± 156.4 responses/20 min after testosterone), and the lowest response rates at 15 mg/kg testosterone (1082.8 ± 114.0 responses/20 min vs 1476.2 ± 111.3 responses/20 min after vehicle). There was no effect of drug or time on total responses when rats were tested 30 or 60 min after injection of vehicle or testosterone at 2 mg/kg ($p>0.05$).

Across a range of testosterone doses, rats easily met one criterion for successful drug discrimination (>90% of total responses on the correct lever). However, they were less successful in their initial selection of the correct lever. Fig. 4 illustrates testosterone discrimination in individual rats according to criteria of Solinas et al. (2006) and Overton et al. (1986). By the strictest criteria (≤5 responses on the incorrect lever prior to obtaining the first food reinforcer in 9 of 10 consecutive sessions; Solinas et al. (2006)), only two rats were successful. Rat #5 met the criteria for successful testosterone discrimination on the 14th day of study, when tested 30 min after vehicle or 1 mg/kg testosterone. Rat #9 demonstrated discrimination of testosterone on the 54th day, when tested 60 min after vehicle or 2 mg/kg testosterone. None of the other 7 rats met this criterion within 110 days. Importantly, discrimination was not stable in Rat #5 and Rat #9. After meeting the criteria for successful discrimination, Rat #5 correctly discriminated the active lever on 44 of 96 (41.9%) of test days; Rat #9 was only marginally more successful (49.1%). When evaluated using less-stringent criteria (8 of 10 or 9 of 12 consecutive days with <10 responses on the incorrect lever before food reward [Overton et al., 1986]), 4 additional rats discriminated testosterone after 29, 38, 58, and 98 sessions. However, their continued performance was not stable (successful discrimination on 45.3% of the remaining test days) Furthermore, 3 of 9 rats did not meet criteria for discrimination of testosterone within 110 days.

4. Discussion

In the present study, high-dose testosterone was unable to serve as a discriminative stimulus in a standard 2-choice test of operant behavior. When tested on an FR10 schedule across a range doses (1–

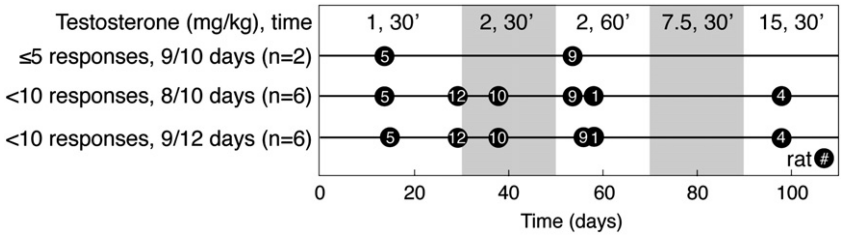

Fig. 4. Successful discrimination of testosterone vs vehicle to obtain a food reward in male rats. Testosterone dose and timing during 110 days of testing is illustrated at top. Black circles represent the completion of successful discrimination in individual rats according to established criteria (>90% of total responses on the correct lever, and either ≤5 responses on the incorrect lever prior to obtaining the first food reinforcer for 9 of 10 consecutive days, <10 responses for 8 of 10 consecutive days, or <10 responses for 9 of 12 consecutive days).

15 mg/kg) equivalent to those used by athletes and other AAS abusers, and at 30 and 60 min after injection, rats were unable to consistently select the correct lever to receive a food reward. Drug discrimination is a core test of abuse liability (Solinas et al., 2006). Although rats in the present study were unable to meet the criteria for successful discrimination of testosterone, reinforcing effects of testosterone and other AAS have been demonstrated by CPP (Alexander et al., 1994; Arnedo et al., 2000, 2002; Frye et al., 2002; Packard et al., 1997; Schroeder and Packard, 2000) and self-administration (Ballard and Wood, 2005; DiMeo and Wood, 2006; Johnson and Wood, 2001; Peters and Wood, 2004; Sato et al., 2010; Triemstra and Wood, 2004; Wood, 2002; Wood et al., 2004). Together, these findings argue that testosterone is reinforcing, but is not accompanied by rapid subjective effects.

Many studies of drug discrimination follow a fairly consistent methodology. Where published reports vary is in the specific criteria for successful drug discrimination. Craft et al. (1999) applied the criteria of Solinas et al. (2006) to test sex differences and effects of gonadal steroids on morphine discrimination. In their study, 7 gonad-intact male rats met the criteria after 33.3 ± 5.1 days (range 20–57 days), compared with only 2 of 9 male rats over 110 days in the present study. For our rats discriminating testosterone vs vehicle, the problem was not in achieving >90% of total responses on the correct lever. Rats in the present study were highly motivated to obtain food reward, and responded vigorously on the correct lever. In fact, the number of reinforcements obtained per minute in the present study (7.7 ± 0.4 after vehicle, 6.2 ± 0.6 after testosterone) was higher than reported previously [4.7 ± 0.3 for gonad-intact males after saline injection; Craft et al. (1999)]. Previous studies have found no effect of chronic high-dose testosterone (2.5 mg/kg) on food intake in male rats (Nunez, 1982), suggesting that testosterone is unlikely to compromise the willingness to work for food in a test of drug discrimination.

Rats had difficulty in initially selecting the correct lever, suggesting that they were unable to detect subjective effects of testosterone. When the less-stringent criteria of Overton et al. (1986) were applied, 33% of the rats in the present study were unable to discriminate testosterone from vehicle. Even in rats that met the initial criteria, continued successful discrimination was inconsistent. Therefore, it appears that testosterone does not produce a strong interoceptive cue.

Furthermore, it seems unlikely that rats would have achieved successful drug discrimination in response to another AAS. Testosterone was used here, both because of chemical similarities to other AAS and relevance to human AAS use. All synthetic AAS are derived from testosterone (reviewed in Wood, 2008). Nonetheless, different AAS have a spectrum of behavioral effects. In this regard, testosterone stimulates sexual and aggressive behavior in rats (Clark and Harrold, 1997; Farrell and McGinnis, 2003), nandrolone (a long-acting AAS) has more modest effects (Clark and Fast, 1996; Farrell and McGinnis, 2003), and stanozolol (an orally-active AAS) inhibits both mating and aggression (Clark et al., 1997; Farrell and McGinnis, 2003). Hamsters show similar rates of self-administration for testosterone (Wood et al., 2004), dihydrotestosterone (DHT, the major non-aromatizable androgenic metabolite of testosterone; DiMeo and Wood, 2006), nandrolone, and drostanolone (a short-acting AAS; Ballard and Wood, 2005). They do not self-administer stanozolol. From a clinical standpoint, testosterone itself is a drug of abuse. In the 2011 Prohibited List of the World Anti-Doping Agency (WADA), testosterone is included among banned anabolic agents that are "Endogenous AAS when administered exogenously" (WADA, 2011). Because native testosterone has a relatively short half-life (1–2 h; Sommerville and Tarttelin, 1983), most human AAS users take long-acting testosterone esters (e.g. testosterone cypionate, Summers, 2002). However, the active steroid is still testosterone. In 2006, testosterone was the single most-common banned substance detected in urine tests at WADA-accredited laboratories, representing 26% of all "adverse analytical findings" (WADA, 2006). Nandrolone was 4th (5.5% of positive tests), behind β2-adrenergic receptor agonists (used as bronchodilators) and cannabinoids. Likewise, testosterone accounted for the largest fraction (34%) of AAS-positive urine tests at the 2000 Sydney Olympic Game (Van Eenoo and Delbeke, 2003).

The doses of testosterone in the present study (up to 15 mg/kg/day) are far above the requirements for physiologic replacement in castrated males (up to 30 μg/kg/day; Hernandez et al., 1994; Lima et al., 2000). Extended exposure to exogenous testosterone at high doses in the present study would be expected to reduce endogenous testosterone production through negative feedback on the hypothalamus and pituitary (Friedl, 2000), as it does in human AAS users (Summers, 2002). Nonetheless, the doses used here would be considered supra-physiologic in castrated males. Previous studies in laboratory rodents have demonstrated effects of androgens on reward and social behavior in a dose range from 1 to 7.5 mg/kg. At 0.8–1.2 mg/kg, testosterone induces CPP (Alexander et al., 1994) and LiCl-conditioned taste aversion (DeBeun et al., 1992) in male rats. This suggested that our initial testosterone dose of 1 mg/kg should be sufficient for discrimination. Similarly, male hamsters (ca. 200 g BW) will self-administer 600 μg of testosterone iv (3 mg/kg) over a 4-hour test session (Wood et al., 2004). With chronic exposure at 5–7.5 mg/kg, testosterone stimulates sexual and aggressive behavior in male rats (Clark and Harrold, 1997; Farrell and McGinnis;, 2003). In the present study, only 15 mg/kg testosterone was beyond the range normally tested in the laboratory. Even so, there were no significant effects on operant behavior.

It is difficult to scale testosterone injections in rats to AAS use in humans. When adjusted according to body surface area according to FDA guidelines (FDA.gov), the doses tested here appear to fall within the range of human AAS use. Clinical studies of human volunteers typically give AAS at up to 600 mg/week. Within this range, subjective drug responses ("feel the drug", "feel high") have not been observed (Fingerhood et al., 1997). However, the amounts that can ethically be administered to human volunteers are much lower than the doses advocated on body-building websites (over 2 g/week, as in "Advanced Cycle #2–3" at Steroid.com). Indeed, we suspect that the true patterns and doses of human AAS use far exceed even these internet recommendations. However, based on the results of the present study, it would appear that, even at high doses, testosterone fails to produce a rapid and pronounced interoceptive cue.

There is a final consideration of drug delivery that is relevant to testosterone discrimination. Most human users take AAS orally,

transdermally or by im injection (Summers, 2002). Their goal is to obtain a lasting elevation in systemic androgens, rather than a rapid increase. However, users also acknowledge that im steroid injections are painful. Previously, our laboratory has demonstrated testosterone self-administration in laboratory rodents by oral, iv or icv routes (DiMeo and Wood, 2006; Johnson and Wood, 2001; Peters and Wood, 2004; Sato et al., 2010; Triemstra and Wood, 2004; Wood, 2002; Wood et al., 2004). CPP has been demonstrated by testosterone injection sc (Alexander et al., 1994), similar to the present study. In terms of the rate of androgen delivery to the brain, the sc and oral routes are intermediate between the slow release of im injection vs the acute bolus of icv delivery. And while rapid testosterone delivery might produce a stronger discriminative cue, there is no clinical human counterpart to icv self-administration. Thus, the sc injection used here is a reasonable approximation of clinical AAS use.

While the classic model of steroid action on behavior argues for a slow time-course (days–weeks), rats develop CPP when paired on 4 occasions for only 30 min after testosterone injection (Alexander et al., 1994; Schroeder and Packard, 2000). Accordingly, it seemed that 30 min should be sufficient to detect subjective effects of testosterone in the present study. Instead, only 2 rats discriminated testosterone 30 min after injection, and there was no increase in performance when the duration was increased to 60 min. In trying to reconcile the discordant results from CPP and drug discrimination, it is important to note that drug discrimination requires an immediate selection of the correct lever, while CPP can reflect an accumulated sense of positive reinforcement over multiple testosterone pairings. This is consistent with self-reports of mood among human AAS users. Users do not report a "rush" associated with injection. Indeed, AAS are most often delivered by im injection to achieve a slow release rate (Summers, 2002). With chronic steroid use, many users feel a long-term sense of power and well-being, and a corresponding sense of depression and withdrawal when steroid use is discontinued (Kanyama et al., 2009). The delayed behavioral action of testosterone is similar to that of fluoxetine, which has a long long-half life and is relatively ineffective as a training agent in drug discrimination (Dekeyne and Millar, 2003).

It remains possible that rats might discriminate testosterone only after a much longer post-injection interval. In this regard, hamsters show both hypothermia and bradykinesia after a 4 h testosterone infusion icv (Peters and Wood, 2004). This time-course is consistent with testosterone action either via classical or non-genomic mechanisms. However, a recent study showed that male Tfm rats lacking classical androgen receptors would nonetheless self-administer DHT (Sato et al., 2010), suggesting that classical androgen receptors are not essential for androgen reward. Ultimately, the lack of discrimination in the present study suggests that testosterone does not impart rapid interoceptive effects comparable to other drugs of abuse. Instead, it would appear that the subjective reinforcing effects of testosterone may develop slowly via mechanisms that do not require androgen receptor binding.

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