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ANABOLIC-ANDROGENIC STEROID ABUSE AND COGNITIVE IMPAIRMENT: TESTOSTERONE IMPAIRS BICONDITIONAL TASK PERFORMANCE IN MALE RATS.

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Abstract

Our goal is to understand the consequences of anabolic-androgenic steroid (AAS) abuse on cognitive function, using rats as a model. There is relatively little research on how AAS abuse impacts cognition. In the present study, rats were tested for their ability to use contextual information to guide decision-making in biconditional discrimination. The Stroop task is a classic human test for contextual decision-making. In rodents, biconditional discrimination challenges subjects to use contextual cues in the operant chamber to resolve the correct lever response when auditory and visual cues are incongruent. The hypothesis is that chronic high-dose testosterone impairs biconditional discrimination. Rats were trained in 24 trials/day over 14 days, in alternating sessions with each environment. On a flat floor with houselight illuminated, auditory cues (clicker vs tone) signified the active lever. On a barred floor with no light, visual cues from 2 stimulus lights (constant vs blinking) identified the active lever. Rats treated chronically with testosterone (7.5 mg/kg) were unimpaired in task acquisition, and all rats learned to select the correct lever in response to auditory or visual cues. During extinction, controls made significantly more correct than incorrect responses in congruent trials ($p < 0.05$ by paired t-test), but testosterone-treated rats failed to show a similar preference. This was reflected by significant interactions of drug x cue agreement ($F_{1,18} = 5.21$, $p < 0.05$) and drug x cue agreement x response accuracy ($F_{1,18} = 8.95$, $p < 0.05$). These results suggest that testosterone impairs cognitive flexibility, and demonstrates potential for AAS abuse to impair cognitive function in humans.

Keywords

anabolic agents; biconditional discrimination; discrimination learning; operant behavior; testosterone

1. INTRODUCTION

A central theme of our research is to understand the consequences of anabolic-androgenic steroid (AAS) abuse on executive function, using rats as a model. Although AAS abuse is

now widespread among both professional and rank-and-file athletes [1], there has been relatively little research on how steroid abuse impacts decision-making and cognitive flexibility. The present study extends earlier work showing AAS-induced alterations in decision-making and deficits in cognitive flexibility [2]. In the present study, rats were tested for their ability to use contextual information to guide decision-making in biconditional discrimination. Chronic high-dose testosterone reduced performance in this task. This demonstrates the potential for AAS abuse to impair executive function in humans.

Executive function reflects processes responsible for cognitive control of behavior, including selective attention, memory and cognitive flexibility. Recent studies have shown that human AAS users have diminished visuospatial memory compared to non-users, and the level of impairment is correlated with lifetime AAS use [3]. Other studies report difficulties with working memory, problem solving, speed of processing [4] and attention [5]. AAS users also show increased amygdala volume, but decreased connectivity with cortical brain areas involved in cognitive control [6]. Likewise, in non-laboratory settings, AAS users report deficits in prospective and retrospective memory, and executive function [7].

However, there are significant limitations to studies of AAS in humans [8]. AAS users are unusually covert about their use of steroids, and are unlikely to disclose this information to physicians [9]. Furthermore, it is unethical to treat volunteers with the massive doses of AAS relevant to human users, thereby precluding the possibility of randomized controlled human trials. Instead, animal studies can explore consequences of AAS in an experimental context where appearance and athletic performance are irrelevant. Animal studies also eliminate potential confounds such as poly drug abuse and preexisting behavioral tendencies to specifically address androgen effects on health and behavior.

Recent studies in animals have explored how AAS modify executive function. Spatial memory in the Morris water maze is impaired in rats treated with high-dose testosterone [10, 11], and exercise does not overcome this deficit [12]. Furthermore, high-dose testosterone alters decision-making as measured in operant discounting tasks [2]. In these studies, testosterone-treated rats will work harder, wait longer and accept punishment relative to controls. However, they do not tolerate uncertainty. Not surprisingly, AAS also impact the mesocorticolimbic dopamine system [13], which is central to many of these processes.

Cognitive flexibility is necessary for appropriate behavioral adaptations to dynamic environments. In tests of cognitive flexibility, human and animal subjects first learn stimulus-response rules to earn reward. When the rules change, subjects must shift response strategies to continue earning reward. Set shifting is evaluated in humans by the Wisconsin Card Sorting Task [14], and in rats by shifts from one stimulus dimension to another [15]. In our recent study of reversal learning (rule inversion) and extra-dimensional set shifting [16], rats treated with chronic high-dose testosterone were unimpaired in the initial learning of an operant task. But when the rules changed, they required at least 50% more trials to adjust successfully to the new rules. Deficits in behavioral flexibility often result from an increase in perseverative behavior, the inability to cease use of a response strategy when it is no longer relevant. Perseveration has been associated with high levels of testosterone in humans [17] chicks [18], and rats [19].

Here we used biconditional discrimination to test contextual modulation of decision-making. The Stroop task is a classic human test for contextual decision making [20]. Subjects are asked to read a color word (e.g. “green”, “orange”) and name the ink color in which the word is printed (green or orange). Subjects respond faster when the word and ink are congruent (“green” printed in green ink), than when they are incongruent (“green” printed in orange ink). The context is the instruction to name the ink color, and subjects must inhibit the tendency to read the word when the word-ink combination is incongruent. Impairments in the use of contextual information to guide behavior are seen in patients with Parkinson’s disease [21] and schizophrenia [22]. In rodents, biconditional discrimination challenges subjects to use contextual cues in the operant chamber to resolve the correct lever response when auditory and visual cues are incongruent [23]. The hypothesis is that chronic high-dose testosterone impairs biconditional discrimination in rats.

2. MATERIALS AND METHODS

2.1. Animals

20 Male Long-Evans rats (5 weeks of age at the start, Charles River Laboratories, MA) were treated chronically with either vehicle or testosterone (n=10/group). Rats were pair-housed with another rat from the same treatment group with *ad libitum* access to water under a reversed 14L:10D photoperiod, and performed daily behavioral testing (5 days/week) during the dark phase. Rats remained gonad-intact in order to approximate human AAS use. To maintain a slow rate of growth (3–4 g/day) and facilitate operant responding, rats were food restricted as in our previous studies [16, 24]. Vehicle- and testosterone-treated rats received the same amount of food, and did not differ in body weight at the start of the study or throughout testing. Experimental procedures were approved by USC’s Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Ed (National Research Council, National Academies Press, Washington DC; 2011).

2.2. AAS treatment

As in our previous studies [16, 24], rats received daily injections sc of 7.5 mg/kg testosterone (Steraloids, RI) or vehicle (3% ethanol and 13% cyclodextrin (RBI, MA) 5 d/wk, beginning at 5 weeks of age. Injections were delivered immediately before rats were placed in the operant chambers. Rats received injections for at least two weeks prior to behavioral testing, and daily injections continued for the duration of the experiment. Testosterone was used because it is the prototypical AAS. All AAS are derived from testosterone, and bind to the androgen receptor [25]. At 7.5 mg/kg, this dose approximates a heavy steroid dose in humans, and has been used to test effects on rodent discounting behavior [16, 24, 26, 27]. Pubertal treatment mirrors patterns of human use, where 4–6% of high school boys in the United States have used AAS [28]. Furthermore, AAS have the strongest behavioral effects in rodents when introduced in adolescence [29]. In particular, because prefrontal cortex (PFC) circuitry is still developing during adolescence [30], it is important to understand how adolescent steroid use may impair behavioral flexibility and its underlying neurobiological mechanisms.

2.3. Biconditional discrimination training

Rats were trained to respond for 45 mg sucrose pellets (Bio-Serv Inc., Frenchtown, NJ) in operant chambers (Med Associates, St. Albans, VT) enclosed in sound-attenuating boxes with fans for ventilation. Each chamber was equipped with a food pellet dispenser with trough flanked by two retractable levers with stimulus lights, a houselight, clicker, and Sonalert module (2,900Hz at 65db, Figure 1A).

Rats were tested for biconditional discrimination according to modifications of Hadden et al. [23]. Daily test sessions lasted 48 min, and consisted of 24 trials (12 trials/lever) of 60 sec each, with a mean intertrial interval (ITI) of 60 sec (range: 30–90 sec). On alternate days, rats were tested in 1 of 2 environments (flat floor with houselight; barred floor with no light), and the order of presentation was balanced (half the rats in each group began with bars, half began with flat).

Initially, each rat received 4 sessions of magazine training (2 sessions/environment) on a 120 sec variable-time (VT) schedule. This was followed by 2 sessions of lever training on an FR1 schedule, and 2 sessions on a 15-sec random-interval (RI) schedule. During lever training, 1 lever was inserted in each 60-sec trial on an alternating basis (left/right lever), and responses were reinforced with a single sugar pellet.

Next, rats were trained for biconditional discrimination over 14 days, in alternating daily sessions with each environment (flat floor with houselight; barred floor with no light). In each trial, both levers were inserted into the chamber, and rats used visual or auditory cues to discriminate the active lever (Figure 1). No pellets were available during the first 10 sec of each trial, and responses on the active lever were reinforced with a single pellet on a 15-sec RI schedule during the last 50 sec. On a flat floor with the houselight illuminated, auditory cues signified the active lever (clicker: left lever; tone: right lever). On a barred floor with no light, rats used visual cues from the two stimulus lights (constant illumination: left lever; blinking 1 sec on/off: right lever) to identify the active lever.

2.4. Biconditional discrimination extinction

Rats were tested for extinction in 1 daily session in each environment (flat floor with houselight; barred floor with no light) with the 2 environments presented in a balanced order. Rats received no pellets during extinction sessions. Each session was divided into 6 blocks of 4 trials each. Each block included 2 congruent trials, with auditory and visual cues aligned (clicker/constant light, tone/blinking light), and 2 incongruent trials, where auditory and visual cues were mismatched (clicker/blinking light, tone/constant light), in random order. In the incongruent trials, responses on the lever associated with the cue (auditory or visual) corresponding to the environment during discrimination training were considered correct. On a flat floor, the auditory cue should signify the correct response; on a barred floor, the rat should respond to the visual cue. Accordingly, rats should respond on the left lever for the clicker/blinking light combination when tested on the flat floor, but should respond on the right lever for the same stimuli when tested on the barred floor (see Figure 1).

2.5. Novel object recognition

To evaluate recognition memory in testosterone- and vehicle-treated rats, we analyzed novel object recognition (NOR) according to Ennaceur and Delacour [31]. This test is conducted over a 3-day period, and takes advantage of rats' normal preference to investigate a novel object. NOR testing took place in a standard plastic arena ($38 \times 78 \times 30$ cm). Objects did not resemble living stimuli [32], and could not be displaced by the rats. Objects were adhered with Velcro to the floor 12cm from the back wall, and spaced 38cm apart. At the start of each presentation, rats were placed towards the center, near the front wall of the arena with their bodies positioned away from the objects. During habituation on Day 1, rats were given 10 minutes to freely explore the empty arena. On pre-test day (Day 2), rats were exposed to 2 identical objects (unopened cans of soft-drink) for 5 minutes. On test day (Day 3), they were exposed to 1 familiar object from the pre-test day, and 1 novel object (stapler), again for 5 minutes.

Data were recorded on video, and analyzed by an observed blinded to treatment groups. For each rat, we recorded the number and duration of each interaction with each object, total duration of interaction with each object, and the latency to first interaction. Group means were compared by RM-ANOVA, with drug (testosterone and vehicle) as between-subjects factors, and day (pre-test and test) as the repeated within-subjects factor.

2.6. Data analysis:

Data were analyzed during the last day of biconditional discrimination training in each environment, and from 1 day of extinction testing in each environment. For each rat in each environment, we determined the number of pellets earned, and the number of correct and incorrect responses. Individual responses were averaged across the two experimental groups (vehicle and testosterone). Using JMP Pro 14 statistical software (SAS Institute, NC), data were analyzed by RM-ANOVA with drug (vehicle vs testosterone) as a between-subjects effect, and environment (flat vs. barred floor), response choice (correct vs incorrect), and cue agreement (congruent vs incongruent, for extinction sessions) as repeated measures. Where there was a significant effect of response choice, correct and incorrect responses were compared by paired t-test for testosterone- and vehicle-treated rats. For novel object recognition, time spent with each object during pre-test and test days was evaluated by RM-ANOVA, comparing the effects of drug (testosterone vs vehicle) as a between-subjects effect, and object (familiar vs novel), and day (pre-test vs test) as repeated measures. Where there was a significant effect of object, time with novel and familiar objects during pre-test and test days was compared by paired t-test for testosterone- and vehicle-treated rats.

3. RESULTS

3.1. Biconditional discrimination training

By the end of training, testosterone- and vehicle-treated rats learned the response requirements to receive sugar pellets in both environments (flat floor with houselight; barred floor with no light). There was no significant effect of testosterone during training on operant responding ($F_{1,18}=1.50$, n.s.) or pellets received ($F_{1,18}=2.66$, $p<0.05$), and no interaction of drug with environment or response choice. Overall, rats made 3x as many

correct responses (104.6 ± 7.7 responses/session) as incorrect responses (30.3 ± 2.1 responses; significant effect of choice: $F_{1,18}=78.07$, $p<0.05$, Figure 2). Interestingly, they made significantly more correct responses for visual cues on the barred floor (121.6 ± 11.5 responses; significant environment $\times$ choice interaction: $F_{1,18}=12.56$, $p<0.05$) and received more pellets (106.9 ± 9.9 pellets; $F_{1,18}=24.49$, $p<0.05$), than in response to auditory cues on the flat floor (87.6 ± 9.1 responses, 79.0 ± 8.8 pellets). Similar findings have been previously reported by [23]. Nonetheless, there were no differences in the number of correct responses for the two cues (tone vs click; still vs flashing light) in each environment (data not shown).

3.2. Biconditional discrimination extinction

In contrast to their enhanced response to visual cues on the barred floor during training, rats performed equally well in the two environments (flat and barred floors) during extinction ($F_{1,18}=0.28$, n.s.). There was still an overall significant effect of response accuracy (correct > incorrect; $F_{1,18}=6.36$, $p<0.05$, Figure 3). However, there was also a significant effect of cue agreement (congruent > incongruent cues, $F_{1,76}=6.66$, $p<0.05$), and interactions of drug $\times$ cue agreement ($F_{1,18}=5.21$, $p<0.05$) and drug $\times$ cue agreement $\times$ response accuracy ($F_{1,18}=8.95$, $p<0.05$). In congruent trials, vehicle controls made significantly more correct responses (39.0 ± 4.9 /session) than incorrect responses (27.3 ± 3.4 /session; $p<0.05$ by paired t-test). With incongruent trials, there was no significant difference between correct and incorrect responses. However, testosterone-treated rats were unsuccessful in both congruent and incongruent trials. In general, testosterone-treated rats responded at high levels on both levers, and averaged 172.3 ± 19.6 responses/session vs 133.7 ± 7.1 responses for vehicle controls ($p=0.08$ by Student's t-test).

3.3 Novel object recognition

During testing for novel object recognition, vehicle- and testosterone-treated rats increased the amount the amount time spent with the novel object vs the familiar object, compared with pretest baseline (object $\times$ day interaction: $F_{1,18}=66.69$, $p<0.05$, Figure 4). Likewise, the average latency to first interaction with the novel object (6.1 ± 1.2 sec) was half that of the latency to contact the familiar object (13.3 ± 3.4 sec, $p<0.05$). Furthermore, both groups of rats spent significantly more time with the novel object than the familiar object ($p<0.05$ by paired t-test). However, testosterone-treated rats spent only 61.0 ± 6.5 sec interacting with the novel object, vs 81.3 ± 7.9 sec for vehicle controls. Thus, there was a significant interaction of drug $\times$ day ($F_{1,18}=1.86$, $p<0.05$) as well as drug $\times$ object ($F_{1,18}=5.82$, $p<0.05$).

4. DISCUSSION

The present study tested rats in a biconditional discrimination task to evaluate AAS effects on cognitive flexibility. Rats treated chronically with high-dose testosterone were unimpaired in initial task acquisition, and all rats learned to select the appropriate lever in response to auditory or visual cues. During extinction, vehicle-treated control rats continued to respond preferentially on the correct lever when auditory and visual cues were congruent. With incongruent cues, they were unable to use contextual information to select the correct lever. However, testosterone-treated rats failed to show a preference for the correct lever in

all extinction trials (congruent or incongruent). These results suggest that testosterone impairs cognitive flexibility.

The biconditional discrimination task used here is modified from Hadden et al [23] and Gonzalez et al [33]. In both studies, animals used visual and auditory cues to choose the correct lever to earn reward. Patterned wallpaper signified context in Hadden et al [23]; the present study used floor texture, as in Gonzalez et al [33]. Unlike vehicle controls in the present study, rats in Hadden et al [23] continued to show a modest preference for the correct lever in response to incongruent cues, albeit substantially lower than for trials with congruent cues. However, their study incorporated different reinforcers (sucrose solution vs pellets) in the two contexts, thereby facilitating performance during incongruent trials. It is possible that vehicle controls in the present would have learned correct responses to incongruent cues with continued testing, since these tasks are difficult for rodents to master [34]. In other rodent studies, biconditional discrimination has been evaluated using olfactory stimuli to signify context in an operant task [34], or by challenging rats to dig for buried food in different cups [35].

Demonstrating negative effects of chronic high-dose testosterone on biconditional discrimination builds on our previous studies to explore potential cognitive changes induced by AAS abuse. Together, these studies show that the effects of AAS on executive function are highly selective. In the present study, learning in the operant chamber was unimpaired. After 14 training sessions, testosterone- and vehicle-treated rats showed a significant preference for the correct lever over the incorrect lever in response to auditory and visual cues. As in our previous study of set shifting and reversal learning, testosterone treatment did not delay initial task acquisition [16]. Memory in testosterone-treated rats from the present study, for an object or for the cue-associated lever in the operant chamber, was likewise intact. Instead, testosterone appears to interfere with rats' abilities to use contextual information to resolve complex cues (biconditional discrimination in the present study) or to adjust their behavior in response to a new rule (set-shifting) [16]. Even so, when faced with a task that they are unable to perform correctly, testosterone-treated rats continued to respond vigorously on both levers, signifying that they remain highly motivated for the task. This mirrors our previous studies of discounting behavior, where testosterone-treated rats were willing to work harder (more lever presses) [26] or accept discomfort (mild footshock) [16] to obtain a large reward. Indeed, testosterone-treated rats tend to prefer larger payoffs in each trial, even when the total number of rewards obtained during the session is disadvantageous [36].

Considering that learning, memory and motivation are all intact, why are testosterone-treated rats unable to successfully perform tasks requiring cognitive flexibility? AAS have been implicated in changes to dopamine function in the prefrontal cortical-striatal circuitry on which cognitive flexibility depends [27, 37]. Studies of brain lesions in biconditional discrimination implicate the medial prefrontal cortex (mPFC) [38] and ventral hippocampus [33] in biconditional discrimination task performance. In particular, ventral hippocampus appears to be important for context-dependent appetitive behaviors [33], especially in response to proximal contextual cues (e.g. the floor in an operant chamber), rather than distal cues in a testing room. However, the effects of supraphysiologic androgens

specifically on ventral hippocampus have not yet been studied. Nonetheless, hippocampal CA1 has abundant androgen receptors [39], and pubertal exposure to AAS increases dendritic spine density in CA1 of dorsal hippocampus [40]. The increased spine density persists weeks after treatment cessation, suggesting long-term effects of AAS on neural anatomy.

With regard to mPFC, inactivation of the prelimbic (PrL), but not the infralimbic cortex (IL), prevents discrimination of the correct response to incongruent cues [38]. PrL receives projections from ventral hippocampus that regulate contextual responses [41], thereby linking results of PrL and ventral hippocampal lesions on biconditional discrimination. Importantly, PrL sends a projection to the nucleus accumbens (Acb) [42]. Set shifting (another test of cognitive flexibility) is dependent upon dopamine activity in mPFC and Acb [43]. Dopamine release increases in mPFC and Acb during set shifting [44, 45]. Manipulations of dopamine receptors in mPFC and Acb impair set shifting [46, 47], particularly blocking dopamine D1-like receptors [47, 48]. AAS reduce D1-like receptors in Acb [13], but AAS effects on dopamine receptors in mPFC have not been determined. However, castration decreases dopaminergic afferents to mPFC in male rats, and this decrease is attenuated by androgen replacement [49]. Together, these findings suggest that the dopaminergic circuitry on which cognitive flexibility depends is sensitive to steroid hormones, and that AAS impair the function of this circuit.

The implication of the present study is that AAS abuse may impair aspects of higher cognitive function related to contextual decision making in humans. The potential interaction of exercise with chronic high-dose testosterone was not determined. Although there is ample evidence for beneficial effects of exercise on cognitive function [50], the potential interaction of exercise and anabolic steroids remains largely unexplored. AAS users typically engage in strenuous exercise [1]; most animal studies do not include exercise as an experimental variable (but see Tanekar et al [12]). Nonetheless, the effects of AAS on cognitive function in human users who do exercise complement the effects in sedentary animals. This suggests that high-resistance training does not overcome effects of AAS on executive function.

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Figure 1:

Experimental model to test biconditional discrimination in an operant chamber. Rats respond on retractable levers to earn sugar pellets in response to auditory cues (clicker/tone) on a flat floor with house-light (*top*), or in response to visual cues from two stimulus lights (constant/blink) on a barred floor (*middle*). During extinction (*bottom*), auditory and visual cues are presented together, either as congruent or incongruent pairs. With incongruent cues (e.g. clicker + blinking lights, *bottom right*), rats use contextual cues from the environment (flat vs barred floor) to determine the correct response. See Methods for experimental details.

Figure 2:

Operant responses/session (mean±SEM, *left*) and total sugar pellets earned (*right*) during the final daily training sessions for biconditional discrimination. Data from testosterone-treated rats are shown in *closed bars*; vehicle controls are in *open bars*. Rats were tested with auditory cues on a flat floor with house-light (*top*), or with visual cues on a barred floor (*bottom*). Correct responses are in *solid bars*; incorrect responses are in *striped bars*. *Daggers* indicate significant effect of environment (flat vs barred floor) and response choice (correct vs incorrect) by RM-ANOVA. *Asterisks* indicate significant differences between correct and incorrect responses by paired t-test.

Figure 3: Operant responses/session (mean±SEM) during 2 daily testing sessions for extinction with biconditional discrimination. Data from testosterone-treated rats are shown in *closed bars*; vehicle controls are in *open bars*. Rats were exposed to auditory and visual cues in 2 environments: flat floor with house-light (*top*), or barred floor (*bottom*). In each trial, auditory and visual cues were congruent (left) or incongruent (right). Correct responses are in *solid bars*; incorrect responses are in *striped bars*. *Daggers* indicate significant effect of cue agreement (congruent vs incongruent) and response choice (correct vs incorrect) by RM-ANOVA. *Asterisks* indicate significant differences between correct and incorrect responses by paired t-test. See Methods for experimental details.

Figure 4:

Time with 2 stimulus objects (mean±SEM) during pretest and testing for novel object recognition in male rats. Data from testosterone-treated rats are shown in *closed bars*; vehicle controls are in *open bars*. During the pretest, rats were exposed to 2 identical unfamiliar objects (*solid bars*). During testing, 1 object was replaced with a novel object. Investigation of the familiar object is in *striped bars*. *Dagger* indicates significant object (familiar vs unfamiliar) x day interaction by RM-ANOVA. *Asterisks* indicate significant differences in interaction with novel and familiar objects by paired t-test.