Research report

Nandrolone decanoate and testosterone undecanoate differently affect stress hormones, neurotransmitter systems, and general activity in the male rat

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ABSTRACT

Anabolic androgenic steroids (AAS) are frequently used to improve physical appearance and strength. AAS are known to affect muscle growth, but many AAS-users also experience psychiatric and behavioral changes after long-term use. The AAS-induced effects on the brain seem to depend on the type of steroid used, but the rationale behind the observed effect is still not clear. The present study investigated and compared the impact of nandrolone decanoate and testosterone undecanoate on body weight gain, levels of stress hormones, brain gene expression, and behavioral profiles in the male rat. The behavioral profile was determined using the multivariate concentric square field (MCSF) test. Blood plasma and brains were collected for further analysis using ELISA and qPCR. Nandrolone decanoate caused a reduction in body weight gain in comparison with both testosterone undecanoate and control. Rats receiving nandrolone decanoate also demonstrated decreased general activity in the MCSF. In addition, nandrolone decanoate reduced the plasma levels of ACTH in comparison with the control and increased the levels of corticosterone in comparison with testosterone undecanoate. The qPCR analysis revealed brain region-dependent changes in mRNA expression, where the hypothalamus was identified as the region most affected by the AAS. Alterations in neurotransmitter systems and stress hormones may contribute to the changes in behavior detected in the MCSF. In conclusion, both AAS affect the male rat, although, nandrolone decanoate has more pronounced impact on the physiological and the behavioral parameters measured.

1. Introduction

Today, misuse of anabolic androgenic steroids (AAS) among adolescents and young adults in the general population is a health problem [1]. The worldwide prevalence of AAS use is difficult to estimate but several countries have in the last years reported increased problems with these substances [2–5]. In many cases, different subpopulations, for example, certain gym communities have reported prevalence as high as almost 10% [6]. AAS are most commonly used by males in order to enhance physical appearance, but also in some cases to boost self-esteem, or enhance braveness [7]. Nevertheless, it is well-known that AAS use also may lead to severe side effects. For example, AAS in supraphysiological concentrations affect the central nervous system (CNS) resulting in psychiatric disabilities such as aggression, irritability, anxiety, and depression [8–11]. In the last decade, structural changes in the brain such as volumetric changes [12,13], decreased cortical thickness [12], and altered functional connectivity [13,14] of weight lifters using AAS have been reported. In addition, animal studies investigating AAS-induced effects on the CNS demonstrate the influence of AAS on a broad range of neurochemical systems, such as the endogenous opioid system [15,16], monoaminergic system [17–19], glutamnergic system [20], GABAergic system [21], and peptidergic systems.
[22], which may all affect behavior.

There are several different AAS on the market and we recently reported that some of these structurally diverse AAS cause toxic effects in primary cortical cell cultures, but to various degrees and probably through different mechanisms [23,24]. Because of differences like these, it is of importance to distinguish and compare the effects induced by structurally diverse AAS, especially the long-term effects on the brain that may cause alterations in behavior. The increased knowledge and understanding of all AAS-induced effects are essential in order to offer individualized treatments for substance use disorders and overall to provide better healthcare for the AAS patients.

In order to study the potential behavioral alterations caused by different AAS the multivariate concentric square field™ test (MCSF-test) was chosen [25]. The MCSF-test provides an arena where diverse types of functional behaviors (i.e., general activity, exploratory activity, shelter seeking, risk assessment, and risk-taking) can be monitored simultaneously. The MCSF test is considered a multivariate method as the animal has a free choice to visit areas with different qualities in the same apparatus and trial session. The aim of the present study was to compare and examine the impact of two commonly used AAS, nandrolone decanoate and testosterone undecanoate. Based on our previous findings, where nandrolone displayed larger neurotoxic impact in primary cortical cells compared to testosterone [23,24], we hypothesize nandrolone decanoate to affect the brain and body to larger extent compared to testosterone undecanoate. Previous studies have reported nandrolone to have a higher binding affinity to the androgen receptor (AR) and also higher ratio regarding anabolic versus androgenic properties [26,27], but nandrolone may also induce its effects by an alternative mechanism other than that through the classical AR pathway [28]. Thus, the present study compared the AAS with regard to their effects on body weight gain, selected neurotransmitter systems, stress hormones, and behavioral profiles in the male rat.

2. Material and methods

2.1. Animals and housing

Male Wistar rats (n = 36) were obtained from Envigo (Netherlands). At arrival the rats were seven weeks old and allowed two weeks of acclimatization in order to adapt to the facility and the reversed 12 h light/dark cycle, which was separated with a dusk/dawn shift (lights on at 18:00 h). The rats were housed in groups of three animals per cage (cage type IV, 59 × 38 × 20 cm) with bedding consisting of wood-chip, paper sheets, and a wooden house as enrichment under standardized conditions (i.e., 20–24 °C and 45–65% humidity). The test rooms were kept at similar conditions and all rooms had masking background noise to minimize disturbance of the animals. The rats were monitored daily and provided with pelleted food (type R36, Lantmännen, Kimstad, Sweden) and tap water ad libitum. Animal health was monitored by weight (every third day), posture, eye, and fur condition. Prior to the start of any experimental procedures the rats were handled by the main experimenter in order to habituate to the experimental situations. After the two weeks of acclimatization, the animals were randomly divided in three treatment groups, with 12 animals per group.

2.2. Ethical statement

All animal experimental procedures were approved by the local animal ethics committee at Uppsala University [5.8.18–02249/2017] and were consistent with Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS 1998:56) and the European Union Directive on the Protection of Animals Used for Scientific Purposes (2010/63/EU).

2.3. Drug treatment

The nandrolone decanoate (Deca-durabol®) and testosterone undecanoate (Nebido®) were manufactured by Organon (Netherlands) and Bayer AG (Germany), respectively. The peanut oil was obtained from APL (Sweden). The animals received subcutaneous injection of 15 mg/kg of either nandrolone decanoate (50 mg/mL, in peanut oil) or testosterone undecanoate (50 mg/mL in peanut oil and castor oil, 80:20 v/v), every third day throughout the experiment (experimental day 1–18). The control group received subcutaneous injections of peanut oil, following the same administration schedule as the experimental groups. All animals received a total of six injections, each injection in a maximum volume of 100 μL, on the upper back. To avoid skin irritation the injection site was varied.

2.4. Behavioral testing

2.4.1. Multivariate concentric square field™ test (MCSF)

The multivariate concentric square field test has been explained in detail elsewhere [25]. Briefly, the MCSF arena consists of several different zones, built up by open, elevated, sheltered, and highly illuminated areas, which are to be explored free of choice by the animals. Therefore, this behavioral test allows different types of functional behavior (general activity, exploratory activity, risk assessment, risk taking behavior, and shelter seeking) to be monitored. The arena consists of a square field (100 cm × 100 cm) with outer walls and a smaller inner square field (70 cm × 70 cm) located in the middle of the arena, called center. The open area in the middle of the center is called central circle. From the center three corridors can be accessed through openings in the walls. In one of the corners, an elevated hole-board device (hurdle) was located. Underneath the floor of the hurdle a photo cell was placed in order to register nose-pokes. In the opposite corner a dark corner room was located, only to be accessed from one corridor. In the third corner a slope leads up to a brightly illuminated (600 lux) bridge. An illustration of the MCSF arena and these zones are illustrated in Fig. 2b. At 12 weeks of age (experimental day 17) the rats were tested in the MCSF and they were transferred in a transportation-bucket from their home cage to the MCSF arena. The animals were released in the center facing the wall without openings and allowed to freely move in the arena for 20 min. The behavioral testing was carried out during the dark period of the light/dark cycle. After each session, the floor of the MCSF arena was wiped with a cloth containing 10% (v/v) ethanol, and allowed to dry before the next animal was placed in the arena.

2.4.2. Behavioral recording

The MCSF trial for each animal was recorded and monitored from an adjacent room. Specific animal behavior (rearing, grooming, and stretched attend posture), was directly scored by the observer, and fecal boli and urinations were noted after each trial. The EthoVision system version 13 (Noldus Information Technology, Wageningen, Netherlands) was used to score distance moved (cm) and velocity (cm/s) for the total MCSF trial. In addition, EthoVision was used to score the latency (L, seconds) to first visiting a zone, frequency (F) of visits in specific zone, and duration (D, seconds) of total time spent in specific zone. Furthermore, following descriptive parameters were obtained; latency to leave center (L leave), the mean duration per visit in specific zone (D/F), the total activity (sum of all frequencies), total corridors (sum of frequencies, duration and D/F, respectively, for the corridors), the frequency and duration of risk/shelter indices ((F bridge-F dark corner room)/(F bridge+F dark corner room) and (D bridge-D dark corner room)/(D bridge+D dark corner room)), and the slope/bridge interval (L slope-L bridge)/L slope).

2.4.3. Trend analysis

In the MCSF test, descriptive parameters that correlates are grouped together to form a functional category, i.e., general activity, exploratory activity, risk assessment, risk-taking, and shelter seeking [29]. In the present study following parameters were selected for the different functional categories; general activity (total activity, distance arena, F
center, F and D/F total corridors), exploratory activity (D total corridors, D center, D hurdle, rearing, nose pores), risk assessment (D, F and D/F for slope and bridge entrance, stretched attend posture in center and slope), risk-taking (D, F and D/F for bridge and central circle), and shelter seeking (D, F and D/F dark corner room). To measure and evaluate variation in behavioral traits a trend analysis was performed. The trend analysis is a rank-order procedure where the individual animals are ranked against each other for each descriptive parameter. The animal with the lowest score gets the lowest rank and the animal with the highest score is given the highest rank. The rank values are then summed together for each animal resulting in a summed rank value for each functional category. Thus, the group-wise comparison is based on the relative position of the animals within the entire population. The parameters that are negatively related to the functional category (D center, D and D/F total corridors) were inversely ranked before the rank values were summed.

2.5. Tissue collection

At baseline (the day before first AAS injection) and the day before behavioral testing (experimental day 16) a blood sample (max 200 μL) was taken from the hind paw of all rats (used in a separate metabolomic study). The day after the behavioral testing (experimental day 18) the rats were euthanized by decapitation. Blood was collected in lithium/heparin tubes (Sarstedt) and centrifuged at 3000 rpm for 10 min at 4 °C to obtain blood plasma. Selected brain areas, including the hypothalamus, frontal cortex, amygdala, striatum, and hippocampus were dissected using a brain matrix and immediately frozen on dry ice. The plasma samples and brain areas were stored in −80 °C until further biochemical analysis.

2.6. Stress hormone levels

2.6.1. Corticosterone plasma concentrations

Corticosterone concentrations were measured in rat plasma using the commercial kit, corticosterone ELISA kit (ab108821), obtained from Abcam (UK). Briefly, all reagents were equilibrated to room temperature and prepared in accordance with the protocol from the manufacturer. All standards, controls and samples were assayed in duplicates. The mean absorbance was detected at 450 nm and 570 nm using the FLUOstar Omega spectrophotometer (BMG Labtech, Germany).

2.6.2. Adrenocorticotropic hormone (ACTH) plasma concentrations

Plasma concentrations of adrenocorticotropic hormone (ACTH) were analyzed using a mouse/rat ACTH ELISA kit (ab263880) from Abcam. The assay was conducted in duplicates and performed in accordance to the instructions provided by the manufacturer with minor modifications. Briefly, standards, undiluted samples, and the antibody cocktail were incubated for 1.5 h. After washing, the development solution was added and incubated for 30 min in the dark. After the stop solution was added the OD was recorded at 450 nm using the FLUOstar Omega spectrophotometer (BMG Labtech).

2.7. Brain mRNA expression

2.7.1. RNA extraction

RNA extractions from frozen brain regions were performed using Qiagen RNeasy Lipid tissue kit (Qiagen) according to the instructions from the manufacturer with minor modifications. Briefly, brain tissue was homogenized in QIAzol Lysis reagent (Qiagen) and chloroform was added to each sample. Thereafter, the samples were centrifuged at 4 °C for 15 min in 12000 × g and to the supernatant a 1:1 vol of 70% (v/v) ethanol was added. RNase free mini spin columns were used to elute the samples and the concentration of total RNA was determined using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., USA). Experion RNA analysis kit (Bio-rad instruments) was used to examine the quality of the RNA. Samples having a RNA quality indicator between 7 and 10, and displaying clear 18 S and 28 S ribosomal RNA peaks were used for further analysis.

2.7.2. cDNA synthesis

RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad). The reactions were performed in a final volume of 20 μL, including 250 ng RNA, 5 × iScript reaction mix, iScript reverse transcriptase, and RNase free water. The following cycling parameters were used; 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min. A control reaction without reverse transcriptase was also conducted.

2.7.3. Quantitative polymerase chain reaction (qPCR) SyBr Green

The gene expression (i.e., mRNA levels) of the genes encoding the oxytocin receptor (OXTR), catechol-O-methyltransferase (COMT), monoaminooxidase A (MAO-A), and monoaminooxidase B (MAO-B), androgen receptor (AR), neuropeptide Y (NPY), neuropeptide Y receptor 1 (NPY1R), neuropeptide Y receptor 2 (NPY2R), were investigated using qPCR. The qPCR analysis was conducted in a 96-well plate with 2 μL of each cDNA sample (2.5 ng/μL). 23 μL master mix containing Q-SyBr Green Supermix (Bio-Rad), 20 μM forward primer, 20 μM reverse primer and RNase free water. Primer sequences were designed using the Primer-BLAST tool (NCBI), and primers were validated in silico using the UCSC genome browser. A list of the primer sequences can be found in the Supplementary material (table S1). PCR-reactions were performed in duplicates including samples, and negative controls. Following protocol was used for amplification on the CFX96 real-time PCR detection system, version 3.1 (Bio-Rad); 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, 72 °C for 10 s. A melt curve was incorporated after each run, in order to ensure specific amplification. LinRegPCR software was used to calculate the amplification efficiency, with a mean value for each primer set. Cq-values were received from CFX managing software and the qBASEPlus software, version 3.2 (Biogazelle) was used together with the three reference genes ribosomal protein 19 (rpl19), actin beta (Actb), and ribosomal protein large (rppl0), which were chosen after evaluation of a set of reference genes with GeNorm (a part of qBASEplus), to obtain normalized gene expression levels.

2.8. Statistical analysis

Classical statistical calculations were performed using GraphPad software (Prism 9.0). MCSF measurements did not follow a normal distribution according to the Shapiro-Wilk’s W test and therefore non-parametric statistics were used. The Kruskal-Wallis test was used for overall comparison between the experimental groups. When a significant difference was found, further group-wise analysis was made using Dunn’s multiple comparison test. In order to investigate differences in the functional categories between the groups, the results from the trend analysis were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test when appropriate. The body weight gain measurements and the MCSF data analyzed over time were statistically evaluated using the repeated measurement two-way analysis of variance (RM two-way ANOVA), with time and treatment as factors to account for differences. When an overall effect was found, on either time or treatment, further comparison was made using Tukey’s multiple comparison test when appropriate. Measurements of stress hormone levels did not follow a normal distribution. Therefore, the Kruskal-Wallis test was used for overall comparison, followed by Dunn’s multiple comparison test. As the data followed a gaussian distribution, from the qPCR analysis were statistically analyzed using the one-way analysis of variance (one-way ANOVA), followed by Tukey’s multiple comparison test when appropriate. Potential outliers in the data were identified using the ROUT outlier test (with Q=1%). Correlation analysis was performed using Pearson correlation or Spearman correlation based on the distribution of the data. The MCSF data and stress hormone levels are presented as median and
interquartile range. The data following a gaussian distribution, i.e., weight data, qPCR measurements, and trend analysis are presented as mean ± SD or SEM. A p-value < 0.05 was considered statistically significant and no corrections for multiple testing were performed.

2.8.1. Multivariate data analysis

Multivariate data analysis was performed using SIMCA 15 (Umetrics AB, Umeå, Sweden) as a complement to the conventional statistic and to visualize the data. The partial least square-discriminant analysis (PLS-DA) is a supervised method examining the relationship between several X: s (study parameters) and a class-Y variable (treatment group), which suggest which study parameters are most likely to be responsible for discrimination between groups. The loading plot displays the relation between the study parameters and the treatment groups. The study parameters that load in close association with a study group indicate positive association with that group. Conversely, study parameters located on the opposite side of the origin of the graph indicate negative association. The VIP (Variable Importance for the Projection) plot was derived from the model, which summarizes the importance of the parameters to explain the model. All experimental data from the study were included in the analyses, except latencies, risk/shelter indices, slope/bridge interval, and rank values for the functional categories. The autofit option was used to create the models resulting in the maximal number of significant components.

3. Results

The multivariate data analysis was performed with the intention to visualize the data, get an overview of the results, and identify the key findings. The PLS-DA analysis identified which parameters that are most likely to be responsible for discrimination between the groups. The nandrolone decanoate treated animals were separated from the control group primarily by component 1 in the score plot (PC, t [1]), while the testosterone undecanoate group was separated from the control group primarily by component 2 (PC, t [2]). There was not as clear separation between the two AAS groups, but they mainly differed in the first component (PC, t [1]). The nandrolone decanoate treated rats had negative contribution of component 1 and positive contribution to component 2. In addition, one outlier was identified. The testosterone undecanoate treated rats had positive contribution of component 2 and low contribution to component 1. The control group had relatively equal contribution of component 1 and 2. In the loading plot the relationship between the class (Y) and parameters (X) is visualized (Fig. S1b). X-variables situated in the vicinity of the dummy Y-variables have the highest discriminatory power between the classes. The VIP score summarizes the parameters important to explain the model for each component (Fig. S1c-d). Parameters contributing to the first component were primarily body weight gain and MCSF parameters associated with general activity (e.g., velocity center, velocity and distance in the arena), together with the gene expression of OXTR and NPYSR in the hypothalamus. Parameters contributing to the second component were body weight gain, changes in brain gene expression of OXTR, COMT, and NPYSR in the hypothalamus, and velocity in center a MCSF parameter associated with general activity.

3.1. Body weight gain

The animals receiving nandrolone decanoate had a significantly slower weight development compared to the other groups. The result revealed, as indicated by ANOVA, a difference between the treatment groups (p < 0.0002, F (2, 33) = 11.50) and Tukey’s multiple comparison test showed that the nandrolone decanoate treated group displayed lower body weight gain compared both to the control group and testosterone undecanoate group as from day 13 and throughout the study (Fig. 1). There were no differences in weight between the experimental groups at the start of the experiment (experimental day 0).

3.2. Multivariate concentric square field™ test

The descriptive parameters analyzed in the MCSF test are presented in the Supplementary material (table S2). Differences were mainly found for parameters associated with general activity; total activity (p = 0.04); distance in the arena (p = 0.02); velocity in the arena (p = 0.02); visits in center (p = 0.03); duration per visit in center (p = 0.001); velocity in center (p = 0.0006); and visits in corridors (p = 0.04). In addition, duration in the corridors (D total corridor), a parameter of relevance for exploratory activity, was found to be significantly different between the groups (p = 0.02). The post hoc analysis revealed that the nandrolone decanoate group had reduced total activity, traveled shorter distance in the arena, with lower speed both in the arena and in the center, and spent more time per visit in the center compared to the control group. In addition, they spent less time exploring the corridors compared to the controls. These results were supported by the PLS-DA as the nandrolone decanoate treated group load far to the left in opposite to the parameters total activity, distance arena, velocity arena, velocity center, and duration total corridors which load far to the right, indicating negative association. The parameter duration center load in vicinity of the nandrolone decanoate treated group, indicating positive association. Moreover, there was a trend towards difference for some parameters associated with general activity (duration center, p = 0.08), shelter seeking (frequency DCR, p = 0.07), risk taking (duration per visit on the bridge, p = 0.054), and other (urinations, p = 0.058). In the PLS-DA loading plot, these descriptive parameters load relatively far away from the plot origin, indicating strong impact on the model. The statistical data is provided in table S2.

These findings were further supported by the trend analysis which demonstrated nandrolone decanoate to cause a reduction in general activity when compared to control (p = 0.039, F (2, 33) = 3.58) as indicated by ANOVA (Fig. 2a). The trend analysis could not reveal differences between the groups in the other functional categories described (exploratory activity, risk assessment, risk-taking and shelter seeking).

3.3. Stress hormone levels in blood plasma

Nandrolone decanoate significantly reduced the plasma levels of ACTH (p = 0.03) in comparison to the control (Fig. 3a). The AAS
treatment also induced an overall effect on the corticosterone plasma levels ($p = 0.005$). Interestingly, Dunn’s multiple comparison test showed a significant higher levels of corticosterone in nandrolone decanoate treated rats compared to testosterone undecanoate treated rats (Fig. 3b).

3.4. Brain mRNA expression (qPCR)

The effect of nandrolone decanoate and testosterone undecanoate on brain mRNA expression of genes encoding OXTR, AR, MAOA, MAOB, COMT, NPY, NPY1R, and NPY5R were investigated in different brain regions; amygdala, hypothalamus, frontal cortex, hippocampus, and striatum (Table 1). In the hypothalamus both AAS demonstrated an increase in the expression of the OXTR. In addition, the COMT and NPY5R genes were increased by nandrolone decanoate and the AR was increased by testosterone undecanoate. In the frontal cortex the expression of the MAO-B gene was reduced by testosterone undecanoate. In addition, an overall effect was found in the expression of NPY1R in the same region. In the striatum testosterone undecanoate treated rats had a reduction in AR expression compared to the nandrolone decanoate treated group. An overall difference in NPY1R expression were identified in the amygdala. No changes in mRNA expression of any of the genes investigated could be detected in the hippocampus. The statistical data is summarized in Table 1. In accordance with the conventional statistics, the multivariate data analysis indicated that the hypothalamus was the brain region most affected by the AAS-treatments, as several of the genes expressed in the hypothalamus (OXTR, COMT, NPY1R, NPY5R, and AR) load far away from the plot origin which indicated these descriptive parameters to have strong impact on the model. Based on the conventional statistics and the multivariate data analysis, the AAS-induced elevation of OXTR was considered the most robust finding. In the PLS-DA OXTR expression in the hypothalamus load in opposite to the body weight gain and parameters associated with general activity (total activity, F total corridor, distance and velocity arena, distance, velocity and F center), indicating a negative relationship between these parameters. In addition, the

![Fig. 2. Behavioral profiling in the Multivariate Concentric Square Field (MCSF). A) The MCSF trend analysis. Rats were treated with either 15 mg/kg nandrolone decanoate, testosterone undecanoate, or peanut oil (control). In the MCSF-test, descriptive parameters that correlate are grouped together to form a functional category, i.e., general activity, exploratory behavior, shelter seeking, risk assessment, and risk-taking. The individual rank values for parameters included in the functional categories are summed, and the group-wise comparison is based on the relative position of the animals within the entire population. Values are presented as mean with SEM, $n = 12$ rats per group. Statistical analysis was performed using the one-way ANOVA followed by Tukey’s multiple comparison test and $p < 0.05$ was considered statistically significant. B) Illustration of the MCSF arena. The arena is divided into zones by walls or imagined boundaries (dashed lines). Zones in the arena: central circle, center, corridors, dark corner room, hurdle, slope, bridge entrance, and bridge. The saturation levels in the figure reflect the illumination levels in respective zone.](image1)

![Fig. 3. Stress hormone levels in plasma detected with ELISA. A) ACTH levels and B) corticosterone levels in rats treated with either 15 mg/kg nandrolone decanoate, testosterone undecanoate, or peanut oil (control). Statistical analysis was performed using Kruskal-Wallis test followed by Dunn’s multiple comparison test, and a p-value less than 0.05 was considered statistically significant. Data is presented as percent of control with median and interquartile range. Abbreviations: adrenocorticotropic hormone (ACTH), nandrolone decanoate (ND), and testosterone undecanoate (TU).](image2)
Table 1
Gene expression levels in brain regions analyzed following AAS treatment.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Gene</th>
<th>Control Mean ± SD</th>
<th>ND Mean ± SD</th>
<th>TU Mean ± SD</th>
<th>ANOVA p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamus</td>
<td>OXTR</td>
<td>1.0 ± 0.12</td>
<td>1.39 ± 0.19</td>
<td>1.27 ± 0.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>MAO-A</td>
<td>1.0 ± 0.16</td>
<td>1.02 ± 0.19</td>
<td>1.06 ± 0.24</td>
<td>0.63</td>
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<td>MAO-B</td>
<td>1.0 ± 0.11</td>
<td>1.06 ± 0.12</td>
<td>1.05 ± 0.17</td>
<td>0.25</td>
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<tr>
<td></td>
<td>COMT</td>
<td>1.0 ± 0.10</td>
<td>1.17 ± 0.11</td>
<td>1.17 ± 0.14</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>AR</td>
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<td>1.07 ± 0.3</td>
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<td>0.02</td>
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<tr>
<td></td>
<td>NPY</td>
<td>1.0 ± 0.06</td>
<td>0.97 ± 0.11</td>
<td>0.9 ± 0.11</td>
<td>0.09</td>
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<td></td>
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<td>1.14 ± 0.28</td>
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<td></td>
<td>NPY5R</td>
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<td>1.4 ± 0.3</td>
<td>1.29 ± 0.36</td>
<td>0.02</td>
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<td>Frontal cortex</td>
<td>OXTR</td>
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<td>0.93 ± 0.27</td>
<td>0.86 ± 0.19</td>
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<td>1.0 ± 0.16</td>
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<td>0.9 ± 0.06</td>
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<tr>
<td></td>
<td>MAO-B</td>
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<td></td>
<td>COMT</td>
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<td>AR</td>
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<td>0.9 ± 0.15</td>
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<td>0.49</td>
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Statistical analysis was performed using the one-way ANOVA, followed by Tukey’s multiple comparison test when appropriate. Outliers were identified using the ROUT outlier test. Values are presented as mean ± SD. A p-value less than 0.05 was considered significant, where *** indicates significance in comparison with the control group and # indicates significance in comparison with the ND group. *p < 0.05, **p < 0.01, and ***p < 0.001. Abbreviations: nandrolone decanoate (ND), testosterone undecanoate (TU), oxytocin-receptor (OXTR), monoaminoxidase A (MAO-A), monoaminoxidase B (MAO-B), catechol-O-methyltransferase (COMT), androgen receptor (AR), neuropeptide Y (NPY), neuropeptide Y1 receptor (NPY1R), neuropeptide Y5 receptor (NPY5R), standard deviation (SD).

expression of OXTR in the hypothalamus and corticosterone levels both load on the left side of the loading plot suggesting a positive relationship. Therefore, correlations based on the entire population (n = 36) between the OXTR mRNA expression in the hypothalamus and the other key findings (body weight gain, corticosterone plasma levels, and general activity) were investigated. Significant correlations of medium strength were found between OXTR and body weight gain (p = 0.0004; r = -0.57), OXTR and corticosterone levels (p = 0.03; r = 0.37), and

Fig. 4. Relationship between the OXTR gene expression in the hypothalamus (presented as % of control) and A) body weight gain (presented as % of initial body weight), B) corticosterone levels (presented as % of control), and C) general activity (presented as summed rank values from the trend analysis). n = 36 and a p < 0.05 was considered significant.
OXTR and general activity (p = 0.046; r = -0.34), which can be seen in Fig. 4.

4. Discussion

In the present study a multivariate approach (the MCSF test) was used with the aim to study the behavior in male rats treated with the AAS, nandrolone decanoate and testosterone undecanoate, in supra-physiological doses. The PLS-DA identified the study parameters most likely to be responsible for the discrimination between the groups i.e., differences in body weight gain, altered general activity, and changes in mRNA expression of the hypothalamus. These findings are acknowledged as the key findings of the study and will be prioritized in the discussion.

In the present study several brain region-dependent differences of gene expression were identified which possibly account for some of the behavioral changes detected. Of the brain regions analyzed, hypothalamus was the brain region most affected by the AAS treatments. Testosterone induced a particular pronounced elevation of OXTR mRNA, a finding that the PLS-DA support. Oxytocin is a neuromodulator well known for its involvement in reproductive processes and behaviors [30], but also for contributing to the regulation of a wide range of other behaviors [31]. Oxytocin is known for its involvement in the regulation of food intake and energy balance, where it reduces appetite and stimulates satiety [32,33]. Another neuropeptide known to regulate feeding behavior and energy homeostasis is the NPY [34,35]. In addition to the elevated expression of the OXTR in the hypothalamus, significant changes in mRNA expression of the NPY-receptors were identified in the AAS-treated rats. The most pronounced effect was the increase in NPYSR discovered in the hypothalamus of nandrolone decanoate treated rats. Interestingly, rats exposed to nandrolone decanoate also demonstrated reduced weight gain in comparison both to the control and rats treated with testosterone undecanoate. The impact of nandrolone on body weight is previously known [36,37], and in fact Lindblom et al., suggest that the reduced body weight in animals receiving nandrolone decanoate is due to reduced food intake [37]. The altered receptor expression indicates a dysregulation of the oxytocin system and NPY system within the hypothalamus, which at least in the nandrolone decanoate treated rats, may cause a modified appetite regulation and feeding behavior, and thereby account for the effect seen on body weight development in the present study. This theory is further supported by the negative relationship found in the PLS-DA and confirmed by the correlation analysis between the OXTR expression in hypothalamus and body weight gain. However, the elevation of OXTR mRNA was found in the hypothalamus of rats treated with testosterone undecanoate as well. These animals did not display any significant alterations in body weight gain, but followed the same trend as rats treated with nandrolone decanoate. This could be explained by the fact that testosterone has lower relative binding affinity to the AR compared to nandrolone [26], and therefore causes milder effects. Alternatively, the increased expression of AR mRNA in the hypothalamus caused by testosterone undecanoate could possibly counteract the effect, as genes related to oxytocin are previously described to be regulated by the AR in the hypothalamus [38].

The reduction in body weight gain detected in the nandrolone decanoate treated rats may also indicate an impact on the well-being of the animals, something that is further supported by the altered stress hormone levels and reduction in general activity discovered in these animals. Nandrolone decanoate treated rats had reduced plasma levels of ACTH compared to the control, which is in accordance with previous published data [29], but also displayed increased levels of corticosterone compared to the testosterone undecanoate treated group. The oxytocin system has been implicated to contribute to several neuropsychiatric conditions [40] and is suggested to modulate the stress response [33,41,42]. Oxytocin is mutually regulated with the HPA system [43,44] and the release of oxytocin within the brain is shown to increase after stressful stimuli [45]. Furthermore, oxytocin has been reported to reduce locomotor activity in the open field and is suggested to have sedative effects [46]. The decreased general activity identified in the nandrolone decanoate treated rats indicates a reduction in locomotor activity, as associated parameters such as, distance moved, velocity, and total activity, in the MCSF-test were affected. The link between the oxytocin-system, stress response, and contribution to the behavioral effects detected is further supported by the relationship identified in the PLS-DA between the OXTR expression in hypothalamus, the corticosterone levels, and general activity, which was confirmed by the correlation analysis. As other types of drug exposure previously have been suggested to impair the functioning of the oxytocin system and to contribute to dysfunctional stress-related behaviors [47], one explanation to the nandrolone-induced reduction in general activity could be altered oxytocin-signaling, resulting in increased stress vulnerability. However, the AAS-effect on locomotor activity is contradictory. In the literature there are studies describing both AAS-induced and reduced effects on activity [48,49]. In fact, McGinnis et al., reported differences in activity between nandrolone and testosterone, where testosterone increased the activity whereas the nandrolone treated rats displayed a reduced activity [50].

Stress and dysregulation of the HPA-axis are described as risk factors for developing mood disorders, like depression [51]. As an example, exposing rats to stress is a commonly used method to model depression in rodents [52]. Chronic stress is suggested to cause depressive-like behavior in rats [53], and when exposed to early life stress an altered HPA-axis activity and reduced locomotor activity have been identified [54]. Interestingly, increased OXT mRNA levels [55], number of oxytocin neurons [56], and elevated plasma levels of oxytocin [57] have been encountered in depressed patients. In addition, abnormal weight gain, appetite, energy, and locomotor behavior are symptoms that can be associated with depressive-like behavior in rodents [52]. Also, AAS use in supraphysiological doses has been associated with mood disorders [11], and previous studies have identified that AAS-dependent users suffer from higher risk of developing major depression compared to non-users [8,10] and that AAS can cause a depressive-like behavior in rodents [18,58–61]. Although, depression among human AAS users usually arises as a withdrawal symptom [1] when ending a steroid cycle, it is still intriguing to speculate whether it is a depressive-like behavior identified in the nandrolone decanoate treated rats in the present study. Modeling human neuropsychiatric disorders, like depression, in rodents is difficult and the use of tests allowing multidimensional strategies are warranted. The MCSF test allows the animal to express a more comprehensive behavioral repertoire and has previously been used for experimental depression research [62]. In a previous study, the Flinders Sensitive Line rats (FSL; an animal model of depression) were examined and demonstrated altered behavior in descriptive parameters associated with general activity and exploratory behavior (distance moved in center, velocity and duration in corridors, and time per visit in hurdle) [62]. Rats treated with nandrolone decanoate displayed similar behavior in the MCSF as descriptive parameters associated with the same type of functional behaviors were affected (distance moved and velocity in the arena and center, total activity, number of visits and duration in center and corridors). However, the FSL rats also have higher risk assessment, something we were not able to identify in nandrolone decanoate treated rats. Whether the AAS-induced changes in behavior demonstrated in the present study are caused by neuroadaptive alterations in the brain, by exacerbating stress vulnerability, or by a combination of both is open for speculation.

There is a higher prevalence of psychiatric symptoms among AAS-users compared to non-users [63,64]. Although, the reason is not fully elucidated, several factors such as concomitant drug use, pre-existing psychiatric disorders, and family background have been suggested to be involved [65]. The present study was able to identify differences in behavioral profiles and brain gene expression which indicates that the investigated substances, especially nandrolone decanoate, have a direct
or alternatively indirect impact on the brain and behavior of male Wistar rats. Nevertheless, it is possible that the psychological adverse effects are enhanced in humans due to additional factors such as polydrug use, psychiatric pathology, medical and social background. In addition, AAS-users commonly combine several different types of AAS in order to receive optimal results. For example, it is common that depot steroids, such as nandrolone decanoate, are combined with orally administered AAS [66] which probably also contribute to more severe side-effects. To reduce unwanted effects the AAS administration pattern usually also occurs in cycles. The AAS users gradually increase the steroid amounts administered and thereafter gradually decrease the administration. This is followed by a wash-out period without AAS before the next cycle starts [66,67]. During this period of time several withdrawal symptoms may occur, including depression as previously described, and AAS-users experiencing psychological adverse effects often report these to occur immediately after discontinuation of AAS [64]. It would be interesting to mimic this administration pattern, but there are practical difficulties and extensive change of study design needed.

We believe the present study provides important information on differences in behavioral and biochemical effects of structurally diverse AAS, which is important in order to improve the limited understanding of such adverse effects. However, we would like to highlight some potential limitations of the findings. Firstly, there were relatively large differences within the experimental groups, especially considering the effects on behavior, which could be explained by individual variation between the animals. In humans, the psychiatric symptoms caused by AAS seem to be idiosyncratic, as steroid users may have an individual response to AAS usage [1]. Therefore, it is possible that susceptible individuals develop changes in the brain and behavior to a greater extent when exposed to AAS. Also, AAS users with a personality disorder report more aggressive feelings, suicidal thoughts, and criminality compared to those without a personality disorder [65]. It is possible that dependent on the behavioral profile of the animal it might respond differently to the specific AAS treatment resulting in diverse behavior in the MCSF. If this hypothesis is true, it is not characterizing the behavioral profile before treatment could explain the large variance within the experimental groups. Secondly, only male rats were investigated in the study. Although young adult males are the typical AAS user [68], AAS-use occur among women as well but to a lesser extent. Interestingly, a previous study has however indicated that women experience even more severe side-effects compared to male users [69]. Therefore, AAS-studies including females are warranted. Thirdly, the MCSF is validated with regard to recognition of risky as opposed to safe areas [25], and to the best of our knowledge not specifically for psychiatric disorders, like depression. Therefore, it would be interesting to study the nandrolone-induced depressive-like behavior in a traditional behavioral test investigating depression, possibly during a wash-out period. Fourthly, the use of different esters (decanoate and undecanoate) may affect the results as previous study demonstrates that shorter esters are followed by a wash-out period without AAS before the next cycle starts [64,67]. During this period of time several withdrawal symptoms may occur, including depression as previously described, and AAS-users experiencing psychological adverse effects often report these to occur immediately after discontinuation of AAS [64]. It would be interesting to mimic this administration pattern, but there are practical difficulties and extensive change of study design needed.

In conclusion, the present study identified nandrolone decanoate to suppress body weight gain, general activity in the MCSF test, and alter stress hormone levels in male Wistar rats. The same effect could not be identified in rats treated with testosterone undecanoate. These results, together with the changed expression of genes found in the hypothalamus, may imply a depressive-like-behavior to be caused by nandrolone decanoate. We therefore conclude that nandrolone decanoate alters the behavioral profile of male rats, but also has a larger impact on physiological effects compared to testosterone undecanoate.

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CRediT authorship contribution statement

Sofia Zelleroth: Conceptualization, Investigation, Formal analysis, Writing – original draft, Visualization. Erik Nylander: Investigation.
Ellinor Kjellgren: Investigation. Alfhild Gronbladh: Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition. Mathias Hallberg: Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Conflict of interest
The authors display no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2022.113971.

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