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Anabolic Androgenic Steroids

Effects on Neuropeptide Systems in the Rat Brain

MATHIAS HALLBERG

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Abstract
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Anabolic-androgenic steroids (AAS) have been used in clinics for decades. The misuse of AAS has previously been attributed merely to sport athletes, taking AAS with intentions to increase muscle mass, enhance physical performance and to improve results in competitions. Today, the misuse of AAS has spread to adolescents and young adults not connected to sports. Alarmingly, many reports are pointing at severe psychiatric adverse effects among AAS abusers, which include mood swings, mania, anxiety, depression and aggression. Numerous examples of severe and often unprovoked violence and brutal crimes have been connected to AAS abuse and there is a strong need for a better understanding of the underlying biochemical events that might account for the adverse behaviors induced by AAS. The general aim of this thesis was to study the effect of chronic AAS administration on neuropeptide circuits in the rat brain associated with the regulation of rewarding effects, memory, anxiety, depression and aggression, using nandrolone decanoate as a prototype AAS.

Results demonstrated that daily administration of AAS to rats in doses comparable to those taken by AAS abusers, in certain brain structures significantly affected, *a*) the levels of the opioid peptides dynorphin B and Met-enkephalin-Arg^{6}Phe^{7}, *b*) the levels of the tachykinin substance P (SP), *c*) the density of the SP neurokinin 1 (NK1) receptor, *d*) the level of the SP metabolite SP_{1-7} that frequently exerts opposite effects to SP, *e*) the SP_{1-7} generating enzyme substance P endopeptidase (SPE) and finally, *f*) the levels of the neuropeptide calcitonin gene-related peptide (CGRP) often co-localized with SP. The alterations seen in the levels and activities of these neurochemical components are in many aspects compatible with behaviors typified among AAS abusers.

Keywords: Anabolic androgenic steroids, Nandrolone decanoate, Substance P, NK1 receptor, Substance P(1-7), Endopeptidase, Opioids, Calcitonin gene-related peptide, Radioimmunoassay, Autoradiography, Central nervous system, Rats

Mathias Hallberg, Department of Pharmaceutical Biosciences, Box 591, Uppsala University, SE-75124 Uppsala, Sweden

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Supervisor
Professor Fred Nyberg

Co-supervisor
Associate Professor Pierre Le Grevès

Faculty opponent
Professor James Zadina

Members of the examining board
Professor Kjell Wikvall
Professor Lisa Ekselius
Associate Professor Johan Franck
Associate Professor Mats Stridsberg
Associate Professor Jonas Bergquist

List of Papers

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

I. Johansson P, Hallberg M, Kindlundh AMS, Nyberg F. The effect on opioid peptides in the rat brain, after chronic treatment with the anabolic androgenic steroid, nandrolone decanoate. Brain Res Bull. 2000: 51 (5): 413-8.

II. Hallberg M, Johansson P, Kindlundh AMS, Nyberg F. Anabolic-androgenic steroids affect the content of substance P and substance P(1-7) in the rat brain. Peptides. 2000: 21 (6): 845-52.

III. Hallberg M, Kindlundh AMS, Nyberg F. The impact of chronic nandrolone decanoate administration on the expression of the NK 1 receptor in rat brain. Peptides 2005 *In press*

IV. Magnusson K, Hallberg M, Kindlundh AMS, Nyberg F. Administration of the anabolic androgenic steroid nandrolone decanoate affects substance P endopeptidase-like activity in the rat brain. *Manuscript*

V. Hallberg M, Magnusson K, Kindlundh AMS, Steensland P, Nyberg F. The effect of anabolic androgenic steroids on calcitonin gene-related peptide (CGRP) levels in the rat brain. *Manuscript*

Contents

Abbreviations

AAS	Anabolic androgenic steroids
ACE	Angiotensin converting enzyme
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CRF	Corticotropin releasing factor
DOP	Delta opioid receptor
GABA	Gamma-aminobutyric acid
HCG	Human chorionic gonadotropin
HPLC	High Performance Liquid Chromatography
i.m.	Intramuscular
KOP	Kappa opioid receptor
MEAP	Met-enkephalin-Arg^6-Phe^7
MOP	Mu opioid receptor
NEP	Neutral endopeptidase
NK	Neurokinin
NMDA	N-methyl-D-aspartate
PAG	Periaqueductal gray
POMC	Proopiomelanocortin
RIA	Radioimmunoassay
S.E.M.	Standard error of the mean
SP	Substance P
SPE	Substance P endopeptidase
TFA	Trifluoroacetic acid
VTA	Ventral tegmental area

Introduction

Anabolic androgenic steroids in society

Anabolic androgenic steroids (AAS) have been used as enhancers of skills since the 1950s by athletes, and in the early 1970s, approximately 30% of the Swedish track- and field athletes admitted to have used AAS (Ljungqvist 1975). During the 1980s, these steroids also became more commonly used outside the arena of sports (Yesalis et al 1989) and today, not only athletes and bodybuilders, but also adolescents and young adults not connected to sports are unfortunately often frequent steroid users. Several epidemiologic studies have been conducted in recent years to determine the prevalence of AAS abuse among adolescents and a typical reported life-time prevalence is in the range of 1-5% among males in these studies (DuRant et al 1993; Lambert et al 1998; Scott D. M. et al 1996; Tanner et al 1995), for a review see (Thiblin and Petersson 2005). For example, in a study conducted in 1995, in Uppsala, Sweden, 2.7% of the male and 0.4% of the female senior high school students reported life-time use of AAS (Kindlundh et al 1998). Seven years later, using an identical multiple-choice questionnaire, the reported life-time use of AAS had increased to 4.7% among the males in the first grade of senior high school (Hallberg et al 2004). In certain subpopulations, such as bodybuilders or male prisoners, the prevalence of AAS is reported to be approximately 10% or in some cases even higher (Korkia and Stimson 1997; Korte et al 1998; Lindstrom et al 1990).

Although adolescents participating in sports still are slightly more likely to use AAS (Scott D. M. et al 1996; Tanner et al 1995), other groups characterized by low self-esteem, truancy and often bad school achievements are also more frequently using AAS (Kindlundh et al 2001a). Eating disorders, depressed mood and substance abuse are reported to be more frequent among AAS users (Irving et al 2002). Interestingly, as compared to non-users, male adolescents using AAS believe to a higher extent that girls prefer big muscles (Nilsson et al 2001). Adolescents males were reported to use anabolics in order to get larger muscles, become braver, become intoxicated, because it is fun to try or because friends do so (Kindlundh et al 1998). In Sweden, the higher prevalence of AAS use among teenagers has become a major concern. Furthermore, one should be worried about that there are studies reporting that AAS are administered in order to become more aggressive (Thiblin et al 1997). In fact, there is also a strong connection between AAS abuse and violence (Pope and Katz

1994; Schulte et al 1993; Thiblin and Parlklo 2002) and wives and girlfriends often become victims of physical abuse (Choi and Pope 1994). The AAS related violence has been subdivided into three different behavioral violence patterns (Thiblin et al 1997). The first pattern is termed "roid rage" and is characterized by the perpetrator being slightly provoked, leading to impulsive, intense and long lasting violence. The second pattern is the "terminator", an AAS abuser who commits executioner-like homicides in a non-impulsive manner and finally the third "stürmschnapps" behavior, which is characterized by a person taking AAS in advance to planned criminal acts in order to become more aggressive (Thiblin et al 1997).

The anabolic androgenic steroids are synthetic derivatives of the endogenous steroid testosterone and exert their effects via activation of the same androgen receptor. Therefore, testosterone and its biological effects, biosynthesis and further conversion into metabolites are first addressed in this thesis summary and thereafter the AAS on the illegal market with special focus on nandrolone decanoate, one of the most commonly used AAS.

Testosterone; biological effects, chemical structure and prodrugs

In 1931, Butenandt presented the first report on the isolation of a substance with androgenic activity (Butenandt 1931; Butenandt and Tscherning 1934). This compound androsterone was isolated in an amount of 15 mg from 15000 liter of male urine. Testosterone was later isolated in a small amount, 5 mg from nearly one ton of testicles from bulls in 1935 by David et al (David et al 1935), and in the same year the groups of Butenandt (Butenandt and Günter 1935) and Ruzicka (Ruzicka and Wettstein 1935) independently determined the chemical structure and testosterone was synthesized. For this accomplishment the latter two scientists were awarded the Nobel prize in 1939. Testosterone is an androgen hormone, the class of steroids that are responsible for primary and secondary male sex characteristics. Testosterone that constitutes the principal circulating androgen is formed in the Leydig cells of the testis, although minor amounts are also provided from precursors in other tissues, e.g. adrenal cortex, the liver and the prostate. In females, the adrenal cortex and ovary produce small amounts of testosterone. In male plasma, the testosterone levels are normally ranging from 2.5 to 14 ng/ml (Starcevic et al 2003), while in female these numbers are 5-100 times lower (Prunty 1966). The sex characteristics are seen already in the male fetus, where the embryonic testis secretes testosterone, which is important for the development of the fetus. Later, at puberty, androgens stimulate further development of sex organs, such as the prostate and penis and also stimulate maturation of spermatozoa, which are produced in the testis. The androgen promoted hair growth and the deepening of the voice are examples of typical secondary sex characteristics, as well as the thinning of the hair and hairline recession in some adults later in life. Furthermore, the testosterone secretion has an impact on the behaviors related to sexuality and have been linked

to social dominance (Schaal et al 1996), as well as aggressiveness in males at puberty (Finkelstein et al 1997; Olweus et al 1988).

Testosterone affects many organs and beside its androgenic (masculinizing) actions, the hormone exhibits powerful anabolic properties. These anabolic (myotropic) effects are manifested in an increased protein synthesis and decreased protein catabolism, a larger muscle mass and an increased skeletal maturation and mineralization. In addition, testosterone induces a loss of subcutaneous fat. The anabolic properties of the androgens were reported already in 1935 (Kochakian and Murlin 1935) and clinical studies in the 1950s showed that testosterone increased the muscle mass considerably (Leonard 1952; Loring et al 1961; Meyer and Hershberger 1957). The anabolic effects that result from androgen receptor stimulation have attracted great interest among some sport athletes and bodybuilders. For reviews on the biological actions of androgens, see (Hartgens and Kuipers 2004; Mooradian et al 1987).

The chemical structure of testosterone consists of an androstane four-ring skeleton where the rings are denoted A, B, C and D. This C_{19} steroid carries two axial methyl groups, C-18 and C-19. The anabolic androgenic steroid nandrolone consists of a C_{18} skeleton, where the C-19 methyl group present in testosterone is missing. Therefore, nandrolone is frequently named 19-nortestosterone, Figure 1. To prolong the activity and/or to achieve a local depot effect after injections, the hydroxyl group attached to C-17 in the D ring of the two compounds is often reacted with long chain fatty acid derivatives to provide lipophilic esters. Such molecules are serving as prodrugs and are slowly released from intramuscular depots. Once released the esters are hydrolysed by esterases (van der Vies 1985), delivering the corresponding bioactive steroids, as exemplified with nandrolone decanoate in Figure 1. Metabolic ring transformations might take place prior to ester hydrolysis, at least after per oral administration of testosterone undecanoate, which is absorbed via the intestinal lymphatic ducts and not via the portal system. Thus, both testosterone undecanoate and dihydrotestosterone undecanoate are released from the lymph system to the plasma where the esters are hydrolysed to testosterone and dihydrotestosterone, respectively (Horst et al 1976; Shackleford et al 2003). Testosterone itself exhibits a very low bioavailability after oral administration. With regard to esters of nandrolone such as nandrolone decanoate, the compound that has been used in the studies described in this thesis, the half-life in the intramuscular depot in rat has previously in one study been estimated to 130 hours (5.4 days) as compared to the nandrolone with a half-life of 0.6 hours (van der Vies 1985). This finding is in good agreement with a corresponding study involving healthy volunteers where the half-life was determined to 6 days (Wijnand et al 1985). In Sweden, testosterone (in Atmos®, Astra) and the prodrugs testosterone enantate (Testoviron-Depot®, Schering Nordiska), testosterone undecanoate (Undestor®, Organon) and nandrolone decanoate (Deca-Durabol®, Organon) are approved by the Medical Product Agency.

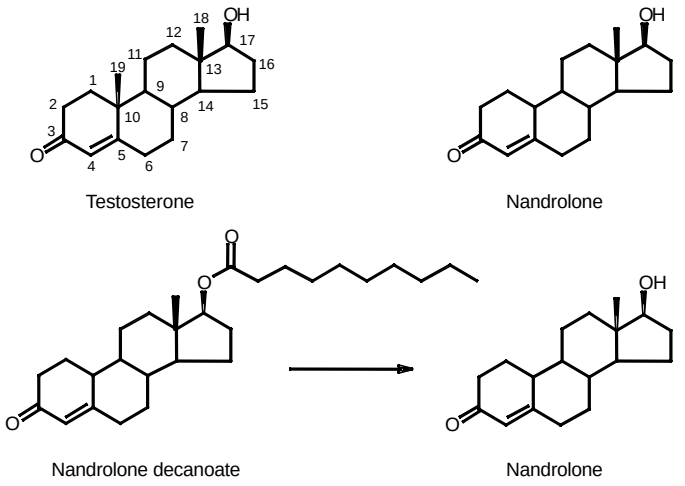


Figure 1. The chemical structures of testosterone and nandrolone (top). Conversion of the prodrug nandrolone decanoate to nandrolone (bottom).

Testosterone; biosynthesis and metabolism

The biosynthesis of testosterone is summarized in Figure 2. With cholesterol as the precursor, pregnenolone and progesterone are formed after oxidative cleavage of the C-17 side chain by a cytochrome P-450 mixed-function oxidase system, named cholesterol side chain cleavage. The enzyme system consists of three proteins, cytochrome P450 11A ($P450_{SCC}$), adrenodoxin and adrenodoxin reductase and requires NADPH and oxygen. The conversion of pregnenolone to progesterone involves 3€-hydroxysteroid dehydrogenase and is an oxidative process resulting in a double bond migration and formation of the/ ,€-unsaturated system. Androstenedione, a key intermediate in the formation of testosterone, is a product from 17/ -hydroxylase/17,20-lyase cytochrome P450 (cytochrome P 450 17) catalyzed conversions of progesterone and pregnenolone. In the latter case, 3€-hydroxysteroid dehydrogenase is also needed to obtain the androstenedione (androst-4-ene-3,17-dione). Androstenedione can be transformed to estrone by cytochrome P450 19 (aromatase) and by 17€-hydroxysteroid dehydrogenase finally to testosterone. Aromatase is an important target for breast cancer therapy (Brodie and Njar 2000) and recently cytochrome P450 17 was identified as a promising target for prostatic androgen dependant diseases (Cavalli and Recanatini 2002; Van Wauwe and Janssen 1989).

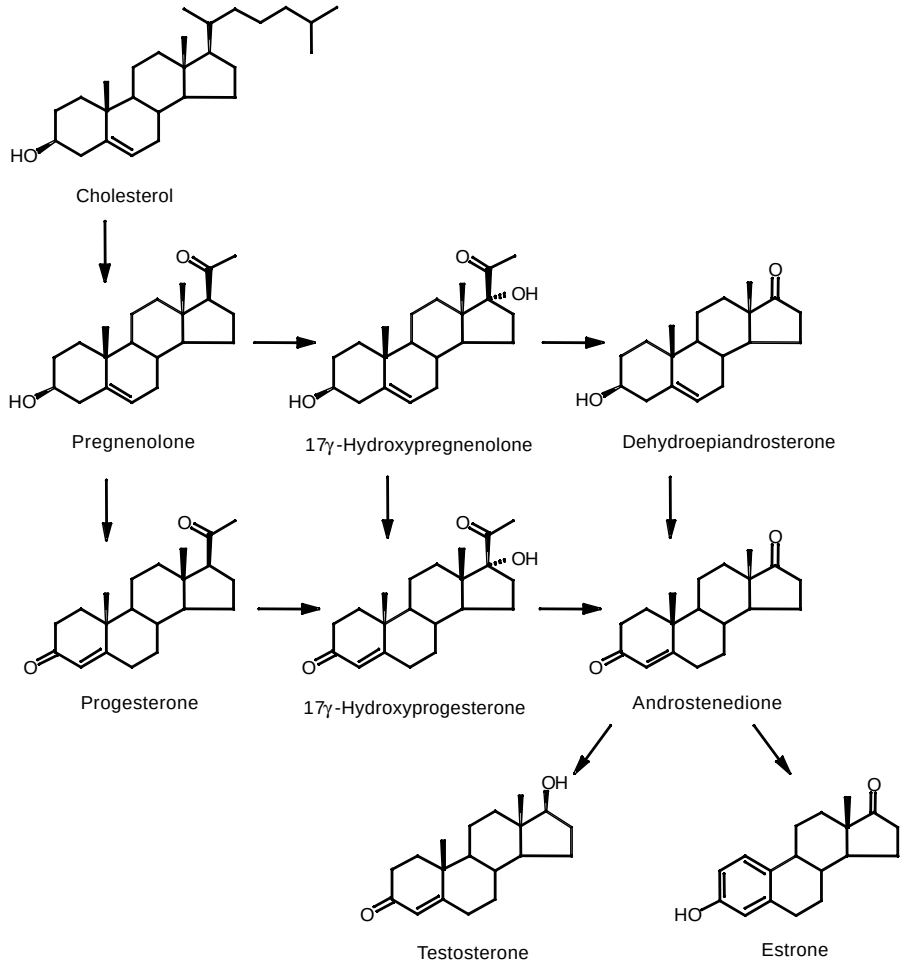


Figure 2. Biosynthesis of testosterone from cholesterol.

Two metabolites of testosterone have attracted a particular interest due to their pronounced bioactivity, although numerous of metabolites have been identified. These two metabolites are the reductive metabolite 5 -dihydrotestosterone and the oxidative metabolite estradiol. The two enzyme systems responsible for the conversions are present in the brain and could be important with regard to the mechanism of action of hormonal steroids in the brain (Celotti et al 1997). The active metabolites 5 -dihydrotestosterone and estradiol as well as the major excretory products, the less bioactive androsterone and etiocholanolone as well as 5 -hydroxytestosteron, are shown in Figure 3.

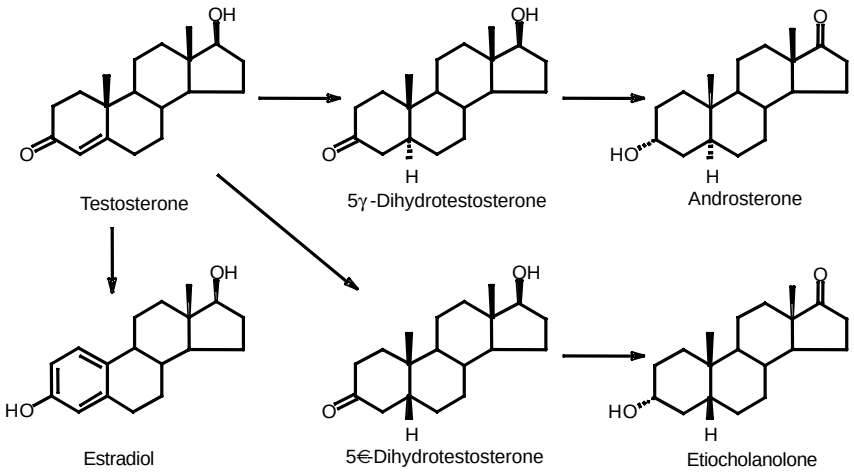


Figure 3. Central Phase I metabolites of testosterone.

The conversion of testosterone to 5⁄ -dihydrotestosterone is of importance since the latter derivative is the major intracellular mediator of the testosterone effects. It has a higher potency and binds more tightly than testosterone to the androgen receptor. It was not until in 1968, it was revealed that dihydrotestosterone, was the active androgen in target tissues e.g. in the prostate and that the reduction can take place in these tissues (Anderson and Liao 1968; Bruchovsky and Wilson 1968). The reduction of the C-4,5 double bond that creates an asymmetric centre at the C-5 carbon is the rate-limiting step in the testosterone metabolism. The double bond is reduced in an irreversible reaction by either 5⁄ -reductase or by 5€-reductase, two enzymes that are found mainly in the liver, primarily in the endoplasmatic reticulum and the cytoplasm, respectively (Schanzer 1996). In the steroid with the 5⁄ -configuration, the hydrogen in the ring junction is located below the planar molecule skeleton. The C-3 carbonyl group is subsequently reduced by hydroxysteroid dehydrogenases (Leonard 1952). After oral administration or intramuscular injection, the 3⁄ -hydroxy isomers are predominantly produced (Schanzer 1996). The formation of estradiol from testosterone involves the previously mentioned aromatase, a membrane-bound cytochrome P-450 monooxygenase comprising aromatase cytochrome P-450 and NADPH-cytochrome P-450 reductase. The reaction proceeds via an initial hydroxylation at C-19. After further oxidations and water elimination, the aromatic A-ring system is generated. The enzymes involved in testosterone metabolism are also to a large extent engaged in the metabolism of anabolic androgenic steroids as nandrolone although the latter lacking C-19 is a poor substrate for aromatase. AAS often undergo Phase II metabolism e.g. conjugation reactions with glucuronic acid. For example, testosterone after C-3 reduction to alcohols forms 3⁄ -€-glucuronides but also sulfate conjugates.

Anabolic androgenic steroids on the market and administration patterns

In the present study we used nandrolone decanoate as a prototype AAS. This steroid is often seen in connection with use among sportsmen but there are numerous of different AAS on the illegal market today. Interestingly, due to the limited availability of approved AAS, there also seems to be many counterfeit products on the illegal market (Madea et al 1998). In fact, 35% of the AAS analyzed in a German study did not contain the expected substances (Musshoff et al 1997). However, not knowing what is injected seems to be of little concern since AAS abusers often perceive themselves as being invulnerable.

Bodybuilders and athletes usually administer the steroids in cycles 2-3 times per year, each cycle lasting 6-12 weeks. However, some steroid users also go year round in the hope for optimal results. During cycling it is often common to use 2-3 different steroids at the time, so called stacking (Pope and Katz 1994; Yesalis and Bahrke 1995). Stacking often involves a depot steroid, such as nandrolone decanoate together with an orally administered AAS such as methandrostenolone. According to "Anabolics 2002" (Llewellyn 2002), an anabolic steroid reference manual, the particular combination mentioned will give extremely good results. Another combination recommended by steroid users is stanozolol in the combination with trenbolone acetate.

A recent study conducted in Sweden showed that four of all available AAS seemed to be considerably more used than others (Eklof et al 2003); testosterone, nandrolone decanoate, methandrostenolone and stanozolol. These derivatives will be discussed in some more detail below. AAS can be bought legally in some parts of the world, whereas in other countries AAS are classified as illegal narcotic substances.

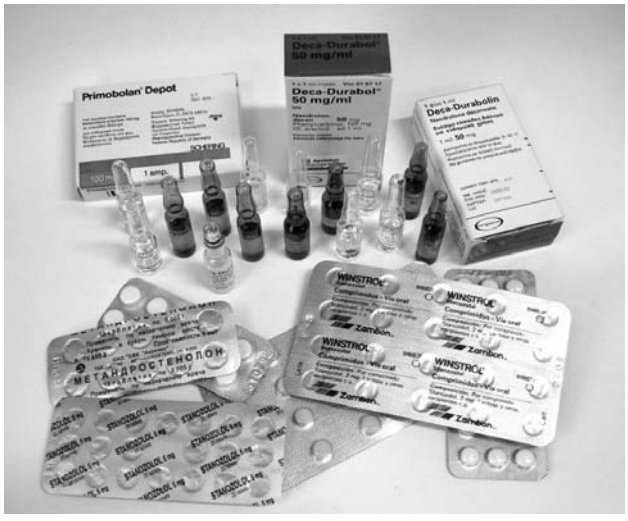


Figure 4. Some of the most common steroids on the market.

In Figure 5 some of the most commonly used AAS are drawn. These compounds are often administered as prodrugs. The rationale behind the alterations of the chemical structure starting from the native testosterone has been a demand for higher potency and selectivity, prolonged action and an improved bioavailability. The simplest approach from the synthetic point of view is to make prodrugs to prolong action and to achieve depot effects. Nandrolone decanoate and the testosterone esters discussed previously provide typical examples where the hydroxyl groups at C-17 are used as handles for modifications. Nandrolone decanoate remains one of the most popular anabolics in circulation among abusers of AAS in the world. The drug is easily available and information on nandrolone decanoate and a large variety of other AAS can be found in the anabolic steroid reference manuals that are popular among AAS users. Concerning nandrolone decanoate, for mentioning one example, it is stated, "The major drawback for competitive purposes is that in many cases nandrolone metabolites will be detectable in a drug screen for up to a year (or more) after use" (Llewellyn 2002).

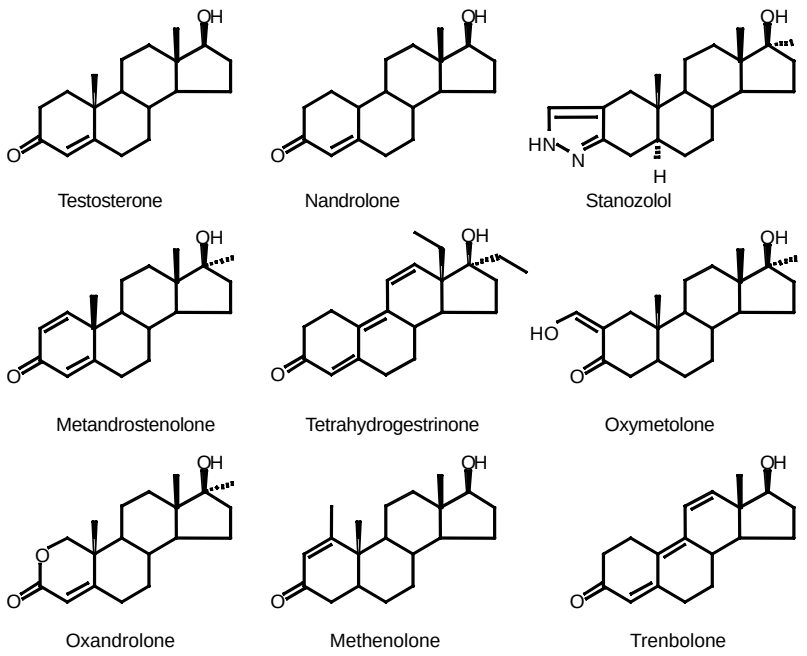


Figure 5. Examples of anabolic androgenic steroids on the market. In some cases frequently administered as ester prodrugs.

Methandrostenolone, also referred to as metandienone ("Russian"), is characterized by the added 17′ methyl group that eliminates the potential oxidation of the 17′ hydroxyl group. This manipulation results in a better bioavailability and similar operations to improve bioavailability were previously successfully applied in the development of the oral contraceptives. Furthermore, the C-19 methyl group in

testosterone is retained in this steroid and an extra double bond has been introduced in the A-ring. A large number of metabolites of methandrostenolone have been identified, (Schanzer 1996) despite the metabolism block at the C-17 position. Stanozolol provides a third example of structural modifications that deliver potent AAS. The pyrazole ring linked to the A-ring, creating a five-ring system, is the characteristic feature of this molecule. Thus, testosterone, nandrolone decanoate, methandrostenolone and stanozolol, which all are popular steroids among AAS abusers differ considerably from a chemical point of view. Below, nandrolone decanoate will be discussed in more detail.

Nandrolone; synthesis and metabolism

Nandrolone was first synthesized by Birch in 1950 (Birch 1950) and is prepared from estradiol-3-methyl ether, obtained via reduction of the C-17 carbonyl group in the corresponding estrone derivative. The latter compound also serves as a precursor in the preparation of the oral contraceptive mestranol. A so-called Birch reduction followed by acid hydrolysis gives nandrolone and subsequent esterification provides the prodrug nandrolone decanoate. Thus, synthetic nandrolone is prepared from an estrogen derivative while testosterone is biosynthetically partly metabolized to estradiol.

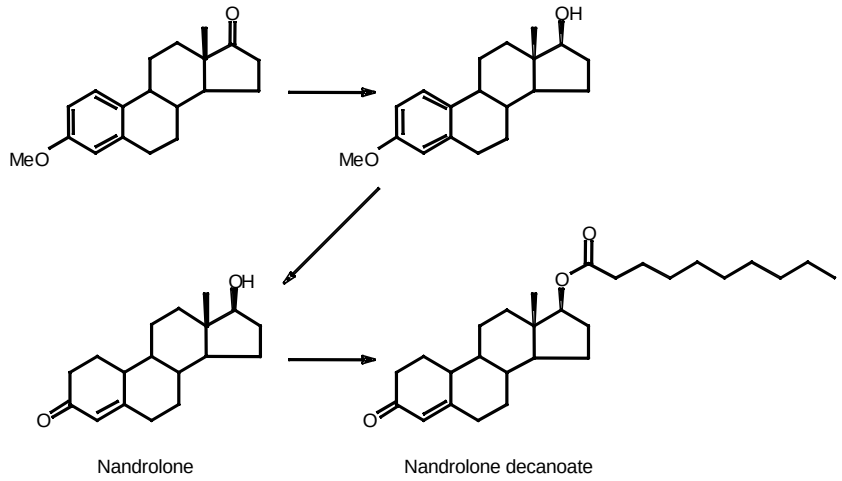


Figure 6. Synthesis of nandrolone and nandrolone decanoate.

The A-ring of nandrolone is metabolized by several different enzymes, including 5 -reductase and 5 -reductase as well as 3 -hydroxylase and 3 -hydroxylase. The metabolism pattern follows essentially the same pathways as testosterone although the oxidative metabolism involving C-19 cannot occur. The stereoisomers 3 -hydroxy-5 -estran-17-one (19-norandrosterone), 3 -hydroxy-5 -estran-17-one

(19-noretiocholanolone) and 3€-hydroxy-5 -estran-17-one are all less potent than nandrolone (Marshall 1988). As compared to testosterone, oxidative metabolism leading to aromatization is much less common. Notably, the 17-keto metabolites are predominant among the excreted metabolites in AAS with a secondary 17€-hydroxyl group that can be oxidized. According to the protocols from the international national committee (IOC), the anti-doping analysis for nandrolone is relying on the identification of two major Phase II metabolites in urine: the glucoronides of 19-norandrosterone and 19-noretiocholanolone, where limits of 2 ng/mL and 5 ng/mL have been fixed for males and females, respectively (Ozer and Temizer 1997).

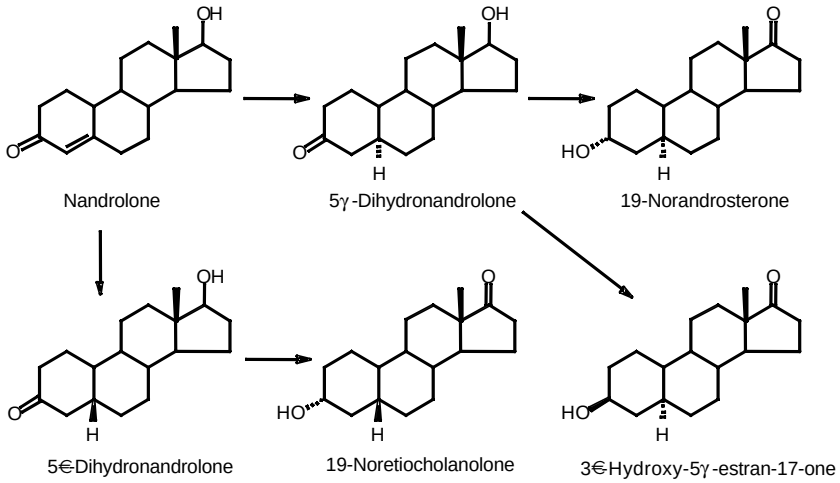


Figure 7. Central Phase I metabolites from nandrolone.

Nandrolone and other anabolic androgenic steroids; biological effects

The androgen receptor is a member of the nuclear receptor superfamily and is located in the cell nucleus (Mangelsdorf et al 1995). After reduction of testosterone to 5 -dihydrotestosterone, the latter binds to the receptor and induces the formation of a homodimer (Evans R. M. 1988). Binding of the homodimer in an active conformation to Androgen Response Elements (ARE) stimulates the gene transcription (Mangelsdorf et al 1995). Notably, both testosterone and 5 -dihydrotestosterone bind to the receptor but the latter more tightly and activates the gene expression more efficiently (Deslypere et al 1992; Wilbert et al 1983). It has been known for a long time that different AAS display different anabolic/androgenic ratios. Research programs have been devoted to achieving anabolic steroids with a minimum of androgenic properties. The programs have been successful to some

degree but are hampered by the inherent problem that the androgenic and anabolic effects are mediated by the same receptor. Nandrolone shows higher myotropic potency and exhibits also a higher affinity for androgen receptors than testosterone. In experiments with castrated rats, nandrolone was twice as potent as testosterone but was found to be five times less androgenic than testosterone (Sundaram et al 1995). On the contrary, while the major testosterone metabolite 5′ -dihydrotestosterone is a potent ligand to the receptors, the corresponding 5′ -dihydronandrolone is less potent than nandrolone (Bergink et al 1985; Toth and Zakar 1986). As mentioned previously, the reduction of testosterone to 5′ -dihydrotesterone can take place in target tissues. It should therefore in this context be emphasized that it has been reported that 5′ -reductase activity is less pronounced in muscle (Sundaram et al 1995) which implies that a high nandrolone activity can be better retained in this tissue while in the case of testosterone the active 5′ -dihydrotesterone is less prone to be formed.

Nandrolone and other anabolic androgenic steroids; physical and psychological effects

Besides the desired anabolic effects leading to an increased strength and larger muscle mass (Bhasin et al 1996), there are many adverse effects associated with the use of AAS, especially when administering high doses of the steroids. For example, acne and gynecomastia are commonly seen among AAS users (Pope and Katz 1988; Pope and Katz 1994; Strauss and Yesalis 1991). The latter, being an effect of aromatization of the A-ring of the steroids, delivering compounds with estrogen activity (the reference compound testosterone is aromatized to estrogen as discussed above). However, as previously described, not all AAS serve as good aromatase substrates and consequently the aromatization of AAS takes place to various extent as a function of the steroid structure. Interestingly, to avoid gynecomastia, aromatase inhibitors are also frequently sold on the black-market. Baldness and striae represent other side-effects (Scott M. J., Jr. et al 1994; Strauss and Yesalis 1991), but also adverse effects such as testicular atrophy, reduction of sperm production and impotence are reported (Korkia and Stimson 1997). The effects on testes and sperm production are due to AAS induced suppression of the follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels. The LH and FSH levels, regulating the testosterone production, have been reported to return to normal after withdrawal of the AAS, whereas the concentration of endogenous testosterone remains reduced for a longer period of time (Alen et al 1987; Alen et al 1985). These phenomena are well known among the AAS abusers and thus most of the "recommended" cycle-schedules with steroid stacking end with three weeks of human chorionic gonadotropin (HCG) administration in order to "kick-start" the endogenous testosterone production (Llewellyn 2002).

Administration of AAS affects serum lipoprotein levels, blood coagulation and triglycerides. In addition, fluid retention, hypertension, myocardial infarction,

arrhythmia and stroke have been reported in connection to AAS abuse (Dickerman et al 1996; Dickerman et al 1995; Fineschi et al 2001; Huie 1994). Concerning orally administered 17⁄ -alkylated steroids, such as methyl-testosterone, stanozolol and metandrostenolone, those steroids have been reported to increase the risk for jaundice, hepatic carcinomas and hepatic malignancy (Cabasso 1994; Creagh et al 1988).

In women using AAS, deepening of the voice, clitoromegaly and hirsutism but also acne and fluid retention constitute frequent side-effects (Gruber and Pope 2000).

Furthermore, among adolescents who have used anabolics, 25% have shared needles (DuRant et al 1993). Thus, AAS users might be exposed to a higher risk of attracting human immunodeficiency virus (HIV) and hepatitis infections.

In addition to physical effects, the use of AAS also induces several psychological effects. However, the biochemical mechanisms accounting for the alterations in psychological behaviors are in most cases considerably less understood than those associated with physical effects. Administering high dose of the AAS methyltestosterone has been shown to cause both positive mood such as euphoria, energy and sexual arousal as well as negative mood, including irritability, hostility, violent feelings and mood swings (Daly et al 2001; Su et al 1993). Furthermore, not only methyltestosterone, but also other AAS, exert similar actions. However, as reported in most studies, a cocktail of different steroids are being used, making it difficult to establish a correlation between certain psychological behaviors and specific steroids.

Suspiciousness, anxiety and irritability are other psychological side effects that have been associated with AAS administration at high doses (Parrott et al 1994). Furthermore, aggression and violent behavior are commonly reported in connection to AAS abuse (Galligani et al 1996; Parrott et al 1994; Pope et al 2000; Thiblin et al 1997). During periods of chronic AAS exposure mania have been observed, while after discontinuation of long-term AAS abuse depression and suicidal ideas have appeared (Brower et al 1989b; Brower et al 1990; Pope and Katz 1988; Pope and Katz 1994). It also seems that AAS abuse can lead to cognitive dysfunctions such as distractibility, forgetfulness and confusion (Su et al 1993). Furthermore, according to a case report, balance disorders and long lasting vertigo in connection to AAS administration were observed (Bochnia et al 1999).

Several studies have also indicated that AAS might lead to dependence (Brower et al 1989a; Hays et al 1990; Yesalis et al 1990). In a study of 100 Australian AAS users approximately 25% met the DSM IV criteria for AAS dependence, as well as for AAS abuse (Copeland et al 2000). In another study, 57% of male weight lifters using AAS displayed several symptoms consistent with dependence (Brower et al 1991). Thus, these studies support the claim that AAS are drugs of dependence.

The biochemical events responsible for the alterations of behaviors that are so frequently observed in connection to AAS abuse are not fully understood and neither are the roles of the various neurochemical systems in the brain. It has previously been demonstrated that chronic AAS treatment in rats affects both the dopaminergic as

well as the serotonergic systems of the brain (Kindlundh et al 2002; Kindlundh et al 2003; Kindlundh et al 2001b; Kindlundh et al 2004; Lindqvist et al 2002; Thiblin et al 1999). Furthermore, nandrolone decanoate induces alterations in the gene transcripts of both corticotropin releasing factor (CRF) and proopiomelanocortin (POMC) (Lindblom et al 2003; Schlussman et al 2000). AAS also affect the gamma-aminobutyric acid (GABA) (Bitran et al 1996) as well as the glutamate system (Le Greves et al 1997; Le Greves et al 2002).

Several adverse behavioral effects, characterizing AAS abusers, could result from alterations in the above-mentioned neurotransmitter systems but also in part be attributed to a disturbance of the delicate balance within neuropeptide systems in the central nervous system. The opioid system, tachykinin system and systems regulating the calcitonin gene-related peptide levels are of special relevance in this context.

Peptidergic systems

Opioid peptides

The first opioid peptides to be discovered were the enkephalins (Hughes et al 1975). The enkephalins are pentapeptides with binding affinity for the delta opioid peptide (DOP) receptor and also to some extent for the mu opioid peptide (MOP) receptor. The opioid peptides, including enkephalins, €-endorphin, dynorphin A, dynorphin B and / -neoendorphin, are all based on the enkephalin N-terminal amino acid sequence, as can be seen in Table 1. The dynorphins prefer the kappa opioid peptide (KOP) receptor whereas €-endorphins are less selective but mainly bind to the MOP receptor. The above-mentioned opioid peptides share a common N-terminal sequence, which is essential for their opioid action (see Table 1) and they are referred to as the *classical* endogenous opioids. However, even shorter opioids lacking the classical N-terminal sequence have been discovered. For example, the endomorphins that consist of only four amino acid residues have been shown to act as potent and selective MOP receptor agonists (Zadina et al 1997). Hemorphins and €-casomorphins are other examples of peptides with opioid receptor activity (Brantl et al 1986; Henschen et al 1979; Nyberg et al 1997).

The classical opioid peptides, i.e. enkephalins, dynorphins and €-endorphin are derived from three different propeptides; proenkephalin, prodynorphin and proopiomelanocortin (POMC), respectively (Kakidani et al 1982; Nakanishi et al 1979; Noda et al 1982).

Interestingly, certain enzymes have been reported to be capable of generating enkephalins from dynorphin peptides (Chesneau et al 1994; Nyberg et al 1985; Nyberg and Silberring 1990; Silberring et al 1992). Thus, transformation of a KOP receptor agonist into a DOP receptor agonist can occur. This processing can be of interest since actions mediated by the KOP receptor in some cases can oppose those

resulting from DOP/MOP receptor activation (Koob 1996). Morphine is one of several examples of non-peptide opioid receptor agonists that have been used in the clinics for a very long time as an analgesic drug. Beside the modulatory action of opioids in the processing of pain, opioid neuropeptides are also associated with several other behavioral processes. These include dependence, reward, sedation and response to stress.

Substance P, substance P_{1-7}, substance P endopeptidase and the NK1 receptor

Substance P (SP) was discovered 1931 by von Euler and Gaddum (Von Euler and Gaddum 1931) and is an undecapeptide that belong to the tachykinin family. The most well known members of this family are SP, neurokinin A and neurokinin B, which all bind to neurokinin (NK) receptors. Neurokinin A and neurokinin B prefer the NK2 and NK3 receptors, respectively, whereas SP primarily binds to the NK1-receptor. Recently, studies reported that hemokinin and endokinins are two other new putative members of the tachykinin family (Kurtz et al 2002; Page et al 2003; Zhang et al 2000). SP originates from at least three different gene transcripts, including / -, €, preprotachykinin. After being released from the precursors, SP is amidated at its C-terminal end (Eipper et al 1992), a feature that together with the two N-terminal proline residues (Pro^2 and Pro^4) contributes to the stability of the peptide. SP is degraded by different enzymes to smaller fragments (Persson et al 1995; Skidgel et al 1984; Yokosawa et al 1983), for reviews see (Hallberg et al 2005; Nyberg and Terenius 1991). One of these fragments, SP_{1-7} (Sakurada et al 1985), that is addressed in this thesis, is partly produced via action of substance P endopeptidase (SPE).

Historically the role of SP in connection with pain transmission has attracted most interest (Zubrzycka and Janecka 2000), but the role of the neuropeptide for induction and progression of inflammatory response has also been intensively studied (Barnes 1986; Lembeck and Holzer 1979; Levine et al 1986; Mantyh C. R. et al 1988). Furthermore, and particularly in the context of AAS abuse, SP is associated with aggression (Shaikh et al 1993) and NK1 receptor activation also with depression (Kramer et al 1998). The N-terminal fragment of SP, SP_{1-7}, a bioactive metabolite is of special interest since it often exerts opposite effects to SP, for a review see (Hallberg and Nyberg 2003).

Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP), a 37 amino acid residue long peptide, is part of the calcitonin family together with peptides such as amylin and adrenomedullin, but also the newly discovered intermedin (Roh et al 2004). CGRP, derived from the same primary transcript as calcitonin (Amara et al 1982; Rosenfeld et al 1983), is widely distributed in the CNS and is also present in the cardiovascular system. CGRP exist in two different forms referred to as / -CGRP and €-CGRP, in rat only

differing by one amino acid. The peptides act through the CGRP-1 and CGRP-2 G-protein coupled receptors (Wimalawansa 1996). CGRP is known to be a potent vasodilator (Brain et al 1985) but seems also to play a role in the drug reward system (Salmon et al 2004; Zhou et al 2003). In addition, CGRP serves as a regulator of food intake (Lutz et al 1997). The neuropeptide interacts with both dopamine and noradrenaline systems in the brain (Deutch and Roth 1987; Tsuda et al 1992) and there is support for a role of CGRP in psychiatric disorders (Mathe et al 1994; Mathe et al 1996).

Table 1. *The amino acid sequence of selected peptides discussed in the present thesis. *indicates a disulfide bond between the Cys[2] and Cys[7] amino acid residues.*

Peptides	Amino acid sequence
Opioids	
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu
Met-enkephalin	Tyr-Gly-Gly-Phe-Met
Met-enkephalin-Arg^6-Phe^7	Tyr-Gly-Gly-Phe-Met-Arg-Phe
Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
Dynorphin B	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
-Neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
! -Endorphin (human)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Endomorphin-1	Tyr-Pro-Trp-Phe-NH_2
Endomorphin-2	Tyr-Pro-Phe-Phe-NH_2
Tachykinins	
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
SubstanceP_{1-7}	Arg-Pro-Lys-Pro-Gln-Gln-Phe
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
CGRP	
-CGRP (human)	Ala-Cys*-Asp-Thr-Ala-Thr-Cys*-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2
! -CGRP (human)	Ala-Cys*-Asn-Thr-Ala-Thr-Cys*-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Met-Val-Lys-Ser-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2
-CGRP (rat)	Ser-Cys*-Asn-Thr-Ala-Thr-Cys*-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-Phe-NH_2
! -CGRP (rat)	Ser-Cys*-Asn-Thr-Ala-Thr-Cys*-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2

Comments on the introduction to the peptidergic systems

As presented briefly above in the short summaries of the opioid, tachykinin and CGRP systems, these neuropeptide systems are all involved in modulating biochemical events that to various degrees could be of relevance for the adverse behaviors often recognized in connection with AAS abuse. However, to the best of our knowledge and with the exception for the opioids, the impact of AAS on these neuropeptide systems in the rat brain was not known at the time the studies presented in this thesis were initiated. Thus, no information was available neither on which brain structures

that might be affected and if so if those anatomic structures could be associated in any way with adverse behaviors nor on neuropeptide concentrations that might be altered as a result of chronic AAS administration. In a longer perspective such data could be valuable in comparisons of AAS with different characteristic features in human, e.g. nandrolone with stanozolol, the latter reducing aggressive behavior whereas nandrolone induces this type of behavior. Data on neuropeptide levels and the brain structures affected could possibly enable correlations between peptide levels, affected brain structures and behaviors, e.g. aggression.

Aims

The general aim of this thesis was to study the effect of chronic anabolic androgenic steroid administration on peptidergic systems in the rat brain. Special focus was devoted to regions in the brain that are considered to be associated with the regulation of rewarding effects, aggression, depression, memory and anxiety, using nandrolone decanoate as a prototype anabolic androgenic steroid.

The specific aims were;

- To study the effect of nandrolone decanoate on the levels of opioid peptides MEAP and Dyn B in the rat brain.
- To study the effect of nandrolone decanoate on the levels of SP and its bioactive N-terminal fragment, $SP_{1\text{-}7,}$ in the rat brain.
- To study the effect of nandrolone decanoate on the density of the NK1 receptor in the rat brain.
- To study the effect of nandrolone decanoate on substance P endopeptidase-like activity in the rat brain
- To study the effect of nandrolone decanoate on the levels of CGRP in the rat brain.

Materials and methods

General procedures

In order to test the hypothesis that AAS affect peptidergic systems in the brain, studies in which male Sprague-Dawley rats were exposed to AAS were conducted. To obtain comparable data in the different investigations, the same steroid and dose-regime were used in all reports included in this thesis. We selected nandrolone decanoate as a proper representative AAS prototype molecule. The reason for this choice was the fact that nandrolone is one of the most common and popular steroids on the illegal AAS market and that the compound is classified by the AAS users as a "good" steroid (Evans N. A. 1997; Llewellyn 2002; van Marken Lichtenbelt et al 2004), due to its high anabolic and low androgenic effects. The nandrolone decanoate was administered during 14 days at a dose of 15 mg/kg/day, a dose that we found relevant. This dose is estimated to be approximately 40 times higher than the therapeutic dose used in clinic but notably this dose mimics heavy AAS abuse, which frequently is reported to be 10-100 times higher than the therapeutical doses (Brower 1993; Pope and Katz 1988).

Animals and drug treatment

Adult male Sprague-Dawley rats, purchased from Alab, Sollentuna, Sweden, were used in all investigations. The rats, weighing 320-400 g (paper I, II and V), 305-335 g (paper III) and 480-520 g (paper IV) at the start of the experiments, were housed in air-conditioned rooms at a temperature of 22 ± 2°C and a humidity of 50 ± 10% with a 12 hour light/dark cycle (lights on 6.00 a.m.). Standard pellet food (R36 Labfor; Lactamin, Vadstena, Sweden) and water were freely available.

At the arrival to the animal facilities the rats were randomly housed four by four in standard macrolon cages (59x38x20 cm). The rats were adapted to the novel laboratory environment for 11 ± 4 days before the experiments started. The treatment in all animal experiments consisted of daily intramuscular (i.m.) injections (left and right hind leg every other day, respectively) of nandrolone decanoate (15 mg/kg day) or oil vehicle (sterile arachidis oleum). The injections, 0.1 ml/day, were administered during 14 days. On the 15:th day of the experiments (paper III and IV), approximately 24 hours after the last injection, all animals were sacrificed by

decapitation. In the studies described in paper I, II and V, half of the animals (eight nandrolone decanoate and eight control treated rats) were sacrificed by decapitation on the 15:th day whereas the other half were thereafter undergoing a three-week long recovery period before decapitation. During this recovery period the rats were neither exposed to AAS nor vehicle injections.

All experimental animal procedures presented in this thesis were approved by the local ethical committee of the Swedish National Board for Laboratory Animals.

Dissection

In the studies reported in paper I, II, IV and V the brains were rapidly taken out and dissected using a rat brain matrix (Activational System Inc., Mortella Drive Warren, MI, U.S.A.) following decapitation. The frontal cortex, hypothalamus, nucleus accumbens, striatum, amygdala, hippocampus, substantia nigra, VTA, PAG, pituitary anterior, pituitary posterior and spinal cord were collected and immediately put on dry ice. The tissues were kept at -80˚C until further use. Regarding the study on NK1 receptors presented in paper III the brains were rapidly removed and frozen in 2-methyl-butane at –25 ± 5°C before being stored at –80°C until further used.

Radioimmunoassay

The radioimmunoassay technique is well suited for determining peptide concentrations in brain tissue due to its ability to detect peptides down to the low femtomolar range. The radioimmunoassay technique also offers good selectivity between different related peptides, especially when pre-separation is performed. Furthermore, the radioimmunoassay technique is practical when handling large numbers of samples. The drawbacks of the technique are primarily problems to obtain high reproducibility and comparable results after running the radioimmunoassays at different times.

Peptide extraction

The dissected brain tissues from each animal were added to preheated (90˚C) 1.0 M acetic acid in order to avoid enzymatic degradation of the peptides. The tissues were heated in a water bath (90˚C) for 5 minutes and chilled on ice for 10 minutes. The brain tissues were subsequently homogenized using ultrasonification and thereafter heated (90˚C) for another 5 minutes. The homogenates were centrifuged (12 000 x g) and the supernatant fractions were diluted with 0.1 M formic acid and 0.018 M pyridine (pH 3.0).

Separation procedures

The diluted samples from the peptide extraction were purified by ion exchange chromatography. The samples were added to columns packed with SP-Sephadex C-25 gel. After washing the columns with 0.1 M formic acid and 0.018 M pyridine the fractions containing relevant peptides were eluted using buffers (formic acid/pyridine) with three step-wise increases in ionic strengths. The eluates were evaporated in a Speed Vac centrifuge (Savant, Hicksville, NY, U.S.A.) and stored at –20°C until analyzed by RIA.

Radioimmunoassay technique

The radioimmunoassay (RIA) technique used for all peptides, except Dyn B, was based on the charcoal adsorption technique (Eriksson et al 1996; Sharma et al 1990). Briefly, samples or standards were added in triplicates to incubation tubes together with the diluted antibody and the labeled iodinated peptide (4,500-5,500 cpm/100 µl). The antibody and the labeled peptide were each diluted in a 50 mM sodium phosphate buffer (pH 7.4) containing 0.1% gelatin, 0.1% bovine serum albumin, 0.82% NaCl and 0.93% EDTA. The samples were incubated for 24 hours (4˚C) and subsequently incubation was terminated by adding active charcoal solution (charcoal and dextran T-70 dissolved in 50 mM sodium phosphate buffer, pH 7.4). After 10 minutes of incubation the mixture was centrifuged for 1 minute in a Beckman Microfuge in order to separate the bound and the free peptides. The supernatant, 300 µl, was collected and the radioactivity was determined in a gamma-counter.

The radioimmunoassay technique used for Dyn B is based on the double antibody precipitation. Briefly, the samples, antibody and the radioactive iodine labeled dynorphin were incubated for 24 h before sheep anti-rabbit serum was added and the samples were incubated for an additional hour. The samples were subsequently centrifuged and the supernatant discarded before the radioactivity in the remaining pellet was determined using a gamma-counter.

The detection limits of the RIA were about 5 fmol/tube and 2 fmol/tube for SP and SP_{1-7}, respectively. For CGRP, MEAP and Dyn B the corresponding values were 5 fmol/tube, 2 fmol/tube and 2 fmol/tube, respectively. The 50% inhibition of tracer binding was about 20 fmol/tube and 10 fmol/tube for SP and SP_{1-7}, respectively. For CGRP, MEAP, Dyn B the corresponding values were 20 fmol/tube, 10 fmol/tube and 10 fmol/tube, respectively. Cross reactivity in the RIAs with other tachykinins, opioids and related peptides were as given elsewhere (Eriksson et al 1996; Ploj et al 2003; Sakurada et al 1991; Sharma et al 1990).

Antibodies

All antibodies were developed in rabbit through injection of the peptide-thyroglobulin conjugate. Specific fragments of the peptides were selected for injection in order to obtain selective antibodies. For example, MEAP was injected as an oxidized

analogue and/ -rCGRP(23-37) was selected in order to obtain a selective antibody for/ -rCGRP.

Labeling of peptides

The peptides used for the ^{125}I-labeling were MEAP, Dyn B, Tyr^{8}-SP, Tyr-SP$_{1-7}$ and Tyr-/ -rCGRP$_{23-37}$. Briefly, each peptide and the radioactive iodide (^{125}I) were added to 0.2 M sodium phosphate buffer. The reaction started by the addition of chloramine-T and was terminated after approximately 40 seconds by adding 15% acetonitrile. The reaction times were adjusted depending on peptide in order to avoid diiodination in the second ortho position of the hydroxyl group of the tyrosine or at other positions of the peptide that otherwise easily could occur. The monoiodinated peptides were purified using a HPLC system equipped with a reversed phase column. Elution was carried out using a linear gradient of 15%-40% acetonitrile for 40 minutes at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and the radioactivity profile was determined by a gamma-counter. The fractions containing the labeled peptides were then diluted in a gelatin buffer, aliquoted and stored at -20˚C until needed. A more detailed description of the procedure can be found in paper II, IV and V or elsewhere (Persson et al 1992).

Autoradiography

The rat brains were frozen in 2-methyl-butane and stored at –80°C until further used. Coronal frozen sections (14 µm) of relevant brain areas were cut in a cryostat, thaw-mounted on gelatin-coated slides and thereafter stored at –80°C until used for autoradiography.

The sections were pre-incubated in 50 mM Tris-HCl (pH 7.4) buffer, containing 0.9% NaCl and 0.02% bovine serum albumin (BSA) and subsequently incubated with 100 pM [^{125}I]-BHSP in 50 mM Tris-HCl (pH 7.4) buffer containing 3 mM $MnCl_2$, 0.02% BSA, bacitracin, leupeptin, and chymostatin. The concentration of the radioactive ligand [^{125}I]-BHSP was adopted from previous studies in order to obtain comparable results (Croul et al 1998; Mantyh P. W. et al 1989; Schoborg et al 2000). The non-specific binding was determined using 1 µM SP. The incubation was terminated by washing the slides in 50 mM Tris-HCl buffer (pH 7.4). The slides were thereafter co-exposed with autoradiographic [^{125}I]-micro-scales to hyperfilm for 48 hours. The films were manually developed, fixed and digitalized using an Epson perfection 4870 photo scanner. The optical densities were converted to fmol/mg using NIH Image program. The mean values of the measurements in each region from duplicate coronal sections were used as entrance for the statistical evaluation between the control group and the nandrolone treated group. The brain regions of interest were identified using a rat brain atlas (Paxinos and Watson 1997).

Enzyme activity

Tissue extraction

The dissected brain tissues were each homogenized by ultrasonification in 20 mM Tris HCl (pH 7.8) and subsequently centrifuged for 20 min at 8000 x g. The supernatants were re-centrifuged for 20 min at 10000 x g and the new supernatants were collected and stored at -80°C until further used.

Enzyme assay

The SPE-like activity was determined by studying the conversion of SP to its N-terminal fragment SP_{1-7} in the specific brain regions. Briefly, a 20 mM Tris HCl (pH 7.4) buffer containing the enzyme inhibitors phosphoramidon and captopril as well as the enzyme homogenate were preincubated at 37°C for 20 minutes before the substrate SP was added. SP was incubated in the enzyme homogenate and fractions were withdrawn at different time points (20 min and 40 min) in order to create a SPE-like activity profile. Ice-cold methanol was added to the withdrawn samples in order to terminate the enzymatic activity in the fractions. The methanol containing fractions were evaporated in a Speed Vac centrifuge and stored at -20°C until analyzed. The dried fractions were re-dissolved and the concentration of SP_{1-7} was assessed in each sample by RIA. A more detailed description of the procedure can be found in paper IV.

HPLC characterization

The SP metabolites, generated by the SPE-like activity, were also studied by HPLC. During similar incubations as described above, fractions were withdrawn, evaporated and redissolved in 0.01% trifluoroacetic acid (TFA). The samples were analyzed by reversed phase HPLC using a Pharmacia SMART system (µRPC C2/C18, SC 2.1/10 column). An acetonitrile (0.01% TFA) gradient was run and the peaks were detected by UV-absorbance at 214 nm. A more detailed description of the HPLC characterization can be found in paper IV.

Statistics

Statistical analyses of difference between groups were performed using the unpaired Student's t-test. P-values below 0.05 were considered significant.

Results

Met-enkephalin-Arg^6-Phe^7 and dynorphin B

The levels of the opioid peptides, MEAP and Dyn B, were both significantly elevated in hypothalamus, striatum and PAG after 14 days of chronic nandrolone decanoate treatment as shown in Figure 8. The observed differences did not remain significant after a three-week recovery period, although the Dyn B levels in PAG and hypothalamus still tended to be higher after this treatment-free period. Comparing the recorded levels of MEAP and Dyn B in certain brain regions, e.g. nucleus accumbens, a significant positive correlation was found in control animals, however, in rats treated with AAS this correlation was abolished. This pattern remained also after three weeks recovery.

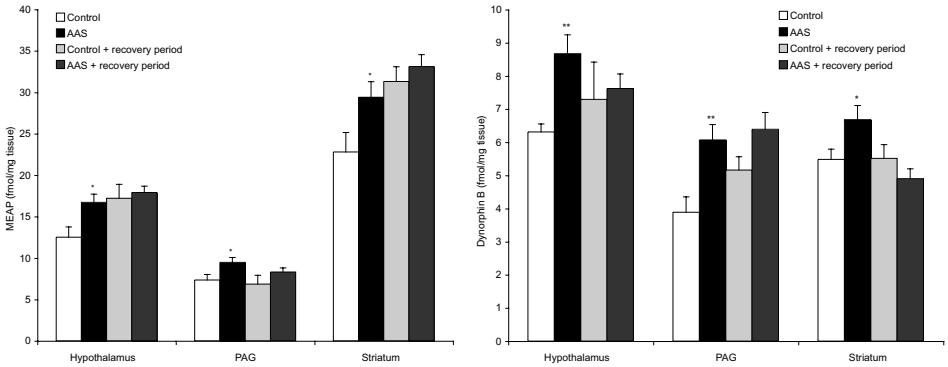


Figure 8. The MEAP and Dyn B concentrations (fmol/mg tissue) in male Sprague-Dawley rat brain after two weeks of daily treatment with nandrolone decanoate and after a three-week recovery period. The columns and error bars represent mean ± S.E.M. concentrations. Significant levels, according to Student's t-test are denoted by *P < 0.05 and **P < 0.01.

Substance P and substance P$_{1\text{-}7}$

Chronic treatment of rats with nandrolone decanoate induced a significant increase in the levels of substance P in amygdala, hypothalamus, PAG and in striatum. In PAG the increase also remained after the treatment free recovery period. The results are summarized in Table 2.

The concentrations of SP_{1-7} also increased in two of the areas studied. In the nucleus accumbens, the levels increased by 47% and in the PAG by 40%. However, in the striatum the level of SP_{1-7} was found to display a significant decrease after nandrolone decanoate administration both directly after the two weeks of treatment and also after the three-week recovery period. After the two weeks of steroid treatment the ratio between SP and SP_{1-7} significantly increased in both the striatum and the amygdala, alterations that also remained after the three-week recovery period.

Table 2. *The SP and SP_{1-7} concentrations (fmol/mg tissue) in male Sprague-Dawley rat brain after two weeks of nandrolone decanoate treatment and after a three-week recovery period. Significant levels, according to Student's t-test are denoted by *P < 0.05.*

Region	Control	AAS	Control + recovery period	AAS + recovery period
		Substance P (SP)		
Amygdala	11.6 ± 0.46	14.8 ± 1.1*	16.6 ± 1.15	19.2 ± 1.9
Hippocampus	1.32 ± 0.24	1.15 ± 0.13	1.26 ± 0.13	1.24 ± 0.1
Hypothalamus	72.6 ± 3.2	82.6 ± 2.9*	82.1 ± 6.0	81.0 ± 4.8
Nucleus accumbens	18.8 ± 2.1	19.6 ± 2	18.9 ± 1.4	21.1 ± 1.6
PAG	102 ± 10	125 ± 3.2*	94 ± 7.8	118 ± 6.6*
Striatum	198 ± 11	243 ± 13*	235 ± 10	248 ± 15
Substantia nigra	196 ± 32	256 ± 27	163 ± 25	154 ± 7.3
		Substance P_{1-7} (SP_{1-7})		
Amygdala	1.03 ± 0.1	0.89 ± 0.08	1.42 ± 0.13	1.05 ± 0.12
Hippocampus	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.15 ± 0.01
Hypothalamus	1.81 ± 0.06	1.76 ± 0.09	2.27 ± 0.17	2.12 ± 0.14
Nucleus accumbens	1.05 ± 0.06	1.54 ± 0.15*	2.27 ± 0.11	2.69 ± 0.16
PAG	2.68 ± 0.31	3.76 ± 0.24*	3.24 ± 0.18	2.98 ± 0.25
Striatum	0.84 ± 0.07	0.63 ± 0.03*	0.79 ± 0.02	0.65 ± 0.04*
Substantia nigra	13.4 ± 1.6	12.7 ± 1.2	8.87 ± 1.1	7.1 ± 0.71

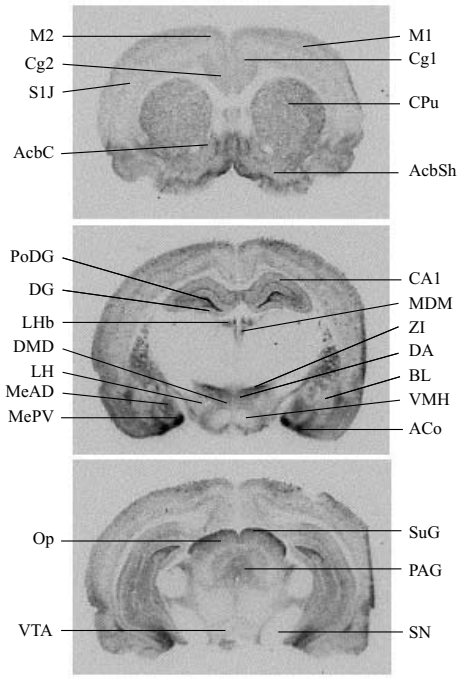
The NK1 receptor

Treatment with nandrolone decanoate was shown to induce significant down-regulations in the density of NK1 receptors, as deduced by [^{125}I]-BHSP (i.e. [^{125}I]-Bolton Hunter Substance P), in certain brain regions of the rat brain. Thus, as shown in Table 3, the density of NK1 receptors was significantly decreased in the nucleus accumbens core, dentate gyrus, basolateral amygdaloid nucleus, ventromedial hypothalamic nucleus, the dorsal part of the dorsomedial hypothalamic nucleus and PAG. Although no statistical significance was observed in all structures, the chronic

AAS treatment tended to induce an overall trend of receptor down-regulation in most brain areas studied. An exception was the cortex region where the AAS brains on the contrary tended to exhibit higher NK1 receptor densities.

Table 3. *The NK1 receptor density (fmol/mg) in male Sprague-Dawley rat brain after two weeks of nandrolone decanoate treatment. Significant levels, according to Student's t-test are denoted by *P < 0.05. The abbreviations used can be found in Figure 9.*

Region	**Bregma**	**Control** Mean ± S.E.M.	**AAS** Mean ± S.E.M.
Basal ganglia			
Caudate putamen	+1.60 mm	9.74 ± 1.35	8.27 ± 0.81
Accumbens nucleus, core	+1.60 mm	10.73 ± 1.22	6.81 ± 0.88 *
Accumbens nucleus, shell	+1.60 mm	12.81 ± 2.57	9.83 ± 0.73
Cortex			
Cingulate cortex, area 1	+1.60 mm	1.36 ± 0.14	1.58 ± 0.11
Cingulate cortex, area 2	+1.60 mm	1.58 ± 0.10	1.81 ± 0.17
Primary motor cortex	+1.60 mm	1.12 ± 0.11	1.16 ± 0.14
Secondary motor cortex	+1.60 mm	1.63 ± 0.23	1.76 ± 0.18
Primary somatosensory cortex, jaw region	+1.60 mm	0.83 ± 0.06	0.71 ± 0.05
Amygdala			
Basolateral amygdaloid nucleus	-2.56 mm	2.62 ± 0.16	2.01 ± 0.23 *
Medial amygdaloid nucleus, anterodorsal part	-2.56 mm	15.29 ± 1.72	14.45 ± 0.74
Medial amygdaloid nucleus, posteroventral part	-2.56 mm	321.51 ± 56.33	353.67 ± 46.66
Anterior cortical amygdaloid nucleus	-2.56 mm	305.91 ± 21.05	284.36 ± 14.84
Hippocampus			
Field CA1 of hippocampus	-2.56 mm	1.73 ± 0.27	1.52 ± 0.25
Dentate gyrus	-2.56 mm	1.36 ± 0.11	1.00 ± 0.10 *
Polymorph layer of the dentate gyrus	-2.56 mm	98.67 ± 14.92	93.13 ± 8.70
Hypothalamus			
Ventromedial hypothalamic nucleus	-2.56 mm	0.14 ± 0.02	0.09 ± 0.01 *
Dorsomedial hypothalamic nucleus, dorsal part	-2.56 mm	1.76 ± 0.28	1.01 ± 0.17 *
Lateral hypothalamic area	-2.56 mm	0.73 ± 0.08	0.60 ± 0.07
Dorsal hypothalamic area	-2.56 mm	9.81 ± 1.15	8.86 ± 0.94
Thalamus			
Lateral habenular nucleus	-2.56 mm	4.18 ± 1.09	4.18 ± 0.60
Mediodorsal thalamic nucleus, medial part	-2.56 mm	4.62 ± 0.81	5.16 ± 1.19
Zona incerta	-2.56 mm	9.87 ± 2.16	9.20 ± 1.93
Miscellaneous			
Superficial gray layer of the superior colliculus	-5.80 mm	17.90 ± 4.83	21.43 ± 3.58
Optic nerve layer of the superior colliculus	-5.80 mm	2.18 ± 0.28	2.26 ± 0.36
Periaqueductal gray	-5.80 mm	6.23 ± 0.56	4.71 ± 0.36 *
Ventral tegmental area	-5.80 mm	0.06 ± 0.01	0.06 ± 0.01
Substantia nigra	-5.80 mm	not detectable	not detectable



Abbreviation	Region
AcbC	Accumbens nucleus, core
AcbSh	Accumbens nucleus, shell
ACo	Anterior cortical amygdaloid nucleus
BL	Basolateral amygdaloid nucleus
CA1	Field CA1 of hippocampus
Cg1	Cingulate cortex, area 1
Cg2	Cingulate cortex, area 2
CPu	Caudate putamen
DA	Dorsal hypothalamic area
DG	Dentate gyrus
DMD	Dorsomedial hypothalamic nucleus, dorsal part
LH	Lateral hypothalamic area
LHb	Lateral habenular nucleus
M1	Primary motor cortex
M2	Secondary motor cortex
MDM	Mediodorsal thalamic nucleus, medial part
MeAD	Medial amygdaloid nucleus, anterodorsal part
MePV	Medial amygdaloid nucleus, posteroventral part
Op	Optic nerve layer of the superior colliculus
PAG	Periaqueductal gray
PoDG	Polymorph layer of the dentate gyrus
S1J	Primary somatosensory cortex, jaw region
SN	Substantia nigra
SuG	Superficial gray layer of the superior colliculus
VMH	Ventromedial hypothalamic nucleus
VTA	Ventral tegmental area
ZI	Zona incerta

Figure 9. Representative autoradiograms from control rats illustrating the NK1 receptor density, as deduced by [^{125}I]-Bolton Hunter SP, in regions measured at a) bregma +1.60 mm, b) bregma -2.56 mm and c) bregma –5.80 mm.

Substance P endopeptidase

The SPE-like activity was determined by measuring the amount of SP_{1-7} generated over time using SP as an enzyme substrate. The results demonstrated that SPE-like activities are exerted in all brain regions studied. Furthermore, the study revealed that the predominantly membrane bound enzymes ACE and NEP to some extent exist also in a cytosolic soluble form in the brain. The two-week treatment with daily i.m. injections of nandrolone decanoate, significantly decreased the SPE-like activity in hypothalamus, striatum, substantia nigra and ventral tegmental area in the rat brain as summarized in Figure 10.

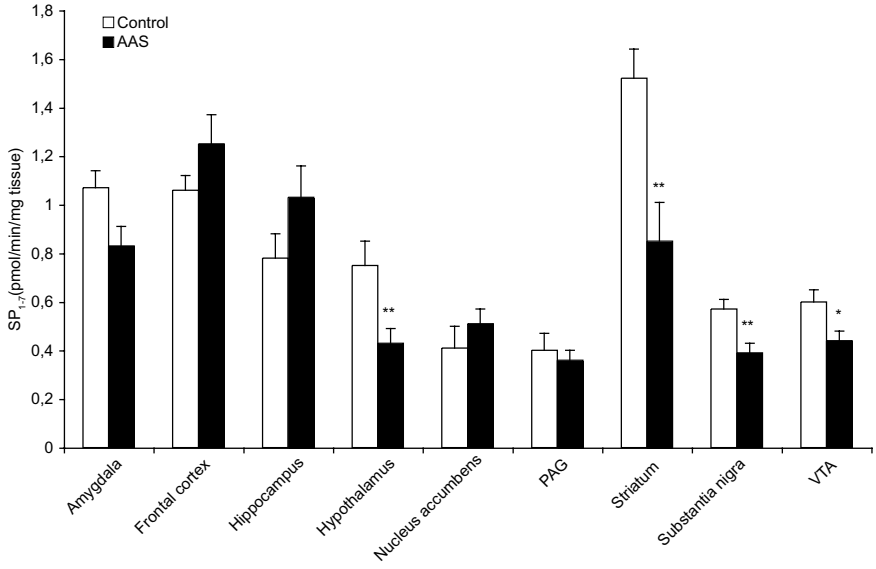


Figure 10. The SPE-like activity in male Sprague-Dawley rat brain after two weeks of daily treatment with nandrolone decanoate. The columns and error bars represent mean ± S.E.M. concentration (fmol/mg tissue) of SP_{1-7} generated in various brain regions after addition of the SPE substrate SP. Significant levels, according to Student's t-test are denoted by *P < 0.05.

Calcitonin gene-related peptide

High concentrations of CGRP were measured in the anterior pituitary, dorsal spinal cord and in the amygdala. Data are summarized in Table 4. Chronic treatment with nandrolone decanoate was found to induce significantly increased levels of CGRP in the amygdala and nucleus accumbens. The increased level of CGRP in nucleus accumbens also remained elevated after a treatment-free recovery period. However, in the anterior pituitary, the levels of CGRP were significantly lower in the nandrolone treated rats compared to the rats in the control group. This pronounced lower level of immunoreactivity in the anterior pituitary was also encountered after the three-weeks of recovery.

Table 4. *The CGRP concentrations (fmol/mg tissue) in male Sprague-Dawley rat brain after two weeks of nandrolone decanoate treatment and after a three-week recovery period. Significant levels, according to Student's t-test are denoted by *P < 0.05.*

Region	Control	AAS	Control + recovery period	AAS + recovery period
		Calcitonin gene-related peptide (CGRP)		
Amygdala	2.6 ± 0.29	4.6 ± 0.62*	6.8 ± 0.51	8.1 ± 0.67
Hypothalamus	0.48 ± 0.03	0.56 ± 0.04	0.56 ± 0.04	0.66 ± 0.06
Nucleus accumbens	0.13 ± 0.01	0.18 ± 0.02*	0.21 ± 0.02	0.29 ± 0.03*
PAG	1.0 ± 0.08	1.1 ± 0.14	0.6 ± 0.13	0.8 ± 0.06
Pituitary anterior	5.6 ± 0.94	2.5 ± 0.46*	5.0 ± 0.37	2.6 ± 0.32*
Spinal cord dorsal	4.2 ± 0.70	5.0 ± 0.8	4.6 ± 0.67	4.9 ± 0.73
Striatum	0.57 ± 0.07	0.65 ± 0.02	1.3 ± 0.15	1.8 ± 0.21

Body weight

Chronic nandrolone decanoate (15 mg/kg/day) administration has an impact on the weight of the rats. The less pronounced gain in weight observed after AAS treatment is demonstrated in Figure 11. The weight differences remained throughout the three-week recovery period.

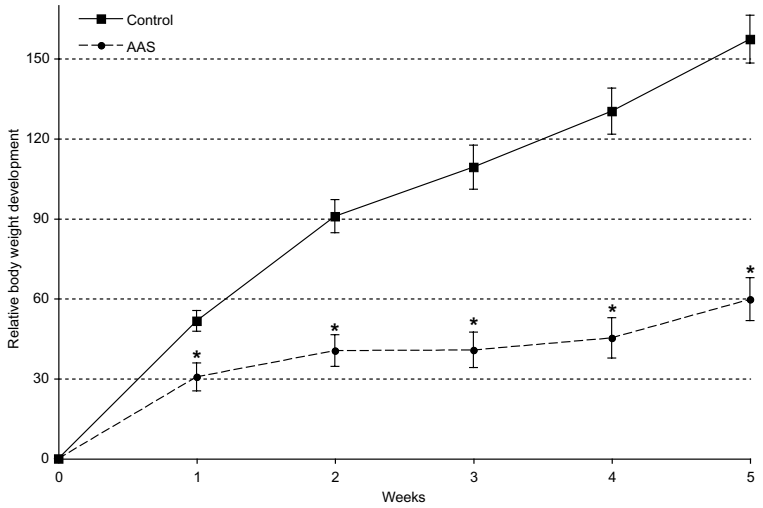


Figure 11. Body-weight development in male Sprague-Dawley rats relative to the weight the first day of the experiment (week 0) during and after two weeks of nandrolone decanoate treatment. Significant levels, according to Student's t-test is denoted by *P < 0.05.

Discussion

Alterations in the endogenous opioid system, as a result of AAS administration, have previously been reported (Johansson et al 1997; Menard et al 1995). In fact, a remarkable increase in the concentration of €endorphin in the VTA was reported after nandrolone decanoate administration to rats (Johansson et al 1997). Furthermore, alterations of opioid peptide levels have also been reported eight weeks after chronic nandrolone decanoate treatment (Johansson et al 2000). Furthermore, AAS have been shown to down-regulate the DOP receptor mRNA in cells, through a mechanism suggested to be independent of the androgen receptor (Pasquariello et al 2000).

MEAP and Dyn B serve as markers of the activity in two genetically different opioid peptide systems. The immunoreactivities of the two peptides in hypothalamus, the major control center in the brain, were significantly higher after AAS administration. This finding might have implications in the context of AAS abuse since hypothalamus regulates not only autonomic and endocrine responses, but also defensive and aggressive behaviors as well as emotions (Dielenberg and McGregor 2001; George et al 2004; Hassanain et al 2003). Significantly higher levels of MEAP and Dyn B were also encountered in the striatum, which is also engaged in controlling emotions, fear and aggression (Calder et al 2004). However, one should bear in mind in this context that the relation between endogenous opioids and aggressive behavior in rodents, as documented in literature, is somewhat controversial (Tordjman et al 2003). PAG, the third structure dealt with in this context, also displayed significantly higher levels of MEAP and Dyn B after chronic nandrolone decanoate administration. These results further support that high doses of AAS affects areas regulating aggressive behavior.

In addition, the changes in peptide concentrations induced by the nandrolone administration could probably also affect the sensitive brain reward system. Dynorphin and enkephalin peptides both have regulatory roles with regard to the dopamine transmission in the mesolimbic reward system (Bals-Kubik et al 1993; Wise and Bozarth 1982). Thus, the euphoria and increased self-esteem often observed after AAS use may partly be attributed to stimulation of this system and an increased dopamine activity. The study summarized in this thesis demonstrated that nandrolone decanoate affected the dynorphin and the enkephalin systems not only in striatum, hypothalamus and PAG but also induced an imbalance in nucleus accumbens, a structure reported to be involved in reward mechanisms (Herz 1998). Concerning pain perception, opioids are effective as analgesics. While the enkephalins induce their effects mainly via activation of DOP receptors, the

dynorphins activate mainly the KOP receptors. Through their action on receptors in PAG, the endogenous opioids are able to suppress nociceptive spinal reflexes by acting on the serotonin pathway projecting to the dorsal horn of the spinal cord from nucleus raphe magnum. At the spinal level, substance P release from dorsal horn neurons is inhibited leading to reduced transmission of nociceptive impulses. Hence, the significantly higher levels of MEAP and Dyn B observed in PAG after AAS administration might indirectly suppress SP release from the dorsal horn neurons and possibly as a consequence and provided that the biochemical alterations can be translated into man, affect the pain perception experienced by AAS abusers.

Substance P (SP), probably the most studied member of the tachykinin family, is the preferred endogenous agonist for the NK1 receptor. Thus, SP exerts its effects as a neurotransmitter and neuromodulator via activation of the G-protein coupled NK1 receptor. However, SP effects can also indirectly be achieved through fragmentation of the peptide to its N-terminal bioactive fragment SP_{1-7}. As mentioned previously, this heptapeptide sometimes exerts similar effects as the parent peptide but also in many cases completely opposite actions from that of SP (Hallberg and Nyberg 2003). Several enzymes, including SPE, NEP and ACE, are capable of generating SP_{1-7} from SP in the CNS (Persson et al 1995; Skidgel et al 1984; Yokosawa et al 1983). Whereas both NEP and ACE are predominantly membrane bound enzymes, SPE, a fairly SP specific enzyme responsible for the formation of both SP_{1-7} and SP_{1-8} has been shown to be present in the CNS in a soluble form.

The AAS nandrolone decanoate is, as demonstrated in this thesis, affecting the SP system at several levels, including peptide concentrations, receptor densities and enzymatic processing. The nandrolone-induced effects were observed in regions of the brain such as hypothalamus, amygdala, PAG, nucleus accumbens and striatum. Thus, chronic treatment with nandrolone decanoate not only has a strong impact on the opioid concentrations but also on the tachykinin levels in brain regions associated with the regulation of emotional behaviors such as aggression, depression and reward.

The NK1 receptor antagonists have recently attracted attention as new potential antidepressive agents (Argyropoulos and Nutt 2000; Kramer et al 1998). Although the observed effects in the clinical trials did not fully meet the expectations, this does not necessarily mean that SP might not play an important role in the regulation of depression. It is possible that the depression that is frequently reported as a common side-effect after prolonged usage of AAS is originating from alterations in the SP systems. The higher concentrations of SP observed in the brain of AAS treated rats might be compensated for to some degree by the down-regulation of NK1 receptors. The impact of AAS treatment on the SP/NK1 system in man is not known but one might speculate that symptoms of depression are emerging from long-term NK1 receptor stimulation. In fact, depression is the second most reported adverse effect linked to use of AAS according to the Swedish anti-doping hotline (Eklof et al 2003).

In the amygdala, an important region for the regulation of affective behaviors, significantly enhanced levels of both SP (28%) and CGRP (77%), although not of

the opioids Dyn B and MEAP, were observed after two weeks of chronic nandrolone decanoate administration. After the same period, the concentration of SP_{1-7} tended to be decreased. When comparing the levels of SP and SP_{1-7}, a higher ratio was found between the two peptides in the amygdala, indicating a possible decreased enzymatic activity in this structure. In fact, a tendency towards a lower SPE-like activity in amygdala was also observed. However, suppressed activities of other SP degrading enzymes such as NEP and ACE could also possibly contribute to the higher SP/SP_{1-7} ratio. Both NEP and ACE have been identified in moderate concentrations in amygdala (Chai et al 1987; Waksman et al 1986). Furthermore, the density of NK1 receptors in basolateral amygdala was attenuated by 23% after nandrolone administration, an observation that can be attributed to down-regulation / internalization of the NK1 receptor in response to the elevated SP levels. In gerbils, immobilization stress induced a pronounced NK1 internalization in the basolateral amygdala, an effect that was concluded to be due to SP release. This internalization was also reduced by pretreatment with a SP antagonist (Smith D. W. et al 1999). Furthermore, SP seems to be released in amygdala in guinea pig pups in response to psychological stress caused by maternal separation (Kramer et al 1998). SP has been shown to play a role not only in affective disorders such as depression, as mentioned previously, but also in anxiety. Hence, microinjection of SP into the medial amygdala induce a dose dependent anxiogenic behavior (Ebner et al 2004). Amygdala is not only an important region for the regulation of anxiety and depression but also for aggression. In fact, SP neurons originating in the medial amygdala project to the hypothalamus, a brain region where SP release was shown to induce defensive rage in cats (Shaikh et al 1993). Although, there appears to be some differences between the cat and rat with regard to systems involved in mediating aggression, the importance of amygdala and hypothalamus for aggressive responses seems to be similar in both species (Siegel et al 1999). Thus, with regard to the nandrolone decanoate induced biochemical alteration observed in amygdala it is tempting to suggest that side-effects such as anxiety, depression and aggression often reported in connection to AAS abuse might partly be due to altered SP and CGRP levels in this region.

In hypothalamus, the levels of SP, MEAP and Dyn B increased after nandrolone administration, but neither of the peptide levels analyzed remained elevated after the treatment-free recovery period. Although the SPE-like activity was decreased in this region after nandrolone treatment no alteration was observed in the concentration of SP_{1-7}. The NK1 receptor density was decreased in both the ventromedial hypothalamic nucleus, as well as the dorsal part of the dorsomedial hypothalamic nucleus, reflecting a possible decreased biosynthesis or enhanced internalization of the NK1 receptor in response to the higher concentrations of SP induced by the AAS treatment.

Aggression is the most commonly reported side-effect according to the Swedish anti-doping hotline (Eklof et al 2003). Rats chronically treated with nandrolone decanoate display an increased defensive aggression (Johansson et al 2000) and are in addition more dominant in a competitive situation (Lindqvist et al

2002). Pretreatment with nandrolone decanoate has also been shown to enhance amphetamine-induced aggression (Steensland et al 2005). Furthermore, chronic treatment with AAS increased aggression in male adolescent hamsters (Harrison et al 2000). Thus, animal models provide good support for the hypothesis that AAS induce aggression. However, in this context it is important to emphasize that not all AAS induce aggression (Clark and Barber 1994). In fact, the AAS stanozolol seems to suppress aggression (Breuer et al 2001).

In the PAG, the concentrations of both SP and SP_{1-7} became higher after two weeks of chronic nandrolone treatment. The higher level of SP remained after the three-week recovery period, whereas the increased level of SP_{1-7} was abolished after this period. Interestingly, SP has previously been shown to produce anxiogenic effects in rats when injected into the PAG, whereas the N-terminal fragment SP_{1-7} displayed an opposite action (De Araujo et al 2001). This anxiolytic effect attributed to SP_{1-7} was however reported not to be mediated by the NK1 receptor. Considering the opposite actions of the two peptides i.e. anxiogenic versus anxiolytic effects and that the SP_{1-7} concentration increased by 40% (SP increased by 23%), and provided that the efficacy of the two peptides were the same, one could speculate that the sum of the two effects would be an anxiolytic outcome after the two-weeks of nandrolone treatment. In fact, rats treated with nandrolone during 14 days have been shown to be less anxious as deduced from studies of fleeing and freezing behavior (Johansson et al 2000). On the other hand, after the recovery period as reported in this thesis, only the elevated levels of SP remained and thus one would presume an overall anxiogenic outcome after a recovery period. However, since anxiety has been reported both during ongoing and after AAS abuse in humans, this effect might be individual or species specific. The impact of AAS on SP/SP_{1-7} ratios in human is not known and not only SP, but also those in an anxiety context more well known GABA and serotonergic systems, play central roles in the modulation of anxiety. Notably, it has been reported that AAS in rats can induce significant modulation of GABAergic transmission in rat brain regions essential for endocrine functions (Jorge-Rivera et al 2000). Both stanozolol and 17⁄ -methyltestosterone significantly inhibited the binding of flunitrazepam to the benzodiazepine site in rat brain (Masonis and McCarthy 1995). Thus, AAS can directly have an influence on anxiety by modulating the $GABA_A$ receptor, for a review see (Clark and Henderson 2003).

Whereas SP exerts a nociceptive action in the spinal cord, SP can produce an analgesic effect when injected into the PAG (Malick and Goldstein 1978). This could be an indirect effect mediated through SP induced release of enkephalins (Del Rio et al 1983). Indeed, the concentrations of MEAP, a biomarker for enkephalin activity, were increased after chronic AAS treatment in our experiments. Thus, it is possible that the increased levels of MEAP could be attributed to increased levels of SP in PAG as a result of nandrolone exposure. Furthermore, the attenuated density of NK1 receptors observed could be a secondary effect of feedback regulation, or possible internalization of the receptor, in response to the increased SP levels observed.

AAS have been reported to have rewarding properties and studies have shown both conditioned place preference when administering testosterone and also oral self-administration to rodents (Alexander et al 1994; Arnedo et al 2000). Interestingly, a study reported that the conditioned place preference could be blocked by administering a dopamine antagonist in the nucleus accumbens in the rat (Packard et al 1998), indicating that AAS might have a stimulatory action in the brain reward system. The nucleus accumbens, an important region for the brain reward system, can be subdivided into the nucleus accumbens core and shell. Whereas the shell is coupled to emotion, the nucleus accumbens core is coupled to motor functions (Di Chiara 1999). Nandrolone decanoate had a significant impact on the level of SP_{1-7} in nucleus accumbens, increasing the SP_{1-7} content by a remarkable 47%. After a treatment-free recovery period, the increased SP_{1-7} concentration still tended to remain elevated. Since neither SP nor the SPE-like activity was altered in this region after nandrolone administration, it is tempting to suggest that other endopeptidases have been engaged in the generation of SP_{1-7}. Thus, a nandrolone induced biosynthesis of SP, combined with an enhanced enzymatic processing of SP for example by NEP or ACE might account for the high levels of SP_{1-7}. If this would be the case, the net-effect in the nucleus accumbens could be unaltered SP levels and higher concentrations of SP_{1-7}. Furthermore, nandrolone decanoate down-regulated the NK1 receptors in the nucleus accumbens core, but not in the shell.

The brain reward system consists of several important brain regions including nucleus accumbens, VTA, frontal cortex and amygdala. Some dopaminergic neurons originating in nucleus accumbens project to the VTA. Thus, one can speculate that the pronounced increase of SP_{1-7} levels might exert an impact on the reward system. When injecting SP or SP fragments into the nucleus accumbens in rats, SP and its C-terminal fragment attenuated passive avoidance behavior while SP_{1-7} was shown to exert the opposite effect, enhancing this behavior (Gaffori et al 1984).

Striatum is a region displaying a high content of SP and a high density of NK1 receptors. Furthermore, the striatum seems to possess a considerable SPE-like activity. Striatum is primarily associated with the motoric system but also with altered behaviors as results of drug abuse (Karler et al 1995). Chronic treatment with nandrolone decanoate increased the levels of SP, Dyn B and MEAP in the striatum, effects that disappeared after the three-week recovery period. Notably, the level of the bioactive SP_{1-7} decreased in the same region and in this case, the level of SP_{1-7} also remained attenuated after the recovery period. In addition to the reduced level of SP_{1-7}, the AAS administration was found to significantly reduce also the SPE-like activity in striatum. Hence, as expected, a low SPE-like activity should provide lower levels of SP_{1-7} and higher levels of SP. Thus, it seems like SPE is an important endopeptidase regulating the balance between SP and SP_{1-7} in striatum.

Nandrolone decanoate treated rats displayed a 26% density reduction of NK1 receptors in dentate gyrus, an important relay through which cortical projections reach the hippocampal formation. Interestingly, no other biochemical changes were observed in the hippocampus. However, it is possible that the SP levels in certain regions of the hippocampus could be altered by nandrolone treatment, although

the overall SP concentration in hippocampus remained unaffected. It has also been reported that the number of SP containing neurons in dentate gyrus are lower in patients suffering from Alzheimer's disease (Beal and Mazurek 1987; Quigley and Kowall 1991). Nandrolone is known to suppress testosterone plasma levels by feedback regulation (Minto et al 1997), and men who develop Alzheimer's disease have also been reported to have lower testosterone levels (Moffat et al 2004). Thus, although nandrolone activates the same receptor as testosterone and potentially should compensate well for the lower testosterone levels during long-term use of AAS, nandrolone could still have an effect on the biochemical events linked to memory and dementia. In fact, AAS abuse has been reported to be associated with forgetfulness and distractibility (Daly et al 2003), although in rats AAS does not seem to affect spatial working memory (Clark et al 1995; Smith S. T. et al 1996). However, the relation between memory and the activation of androgen receptors and subsequent gene expression is still unclear.

In the anterior pituitary, the levels of CGRP were reduced by 45% after the nandrolone administration, an effect that remained after a three-week recovery period. Both SP and CGRP nerve fibers innervate the anterior pituitary in the rat, although at a smaller extent than in other species (Ju et al 1993; Ju and Zhang 1992). The neuropeptide CGRP seems to have a regulatory role in the region and induces ACTH release from anterior pituitary cells from rat (Iino et al 1998). Thus, considering the low levels of CGRP found in this structure it is less surprising that the levels of ACTH were reported to be reduced in power athletes using AAS (Alen et al 1985).

It has been demonstrated that SP and CGRP frequently co-exist in the CNS (Tuchscherer and Seybold 1989). CGRP has also been reported to act as an inhibitor of SPE (Le Greves et al 1985). Thus, increased levels of CGRP in regions with SPE-like activity and where SP is present will probably lead to a decreased formation of SP fragments. Since SP_{1-7} is the major metabolite formed from SP by a SPE-like activity it is tempting to suggest that some of the outcome attributed to CGRP in the CNS might as well be effects mediated by increased levels of SP or alternatively be due to a decreased formation of SP_{1-7}. CGRP has further been reported to serve as a signaling molecule that induces expression of NK1 receptors in the CNS (Seybold et al 2003). Thus, it seems that increased levels of CGRP can both inhibit the degradation of SP as well as increase the NK1 receptor density.

Although the impact of AAS on neuropeptide systems has been the focus for the research summarized in this thesis it should be emphasized that it is known that AAS administration to rats also effect other systems with high relevance for the altered behaviors attributed to AAS abuse. These include AAS impact on e.g. the serotonin, dopamine and glutamate systems (Kindlundh et al 2002; Kindlundh et al 2003; Le Greves et al 1997). All these neuroactive compounds together with the neuropeptides studied in this thesis may in various ways interact with each other. It should also be emphasized that the high doses and accumulated levels of nandrolone could lead to activation also of other related steroid receptors such as estrogen, progesterone and mineralcorticoid, as well as glucocorticoid receptors. In addition,

it should be remembered that the half-life of nandrolone decanoate in intramuscular depots is almost a week and that after a three-week recovery period, nandrolone is still present in the target tissues. Furthermore, activation of membrane bound steroid receptors or neurosteroid receptors, e.g. $GABA_A$ and NMDA receptors, could all contribute to the observed alterations of the neuropeptide systems reported in this thesis. AAS or their sulfate conjugates could also interact with neurosteroid receptors or alternatively AAS could indirectly modulate levels of endogenous neurosteroids.

The impact of biological and behavioral data recorded in experimental animal models on the human situation has indeed been widely discussed. In many aspects, it has been questioned whether it is possible to extrapolate observations seen e.g. in the rat to human. Are the effects induced by AAS on brain biochemistry and behavior in male rats, as described in this thesis, in agreement with those the steroids would give rise to when they are injected in young men?

Effects, which are comparable between rat and man would occur in behaviors that results from alterations in brain circuits that are similar in rat and human. On the other hand, it would be difficult to extrapolate changes in behaviors and related chemistry in rat without corresponding behaviors and neurochemistry in human. Regarding primitive functions and behaviors related to the emotional centers in the brain, e.g. the limbic area, there are close similarities between rats and primates. For instance, defensive aggression, reward and factors involved in memory and cognition. On the other hand, factors that account for evaluation, interpretation and conclusion of events that appear in connection with intake of drugs such as AAS may be different. The more extended cortical regions in man compared to rat may include brain circuits and mental capabilities which highly exceed those in rodents and therefore the steroids may affect the human brain in a more sophisticated mode, both with regard to brain chemistry and behavior. Therefore, in attempts to extrapolate data recorded in animals to human it is essential which type of behaviors and which kind of brain circuits the researcher has taken under consideration.

Conclusion

The present thesis reports that intramuscular injections of the commonly used anabolic androgenic steroid nandrolone decanoate (15 mg/kg) once daily for two weeks to rats, significantly affect important peptidergic systems in brain structures that are often associated with anxiety, depression, emotions, memory, reward effects and dependence, as well as defensive reactions and aggression.

- The levels of the opioid peptides Met-enkephalin-Arg^{6}-Phe^{7} (MEAP, a MOP- and DOP receptor agonist) and dynorphin B (Dyn B, a KOP receptor agonist) representing biomarkers of two distinct opioid systems, were measured in the rat brain using radioimmunoassay (RIA). In hypothalamus, MEAP and Dyn B had increased 34% and 37%, respectively; in striatum, 29% and 22%, respectively; and in periaqueductal gray (PAG), 29% and 56%, respectively, as compared to controls two weeks after the nandrolone decanoate administration had started.

- The levels of the tachykinin substance P (SP) and its bioactive N-terminal fragment SP_{1-7} were measured in the rat brain using radioimmunoassay. In hypothalamus, SP had increased 14%; in PAG, SP had increased 23% and SP_{1-7} 40%; in amygdala, SP had increased 28%; and in striatum, SP had increased 23% but in striatum the levels of SP_{1-7} had decreased 25%; and in nucleus accumbens the level of SP_{1-7} had increased by 47% as compared to controls two weeks after the nandrolone decanoate administration had started.

- The density of the NK1 receptor in the rat brain was examined with autoradiography. The density was significantly lower in dorsomedial hypothalamic nucleus, 43%; in ventromedial hypothalamic nucleus, 36%; in nucleus accumbens core, 37%; in dentate gyrus, 26%; in PAG, 24%; and in basolateral amygdaloid nucleus, 23%, as compared to controls two weeks after the nandrolone decanoate administration had started.

- The substance P endopeptidase-like activity (SPE) was examined by using radioimmunoassay and measuring the level of SP_{1-7}, formed in SPE-catalyzed fragmentation of SP. In hypothalamus the SPE-like activity had decreased 43%; in striatum, 44%; in substantia nigra, 32%; and in ventral tegmental area 27%, as compared to controls two weeks after the nandrolone decanoate administration had started.

- The level of the calcitonin gene-related peptide (CGRP) was measured in the rat brain using radioimmunoassay. In amygdala, CGRP had increased 77%; in nucleus accumbens, 39%; but in anterior pituitary the immunoreactivity had decreased 55%, as compared to controls two weeks after the nandrolone decanoate administration had started.

- After recovery periods of three weeks, effects of the nandrolone decanoate treatments on neuropeptide systems were still observed in some regions of the brain.

We conclude that the supra-therapeutic doses of nandrolone decanoate given to rats in the studies presented in this thesis, which should be comparable to those injected by AAS abusers, have a significant impact on the neuropeptide systems in important brain areas. We speculate that the adverse behaviors frequently connected to AAS abuse, at least in part could be attributed to alterations in the opioid, tachykinin and CGRP circuits, although we are aware that caution should be taken when extrapolating results from animal to man.

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