ANABOLIC-ANDROGENIC STEROID EFFECTS ON ACUTE & CHRONIC NOCICEPTION AND MORPHINE ANTINOCICEPTION

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of KIMBERLY T. TSUTSUI find it satisfactory and recommend that it be accepted.

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Abstract

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Use of anabolic-androgenic steroids (AAS) has been anecdotally associated with pain reduction as well as opioid abuse. The purpose of the current study was to investigate the effects of AAS on nociception and morphine antinociception in acute pain models, as well as on chronic nociception in an arthritis model, in adult male Sprague-Dawley rats. Rats were injected s.c. for 28 days with either 5 mg/kg dihydrotestosterone proprionate (DHT), testosterone proprionate (T), or stanozolol proprionate (STAN), or safflower oil vehicle (N=16-20/group). Half of the rats in each group were tested on acute thermal and mechanical nociceptive assays on day 28, without and then with morphine. The other half of the rats in each group were injected with mineral oil or complete Freund's adjuvant (CFA) into one hindpaw, and then tested for thermal hyperalgesia, mechanical allodynia, inflammation and locomotor suppression intermittently for 28 days. Body weight was recorded at least weekly, and reproductive organs were harvested on the last day of testing.

While AAS affected body weight and reproductive organ weights in a mostly expected manner, AAS did not significantly alter acute nociception nor did they significantly attenuate the development of various pain parameters after administration

of CFA. Further, morphine antinociception was only modulated by DHT, only on the hotplate test, and in the opposite direction to what was predicted: rather than potentiating morphine antinociception, DHT decreased morphine potency. The findings of this study have produced results that conflict with many past findings of experiments that utilized gonadectomized subjects, implicating a need for further pain studies done in gonadally intact subjects that more accurately model human AAS users.

TABLE OF CONTENTS

Page

CHAPTER ONE
INTRODUCTION

Our understanding of the role of natural androgens in reproduction dates back to the time when farmers first realized the benefit of castration in the domestication of animals, more than 6,000 years ago (Dotson & Brown, 2007). It was not until 1849, however, that Arnold Bethold discovered that the physiological and behavioral changes elicited by castration were either correlated with or caused by an actual secretion from the testes (Freeman et al., 2001). Charles Brown-Sequard then daringly assessed the chemical contents of dog and guinea pig testes via self-injection of the substance into his own body: Brown-Sequard claimed that this substance increased physical strength, cognitive performance, and appetite (Freeman et al., 2001). Finally, in 1929, the Nobel Prize winner Adolf Butenandt isolated estrone, the first sex hormone, and subsequently isolated androsterone (the first androgen) from urine (Freeman et al., 2001).

Following their chemical identification, anabolic-androgenic steroids (AAS) have gained popularity as a means to improve athletic performance. Individuals strive to achieve a high anabolic-to-androgenic ratio, in which the anabolic component refers to myotrophic effects (an increase in muscle mass and strength), as opposed to virilizing effects caused by the androgenic component. In 1991, the National Household Survey on Drug Abuse reported that over a million individuals (predominantly males) were either currently using AAS or had used it previously at some point in their life, with a majority of users 26 years old or older (Yesalis et al., 1993). The popularity of AAS has persisted despite Congress's Anabolic Steroid Control Act of 1990, which formally classified steroids as controlled substances, and the formation of the World Anti-Doping Agency in 1999 to regulate steroid use within national sporting federations (Wood,

2008). Despite these measures, AAS have achieved notoriety as the most frequently detected category of prohibited substances within athlete urine samples at the Olympic Games (Fitch, 2008).

Profile of AAS Users

AAS use by Olympic athletes spawned many studies of AAS use among non-professional athletes. Such studies showed that the primary reasons for AAS use were athletic or aesthetic; also, there was a trend towards increased psychiatric disorders among users (Malone et al., 1995), as well as higher muscular tension, impulsiveness, indirect aggression, verbal aggression, and significantly lower social desirability (Galligani et al., 1996). Kanayama et al. (2006) found that chronic AAS users are more likely to be advocates of conventional male roles, and to suffer from muscle dysmorphia: these individuals believe their bodies are too small, and they forego social and occupational events to exercise, while also avoiding all situations in which their bodies may be seen in public.

Cohen et al. (2007) raised the possibility that AAS use has extended beyond the stereotypical adult athlete or bodybuilder. Rather than recruiting subjects from a gym, these investigators recruited via internet posts and mass emails, through message boards where steroid discussion was a common topic, through printed media, and through word of mouth. They found that the typical non-medical AAS user is 31.1 years old, Caucasian (88.5%), well-educated with a post-secondary degree (72.1%), and employed full-time (77.7%) with a household income of $60,000-$79,999. A majority of subjects expressed that their motivation for AAS use was not recreational weightlifting, amateur bodybuilding, amateur/recreational sports and powerlifting, but rather to

increase muscle mass, increase strength and enhance physical appearance, and to a slightly lesser degree to decrease fat, increase confidence, improve mood and attract sexual partners. Involvement in an organized sport was rare (11%).

Recent research suggests that AAS use extends to adolescents, college students and females as well. A 5-year longitudinal study called Project Eat-II (Eating Among Teens) found that out of 2,516 high school students, 1.7% of male and 1.4% of female adolescents reported having used AAS within the last year (van den Berg et al., 2007). These prevalence rates are comparable to those reported in the 2007 Monitoring the Future survey (approximately 50,000 students nationwide), which showed annual AAS prevalence rates of 1.1%, 1.7% and 2.3% in males and 0.4%, 0.4% and 0.6% in females in 8^{th}, 10^{th} and 12^{th} grade, respectively (U.S Department of Health and Human Services, 2008). Similarly, McCabe et al. (2007) reported that prevalence of lifetime, past-year and past-month non-medical AAS use was 1% or less, based on a sample of 55,000 students from 119 four-year U.S. colleges and universities. In contrast, Berning et al. (2008) found that 8.7% of 485 non-athlete college students (88% male and 12% female) reported having used AAS, with the highest use among seniors (35.7%). It has been suggested that following the Anabolic Steroids Control Act of 1990, users have been less inclined to report their AAS use, resulting in fewer reports. The high reported use in the study by Berning and colleagues might be due to strongly emphasized confidentiality and anonymity in that study. At any rate, there appear to be significant numbers of people currently using AAS, or having a history of use.

Physiological Consequences of AAS Use

One area of concern regarding non-medical AAS use is reproductive function, in both males and females (Hartgens & Kuipers, 2004). AAS suppress the hypothalamic-pituitary-gonadal axis, thereby disrupting regulation of testosterone and gonadotrophin production. Resulting adverse effects in males include testicular atrophy and compromised semen production and quality. For example, Torres-Calleja et al. (2001) reported that eight of fifteen AAS users had sperm counts below normal, and only three had morphologically normal spermatozoa. Another adverse effect resulting from compromised endogenous secretions is hypogonadotrophic hypogonadism (characterized by reduced testicular size, azoospermia or impotence) due to decreased secretion of follicle-stimulating hormone and luteinizing hormone from the pituitary (Gill, 1998).

Adverse effects of AAS may manifest differently in males and females. For example, in males, excess estrogens (which are metabolites of some AAS) may produce gynaecomastia, which in certain cases may be remedied only with surgical correction (Kicman, 2008; Hartgens & Kuipers, 2004). Males may also experience increased baldness and severe acne. In contrast, females may experience masculinizing effects such as lowering of the voice (due to lengthened vocal chords), increased facial hair, enlargement of the clitoris, breast atrophy, and increased appetite (Kicman, 2008). AAS can also affect children differently from adults: excess estrogens aromatized from AAS may lead to premature closure of epiphyseal growth plates in long bones, decreasing the final height reached by adulthood (Basaria et al., 2001).

AAS may also seriously affect both the cardiovascular system and the liver. There is an increased tendency for left ventricular hypertrophy in AAS users compared to non-AAS users (Dickerman et al., 1997), and increased risk of stroke (Santamarina et al., 2008), myocardial infarction (Petersson et al., 2006), thrombosis, and ventricular fibrillation (Nieminen et al., 1996). Risk of cardiac mortality increases with AAS-induced hypertension (Payne et al., 2004) and with the increased atherogenic lipid profile characterized by reduced high-density lipoprotein and increased low-density lipoprotein (Hartgens et al., 2004). AAS may lead to significant liver disorders and malfunction, such as liver fibrosis (Vieira et al., 2008), hepatic cholestasis (Kicman, 2008), peliosis hepatitis (Wakabayashi et al., 1984), hepatic carcinomas, hepatocellular hyperplasia and subcellular changes of hepatocytes (Hartgens & Kuipers, 2004).

Psychological Consequences of AAS Use

Aggression (or “roid rage”) is the most well-known psychological effect of AAS. Most data demonstrating a link between aggression and AAS use have been acquired through correlational surveys (Choi et al., 1990) and self-reports (Beaver et al., 2008), or via experimental rat models. One such rat model involved a resident-intruder paradigm of aggression, in which gonadectomized rats were treated with either methyltestosterone, testosterone propionate, stanozolol or vehicle, and then introduced to an intruder male. Methyltestosterone and testosterone propionate increased aggression in resident males, whereas stanozolol did not (Clark & Barber, 1994). These findings suggest that the psychological effects manifested in human AAS users may depend on the chemical structure of the steroid used. Another study showed that testosterone propionate heightened sensitivity of gonadally intact males to external

stimuli (such as a brief tail pinch) and decreased the threshold at which a stimulus evoked aggression and dominance (McGinnis et al., 2002). In this study, the AAS nandrolone had no effect, whereas stanozolol inhibited aggression. A handful of experimental studies in human participants have also been done; in one study, testosterone administration increased aggressive responding in a computer paradigm (Kouri et al., 1995). Taken together, these studies suggest that AAS can increase aggressive behavior in both rats and humans.

It has also been proposed that AAS use pre-disposes users to abuse of other drugs. Yesalis et al. (1993) found that among 12- to 34-year-olds, AAS use was significantly and positively associated with the use of other illicit drugs, cigarettes and alcohol. Petersson et al. (2006) report a similar tendency towards substance abuse in AAS users. However, Skarberg et al. (2008) also draw attention to the likelihood that social background, age of onset of drug use, relationship to AAS use and the experience of AAS effects all play a role in the development of further substance use.

To understand AAS psychological effects from a biological perspective, some researchers have examined the effects of AAS on brain function. There is evidence that AAS modulate various neurotransmitters involved in drug reward and reinforcement, such as dopamine (DA), gamma-aminobutyric acid (GABA), and opioids (Wood, 2008). Male rats will form a conditioned place preference when testosterone is injected into the nucleus accumbens (Packard et al., 1997), and animals will self-administer testosterone (or its metabolites) by various routes, such as oral (Johnson & Wood, 2001; Frye et al., 2007), intravenous, and intracerebroventricular (Wood, 2004; Wood et al., 2004; Ballard & Wood, 2005). AAS exhibit similarities with drugs that modulate the GABAergic

system (e.g., barbiturates and benzodiazepines): testosterone, dihydrotestosterone, androsterone and androstenedione are anxiolytic, as evidenced by AAS-treated mice spending more time than controls in the open arms of an elevated plus-maze (Aikey et al., 2002). AAS have additionally been postulated to have opioidergic effects. High doses of testosterone may produce overdose symptoms extremely similar to those of opioid intoxication, such as autonomic depression; furthermore, testosterone's depressant effects were blocked by naltrexone, an opioid antagonist (Peters & Wood, 2004).

AAS & Pain

AAS may also affect pain. Research on pain and AAS is primarily limited to testosterone's effects on animal models, most commonly acute thermal nociception using the tail withdrawal and hot plate tests, or employing the formalin model to examine inflammatory pain. In the hotplate and tail withdrawal tests, administration of testosterone or its metabolites 3α-androstanediol and dihydrotestosterone (DHT) increased latency to respond to nociceptive stimuli (Hau et al., 2004; Frye et al., 2007; Edinger & Frye, 2004), suggesting that AAS have analgesic properties. However, several studies have reported little or no effect of testosterone on nociception in the hot plate or tail withdrawal tests (Negus et al., 2001; Sumner et al., 2006). In the formalin model, testosterone administered to gonadally intact rats decreased nociceptive responses, such as licking or flinching of the paw (Aloisi et al., 2004; Fischer et al., 2007). Further, the depletion of testosterone resulting from gonadectomy has been shown to increase formalin-induced responses (Aloisi & Ceccarelli, 2000), while testosterone replacement appears to decrease nociception in the formalin test

(Gaumond et al., 2005), although results may depend on the particular phase of the formalin response (Gaumond et al., 2005).

The relationship between androgens and pain in humans has been investigated in an elegant study by Aloisi et al. (2007), in which male-to-female and female-to-male transsexuals chronically received cross-sex hormones to develop and maintain characteristics of the opposite sex. About one third of the male-to-female subjects developed chronic pain with their estrogen treatment, whereas approximately half of the female-to-male subjects' chronic pain disappeared after being treated with testosterone, suggesting that testosterone also may be analgesic in humans, as some rat studies suggest.

In addition to testosterone's effects on nociception, testosterone may modulate opioid-induced antinociception. As mentioned earlier, AAS have been previously linked to the opioidergic system as evidenced by overdose symptoms resembling opioid intoxication, which can be reversed with an opioid antagonist (Peters & Wood, 2004). However, findings have been inconsistent across studies investigating the nature of the relationship between testosterone and opioid antinociception. While some studies report that testosterone enhanced opioid antinociception (Cicero et al., 2002; Stoffel et al., 2003) and that testosterone depletion via castration decreased opioid antinociception in male rats (Stoffel et al., 2003; Borzan & Fuchs, 2006), others report no significant testosterone effect on opioid antinociception (Celerier et al., 2003; Negus et al., 2001; Sumner et al., 2006), and some report a decrease in opioid antinociception following AAS administration (Philipova et al., 2003) or an increase in opioid antinociception after testosterone depletion via castration (South et al., 2001). Such

contradictory findings may reflect inconsistencies of experimental methods between studies, or elaborate mechanisms that have yet to be fully understood. Further research on this topic is thus necessary.

Purpose of Study

The purpose of the current study was to investigate the effects of AAS on nociception and morphine antinociception. The majority of studies on AAS and pain have used hormone removal and replacement procedures to examine the effects of androgens on pain. This study, in contrast, modeled the typical human male AAS user by using gonadally intact, male Sprague-Dawley rats that were young adults at the beginning of the study. Typical human AAS abusers and individuals suffering from muscle wasting disorders desire a high anabolic-to-androgenic AAS ratio, with prolonged and enduring effects. For this reason, over a hundred synthetic steroids have been created by modifying the testosterone molecule. To test the generality of AAS effects on nociception, three AAS were tested: testosterone (T), DHT and stanozolol (STAN). Testosterone is a pro-hormone that is typically converted to DHT via 5α-reductase, and to estradiol via aromatase. We examined T as a naturally occurring androgen and the precursor to DHT. DHT was chosen because it is both a non-aromatizable and more potent androgen than T, binding with greater affinity than T to androgen receptors (Kicman, 2008). Because DHT is non-aromatizable, we can be more certain that any effects are due to DHT itself and not due to an estrogen metabolite. The third AAS examined, STAN, is a synthetic, 17α-alkylated AAS with weak androgen receptor binding affinity due to its inability to be reduced by 5α-reductase (Kicman, 2008). The 17α-alkylation refers to the addition of a methyl group to

the C17 α position, which prevents quick deactivation of the steroid in first-pass metabolism, resulting in a compound that is active when taken orally (Kicman, 2008). STAN is also incapable of being aromatized to estradiol, resulting in decreased estrogenic side effects; for these reasons it is popular among AAS users (Wood, 2004). Thus, we chose STAN because of its common use and the fact that we may be fairly certain that any effects are due to STAN itself and not due to an estrogen metabolite.

Both acute and chronic nociception were measured. For acute nociception, hotplate and tail withdrawal tests were used to measure thermal nociception, and a paw pressure test was used to measure mechanical nociception (Whiteside et al., 2008). For chronic nociception, the Complete Freund's Adjuvant (CFA) model was used as an inflammatory pain test that elicits a heightened sensitivity to otherwise non-noxious stimuli (allodynia), as well as heightened sensitivity to noxious stimuli (hyperalgesia). The inflammatory nature of the CFA results from the sustained release of immunogen which stimulates an immune response. CFA is most commonly injected into either the base of the tail or directly into the hindpaw, which may result in localized inflammation within the injected paw or inflammation in both the injected paw and the contralateral paw. The latter situation occurs more readily in Lewis rats (as opposed to various other rat strains, such as Sprague-Dawley) due to their genetic susceptibility to various T-cell mediated autoimmune disease models (Yoshida et al., 1998). To measure CFA-induced thermal hyperalgesia and mechanical allodynia the Hargreaves and von Frey methods were used (Nagakura et al., 2003). The first hypothesis of this study was that each AAS would attenuate thermal and mechanical nociception compared to control rats treated with vehicle, in acute models of pain, as well as attenuating the

development of thermal hyperalgesia, mechanical allodynia and inflammatory measures in the chronic pain model. The second hypothesis of this study was that administration of AAS would enhance morphine antinociception.

CHAPTER TWO

METHODS

Subjects

Subjects were 70-90 day old Sprague-Dawley rats (offspring from breeders purchased from Taconic Farms (Germantown, NY)). Rats were weighed before assigning to groups so that mean body weight could be balanced initially among groups; rats were then housed in pairs according to hormone administration group, either vehicle or AAS-treated (*n* = 10/group). Rats were maintained on a 12/12-hour light/dark cycle (lights on at 0600 h) in a vivarium room maintained at 21±2 °C. Rats were provided with food (Teklad) and water *ad libitum* except during testing. Animals were housed in an AAALAC-accredited vivarium and all procedures approved by the WSU IACUC.

Apparatus

To measure acute thermal nociception, a hotplate analgesia meter (Columbus Instruments, Columbus, OH) and tail-withdrawal water bath (Precision Scientific, Winchester, VA) were used. The hotplate was set to 50 (±0.1) °C and the water bath set to 50 (±0.5) °C. To measure acute mechanical nociception, the Randall-Selitto paw pressure test was conducted with an analgesy-meter (Ugo Basile, Varese, Italy), with a gradual weight increase of 48 g/sec from 30 to 1230 g.

An electric von Frey anesthesiometer (IITC Life Science, Woodland Hills, CA) was used to measure mechanical allodynia. The von Frey test involves a probe tip being pushed up against the ventral side of the hindpaw, and the pressure necessary to elicit foot withdrawal is recorded. To measure thermal hyperalgesia, an 85x40x35 cm

Basile Plantar (Hargreaves) test (Ugo Basile, Varese, Italy) was used, with an infrared intensity of 32 mW/cm^2. The Hargreaves test measures latency for paw withdrawal (in sec) from an infrared beam projected upwards towards the plantar surface of the hindpaw. Mobility was measured using a photobeam test chamber (Opto-varimex, Columbus Instruments, Columbus, OH) consisting of 15 photobeams that cross the width of a 20 cm X 40 cm X 23 cm clear Plexiglas rodent cage. Photobeams are 2.5 cm apart and 8 cm above the cage floor. Whole-paw inflammation was assessed as displacement of tap water in a beaker filled to the 20-ml mark, and maximal dorsal-plantar foot thickness was measured with a caliper. Tests were conducted on both the left and right paw, with half of the rats being tested on the left hindpaw first and half on the right hindpaw first.

Drugs

Morphine sulfate (Sigma-Aldrich, Inc., St. Louis, MO) was dissolved in 0.9% physiological saline and administered s.c. in a volume of 1 ml/kg. T propionate, DHT propionate and STAN (Steraloids Inc., Newport, RI) were dissolved or suspended in safflower oil, and administered s.c. at a dose of 5 mg/kg, in a volume of 0.5 ml/kg. Paw inflammation was induced with 0.1-ml injection of a 5 mg/ml CFA solution (*Mycobacterium butyricum* suspended in mineral oil: Fisher Scientific, Pittsburgh, PA).

Procedure

For 28-56 days, either an AAS (T, DHT, or STAN, 5 mg/kg) or vehicle (safflower oil) was administered s.c. daily at approximately 0900 - 1100 h. Body weight was recorded on the first day of treatment and thereafter at weekly intervals. Injection volume was adjusted weekly according to body weight. Acute nociception was tested

on the 28th day of treatment, beginning approximately 3 hr after the vehicle or AAS injection. Chronic nociception was tested starting on the 28th day of AAS treatment: immediately before and then 1, 3, 7, 10, 14, 21 and 28 days post-CFA or mineral oil injection. Immediately following the last nociceptive test, in order to confirm AAS effect and to assess physiological effects of AAS on steroid-sensitive organs, trunk blood, seminal vesicles, and testes were harvested. Testes were weighed wet. Seminal vesicles were stored in Bouin's solution for approximately two weeks to fix the tissue, after which they were trimmed, blotted and weighed. Trunk blood was centrifuged at 4°C and 2000 rpm for approximately 25 min, and plasma was aliquotted into microfuge tubes for later analysis of hormone levels.

Experiment 1: AAS effects on acute nociception and opioid antinociception

Procedure

On the 28th injection day, nociception was assessed in the following order: hotplate (latency to lick hindpaw or jump off plate, in sec), tail withdrawal (latency to tail flick, in sec), and paw pressure (latency to retract or attempt to retract foot, in sec), with cutoff latencies of 60 sec, 20 sec and 25 sec, respectively, to prevent tissue damage. Three baseline (non-drug) tests were conducted. Immediately following the second baseline test, saline (1.0 ml/kg) was administered s.c., and 20 min later the third baseline test was conducted. Cumulative dosing of morphine commenced immediately after completion of the third baseline test, with the following actual doses injected at 20-min intervals: 1.0 mg/kg, 0.8 mg/kg, 1.4 mg/kg, 2.4 mg/kg, 4.4 mg/kg and 8.0 mg/kg, reaching a total cumulative dose of up to 18 mg/kg. Twenty min after each injection, rats were re-tested on all 3 nociceptive tests in the order noted above. Testing

continued until the rat reached cutoff on all three tests. If a rat reached cutoff on one particular test, the rat was no longer tested on that test.

Statistical Analyses

Differences in body weights between vehicle and AAS-treated groups over time were determined using a repeated-measures ANOVA with treatment (vehicle or AAS) as the between-subjects factor and week of administration as the within-subjects factor. Nociceptive baseline for each rat was the mean latency time of the second and third baseline tests; the first test is dropped due to the potential confoundment of exploration on the hotplate test (Craft and Bernal, 2001). The baseline nociception difference between vehicle and AAS-treated groups was assessed with a one-way ANOVA, followed by post-hoc analysis with Dunnett's *t*-test (2-sided). To account for individual differences in baseline latencies, response latencies following each cumulative morphine dose for each rat was calculated as % Maximum Possible Effect (%MPE): [(post-injection latency – baseline latency)/(cutoff latency – baseline latency)] x 100. To calculate the point at which morphine antinociception reached 50% MPE (ED_{50}) for each rat, the estimated log-unit dose was calculated using at least one %MPE point falling under 50% and at least one point above 50%. The difference in morphine ED_{50} values between AAS-treated groups and the vehicle group was analyzed with a one-way ANOVA and post-hoc Dunnett's *t*-test, for each nociceptive test. Lastly, because organ weight increases with body weight, organ weight was adjusted by body weight: (organ weight (g)/BW (kg)), and then group differences were assessed with a one-way ANOVA and post-hoc Dunnett's *t*-test. Significance was set at $p \leq 0.05$.

Experiment 2: AAS effects on chronic inflammatory pain using the CFA test

Procedure

On the 28th day of AAS or vehicle treatment, baseline responses were measured on the von Frey and Hargreaves apparatuses, as well as mobility and paw inflammation (i.e., paw displacement and paw thickness). Immediately following baseline measurements rats were lightly anesthetized with isoflurane and either 0.1 ml of CFA or mineral oil was injected into the plantar surface of the right hindpaw. The same behavioral and inflammation measurements were taken on days 1, 3, 7, 10, 14, 21 and 28 following injection of CFA or mineral oil. Vehicle and AAS daily injections were continued throughout this testing period. Rats were euthanized by rapid decapitation after the last measurement on the 28th day of testing, when blood and reproductive organs were harvested.

Statistical analyses

Body weight over time was assessed for both weekly body weight changes and test day body weight changes with a repeated measures ANOVA, with test day as the within-subjects factor and steroid and CFA/mineral oil treatment as the between-subjects factors. Differences in nociceptive thresholds and paw inflammation were determined using a repeated measures ANOVA with test day and foot (left vs. right) as the repeated factors, and AAS and CFA/mineral oil as the between-subjects factors. Paw displacement was determined by subtracting 20 ml (the initial volume) from the final volume. Mobility (# of photobeam breaks) was assessed with a repeated measures ANOVA as well, with test day as the within-subjects factor and AAS and CFA/mineral oil treatment as the between-subjects factors. A difference statistic was

also calculated between the left foot and right foot by subtracting the left foot responses from the right foot responses, thereby simplifying data presentation and analysis. Lastly, differences in organ weights between vehicle and AAS-treated rats were compared with a one-way ANOVA and Student-Newman Keuls post-hoc.

CHAPTER THREE

RESULTS

Experiment 1

Body Weight. On day 1 of injections, mean body weight in all groups was approximately 430 g (Figure 1, Appendix A, p.42). Body weight increased significantly over the four weeks of AAS or vehicle administration (Week: $F(4,236)=120.20$, $p<0.001$). However, while the body weight of most groups increased steadily, body weight of T-treated rats increased only slightly across the four weeks, such that T-treated rats were significantly lighter than vehicle-treated rats by weeks 3-4 (AAS x week: $F(12,236)=16.60$, $p<0.001$; Figure 1, Appendix A, p.42).

Nociceptive and antinociceptive behavior. Figure 2 (Appendix B, p.43) shows baseline data for hotplate, tail withdrawal and paw pressure tests. Administration of an AAS for 28 days did not significantly alter basal nociception on any test. However, Figure 3 (Appendix C, p.44) shows that AAS treatment did significantly alter morphine's antinociceptive potency, on the hotplate test: specifically, ED_{50} values for DHT-treated rats were significantly higher than those of vehicle-treated controls ($F(3,59)=3.58$, $p=0.02$), suggesting that DHT treatment significantly decreased the potency of morphine on the hotplate test.

Reproductive organs. Figure 4 (left panel; Appendix D, p.45) shows that testis weight was somewhat lower in DHT-treated rats, and significantly lower in STAN-treated rats when compared to controls ($F(3,44)=3.12$, $p=0.03$). Also shown in Figure 4 (right panel; Appendix D, p.45), the seminal vesicles of T- and DHT-treated rats were

significantly heavier than those of controls, while the seminal vesicle weight of STAN-treated rats was similar to that of controls ($F(3,57)=78.90$, $p<0.001$).

Experiment 2

Body weight. Figure 5 (left panel; Appendix E, p.46) shows body weight of all groups from the first week of AAS or vehicle injection through the end of testing at week 8 (either CFA or mineral oil was administered into a hindpaw at week 4). Mean starting weights ranged from approximately 425-440 g. Overall, body weights increased across the 8 weeks of treatment (week: $F(8,560)=31.60$, $p<0.001$). However, T-treated rats (during weeks 4-6) and DHT-treated rats (during weeks 6-8) gained less weight than vehicle-treated controls (AAS x week: $F(24,560)=6.05$, $p<0.001$).

Figure 5 (right panel; Appendix E, p.46) shows body weight gain during weeks 4-8, when rats were being tested at various time points after CFA or mineral oil injection into a hindpaw. In general, body weight decreased within the first few days after CFA injection, and then began to increase again (Time: $F(7,490)=198.80$, $p<0.001$). Similar to Figure 5 (left panel), Figure 5 (right panel) shows that T-treated rats weighed significantly less than vehicle-treated controls regardless of test day (AAS: $F(3,70)=5.32$, $p=0.002$); additionally, DHT-treated rats weighed less than controls on test days 14-28 (AAS x day: $F(21,490)=6.86$, $p<0.001$).

Nociception and mobility. An initial assessment of the mineral oil groups alone revealed no effects of AAS treatment on any nociceptive test. For example, in mineral oil-injected rats, the von Frey (Figure 6, top panel; Appendix F, p.47) and Hargreaves (Figure 6, bottom panel; Appendix F, p.47) tests yielded no significant response

threshold differences between the right and left paw in any group ($F(3,34)=0.68$, n.s.). Similarly, Figure 7 (Appendix G, p.48) shows no significant difference among mineral oil-injected groups treated with vehicle vs. any of the three AAS on locomotor activity. In contrast, in the CFA-injected groups (Figure 6, right panels) there was a dramatic decrease in pain threshold for the von Frey (Day: $F(7,238)=8.34$, $p<0.001$) and Hargreaves tests (Day: $F(7,238)=9.30$, $p<0.001$) and a decrease in locomotor activity (Day: $F(7,238)=24.20$, $p<0.001$) immediately following administration of CFA. However, there were no significant differences in allodynia, hyperalgesia or locomotion between AAS-treated rats and controls.

Paw inflammation. Inflammation was demonstrated by increases in paw volume and paw thickness, as shown in Figure 8 (top panel; Appendix H, p.49) and (bottom panel; Appendix H, p.49), respectively. In rats injected with mineral oil (left panels), right hindpaw thickness increased slightly (by approximately 0.1 mm) in comparison to the left hindpaw in all groups, and it remained elevated ($F(7,252)=24.50$, $p<0.001$). There were no AAS group differences in this effect. In CFA-injected rats (right panels), right paw volume displacement ($F(7,238)=65.00$, $p<0.001$) and paw thickness ($F(7,238)=222.90$, $p<0.001$) increased significantly over time after CFA injection, but no significant differences were found between AAS groups and controls.

Reproductive organs. Figure 9 (Appendix I, p.50) shows the effect of AAS on reproductive organs after 56 days of AAS administration. Similar to Experiment 1, only STAN-treated rats had lower testis weights in comparison to controls ($F(3,74)=14.00$, $p<0.001$). Also similar to Experiment 1, T- and DHT-treated rats had substantially

heavier seminal vesicles than controls; however, in addition, STAN-treated rats had significantly heavier seminal vesicles compared to controls ($F(3,74)=155.80$, $p<0.001$).

CHAPTER FOUR

DISCUSSION

Effect of AAS on acute nociception

Experiment 1 showed no significant differences in basal nociception in AAS-treated rats in comparison to controls on the hotplate, tail withdrawal and paw pressure tests. This result is consistent with findings from other studies showing that modulating androgen levels either through castration (Liu & Gintzler, 2000; Ali et al., 1995; Stoffel et al., 2003) or castration with T replacement via hormone capsules (Sumner et al., 2006) did not alter basal nociception. However, it is important to note that manipulation of gonadal hormones in males has produced a wide range of results, with castration sometimes increasing basal nociceptive thresholds (Nayebi & Ahmadiani, 1999) while at other times decreasing it (Aloisi & Ceccarelli, 2000; Gaumond et al., 2002), and T replacement following castration either decreasing (Nayebi & Rezazadeh, 2004) or increasing (Frye & Seliga, 2001; Gaumond et al., 2005) nociceptive threshold. The large variation in findings across experiments may be due to differences in methodology, such as use of varying rat strains, ages of rats, dosage and duration of T administration, and differing stimulus intensities in the nociceptive tests. At any rate, the present results do not support the hypothesis that AAS administration attenuates acute mechanical and thermal nociceptive responses.

Effect of AAS on chronic pain

Pain measurements in Experiment 2 also did not reveal any differences between AAS groups and controls. As such, the hypothesis that AAS administration would attenuate the development of thermal hyperalgesia, mechanical allodynia, and

inflammation was not supported. This result contrasts research findings that implicate a protective role of androgens in the development and severity of chronic inflammatory pain, as shown in formalin studies (Gaumond et al, 2002; Aloisi et al., 2004) and CFA-induced arthritis models. In one CFA study, castration caused the onset of arthritic symptoms to occur sooner and administration of T decreased inflammation as measured by paw volume (Harbuz et al., 1995). Furthermore, the lower frequency of inflammatory disease occurring in human males in comparison to females has been attributed to greater immunosuppressive effects of androgens in males (Green et al., 1999; Da Silva, 1999). It should be noted that in the present study, the time course of hyperalgesia, allodynia and inflammation was consistent with those reported in previous studies: immediately following administration of CFA, all rats showed a significant decrease in threshold on both Hargreaves and von Frey tests and an increase in paw inflammation; furthermore, allodynia and inflammation waned but did not return to baseline throughout the course of the 28-day experiment (Nagakura et al., 2003; Cook & Nickerson, 2005). All CFA-treated rats showed these effects in the present study, even though the Sprague-Dawley strain of rat was used instead of the more commonly used Lewis strain, which is known to be more susceptible than the Sprague-Dawley to CFA-induced arthritis (Cook & Nickerson, 2005; Nagakura et al., 2003). Thus, the failure to observe anti-allodynic, anti-hyperalgesic and anti-inflammatory effects of AAS is not likely due to a general failure of the inflammatory pain procedure. It is also unlikely that the study's AAS treatment regimen was insufficient either in dosage or duration, as T and DHT increased seminal vesicle weight in both experiments, as did STAN in Experiment 2. It is also likely that administration of 5 mg/kg daily for 4-8 weeks

was sufficient to observe an effect on nociception. Previous studies have demonstrated antinociceptive effects of T in response to formalin-induced temporomandibular joint pain in male rats given 1 mg/kg T for only 7 days (although subjects were gonadectomized: Fischer et al., 2007). Additionally, 5 mg/kg T administered 5 times/week for 12 weeks to gonadally intact male rats increased aggression and dominance (Breuer et al. 2001). Together with the clear effects of AAS on reproductive organ weight observed in the present study, these previous studies suggest that the dose and duration of AAS administration in the present study were most likely sufficient in order to produce physiological and behavioral effects.

A possible explanation as to why AAS effects were not observed may be due to the fact that in the present study, gonadally intact males were used to model the typical human AAS user with normal sex steroid levels, whereas in many previous studies, subjects were gonadectomized (Pednekar & Mulgaonker, 1995; Frye & Seliga, 2001; Gaumond et al., 2002). Gonadectomy results in a profound depletion of sex steroids and steroid production. Thus, comparing steroid-treated rats to gonadectomized rats would maximize group differences in behavior due to the drastic group difference in sex steroid levels, whereas a more modest difference would be expected when comparing intact rats to those treated with supraphysiological doses of AAS. In a study by Frye & Seliga (2001), rats were either gonadectomized, gonadectomized with T-replacement or left intact. T-replaced rats had significantly longer tail withdrawal latencies than gonadectomized rats *and* intact rats, indicating that gonadectomy with T-replacement produced accentuated effects in comparison to the effects produced by physiological hormone levels alone. Also, Aloisi et al. (2004) reported that administration of T to

intact male rats produced only small differences in paw-lick latencies in the formalin model in comparison to intact controls given oil vehicle. However, when the steroid 3α-androstanediol (a T metabolite) was administered to intact male hamsters, it significantly lengthened paw-lick latencies when compared to intact vehicle-treated controls (Frye et al., 2007), suggesting that significant AAS effects can be observed when comparing subjects with physiological vs. supraphysiological levels of androgens. The potential effect of gonadectomy could be examined by replicating Experiments 1 and 2 using gonadectomized rats in place of gonadally intact rats. This may allow for more valid comparison of findings between the present and previous studies.

Effect of AAS on morphine antinociception

In Experiment 1, only minimal differences between AAS-treated groups and controls were found in regards to morphine antinociception. Specifically, only DHT treatment altered morphine potency, and only on the hotplate test. This finding does not support the hypothesis that administration of all AAS would potentiate morphine antinociception, as antinociception in T- and STAN-treated groups did not differ from controls, and DHT-treated rats expressed a rightward shift in the dose response curve, thereby indicating a *decrease* in the potency of morphine. It may be the case that the exogenous DHT administered daily for 28 consecutive days suppressed the HPG axis, thereby causing testicular atrophy and decreasing endogenous gonadotrophin production (Hartgens & Kuipers, 2004). A depletion of endogenous T via gonadectomy has been shown to *decrease* the potency of morphine in comparison to intact controls and T-treated gonadectomized male rats (Stoffel et al., 2003). However, if this were true, the T-treated group should also have shown decreased morphine potency similar

to that of the DHT group, yet the T-treated group did not differ from controls. In addition, significant suppression of testis weight -- which would be expected if excessive exogenous androgen levels were exerting a negative feedback to the HPG axis – was not observed in either the DHT- or T-treated groups. Instead, it is possible that chance may account for the decrease in morphine antinociceptive potency in the DHT group. In that case it would be predicted that replication of the experiment would fail to yield the same result, or the addition of more subjects to the group would eliminate the effect.

In the event that the DHT finding is reliable, the question arises, what is the mechanism of androgen modulation of opioid antinociceptive sensitivity? Mechanisms underlying AAS modulation of opioid antinociception are still much debated. One proposed mechanism is the modulation of brain opioid peptides by AAS. For example, the AAS nandrolone administered daily for two weeks increased levels of kappa, mu and delta opioid agonist immunoreactivity in the hypothalamus, striatum and PAG (Johansson et al., 2000). The PAG is a brain structure well-known for its modulation of pain and has been shown to mediate morphine antinociception as well as sex differences and gonadectomy effects on morphine antinociception (Krzanowska & Bodnar, 1999). Likewise, chronic administration of nandrolone also increased beta-endorphin levels in the VTA (Johansson et al., 1997). In addition to the increase in opioid peptides, sex hormones may also stimulate an increase in mu-opioid receptor mRNA (Petersen & LaFlamme, 1997; Quinones-Jenab et al., 1997). Conversely, Hammer et al. (1993) found that hormone-depleted rats experienced reduced proenkephalin mRNA expression in various brain structures, and this reduction was not reversed with administration of DHT. Pluchino et al. (2009) also found that depletion of

gonadal hormones decreased brain beta-endorphin levels, and T administration significantly increased beta-endorphin levels in various brain areas and in the plasma of gonadectomized rats, but administration of DHT failed to restore beta-endorphin levels (a similar regimen of 5 mg/kg/day was used in their experiment). If it is in fact the case that supraphysiological levels of DHT activated a negative feedback loop to the hypothalamus, thereby causing hypogonadism (decreased T production) and thus a decrease in morphine antinociceptive potency consistent with rats depleted of hormone via gonadectomy (Stoffel et al., 2003), perhaps exogenous administration of DHT was not sufficient as T or STAN in reversing the inhibitory effect of hormone depletion on endogenous opioid levels in the brain. Together these studies suggest that exogenous T but not DHT can restore decreased endogenous opioid levels that result from hormone depletion. However, as noted earlier, testis weight did not decrease in the DHT groups, which would have been expected if DHT was activating the negative feedback loop to the HPG axis. This study's ambiguous findings demonstrate that further investigation on the effect of DHT on morphine potency and antinociception may be warranted, as DHT may act differently than T.

Effect of AAS on other pain indices and reproductive organs

Body Weight. Body weight was recorded in Experiments 1 and 2 to show the physiological effects of AAS administration on weight gain or loss. In both experiments, T-treated rats gained significantly less weight than controls (at week 3 and 4 in Experiment 1, and after week 4 in Experiment 2). This result is consistent with studies demonstrating that AAS reduce fat mass (Hartgens & Kuipers, 2004) and T administration decreases weight gain (Clark et al., 1997). DHT also slightly decreased

body weight gain, but was less effective than T. Not surprisingly, STAN, being the weakest of the androgens, inhibited weight gain the least out of the three AAS used in this study. In Experiment 2, body weight was also used as an indicator of pain severity after CFA injection into the hindpaw. CFA protocols typically stipulate that if a rat's body weight decreases by 15% from its initial starting weight, the rat should be euthanized. T groups continued to be significantly lighter than controls from week 4 onwards and DHT groups were significantly lighter than controls starting at week 6. However, these AAS effects were independent of CFA injection, which would imply that the decrease in weight gain in T and DHT groups was most likely a reflection of AAS effects rather than an indication of pain.

While pain may elicit particular behaviors (i.e. paw favoring, quicker tail or paw withdrawal, flinching, etc.) it may also suppress normal behaviors, such as feeding and locomotion (Negus et al., 2006). Thus, the locomotor test was used to measure mobility in Experiment 2 as an additional reflection of pain following CFA injection. The dramatic decrease in the number of beam breaks on the day immediately following CFA administration was coincident with the drop in thresholds obtained on the von Frey and Hargreaves tests, suggesting that pain suppressed locomotion. On day 3 after CFA injection, locomotor scores began to recover towards that observed in mineral oil-injected rats, roughly paralleling the recovery of mechanical and thermal response thresholds, as well as paw inflammation. However, it is apparent that AAS administration failed to attenuate the decreases in locomotor scores resulting from CFA, as locomotor scores belonging to groups treated with an AAS did not differ from that of controls. Also of note was that paw thickness increased immediately following mineral

oil administration as well, though to a substantially lesser degree than after CFA administration. This change in thickness remained constant across all test days. Because it is unlikely that a 0.1 ml injection of mineral oil could cause lasting inflammation, this effect may reflect biased measurement by the experimenter.

Reproductive organs (testes and seminal vesicles) were harvested as an indication that steroid treatment was physiologically relevant (i.e., sufficient dose and duration of AAS administration to produce a known effect). Testis weight was not significantly different between T- and DHT-treated groups when compared to controls, a finding that is *inconsistent* with many other studies in which T administration decreased testis weight (Banasal & Davies, 1986; Bronson, 1996). This finding contrasts previous expectations that all three AAS (but particularly T and DHT due to their greater androgenic properties) would cause a decrease in testis weight via negative feedback to the hypothalamus (Hartgens & Kuipers, 2004). Excessive levels of T generally inhibit gonadotropin releasing hormone (GnRH) from the hypothalamus as well as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary, thereby ultimately decreasing the production of T and testicular size. Previously cited studies assessing testis weight administered AAS for 12 weeks to 6 months instead of only 4 to 8 weeks. STAN on the other hand decreased testis weight at both 4 and 8 weeks (Experiments 1 and 2, respectively), but only increased seminal vesicle weight after 8 weeks, compared to controls. This finding is consistent with a number of other studies, corroborating previous findings that STAN is inefficient or weakly effective in increasing the weight of reproductive organs such as the prostate and seminal vesicles (Tingus & Carlsen, 1993; Bauman et al., 1988; Clark & Barber, 1994). It has also been shown by

Breuer et al. (2001) that STAN may significantly decrease testis weight in comparison to controls, even more so than administration of T.

Although T and DHT failed to decrease testis weight significantly, all three AAS significantly increased seminal vesicle weights in comparison to controls in Experiment 2, and in Experiment 1 seminal vesicle weights were heavier than controls in T- and DHT-treated rats. Perhaps administration of AAS in this study was sufficient to produce direct androgenic effects (on the seminal vesicles) but not enough to produce a negative feedback on the hypothalamus and pituitary (as is evident by the lack of decreased testis weight in T- and DHT-treated rats compared to controls). A second possibility is that testis weight is not a perfect reflection of serum LH levels (which would be decreased in AAS-treated rats if negative feedback occurred). A previous study reported that T-treated rats showed a greater decrease in LH levels than STAN-treated rats in comparison to controls, while STAN-treated rats had lighter testicles than T-treated rats (Breuer et al., 2001). Thus, AAS modulation of behavioral vs. physiological effects could be better characterized by measuring both androgen and LH plasma levels in the future.

Conclusion

In sum, despite apparently sufficient administration of hormone, the AAS used in this study did not significantly alter acute nociception nor did they significantly attenuate the development of various pain parameters after administration of CFA. Further, morphine antinociception was only modulated by DHT, and only on the hotplate test and in the opposite direction to what was predicted: rather than potentiating morphine antinociception, DHT decreased morphine potency. The findings of this study have

produced results that conflict with many past findings of experiments that utilized gonadectomized subjects, implicating a need for further pain studies done in gonadally intact subjects that more accurately model human AAS users.

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APPENDIX A

EXPERIMENT 1: BODY WEIGHT

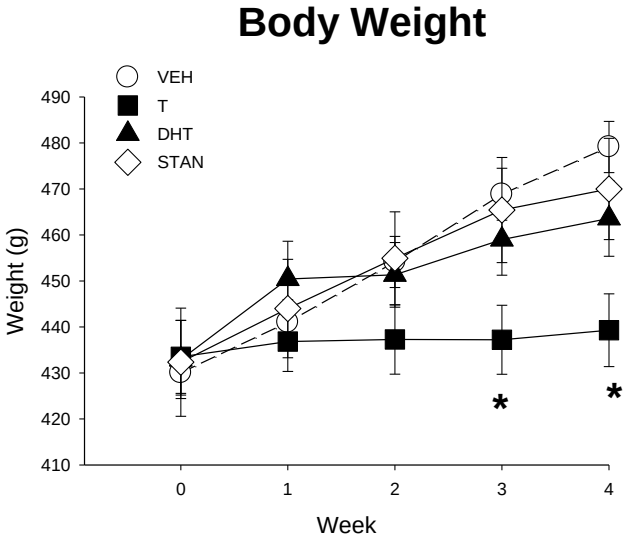

Figure 1. Body weight from week 0 (first day of AAS or oil administration) to week 4 in gonadally intact male rats treated for 28 days with vehicle (VEH), testosterone (T), dihydrotestosterone (DHT), or stanozolol (STAN) (5 mg/kg/day) (Experiment 1). Each point is the mean ± 1 S.E.M., N=25 (vehicle group) or N=12-14 rats (AAS groups). *significantly different from vehicle group, p≤0.05.

APPENDIX B

EXPERIMENT 1: NOCICEPTIVE BASELINES

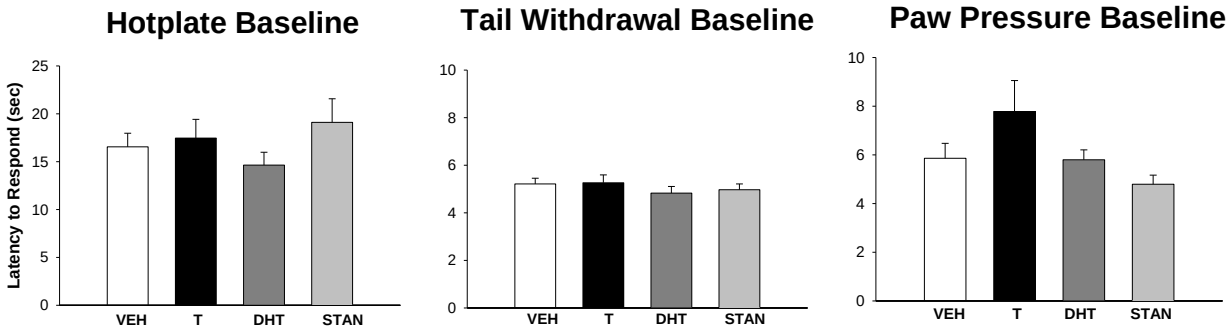

Figure 2. Nociceptive baselines on the 50ºC hotplate test (left panel), 50ºC tail withdrawal test (middle panel), and paw pressure test (right panel) on day 28 of daily treatment with vehicle (VEH), testosterone (T), dihydrotestosterone (DHT), or stanozolol (STAN). Nociceptive baselines were obtained immediately prior to administration of morphine. Each bar is the mean + 1 S.E.M., N=25 (vehicle group) or N=12-14 rats (AAS groups).

APPENDIX C

EXPERIMENT 1: MORPHINE ANTINOCICEPTION

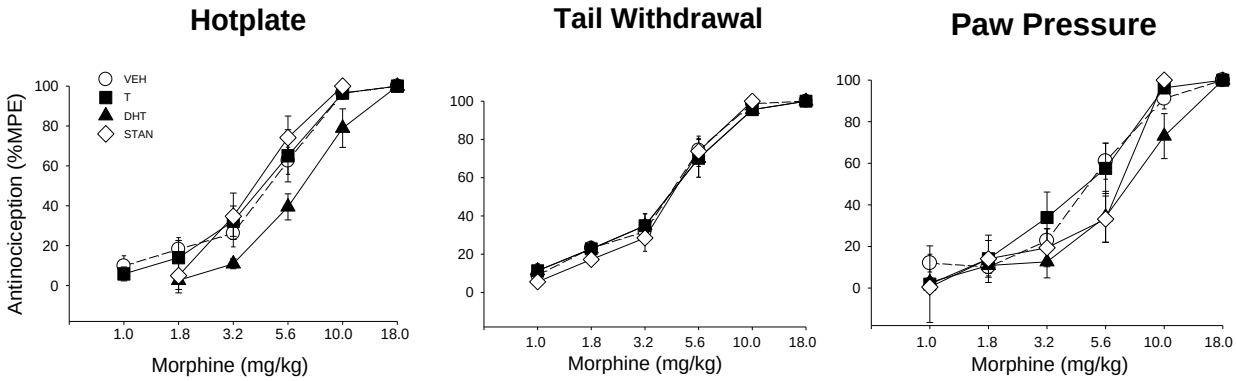

Figure 3. Morphine antinociception (%MPE) using a cumulative dosing regimen on the hotplate test (left panel), tail withdrawal test (middle panel) and paw pressure test (right panel) in gonadally intact male rats treated for 28 days with vehicle (VEH), testosterone (T), dihydrotestosterone (DHT), or stanozolol (STAN). Each point is the mean ± 1 S.E.M., N=25 (vehicle group) and N=12-14 rats (AAS groups). The hotplate ED50 was significantly greater in the DHT group than in the vehicle group.

APPENDIX D

EXPERIMENT 1: REPRODUCTIVE ORGANS

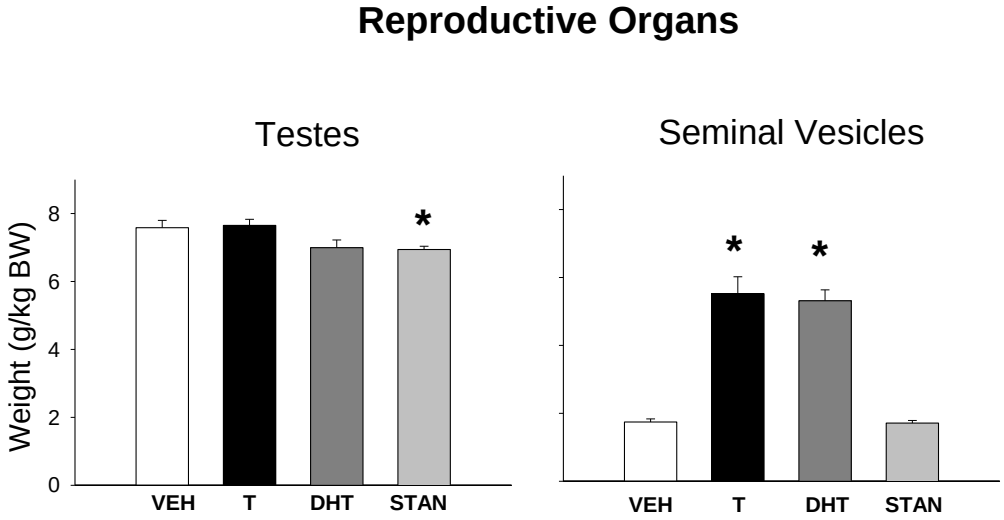

Figure 4. Reproductive organ weights of gonadally intact males after 28 days of treatment with vehicle (VEH), testosterone (T), dihydrotestosterone (DHT), or stanozolol (STAN). Organs were harvested immediately following morphine antinociceptive testing. Organ weights were adjusted for individual differences in body weight (BW). Each bar is the mean + 1 S.E.M., N=18 (vehicle group) and N=6-12 rats (AAS groups). *significantly different from vehicle group, p≤0.05.

APPENDIX E

EXPERIMENT 2: WEEKLY AND TEST DAY BODY WEIGHT

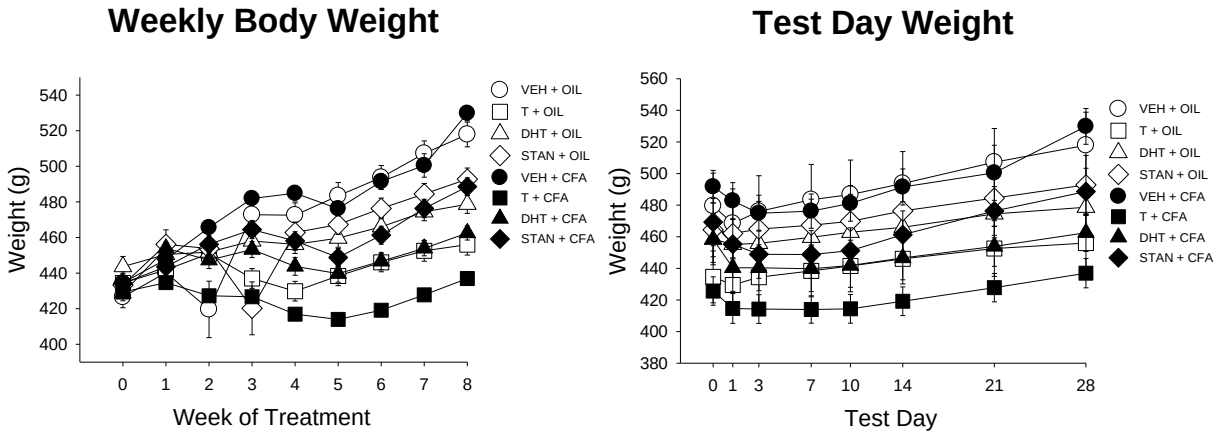

Figure 5. Body weight at each week of AAS treatment (left panel) and on test days 0, 1, 3, 7, 10, 14, 21 and 28 for rats treated with vehicle (VEH), testosterone (T), dihydrotestosterone (DHT), or stanozolol (STAN) in Experiment 2. CFA or mineral oil was injected into the hindpaw at week 4 (i.e., test day 0). Each point is the mean ± 1 S.E.M., N=8-10 rats/group. T-treated rats were significantly lighter than vehicle groups after week 4 and DHT rats were significantly lighter than vehicle groups after week 6 (left panel). AAS groups gained less weight than vehicle groups across various test days: TP groups across all days, DHT groups on test days 14, 21, 28, and STAN groups on day 28 (right panel).

APPENDIX F

EXPERIMENT 2: VON FREY AND HARGREAVES

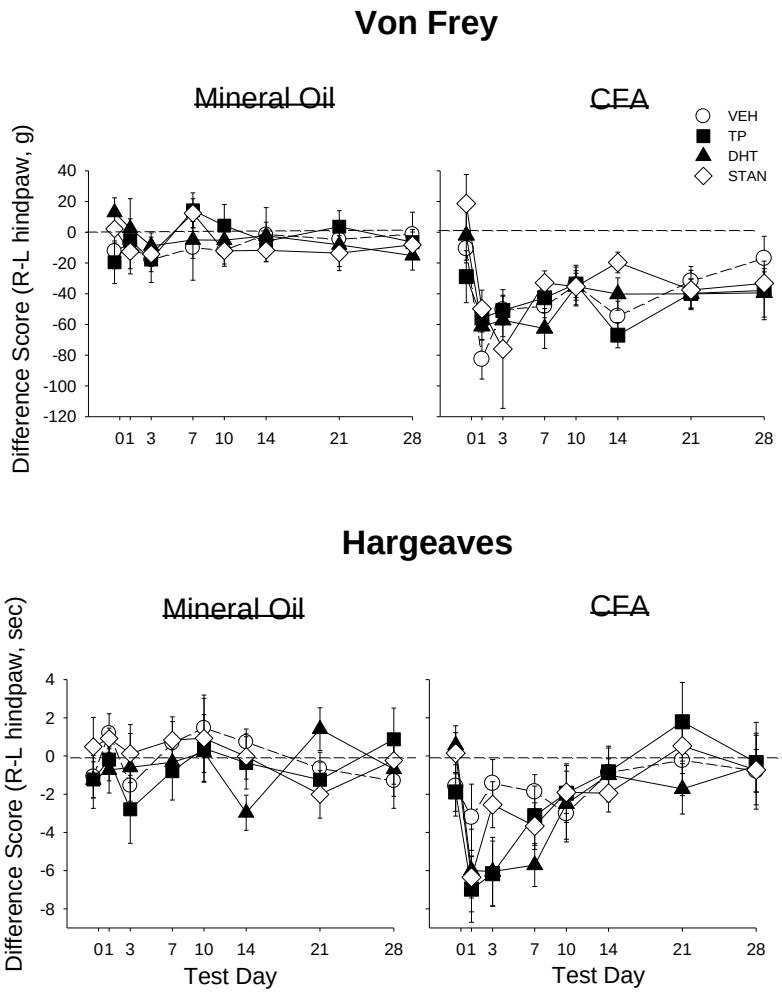

Figure 6. Effect of mineral oil (left panels) vs. CFA injection (right panels) on mechanical response threshold (von Frey test, top panel) and thermal response threshold (Hargreaves test, bottom panel) in rats treated with vehicle (VEH), testosterone (T), dihydrotestosterone (DHT), or stanozolol (STAN). Data are presented as difference scores between the left (uninjected) and right (injected) paw for the von Frey and Hargreaves tests; the dashed line indicates a difference score of 0 for the right paw compared to the left, meaning response thresholds were the same in each paw, whereas points below the dotted line indicate heightened sensitivity (lower threshold) in the injected paw compared to the uninjected paw. Each point is the mean ± 1 S.E.M., N=8-10 rats/group.

APPENDIX G

EXPERIMENT 2: LOCOMOTOR SCORES

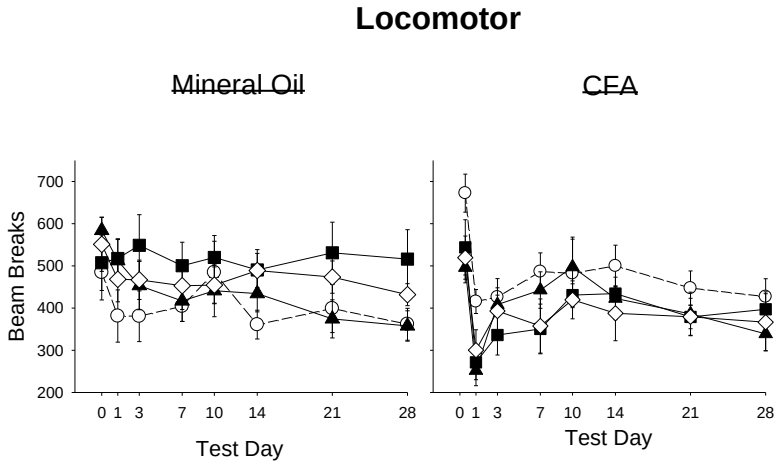

Figure 7. Effect of mineral oil (left panels) vs. CFA injection (right panels) on locomotor scores in rats treated with vehicle (VEH), testosterone (T), dihydrotestosterone (DHT), or stanozolol (STAN). Each point is the mean ± 1 S.E.M., N=8-10 rats/group.

APPENDIX H

EXPERIMENT 2: INFLAMMATORY MEASUREMENTS

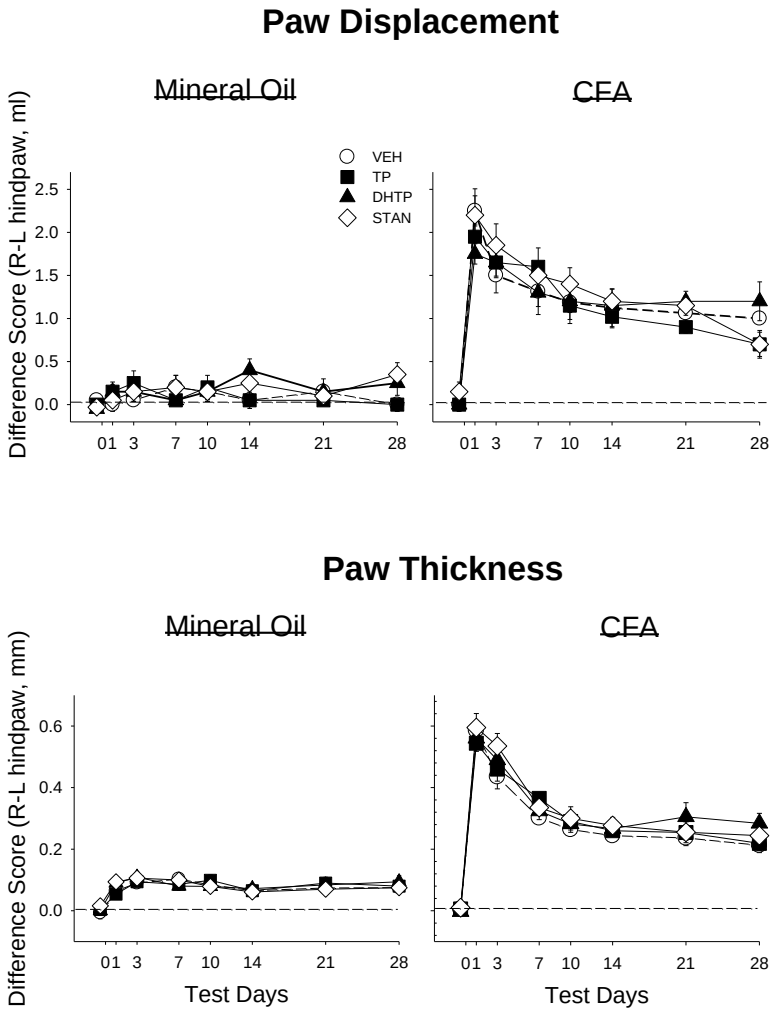

Figure 8. Effect of mineral oil (left panels) vs. CFA injection (right panels) on measures of inflammation: paw displacement (top panel) and paw thickness (bottom panel) across test days. 0, 1, 3, 7, 10, 14, 21 and 28 for rats treated with vehicle (VEH), testosterone (T), dihydrotestosterone (DHT), or stanozolol (STAN). The dotted line indicates a difference score of 0 for the right paw compared to the left, meaning response thresholds were the same in each paw, whereas points below the dotted line indicate heightened sensitivity (lower threshold) in the injected paw compared to the uninjected paw. Each point is the mean ± 1 S.E.M., N=8-10 rats/group. *significantly different from vehicle group, p≤0.05.

APPENDIX I

EXPERIMENT 2: REPRODUCTIVE ORGANS

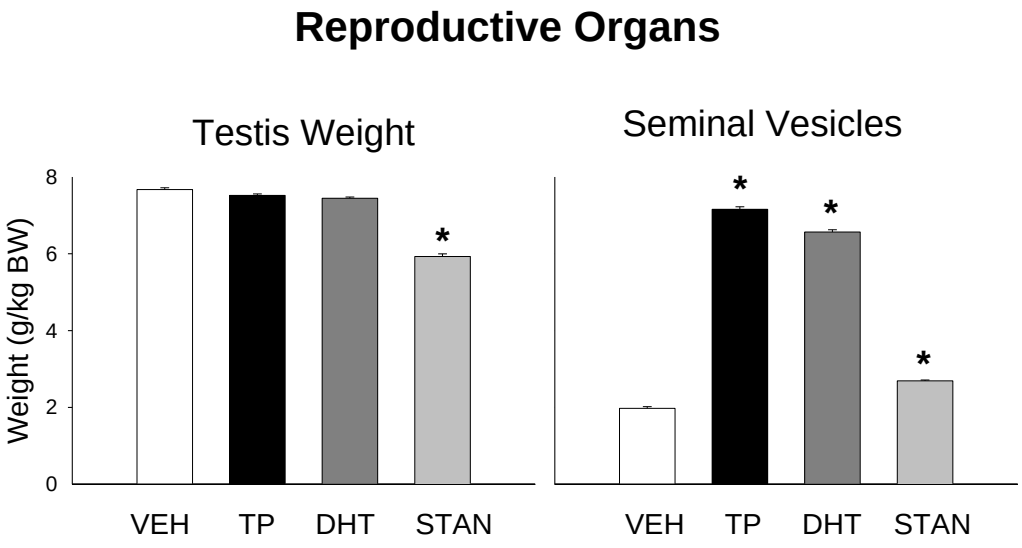

Figure 9. Reproductive organs weights of gonadally intact males following the last period of testing on day 56 for rats treated with vehicle (VEH), testosterone (T), dihydrotestosterone (DHT), or stanozolol (STAN). Organs weights were adjusted for individual differences in body weight (BW). Each bar is the mean + 1 S.E.M., N=18-20 rats/group. *significantly different from vehicle group, p≤0.05.