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Anabolic androgenic steroids and central monoaminergic systems

Supratherapeutic doses of nandrolone decanoate affect dopamine and serotonin

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Abstract
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Supratherapeutic doses of anabolic androgenic steroids (AASs) are administered, not only as performance-enhancing drugs in the world of sports, but also in order to modify behaviour. AAS abusers are at risk of developing serious physical and psychological side effects such as dependence and aggressive behaviour. The aim of this thesis was to investigate the impact of supratherapeutic doses of nandrolone decanoate after subchronic administration on dopamine and serotonin pathways involved in drug dependence and aggression, in the male rat brain.

Adult male Sprague-Dawley rats received intramuscular injections of nandrolone decanoate (3 or 15 mg/kg) or vehicle once daily for 14 days. Nandrolone decanoate pre-exposure abolished the effect of amphetamine on the 3,4-dihydroxyphenylacetic acid (DOPAC) tissue level in the hypothalamus and on the DOPAC/dopamine ratio in the hypothalamus and the hippocampus. A significant decrease of the basal extracellular DOPAC and homovanillic acid (HVA) levels could be detected in the nucleus accumbens, which remained low during the first hour following the amphetamine challenge. Nandrolone decanoate significantly reduced the activity of both monoamine oxidase A and B (MAO-A and -B) in the caudate putamen and amygdala. The gene transcript levels of MAO-B, and the dopamine D1 and D4 receptors were altered in limbic regions. No changes in transcriptional levels could be detected among the serotonin receptor genes examined. However, the density of the serotonin transporter protein was elevated in a range of aggression-related brain regions.

Taken together, subchronic administration of nandrolone decanoate causes dopaminergic and serotonergic dysregulations in distinct brain regions. These areas of the brain are involved in the development of drug dependence and expression of impulsive and aggressive behaviours. These results may contribute to explain some of the behavioural changes often reported in AAS abusers, such as polydrug use and impaired impulse control.

Keywords: Pharmacology, anabolic androgenic steroids, nandrolone decanoate, dopamine, serotonin, rat, central nervous system

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List of papers

This doctoral thesis is based on the following original papers, in the text referred to by their Roman numerals (I-V). Papers II and III are reprinted with permission from Elsevier Ltd.

I Birgner, C., Kindlundh-Högberg, A.M.S., Ploj, K., Lindblom, J., Nyberg, F., Bergström, L. Effects on rat brain dopamine and DOPAC levels after sub-chronic nandrolone administration followed by an amphetamine challenge.
Pharmacologyonline 3, 99-108 (2006)

II Birgner, C., Kindlundh-Högberg, A.M.S., Nyberg, F., Bergström, L. Altered extracellular levels of DOPAC and HVA in the rat nucleus accumbens shell in response to sub-chronic nandrolone administration and a subsequent amphetamine challenge.
Neuroscience Letters 412, 168-172 (2007)

III Birgner, C, Kindlundh-Högberg, A.M.S., Oreland, L., Alsiö, J., Lindblom, J., Schiöth, H., Bergström, L. Reduced activity of monoamine oxidase in the rat brain following repeated nandrolone decanoate administration.
Brain Research 1219, 103-110 (2008)

IV Birgner, C, Kindlundh-Högberg, A.M.S., Alsiö, J., Lindblom, J., Schiöth, H., Bergström, L. The anabolic androgenic steroid nandrolone decanoate affects mRNA expression of dopaminergic but not serotonergic receptors.
In progress

V Birgner, C, Kindlundh-Högberg, A.M.S., Bergström, L. Nandrolone decanoate increases serotonin transporter density in rat brain.
In manuscript

Carolina Birgner's contribution to the papers:

Participated in the planning of the experiments together with the last author (Papers I, II, III and V), and fourth and last authors (Paper IV). Performed all the laboratory work and analyses in Paper II and the major part of the laboratory work and analyses in Papers I, III and IV. Performed image analysis in Paper V. The evaluation of results and writing the manuscripts was mainly performed by the first, second and last authors (Papers I and II), first, second and third authors (Paper III), and first author (Papers IV and V).

Contents

Abbreviations

5-HIAA	5-hydroxyindole acetic acid
$5HT_x$	Serotonin receptor, subtype x
AAAD	Aromatic amino acid decarboxylase
AAS	Anabolic androgenic steroid
ACT	β-actin
AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
CCP	Conditioned place preference
CYCLO	Cyclophilin
D_x	Dopamine receptor, subtype x
DAT	Dopamine transporter
DDR	Deutsche Demokratische Rebublik
DHT	Dihydrotestosterone
DOPA	Dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenyl acetic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, fourth edition
EDTA	Ethylenedinitrilotetraacetic acid disodium salt dihydrate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GOI	Gene of interest
H3b	Histone H3b
HCG	Human chorionic gonadotropin
HKG	Housekeeping gene
HVA	Homovanillic acid
ICD-10	International Statistical Classification of Diseases and Related Health Problems
i.c.v.	Intracerebroventricular
i.m.	Intramuscular
i.v.	Intravenous
i.p.	Intraperitoneal
LTD	Long-term depression
LTP	Long-term potentiation
MAO-A	Monoamine oxidase A

MAO-B	Monoamine oxidase B
MDMA	Methylenedioxymethamphetamine
PET	Positron emission tomography
RPL19	Ribosomal protein L19
s.c.	Subcutaneous
SDCA	Succinate dehydrogenase complex A subunit
SERT	Serotonin transporter
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
TUB	β-tubulin beta 5
VAS	Visual analogue scale
VMAT2	Vesicular monoamine transporter 2

Introduction

Anabolic androgenic steroids (AASs)

Anabolic androgenic steroids (AASs) are defined as synthetic derivatives of the endogenous sex hormone testosterone. Testosterone is the primary male sex hormone, mainly synthesized in the testicles of men and to some extent in the ovaries of females. Testosterone is responsible for sexual differentiation *in utero* and the development of secondary sexual characteristics during male adolescence. In the adult male body, testosterone is required for maintenance of sexual function and fertility [97, 183, 228, 266]. Androgens exert many of their effects through binding of androgen receptors (ARs). The receptor-androgen complex is classically translocated to the cell nucleus functioning as a transcription factor [183, 210]. Recently, evidence of non-genomic ARs functioning outside the nucleus, have emerged. These receptors most likely mediate some of the rapid effects of androgens [221]. AASs were originally developed to have maximal anabolic and minimal androgenic effects. However, so far no anabolic steroid is entirely devoid of androgenic properties. The AAS used in the studies included in this thesis is nandrolone decanoate, which is dissolved in sterile arachis oil and intramuscularly (i.m.) injected as a depot.

History

In 1889, Charles E. Brown-Séquard subcutaneously (s.c.) injected an extract from dog and guinea pig testicles, and described it as a "rejuvenating elixir" which could e.g. increase his strength and intellectual energy [28]. Today, his experiences are considered the result of placebo effect. However, 20 mg of testosterone was indeed isolated in 1927 by F. C. Koch and L. McGee from 40 pounds of bovine testicles. They could also show that this substance could restore masculine characteristics in several species after castration [132]. In 1935, testosterone had been isolated from human testicle and chemically characterized, work contributed by E. Laqueur and A. Butenandt, among others. The same year, testosterone was first synthesized by two groups independently, and this earned the group leaders A. Butenandt and L. Ruzicka the joint Nobel Prize in Chemistry in 1939 [104].

In the late 1930s, C. Kochakian and other researchers demonstrated both the anabolic and androgenic effects of testosterone. This hormone and syn-

thetic derivatives have been used clinically to treat e.g. hypogonadism, anaemia and patients suffering from protein deficiency due to severe burns or extensive surgery [10, 228]. The use of AAS in clinical medicine has declined during the years and been replaced by other therapies for the disorders formerly treated with testosterone and its derivatives. However, the anabolic effects of AASs made these substances attractive outside the clinic and the first reports of athletes using AASs for non-medical reasons appeared in the literature during the 1950s [104]. Sports organizations have banned these substances and perform tests regularly but the Olympic Games and other large sports tournaments still meet with doping scandals. After the fall of the "Iron Curtain", it was even revealed that the Deutsche Demokratische Republik (DDR) treated thousands of athletes with androgens and other doping agents to increase athletic performance, a program supported and financed by the DDR government during the 1960s, 70s and 80s [77].

Abuse of AASs

Apart from elite athletes aiming at increasing performance during competition, both professional and amateur bodybuilders administer AASs to gain maximal muscle volume. It has also become apparent through survey studies that AASs are used as recreational drugs, primarily by adolescents, to become bold, boost self-esteem, or just to become intoxicated [123, 124, 188]. Abuse of AAS among adolescents is also associated with heavy alcohol consumption and use of other illicit drugs [4, 83, 117, 124, 187, 188, 201, 248]. There are also numerous reports of AAS abusers involved in criminal activity and violent acts [130, 131, 201, 248].

The amounts of AASs administered by abusers have been reported to widely exceed those used in clinical medicine. Reports range from 10 to 100 times more [269], and in a case study of seven AAS abusers who where followed during a year, nandrolone decanoate doses ranged from 5 mg/kg/day in the beginner to 19 mg/kg/day in the experienced heavy user, in cycles of typically 8 weeks [83]. In a survey of 500 AAS abusers, 60% of participants administered more than 1000 mg of testosterone, or its equivalent, per week [197]. To optimize effect and avoid side-effects due to cessation of endogenous testosterone production, different dose regimens are applied, e.g. "stacking" (mixing and alternating between different steroid compounds and routs of administration), "pyramiding" (gradually increasing steroid dose in early phase of the cycle and gradually decreasing it in the end) and administration of additional substances (growth hormone, insulin, ephedrine, clenbuterol etc.) [65, 66, 197]. AAS abusers are at risk of several physical side effects, such as cardiovascular disease [252], alterations of lipoprotein levels, testicular atrophy and gynecomastia [203]. Additionally, women might experience deepening of the voice, hirsutism, amenorrhea and clitoral hypertrophy [210, 228]. However, the scope of this thesis is neurochemical altera-

tions possibly underlying some of the psychiatric/behavioural side effects reported in AAS abusers.

Behavioural effects of supratherapeutic doses of AASs

AASs and aggression

Abusers of AASs experience increased aggression and irritability [7] and are reported to engage in criminal activity, violent acts [201, 248], and even homicide [48]. There have been several attempts to demonstrate a correlation between AAS administration and human aggression through controlled studies on male volunteers. After 40 mg of methyltestosterone daily for 3 days followed by 240 mg/day for 3 days, test objects showed increased energy, libido and aggressiveness whereas cognitive symptoms were impaired, assessed by the visual analogue scale (VAS) [51, 52]. Pope and co-workers conducted a test of aggressive responding in a controlled laboratory setting on male volunteers. Male subjects received increasing doses of testosterone cypionate (150 mg/week for 2 weeks, 300 mg/week for 2 weeks and finally 600 mg/week for 2 weeks), or placebo in a double-blind crossover design. Aggressive and non-aggressive responses were assessed as the test persons played a computer game with a fictive opponent under the impression that motor response would be investigated. One could either gain 100 points, exchangeable for money, by pressing button A or subtract 10 points from the opponent by pressing button B, but then without personal gain. Testosterone administration significantly increased the number of aggressive responses (~130%) whereas non-aggressive remained unchanged [136, 204]. On the other hand, in two studies were 200 mg of testosterone enanthate was administered weekly for 8 and 20 weeks, respectively, no mood changes were found. Behaviour was recorded in diaries and with self-rating scales [1, 6]. 200 mg of testosterone weekly corresponds to the dose used in clinical trials for male contraception and does not come close to the doses abused. Due to ethical considerations, AASs in doses abused cannot be allowed for administration to human volunteers. Therefore, the controlled studies presented above can never be entirely representative.

However, in animal studies AAS doses corresponding to those abused have been evaluated with regard to aggressive behaviour. Several studies performed on male Long-Evans rats have demonstrated increased intermale aggression after 12 weeks of testosterone propionate administration [49, 72, 73]. However, nandrolone decanoate had no effect on aggression and stanozolol even reduced such behaviour [24, 169, 263]. The same results were obtained after AAS withdrawal [170] and with castrated animals [43]. On the other hand, nandrolone decanoate caused heightened aggressive response in male Sprague-Dawley rats after both 2 mg/day and 20 mg/week for 4 weeks. Except for rat strain, the test conditions differed from the before

mentioned in the sense that the animals were experienced with the resident-intruder paradigm before AAS administration [156].

With mice, test results have been diverse, once again likely to depend on strain and test conditions. A recent study by Pinna *et al.* have demonstrated isolation-induced aggression in male mice to be dependent on brain testosterone and allopregnanolone content [202]. However, in studies by Martinez-Sanchis *et al.* and Bonson *et al*., female mice have been more prone to react with aggression after AAS administration than male mice, and only after prolonged exposure (10 weeks) have males displayed isolation-induced aggression [25, 26, 165, 166].

The only species tested for AAS induced aggression showing unambiguous results are adolescent Syrian hamsters. Adolescent hamsters receiving a cocktail of AAS (2 mg/kg/day of testosterone cypionate, 2 mg/kg/day of nandrolone decanoate and 1 mg/kg/day of boldenone undecylenate) increased number of attacks and decreased latency to attack in the resident-intruder paradigm after both 14 [57, 92, 220], 28 [92] and 30 days of administration [76, 90, 91, 93, 94, 175, 212, 213]. Additionally, this persists after 4 and 11 days of withdrawal, but is no longer present after 18 or 25 days after cessation of the 30-day administration period [94]. In the one study reporting effects of AAS administration on *adult* hamsters, no effects were observed [220].

AASs and reward

It has been suggested that AAS abuse can lead to dependence in humans [159], and even function as a gateway to abuse of other substances [4, 117]. Approximately 25% of examined AAS abusers fulfil the DSM-IV criteria for steroid dependence [47, 203]. Hypomania is a common symptom in early phases of AAS abuse and depression often occurs during withdrawal, leading to resumed AAS intake [203].

Androgen induced reward has been studied in experimental animal models, where conditioned place preference (CPP) and self-administration are the most important methodologies. CPP has been produced by 0.25 and 0.5 μg of testosterone injected directly into the nucleus accumbens [195], and 0.8 mg/kg of testosterone administered intraperitoneally (i.p.) to male Long-Evans rats [196, 224]. In the two latter studies, CPP could be blocked by the mixed dopamine D1/D2 receptor antagonist flupenthixol, administered both systemically [224] and directly into the nucleus accumbens [196]. I.p. injections of the specific dopamine D1 antagonist SCH23390 or D2 antagonist sulpiride could also block testosterone induced CPP, indicating involvement of both dopamine D1 and D2 receptors [224]. However, unilateral injections of testosterone into the medial preoptic area of Long-Evans rats, produced CPP at the dose 0.1 μg, but conditioned place *aversion* at 0.2 μg [129]. When administered s.c. to male Long-Evans rats, 1 mg/kg of testosterone failed to produce CPP, whereas its metabolites dihydrotestosterone (DHT)

and 3α-androstanediol did [81]. However, when implanted bilaterally into the nucleus accumbens shell, all three substances produced CPP [82]. Based on these results, the temporal onset of CPP for the individual substances, and brain as well as plasma levels, the authors conclude that the testosterone metabolite and neurosteroid 3α-androstanediol is responsible for the CPP [80-82, 216]. Testosterone administerd s.c. to mice have also been shown to produce CPP at doses ranging from 0.8-2 mg/kg [2, 3].

In self-administration paradigms, male adult Syrian hamsters have been shown to prefer up to 800 μg/ml of testosterone in an oral solution, over vehicle, in 2-bottle choice tests [80, 116]. Both Sprague-Dawley rats and Syrian hamsters self-administer testosterone (50 μg/nose poke) intravenously (i.v.) [265]. Adult Syrian hamsters have also been tested for intracerebroventricular (i.c.v.) self-administration. Number of nose pokes for testosterone [60, 265], drostanolone and nandrolone (0.1-2 μg/nose poke) significantly increased [8], as well as for DHT and estradiol, an estrogen metabolite of testosterone [61]. The oral steroid compounds oxymetholone and stanozolol were, however, not self-administered i.c.v. by hamsters [8]. In a study by Peters and Wood, hamsters were allowed to self-administer testosterone i.c.v. for up to 56 days. 24% of the animals infused testosterone to the point of death, which correlated with peak daily intake (>60 μg/day) [200]. To summarize, AASs are self-administered by rodents, although the effect is modest. Still, it is comparable with other mild reinforcers such as benzodiazepines [185] and nicotine [164].

Pre-exposure to AASs has been demonstrated to affect the response to other substances of abuse in experimental animals. Nandrolone decanoate administered at a dose of 15 mg/kg (i.m.) to CD-1 mice daily for 14 days, blocked food-, tetrahydrocannabinol (THC)- and morphine-induced CPP. Nandrolone decanoate also heightened THC- and morphine-withdrawal [36, 37]. Male adult Wistar rats receiving the same dose of nandrolone decanoate s.c., increased their voluntary intake of alcohol the second and third week after cessation of AAS administration, exceeding 0.5 mg/kg/day [114]. It was also demonstrated that pre-exposure to nandrolone decanaote prevented alcohol-induced decrease in locomotion when ethanol was injected i.p. at the sedentary dose 0.5 mg/kg [152].

Neurochemical effects of supratherapeutic doses of AASs

Neurochemical alterations after AAS administration has been examined in several species, covering many transmitter systems, such as excitatory [143, 144, 217] and inhibitory [17, 93, 171, 199, 267, 268] amino acids, various peptides [99, 100, 102, 113-115, 160, 161], and the focus of this thesis; monoamines.

AASs and dopamine

The impact of AASs on the dopaminergic systems has been studied to some extent in the rodent brain. 14 days of i.m. nandrolone decanoate administration at 15 mg/kg gave rise to decreased density of dopamine D1-like receptor protein in the caudate putamen and nucleus accumbens core and shell [126]. Similar results were later obtained for mRNA levels [127]. Dopamine D2-like receptor densities, on the other hand, were upregulated in the caudate putamen, ventral tegmental area and nucleus accumbens core, while down-regulated in the shell [126]. Alterations in mRNA levels for the dopamine D2 receptor was heading the same direction as the protein in the caudate putamen, however at lower doses only (1 and 5 mg/kg). 1 and 5 mg/kg also *increased* the mRNA tissue content in the nucleus accumbens shell [127]. The increased transcription of the D2 receptor might be a compensation for the decreased protein expression. Alternatively, the discrepancies between mRNA and protein levels are due to posttranslational processing. No changes in gene expression for the dopamine synthesizing enzymes tyrosine hydroxylase (TH) or aromatic amino acid decarboxylase (AAAD) were detected [127]. All three doses 1, 5 and 15 mg/kg of nandrolone decanoate daily for 14 days significantly increased the protein density of the dopamine transporter (DAT) in the caudate putamen [128], later confirmed for the higher dose by positron emission tomography (PET) in live animals [122].

The tissue content of dopamine and its metabolites has been examined in the striatum of male rats receiving 5 mg/kg either of testosterone, nandrolone, methandrostenolone or oxymetholone, s.c. once weekly for 6 weeks. Dopamine increased in the oxymetholone group, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) increased in all treatment groups, as did the (DOPAC+HVA)/dopamine ratio apart from the group receiving oxymetholone. The latter was however the only substance which affected monoamine oxidase (MAO) activity, causing an increase of MAO-A activity in the hypothalamus [247]. Nandrolone decanoate at the doses 5 and 20 mg/kg, 5 days a week for 2 weeks, increased DOPAC in the cerebral cortex and the lower dose increased the DOPAC/dopamine ratio in the hypothalamus of Wistar rats [139], whereas Lindquist *et al.* reported no dopaminergic changes after 15 mg/kg of nandrolone decanoate daily for 14 days [152].

AASs and serotonin

Studies on serotonin and 5-hydroxyindole acetic acid (5-HIAA) tissue content have reported diverse results, likely due to differences in dosing regimens and choice of animal model. 5 mg/kg/week for 6 weeks of oxymetholone increased serotonin, 5-HIAA and the 5-HIAA/serotonin ratio in the hippocampus. The ratio was also elevated by testosterone, nandrolone and methandrosteneolone in the hippocampus. Additionally, modest but

significant effects were observed in the frontal cortex and hypothalamus. As mentioned above, this dose regimen increased hypothalamic MAO-A activity in the oxymetholone group [247]. With the doses 5 and 20 mg/kg of nandrolone decanoate (s.c.) administered for 14 days to male Wistar rats, Kurling *et al.* also observed an increased 5-HIAA level in the frontal cortex by 5 mg/kg. Additionally, serotonin tissue content in the cerebral cortex and hypothalamus was elevated in the group receiving 20 mg/kg, however decreased it in the hippocampus [139]. In a similar study, Lindquist *et al.* demonstrated decreased levels of serotonin in the forebrain and dorsal striatum, and 5-HIAA in the striatum by 15 mg/kg of nandrolone decanoate, but no alterations in the hippocampus [152]. A single dose of nandrolone decanoate (3.75 mg/kg) increased 5-HIAA and the 5-HIAA/serotonin ratio in the hypothalamus, whereas 0.375 and 37.5 mg/kg did not [246].

The impact of chronic AAS exposure on serotonin receptors has been investigated in both rats and hamsters. 30 days of daily administration with a steroid cocktail consisting of 2 mg/kg testosterone cypionate, 2 mg/kg nandrolone and 1 mg/kg boldenone undecylate (s.c.), decreased the density of both serotonin 5HT1A [213] and 5HT1B receptors in the anterior hypothalamus [91]. 5HT1B was also reduced in the ventrolateral hypothalamus, and central and medial amygdala [91]. The same dosing regimen also decreased the density of serotonin fibres in the medial and central amygdala, as well as in the ventrolateral and anterior hypothalamus of male adolescent hamsters [90]. In the anterior hypothalamus, this reduction could be detected already after 7 days of AAS administration [92]. In rats receiving 1, 5 or 15 mg/kg of nandrolone decanoate daily for 14 days, a reduced density of 5HT1B was observed in the medial globus pallidus and field CA1 of the hippocampus, as well as a downregulation of 5HT2 in the frontal association cortex and amygdala. 5HT2 was however increased in the nucleus accumbens shell and ventromedial hypothalamus [125].

Dopamine

The dopamine system

Dopaminergic cellbodies originate in the ventral tegmental area, substantia nigra and hypothalamus. The ventral tegmental area largely innervates the nucleus accumbens, lateral septum and prefrontal cortex, and other cortical areas such as the cingulate and piriform cortices. The amygdala, bed nucleus stria terminalis, hippocampus and olfactory tubercle are also targeted by the ventral tegmental area. The cortical projections are termed the mesocortical dopamine system and the projections targeting other limbic areas the mesolimbic dopamine system. The substantia nigra projects to the striatum and is thus called the nigrostriatal dopamine system, and finally, there are

short projections from the hypothalamus to the pituitary gland (tuberohypophyseal dopamine system) [18], where dopamine modulates prolactin release [78].

Dopamine is synthesized from tyrosine by the rate-limiting enzyme TH, forming the intermediate dihydroxyphenylalanine (DOPA), which is subsequently converted to dopamine by the enzyme AAAD [45]. Newly synthesized dopamine is transported into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2), and stored in these vesicles awaiting release [45, 105]. When released into the synaptic cleft, dopamine homeostasis is maintained through several mechanisms, one being uptake by the plasma-membrane bound DAT [186]. MAO metabolizes cytosolic dopamine into DOPAC, which is the main metabolite in rat. MAO-A is the main dopamine-degrading enzyme in rat, whereas MAO-B dominates in man [45, 193]. In the extracellular space, dopamine is metabolized by catechol-*O*-methyltransferase (COMT) into HVA, which is the major metabolite in humans [45].

Dopamine exerts its actions via two classes of G-protein coupled receptors: D1-like and D2-like. The former consists of the dopamine D1 [56, 181, 244, 272] and D5 [88, 243, 250, 262] receptors that are situated postsynaptically to dopamine neurons. D1 and D5 are G_s-coupled and increase cAMP formation and DARPP-32 phosphorylation upon stimulation. The affinity for dopamine is in the micromolar range [45]. The dopamine D1-like receptors are distributed throughout the mesocorticolimbic and nigrostriatal terminal areas, subtype D1 being the dominating one [23, 56, 62, 79, 174, 223, 250, 261]. The D2-like receptors mainly function as autoreceptors, slowing firing rate and inhibiting further dopamine synthesis and release, depending on receptor subtype and location [45]. Three distinct receptor subtypes have been cloned, namely the dopamine D2 [29, 42, 50, 86, 87, 182, 211, 241], D3 [239] and D4 [255] receptors. As opposed to the dopamine D1-like receptors, D2-like are G_i-coupled, and thereby decrease cAMP formation. These receptors also have higher affinity for dopamine than D1 and D5, and can be found on the soma, dendrites and terminals of dopamine neurons [45]. Dopamine D2-like receptors are also heteroreceptors, i.e. localized at axon terminals of non-dopaminergic neurons, postsynaptic to the dopamine neuron. The D2 subtype is widely distributed throughout the dopamine system, present in both origin and terminal areas [20, 23, 39, 145, 163, 173, 261]. D3 [21, 239] and D4 [255] have more restricted distributions.

Dopamine and development of dependence

The American Psychiatric Association and the World Health Organization have outlined diagnostic criteria for substance dependence, entitled Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) and International Statistical Classification of Diseases and Related Health Prob-

lems (ICD-10), respectively. According to these protocols, substance dependence is characterized by at least three of the following criteria being fulfilled in the last 12 months: tolerance, withdrawal symptoms, inability to cease drug consumption even for short periods of time, neglect of social and occupational activities, substantial time spent on obtaining, using and recovering from the drug, and compulsive drug intake despite harmful effects [135].

The role of dopamine in the development of dependence is heavily debated in the literature, and several hypotheses have been presented. Dopamine has received much attention since many drugs of abuse have the ability to directly or indirectly increase the release of dopamine in, primarily, the nucleus accumbens. Examples of such substances are amphetamine, cocaine and ethanol [11, 30, 31, 34, 59, 209, 229]. Initially dopamine was hypothesized to solely mediate the hedonia (pleasure) when consuming the drug, or alternatively, that dopamine had the ability to relieve withdrawal symptoms (negative reinforcement) [264]. It soon came apparent that the simplicity of these explanations would not last. Based on both behavioural and pharmacological studies, other theories of dopamine's role in the development of dependence have been presented since [14, 120]. These include associative reward-learning [58, 109], incentive salience, postulating that dopamine is necessary for "wanting", but not "liking" the drug [214], and reward prediction [225].

However, the neurobiological basis for the development and behavioural manifestation of drug dependence is complex, and involves several neurocircuitries and transmitters [119, 120, 134]. These neurocircuitries are modulated by monoamines, opioid peptides, amino acids and corticotropin-releasing factor [135]. The extended amygdala is involved in positive and negative reinforcement [133]. The prefrontal cortex and basolateral amygdala is important for craving [133, 134] and the striatal-pallidal-thalamic loop for the compulsive component of substance dependence [112]. Memory processing and associative learning involves the hippocampus [119]. The hypothalamic-pituitary-adrenal axis is important for stress-induced drug intake and relapse [138].

Serotonin

The serotonin system

Serotonin is widely distributed throughout the central nervous system. Serotonergic cell bodies can be found in the spinal cord, brain stem and raphe nuclei. From the raphe, neurons project to limbic areas, the neostriatum, cerebral cortex and cerebellum [46, 110, 149, 184, 240].

Serotonin is derived from dietary tryptophan, which is converted into 5-hydroxytryptophan by tryptophan hydroxylase (TPH) [260, 271] and sequentially converted to serotonin by AAAD [46]. Like dopamine, newly synthesized serotonin is transported into synaptic vesicles by VMAT2 and is stored in, and released from these vesicles [46, 105]. When released, serotonin homeostasis is maintained by reuptake into the terminal by the plasma membrane bound carrier SERT (serotonin transporter) [46, 186]. Cytosolic serotonin is metabolized by MAO-A to 5-HIAA [46, 259].

Seven families of serotonin receptors have been identified (5HT1-7) and several of these are further subdivided [9]. In general, 5HT1 receptors are negatively coupled to adenylate cyclase and are considered autoreceptors. 5HT1A is found on somas and dentrites in the raphe nuclei, regulating firing rate, whereas the release modifing 5HT1B receptor is situated presynaptically in e.g. the substantia nigra. 5HT1 receptors are also heteroreceptors, i.e. localized on axon terminals of non-serotonergic neurons, postsynaptic to the serotonin releasing neuron [9, 273]. 5HT2-7 receptors, on the other hand, are postsynaptic receptors. 5HT2A can be found in high quantity in cortical and limbic areas, as can the 5HT2C receptor, which is also abundant in the basal ganglia [9]. The 5HT3 receptor differs from the others by being the only ligand gated ion-channel, as opposed to the other families that are G-protein coupled. It is highly expressed in the brain stem but can also be detected in e.g. the hippocampus and amygdala [9]. 5HT4 can be found in the nigrostriatal and limbic systems [9, 95]. 5HT5 receptors are the least characterized, but seem to be widespread within the central nervous system [9, 67, 167]. The 5HT6 receptor has been detected in the limbic system [9, 218] and 5HT7 expression is restricted to thalamic, hypothalamic and hippocampal areas [9, 231]. 5HT4-7 have only recently been cloned and characterized. Their functional significance remains to be evaluated in detail; however, it is presumed that they are G_s-coupled, postsynaptic receptors [9, 46].

Serotonin and aggression

It is well known that serotonin is a potent modulator of mammalian aggressive behaviours and serotonin deficiency has so far been hypothesized to underlie aggressive and impulsive behaviours [27, 85, 153, 162, 191]. This hypothesis was originally based on the findings that serotonin depletion by means of neurotoxins increase aggressive responses [257, 258] and that CSF levels of 5-HIAA were reduced in aggressive and violent humans [27, 85, 153, 162]. Serotonin reuptake inhibitors suppress aggression in several species (including humans) [41, 233] and SERT knockout mice display reduced aggression in behavioural paradigms [106, 108], together with markedly elevated brain tissue levels of serotonin [107, 121].

The serotonin deficiency hypothesis has recently been challenged, though. Based on new pharmacological and molecular evidence this hypothesis has

to be partly revised. It is instead proposed that there is a positive correlation between normal offensive aggression and serotonin, whereas there is an inverse correlation between pathological violent aggression and serotonin activity [55, 192]. MAO-A knockout mice, for instance, display enhanced aggression together with *increased* brain levels of serotonin and marked decreases of monoamine metabolite levels [35, 69, 205, 232]. These effects are accompanied by a lower density of 5HT2A receptors [35], a dysfunction suggested to underlie impulsive behaviour in humans [189]. 5HT1A agonists attenuate aggressive responses in several species [54, 176, 178, 179], and animals bred for aggressive behaviour have reduced levels of the 5HT1A receptor in the central nervous system [206]. 5HT1B knockout mice are more aggressive than wildtype mice [22, 222] and 5HT1B agonists have been shown to reduce aggression and impulsivity in rodents [191, 192].

Aims

It has been hypothesized that abuse of supratherapeutic doses of AAS can lead to dependence and function as a gateway to abuse of other illicit drugs. This is supported by behavioural studies on animal models and psychiatric evaluations of human subjects. Alteration of dopamine function is indicated as a possible neurochemical basis for these behavioural effects. Additionally, AAS abusers are often reported to display increased irritability and aggression, and links between serotonin, androgens and aggression have been presented in the literature. However, the effects of supratherapeutic doses of AAS on the dopamine and serotonin systems are not yet fully evaluated. The specific aims of this thesis are to investigate:

- Dopamine and DOPAC tissue levels in brain areas primarily involved in drug dependence, after subchronic administration of nandrolone decanoate (15 mg/kg) to adult male Sprague-Dawley rats (Paper I).
- The basal extracellular levels of dopamine, DOPAC and HVA in the nucleus accumbens, after subchronic administration of nandrolone decanoate (15 mg/kg) to adult male Sprague-Dawley rats (Paper II).
- The influence of nandrolone decanoate pre-exposure (15 mg/kg) on amphetamine-induced dopamine tissue levels and dopamine release in the nucleus accumbens of adult male Sprague-Dawley rats (Papers I and II).
- The enzymatic activities of MAO-A and MAO-B in brain areas primarily involved in drug dependence and aggressive behaviour, after subchronic administration of nandrolone decanoate (3 or 15 mg/kg) to adult male Sprague-Dawley rats (Paper III).
- mRNA levels corresponding to dopamine synthesizing and metabolizing enzymes, and dopamine transporter proteins (plasma membrane bound and vesicular) in brain areas primarily involved in drug dependence, after subchronic administration of nandrolone decanoate (3 or 15 mg/kg) to adult male Sprague-Dawley rats (Papers III and IV)
- mRNA levels corresponding to dopaminergic and serotonergic receptors in brain areas primarily involved in drug dependence and aggressive behaviour, after subchronic administration of nandro-

lone decanoate (3 or 15 mg/kg) to adult male Sprague-Dawley rats (Paper IV).

- The protein density of SERT in brain areas primarily involved in aggressive behaviour, after subchronic administration of nandrolone decanoate (3 or 15 mg/kg) to adult male Sprague-Dawley rats (Paper V).

Methods

Below, general descriptions of the methods used in this thesis are given. For details on equipment, manufacturers and retailers, the author refers to the original papers. The same applies for statistical considerations and methods.

The dose

The differences, regarding experimental design, between existing studies of neurochemical changes after supratherapeutic AAS exposure, constitute type of steroid, dose (1-40 mg/kg), dose interval (1-7 days), duration of administration (1 day-6 weeks), route of administration (i.m. or s.c.) and time from last injection to euthanasia (24-72 h). We chose the doses 3 and 15 mg/kg nandrolone decanaote administered i.m. once daily for 14 days, corresponding to one cycle of use during early and experienced AAS abuse respectively, based on a one year follow-up study and a survey study of 500 AAS abusers [83, 197]. Nandrolone decanoate has a half-life of 5.4 days in rat and 6 days in human when administered as an i.m. depot [253]. A single injection of 20 mg/kg of nandrolone decanoate to rats, gave rise to a plasma level of 2.6 µg/l after 4 days and nandrolone was still detectable in plasma 16 days after administration (1.3 µg/l) [141]. In humans, plasma levels peaked after 24 hours following a single i.m. injection of 100 mg nandrolone decanoate, and remained on that level for 10 days [180]. 24 hours after the last injection of nandrolone decanoate administered in the papers presented in this thesis, the depot is calculated to containe 6 mg of nandrolone decanaote in the 3 mg/kg group and 30 mg in the 15 mg/kg group, and thus still release nandrolone into the circulation [253].

We consider the doses 3 and 15 mg/kg administered i.m. once daily for 14 days to rats, to be equivalent of one cycle of human use during early and experienced AAS abuse respectively. These doses correspond to approximately 10 and 50 times the highest clinically recommended dose of nandrolone decanoate of 100-200 mg/week for anaemia, if presumed a 70 kg patient.

Animals

All studies were performed on male Sprague-Dawley rats, ten weeks of age. The animals were housed three or four in each cage at an average temperature of 22°C and 60% humidity. The twelve-hour light/dark circle started at 6 a.m. and food and water were provided *ad libitum*. After being allowed to adapt to the new environment for 7-14 days, the rats were randomly divided into treatment groups. Nandrolone decanoate was administered as i.m. injections once daily for 14 days, whereas control groups were administered i.m. injections of vehicle (arachidis oleum) once daily for 14 days. In Papers I and II nandrolone decanoate was administered at a dose of 15 mg/kg and in Papers III, IV and V an additional treatment group receiving 3 mg/kg was added. Injections were given in the hind leg, alternating between left and right (injection volume 0.1 ml). The Animal Care and Ethical Committee in Uppsala, Sweden approved the experimental procedure.

Paper I

Of the four treatment groups in this study, two were administered nandrolone decanoate (15 mg/kg) and two received vehicle. On day 15, one vehicle treated and one nandrolone treated group received a single i.p. injection of amphetamine (5 mg/kg). The two remaining groups received saline as vehicle. The animals were sacrificed by decapitation one hour after the amphetamine injection. The brains were rapidly removed for dissection. The frontal cortex, medial prefrontal cortex, nucleus accumbens, amygdala, caudate putamen, hypothalamus, hippocampus, and the periaqeductal grey were collected, put on dry ice and kept in Eppendorf tubes at –80°C, until prepared for analysis.

Paper II

Two treatment groups were included in this study; one was administered nandrolone decanoate (15 mg/kg) and one vehicle. On day 15, animals were anaesthetized with isoflurane (4.5% mixed with air at ca 550 ml/min) and mounted in a stereotaxic frame. During surgery the amount of isoflurane was decreased to 3.5%. An incision was made over the scull midline, uncovering the bone. A hole was drilled in the scull and the dura was carefully punctuated using a syringe. After insertion of the microdialysis probe, the maintenance dose of isoflurane was kept between 2.2 and 2.4% depending on the individual animal's reaction to pinching between the toes with a pair of tweezers. The isoflurane dose was kept to the lowest level were no reaction could be detected by the pinch test.

Papers III and IV

24 hours after the last injection of nandrolone decanoate, animals were sacrificed by decapitation and the brains were rapidly removed and dissected using a rat brain matrix. The prefrontal cortex, caudate putamen, nucleus accumbens, hippocampus, hypothalamus, amygdala, ventral tegmental area and the substantia nigra were collected. The parts from one hemisphere were put on dry ice, and transferred to Eppendorf tubes when frozen. The parts from the other hemisphere were immersed in RNAlater allowing the solution to infiltrate the tissue for 1 hour in room temperature. The ventral tegmental area and substantia nigra were too small to be divided and were stored in RNAlater only. All samples were stored in –80°C until prepared for analysis.

Paper V

On day 15, animals were sacrificed by decapitation and the brains were rapidly removed, frozen in 2-methyl butane (average temperature –25°C) and stored in –80°C until further prepared.

The whole brains were mounted and sliced in a cryostat at –19°C into 14 µm coronal sections and thaw mounted on polysine slides. Sections were collected at +4.2, +1.2, –0.92, –2.8 and –5.8 mm from bregma [198]. The slides were stored in –80°C until used for *in vitro*-autoradiography.

Extraction of monoamines from wet tissue (Paper I)

The brain tissues were homogenized by sonication in 500 µl of a chilled mixture of 0.1 M perchloric acid with 2 mg/ml ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA). The tissues and homogenates were kept on ice. The homogenates were then centrifuged for 15 minutes at 12 000 x g in 4°C. The supernatants were collected in new Eppendorf tubes and stored at –80°C until analyzed.

HPLC with electrochemical detection (Papers I and II)

The levels of dopamine, DOPAC and HVA were measured using a reversed-phase high performance liquid chromatography (HPLC) system and electrochemical detection (EC). The system consisted of a ReproSil-Pur C18-AQ, 150x3 mm, 5 µm column, an injector with a 150 µl loop, an analytical cell set at an oxidation potential of 350 mV and a guard cell set to 400 mV. Isocratic eluation was applied at a flow rate of 0.8 ml/min and the chromatograms were obtained and analysed with a chromatographic software.

The following mobile phase was used in Paper I: 55 mM sodium acetate trihydrate, 180 µM 1-octansulphonic acid sodium salt, 10 µM EDTA and

10% methanol dissolved in purified water. pH was set to 4 by addition of 70% acetic acid and the buffer was then filtered (0.22 µm) and degassed with helium [151]. In Paper II the concentration of 1-octansulphonic acid sodium salt was increased to 520 µM and the methanol content to 12%.

The sample volume injected ranged from 20 to 40 µl and the amounts of the monoamines were determined using the external standard method. Calibration curves were created with pure dopamine, DOPAC and HVA dissolved and diluted in 0.1 M perchloric acid containing 2 mg/ml EDTA (Paper I) or in 0.1 M perchloric acid containing 0.5 mg/ml glutathione (Paper II). The chemicals were all of analytical grade.

Microdialysis (Paper II)

A microdialysis probe with an outer diameter of 0.6 mm, a membrane length of 2 mm and a 15 kDa cut off was inserted into the nucleus accumbens shell (coordinates: anterior +2.2 mm, lateral +1.4 mm with bregma as reference and –7.5 mm ventral to the brain surface) [198]. A sterile solution of sodium chloride (147 mM Na^+), calcium chloride (1.2 mM Ca^{2+}), magnesium chloride (0.9 mM Mg^{2+}) and potassium chloride (2.7 mM K^+) was perfusing the probe at 2 µl/min. Samples were collected with 15-minute intervals in tubes kept cool (4°C). Samples collected during the first 105 minutes after surgery were discarded before beginning baseline monitoring for one hour. An i.p. injection of 5 mg/kg amphetamine was administered to every other animal and saline to the once remaining. Dialysate was collected for an additional three hours. Body temperature was maintained at 38°C during the procedure. All fractions contained 10 µl of 0.1 M perchloric acid with glutathione (0.5 mg/ml) and were stored at –80°C until further analyzed.

Radiometric assay determining the enzymatic activity of MAO (Paper III)

The enzyme activity was determined with a radiometric assay described in detail elsewhere, with slight modifications [101]. In short, the brain tissues were homogenized by sonication in 0.01 M sodium phosphate buffer (pH 7.4) and diluted to 2.5% of the wet weight. The homogenates were first pre-incubated for 20 minutes with an enzyme inhibitor in glass tubes. 100 nM deprenyl, also known as selegiline, was added to inhibit MAO-B, and 100 nM clorgyline to inhibit MAO-A. After the pre-incubation, the reaction was terminated by addition of 3 M HCl in the samples for blank activity, while substrates were added to the samples for total activity. 0.1 nM ^{14}C-serotonin (1.0 µCi/ml) was used as a substrate for MAO-A, and 0.05 nM ^{14}C-2-phenylethylamine (0.5 µCi/ml) as a substrate for MAO-B. The samples for

MAO-A activity were incubated for 20 minutes and the samples for MAO-B activity for 5 minutes, in a water bath (37°C). The reaction was terminated by acidification. The radioactive compound was extracted with a water saturated mixture of toluene:ethylacetate (1:1 vol/vol), and the organic phase was transferred to scintillation vials and mixed with scintillation fluid. Standard samples were prepared and all samples were then measured for 5 minutes in a liquid scintillation apparatus. The remaining homogenates were used for protein determination [157]. Enzyme activities were then calculated as nmol/minute/mg protein. All samples were made in triplicates.

Quantitative real-time polymerase chain reaction (qPCR) (Papers III and IV)

Primers

Forward and reverse primers, together with accession numbers, for both housekeeping genes (HKGs) and genes of interest (GOIs) are presented in Papers III and IV. The Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) was used to verify that no homologies were shared between amplified sequences and other cDNA in the database. Primer efficiencies ranged between 80 and 100%.

RNA isolation

Total RNA was isolated from individual brain tissue samples by phenol-chloroform extraction. Tissue samples were homogenized in 500 µl TRIzol® by ultrasonication. 100 µl chloroform was added and the homogenate was centrifuged at 12 000 x g for 15 minutes (4°C). The supernatant was transferred to a new tube and RNA was precipitated in isopropanol. The pellet was washed twice with 75% ethanol, thereafter air-dried and dissolved in DNAase buffer. DNAase treatment was performed at 37°C for 2 hours in order to remove DNA contamination, followed by inactivation of the DNAase at 75°C for 15 minutes. RNA purity was validated by PCR and gel-electrophoreses using primers for a 300 bp cDNA of GAPDH (se below under headline "cDNA synthesis"). RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer.

cDNA synthesis

cDNA synthesis was performed with M-MLV reverse transcriptase according to the manufacturer's protocol, using random hexamer primers. The cDNA synthesis was validated by PCR and gel-electrophoresis. PCR was performed in a final reaction volume of 10 µl containing 20 mM Tris-HCl

(pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.025% Tween, 0.2 mM dNTP, 1 pmol/µl of forward and reverse primers for a 300 bp cDNA of GAPDH and 3.4 U/µl Taq DNA polymerase. Annealing temperature was 58°C and 35 cycles were performed. Genomic DNA was used as positive control and water as negative. Product formation was visualized with gel-electrophoresis. Subsequent to PCR, samples were mixed with 6x DNA loading dye and applied to a 2% agarose gel made with TAE buffer and with addition of ethidium bromide (0.5 µg/ml). A 100 bp DNA ladder was applied to the first and last well of each row. Separation was run on 130 V for 20 minutes. Finally, the gel was photographed on a UV light table.

Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed in a final reaction volume of 25 µl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dNTP, SYBR Green 1:50 000, 10 nM fluoroscein, 0.8 pmol/µl each of reverse and forward primer, 0.02 U/µl Taq DNA polymerase. 50 cycles were performed. Annealing temperatures were 62°C for all GOIs except MAO-A for which both 62°C and 55°C were tried. The annealing temperature used for the HKGs was 60°C. Melting point curves were included to confirm that only one product was formed. Each assay included individual samples in duplicate and a negative control in triplicate.

qPCR data where analysed accordingly: the starting quantity means were normalized to the maximum sample value of each plate, resulting in values falling between 0 and 1. For the nucleus accumbens, caudate putamen, ventral tegmental area, hippocampus and prefrontal cortex a standard curve of four dilution points in triplicates was included, and for the hypothalamus, amygdala and substantia nigra the sample values were corrected for primer efficiency using the LinRegPCR protocol [208]. Sample values were then divided by the normalization factors created according to the $2^{-\Delta\Delta Ct}$ method [155]. The most stable set of HKGs out of seven tested in each tissue was selected using the GeNorm protocol [256], discussed in [150]. The HKGs included in the normalization factors were, for the prefrontal cortex: ACT, H3b, TUB; nucleus accumbens: CYCLO, GAPDH, SDCA; caudate putamen: H3b, RPL19, TUB; hippocampus: ACT, H3b, RPL19; hypothalamus: ACT, CYCLO, TUB; amygdala: ACT, GAPDH, RPL19; ventral tegmental area: CYCLO, H3b, RPL19; and substantia nigra: GAPDH, H3b, RPL19. See page 7 for abbreviations.

In vitro-autoradiography (Paper V)

The tissue sections were labelled with [^{3}H]-citalopram as described previously [40, 64], with slight modifications. In short, the pre-incubation of

slide-mounted sections was performed in 50 mM Tris buffer (pH 7.4), containing 120 mM NaCl and 5 mM KCl for 15 minutes in room temperature. Sections were then incubated with 1 nM [^{3}H]-citalopram (83 Ci/mmol) for 120 minutes in room temperature in buffer with the same composition as for pre-incubation. 10 μM fluoxetine was used to determine unspecific binding. Washing was performed 4 x 2 minutes in ice-cold Tris buffer and the slides were then quickly rinsed in ice-cold water. After the washing procedure, sections were dried with a stream of cool air from a table fan for one hour and then left in room temperature overnight. When dry, the slides and Hyperfilms were placed in X-ray cassettes and co-exposed with plastic standards (3-110 nCi/mg) at 4 °C for 35 days. The films were manually developed and the autoradiograms were digitized using a photo scanner. The optical densities were converted to fmol/mg wet weight based on the co-exposed standards using NIH Image software. Brain regions were identified with a rat brain atlas [198].

Results and discussion

Effects on dopamine

Dopamine levels (Papers I and II)

Of the eight brain regions analysed for dopamine and DOPAC tissue content after 14 days of nandrolone decanoate pre-treatment (15 mg/kg/day) and a subsequent amphetamine challenge (5 mg/kg), the hypothalamus and hippocampus were significantly affected. The effect of amphetamine on the DOPAC/dopamine ratios in the hippocampus and hypothalamus was abolished by the nandrolone pre-exposure (*Figure 1*). The hypothalamic DOPAC level was likewise affected. Nandrolone decanoate *alone* also had the ability to decrease the DOPAC tissue content in the hypothalamus compared to vehicle, as well as the DOPAC/dopamine ratio (*Figure 1*). No significant changes were observed in the amygdala, frontal cortex, nucleus accumbens or periaqeductal grey (Paper I). In the following study, animals were subjected to the same treatment as in Paper I. Dopamine release in the nucleus accumbens was evaluated before and during the amphetamine challenge. Nandrolone decanoate pre-exposure caused a decrease in baseline levels of extracellular DOPAC and HVA, however left the baseline level of dopamine unchanged, in comparison to controls (*Figure 2*). The differences in metabolite levels lasted one hour after the amphetamine administration (Paper II).

The ability of nandrolone pre-exposure to prevent or attenuate the effect of amphetamine is in congruence with several animal behavioural studies. Self-administration of ethanol increased [114] and the ethanol-induced sedation was prevented [152] in rats pre-treated with nandrolone decanoate (15 mg/kg/day for 14 days). Attenuated anxiolytic and rewarding effects of THC have been shown in mice after receiving the same dose of nandrolone decanoate as in the before mentioned studies on rats, whereas withdrawal symptoms increased. Development of tolerance was unaffected [36]. Attenuated morphine-induced CPP was also observed in mice, together with enhanced withdrawal reaction. Tolerance and sensitization remained unaffected [37]. Furthermore, a combined behavioural and microdialysis study has been published recently, where the behavioural and locomotor responses to both amphetamine and MDMA were attenuated or prevented following nandrolone pre-exposure (5 or 20 mg/kg 5 times every 48 hours). These effects were accompanied by attenuated dopamine release in the nucleus accumbens, and in the case of the MDMA, the extracellular metabolite levels

were increased. However, no intrinsic effect of nandrolone decanoate itself could be detected [140]. Also, the *acute* effect of testosterone on dopamine release has recently been tested in male hamsters. As expected, s.c administration of neither 7.5 nor 37.5 mg/kg of testosterone affected dopamine release in the nucleus accumbens within 4 hours from administration. However, acute administration of testosterone i.c.v. (2 µg every 6 minutes for 4 hours) decreased dopamine release in the nucleus accumbens. Following 14 days of this dosing regimen the effect on dopamine was however no longer present [251]. Although the microdialysis results published so far points towards decreased dopamine release in the nucleus accumbens after nandrolone exposure, there are still issues that have to be addressed in future studies. Discrepancies can most likely be attributed to differences in experimental designs, such as dose, route of administration, duration of the experiment and choice of animal model.

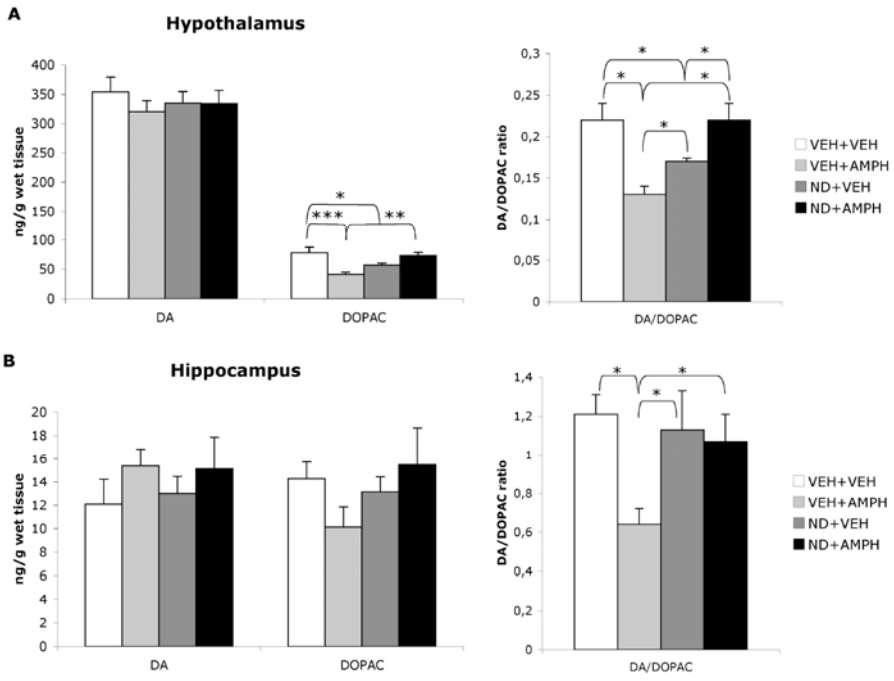

Figure 1. Tissue content of dopamine and DOPAC, and the ratio between them, in the hypothalamus (A) and hippocampus (B) after 14 days of nandrolone decanoate administration (15 mg/kg) to male rats, followed by an amphetamine challenge (5 mg/kg). The data is presented as mean ± SEM and was statistically analysed with one-way ANOVA and Fisher's PLSD. *p<0.05 **p<0.01 ***p<0.001. Abbreviations: AMPH amphetamine; DA dopamine; DOPAC dihydroxyphenyl acetic acid; ND nandrolone decanoate; VEH vehicle.

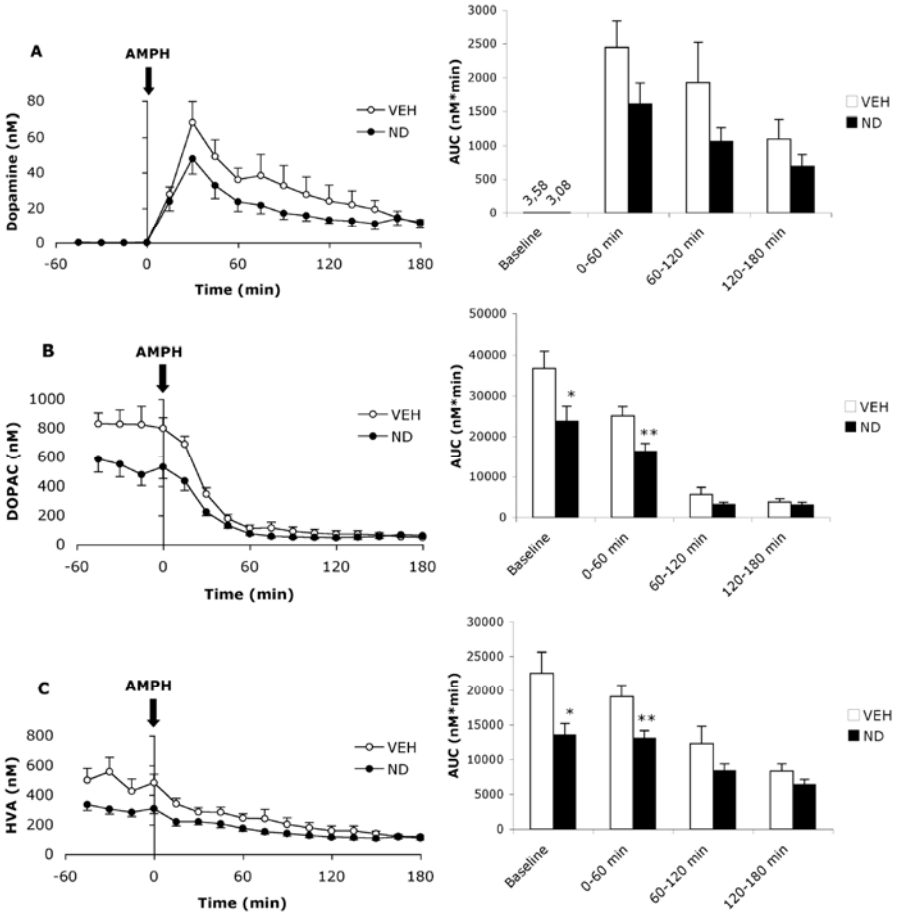

Figure 2. Microdialysate content of dopamine (A), DOPAC (B) and HVA (C) after administration of nandrolone decanoate (15 mg/kg/day for 14 days) and a subsequent amphetamine challenge (5 mg/kg). Dialysate contents are given as mean ± SEM in the left panel (dopamine baseline values ranged between 0.06 and 0.10 nM). AUC values and significance levels are given in the right panel. AUC values were statistically evaluated using Student's t-test. *p<0.05 **p<0.01. Abbreviations: AMPH amphetamine; AUC area under the curve; DOPAC dihydroxyphenyl acetic acid; HVA homovanillic acid; ND nandrolone decanoate; VEH vehicle.

The tissue content of dopamine and metabolites is rather a blunt tool in evaluating dopamine activity compared to microdialysis, however fully adequate as a screening method. Total tissue content of dopamine, DOPAC and HVA after AAS administration have been presented in a scarce number of studies, were Paper I is the only study investigating the impact of an AAS on the effect of a second drug. In accordance with our study, Lindqvist *et al.* found no effects on the dopaminergic system in the regions investigated (hypothalamus not included), by nandrolone decanoate alone administered as in Paper I [152]. Kurling *et al*. reported an *increased* DOPAC/dopamine

ratio in the hypothalamus, after the slightly different dosing regimen 5 mg/kg of nandrolone decanoate 5 days per week for 2 weeks, together with elevated levels of DOPAC in the cerebral cortex (5 and 20 mg/kg) [139]. In a study by Thiblin *et al.* different AASs were administered once weekly for 6 weeks at a dose of 5 mg/kg. Dopamine parameters were then investigated in the striatum only, where testosterone, nandrolone, methandrostenolone and oxymetholone increased the tissue content of DOPAC and HVA. The former three steroids also increased metabolite/dopamine ratio and the latter increased dopamine [247].

In early studies of dopamine tissue content after AAS administration, effects have been modest and inconclusive. The reasons are most likely due to diverse dosing regimens and methodological limitations. The microdialysis studies published recently point towards AASs reducing dopamine release in the nucleus accumbens. In Paper II, a dysfunctional dopamine activity is reflected by a compensatory decrease of dopamine metabolite levels, and attenuated psychostimulant-induced dopamine release after subchronic AAS administration.

Dopamine synthesis and metabolism (Papers III and IV)

The decreased DOPAC and HVA levels (Paper II), and metabolite/dopamine ratios (Paper I) observed after subchronic administration of supratherapeutic doses of AASs, might be due to altered dopamine synthesis, metabolism, reuptake, release rate, or any combination of these events. So far, there are no evidence for nandrolone decanoate having the ability to affect dopamine synthesis. The mRNA contents of TH and AAAD in the substantia nigra and ventral tegmental area, have been shown not to change in the rat brain after repeated nandrolone decanoate administration, neither using *in situ*-hybridization [127] nor qPCR (Paper IV). Additionally, the accumulation of L-DOPA in the striatum was found to be unaffected in the study by Thiblin *et al.,* although not fully comparable since dosing regimens differed significantly [247]. However, studies of TH and AAAD activities, and protein levels, as well as TH phosphorylation needs to be performed before definite conclusions can be drawn in this matter.

The decreased extracellular baseline levels of DOPAC and HVA in Paper II, indicates reduced metabolism of dopamine by MAO and/or COMT. Therefore, MAO-A and -B activities, together with MAO-A, -B and COMT mRNA expression levels were investigated (Paper III). Nandrolone decanoate at a daily dose of 3 mg/kg for 14 days, significantly reduced the MAO-A and the MAO-B activities in the caudate putamen, compared to controls (*Figure 3*). The enzyme activities, in the group receiving 15 mg/kg, were not different from the control group. Biphasic dose-response relationships have been described earlier in the literature for several transmitters and hormones, including androgens [32] and estrogens [33]. The gene expression

level of MAO-B was also significantly increased in the substantia nigra at the dose 3 mg/kg. No significant changes were found in the prefrontal cortex, nucleus accumbens, hippocampus, hypothalamus, or the ventral tegmental area. COMT mRNA was not affected by the nandrolone decanoate administration (Paper III). There are no previous studies of COMT and only one on MAO activities in connection to supratherapeutic doses of AAS. MAO-A activity was increased by oxymetholone, but again, the dosing regimen differed greatly from that in Paper II, and only the hypothalamus was investigated [247].

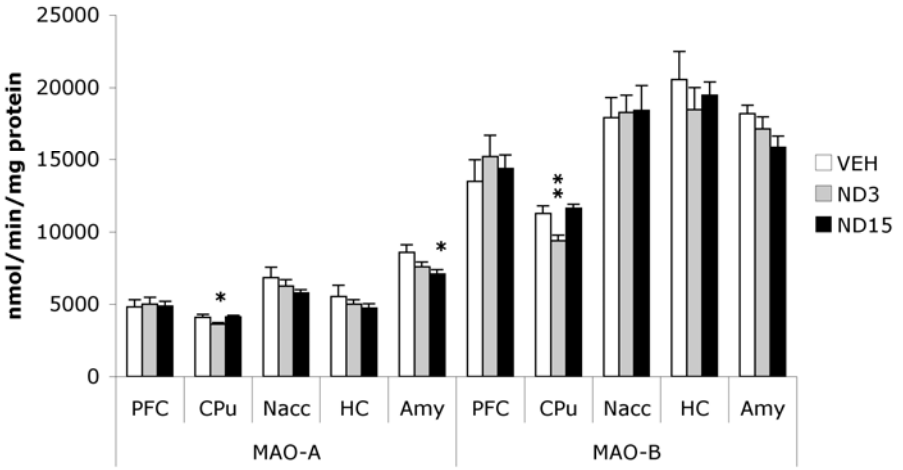

Figure 3. Enzymatic activity of MAO-A and -B in male rat brain after 14 days of daily nandrolone decanoate administration. Values are expressed as mean ± SEM and were statistically analysed with one-way ANOVA and Newman-Keuls *post-hoc* test. *p<0.05 **p<0.01 (only comparisons against the vehicle treated group are displayed). Abbreviations: Amy amygdala; CPu caudate putamen; HC hippocampus; Nacc nucleus accumbens; ND3 nandrolone decanoate 3 mg/kg; ND15 nandrolone decanoate 15 mg/kg; MAO monoamine oxidase; PFC prefrontal cortex; VEH vehicle.

Given the reduced extracellular levels of DOPAC and HVA shown in Paper II, reduced MAO activities were expected in the nucleus accumbens. There is a tendency towards a reduction in the nucleus accumbens, however not statistically significant. The decrease of both MAO activities in the caudate putamen of the group receiving 3 mg/kg of nandrolone decanoate, are interesting since this region, like the nucleus accumbens, plays an important role in the development and behavioural manifestations of drug dependence [68]. *Ex vivo* experiments, using MAO-A inhibitors, have showen that rat striatal HVA decrease almost linearly even at a MAO-A inhibition of less than 20%. Interestingly, consistent with the data in Paper II the dopamine level was not affected, possibly due to compensatory mechanisms [259]. The increased gene transcript level of MAO-B in the substantia nigra, indicated

in this study, might be a compensatory response to the reduced MAO-B activity observed in the caudate putamen, since the cellbodies originating in the substantia nigra extensively innervate the caudate putamen. The lack of linearity between mRNA level and protein level has been shown earlier in studies of rat brain MAO-B [96, 237].

Taken together, the decreased extracellular levels of DOPAC and HVA observed in the nucleus accumbens after nandrolone decanoate administration are most likely due to reduced metabolism of dopamine by MAO.

Dopamine transport (Paper IV)

One of the most important ways of regulating monoamine action and maintaining homeostasis in the extracellular space, is through reuptake mechanisms. Previously, the density of the DAT has been shown to increase in the caudate putamen, by 1, 5 and 15 mg/kg of nandrolone decanoate administered daily for 14 days [128]. The same results were obtained in a PET study on live animals receiving the highest dose [122]. If this reflects an increase of dopamine release in the initial administration period, or an upregulation directly induced by the steroid, independent of dopamine function, remains to be evaluated. As mentioned above, acute i.c.v. administration of testosterone to hamsters *decreased* dopamine release in the nucleus accumbens, indicating that initially increased dopamine release by nandrolone is a less likely explanation to the upregulated DAT density after subchronic nandrolone decanoate administration [251]. The mRNA contents of DAT and VMAT2 were found unaffected in the regions investigated in Paper IV. Thus, the increased protein density might be independent of transcriptional activity, or the temporal onset of mRNA regulation differs from that of translation. The effect of supratherapeutic doses of AAS on VMAT2 has not been previously examined. However, testosterone replacement in castrated mice has been shown to inhibit VMAT2 function in striatal tissue slices compared to untreated castrated animals [230].

Dopamine receptors (Paper IV)

It has previously been reported that supratherapeutic doses of nandrolone decanoate affect dopamine receptors on a translational level in several brain areas. However, on transcriptional level the reports are scarce. Two weeks of AAS administration caused alterations regarding both dopamine D1 and D2 receptors [126, 127]. The levels of dopamine D2 receptor transcript and D2-like protein increased, together with decreased levels of the dopamine D1 receptor transcript and D1-like protein, in the striatum, shown by *in situ*-hybridisation and autoradiography [126, 127]. These alterations resemble those observed following lesions of the nigrostriatal dopamine pathway [84] and might thus reflect reduced dopamine activity as a result of subchronic

AAS administration. Reduced dopamine modulation of striatopallidal outputs results in diminished behavioural activity [84, 234, 245] and could contribute to explain the attenuated effects of amphetamine after nandrolone pre-exposure, as shown in Papers I, II and [139]. In Paper IV, the gene transcript content of the dopamine receptor family was investigated in rat brain with qPCR. This method offers the advantages of highly specific primers, low limit of detection and the ability to analyze a large number of transcripts in the same individuals. This is the first time the dopamine D3, D4 and D5 receptors are analyzed in connection to AAS abuse.

Two weeks of daily nandrolone decanoate administration significantly elevated the dopamine D1 receptor mRNA expression in the amygdala at a dose of 15 mg/kg, but decreased it in the hippocampus at both doses of 3 and 15 mg/kg, respectively. The dopamine D4 receptor mRNA expression was significantly increased in the nucleus accumbens by nandrolone decanoate at a dose of 3 mg/kg/day for 14 days, both compared to controls and the group of rats administered the highest dose of 15 mg/kg/day. No statistically significant changes were detected in the prefrontal cortex, ventral tegmental area or substantia nigra. The hypothalamus and caudate putamen were also analyzed but in the end excluded from Paper IV since results from too few individuals were obtained to create proper basic data for a statistical evaluation. No significant alterations were found in those two regions.

This is the first time the dopamine D4 receptor is investigated in connection to AAS abuse. The dopamine D4 receptor has been implicated in the pathophysiology of ADHD [71], and behaviours associated with schizophrenia and substance abuse, such as novelty seeking [63] and impaired impulse control [5]. Dopamine D4 receptor antagonists display antipsychotic effects [19] and several atypical neuroleptics have high affinity for the dopamine D4 receptor [227]. D4 receptor knockout mice display decreased basal levels of dopamine, DOPAC and HVA in the striatum, as measured by microdialysis [249], and unaltered or even increased sensitivity to ethanol, cocaine and methamphetamine, compared to wild type mice [219]. This is consistent with the results of increased dopamine D4 transcription in the present study and decreased sensitivity to other drugs, shown repeatedly after AAS treatment [15, 16, 36, 37, 140, 152]. However, chronic AAS administration also decreases extracellular levels of dopamine, DOPAC and HVA [15, 140], not in concordance with the dopamine D4 knockout phenotype. If the dopamine D4 receptor is involved in the mechanisms behind AAS-induced behaviours remain to be evaluated.

The transcription level of the dopamine D1 receptor was increased in the amygdala after administration of 15 mg/kg of nandrolone decanoate. The amygdala is innervated by the ventral tegmental area, and is together with the nucleus accumbens involved in stimulus-reward learning, as part of the extended amygdala [58, 133]. In the basolateral amygdala, dopamine D1 receptor activation contributes to acquisition of cocaine-cue association [12]

and drug seeking behaviour [226]. Thus, the increased transcription of the dopamine D1 receptor in the amygdala might contribute to explain the poly-drug use often seen among AAS abusers [4, 83, 117, 124, 187, 188, 201, 248]. In the hippocampus, on the other hand, the transcription level of the dopamine D1 receptor was *decreased* by both doses of nandrolone decanoate. In addition to glutamate, dopamine has the ability to modulate hippocampal plasticity, in the form of long-term potentiation (LTP) and long-term depression (LTD), particularly during novelty detection [154]. D1/D5 antagonists impair late-phase LTP [146] and long-term memory [190] whereas D1/D5 agonists facilitate late-phase LTP [148]. There are indications of cognitive impairment due to testosterone administration in humans [51, 52]. A dose of 15 mg/kg/day of nandrolone decanoate for 6 weeks impaired memory function in rats [137]. However, spatial memory and hippocampal plasticity have been found unaffected in rodent studies using 5 mg/kg/day of a steroid cocktail consisting of testosterone, nandrolone and boldenone, for either 4 or 12 weeks [44, 238]. The possible effects of AAS on learning and memory need to be further evaluated.

Why these two limbic areas display opposing effects on dopamine D1 receptor transcription after nandrolone decanoate administration, would be interesting to explore further. It has been suggested though, that D1 receptor activation differentially affect the excitatory responses of hippocampal and amygdaloid afferents in the nucleus accumbens. D1 agonists attenuate amygdala-evoked responses, whereas hippocampal evoked responses show a bimodal form of modulation by D1 stimulation. The authors suggest that this property of the hippocampal afferents have a functional importance in gating inputs during exploration, and might thus in the end affect memory processing [38]. The qPCR did not detect changes in mRNA levels shown earlier in the nucleus accumbens and ventral tegmetal area by *in situ*-hybridisation. These discrepancies are most likely due to methodological differences such as higher anatomical resolution in *in situ*-hybridisation and the use of different standard methods.

Taken together, an increasing body of evidence points towards AAS induced dopamine dysregulation in limbic areas of the rat brain, possibly explaining many of the behaviours reported in human AAS abusers. These include development of drug dependence, both to AAS and other drugs, and impulsivity.

Effects on serotonin

Serotonin reuptake (Paper V)

Both 3 and 15 mg/kg/day of nandrolone decanoate administration for 14 days could dose-dependently increase the SERT protein in the prefrontal and

orbital cortices, caudate putamen, lateral preoptic area and medial division of bed nucleus of the stria terminalis. The density of the serotonin transporter was elevated by 15 mg/kg/day of nandrolone decanoate only, in the following regions: prelimbic and secondary motor cortices, lateral septal nucleus, olfactory tubercle, lateral globus pallidus, CA1 field of the hippocampus (radiatum and oriens layers), zona incerta, dorsomedial hypothalamic nucleus, lateral amygdaloid nucleus, paraventricular and laterodorsal thalamic nuclei, substantia nigra pars reticulata, periaqueductal grey and the superior colliculus (Paper V).

Electrical stimulation of the hypothalamus elicits attack behaviour in both rats and cats, whereas lesions suppress it [89, 233]. More precisely, the rat "hypothalamus aggression area" consists of the ventrolateral part of the ventromedial hypothalamus and adjacent areas [142]. Interestingly, this part of the hypothalamus projects to several of the areas in the rat brain, in which we have demonstrated increased SERT protein density after subchronic nandrolone decanoate administration, e.g. the lateral septum, lateral preoptic area, bed nucleus stria terminalis, zona incerta, dorsomedial hypothalamus, lateral amygdala, paraventricular thalamic nucleus and periaqueductal grey [215].

Increased density of the SERT is most likely resulting in increased serotonin reuptake and thereby a reduction of extracellular serotonin levels and serotonin function. It has repeatedly been shown that SERT knockout mice and rats have elevated brain levels of serotonin [107, 121] and display attenuated aggression in resident-intruder tests [106, 108]. This is consistent with the serotonin deficiency hypothesis of aggression, which is primarily based on the decreased levels of the serotonin metabolite 5-HIAA in CSF of impulsive and violent individuals [103, 153], and that serotonin depletion facilitates these behaviours in several mammalian species [191, 233, 257, 258]. In pharmacological studies, suppression of aggressive behaviours has been observed after administration of serotonin agonists or reuptake blockers. There are also polymorphisms in the SERT gene that have been associated with affective disorders [98, 147].

The increased protein density of the SERT might lead to decreased serotonin function. This result might contribute to explain the impulsive and aggressive behaviours reported in connection to AAS abuse.

Serotonin metabolism (Paper III)

As shown in Paper III, nandrolone decanoate at a dose of 15 mg/kg decreased the enzyme activity of MAO-A and -B in the amygdala (*Figure 3*). MAO-A knockout mice deviate from the serotonin deficiency hypothesis of aggression by displaying increased aggressive behaviour accompanied by *elevated* brain serotonin levels [35, 205, 207]. Some have tried to explain this discrepancy by suggesting a negative correlation between serotonin and

pathological violent aggression and a positive correlation with "normal" aggression [147, 191]. There is also evidence of differential roles for serotonin in the different phases of aggressive behaviour (anticipation, performance and termination) [74, 254].

The increased serotonin levels during foetal life observed in MAO-A knockout mice causes a reduction in density of serotonergic fibres [35]. Interestingly, adolescent hamsters treated with AASs displayed increased offensive aggression together with a reduced number of serotonin fibres in the amygdala and the hypothalamus, detectable already after 7 days of AAS exposure [90, 92]. The MAO-A promoter contains glucocorticoid/androgen response elements with an enhancing effect on enzyme expression. Glucocorticoids have a stronger effect on enzyme expression and activity compared to androgens [194]. Thus, the net result of increased androgen levels competing for the promoter, might be a reduced expression of the enzyme when androgens dominate over glucocorticoids [236]. The finding of reduced MAO-A activity in the amygdala (Paper III) might thus also contribute to explain AAS-induced aggressive behaviour.

Serotonin receptors (Paper IV)

There are several reports supporting AAS induced aggression due to serotonin receptor alterations in the hypothalamus and amygdala. In steroid treated hamsters, aggressive behaviours have been elicited by AASs, accompanied by decreased density of 5HT1A receptors in the anterior hypothalamus [213], and 5HT1B receptors in the anterior and ventrolateral hypothalamus, and the central and medial amygdaloid nuclei [91]. Either a 5HT1A or a 5HT1B receptor agonist could reverse the AAS induced aggression [91, 213], consistent with earlier behavioural studies of aggression and impulsivity [54, 176, 178, 179, 235]. The 5HT1B receptor density has also been shown to decrease in the hippocampus of male rats subjected to 14 days of nandrolone decanoate administration [125].

The 5HT2A receptor in the nucleus accumbens showed a trend towards an increase, however not reaching statistical significance (Paper IV). Previously, two weeks of nandrolone decanoate administration to male Sprague-Dawley rats has been shown to increase 5HT2 receptor densities in the nucleus accumbens shell (at 1, 5 and 15 mg/kg) and the ventromedial hypothalamus (at 1 and 5 mg/kg) using *in vitro*-autoradiography [125]. Testosterone replacement in castrated male rats has also been shown to increase the 5HT2A receptor mRNA content in the ventromedial hypothalamus [270] and dorsal raphe nucleus [75]. The 5HT2 receptor density was decreased in the frontal cortex, hippocampus and amygdala [125]. No corresponding alterations in mRNA levels of 5HT2 receptors could be detected with qPCR in this study. Differences between mRNA and protein data can sometimes be explained by posttranslational processing, independent of gene transcription.

The lack of linearity between mRNA level and protein level in the serotonin system has been reported earlier, e.g. for the SERT in the macaque brain following ovarian steroid treatment [158, 237]. Also, the temporal onset of mRNA regulation might be different from protein regulation.

It has been difficult to establish a uniform pattern of AASs' effects on rat brain serotonin, possibly due to differences in study designs [139, 152, 247]. However, androgens have been shown to both increase aggression and to modulate serotonin induced suppression of aggressive behaviour. For example, testosterone induced aggression is facilitated if serotonin lesions have been performed prior to the testosterone administration [118]. It has also been suggested that aggressive behaviour is dependent upon the conversion of testosterone to estradiol by aromatase, present in the brain. For instance, 5HT1A and 5HT1B agonists reduce intermale aggression in mice pre-treated with the non-aromatizable androgen DHT and to a lesser extent when pre-exposed to testosterone. In the presence of estradiol, 5HT1A and 1B agonists fail to prevent aggressive behaviour. Additionally, estradiol is equally, or even more in some strains, efficient as testosterone in restoring male aggressive behaviour in castrated male mice [235]. 5HT2A and SERT mRNA contents as well as protein densities have been shown to decrease upon castration of rats. The levels are restored by testosterone and estradiol, but not by DHT [172, 242].

In conclusion, the results of increased SERT density and decreased MAO-A activity in areas of the rat brain involved in the expression of impulsive and aggressive behaviours might support the hypothesis of AAS induced aggression due to serotonin dysregulation.

Conclusions

This thesis reports alterations in the male rat brain dopaminergic and serotonergic systems, induced by subchronic nandrolone decanoate administration at the supratherapeutic doses often abused. The main outcomes from the studies included in this thesis are:

- Nandrolone decanoate reduced the extracellular levels of DOPAC and HVA in the nucleus accumbens
- Nandrolone decanoate reduced MAO activities in the caudate putamen and amygdala
- Nandrolone decanoate reduced the gene transcript level of the dopamine D1 receptor in the hippocampus and increased D1 in the amygdala as well as D4 in the nucleus accumbens
- Nandrolone decanoate prevented or attenuated the amphetamine induced effects on dopamine metabolism and turnover in the hypothalamus, hippocampus and nucleus accumbens
- Nandrolone decanoate increased the density of the SERT protein in a vast number of aggression related brain areas

This thesis concludes that subchronic administration of the AAS nandrolone decanoate in supratherapeutic doses, induced dopaminergic dysregulations in limbic areas of the male rat brain. These alterations might be underlying some of the behavioural changes observed during the different phases of AAS abuse in humans, such as development of drug dependence and polydrug use. The widespread increase of SERT protein density might contribute to explain the androgen-induced impulsivity and aggression observed among human AAS abusers and in animal models of AAS abuse.

Future perspectives

This thesis provides evidence for reduced dopamine activity and increased SERT density in the male rat brain induced by the supratherapeutic doses of AAS abused in society. Here follows a few suggestions on how to evaluate the behavioural consequences and the underlying mechanisms of the monoaminergic alterations induced by AASs.

The correlation between reduced dopamine function in the striatum and behavioural changes needs to be evaluated.

As mentioned before, several authors have suggested that prolonged AAS abuse function as a gateway to other drug abuse [4, 117]. There is now evidence of AAS exposure causing attenuated behavioural responses to several drugs of abuse [36, 37, 114, 152], dopaminergic dysregulation [e.g. 15, 140, 251], and correlations between them [140]. Experiments combining microdialysis and behavioural evaluations in freely moving animals should prove helpful in expanding our knowledge of AAS-induced neurochemical alterations and the behavioural consequences. To test the hypothesis that AASs function as a gateway to abuse of e.g. psychostimulants and opioids, self-administration tests of these drugs should be conducted after AAS pre-exposure.

The link between androgens, serotonin and aggression deserves further attention.

It is shown in this thesis that subchronic administration of nandrolone decanoate in supratherapeutic doses increases the density of the SERT and decreases MAO-A activity in aggression related brain areas. Once again, a combination between microdialysis and behavioural tests in freely moving animals could reveal if this results in reduced extracellular serotonin levels and increased aggression.

How to evaluate aggressive behaviours in appropriate paradigms and how to distinguish between "normal" aggression and pathological aggression in animal models is still debated in the literature [13, 53, 147, 168, 191]. However, while these issues are resolved, the link between androgens, serotonin and aggressive behaviour should be further evaluated. One issue to explore further is the role of estradiol in these behaviours and the impact that might have on AAS abusers. Different AASs have different effects on aggression.

Some facilitate aggression, whereas some do not, or even reduce aggressive response [24, 170]. This might be dependent upon the degree of aromatization of the specific steroid compounds, or through non-classical steroid mechanisms.

The mechanism behind the monoaminergic alterations needs to be evaluated.

Androgens, like other steroid hormones, exert their effects through nuclear receptors present inside the cell. The complex between the hormone and receptor function as a transcription factor, thus effecting gene expression [97, 210]. However, rapid effects through allosteric modulation of membrane bound receptors also occur [70, 177] and possibly also via non-genomic androgen receptor mechanisms [221]. Moreover, both androgenic and estrogenic metabolites are active in many tissues [183]. Therefore, adding treatment groups receiving androgen or estrogen receptor blockers, and inhibitors of 5α-reductase or aromatase, could give important information about possible mechanisms behind the central alterations reported, and maybe even give clues about the non-linear effects sometimes observed.

Additionally, stacking (i.e. mixing different kinds of steroids and routes of administration) is a common dosing regimen among body builders. Administration of steroid cocktails to mimic stacking in a controlled laboratory setting makes for an animal model more realistic to the true situation. However, a major drawback is that possible effects cannot be attributed to a single steroid and hence makes the underlying mechanism uncertain. Different androgens have, for instance, been shown to have different, or even opposing, effects on certain behaviours [24, 170, 263]. If this applies for neurochemical parameters as well remains to be evaluated.

The number of doses in dose-response studies needs to be expanded.

The purpose of the nandrolone decanoate dosing regimen used in this thesis was to mimic heavy AAS abuse. The doses of nandolone decanoate administered in this thesis represent early and experienced AAS abuse (3 and 15 mg/kg respectively), based on case [83] and survey [197] studies of body builders. The dose, types of steroids, duration of administration and washout periods might differ when abused by other categories for intoxication, as behaviour modifying drugs, or by athletes with varying demands on performance enhancement depending on kind of sports. There might also be a risk of overestimating the amount of steroid intake by those purchasing AAS on the black market, since counterfeits are not uncommon.

The non-linear effects demonstrated in several reports might be an indication of “spill-over” on receptor systems only affected by androgens in supratherapeutic doses, or other compensatory mechanisms [111]. It would therefore be necessary to cover a larger dose range, and to follow the gradual effects of AAS over time to make a clearer picture of the biological effects

in the acute, subchronic and chronic phases of administration. There is also a need for proper pharmacokinetic evaluations of repeated administration of supratherapeutic doses of steroid esters in oil depot, e.g. to clarify to what extent and rate the steroids pass the blood brain barrier.

To summarize, the research on AASs at supratherapeutic doses has unveiled certain neurochemical and behavioural changes in animal studies, which can be linked to side effects observed in human AAS abusers. Future AAS research would benefit from studies correlating neurochemical and behavioural data, mechanistic evaluation of AAS receptor interactions and metabolite activity, as well as pharmacokinetic evaluations of the dosing regimens used in these studies.

Populärvetenskaplig sammanfattning

Anabola androgena steroider (AAS) är en grupp substanser som liknar det manliga könshormonet testosteron. Dessa framställdes med målsättningen att göra läkemedel med bättre vävnadsuppbyggande men mindre förmanligande effekter än testosteron. AAS används bland annat för att behandla människor som förlorat mycket vävnad i samband med stora operationer eller på grund av brännskador. Unga män med defekt testosteronfrisättning i kroppen kan få hormonbehandlingar och detta möjliggör en normal utveckling under puberteten.

Den vävnadsuppbyggande egenskapen hos AAS har gjort dessa substanser attraktiva även utanför kliniken. Missbrukare av AAS tar doser som vida överstiger de kliniska och upplever initialt positiva effekter såsom ökad muskelmassa och ökat välbefinnande. I en senare fas av missbruket kan en rad biverkningar uppträda, bl.a. aggressivitet. Många blir deprimerade efter avbrutet intag och återupptar därmed missbruket. Det är också vanligt att de biverkningar som AAS ger upphov till leder till missbruk av andra läkemedelssubstanser och illegala droger. I beteendestudier på djur har det visat sig att gnagare självadministrerar hormonet. Det aggressiva beteendet har påvisats både hos djur och människa.

Ungefär som vi människor kommunicerar på olika sätt, använder sig våra celler av olika system för att överföra information till varandra. Nervcellerna i hjärnan använder olika signalämnen för att kommunicera med varandra och därmed förmedla sina budskap som rör allt från basala funktioner som andning, till högre funktioner som minne och medvetande. Nervcellerna signalerar till varandra i speciella kontaktpunkter, så kallade synapser. I synapsen släpper den sändande cellen ut sitt signalämne, som fastnar i mottagare, så kallade receptorer, på närliggande celler. Aktiveringen av dessa receptorer ger upphov till en förändring i mottagarcellens beteende och en biologisk effekt. Det finns en mängd olika signalämnen i nervsystemet, till exempel dopamin och serotonin.

Syftet med studierna i denna avhandling har varit att undersöka hur upprepad administrering av steroiden nandrolondekanoat påverkar delar av hjärnan som är involverade i drogberoende och aggressivt beteende. Dessa hjärnregioner använder sig av just dopamin och serotonin som signalämnen. Dopamin är förknippat med utvecklandet av drogberoende, och serotonin med impulsivitet och aggression. Försöken är utförda på vuxna hanråttor.

Upprepad administrering av nandrolondekanoat sänkte halten av dopamins två nedbrytningsprodukter, dihydroxyfenylätticksyra (DOPAC) och homovallinsyra (HVA), i nucleus accumbens. Många missbrukardroger, t.ex. amfetamin, höjer halten dopamin i nucleus accumbens men nandrolondekanoat lämnade dopamin opåverkat. När nandrolondekanoat kombinerades med amfetamin tenderade dopaminfrisättningen att vara lägre än hos gruppen som bara fick amfetamin. Nandrolondekanoat förtog med andra ord delar av amfetamins effekter. Även i regionerna hypotalamus och hippocampus upphävdes amfetamins effekt på ett liknande sätt. Detta resultat får stöd av beteendestudier som visat att effekten av olika droger minskar efter förbehandling med AAS.

Kan de minskade halterna av DOPAC och HVA förklaras av minskad aktivitet hos det dopaminnedbrytande enzymet monoaminoxidas (MAO)? Aktiviteten av MAO var som förväntat nedreglerad efter behandling med nandrolondekanoat. Denna nedreglering kunde ses i caudatus putamen, som har nära kontakt med nucleus accumbens, och i amygdala. Vidare påverkades genavläsningen av MAO och flera dopaminreceptorer i bl.a. nucleus accumbens och amygdala.

Den sänkta MAO-aktiviteten i amygdala är särskilt intressant eftersom denna region är involverad i både drogberoende och aggressivitet. MAO bryter ner serotonin också. Låg MAO-aktivitet i blodet har t.ex. observerats hos interner dömda för våldsbrott och hos impulsiva personlighetstyper. Dessutom ökade mängden serotonintransportörer i en rad aggressionsrelaterade hjärnregioner så som hypotalamus, amygdala och PAG efter nandrolonadministrering. Serotonintransportören pumpar tillbaka serotonin till cellen när signaleringen är klar. Om dessa pumpar blir fler kan signaleringen avbrytas tidigare och bli svagare än i vanliga fall. Eftersom serotonin bromsar aggressivt beteende så kan minskad serotoninsignalering få konsekvensen att impulskontrollen försämras och benägenheten att få aggressiva utbrott ökar.

Sammanfattningsvis så verkar upprepad administrering av höga doser nandrolondekanoat till hanråttor nedreglera den dopaminerga aktiviteten i hjärnregioner som är involverade i drogberoende. Effekten av andra droger förändras om AAS har funnits i kroppen en tid. Ökningen av mängden serotonintransportörer i aggressionsrelaterade delar av hjärnan leder sannolikt till minskade halter serotonin i synapsklyftan, vilket kan vara en bakomliggande orsak till det aggressiva beteendet som ofta rapporteras hos AAS-missbrukare.

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