Nandrolone Decanoate Enhances Hypothalamic Biogenic Amines in Rats

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ABSTRACT

TAMAKI, T., T. SHIRAISHI, H. TAKEDA, T. MATSUMIYA, R. R. ROY, and V. R. EDGERTON. Nandrolone Decanoate Enhances Hypothalamic Biogenic Amines in Rats. Med. Sci. Sports Exerc., Vol. 35, No. 1, pp. 32–38, 2003. Purpose: To identify possible mechanisms for an anabolic-androgenic steroid induced increase in aggressive behavior and work capacity, the levels of some biogenic amines considered to be closely related to a systemic hyper-adrenergic state were measured in selected regions of the brain. Methods: Wistar male rats were divided randomly into five groups: nontreated (control), oil-vehicle-treated (vehicle) or one of three (therapeutic dose and 10- or 100-fold higher dose) anabolic-androgenic steroid-treated (steroid-1, -2, -3) groups. Rats in the steroid and vehicle groups were given a single dose of nandrolone decanoate or oil vehicle, respectively, one week before tissue sampling. The levels of norepinephrine (NE) and its metabolite, 4-hydroxy-3-methoxyphenylglycol (MHPG), serotonin (5-HT) and its metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) were measured in the cerebral cortex, hypothalamus and cerebellum by high-performance liquid chromatography. Immunostaining for c-fos was performed as a confirmation of increased neural activity. Results: The levels of NE and MHPG were increased by ~2- and ~7-fold in the hypothalamus of the steroid-2 compared with the control and vehicle groups. The levels of 5-HT and 5-HIAA were ~40 and ~50% higher in the steroid-2 compared with the control and vehicle groups. A significantly higher number of c-fos expressing neurons were observed in the periventricular region of the steroid-2 than the control and vehicle groups, indicating enhanced neuronal activity after nandrolone decanoate treatment. Conclusions: The present results, combined with previously reported findings of physical performance enhancement after anabolic-androgenic steroid treatment, are consistent with the interpretation that elevated levels of adrenergic and serotonergic amines in the hypothalamus could contribute to aggressive behaviors as well as improved physical performance. Key Words: NANDROLONE-DECANOATE, HYPOTHALAMUS, NOREPINEPHRINE, SEROTONIN, C-FOS

Anabolic-androgenic steroids (AAS) are widely abused by athletes and bodybuilders to improve physical performance (ergogenic effects) and enhance overall physical strength and muscle mass (38). For these ergogenic effects, one possibility is that there is a direct effect on the work capacity of the skeletal musculature, i.e., there is evidence that AAS treatment may directly improve the endurance capacity of skeletal muscles. For example, an elevated submaximal running capacity of rats (36) and an improved fatigue resistance of the rat extensor digitorum longus muscle tested via electrical stimulation (8) have been observed after 5–6 wk of AAS treatment. Similarly, 1 wk after a single intramuscular injection of nandrolone decanoate in adult male rats, we have reported an enhanced work volume during an exhaustive weight-lifting session and a greater fatigue resistance of the plantaris muscle tested in situ (35).

Another possibility is that they could be operating through some neurally mediated mechanism, which enhances the motivation to train harder, thus improving work tolerance (1). In human subjects, several psychological effects such as a greater tolerance to pain and irritability and aggression after AAS treatment have been reported, and it has been suggested that these behavioral changes may be related to an enhancement of performance (4,30). A relationship between AAS loading and biochemical changes in the brain reward system also has been observed. For example, AAS treatment for 14 d increased β-endorphin levels in the ventral tegmental area (18), increased dynorphin B and met-enkephalin-Arg⁶Phe (7)-ir activity in the hypothalamus, striatum, and periaqueductal gray region (16), and decreased N-methyl-D-aspartate receptor subunit NR2A mRNA expression in the hypothalamus and hippocampus of the brain (23). In addition, the hypothalamus and hippocampus are thought to play important roles in the expression of aggressive behavior (33), a consistent behavioral sequel of AAS abuse (30). Indeed, a primary effect of AAS use on athletic and/or exercise performance could be neurally me-
diated and manifested as greater motivation and/or aggression (9). Furthermore, possible mechanisms of central nervous system fatigue during exercise have been suggested that implicate the neurotransmitters serotonin, dopamine, and acetylcholine, as well as known neuromodulators like ammonia and various cytokines (6).

Therefore, the primary purpose of the present study was to determine how AAS treatment affects the levels of specific biogenic amines and their metabolites in regions of the CNS associated with aggressive behavior and improved motor performance (30,33). In addition, because immunohistochemical mapping of the inducible transcription factors, such as the members of the Jun family (c-Jun, JunB, and JunD) and the Fos family (c-fos, FosB, and the fos-related antigenes Fra-1 and Fra-2) often has been used to investigate the spatial and temporal distribution of brain functional activity (19,31), we used c-fos immunohistochemistry to determine whether inducible transcription factors were activated in the brain by AAS loading.

**METHODS**

**Experimental design.** Specific pathogen and virus antigen-free Wistar male rats (~17 wk old; 400-412 g body weight; N = 50) were divided randomly into five groups: a nontreated control (control; N = 11), a placebo oil-vehicle-treated (vehicle; N = 10), or one of three AAS-treated (steroid-1, -2, or -3; N = 7, 14, and 7, respectively) groups. A single dose of nandrolone decanoate (Deca-Durabolin, Organon, Oss, Netherlands) was administered in the gluteus muscles of steroid-treated rats 1 wk before the terminal experiment. This drug is a long-acting steroid ester, which is slowly hydrolyzed to give a constant tissue level of steroid for more than 4 wk (7). The recommended therapeutic use of nandrolone decanoate is about 0.4 mg-kg⁻¹ body weight (i.m.) in human subjects. It is highly likely that athletes consume 10–100 times the recommended therapeutic dose (26). Therefore, we selected the following dosage: 0.375 mg-kg⁻¹ body weight of nandrolone decanoate for the steroid-1, 3.75 for the steroid-2, and 37.5 for the steroid-3 groups, respectively. In the vehicle group, the same amount of oil vehicle (same solvent used with the Deca-Durabolin) was administered in the same manner. The animals were housed in standard cages and provided food (CE-2, CLEA, Tokyo, Japan) and water *ad libitum*. The room temperature was kept at 23 ± 1°C, and a 12:12-h light-dark cycle was maintained throughout the experiment. All experimental procedures were conducted in accordance with the Japanese Physiological Society Guide for the Care and Use of Laboratory Animals as approved by the Tokai University School of Medicine Committee on Animal Care and Use and followed the policy statement of the American College of Sports Medicine on research with experimental animals.

**Biochemical analyses.** Forty-one rats (control, N = 8; vehicle, N = 8; and steroid-1, -2, -3, N = 7, 11, and 7, respectively) were used for these analyses. One week after the administration of AAS or oil vehicle, the animals were sacrificed by decapitation for brain monoamine analysis (34). This procedure was performed by trained personnel and approved by the local animal use committee. The brains were removed rapidly and dissected into discrete regions on a dry ice-cooled aluminum plate according to the method of Glowinski and Iversen (10). The sampled regions included the cerebral cortex, hypothalamus, and cerebellum. Each sample was placed immediately in liquid nitrogen and stored at −80°C until analysis. Norepinephrine (NE), serotonin (5-HT), 5-hydroxyindole-3-ctetic acid (5-HIAA), and 5-hydroxy-3-methoxyphenylglycol (MHPG) were measured using a coulometric high-performance liquid chromatography system based on the procedures of Takeda et al. (34). Briefly, each tissue sample was weighed and initially mixed with a solution of 200 ng each of deoxyxypinephrine hydrochloride and 5-hydroxy-N-ω-methyltryptamine oxalate-mL⁻¹ of 0.1 M perchloric acid, 30 μL of 0.1 M ethylenediamine-tetraacetate acid, and 30 μL of 1 M sodium hydrogen sulfite as internal standards. The mixture then was homogenized with an ultrasonic cell disruptor (Ultrasonicator, OHTAKE WORKS, Tokyo, Japan) at 0°C for 30 s and centrifuged at 20,000 g for 15 min at 0°C. The supernatant layer was filtered through a 0.45 μm millipore filter (Nihon Millipore, Yonezawa, Japan) to separate the insoluble residue. A portion of the supernatant was injected into a reversed-phase high-performance liquid chromatography system with the redox-reductive screen detection mode by using a series of three coulometric working electrodes for simultaneous determination of NE, MHPG, 5-HT, and 5-HIAA.

**Immunohistochemical staining.** To confirm neural activity within the CNS, we performed c-fos immunohistochemical staining in the control, vehicle, and steroid-2 groups. One week after the administration of AAS or oil vehicle, three rats in each group (total N = 9) were overdosed with sodium pentobarbital (60 mg·kg⁻¹, i.p.) and perfused with warmed (~37°C) 0.01 M phosphate-buffered saline (PBS; pH 7.4) for 15 min. Then the rats were perfused with 4% paraformaldehyde/0.05 M phosphate buffer (pH 7.4) for 20 min at room temperature. The entire brain was removed and immersed in the same fixative overnight at 4°C. After fixation, the brains were cut serially in 40-μm thickness (coronal sections) by using a microslicer (DTK-1500, Do-han EM Co., Kyoto, Japan). Thirty sections from each rat were processed by free-floating staining for immunohistochemistry. Rabbit polyclonal antisemur for c-fos (Ab-5, Oncogene Research Products, Darmstadt, Germany) was used as the primary antibody (1:20,000). Free-floating sections were incubated with 0.3% H₂O₂ in PBS for 30 min, washed with PBS, and then incubated in a blocking solution (PBS/5% horse serum/0.25% Triton X-100) for 2 h at room temperature. The sections were incubated with primary antibody for 48 h at 4°C. The primary antibody was removed, and then the sections were washed in PBS and incubated with a secondary biotinated antirabbit (1:400, Amersham Pharmacia Biotech, Buckinghamshire, UK) for 2 h at room temperature. Sections were washed well in PBS and subjected to an avidin-biotin-peroxidase reaction (Vectastain, Vector Laboratories, Burlingame, CA) for 1 h and visual-
ized with 0.02% 3,3-diaminobenzidine (Wako Pure Chemical, Osaka, Japan)/0.05 M Tris HCl buffer, pH 7.4, containing 0.005% H2O2 for 4 to 6 min. The sections then were transferred to slide-glass, air dried, dehydrated in a graded ethanol series, and mounted. A digital photograph was taken from the periventricular region of the brain of each rat. The number of c-fos positive neurons were counted using image analysis computer software (NIH Image) and expressed as mean ± SE/unit area (1.85 × 1.2 mm squares as shown in Fig. 4).

Statistical analyses. All data are reported as mean ± SE. An ANOVA was used to determine overall differences and Duncan’s post hoc analyses were used to identify individual group differences. Differences were considered statistically significant at a $P \leq 0.05$ level.

RESULTS

Biochemical data. The final body weight of the five groups were similar: 417 ± 15, 412 ± 12, 424 ± 19, 423 ± 17, and 419 ± 21 g for the control, vehicle, and steroid-1, -2, and -3 groups, respectively. The simultaneous quantification of NE and MHPG, a metabolite of NE, in the cerebral cortex, hypothalamus, and cerebellum are shown in Figure 1, A and B, respectively.

The levels of NE and MHPG in the cerebral cortex and cerebellum were similar among the five groups. In the hypothalamus, however, the NE levels in the steroid-2 and -3 (Fig. 1A) and the MHPG levels in all three steroid groups (Fig. 1B) were higher than in the control and vehicle groups, except that the NE level was not higher in the steroid 3 versus control. In addition, the level of MHPG was higher in the steroid-2 than in the steroid-1 and -3 groups (Fig. 1B).

The levels of 5-HT were not significantly different among the five groups in any of the brain regions studied (Fig. 2A). However, there was a tendency for the steroid-2 group to have higher levels in the cerebral cortex (~30%, $P = 0.06$) and hypothalamus (~40%, $P = 0.06$) than in the control and vehicle groups. The levels of 5-HIAA, a metabolite of 5-HT, were higher in the hypothalamus of the steroid-2 group than in all other groups except control (Fig. 2B). Furthermore, the 5-HIAA/5-HT ratio was higher in the hypothalamus of the steroid-2 than all other groups. A higher 5-HIAA/5-HT ratio also was observed in the cerebellum of the steroid-2 and -3 compared with the other three groups (except for the steroid-3 vs control comparison, which was not significant) (Fig. 3). There were no differences between the control and vehicle groups for any measurement. Overall, the most prominent changes were observed in the steroid-2 group (3.75 mg·kg⁻¹ dose) (Figs. 1–3).

FIGURE 1—Levels of norepinephrine (NE) (A) and its metabolite 4-hydroxy-3 methoxyphenylglycol (MHPG) (B) in the cerebral cortex, hypothalamus, and cerebellum of rats in the control, vehicle, and steroid-1, -2, and -3 groups. Values are mean ± SE. Letters indicate a significant difference from that group at $P \leq 0.05$.

FIGURE 2—Levels of serotonin (5-HT) (A) and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) in the cerebral cortex, hypothalamus, and cerebellum of rats in the control, vehicle, and steroid-1, -2, and -3 groups. Values are mean ± SE. Letters, same as in Figure 1.
Immunohistochemistry. To confirm the biochemical results, we performed c-fos immunohistochemical staining in brain sections of the control, vehicle, and steroid-2 groups, i.e., the AAS-treated group showing the largest biochemical responses. A significantly higher number of c-fos-expressing neurons were observed in the periventricular region of the steroid-2 group (Table 1). Basal expression levels of c-fos, i.e., comparable levels to those shown for the control and vehicle groups in Table 1, were observed in the cerebral cortex, septum, amygdala, and corpus striatum in all three groups (data not shown). A representative photograph of the distribution of c-fos expressing neurons in the periventricular region of the brain is shown in Figure 4. A much denser distribution of c-fos expressing neurons was observed throughout the periventricular region in the rats of the steroid-2 group (Fig. 4C) compared with either the control (Fig. 4A) or vehicle (Fig. 4B) groups.

DISCUSSION

An AAS influence on brain reward systems has been suggested previously. For example, increased aggressiveness is one of the typical side effects after AAS treatment in humans (4). Increased aggressiveness also has been observed in AAS-treated animals based on the resident-intruder paradigm test (3,28). Similarly, AAS-induced defensive aggression has been reported in male rats (17). It has been reported that the hypothalamus plays an important role in the expression of aggressive behavior (33) and that the N-methyl-D-aspartate receptor is involved in the induction of some types of aggressive response (25). The N-methyl-D-aspartate receptor subunit mRNA expression levels are significantly reduced after 14 d of AAS treatment (23). The androgen action is linked to its ability to bind and activate specific androgen receptors, and the highest density is in the hypothalamus of the brain (13). This distribution also is consistent with androgenic influences on the regulation of gonadotropin secretion and reproductive behavior (13).

Nandrolone decanoate administration also has been shown to increase the immunoreactivity of substance P, a peptidergic factors associated with enhanced aggression, in several brain areas, to include the hypothalamus, amygdala striatum and periaqueductal gray region (12). All of these results are consistent with the possibility that the hypothal-
amo is affected by AAS treatment and with the hypothesis that AAS loading could influence the level of aggressiveness (3,12,28,30). The interrelationships among AAS treatment, aggressive behavior and elevated NE metabolism in the hypothalamus (Fig. 1, A and B) are unknown. However, functional interactions between dopamine, 5-HT, and NE levels in the hypothalamus and aggressive behavior in DSP-4 (noradrenergic neurotoxin) treated rats have been suggested (39). Further study will be needed to clarify these interrelationships.

Elevated NE metabolism in the hypothalamus has been associated with a systemic adrenergic state (15). In the present study, the levels and metabolic rate of 5-HT and its metabolite 5-HIAA (Fig. 2, A and B, and Fig. 3) were elevated in the hypothalamus after a single dose of AAS, suggesting that AAS loading has a strong influence on the hypothalamic 5-HT system. A systemic hyper-adrenergic state also is observed under stress conditions. For example, the enhancement of brain 5-HT neurotransmission, i.e., an augmentation of postsynaptic 5-HT1A receptor function and 5-HT turnover, contributes to the adaptation to stressful conditions (5,20). Thus, the observed increases in the 5-HT level and metabolic rate in the hypothalamus in the present study may reflect a counter action after AAS induced systemic hyper-adrenergic state. Similarly, AAS treatment directly modulates GABA-A receptor function (27), and both 5-HT and GABA can modulate hypothalamically evoked aggression (32,37), thus contributing to a systemic hyper-adrenergic state.

An enhanced state of activation of the hypothalamus after AAS treatment was further suggested by the immunohistochemical staining patterns of c-fos expression (Figure 4, A–C, and Table 1). The number of c-fos expressing neurons was significantly higher in the periventricular region of the hypothalamus of rats in the steroid-2 than in the control and vehicle groups, suggesting a state of hyper-neuronal activity (Table 1). Although early Fos expression does not simply reflect neuronal activation (29), the enhanced c-fos immunostaining observed in the present study is consistent with the biochemical data suggesting a systemic hyper-adrenergic state after a single dose of AAS. Enhanced c-fos expression has been observed previously in limbic brain regions, areas involved in stress, and behavioral and reward systems, in the guinea pig after nandrolone decanoate administration (21). These results provide further support for a role of AAS treatment to aggressive behavior.

The present data suggest that there is an optimal dose of AAS to produce these effects, i.e., the influences of AAS treatment at a dose of 3.75 mg·kg⁻¹ body weight were less apparent after the administration of a much lower (0.375 mg·kg⁻¹ body weight) dose. Whether the dose used in the present study is the optimal dose is unknown. However, it is likely that the bell-shaped dose-response curve, as observed in the present study, has also been reported on the brain reward system using other type of agents such as substance P (11) and neuropsin (22). The administration of 0.375 mg·kg⁻¹ body weight corresponds to the recommended therapeutic dose for nandrolone decanoate in humans. However, for the purpose of increasing muscle mass or strength, it is highly likely that athletes may consume doses that are 10–100 times this therapeutic dose (26). The rats injected with a single dose of 3.75 mg·kg⁻¹ body weight AAS in the same manner as in present study also can perform a significantly higher work volume during an exhaustive weight-lifting session, and their plantaris muscles have a greater fatigue resistance when tested in situ than age-matched untreated rats (35).

Any relationship between the increase in the metabolism of NE and 5-HT observed in the present study and the suggested enhancement of work capacity (8,36) after AAS treatment remains unclear. One possibility is that cardiac output and muscle blood flow are increased via sympathetic vasodilator and beta-adrenergic receptors in a hyper-adrenergic state. Electrical stimulation of the posterior hypothalamus activates the sympathetic nervous system, which, in turn, results in an increase in mean arterial pressure (MAP), an increase in muscle blood flow via beta-adrenergic receptors and a decrease in mesenteric artery blood flow mediated by alpha-adrenoreceptors (24). In addition, microinjection of NE in the paraventricular nucleus of the hypothalamus produces a dose-dependent increase both in the systolic and diastolic blood pressure (14). Consequently, a hyper-adrenergic state induced by AAS loading may have profound effects on a blood flow of skeletal muscle, as well as other supporting tissues. Another possibility is that an involvement of brain serotonergic system. Generally, fatigue of voluntary muscular effort has been considered on factors that peripheral fatigue (result in dysfunction of the contraction process within the muscle itself) and CNS fatigue (implication the neurotransmitters, especially 5-HT) (6). It is likely that an increase in 5-HT synthesis and/or concentrations in various regions of the CNS reduced the endurance capacity during prolonged exercise, related to an increase in plasma free tryptophan (TRP). In the present study, the levels and metabolic rate of 5-HT and its metabolite 5-HIAA (Fig. 2, A and B, and Fig. 3) in the resting state were elevated in the hypothalamus after a single dose of AAS. It is not clear that how this increase in serotonergic system during resting state affects and/or contributes to 5-HT metabolism during intermittent intense exercise and can result on its increased work volume (35). However, it is possible that increased synthesis of 5-HT in the brain closely related to the plasma TRP metabolism and its transport to brain across blood-brain barrier. This transport occurs via specific receptors that TRP shares with other large neutral amino acids, most

### Table 1. Number of c-fos positive neurons in the periventricular region of the hypothalamus.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of c-fos positive neurons</th>
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<tbody>
<tr>
<td>Control (N = 3)</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>Vehicle (N = 3)</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>Steroid-2 (N = 3)</td>
<td>239 ± 27*</td>
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Values are mean ± SE; *P < 0.05.

Number of c-fos positive neurons were counted and expressed mean ± SE/unit area (1.85 × 1.2 mm²).
notably the branched-chain amino acids (BCAA) such as leucine, isoleucine, and valine. We have reported that the AAS treatment increased leucine uptake in the muscular component (35), and it may possibly that this enhanced uptake of BCAA means decrease in plasma BCAA concentrations, and this maybe contribute to greater fatigue resistance. Elevated blood ammonia, which accumulates quickly in the brain, is found during brief intense exercise (2), and increased ammonia can alter CNS function and be considered to be related to the CNS fatigue. It may also be possible that the AAS-induced enhanced uptake of BCAA also diminishes an increase in plasma ammonia level. Unfortunately, we have no data for plasma BCAA levels in the ventral tegmental area in the male rat brain. 

REFERENCES

18. JOHANSSON, P., A. Z. RAY, Q. W. HUANG, K. KARLSSON, and F. NYBERG. Anabolic androgenic steroids increase beta-endorphin or ammonia levels in the present study. However, it seems that AAS treatment can enhance the adrenergic and serotonergic state in the hypothalamus, which, in turn, can contribute to a highly integrated and complex response that includes an increase in aggression and an augmentation in work capacity. Further studies are needed to clarify a possible relations among AAS loading, brain neurochemistry, and fatigue (and/or enhanced work volume).

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