

**THE EFFECT OF ANABOLIC ANDROGENIC STEROIDS ON CALCITONIN  
GENE-RELATED PEPTIDE (CGRP) LEVELS IN THE RAT BRAIN**

Mathias Hallberg\*, Kristina Magnusson, Anna M.S. Kindlundh, Pia Steensland and Fred Nyberg

Department of Pharmaceutical Biosciences, Division of Biological Research on Drug Dependence, Uppsala University, Biomedical Center, Box 591, 751 24 Uppsala, Sweden

**RUNNING TITLE:** Anabolic androgenic steroids and CGRP

\* Correspondence should be directed to;

Mathias Hallberg

Department of Pharmaceutical Biosciences

Uppsala University

BMC, Box 591

751 24 Uppsala

SWEDEN

Telephone: +46-18-4714141

Fax: +46-18-501920

E-mail: [Mathias.Hallberg@farmbio.uu.se](mailto:Mathias.Hallberg@farmbio.uu.se)

## **ABSTRACT**

*Objective:* The aim of the present study was to determine whether anabolic androgenic steroids (AAS) have an effect on the levels of calcitonin gene-related peptide (CGRP) in the rat brain, using nandrolone decanoate as a prototype steroid. *Method:* Male Sprague-Dawley rats received daily intramuscular injections of nandrolone decanoate (15 mg/kg) for 14 days. The effect of AAS on CGRP immunoreactivity (CGRP-ir) in various regions of the rat brain was investigated using radioimmunoassay. *Results:* The AAS administration caused a significant increase in CGRP-ir in the nucleus accumbens and the amygdala. After three weeks of recovery, a high level of CGRP-ir still remained in the nucleus accumbens. On the contrary, in the anterior pituitary the CGRP-ir levels became significantly lower after administration of nandrolone decanoate. *Conclusion:* The observed alterations of the CGRP-ir levels could have an impact on biochemical events that are associated with the adverse behaviors and physical effects observed in connection to AAS abuse.

## **KEYWORDS**

Anabolic androgenic steroids, nandrolone decanoate, calcitonin gene-related peptide, rat brain, radioimmunoassay.

**INTRODUCTION**

Anabolic androgenic steroids (AAS) are synthetic derivatives structurally related to testosterone. Testosterone is the primary natural male hormone and is responsible for the androgenic (masulinizing) and anabolic (tissue building) effects observed during male adolescence and adulthood (1). Clinically, androgens are used to treat adult men with androgen deficiency, different forms of anaemia, and patients suffering from a number of diseases that reduce protein synthesis or enhance breakdown of proteins (e.g. burns, trauma, severe infections and malignancies) (1). However, it is the non-medical use of these steroids that has evoked concern. During the last decades, AAS have been used by athletes with the hope of improving their performance. Apart from athletes, AAS abuse has also developed among body builders and persons not directly connected to sports. It appears that these drugs are being used for cosmetic purposes as well as for increasing self-esteem (2) and promoting aggressive behavior (3).

This dual role as an effective pharmacotherapy for a variety of disorders and a drug of abuse places AAS in the company of the opiates, sedative-hypnotics and stimulants. In fact, several studies have provided support for the claim that AAS are drugs of dependence, using DSM III and IV criteria (1,4,5). Furthermore, a recent report indicated an alarming trend where AAS are suggested as a gateway to opioid dependence (6).

While users report benefits of AAS use, such as improved appearance and increased size and strength (7), they also experience a number of adverse effects. These include skin disorders, liver abnormalities, gynecomastia and hypertension (8-11). Chronic administration of steroids has been reported to induce a reduction of circulating levels of ACTH (12). In addition, LHRH, LH and FSH levels are all decreased due to negative feedback of AAS on the hypothalamic-pituitary-gonadal axis (13). Apart from these physical changes, psychological symptoms have been associated with AAS misuse. The users often experience a promotion of self-image, increased libido, euphoria and a sense of well-being (9) at the initial stages of the abuse. More importantly, there are also a number of reported negative psychological side effects such as increased irritability, aggression,

anxiety and depression (3,14,15). Furthermore, severe crimes and unprovoked violence are often committed by AAS misusers (16,17).

Recent studies have demonstrated that nandrolone decanoate can affect transmitter systems in the brain and the levels of endogenous neuropeptides (18-23). Thus, it has been reported that AAS affect the concentration of the tachykinin substance P and its N-terminal metabolite substance P(1-7) as well as the density of the neurokinin 1 (NK1) receptor in rat brain (18,22). The neurotransmitter and neuromodulator substance P co-exists with a series of other peptides, including the calcitonin gene-related peptide (CGRP). In some parts of the CNS virtually all neurons with substance P also contain CGRP.

CGRP is a 37 amino acid neuropeptide generated from the alternative splicing of the calcitonin gene (24,25). Hormones and second messengers regulate the expression of the calcitonin/CGRP gene (26,27). The distribution of CGRP mRNA, CGRP immunoreactive cell bodies and nerve fibers in rat brain has been reported (28-30) and reviewed (31). CGRP is one of the most widely distributed peptides both in the CNS and the cardiovascular system in vertebrates (32). High concentrations of CGRP in rat CNS are found in the dorsal spinal cord, amygdala and pituitary (33).

A wide variety of biological effects on various tissues have been reported for CGRP. The peptide is one of the most potent endogenous vasodilatory substances known so far (34,35) and also seems to have a functional role in food intake (36,37). The peptide is further known to take part in the neural regulation of hormone secretion (38) and furthermore CGRP has been shown to interact with both dopamine and noradrenaline systems in the brain. Thus, CGRP administered into rat VTA significantly increased the dopamine metabolite DOPAC concentration in the frontal cortex (39). In addition, CGRP has been suggested to inhibit noradrenaline release in rat hypothalamus (40). In particular the finding that CGRP affects dopamine release and metabolism has raised the question whether the peptide may play a role in neuropsychiatric disorders. Previously, it was reported that the concentration of CGRP in cerebrospinal fluid is increased in depressed patients compared to healthy individuals (41). Furthermore, it has been shown that major antipsychotic drugs appear to reduce CGRP release in brain (42). These findings, together

with a recent study that reported a release of CGRP in rat limbic forebrain after administration of psychotomimetic drugs (43), provide support for a role of CGRP in psychotic disorders.

To the best of our knowledge, no studies have been performed to determine the effect of AAS on CGRP levels in any species. The aim of the present study was to examine the potential impact of nandrolone decanoate administration on CGRP concentrations in regions of the rat brain that can be anticipated to be associated with the adverse behavioral effects connected to AAS abuse in human.

## **MATERIALS AND METHODS**

*Chemicals*

The synthetic peptide,  $\alpha$ -rCGRP and the labeled peptide, Tyr- $\alpha$ -rCGRP(23-37) were acquired from Bachem Chem (Budendorf, Switzerland) and the AAS, nandrolone decanoate from Organon (Oss, Netherlands). The chromatographic material SP-Sephadex C-25 was obtained from Pharmacia Biotech AB (Uppsala, Sweden) and the sterile arachidis oleum from Apoteket AB (Umeå, Sweden). All other reagents were of analytical grade from commercial sources.

*Animal experiments*

The study included 32 male Sprague-Dawley rats. These rats were housed under controlled conditions of temperature, humidity and light, and had free access to food and water. The animals were randomized into two groups (16 animals in each group). One group was given an intramuscular (i.m.) injection of nandrolone decanoate (15 mg/kg) once daily for 14 days. The control group received i.m. injections of oil vehicle sterile arachidis oleum. On the fifteenth day of the experiment the two groups were divided into four subgroups, with eight animals in each. Half of the animals were sacrificed by decapitation and the others continued for an additional three weeks without any drug treatment (recovery period) before decapitation. After decapitation the brains were rapidly dissected. The hypothalamus, nucleus accumbens, striatum, amygdala, hippocampus, substantia nigra, VTA, PAG, pituitary gland (divided into the anterior and the posterior lobe) and the spinal cord (divided into the ventral and dorsal part) were collected. The tissues were stored at -80°C until further processing.

*Homogenization and ion exchange chromatography*

The frozen tissues were weighed and 1 ml of preheated (90°C) acetic acid (1M) was added to each tube. The mixture was incubated in a water bath (90°C) for 5 minutes in order to reduce enzyme activity. After cooling on ice, the tissues were carefully homogenized by ultrasonification and thereafter heated for another 5 minutes in the water bath (90°C). The

homogenates were chilled on ice and centrifuged at 14 000 x g for 10 minutes in 4°C. The supernatant was collected and diluted (1:1) with a cold (4°C) 0.1 M formic acid/ 0.018 M pyridine, pH=3 (Buffer I). The supernatants were further purified with ion exchange chromatography. Briefly, small plastic columns were packed with SP-Sephadex C-25 gel (packed gel volume = 1 ml). Each column was washed with 20 ml Buffer I before the samples were added. After additional washing with 10 ml Buffer I, elution was carried out with 4 ml of Buffer III (0.35 M formic acid/0.35 M pyridine, pH 4.4). The columns were then washed with 6 ml Buffer III before eluting with 4 ml Buffer IV (0.8 M formic acid/0.8 M pyridine pH 4.4). The fraction containing CGRP was eluted with 4 ml Buffer V (1.6 M formic acid/ 1.6 M pyridine pH 4.4). All elutes were collected in plastic tubes and evaporated in a Speed Vac centrifuge overnight. The tubes were then stored at -20°C and subsequently analyzed by radioimmunoassay.

#### *Radioimmunoassay (RIA)*

The samples were redissolved in methanol/0,1 M HCl (1:1). Samples or standards (25 µl) were incubated together with 100 µl of antibody (final dilution 1: 2000000) and 100 µl of iodinated peptide (4700-5200 cpm). The iodinated CGRP was prepared from Tyr- $\alpha$ -rCGRP(23-37) by the chloramine T procedure as described elsewhere (44). The antibody and the <sup>125</sup>I-labeled peptide were diluted in gelatin buffer containing 0.1% gelatin, 0,1% bovine serum albumin, 0.82 % NaCl, 0.93 % EDTA in 50 mM sodium phosphate (pH 7.4). The tubes were incubated at 4°C for 24 h. The incubation was terminated by adding 200 µl active charcoal (750 mg of charcoal / 75 mg dextran dissolved in 200 ml 50 mM sodium phosphate buffer). The tubes were incubated for 10 minutes on ice. The bound and free peptides were subsequently separated by centrifugation for 1 min. The supernatant, containing the bound fraction, was collected and the radioactivity was determined in a gamma-counter.

The CGRP antiserum used was raised in rabbits against the  $\alpha$ -rCGRP-thyroglobulin conjugate and reacts with  $\alpha$ -rCGRP but also shows cross-reactivity with different CGRP analogues, specifically the two C-terminal fragments  $\alpha$ -rCGRP(19-37) and  $\alpha$ -rCGRP(17-

37). However, the latter two are eluted in different fractions (45) as compared to  $\alpha$ -rCGRP and are hereby separated by the ion exchange chromatography step.

#### *High performance liquid chromatography (HPLC) characterization*

In order to characterize the detected CGRP immunoreactivity, pools of homogenates from rat anterior pituitary were separated by SP-Sephadex, evaporated, redissolved in 15% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA), and further analyzed by reversed phase HPLC using a Pharmacia SMART system (Pharmacia Biotech, Uppsala, Sweden). The system was equipped with a  $\mu$ RPC C2/C18, SC 2.1/10 column (particle size 3  $\mu$ m, 120Å; 100×2.1 mm) and UV-absorbance was detected at 214 nm. Elution was carried out with a linear gradient of ACN (15-60%) containing 0.01% TFA at a flow rate of 100  $\mu$ l/min. Fractions (100  $\mu$ l) were collected and subsequently analyzed by RIA.

#### *Statistical analysis*

Statistical evaluation of RIA data was performed with the Student's unpaired t-test. P values less than 0.05 were considered significant.

## **RESULTS**

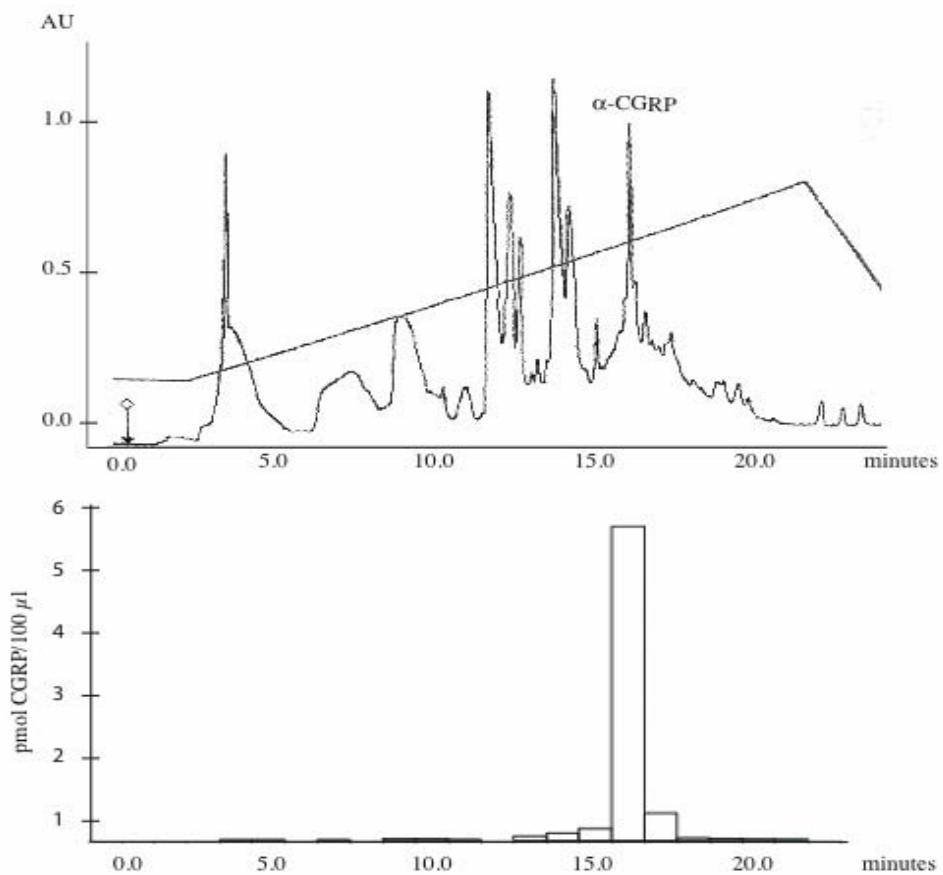
In the present study, the levels of CGRP were measured in all the dissected brain structures. The CGRP concentrations were below the detection limit in the hippocampus, substantia nigra, VTA, posterior pituitary and the ventral spinal cord. High levels of CGRP were found in dorsal spinal cord, amygdala and anterior pituitary. As can be seen in Table 1, the two-week steroid treatment caused a significant increase in the CGRP levels in nucleus accumbens ( $p=0.04$ ) and amygdala ( $p=0.015$ ) compared to the control group. After the three-week recovery period this effect sustained in nucleus accumbens ( $p=0.03$ ) whereas the CGRP-ir in the amygdala was restored to control level. In the anterior pituitary a significant decrease of the CGRP concentration was observed in the AAS treated animals ( $p=0.01$ ). The difference between the two groups remained significant after the three-week recovery period ( $p=0.0002$ ).



Tissue	CGRP		CGRP (with recovery)	
	Control	AAS	Control	AAS
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

**Table 1:** CGRP concentration (fmol/mg tissue ± SEM) in rats treated with AAS (nandrolone decanoate) compared to controls. Measurements were made immediately after 14 days of treatment and after a three-week recovery period. \* p<0.05

The HPLC characterization by the Pharmacia SMART system demonstrated that the highest concentrations of CGRP as determined by RIA after reversed phase HPLC-separation was found in the fraction co-eluting with synthetic  $\alpha$ -rCGRP, as can be seen in figure 1. The retention time for synthetic  $\alpha$ -rCGRP was 16.03 whereas  $\beta$ -rCGRP eluted after 15.24 minutes. Thus, indicating that the major CGRP immunoreactivity observed can be attributed to  $\alpha$ -rCGRP. Considering the fact that the cross-reactivity for  $\beta$ -rCGRP earlier was defined in our lab to be 55% with the CGRP antibody used, less than approximately 5% of the CGRP immunoreactivity in the anterior pituitary is presumed to be  $\beta$ -rCGRP.



**Figure 1:** The upper chromatogram shows pooled homogenates from the rat anterior pituitary (pre-separated on SP-Sephadex columns), with the addition of 3  $\mu\text{g}$  synthetic  $\alpha$ -rCGRP. The lower diagram shows the CGRP immunoreactivity in HPLC fractions (100  $\mu\text{l}$ ) from pooled homogenates from the rat anterior pituitary without the addition of synthetic  $\alpha$ -rCGRP.

**DISCUSSION**

To our knowledge, no studies have been performed to determine the effect of AAS on CGRP levels. The dose nandrolone decanoate used, corresponds to the range of human AAS-abuse and is approximately 40 times larger than the therapeutic dose. This study was initiated since it was previously demonstrated that the levels of substance P and substance P(1-7) were significantly altered after AAS administration in areas of the rat brain anticipated to be of importance for e.g. mediating aggressive behavior (22). As seen in Table 1 nandrolone administration induces significant alterations in the CGRP levels in central structures of the brain. However, although CGRP is often co-localized with substance P, direct correlations of the levels of CGRP and substance P could not be observed in the regions studied.

In amygdala, a region with dense CGRP receptor expression (46), a significant increase of CGRP-ir was encountered after the nandrolone decanoate administration. Amygdala, a center for learning fear and memory functions (47), is also a critical structure for modulation of aggression (48). Furthermore, the central nucleus of the amygdala also mediates cardiovascular and autonomic changes associated with defense or fear responses (49). Although, peripheral administration of CGRP has a powerful vasodilator effect it has also been demonstrated that microinjection of CGRP into amygdala produces an increase of the mean arterial pressure in rat (50,51). Thus, it is possible that the hypertension observed in AAS-users could partially be due to elevated levels of CGRP in the amygdala.

The nucleus accumbens is one of the central structures involved in reward mechanisms and especially the nucleus accumbens shell is rich in CGRP receptors (46). Activation of a dopamine pathway projecting from the VTA to nucleus accumbens seems to cause rewarding effects and there is evidence that drugs of addiction can cause an increase in the release of dopamine in the nucleus accumbens. As reported herein administration of nandrolone decanoate significantly increases the CGRP-ir in the nucleus accumbens, which sustained after three weeks. Since CGRP has been shown to affect dopamine levels in the reward system (39), it is reasonable to assume that the higher CGRP-ir in nucleus

accumbens could indirectly alter the rewarding effects that are suggested to be associated with AAS administration (52,53). In recent studies  $\alpha$ CGRP knock-out mice were shown to display significantly less morphine withdrawal signs (54,55), thus, pointing towards an important role of CGRP in the brain reward system. Considering the fact that CGRP is a potent inhibitor of substance P degradation (56) and that CGRP will inhibit the formation of the bioactive N-terminal fragment substance P(1-7) it is tempting to suggest that the observed effects in the  $\alpha$ CGRP knock-out mice might in fact be due to an increased formation of substance P(1-7), another known inhibitor of morphine withdrawal signs (57-59).

The PAG is connected with numerous brain regions and is involved in the integration of behavioral and physiological responses to threatening or stressful stimuli. The region play a role in the expression of defensive rage behaviors (48) and should be of relevance in context when the influence of nandrolone misuse on behavior is addressed. Notably, in contrast to substance P and its N-terminal fragment substance P(1-7), that both were present in PAG in significantly higher concentrations than in control animals (22) the CGRP levels in the PAG were not affected by nandrolone administration.

High concentrations of CGRP-like material is present in the pituitary gland, a structure reported to have very low density of CGRP binding sites in human (60). The significantly lower CGRP levels observed in the AAS-treated animals might reflect an effect of the involvement of CGRP in the pituitary function; the elevated plasma hormonal levels could cause a feedback inhibition on the pituitary hormones and thereby affecting CGRP as well. It has also been suggested that CGRP nerve fibers of the anterior pituitary respond actively to changes in the plasma hormonal levels (61). AAS administration have previously been shown to reduce levels of circulating ACTH (12). Since CGRP is known to cause an increase in ACTH release (38), the AAS-induced decrease of CGRP-ir observed in the present study is in agreement with these results. Interestingly, the levels of CGRP in the pituitary remained decreased three weeks after the treatment period.

Another observation is that the CGRP levels in some regions are different in the two groups of control animals. Part of the explanation could be that CGRP levels change with age (62,63). For example, in the hypothalamus an age-related increase in CGRP levels has been observed up to 12 weeks of age, whereas in plasma the CGRP concentrations increases continuously with age (62).

In general there is no mismatch between the distribution of CGRP binding sites (pituitary gland one exception) in rat brain (33). It should be emphasized that in the amygdaloid body and nucleus accumbens where there are high levels CGRP receptors in rat, only low levels have been detected in humans (60,64).

## **CONCLUSION**

In conclusion, the present study has demonstrated that chronic treatment with the AAS nandrolone decanoate significantly affects levels of CGRP in areas of the rat brain that are associated with behavior as well as hormonal secretion and cardiovascular function. Although it seems likely that CGRP alterations in response to AAS administration result in physiological consequences in rat, it is unknown if AAS exert a corresponding impact on CGRP levels in humans.

## **ACKNOWLEDGEMENT**

This study was supported by grants from the Swedish Medical Research Council (No. 9459) and by Trygg Hansas Forskningsstiftelse.

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