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# Anabolic Androgenic Steroids and the Brain

*Studies of Neurochemical and Behavioural Changes  
Using an Animal Model*

BY

PIA STEENSLAND



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## **Abstract**

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A new group of anabolic androgenic steroid (AAS) users has developed during the last two decades. This group consists primarily of young men interested in improving their physical appearance. Within this group, AAS are sometimes used together with other illicit drugs, alcohol and nicotine. Brutal and violent crimes have been committed under the influence of AAS, possibly because of AAS psychiatric side effects, ranging from increased aggression and psychosis to depression. Unfortunately, the biochemical mechanisms behind these effects are poorly understood.

In this thesis we used an animal model to study biochemical and behavioural effects of chronic AAS treatment (15 mg/kg/day of nandrolone decanoate for 14 days). The effect on the endogenous opioid peptides and the expression of immediate-early gene protein Fos in various brain regions were studied using radioimmunoassay and immunohistochemistry, respectively. In addition, we studied AAS effect on voluntary alcohol consumption and defensive behaviours, including aggression. The results show that AAS enhance endogenous opioid activity and Fos expression in brain regions regulating reward, aggression and disinhibitory behaviours. An imbalance between two opioid systems with generally opposing effects, the enkephalins with euphoric and the dynorphins with dysphoric effects, was also found. This implies that AAS alter the ability to maintain a stable state of mind and the response to other drugs of abuse. The AAS pre-treated animals enhanced their alcohol intake, were more aggressive and showed lower fleeing and freezing reaction than the controls. In addition, AAS enhanced amphetamine-induced aggression when the amphetamine was given three weeks after the last AAS injection.

The behavioural and biochemical results found in this thesis, support the hypothesis that use of AAS might lead to the development of dependence and may induce changes in the brain leading to disinhibitory behaviours.

Pia Steensland, Department of Pharmaceutical Biosciences, Division of Biological Research on Drug Dependence, Uppsala University, BMC, Box 591, SE-751 24 Uppsala, Sweden

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*To Johan*



## Preface

Brutal and violent crimes have been committed in Sweden under the influence of anabolic androgenic steroids (AAS). This forced the police authority and the politicians to take action and to look more closely into crimes related to steroid use. The legalisation, they concluded, regarding the use of AAS needed to be stricter. As a direct result, a governmental committee was set up with the aim to find out if there were any scientific proof of AAS harming effects. The committee found that little was known about the *psychiatric* side effects of AAS. This was an important finding together with the “usual” results indicating severe *physical* side effects. At the time of this finding, the Swedish police authority contacted my supervisor Professor Fred Nyberg.

With a long tradition in investigating the effects on the brain of drugs of abuse, Fred Nyberg at the Division of Biological Research of Drug Dependence initiated 1995 a project with the aim to establish biochemical proof for AAS harming effects on the brain. A second aim was to survey the use of AAS among adolescents in Uppsala, Sweden. This thesis is the first to be presented from this project.

Many drugs of abuse affect the body's own morphine, the so-called endorphins. The behavioural changes displayed by AAS users, for example possible development of dependence and mood swings, mimic the effects of other drugs of abuse. It was therefore a natural first approach to study how AAS affect the endorphins. This thesis focuses on AAS effect on the endorphins as well as AAS-induced behavioural changes.

Studying the psychiatric effects in humans of a drug such as AAS is difficult. The individuals using these substances often lie about the extension of their use. Moreover, type of AAS, dose and duration of treatment periods differ among users. A combination of different AAS is often used (so-called stacking) to avoid the development of tolerance to a particular AAS. Hence, if one should study a group of real-life AAS users, it would be difficult to compare the results from the different individuals since they most likely use different AAS regimes. It is also difficult to justify ethical approval to a controlled study in healthy volunteers using the extreme doses of AAS as those reported for the abusers. Apart from these methodological and ethical problems, the methods for studying changes in the brain of a living human being are limited. Therefore, the best available option for studying the psychiatric effects of AAS is to use an animal model. Many people outside the “scientific world” have difficulties in accepting the relevance of results obtained from animals. Of course, it is impossible to extrapolate and conclude that what happens in the brain of an animal is exactly what happens in the human brain. Nevertheless, 90-95% of our genes correspond with those of a rat (fortunately, the remaining 5-10% make us a bit different ☺). The brains of animals used in scientific experiments are well characterised and the regions and substances regulating most of our

behaviours including those regulating the effects of drugs of abuse, are basically the same in the brain of rats and humans. These are some reasons why animal studies can help us to understand the mechanisms of both diseases and basal functions of our own bodies.

I believe and hope that the results presented in this thesis will contribute to a deeper understanding of AAS harming effects on the brain. There are understandable circumstances under which primarily young men are tempted to use drugs. I wish to conclude this preface by informing anyone who even remotely considers using AAS that the risk might be greater than the gain.



Pia Steensland  
Uppsala, in November 2001

## List of Original Papers

This thesis is based upon the papers listed below, which are referred to in the text by their Roman numerals I-V.

- I. Johansson P., Ray A., Zhou Q., Huang W., Karlsson K., and Nyberg F. Anabolic androgenic steroids increases  $\beta$ -endophin levels in the ventral tegmental area in the male rat brain. *Neurosci.Res.* 27:185-189, 1997
- II. Johansson P., Hallberg M., Kindlundh A. and Nyberg F. The effect on opioid peptides in the rat brain, after chronic treatment with the anabolic androgenic steroid nandrolone decanoate. *Brain Res. Bull.*, 51(5):413-418, 2000
- III. Johansson P., Lindqvist A-S., Nyberg F. and Fahlke C. The anabolic androgenic steroid nandrolone decanoate affects alcohol intake, defensive behaviors and brain opioid peptides in the rat. *Pharmacol. Biochem. Behav.* 67:271-279, 2000
- IV. Johansson-Steensland P., Nyberg F. and Chahl L. The anabolic androgenic steroid, nandrolone decanoate, increases density of Fos immunoreactive neurones in limbic regions of guinea-pig brain, *Eur. J. Neurosci.*, *in press*
- V. Steensland P., Hallberg M., Kindlundh A., Fahlke C. and Nyberg F. Amphetamine-induce aggression is enhanced in rats pre-treated with anabolic androgenic steroids. *Manuscript*

Reprints of the original articles (I-III) were made with permission from Elsevier Science

## **Abbreviations**

AAS	Anabolic Androgenic Steroid/s
ACTH	Adrenocorticotrophic hormone
CeA	Central nucleus of Amygdala
CNS	Central Nervous System
CRF	Corticotropin-Releasing Factor
DOP	Delta Opioid Peptide
DSM	Diagnostic and Statistical Manual of mental disorders
GABA	Gamma-AminoButyric Acid
KOP	Kappa Opioid Peptide
MAD	Median Absolute Deviation
MEAP	Met-enkephalin-Arg <sup>6</sup> -Phe <sup>7</sup>
MOP	Mu Opioid Peptide
NAcc	Nucleus Accumbens
NMDA	N-Methyl-D-Aspartate
i.m.	intramuscular
i.p.	intraperitoneal
ir	immunoreactivity
PAG	Periaqueductal gray
POMC	Proopiomelanocortin
RIA	Radioimmunoassay
SE	Standard Error
SEM	Standard Error of the Mean
VTA	Ventral Tegmental Area



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## **1 Introduction**

### **1.1 Anabolic Androgenic Steroids**

Doping cases are revealed in seemingly endless streams during major athletic events such as the Olympic Games. The competition is tight and to be able to win and set new records the athletes have to perform at the peak of their ability. Many elite athletes are using forbidden aids, as for example anabolic androgenic steroids (AAS), to get more from their bodies than nature and training would otherwise provide. This is understandable, however not acceptable. New groups of AAS users have developed during the last decades. These groups consist mainly of adolescent boys interested in enhancing their physical appearance and individuals involved in criminal acts.

#### *1.1.1 What are Anabolic Androgenic Steroids?*

Anabolic androgenic steroids are a group of doping agents commonly used by athletes and body builders because of the steroids' ability to promote muscle growth (anabolic effect) and to shorten the recovery period after a hard workout. Apart from the male sex hormone testosterone and other endogenous androgenic hormones, the group consists of synthetic compounds structurally related to testosterone. Attempts to modify the testosterone molecule to promote pure anabolic, rather than androgenic (masculine), effects have been unsuccessful. The agents are therefore called anabolic *androgenic* steroids, rather than anabolic steroids (91).

#### *1.1.2 Who Use Anabolic Androgenic Steroids?*

The use of AAS has accelerated since a Russian weight-lifting team admitted the use of AAS in 1954. In 1972, use of AAS was reported by 99% of the Mr America contestants (42) and in a survey conducted the same year, 31% of Swedish elite track-and-fields athletes admitted that they had used AAS (86). It is estimated that over one million people in the United States use, or have used AAS (168). The use of AAS was also found among non-professional athletes during the 1990's in college and junior and senior high school as well as among recreational athletes (166). However, the use of AAS is no longer limited to athletes and body builders. Studies conducted in several countries during the last decade have shown that adolescents and adults with no primary interest in sports, but to gain weight and improve their physical appearance have started to use AAS (73, 109, 146, 167). This new group of AAS users seems to be heterogeneous with regards to for example social background, academic performance, races and nationalities. The common link between them is that they want to look, perform and feel better, at almost any cost (for review see (9)). Furthermore, some adolescents claim to use AAS to become intoxicated and braver (73). This group of AAS users seems to have common background variables (for example truancy, living alone, heavy alcohol consumption a couple of times per week and tobacco use) with adolescents using psychotropic drugs (74).

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Several studies have found associations with the use of AAS and the use of other illicit drugs, sedatives, alcohol and nicotine (8, 22, 43, 44, 72, 74, 110, 123). Athletes might for example combine AAS with morphine to ease the pain from over-trained muscles and tendons or amphetamine to get an energy bust to be able to train even harder. Anecdotal stories also report that AAS are combined with for example amphetamine or heroine to get a higher kick or euphoric effect from the psychostimulantia (154). Use of AAS has also been suggested to serve as a gateway to abuse of other drugs (7, 37, 154). In addition, AAS have been connected with criminal acts and violent behaviour (27, 37, 117, 122, 125, 154).

The increased and widespread use of AAS is alarming for several reasons. Obviously, it is devastating for the individuals using the drugs, considering the severe side effects that are likely to occur. Furthermore, immediate family and significant others are likely to, at some extent, suffer from the AAS users' change in mental state, like for example mood swings, increased aggression and irritability. Increased domestic violence have been reported to increase after AAS use (27, 37). Finally, the result of crimes committed under the influence of AAS and the increased number of AAS users seeking medical attention (particularly at psychiatric clinics) are costly for society.

### *1.1.3 Mechanisms of Action*

Anabolic androgenic steroids bind to the androgen receptor, which belongs to the nuclear receptor superfamily. It has also been suggested that high doses of AAS can bind to, or affect, other steroid and non-steroid receptor systems as well as possibly interact with the so-called neurosteroids (endogenous steroids synthesised in the brain) (2, 14, 15, 36, 97, 99). The anabolic and androgenic effects of AAS are mediated through the androgen receptors (141). The anti-catabolic effect has been suggested to be mediated through the glucocorticoid receptors (see below) (162). Which receptors that are involved in the psychiatric side effects of AAS are not clarified, however the Gamma-Aminobutyric acid (GABA<sub>A</sub>) and the N-Methyl-D-Aspartate (NMDA) receptors have been suggested as possible candidates (14, 15, 81, 97).

Together with the glucocorticoid-, progesterone- and mineralocorticoid receptors, the androgen receptor forms a subgroup of the superfamily named class I (33). Androgen receptors are found in the central nervous system (CNS) and throughout the body. The mechanism of autoregulation of the receptors in the CNS is unclear. Nevertheless, AAS have been found to upregulate androgen receptors in the brain (93, 101). Testosterone has two active major metabolites, dihydrotestosterone and estradiol. Dihydrotestosterone acts on the androgen receptor but with higher affinity than testosterone, whereas estradiol binds to the estrogen receptor (141). Nandrolone, the AAS examined in this thesis, binds to the androgen receptor with less affinity than dihydrotestosterone but higher than testosterone (130).

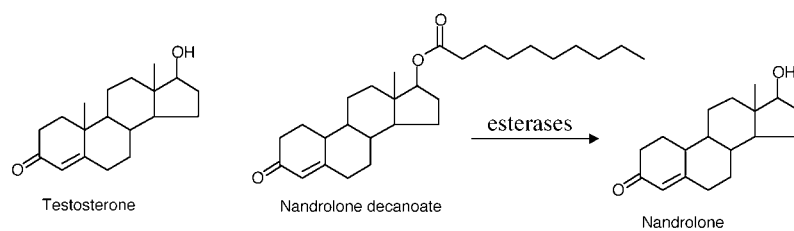
To mediate its action on steroid receptors, AAS passes through the cell wall and the cytoplasm into the nucleus where it binds to androgen receptors. The steroid-receptor complex binds to specific sites on the DNA, which is transcribed to give specific mRNA. The mRNA is then translated into specific new proteins that mediate for example the anabolic and androgenic effects of the steroids (33). This is a simplified description of the receptor activation. Several components such as hormone response elements and androgen-specific enhancers are essential for the receptor activation. For further description of these components and the complexity of the receptor binding, see (33) and references therein.

The anti-catabolic effect of AAS, is suggested to be mediated by blockage of the glucocorticoid receptor (87). Cortisol, or in animals corticosterone, is a stress hormone and the natural ligand for the glucocorticoid receptor. The hormone is secreted during stress, induced by for example intensive and frequent physical training (161), and exerts catabolic effects within the tissue (55). Thus, blockage of the glucocorticoid receptors by AAS, gives the athlete the benefit of shorter recovery times between workouts. As a result, the athlete can have longer and more intense training sessions.

#### *1.1.4 Nandrolone Decanoate*

This thesis is focused on nandrolone, one of the most commonly used AAS (116, 160). Nandrolone is a synthetic modification of the testosterone molecule and lacks a methyl ( $\text{CH}_3$ ) group at the  $\text{C}_{19}$  position (Fig. 1).

The nandrolone molecule is made suitable for injection by conjunction with decanoic acid, creating nandrolone decanoate (Deca Durabol<sup>®</sup> Fig. 1). Nandrolone decanoate is injected intramuscularly (i.m.) and has a long half-life in the depo, approximately 6 days in human as well as in rat. After injection, nandrolone decanoate is converted to nandrolone and is slowly released from the depo into the blood where it has a much shorter half-life (1 hour or less) (157).



**Figure 1.** Structural formula of testosterone, nandrolone (19-nortestosterone) and nandrolone decanoate. Nandrolone decanoate is hydrolysed by esterases to nandrolone after injection.

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Nandrolone has a reduced conversion rate to estrogen compared to testosterone (157) (which for example reduce the risk of development of breast in men, so-called gynecomastia) as well as less androgenic activity compared to the mother molecule testosterone (132). These might be the major reasons for nandrolones popularity.

#### *1.1.5 Physical Side Effects*

To reduce many of the physical side effects of AAS, it is desirable to promote the anabolic rather than the androgenic effect. But as mentioned before, pure anabolic steroids does not exist. However, most of the synthetic AAS exhibit less androgenic activity than testosterone (132). Anabolic Androgenic Steroids induce a variety of physical side effects (for review see (16)), in brief, gynecomastia, baldness, testes and prostate atrophy have been reported after prolonged AAS use in men (see for example (16, 24, 116, 123)). In women, deepening of the voice, menstrual irregularities, increased growth of facial hair and enlargement of clitoris are commonly reported side effects (16, 58). Increased acne, liver dysfunction, as well as increased risk of cardiovascular diseases (for example altered serum lipoproteins, stroke, enlarged heart, blood clotting) are found in both sexes (see for example (16, 24, 58, 116, 123)). When AAS are used by adolescents, the epiphyses can close prematurely and thereby halt bone growth (16).

#### *1.1.6 Psychiatric Side Effects*

Psychiatric side effects of AAS, ranging from depression to psychosis, have been observed more frequently as the use of AAS has become more widespread. Increased aggressiveness, energy and libido, as well as delusions and manic episodes, are often associated with AAS (22, 24, 27, 37, 39, 51, 58, 114, 116, 117, 121-123, 144, 154, 163, 164). Some individuals feel immortal when using AAS. They believe for example that they can step out in front of a car approaching at a high speed or jump of a cliff without getting hurt (123). Furthermore, withdrawal symptoms, characterised by depression, fatigue, insomnia, suicidal thoughts and craving for the steroids, have been reported when the drug intake is stopped (22, 23, 39, 58, 65, 123, 124, 148, 154, 155).

Aggression, particularly in response to provocation, is one of the most commonly reported psychiatric side effects (for review see (9)). This effect may be desirable when training for example strength, but devastating during daily activities and for family life. Verbal aggression and violence against their significant other have been reported among AAS users (27, 37, 126, 154). Case reports show that individuals, without prior drug abuse or psychiatric disorders, turn violent when using AAS. As for example, one AAS user threw a brick at his girlfriend, another picked up his girlfriend and flung her across the room, yet another fractured several bones in his girlfriend's hand by squeezing it (27). The combination of AAS and alcohol made one AAS user lose his temper when his wife said something inappropriate. He beat her up so badly that she died from her injuries (37). Furthermore, AAS has been

associated with violent crimes including homicide, assaults and vandalism. A traffic jam made one AAS user so furious that he started to attack the other cars stuck in the jam using his fists and a metal bar (123). Another user got paranoid ideas about his ex-girl friend and placed a bomb underneath her car (122). Some victims of homicides have also been reported to be AAS users (154). The victims often knew their predator and initiated the fight that lead to their death, themselves. Violent behaviour in an AAS user is often induced by minimal provocation, has great intensity and long duration. They seem to lose their impulse control. These characteristic, unprovoked and sudden outbursts of aggression after AAS abuse are known as roid rage (27, 37). Statement from the Swedish police confirm that AAS are used to increase aggressiveness and or self-confidence by various criminal groups (153).

Controlled laboratory studies of healthy volunteers (61, 78) as well as animal studies confirm that AAS treatment induce aggression (19, 64, 88, 92, 100, 128). The neurochemical mechanisms behind AAS-induced aggression and the other psychiatric side effects are not clear. Nevertheless, animal studies have shown that AAS affect serotonin (50, 84, 152), corticotropin-releasing factor (CRF) (131), substance P (60) and opioid (28, 29, 83, 102, 131) systems as well as NMDA (81), GABA (14, 15, 96-98) and dopamine receptors (75) in brain regions regulating reward, aggression and other emotional states. It can be concluded that AAS action on the CNS is complex and involves many different neurotransmitters.

Placebo controlled studies in humans as well as studies of real-life AAS users, show that not every AAS user will get severe psychiatric side effects (18, 61, 116, 123, 124, 144). The whole spectrum exist, from few mild effects, such as increased energy or anxiety, to psychosis and suicidal attempts. With today's limited knowledge about the mechanisms behind the psychiatric side effects, it is impossible to know who will be affected the most. Disinhibitory psychopathology (personality disorders with behaviours such as for example impulsiveness, aggression and violent behaviour) has been reported among individuals abusing AAS (22, 51, 114, 116, 122, 123, 126, 127, 144, 163-165). Whether AAS abuse induces psychopathological behaviour, or if individuals with these personality disorders abuse AAS as a result of their disorder is still unclear and the question is controversial. It has been suggested that antisocial personality disorder trigger AAS use since common personality characteristics for both alcoholics and AAS users has been found (165). On the other hand, disinhibitory psychopathology has been found among AAS users without prior history of drug abuse or psychiatric disorders, suggesting that use of AAS induce these kind of behaviours (37, 51, 123, 127, 144).

### *1.1.7 Dependence of Anabolic Androgenic Steroids*

The question whether AAS induce dependence or not is controversial. Nevertheless, AAS dependence has been reported in a number of case reports (21, 38, 65, 123, 169). Although AAS is not on the "list of dependent substances", it has been sug-

gested that dependence of AAS could be assessed using the American Psychiatric Association's criteria for substance dependence outlined in the Diagnostic and Statistical Manual of Mental disorders (DSM, Table 1) (22, 23, 39). To be regarded as dependent of a drug, one should have at least three of the symptoms on the DSM IV criteria with in a 12-month period. For the older version of the DSM criteria (DSM III-R) as used in some of the studies cited here, the symptoms should persist during at least one month.

**Table 1 DSM-IV criteria for substance dependence**

A maladaptive pattern of substance use leading to clinically significant impairment of distress, as manifested by three (or more) of the following, occurring at any time in the same 12-month period:

1. Tolerance, as defined by either of the following: a. a need for markedly increased amounts of the substance to achieve intoxication or designed b. effect markedly diminished effect with continued use of the same amount of the substance
2. Withdrawal, as manifested by either of the following: a. the characteristic withdrawal syndrome for the substance b. the same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms
3. The substance is often taken in larger amounts or over a longer period than was intended
4. There is a persistent desire or unsuccessful efforts to cut down or control substance use
5. A great deal of time is spent in activities necessary to obtain the substance (e.g., visiting multiple doctors or driving long distances), use the substance (e.g., chain-smoking), or recover from its effects
6. Important social, occupational, or recreational activities are given up or reduced because of substance use
7. The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance (e.g., current cocaine use despite recognition of cocaine-induced depression, or continued drinking despite recognition that an ulcer was made worse by alcohol consumption).

The incidence of AAS dependence varies among the different studies. At least one symptom of dependence was reported in 78%(39), 94% (22) and 100% (23) of the subjects in the different studies cited here. Three or more symptoms were reported in 75%, 57% and 23% of the subjects, respectively. These subjects met the DSM criteria for dependence. Withdrawal was the dependence symptom that was reported most frequently.

The biochemical mechanisms behind a possible development of AAS dependence are still poorly understood. Self-administration and other reliable animal models for measuring brain reward are difficult to apply to AAS, since AAS generally have slower onset and longer duration than classical drugs of abuse (90). Nevertheless, one way to investigate the abuse potential and the risk of development of dependence after use of AAS is to study the steroids' effect on neurotransmitters known



to regulate the rewarding effects of other drugs of abuse (22, 70). The endogenous opioid peptides are an example of neurotransmitters regulating reward.

## **1.2 Opioids**

*Papaver somniferum*, or opium poppy, has been used for therapeutic and recreational purposes for thousands of years. The active principle in opium was isolated by Sertürner in 1806 and was named morphine after the Greek god of dreams, Morpheus. Morphine and morphine-like drugs, so called opioids or opiates, produce various effects including rewarding, respiratory depression, myosis, analgesia as well as modulation of gastrointestinal functions (59). Tolerance to these effects develops to a varying degree and the opioids are strongly addictive (59). In this thesis the term opioid will be used for all substances with opiate-agonistic action. Endogenous and exogenous opioids can be distinguished, depending on whether the substances are normally present in the body or not.

### *1.2.1 Opioid Receptors*

In 1973, the laboratories of Simon, Snyder and Terenius independently reported the biochemical discovery of opioid receptors (118, 139, 150). The receptors bound opioid drugs in a stereoselective manner and were found to be integral membrane proteins located on the cell surface of neurones in the brain. It was soon postulated that different types of endogenous opioid receptors existed (95) and in the early 1990's the three "classical" opioid receptors ( $\mu$ -,  $\delta$ - and  $\kappa$ ) were cloned (26, 45, 71, 103). All opioid-receptors are G-protein couples receptors with an extracellular N-terminal region, seven transmembrane domains and an intracellular C-terminal structure. They share significant sequence homology with 61% identity at the amino acid level (4). In 2000, the Committee on Receptor Nomenclature and Drug Classification of the International Union of Pharmacology adopted the terms MOP, DOP and KOP to indicate the  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors, respectively (59). The MOP, DOP and KOP nomenclature is used in this thesis.

The MOP receptors are widely distributed throughout the brain and are predominantly found in the amygdala, hippocampus, Nucleus Accumbens (NAcc), striatum, thalamus and the VTA. The DOP receptors are found in the cortical areas, amygdala, NAcc and striatum. The KOP receptors are found in the NAcc, amygdala, olfactory tubercle, striatum and hypothalamus (for review see (94)).

### *1.2.2 Endogenous Opioid Peptides*

The discovery of the opioid receptors and the fact that stimulation-produced analgesia was reversible by the opioid-receptor antagonist naloxone (3) made researchers to believe in the existence of endogenous opioid peptides. The enkephalin peptides was the first of the three classical endogenous opioid peptide families to be discovered (66), and were named after the Greek word for brain, *enkefalos*. Soon after, the other

two family members were discovered, namely the endorphins (from endogenous morphine) (13) and the dynorphins (dyn after the Greek word *dynamis* = power) (56).

Each of the three families is derived from different precursors encoded by three corresponding genes, namely proenkephalin, proopiomelanocortin (POMC) and prodynorphin. Proenkephalin primarily encodes for Met- and Leu-enkephalin and extended forms of this pentapeptide such as for example Met-enkephalin-Arg<sup>6</sup>-Phe<sup>7</sup> (MEAP). The POMC encodes for  $\beta$ -endorphin and  $\beta$ -lipotrophin as well as the non-opioid peptides ACTH and MSH. Prodynorphin encodes for dynorphin A and B and neo-endorphin (for review see (158)). The precursors, typical peptide products and preferred receptor/s for each classical endogenous opioid family, are shown in Table 2. The opioid peptides share the common N-terminal sequence of Tyr-Gly-Gly-Phe (-Met or -Leu). The length of the C-terminal varies from 5 to 31 residues, with enkephalin having the shortest sequence possible for biological activity (59). Immunohistochemical studies have shown that all the classical opioid peptides are found in brain and or spinal cord regions regulating for example pain (for review see (94)). This thesis studies reward and mood regulating actions and opioids in brain regions related to these behaviours.

The anterior and intermediate pituitary glands are the two major sites for POMC biosynthesis. In the brain, POMC is found in regions such as the hypothalamic arcuate nucleus and the nucleus tractus solitarius. From these nuclei, the neurones send projections with their peptide products to reward regulating regions such as the NAcc and Ventral Tegmental Area (VTA) as well as to the Periaqueductal Gray (PAG) and amygdala that regulates affective behaviours (89). The distribution of proenkephalin and prodynorphin products is more widespread through out the brain, compared to that of the endorphins. Furthermore, they are frequently co-located with high density in for example the NAcc, PAG, hippocampus and amygdala (for review see (94)).

**Table 2.** The precursors, typical peptide products and preferred receptor/s for each classical endogenous opioid family

Opioid family	Precursor	Typical peptide	Preferred receptor
Enkephalins	Proenkephalin	Leu-enkephalin Met-enkephalin	DOP, (MOP)
Endorphins	Proopiomelanocortin (POMC)	$\beta$ -endorphin	DOP, MOP
Dynorphins	Prodynorphin	Dynorphin A and B	KOP

### *1.2.3 Biological Function of the Endogenous Opioid Systems*

As mentioned above, the exogenous opioids induce for example reward, analgesia and respiratory depression. Obviously, the endogenous opioid peptides and their receptor systems are involved in the basal regulation of these behaviours. They also involved in the rewarding aspects of other drugs of abuse (see *Section 1.3.1*) and modulate endocrine and immune functions (59). More over, since they are present in brain regions regulating affective and defensive behaviours they are involved in psychopathological conditions and aggression (107, 136, 147, 151).

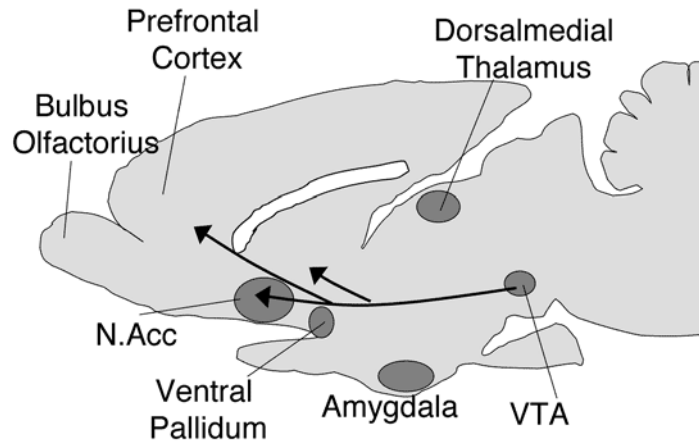
Opioid antagonists are practically inactive in a normal animal (4), thus suggests that the activity in the endogenous opioid systems are generally low. There might be a natural and functional explanation for this. If opioids were constantly released at a high level, their action would not be functional. Pain is a strong signal of injury and feeling of good mood is not functional except as a reward. Terenius (149) therefore suggests that "...opioid peptides may only be released under special, perhaps more extreme conditions such as in 'battlefield analgesia', the lack of pain the soldier feels when wounded and transported to hospital behind the front".

## **1.3 The Brain Reward System**

It is generally believed that most drugs of abuse, if not all, act through the so-called brain reward system, a general name for the mechanisms involving the brain neurotransmitter dopamine and the neural system that it regulates. Basically all drugs of abuse share the common property of increasing dopamine release in the reward system (for review see (156)). The brain reward system is also activated by natural stimuli essential for survival and reproduction, such as eating and sexual activity (119, 145).

The brain reward system, anatomically known as the mesolimbic dopamine pathway, consists of dopaminergic neurones projecting from the VTA to the Nacc and prefrontal cortex, is critical in mediating the reinforcing aspects of drugs of abuse. However, other brain regions such as the olfactory tubercle, amygdala, medialdorsal thalamus and ventral pallidum are important when translating rewarding stimuli into adaptive motor responses (see for example (69, 76)) (Fig. 2). The NAcc, perhaps the most important brain region in the reward system, can be anatomically divided into *core* and *shell*. The shell is connected with the VTA and with other reward-relevant brain regions besides the mesolimbic pathway, for example the lateral hypothalamus and medial prefrontal cortex. Therefore it may be directly involved in the emotional and motivational aspects of drug-induced reward. The core, on the other hand, is connected with the nigrostriatal system and is rather a motoric than reward regulating nuclei (59).

## Brain regions involved in the mediation of reward



**Figure 2.** Brain regions involved in the regulation of reward. The mesolimbic dopamine pathway (shown in black) project from the VTA to the NAcc and to cortical areas. Abbreviations: VTA, ventral tegmental area; NAcc, Nucleus Accumbens.

The brain reward system's involvement in reinforcement is supported by the fact that rats will self-administer microinfusions of for example amphetamine into the NAcc, or morphine directly into the VTA (for review see (158)). Furthermore, lesions of the mesolimbic dopamine pathway result in decreased intravenous self-administration of for example stimulants (for review see (76)).

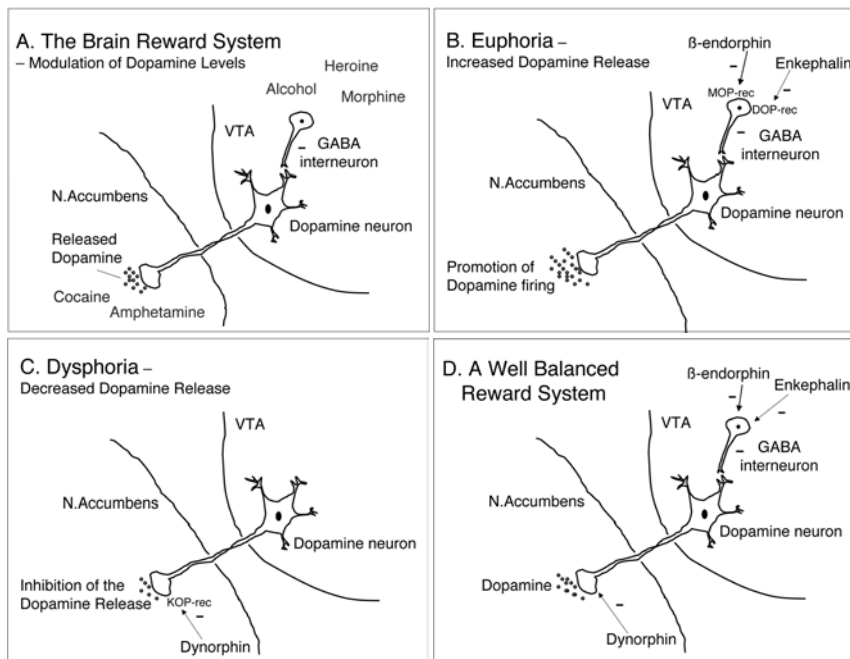
Drugs of abuse can activate the brain reward system on several levels, either by activation of dopamine directly or by altering the activity of other neurotransmitters that have a modulatory effect at various points of the mesolimbic dopamine pathway (Fig. 3A). Opioids, GABA, serotonin and noradrenergic are all neurotransmitters that influence the reward system (for review see (156)). In brief, stimulants such as cocaine and amphetamine inhibit re-uptake of dopamine in the NAcc and thereby activate the reward system. The activation by alcohol is more complex and involves GABA, the endogenous opioids and serotonin. Nicotine also increases the dopamine levels both directly and indirectly via the opioid systems (76). The next section describes in detail how opioids affect the reward system.

### *1.3.1 Regulation of the Brain Reward System by Opioid Peptides*

The opioid peptides deriving from the POMC and proenkephalin precursors are, as the exogenous opioids, addictive. In fact, rats self-administer for example enkephalin and  $\beta$ -endorphin in the same manner as they administer morphine (11, 54, 140, 159). Endogenous MOP receptor agonists, for example  $\beta$ -endorphin and enkephalin, appear to mediate their reinforcing effects by enhancing the dopamine activity

in VTA dopamine neurones. This effect is indirect since the opioids bind to MOP receptors on GABA neurones innervating the VTA. Agonists to the MOP receptors disinhibit the firing of dopaminergic neurones in this region by inhibiting GABAergic neurones that normally suppress their dopaminergic neighbours (Fig. 3B). The disinhibition results in increased dopamine release in the NAcc (for review see (156)). In addition an enkephalinergic neurone has been proposed to project from the NAcc to the VTA, possibly having a positive feedback mechanism on the dopamine firing (68).

The KOP receptor agonists (e.g. dynorphin), on the other hand, are aversive in both human and rat (133). In general, dynorphin has opposing effects than enkephalins and endorphins. By activating KOP receptors in the NAcc, dynorphin cause a *decrease* in dopamine release (Fig. 3C) (142). Thus, instead of euphoria it produces mild dysphoria. It has been speculated that the dysphoria seen during the withdrawal phase after a drug intake might be caused by increased levels of dynorphin in the NAcc (135). There is thus a balance in the normal animal, between the rewarding (i.e. POMC and proenkephalin) and aversive (i.e. prodynorphin) opioid systems to maintain a neutral or stable state of mind (Fig 3D).



**Figure 3.** A simplified sketch over the modulations of the brain reward system. **Panel A.** Dopamine neurones projects from the VTA to the NAcc. These dopamine neurones are tonically inhibited by GABA interneurones. Different drugs of abuse modulate the brain reward system at different levels with the same goal of increasing the dopamine content in the NAcc. **Panel B.** Euphoric effects: MOP and DOP receptors are present on the GABA interneurons. Agonists to these receptors (e.g.

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$\beta$ -endorphin and enkephalin) remove the GABA inhibition and thereby increase the dopamine release in the NAcc. **Panel C.** Dysphoric effects: Agonists to the KOP receptors on the terminals of the dopamine neurones inhibits the release of dopamine in the NAcc. **Panel D.** A well balanced reward system with tonic control of the endogenous opioid peptides. Abbreviations: DOP-rec, delta-opioid receptor; GABA, Gamma-Aminobutyric acid; KOP-rec, kappa-opioid receptor; MOP-rec, mu-opioid receptor; NAcc, nucleus accumbens; VTA, ventral tegmental area.

#### **1.4 Immediate Early Genes**

Detection of c-Fos, the protein product of the immediate-early gene c-fos, provides information about the sites of action of various pharmacological, electrical or physiological stimuli in the brain regardless of the mechanisms of action (67, 106). The basal level of c-Fos expression is usually low or absent. Following a stimulus, the classical activation of c-fos is a rapid and transient induction (106). Nevertheless, a more long-lasting expression of Fos related antigens, for days or weeks, has also been observed (105). Stable proteins of the Fos family, which are variants of Delta Fos B, have now been found following chronic perturbations of the brain, and these stable proteins have been proposed to show long-term neural plasticity and behavioural changes (25, 111).

## **2 Aim of the Thesis**

The general aim of this thesis was to study biochemical and behavioural effects in the rodent after chronic treatment with the anabolic androgenic steroid nandrolone decanoate.

More specifically, this thesis investigates:

- the effect of nandrolone on the endogenous opioid peptides in rat brain regions regulating reward and aggression
- in which brain regions of the guinea-pig nandrolone induce expression of Fos-related antigens
- whether nandrolone affects voluntary alcohol intake in the rat
- if nandrolone affects defensive behaviours such as fleeing, freezing and defensive aggression in the rat
- if amphetamine-induced aggression is enhanced in nandrolone pre-treated rats

## **3 Material and Methods**

### **3.1 Animals**

We used adult male Sprague Dawley (Alab, Sollentuna, Sweden; Paper I, II and V) or Wistar (Møllegaard Breeding Laboratories, Denmark; Paper III) rats that weighed 200 – 300 g at the beginning of the experiments. They were housed in clear plastic cages under standard conditions with constant temperature and humidity with lights on 6 a.m. – 6 p.m. (Paper I, II and V) or 10 p.m. – 10 a.m. (reversed daylight cycle; Paper III). The rats had free access to tap water and food pellets (R36 (Paper I, II and V) or R34 (Paper III) (Labfor, Lactamin, Vadstena, Sweden)) throughout the experiments. The rats were housed four per cage except during alcohol consumption experiments when they were housed individually (Paper III). The animals were kept in the animal room for two weeks before the start of the experiments to get adapted to the laboratory environment. During the adaptation period, each animal was handled for two minutes at least three times to get used to the experimenter.

In Paper IV adult, male, coloured guinea-pigs weighing 450 – 660 g were housed individually before and during the experiment in an animal room kept at 23°C and on a 12-h light-dark cycle. Food and water were available ad libitum and a vitamin supplement (Penta-vite, Nicholas, Australia) was added to the drinking water daily. The animals were acclimatised to the laboratory environment for at least 48 hours before the experiment started.

#### *3.1.1 Ethical Approval*

The experiments conducted in Paper IV was approved by the Animal Care and Ethics Committee of the University of Newcastle, Australia. All other experiments in this thesis were approved by local ethical committees of the Swedish National Board for Laboratory Animals.

### **3.2 Drug Treatment**

#### *3.2.1 Anabolic Androgenic Steroid Treatment*

In all papers, the AAS nandrololone decanoate (Deca Durabol®, Organon, Netherlands), at a dose of 15 mg/kg body weight, was used. In Paper I an additional dose of 5 mg/kg was used. The dose of 15 mg/kg induce plasma levels of 35-40 ng nandrolone/ml at the end of the two-weeks-treatment period (unpublished data) and was chosen to mimic the high doses of AAS that are abused. The animals were treated with one daily injection for 14 days. The drug was given i.m. in all studies except in Paper III where it was given subcutaneously. The control animals received an oil vehicle (arachidis oleum, Apoteket AB, Sweden, Paper I-III and V; peanut oil,



Sigma; Paper IV). In Paper II, III and V we introduced a recovery period, without any drug treatment, after the last AAS injection prior to sacrificing or treatment with amphetamine or alcohol.

### *3.2.2 Voluntary Alcohol Intake (Paper III)*

The voluntary alcohol intake was measured using the two-bottle free-choice paradigm. The rats were given continuous access to a second bottle containing an ethanol solution, in addition to the water bottle, following a one- or three-week-recovery period after the last of 14 daily AAS or oil injections. The rats were habituated to drink ethanol over a two-week period, when the ethanol concentration was gradually increased (2-4-6 % v/v). The animals were subsequently housed individually during three consecutive weeks with free access to two bottles, one containing tap water and one 6% ethanol solution. This particular ethanol concentration stimulates peak levels of consumption in the Wistar rats (62). Fluid consumption was recorded daily at 9.00 a.m. and 3.00 p.m. Ethanol intake is expressed as g/kg/day of absolute ethanol, and ethanol preference as proportion of ethanol solution intake relative to total fluid consumption in percent.

### *3.2.3 Amphetamine Challenge (Paper V)*

Three weeks after the completed two-week-period of AAS or vehicle injections, one group of the AAS-treated and one of the vehicle-treated rats were challenged to a single dose of amphetamine (5 mg/kg). One of the control groups and one group of the AAS-treated rats were given a single dose of saline at the same time. All these four groups of animals were subsequently tested for defensive aggression (see *Section 3.4.1*) and sacrificed by decapitation two hours after the amphetamine or saline injection. The remaining batch, one AAS and one control group were kept in the animal house for an additional three weeks (in total six weeks of recovery after the last AAS injection) and then challenged with amphetamine in the same manner as the former groups. All groups consisted of 8 animals.

## **3.3 Biochemical Analysis**

### *3.3.1 Dissection*

After decapitation, the brains were sliced in coronal sections using a rat brain matrix (Activational System Inc., Mortella Drive Warren, MI, U.S.A). Discrete brain regions were dissected according to the rat brain atlas of Paxinos and Watson (115) and immediately put on dry ice. The tissue from each individual animal was put in an eppendorf tube and kept at -80°C until further processing.

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### *3.3.2 Radioimmunoassay (Paper I-III)*

Tissue samples from each independent rat were homogenised by ultrasonification in 1M preheated acetic acid (90°C) and peptides were extracted using ion-exchange chromatography (SP-Sephadex C-25 gel) as described in Paper I-III. A detailed description of the radioimmunoassays (RIA) can be found in each paper. The technique is described briefly below.

The RIA-technique is a sensitive and specific method to quantify concentrations of various peptides, hormones and steroids in biological tissues. In these assays, the peptide competes with a radiolabelled peptide to bind to a specific antibody. Thus, some antibody-peptide complexes will be radioactive and some non-radioactive. Free radioligand can thereafter be separated from the antibody-bound complex and the total radioactivity of the sample is measured. The concentration of the peptide is calculated from a standard curve with known concentrations.

One of the limitations with the RIA is the difficulty to determine whether the found changes in immunoreactivity correspond to an altered secretion or biosynthesis of the neuropeptides. However, based on the chronic treatment, we believe that the altered levels seen in this thesis correspond to an up or down regulation of the peptide systems and not a sudden change in peptide release.

The MEAP- and  $\beta$ -endorphin-RIA were based on the charcoal adsorption technique (30). Before the MEAP-RIA, the evaporated fraction containing MEAP material was oxidised by reconstitution in 100  $\mu$ l 1M acetic acid and incubation for 30 minutes at 37°C with 10  $\mu$ l 30% H<sub>2</sub>O<sub>2</sub>. The sample was incubated with antiserum and labelled peptide for 24 h. After incubation, a charcoal suspension was added to the samples. The sample was mixed, incubated for ten minutes and then centrifuged for one minute. The radioactivity in the supernate was determined in a gamma counter. Quantitative measurements of peptide in the tissue samples were made by comparison with a standard curve with known concentration of the peptide. The  $\beta$ -endorphin-ir found in the VTA was characterised using reversed-phase HPLC as described in Paper I. It was found that the  $\beta$ -endorphin-ir material eluted at a retention time coinciding with that of synthetic  $\beta$ -endorphin. This result was consistent with previous characterisations of the  $\beta$ -endorphin antibody used in this study (137). A similar identification of MEAP- and dynorphin B-ir in the rat CNS tissues has previously been described (113).

All antisera were raised in rabbits against the peptide-thyroglobulin conjugate as described elsewhere (53). The MEAP antiserum was raised against the oxidised analogue of MEAP and the cross-reactivity was 0.5% with Met-enkephalin-sulphoxide and less than 0.1% with Met-enkephalin, Met-enkephalin-Arg<sup>6</sup>Gly<sup>7</sup>Leu<sup>8</sup>, Leu-enkephalin-Arg<sup>6</sup> and Leu-enkephalin (113). The MEAP peptide was studied as a marker for the proenkephalin system. The cross-reactivity for the  $\beta$ -endorphin

antiserum was 100 % with  $\beta$ -lipotropin and less than 1% for hemorphins, Met-enkephalin, Leu-enkephalin, dynorphin A and B (137).

The dynorphin B-RIA was based on the double-antibody precipitation. Samples, antiserum and labelled peptide were incubated for 24-hours (4°C). To separate free and antibody-bound peptide, the samples were incubated for an additional hour at 4°C with sheep anti-rabbit antiserum (Pharmacia decanting suspension 3, Pharmacia Diagnostics, Uppsala, Sweden). After centrifugation, the radioactivity in the pellets was counted in a gamma counter. The cross-reactivity was 100% with dynorphin 32 and 1% and with dynorphin 29 (113). Dynorphin B was studied as a marker for the prodynorphin system.

### *3.3.3 Immunohistochemistry (Paper IV)*

Immunohistochemistry is a sensitive technique where you can study antibody binding to different antigens in for example the CNS. An advantage over the RIA is that you when slicing the brain into thin sections are able to look into discrete nuclei within specific brain regions rather than the whole brain region content as you measure with the RIA.

Approximately 24 h after the last AAS or oil injection the guinea-pigs were given a lethal i.p. injection sodium pentobarbitone. After transcardial perfusion of heparinised PBS followed by 4% paraformaldehyde, brains were removed, post-fixed, cryoprotected and cut into coronal sections (50  $\mu$ m). Details of the immunohistochemical method (ABC method) are described in Paper IV.

The polyclonal goat Fos antibody (Santa Cruz K-25) used in Paper IV was raised against a conserved domain of c-Fos p62 of human origin, identical to mouse, rat and chicken sequences. This Fos antibody recognises a conserved region common to several members of the Fos family, including c-Fos, Fos B, and Fos related antigens (Fra-1 and Fra-2). The c-Fos(4) antibody from Santa Cruz, on the other hand, is highly selective for c-Fos and does not recognise other Fos proteins or Fos-related antigens. Since the Fos staining observed with the less specific antibody may be produced by the presence of various proteins of the Fos family, each of which may be synthesised and degraded at a different rate, this staining is referred to as Fos-related antigens. Staining with the specific c-Fos antibody is referred to as c-Fos. Sections incubated without primary antibody developed no staining.

For qualitative measurements we stained sections from the VTA from one AAS treated and one control guinea-pig with a  $\beta$ -endorphin antibody. The antibody was raised in rabbits and kindly donated by Professor Roger Smith at the John Hunter Hospital, Newcastle, Australia

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After staining with 3,3'-di-aminobenzidine (0.033%), brain sections were examined using a light microscope. Coronal sections were taken at comparable rostrocaudal levels for cell counting in each animal. All labelled cells in a single section were counted twice and a mean value of the counts was calculated.

#### *3.3.4 Physiological Measurements*

The body weight of the animals in Paper II, III and V was recorded once weekly. In paper V, the body weight of the guinea-pigs was recorded daily. The wet weight of the thymus was recorded at the time of decapitation (Paper II, III and V).

### **3.4 Behavioural Studies**

#### *3.4.1 Defensive Aggression (Paper III)*

Defensive aggression is a form of aggression sometimes called hyper-reactivity or hyperirritability. This defensive reaction, occurs in response to a real threat but also to perceived provocation, for example elicited by innocuous stimuli as reaction towards the experimenter. Defensive aggression, in contrary to social aggression, is suggested to constitute the closest approximation in non-primate species to human aggression (5).

The rat was placed in a Plexiglas cage (50 cm in diameter and 41 cm high) and allowed to habituate for 30 seconds. The rats defensive reactions towards 4 different stimuli (I, II, III and IV) was thereafter recorded (modified from (12, 46, 82)). The different stimuli and the scoring for each test are described in Table 3. The total score across the four tests (maximum total score is 10), as well as the scores from each individual test were used in the statistical calculations.

In Paper III the defensive aggression test was conducted at two different occasions for each animal, first in connection with the last AAS or oil injection and secondly, immediately after the end of the three-week-alcohol-consumption period.

In Paper IV, all animals were subjected to the defensive test immediately after the two-week-treatment period. The animals were thereafter subsequently tested 30 minutes after the amphetamine or saline injection three or six weeks after the last AAS or oil injection.

**Table 3.** Description of the different stimuli that constitute the defensive aggression test

Stimuli	Scoring (points)
<b>I:</b> A wooden rod was moved slowly to approach and touch the snout of the rat	<b>0:</b> No response or sniffs at the rod <b>1:</b> Intermittently bites or attacks of the rod and/or adopts a defensive upright posture <b>2:</b> Continuously bites/attacks of the rod
<b>II:</b> Startle to an air puff at the back (air blown from a 50 ml syringe)	<b>0:</b> No response or some movement <b>1:</b> Jumping response <b>2:</b> Exaggerated jumping response
<b>III:</b> Poking with a wooden rod at the flanks	<b>0:</b> No response or sniffing at the rod <b>1:</b> Defensive upright posture <b>2:</b> Defensive upright posture together with biting/attack
<b>IV:</b> Capturing with a gloved hand	<b>0:</b> Very easy to capture <b>1:</b> Easy to capture but some resistance and/or prolonged vocalisation <b>2:</b> Difficult to capture because of escape <b>3:</b> Difficult to capture because of attacking or biting <b>4:</b> Very difficult to capture because of continuous violent attacks/bites

### 3.4.2 Fleeing and Freezing (Paper III)

Fleeing and freezing are two expressions of the motivational system. They are naturally occurring defence reactions, presumably reflecting fear in a threatening situation. For example rodents freeze to avoid being detected by a raptor circling in the sky. Due to its sensitivity to the anxiolytic drug diazepam (63), the freezing reaction may be regarded as a fear/anxious reaction.

The rat was placed in a circular Plexiglas cage (diameter 39 cm) and allowed to habituate for 5 minutes. The floor in the test cage was divided by two lines to form four 90° sectors. The cage was enclosed in a soundproof test chamber, illuminated by a 15 W white light bulb. The light bulb and a doorbell were placed under the chamber ceiling. The behaviour of the animal was observed through a Plexiglas window on the front wall. During the adaptation period, the following open-field behavioural items were observed: first crossing (latency to leave the sector where the animal was first placed), locomotor activity (number of lines crossed by the hind legs), rearing (number of raisings on hind legs), grooming (cumulative time recorded) and defecation (number of boli deposited). After the adaptation period, the doorbell (95 dB) subsequently sounded for 6 sec and the rat would attempt to flee. The number of lines crossed by the animal during the signal was recorded and

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used as a measure of flight distance. The rat froze concurrently with, or slightly before, termination of the doorbell. In both cases, the duration of the freezing reaction was defined as the beginning of the cessation of the bell sound and ending with the first distinct movement of some part of the body (usually the head), excluding eye blinks and respiratory or vibrissae movements.

### *3.4.3 Behavioural Study in Connection with the Daily Injection (Paper IV)*

Thirty minutes before each injection in the study conducted in Paper IV, the animals were placed individually in activity cages. During these 30 minutes and an additional 30-minute period following each injection, animals were observed by a trained observer who quantified their behaviours (rearing, grooming, face washing, scratching, head/body shakes, biting, vocalising, chewing and sneezing). The guinea-pigs locomotor activity was also measured in the activity cages equipped with a single infrared photocell and detector on the long axis located 5 cm above the floor and 14 cm from either end of the cage. Every crossing of the photo beam that occurred at least 1.5 seconds apart, was recorded on a digital counter. This time-delay was chosen to avoid measurement of small body movements.

## **3.5 Statistics**

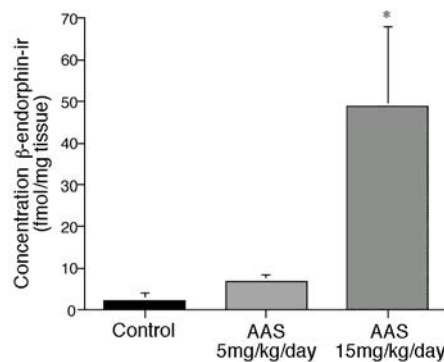
Student's *t*-test (or analysis of variance (ANOVA) followed by Scheffe's *F*-test where appropriate) was used for the statistical analysis of peptide-ir, and biological measurements (Paper I-III and V), immunohistochemical data (Paper IV) as well as for between-group comparisons of defensive behaviours (Paper III and V) (StatView, Abacus®). All values were expressed as mean  $\pm$  SEM or SE. Two-way-analysis of variance for repeated measures was used to analyse behaviour data in Paper IV (GraphPad Prism3<sup>®</sup>, GraphPad Software Inc). Correlation analysis was made using the StatView 512+ software, followed by calculations of the *P*-value (Paper II). Non-parametric methods (Mann-Whitney *U*-test and Wilcoxon matched-pairs signed-ranks test) were used for statistical analysis of drinking data, because the ethanol intake does not follow the normal distribution (62). Drinking data were presented as median  $\pm$  Median Absolute Deviation (MAD).

## 4 Results and Discussion

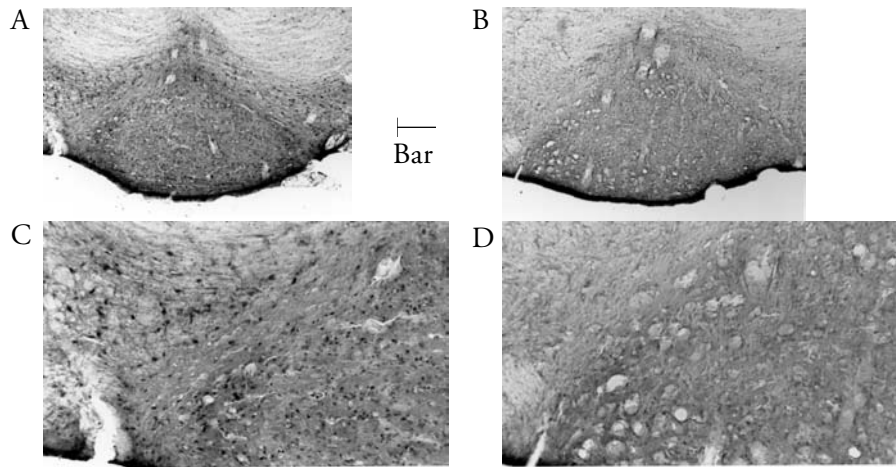
### 4.1 The Effect of Anabolic Androgenic Steroids on Opioid Peptides and Fos-like Immunoreactivity

#### 4.1.1 The Effect of Anabolic Androgenic Steroids in the Brain Reward System (Paper I, II and IV)

The major finding of Paper I was the marked increase of opioid peptide  $\beta$ -endorphin-ir in the VTA, a region in the brain reward system, following 14 days of high-dose AAS treatment (5 and 15 mg/kg/day of nandrolone decanoate) (Fig. 4). The dose of 5 mg/kg/day fail to induce any significant changes. The POMC system has previously been connected with the action of AAS (28, 29, 83, 102, 131). Castration of male rats has been shown to decrease the POMC mRNA signal in the arcuate nucleus of hypothalamus. The decrease was restored by physiological doses of testosterone (28, 29). Another study found that a cocktail of AAS, decrease  $\beta$ -endorphin-ir in the same region and the authors suggested that treatment result in a transport of  $\beta$ -endorphin-ir from the soma of the neurones in the nucleus arcuate to terminal field locations (102). Previously unpublished data gives further support for an AAS-induced  $\beta$ -endorphin-ir in the VTA (Fig. 5). Although it is impossible to quantify the number of neurones (due to methodological considerations and the limited number of animals), there is a trend showing more staining in the AAS treated guinea-pig compared to the control. In conclusion, it is clear that AAS affect the POMC system and Paper I suggest that the increased  $\beta$ -endorphin found in the VTA is a result of an activation of POMC neurones in arcuate nucleus terminating in the VTA.

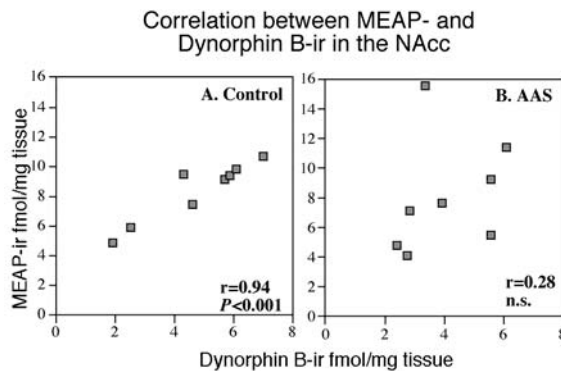


**Figure 4.** The  $\beta$ -endorphin-ir (fmol/mg tissue) in the VTA of rats treated with the AAS nandrolone decanoate (daily i.m. injections of 5 or 15 mg/kg for 14 days) and controls. Measurements were made on the 15th day of the experiment. The values are expressed as mean  $\pm$  SEM. \* $P < 0.05$  compared to controls;  $n = 8$ . Abbreviations: AAS, anabolic androgenic steroids; VTA, ventral tegmental area.



**Figure 5.** Photomicrographs showing  $\beta$ -endorphin-ir staining in the VTA of a nandrolone treated (15 mg/kg/day for 14 days) and a control guinea pig treated with peanut oil vehicle at low (Panel A, AAS; Panel B, control) and high power (Panel C, AAS; Panel D, control). Bar = 200  $\mu$ m, Panel A and B; 100  $\mu$ m, Panel C and D. Abbreviations: AAS, anabolic androgenic steroids; VTA, ventral tegmental area.

In Paper II, using the same steroid treatment as in Paper I, nandrolone induced an imbalance between the dynorphin- and the enkephalin-opioid systems in the NAcc. Thus, we found a positive correlation between dynorphin B-ir and MEAP-ir levels in the control animals (Fig. 6A). The correlation was altered in the AAS-treated animals (Fig. 6B). The imbalance between the two systems remained after a three-week-recovery period without any drug treatment, indicating that AAS induce long lasting changes in the endogenous opioid systems.



**Figure 6.** Correlation between dynorphin B-ir and MEAP-ir (fmol/mg tissue) in the nucleus accumbens of control (Panel A) and (Panel B) AAS-treated animals (daily i.m. injections of 15 mg/kg for 14 days).



In Paper IV we examined which brain regions that expressed Fos protein after 14 days of nandrolone treatment (15 mg/kg/day) in the guinea-pig. We found Fos-related antigen staining in several brain regions including the frontal cortex (layer V) and the shell of NAcc of the AAS treated animals (Table 4).

**Table 4.** Number of neurones expressing Fos-related antigens in regions of guinea-pig brain following chronic treatment with the AAS nandrolone decanoate (15 mg/kg/day for 14 days) or vehicle (controls).

	Control	AAS
N. Accumbens shell (n=5)	0 ± 0	22 ± 6**
Frontal Cortex (n=6)	30 ± 19	153 ± 28**

Analysis was made from comparable sections with strongest staining visualised under bright-field microscopy. Values are means of total number of neurones containing Fos-related antigens ± SEM \*\* $P < 0.01$  compared to controls.

As mentioned earlier, the VTA and NAcc are the two brain regions that are generally believed to mediate reward from drugs of abuse (76). It is interesting that AAS induce changes in these brain regions since case reports suggests that prolonged use of high doses of AAS may induce dependence (21, 38, 65, 123, 148, 169). Furthermore, AAS users have in controlled studies been classified dependent according to the DSM criteria (22, 23, 39). Withdrawal symptoms including depression, suicidal ideas and headaches upon discontinuation of AAS use, was the most commonly reported criterion of dependence. Other criteria were: the AAS user took higher doses over longer periods than initially intended, continued to use AAS despite negative consequences and renounced important events in favour to use of AAS and related physical training. (22, 23, 39). Interestingly, a case report has shown that an AAS abuser exhibit an opioid-like withdrawal syndrome when challenged with the opioid antagonist naloxone (148). Furthermore, opioid abuse and dependence has been reported among AAS users (7).

The neurocircuit from the VTA to the shell of NAcc forms the brain reward system, or the mesolimbic dopamine pathway, and basically all drugs of abuse increase the dopamine firing in this circuit (for review see (156)). The endogenous opioids are some of the neurotransmitters involved in the regulation of this dopamine activity (76). The increased Fos expression in the shell of the NAcc (Paper IV) confirms that AAS induce neural activity in the reward system. The increased  $\beta$ -endorphin-ir in the VTA (Paper I) together with the imbalance between the dynorphin and enkephalin systems in the NAcc (Paper II), suggests a biological explanation to the dependence theory.

*Pia Steensland*

As mentioned in the section *Introduction*, activation of the brain reward system by a MOP receptor agonist is conducted as follows. The VTA contains GABA interneurons on which MOP receptors are located (76). These GABAergic neurons have an inhibitory effect on connecting dopaminergic neurons projection to the NAcc. When for example  $\beta$ -endorphin binds to the MOP receptors, the GABAergic inhibitory effect is disinhibited resulting in increased firing in the dopaminergic neurons and elevated DA release in the NAcc, and thereby reward (76). Thus, the AAS-induced elevation of  $\beta$ -endorphin-ir in the VTA as observed in Paper I, is likely to stimulate the reward system through the MOP receptors with increased dopamine activity and an euphoric feeling as a result (see Fig. 3B under *Introduction*).

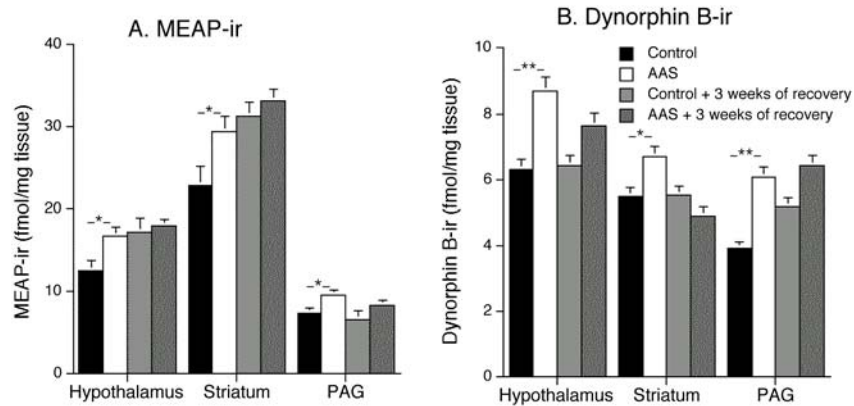
The AAS-induced imbalance between the dynorphin and enkephalin systems in the NAcc (Paper II) is interesting since the two systems generally have opposing effects. Dynorphin inhibits whereas enkephalin promotes dopamine release (142). A fine tonus between these systems, with dynorphin as the brake and enkephalin as the accelerator, helps to keep a balance between dysphoric and euphoric effects. Thus, an imbalance between these systems may cause a dysregulation of the dopamine activity with a malfunctioning brain reward system as a result, possibly making the brain more sensitive to other drugs of abuse. Long-term treatment with other psychoactive substances has previously been shown to sensitise the brain reward system (for review see (143)). One study proposes that AAS have similar properties and act directly on the brain to increase the rewarding aspects of amphetamine (34). Taken together, our results indicate that AAS may, via the action of the endogenous opioids, stimulate or affect the brain reward system and thereby induce dependence and sensitisation. Our results are strengthened by the fact that a case report has shown that AAS withdrawal can be precipitated by the opioid antagonist naloxone (148).

A recent study (75) gives further support for the AAS-dependence-theory. An alteration of the expression of dopamine receptors was found in the NAcc after treatment with nandrolone decanoate (15 mg/kg/day for 14 days). In relation to the rewarding properties of AAS, the dopamine receptor subtype  $D_2$  was downregulated in the shell of NAcc (75). The  $D_2$  receptor subtype has been suggested to be responsible for mediating the reinforcing effects of dopamine (104). The downregulation of  $D_2$  receptors was suggested to be mediated through negative feedback mechanisms induced by increased levels of dopamine. The increased levels of dopamine could possibly be induced by a MOP receptor agonist, as found in Paper I. The increased expression of Fos-related antigens in the shell of NAcc and the frontal cortex presented in Paper IV are of particular importance. It is tempting to speculate that the cortical activation reflects an activity in dopamine neurons in the mesolimbic pathway originating in the VTA and the activation of the NAcc provide direct support for the proposal that nandrolone activates the mesolimbic pathway. Despite clinical and experimental evidence of AAS dependence, the issue is controversial. The pharmacokinetics of AAS with long duration, active metabolites and

long elimination half-life, make it difficult to study their abuse liability in a classical manner. Thus, AAS are not self-administered in rat (90) and in non-steroid using volunteers, acute administration of 200 mg testosterone does not induce euphoria or a sense of well-being. In addition, the volunteers could not detect the drug over the placebo (49). However, one acute dose might not be enough, or the dose was not high enough to activate the brain reward system since euphoria and a sense of well-being in fact has been reported among active athletes after AAS use (22, 58, 144). On the other hand, the reported euphoria among active athletes might be due to pleasurable feelings induced by intense physical exercise, since increased levels of  $\beta$ -endorphin have been reported after intense exercise as for example marathon running (53). A recent study in rhesus monkey, failed to support the hypothesis that treatment with high-dose AAS enhance endogenous opioid activity in a way that produce opioid tolerance or dependence (108). However, only three monkeys were used in that study and the authors suggest that the AAS treatment might have enhanced the endogenous opioid activity, but induced too low levels of tolerance and dependence making them undetectable under the conditions used in their study (108). Nevertheless, accumulating evidence suggest that AAS should be regarded as drugs of abuse. This thesis shows that AAS do affect brain regions regulating reward. The increased  $\beta$ -endorphin-ir in the VTA and the activation of Fos-related antigens in the NAcc gives direct support for the theory that use of AAS might lead to the development of dependence. The imbalance between the enkephalin and dynorphin systems in the NAcc suggests that AAS induce long lasting changes in the reward system, changes that could make the brain more sensitive to other drugs of abuse. This is interesting since it has been suggested that AAS might serve as a gateway to addiction and dependence of other drugs of abuse (7). However, more studies need to be conducted to fully understand the mechanisms behind the activation of the reward system and a possible development of dependence after use of AAS.

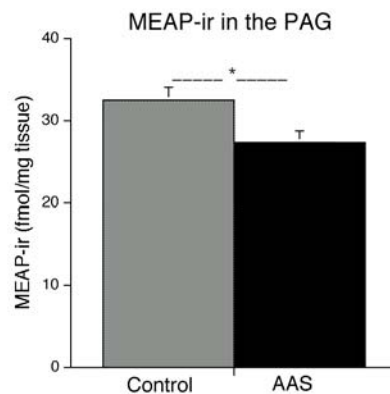
#### *4.1.2 The Effect of Anabolic Androgenic Steroids in Brain Regions Regulating Aggression and Defensive Behaviours (Paper II-IV)*

In Paper II, it was found that chronic AAS treatment (15 mg/kg/day of nandrolone decanoate for 14 days) resulted in increased dynorphin B- as well as MEAP-ir in the hypothalamus, striatum and PAG compared to controls (Fig. 7). In addition, the AAS-induced imbalance between the dynorphin and enkephalin systems, as described in the previous section, was also found in the hypothalamus (control:  $r = 0.77$ ,  $P < 0.05$ ; AAS:  $r = 0.072$ , n.s).



**Figure 7.** The MEAP- and Dynorphin B-ir (fmol/mg tissue), Panel A and B, respectively, in rats treated with the AAS nandrolone decanoate (daily i.m. injections of 15 mg/kg for 14 days) and controls. Measurements were made on the 15th day of the experiment and after a three-week recovery period. The values are expressed as mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$  compared to the control group;  $n = 8$ . Abbreviations: AAS, anabolic androgenic steroids; MEAP, Met-enkephalin-Arg-Phe; PAG, periaqueductal gray.

In Paper III, a reduction of MEAP-ir was found in the PAG in Wistar rats pre-treated with AAS followed by three weeks of voluntary alcohol consumption (Fig. 8).



**Figure 8.** The MEAP-ir (fmol/mg tissue) in the PAG of rats treated with the AAS nandrolone decanoate (daily i.m. injections of 15 mg/kg for 14 days) and controls, followed by a three-week-alcohol consumption period. The values are expressed as mean  $\pm$  SEM. \* $P < 0.05$ ;  $n = 8$ . Abbreviations: AAS, anabolic androgenic steroids; PAG, periaqueductal gray.

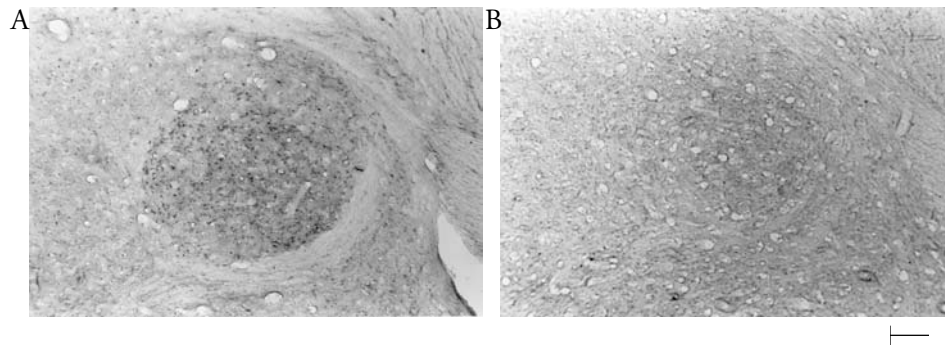
As mentioned in the section *Introduction*, the endogenous opioid system is thought to play a role in psychopathological disorders and aggression (107, 136, 147, 151). Furthermore, AAS' negative feedback control over the hypothalamic-pituitary-axis

has been suggested to be regulated by the endogenous opioids (32). The MOP and DOP receptor agonist enkephalin does not only regulate reward, but also the responses to stress, aggression and dominance (79). Activation of the KOP receptor, on the other hand, leads to dysphoric and aversive effects (134). The imbalance between dynorphin- and enkephalin-ir found in Paper II, remained after the three-week-recovery period, which indicates that AAS induce long-lasting changes in endogenous opioid systems possibly responsible for some of the psychiatric side effects.

Animal studies have previously shown that treatment with AAS induce aggression (19, 64, 88, 92, 100, 128). However, the regulation of aggression is complex and involves different neurotransmitters as for example serotonin, glutamate, vasopressin and substance P. These neurotransmitters or their receptors have previously been shown to be affected by chronic AAS treatment (60, 64, 81, 84). Enkephalin is another neurotransmitter suggested to modulate aggression in various species (77, 79). In cats for example, a powerful suppressive effect on PAG-elicited defensive rage behaviour is suggested to be mediated through MOP receptors (for review see (57)). Thus, the observed alteration in the endogenous opioid systems reported in Paper III, might also be connected to AAS-induced aggression,

In contrast to Paper II, a decrease of MEAP-ir in the PAG in AAS treated animals compared to controls was found in Paper III. This may be due to differences in drug administration, time from last AAS dose to decapitation, combination with alcohol or not. It might also reflect the difference in AAS-induced defensive aggression that we saw in the two strains. *Enhanced* enkephalin activity within the PAG *suppresses* defensive aggression in the cat (for review see (57)). Thus, decreased levels of enkephalin in the PAG should result in increased aggression. This is compatible with our behavioural results as we found increased defensive aggression and decreased levels of MEAP-ir in the PAG of Wistar rats, but no affect on aggression and increased MEAP-ir in the same brain region of Sprague Dawley rats. This suggests that AAS have reduced the ability to control aggression in the Wistar rats, possible via the enkephalin system. For a full description of the aggression results see *Section 4.2*.

The major finding of Paper IV was the marked increase expression of Fos-related antigen and cFos neurones in the central nucleus of amygdala (CeA; Fig. 9) of the AAS treated guinea-pigs compared to controls (Fos-related antigens: AAS,  $109 \pm 23$ ; control  $40 \pm 3$ ,  $P < 0.01$ ; cFos: AAS,  $25 \pm 7$ ; control  $4 \pm 4$ ,  $P < 0.05$ ). The supraoptic nuclei of the AAS treated guinea-pigs also had significantly more neurones stained with Fos-related antigens than the controls (AAS,  $28 \pm 3$ ; control,  $12 \pm 3$ ,  $P < 0.01$ ;  $n=6$ ).



**Figure 9.** Photomicrographs showing Fos-related antigen positive neurones in the CeA of a nandrolone treated (15 mg/kg/day for 14 days) and a control guinea-pig treated with peanut oil vehicle (Panel A, AAS; Panel B, control) Bar = 200  $\mu$ m. Abbreviations: AAS, anabolic androgenic steroids; CeA, Central nucleus of Amygdala.

The CeA regulates behaviours such as defensive aggression, anxiety, and a variety of psychopathological disorders including depression (see for example (1, 41, 57, 80)). Such behaviours are seen after AAS abuse, both in humans and in laboratory animals (see for example (9, 84, 172)). Furthermore, the activation of Fos in the CeA can be related to the increased biting behaviour seen in the AAS treated guinea-pigs (data not shown). It is unknown whether biting behaviour in guinea-pigs is an expression of aggression or a response to stress. Whatever the behavioural response may express, it is likely that the CeA is involved since it also regulates the expression of behavioural, autonomic and neuroendocrine components of stress as well as aggression (17, 57).

The activation of the CeA may be a result of direct action of nandrolone in the CeA or from an indirect effect via activation of other brain regions, since the CeA receives extensive inputs from the brainstem as well as from higher levels such as the cortex and thalamus. However, it is most likely that the effect of nandrolone was exerted directly on CeA, since both androgen and glucocorticoid receptor immunoreactivities are dense in this region (129, 138).

The induction of Fos proteins is often a rapid and acute effect. Nevertheless, the Fos-related antigen Delta Fos B is expressed in the brain following chronic treatments with for example cocaine and morphine (25, 111). Eventhough the identity of the Fos proteins reacting with the non-specific Fos antibody used in the present study is unknown, it is possible that chronic nandrolone treatment induced the expression of Delta Fos B, which would explain the long-lasting changes in behaviour induced by AAS.

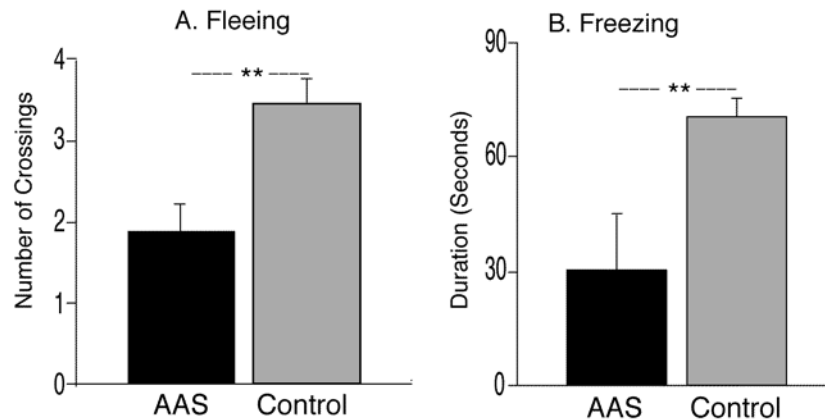
Since AAS affects various neurotransmitters, it is difficult to speculate which specific transmitter being responsible for the Fos induction in each brain region. Nevertheless, it is tempting to speculate that vasopressin is responsible for the increased numbers of Fos-related antigen neurones observed in the supraoptic nucleus in nandrolone treated guinea-pigs since this neurotransmitter has been implicated in social and aggressive behaviours in rodents (170). Vasopressin neurones project from the supraoptic nucleus and terminate in the anterior hypothalamus (47). Microinjections of vasopressin into the anterior hypothalamus increase offensive aggression (48) and microinjection of a vasopressin antagonist into the same region of AAS pre-treated hamsters decreased the AAS-induced aggression (64). Interestingly, an increase in the vasopressin content in neurones in the anterior hypothalamus has been found after AAS treatment (64). The expression of Fos-related antigens in the supraoptic nucleus as found in Paper IV in this thesis may therefore be induced by vasopressin that will concurrently be released downstream in the anterior hypothalamus.

As described in the section *Introduction*, the use of AAS has been connected with psychopathology. Whether AAS induce psychopathological behaviour or if individuals with these personality disorders abuse AAS as a result of their disorder is still unclear and the question is controversial (22, 126, 165). Behaviours common in disinhibitory psychopathology, such as for example impulsiveness, aggression, violent behaviour and excessive alcohol intake characterise a subgroup of alcoholics, so-called type II alcoholism (35). Individuals in this group have an early onset of problem drinking, they abuse other drugs together with alcohol and often display impulsive and violent behaviour (35, 85). A serotonergic dysfunction has been found in these individuals (85). The “type II-behaviour” resemble behaviours seen among AAS users (51, 165). Interestingly, an altered serotonergic activity was recently found in AAS treated animals (84, 152) as well as human AAS users (40). Results presented in this thesis supports the hypothesis that AAS abuse may induce a type II personality. In particular, chronic AAS treatment induce imbalance between two endogenous opioid systems and induced Fos expression in the reward system and brain regions regulating aggression and impulsiveness, behaviours typically seen among both type II alcoholics and AAS users.

## **4.2 The Effect of Anabolic Androgenic Steroids on Aggression and Defensive Behaviours (Paper III and V)**

Fleeing and freezing are naturally occurring defence reactions, presumable reflecting fear. The flight reaction, number of lines crossed during the bell signal, was less in the AAS animals compared to controls as well as the duration of the freezing period (Fig. 10). Thus, the decreased fleeing response and duration of freezing in the AAS animals suggests a lower potential for fear reactions in a threatening situation. Anabolic androgenic steroids have previously been shown to reduce anxiety in an elevated plus-maze test (15) and to reduce vigilance and fear in a modified open-

field model (172). These results indicate that AAS possess anxiolytic-like properties, which may correspond to the hypomani-like symptoms observed in humans. Interestingly, some adolescents claim to use AAS to become braver (73).



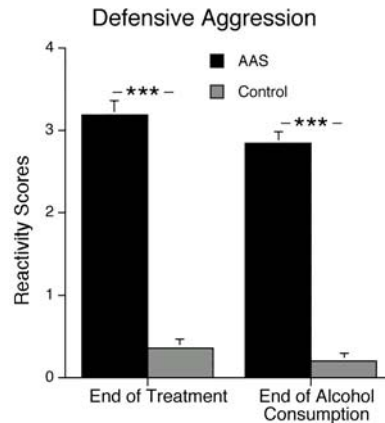
**Figure 10.** The expression of freezing (duration; seconds; Panel A) and flight reaction (number of lines crossed; Panel B) in rats pre-treated with AAS (daily i.m. injections of 15 mg/kg for 14 days) or oil (controls). The values are expressed as mean  $\pm$  SEM. \*\* $P < 0.01$  (Student's  $t$ -test);  $n = 20$ . Abbreviations: AAS, anabolic androgenic steroids.

Defensive aggression, contrary to social aggression, is suggested to constitute the closest approximation in non-primate species to human aggression (5). This defensive reaction, occurs in response to a real threat but also to perceived provocation, for example elicited by innocuous stimuli as reaction towards the experimenter. As described in the section *Material and Methods*, the defensive aggression test employed in this thesis evaluate the animal's reaction towards four different stimuli. The response to the different stimuli could be considered to reflect hyperirritability or hyperactivity rather than aggression, as for example seen after depletion of brain catecholamines when the rat respond by vocalisation or trying to flee away from the stimuli (46). However, the AAS treated animals that received high reactivity scores, in particular to stimuli IV (capturing with a gloved hand), repeatedly attacked and bit the gloved hand that was trying to capture them. Therefore, we refer to the AAS induced response to stimuli IV as defensive aggression.

When defensive aggression was tested in connection with the last AAS or saline injection in Wistar rats (Paper III), the AAS treated animals showed marked increased defensive aggression. Stimuli IV, capturing with gloved hand, gave the most significant results (Fig. 11). When the aggression test was conducted again after the three-week alcohol consumption period, the same results persisted (Fig. 11). Even though the latter test occasion was several weeks after the last AAS injection,

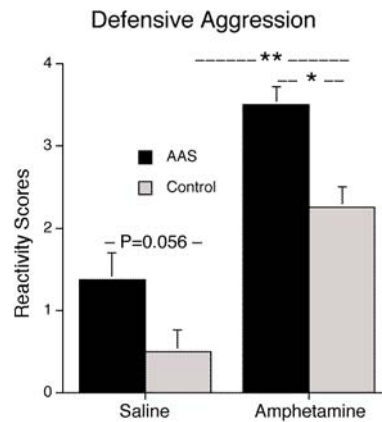


the increased defensive aggression is probably due to the AAS alone, or possibly an interaction between the AAS and ethanol, since ethanol alone does not induce defensive aggression in untreated rats (12). Ethanol might reinforce the AAS-induced aggression, supporting anecdotal stories describing roid rage after concurrent use of AAS and alcohol.



**Figure 11.** Defensive behaviour (stimuli IV: Capturing with a gloved hand) in rats pre-treated with AAS (daily i.m. injections of 15 mg/kg for 14 days) or oil (controls). The test was conducted immediately after the end of the two-week-treatment period (AAS or oil) and immediately following the end of the three-week-alcohol-consumption period. The values are expressed as mean  $\pm$  SEM. \*\*\* $P$ <0.001 (Student's  $t$ -test):  $n$ =20. Abbreviations: AAS, anabolic androgenic steroids.

In Sprague Dawley rats (Paper V) the defensive aggression test, conducted in connection with the last AAS or oil injection, showed no statistical significant difference between the groups (reactivity scores: AAS,  $2.9 \pm 1.6$ ; control,  $2.9 \pm 0.8$ ). After the three-week-recovery period the AAS animals showed a trend, however not quite significant towards being more aggressive (reactivity scores from stimuli IV: AAS,  $1.4 \pm 0.3$ ; control,  $0.5 \pm 0.3$ ,  $P=0.056$ ). However, when amphetamine was given, the total score, as well as the scores from stimuli IV, show that the AAS pre-treated animals were more aggressive than the control animals (Fig. 12). Also the amphetamine alone, induced aggression compared to those control animals that received a saline injection (Fig 12). In contrast, after six weeks of recovery there were no differences in amphetamine-induced aggression between groups (reactivity scores stimuli IV: AAS,  $1.6 \pm 0.3$ ; control  $1.9 \pm 0.3$ ). Hence, pre-treatment with AAS seems to enhance the amphetamine-induced aggression when the amphetamine challenge is given three but not six weeks after the last AAS injection. Consequently AAS may induce long lasting, but not persistent, changes in the behavioural response to amphetamine.



**Figure 12.** Defensive behaviour (stimuli IV: Capturing with a gloved hand) in rats pre-treated with AAS (daily i.m. injections of 15 mg/kg for 14 days) or oil (controls). The test was conducted 30 minutes after an amphetamine challenge (5 mg/kg) or a saline injection after three weeks of recovery. The values are expressed as mean  $\pm$  SE. \* $P$ <0.05; \*\* $P$ <0.01 (ANOVA followed by Scheffe's  $F$ -tests). Abbreviations: AAS, anabolic androgenic steroids.

Increase in aggression was seen in the AAS treated animals compared to controls in connection with the last injection in Paper III. No such difference was seen in Paper V. These conflicting results may be explained by the different rat strains used in the two studies. Sprague Dawley rats are usually less aggressive than the Wistar rats (88). A strain-difference in the defensive aggression response is further supported by the neurochemical results discussed in Section 4.1.2. Another possible explanation would be that the behavioural test in Paper III was conducted during the dark phase of the light-dark cycle, when animals are naturally awake and active, in contrary to Paper V when the test was conducted during the light phase. Thus, it is possible that tests performed during the light phase of the light-dark cycle eliminates possible differences between experimental and control animals. However, during the light phase an extra parameter such as the amphetamine challenge induced differences between the groups in Paper V.

Altered aggression has been reported after co-administration of cocaine and nandrolone using a resident intruder paradigm (88). The combination of the two drugs did not induce more aggression than either drug administered alone. However, a greater percentage of animals receiving both drugs exhibited aggression than did rats receiving either drug alone. In consistence with Paper II in this thesis, the nandrolone alone induced less aggression than expected in Sprague-Dawley rats (88).

Another kind of aggressive behaviour, namely dominant behaviour, has been reported after AAS treatment (20, 84). When competing for a water bottle serving one animal exclusively, the AAS pre-treated animals efficiently controlled the access

to the bottle over the controls. Some of the AAS rats also showed piloerection (84). The same results has been reached in other competitive situations (20).

Aggression is induced by AAS as shown in both clinical and animal studies (see for example (22, 51, 88, 114)). However, previous animal studies report AAS-induced aggression using a resident/intruder paradigm, reflecting *social* aggression (see for example (19, 64, 88)). This thesis presents that AAS induce aggression also in a *defensive* aggression paradigm. Interestingly, as mentioned above it has been postulated that defensive compared to social aggression constitute the closest approximation to human aggression in non-primate species (5). Furthermore, our results support the hypothesis that use of AAS might alter the behavioural responses of other drugs of abuse, as for example ethanol or amphetamine.

### **4.3 Interaction between Anabolic Androgenic Steroids and Alcohol (Paper III)**

In Paper III, we examined voluntary alcohol intake after pre-treatment with nandrolone decanoate (15 mg/kg/day s.c. for 14 days) followed by a recovery period of one or three weeks. The animals were adapted to the alcohol over a two-week period with increasing amounts of ethanol, and thereafter given free access to ethanol (6%) during the following three weeks. Since there were no differences in alcohol consumption between the two subgroups of AAS (one or three weeks of recovery prior the alcohol-adaptation period) or matching control animals, they were combined in their original cohorts. The AAS treated group drank more ethanol than the controls during the second and third week of voluntary alcohol consumption (Fig. 13). The AAS animals significantly increased their preference for ethanol during week three, otherwise there were no differences in ethanol preference, water- or total fluid intake between the groups (Table 5).

A concurrent abuse of AAS and other drugs of abuse have been reported among athletes and body builders (22, 123) as well as teenagers and adults taking AAS to improve their physical appearance (43, 44, 74, 110). Anecdotal stories and case reports show that individuals using AAS react abnormal to large amounts of alcohol, with for example increased impulsivity, verbal and physical aggression and loss of control (37). As a consequence, AAS users are often recommended by other AAS users to refrain from drinking alcohol when on steroids. However, our results show that AAS may constitute a risk factor for the onset of enhanced alcohol consumption later in life when steroids are no longer used. This indicates that AAS would induce long lasting changes in the CNS making the brain sensitive to alcohol. Thus, even if individuals do not drink alcohol until quitting their use of AAS, the risks associated with the combined abuse (for example increased aggressiveness) may remain. These results support the case reports of individuals having become addicted to other drugs of abuse, including alcohol, after the initiation of AAS use (7, 37, 154).



**Figure 13.** The ethanol intake (expressed as g/kg/day of absolute ethanol) during three consecutive week in rats pre-treated with AAS (daily i.m. injections of 15 mg/kg for 14 days) or oil (controls). The values are expressed as median ± MAD. \* $P < 0.05$  compared to controls (Mann-Whitney  $U$ -test);  $n = 20$ . Abbreviations: AAS, anabolic androgenic steroids.

**Table 5.** Median ± MAD ethanol preference (% of total fluid intake), total fluid intake (ml/kg/day) and water intake (ml/kg/day) during three weeks in rats pre-treated with nandrolone decanoate (15 mg/kg/day for 14 days;  $n = 20$ ) or control rats ( $n = 20$ )

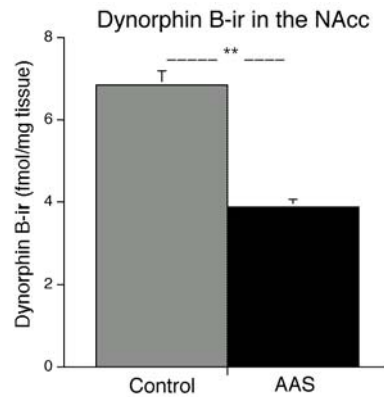
	Week 1	Week 2	Week 3
<i>Ethanol preference</i>			
AAS	39.8 ± 24.6	56.9 ± 26.5	65.4 ± 21.4*
Control	29.5 ± 11.9	34.7 ± 9.9	35.7 ± 8.8
<i>Total fluid intake</i>			
AAS	27.9 ± 5.4	27.8 ± 9.6	37.1 ± 11.0
Control	25.5 ± 3.9	24.7 ± 6.8	26.8 ± 6.6
<i>Water intake</i>			
AAS	16.0 ± 6.3	13.9 ± 9.0	19.9 ± 13.3
Control	21.7 ± 4.6	27.1 ± 4.9	29.8 ± 6.1

\* $P < 0.05$  compared to controls (Mann-Whitney  $U$ -test)

As mentioned earlier in the discussion, AAS induce an imbalance between the dynorphin B- and MEAP-ir in the NAcc. This imbalance in the reward system could sensitise the brain for other drugs of abuse such as alcohol. Thus, the increased alcohol consumption of the AAS pre-treated animals in Paper III could be triggered by an imbalance in the reward system. Furthermore, the fact that the AAS pre-treated animals increase their consumption over the three-week period can be related to the found decreased levels of dynorphin B-ir in the NAcc (Fig. 14). Since

KOP receptor agonists decrease the dopamine release in the NAcc (142), decreased levels of dynorphin B-ir in the NAcc during ethanol intake would be compatible with an increase in the dopaminergic activity, leading to reward and euphoria. Therefore, it is possible that the altered dynorphin B-ir seen in Paper III may, in interaction with the dopaminergic system, promote the rewarding effects of ethanol and reinforce the act of drinking, and thereby reinforce the drug-taking behaviour. Nevertheless, the effect of repeated ethanol administration on the prodynorphin system in the rat is controversial. Some studies show upregulation of the system whereas other studies report downregulation or no effect at all (for review see (52)). It could therefore be argued that the rats in Paper III were undergoing ethanol withdrawal since they were sacrificed three days after the last day of drinking. However, the rats did not show any physical signs of withdrawal and the relatively low daily consumption of ethanol are usually considered too low to induce tolerance and withdrawal (31). Nevertheless, it is possible that the rats actually were undergoing withdrawal, however not strong enough to induce physical signs. In fact, dynorphin B-ir has been shown to be decreased in various brain regions five days after chronic alcohol treatment (120). If hypothesising that the animals in Paper III are actually were undergoing withdrawal at the time of decapitation, the decreased dynorphin B-ir in the NAcc suggests that AAS pre-treated animals would feel less dysphoric than the controls. To get a clearer picture of the cause and effect of the altered dynorphin B-ir levels, it would be necessary to administer the same doses of ethanol to AAS pre-treated and control animals and thereafter study behavioural and neurochemical changes.

Regardless the behavioural response of the altered dynorphin B-ir, is likely that the AAS pre-treatment or the combination of AAS and ethanol is necessary to induce changes in the prodynorphin system. Interestingly, rats genetically predisposed to drink more alcohol have lower basal levels of dynorphin B-ir in the NAcc (112). The results presented in this thesis suggest that AAS induce changes in the reward system of outbred rats, making them, at least in some aspects, more like rats genetically predisposed to drink more alcohol. This would be a possible biological explanation to why some individuals without prior alcohol problems have increased their alcohol consumption during use of AAS.



**Figure 14.** The Dynorphin B-ir (fmol/mg tissue) in the NAcc of rats treated with the AAS nandrolone decanoate (daily i.m. injections of 15 mg/kg for 14 days) and controls, followed by a three-week-alcohol-consumption period. The values are expressed as mean  $\pm$  SEM.  $**P < 0.01$ ;  $n = 8$ . Abbreviations: AAS, anabolic androgenic steroids; NAcc, Nucleus Accumbens.

Other studies confirm that AAS interact with the effects of ethanol. An acute dose of ethanol gives, because of its sedative effects, a decreased locomotor activity in untreated rats (84). However, a single, high dose of ethanol (0.5 g/kg i.p.) given to animals pre-treated with AAS, using the same treatment schedule as in Paper III, did not decrease the locomotor activity in the same manner as ethanol given the controls (84). Furthermore, an increased tolerance to alcohol has been observed in AAS treated animals. The tolerance was observed by measuring how long time the animals needed to wake up from ethanol intoxication (2.5 g ethanol/kg, ip). The AAS-treated animals required less sleep (unpublished data). Preliminary data show that AAS treated rats have lower alcohol levels compared to control despite higher alcohol consumption, indicating that AAS affect the metabolism of alcohol.

In conclusion, pre-treatment with AAS increases the alcohol intake, decreases the sedative and alters the rewarding effects of alcohol. The biochemical results suggest that AAS pre-treated animals get higher euphoria from the ethanol and thereby drink even more. This would be in agreement with case reports showing that AAS users, without prior alcohol problem, have increased their alcohol consumption after the use of AAS (37, 154).

#### 4.4 Physiological Observations

There were no differences between the bodyweights of AAS or control animals at the beginning of the studies. Nevertheless, the body weight of the AAS treated animals were consistently less after the treatment period and at the time of decapitation in all studies, except in Paper V where there were no differences between the groups (data not shown). The AAS treated rats did not lose any weight during the

treatment, but did not gain as much weight as the controls. Representative data from Paper V are shown in Table 6. Decreased food intake has previously been reported after nandrolone administration (171) and is a possible explanation for the weight difference between AAS and control groups. A recent study using the same AAS treatment regime as in this thesis (15 mg nandrolone/kg/day for 14 days), supports this hypothesis (83). It was found that vehicle treated control animals had a weight gain similar to that of the AAS treated animals if they were given as much food as the comparable AAS treated animals consumed the previous day (83).

**Table 6.** Body weights (g) of AAS and control animals throughout the experiment conducted in Paper V.

	AAS	Control
Day 1 (n=24)	389 ± 3	384 ± 3
Day 14 – end of treatment (n=24)	422 ± 7***	460 ± 4
After 3 weeks of recovery (n=24)	423 ± 6***	535 ± 5
After 6 weeks of recovery (n=8)	530 ± 9***	644 ± 13

The values are expressed as mean ± SEM, \*\*\* $P < 0.001$  compared to controls

In humans, thyroid hormones have been found to decrease in serum after use of AAS (6). In this thesis it was found that the thymus was significantly reduced in rats receiving AAS compare to controls, both immediately after the treatment period and after recovery periods of various lengths (measured in Paper II, III and V; Table 7). Anabolic androgenic steroids have previously been shown to affect glucocorticoid receptors (2, 99). Since, glucocorticoid receptors are present in the thymus (10) it is possible that AAS induced the atrophy by blocking these receptors.

**Table 7.** The wet weight of thymus (mg/100g body weight) at various time points after the end of the treatment period.

Number of weeks after last AAS or oil injection (Paper)	AAS	Control
24 hours (Paper II)	87 ± 7***	139 ± 7
Three (Paper II)	46 ± 5 ***	100 ± 1
Three (Paper V)	43 ± 1 ***	109 ± 5
Six (Paper V)	54 ± 2 ***	113 ± 7
Seven (Paper III)	27 ± 9 ***	106 ± 5

Values are expressed as mean ± SEM, \*\*\* $P < 0.001$  compared to controls

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The fact that the body and thymus weight of the AAS group remained lower than the control group even after the recovery period indicates that the steroid has long-lasting effects on the physiology of the rat. Whether this effect is due to the long half-life of nandrolone decanoate (157), or that AAS induces persistent changes in the physiology remains to be clarified.



## **5 Summary and Conclusions**

This thesis studies the effects of chronic AAS treatment on defensive behaviours and alcohol consumption. Biochemical changes in brain regions, related to the AAS-induced psychiatric side effects, are also investigated.

We found that rats pre-treated with AAS drank more alcohol than the control rats. Since the alcohol was introduced to the rats several weeks after the last AAS injection, AAS may increase the risk of excessive alcohol intake long after use of steroids. Furthermore, AAS rats were more aggressive than controls. Both when the steroid was given alone and when it was combined with alcohol or amphetamine. The AAS treated rats were also braver and less frightened in a threatening situation. The behavioural changes found in the animals after AAS treatment, are consistent with the personality changes reported in human AAS abusers (see for example (9, 22, 90, 123, 144)). Interestingly, some adolescents claim to use AAS to become intoxicated and braver (73).

As mentioned above, increased aggression and concurrent use of other drugs such as morphine, amphetamine and alcohol have been reported among AAS users (for review see (9)). The neurochemical analyses in this thesis reveal changes in brain regions and neurotransmitters regulating reward and aggression. This suggests that AAS induce long-lasting changes in brain regions regulating aggression and make the brain more sensitive to other drugs of abuse. Consequently, our studies show that the behavioural responses to alcohol and amphetamine and alcohol are changed with increased aggression as a result. These neurochemical and behavioural changes might be the reason why some AAS abusers lose control over their actions when feeling threatened and responds with sudden and often unprovoked outburst of extreme aggression, so-called roid rage.

Accumulating data suggest that AAS are drugs of abuse. Case reports and controlled studies show that AAS users often exhibit signs of dependence (see for example (23, 39, 148, 169)). Nevertheless, it has been difficult to find scientific proof for the reported dependence symptom. Consequently, the question whether the use of AAS leads to dependence or not is controversial. This thesis provides evidence that AAS affect brain regions regulating reward. In particular, the expression of Fos protein in the NAcc confirms that AAS induce neural activity in the brain reward system. The increased  $\beta$ -endorphin-ir in the VTA of AAS treated animals suggests that AAS activate the brain reward system in the same manner as other drugs of abuse such as morphine and heroin. Furthermore, AAS induced an imbalance between the enkephalin and dynorphin systems that normally uphold a balance between euphoric and dysphoric feelings. This imbalance may cause a dysregulation of the dopamine activity with a malfunctioning reward system and possibly making the brain more sensitive to other drugs of abuse. A sensitisation of the reward system is supported by an animal study showing increased rewarding aspects of ampheta-

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mine. Furthermore, use of AAS has been proposed to serve as a gateway to opioid abuse and dependence (7). The alteration of the endogenous opioid systems found in this thesis provides a possible biological explanation for the hypothesis that usage of AAS may lead to development of dependence.

In summary, the work described in this thesis demonstrates that AAS produce profound effects on endogenous opioid peptides in brain regions regulating reward, disinhibitory behaviours and aggression. The AAS increased the immunoreactivity and elicited an imbalance in opioid systems controlling the activity in the brain reward system, known to be essential for the response to addictive drugs. The AAS-induced effects on the brain were paralleled by an increased sensitivity to alcohol and amphetamine. Behavioural data recorded from animal experiments also indicated a relation between AAS treatment and a decreased reflection of fear in a threatening situation. However, it should be noted that the observed alterations in the endogenous opioid systems may not be the only neurochemical disturbance induced by AAS. Progressive research suggests that several other transmitter systems are affected by high doses of steroids. These systems include tachykinins, monoamines and excitatory amino acids. To fully understand the complexity and the mechanisms by which AAS affect the brain, additional research are requested. Clarification of all neurochemical events behind AAS-induced psychiatric side effects is a great need, in particular for the purpose to develop proper strategies for treatment of the increasing number of AAS abusers seeking medication for mental inconveniences elicited by these steroids.

## **6 Sammanfattning på svenska**

Under de senaste decennierna har bruket av anabola androgena steroider (AAS) spridit sig från elitidrottsmän och kroppsbyggare till amatöridrottare och ungdomar som bygger muskler för att förbättra sitt utseende. Inom den senare gruppen förekommer det ibland ett blandmissbruk där AAS kombineras med andra droger som till exempel amfetamin, alkohol och tobak. Dessutom har våldsbrott och andra kriminella handlingar sammankopplats med bruk av AAS. Ökad aggressivitet och minskad impuls kontroll är exempel på psykiska biverkningarna som kan uppstå efter bruk av steroider, och kan vara en anledning till att de används i kriminella kretsar. Det har också spekulerats i om AAS kan vara beroendeframkallande. Kunskapen om de biokemiska förändringarna i hjärnan som orsakar dessa beteendeförändringar är i dag begränsad.

I denna avhandling presenteras resultat från studier där jag använt en djurmodell för att studera biokemiska förändringar och beteendeförändringar efter långvarigt bruk av AAS. Jag har valt att studera effekter på substanser i hjärnan som är involverade i regleringen av beroende och aggressivitet, de så kallade endorfinerna. Dessutom har jag studerat hur frivillig alkoholkonsumtion påverkas hos djur förbehandlade med AAS och hur defensiva beteenden (inklusive aggressivitet) påverkas av enbart AAS eller AAS i kombination med alkohol eller amfetamin. De biokemiska resultaten visar att AAS ökar halten av endorfiner i hjärnregioner som medierar och reglerar lustkänslor jämför med kontroldjuren. Anabola androgena steroider verkar därmed ge en belönande effekt, vilket kan vara en anledning till det beroende som har rapporterats efter bruk av dessa steroider. Dessutom skapar AAS en obalans mellan endorfinsystem som normalt upprätthåller en balans mellan lust- och olustkänslor. Denna obalans kan göra hjärnan känsligare för andra droger och ger en möjlig biologisk förklaring till varför individer utan tidigare drogmissbruk har blivit beroende av till exempel alkohol sedan de börjat använda AAS. De AAS-behandlade djuren drack mer alkohol, uppvisade mindre rädsla och flyktbenägenhet i en hotfull situation och var mer aggressiva än kontroldjuren. Dessutom förstärktes amfetamin-inducerad aggressivitet ytterligare i de djur som var förbehandlade med AAS.

Sammanfattningsvis ger resultatet från min avhandling stöd för att bruk av AAS kan leda till beroende och skapa långvariga förändringar i hjärnan som kan göra den känsligare för andra droger. Dessutom stämmer det ökade alkoholintaget, den förhöjda aggressiviteten och den minskade rädslan i en hotfull situation, väl överens med beteenden som man sett bland AAS-missbrukare.

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