

Circulating Neuroactive C₂₁- and C₁₉-Steroids in Young Men Before and After Ejaculation

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Summary

Twelve neuroactive and neuroprotective steroids, androgens and androgen precursors i.e. 3 α ,17 β -dihydroxy-5 α -androstane, 3 α -hydroxy-5 α -androstane-17-one, 3 α -hydroxy-5 β -androstane-17-one, androst-5-ene-3 β ,17 β -diol, 3 β ,17 α -dihydroxy-pregn-5-en-20-one (17 α -hydroxy-pregnenolone), 3 β -hydroxy-androst-5-en-17-one (dehydroepiandrosterone, DHEA), testosterone, androst-4-ene-3,17-dione (androstenedione), 3 α -hydroxy-5 α -pregnan-20-one (allopregnanolone), 3 β -hydroxy-pregn-5-en-20-one (pregnenolone), 7 α -hydroxy-DHEA, and 7 β -hydroxy-DHEA were measured using the GC-MS system in young men before and after ejaculation provoked by masturbation. The circulating level of 17 α -hydroxypregnenolone increased significantly, whereas the levels of other circulating steroids did not change at all. This fact speaks against the hypothesis that a decrease in the level of neuroactive steroids, e.g. allopregnanolone, may trigger the orgasm-related increase of oxytocin as it was reported by other authors.

Key words

Allopregnanolone • Ejaculation • 17 α -hydroxypregnenolone • Neuroactive steroids • Testosterone

Introduction

While we have very detailed knowledge of the neural mechanisms, which control the function of male and female genital organs, in particular those mediating erection in males, very little is known of the hormonal mechanisms involved and of potential endocrine regulation of these processes. Nevertheless, several neurotransmitters and neuropeptides, such as dopamine, glutamic acid, nitric oxide, oxytocin, and ACTH-MSH peptides are known to enhance sexual function, while serotonin, gamma-aminobutyric acid (GABA) and opioid peptides reduce it (Argiolas and Melis 2003; Succu *et al.* 2003). In relation to endocrine events at orgasm the attention was paid to oxytocin, the level of which

increases before orgasm and which is believed to be a factor important for ejaculation (Filippi *et al.* 2003, Argiolas and Melis 2004, Vignozzi *et al.* 2004).

Although parturition can proceed in the absence of oxytocin (Nishimori *et al.* 1996), it is well-known that this hormone or at least its receptor plays an important role in parturition. As reported in rats, until close to term, uterine responsiveness of oxytocin is restrained by neuroactive steroid metabolites of progesterone that potentiate GABA inhibitory mechanisms (Leng and Russell 1999, Russell *et al.* 2003). These authors have previously suggested that a decrease in the level of progesterone metabolites, especially allopregnanolone (3 α -hydroxy-5 α -pregnan-20-one), could trigger the production of oxytocin, thus resulting in acceleration of

delivery.

Since oxytocin levels increase in the course of male and female sexual act, it may also be of interest whether the levels of neuroactive steroids are changed at orgasm and ejaculation. Especially allopregnanolone could be a candidate factor contributing to the analgesia induced by sexual act and to sleepiness after it. While the latter steroid would operate predominantly in women, in men the similar effect could be expected in testosterone-derived androstane metabolites with hydroxy-group in 3 α -position. The metabolites were reported to act on GABA_A receptors in a similar way as pregnanolone isomers (Turner *et al.* 1989, Turner and Simmonds 1989, Twyman and MacDonald 1992, Barbaccia *et al.* 2000, Jorge-Rivera *et al.* 2000). For this reason, we attempted to examine whether there would be an analogous effect of both androstane metabolites and pregnanolone isomers in triggering ejaculation as that, which was reported in the case of the role of pregnanolone isomers in oxytocin production in pregnant rats.

The aim of the study was to confirm or to disprove the participation of neuroactive C₁₉- and C₂₁-steroids in male orgasm and ejaculation.

Methods

Subjects

The patient group consisted of 14 healthy men 22-35 years old who were examined for fertility problems of this couple. They had a normal spermogram and normal concentrations of prolactin and they received no hormonal therapy. The local ethical committee of the Institute of Endocrinology and the Charles University approved the protocol for the study. After signing written informed consent the patients underwent blood sampling from the cubital vein before and 5-10 min after ejaculation provoked by masturbation.

Sample collection

Cooled plastic tubes containing 100 μ l of 5 % EDTA and 50 μ l of aprotinin (Antilysin from Spofa, Prague, Czech Republic) were used for blood sampling. The plasma was obtained after centrifugation for 5 min at 2000 \times g at 0 °C. The plasma samples were stored at -20 °C until analyzed.

Steroids and chemicals

The solvents for the extraction and HPLC, were of an analytical grade, from Merck (Darmstadt,

Germany). The derivatization agents Sylon BFT and methoxylamine were purchased from Supelco (Bellefonte, PA, USA) and Sigma (St. Louis, MO, USA), respectively. The internal standard (trideuterated DHEA) was synthesized using our recently published method (Černý *et al.* 2004). The steroids standards were from Steraloids (Newport, RI, USA).

Instruments

The GC-MS system was supplied by Shimadzu (Kyoto, Japan). The system consisted of a GC 17A gas chromatograph equipped with automatic flow control, AOC-20 autosampler and for the MS a QP 5050A quadrupole electron-impact detector with a fixed electron voltage of 70 eV.

GC-MS identification and determination of allopregnanolone and androstanes

Twelve neuroactive and neuroprotective steroids, androgens and androgen precursors i.e. 3 α ,17 β -dihydroxy-5 α -androstane, 3 α -hydroxy-5 α -androstane-17-one, 3 α -hydroxy-5 β -androstane-17-one, androst-5-ene-3 β ,17 β -diol (androstenediol), 3 β ,17 α -dihydroxy-pregn-5-en-20-one (17-hydroxy-pregnenolone), 3 β -hydroxy-androst-5-en-17-one (dehydroepiandrosterone, DHEA), testosterone, androst-4-ene-3,20-dione (androstenedione), 3 α -hydroxy-5 α -pregnan-20-one (allopregnanolone), 3 β -hydroxy-pregn-5-en-20-one (pregnenolone), 7 α -hydroxy-DHEA, and 7 β -hydroxy-DHEA were measured using the GC-MS system principally as described elsewhere (Klak *et al.* 2003).

Extraction

Plasma (1 ml) was extracted with 3 ml of diethyl ether. The polar phase was frozen in a mixture of ethanol and solid carbon dioxide, and the organic phase was separated and evaporated in a vacuum centrifuge. To eliminate the majority of lipids and sterols the dry residue was partitioned between 1 ml of 80 % methanol and 1 ml of pentane. The upper pentane phase was discarded, while the methanol-water phase containing free steroids was evaporated in a vacuum centrifuge, and the dry residue derivatized as described below.

Derivatization

The dry residue of treated sample diluted in 100 μ l of acetonitrile was evaporated under nitrogen; 50 μ l of 2 % methoxylamine in pyridine was added to the dry residue with brief mixing, and the resultant mixture

Table 1. Characteristics of GC-MS analysis of steroids

Substance	Injection No.	Retention time (min)	Effective mass (m/z)
<i>Internal standard (trideuterated DHEA)</i>	1-4	7.26	<u>377</u>
<i>3α,17β-Dihydroxy-5α-androstane</i>	1	5.16	<u>241</u> , 256
<i>3α-Hydroxy-5α-androstan-17-one</i>	1	6.29	<u>270</u> , 360
<i>3α-Hydroxy-5β-androstan-17-one</i>	1	6.39	<u>270</u> , 360
<i>3β,17β-Androstenediol</i>	2	5.87	215, <u>305</u> , 344
<i>17-Hydroxy-pregnenolone</i>	2	7.10	270, <u>305</u> , 360
<i>DHEA</i>	2	7.21, 7.27	260, <u>268</u> , 305
<i>Testosterone</i>	2	7.97, 8.27	268, <u>389</u>
<i>Androstenedione</i>	2	10.05, 10.45	313, <u>344</u>
<i>3α-Hydroxy-5α-pregnan-20-one</i>	3	7.90	100, <u>388</u>
<i>Pregnenolone</i>	3	9.09	100, 296, <u>386</u>
<i>7α-Hydroxy-DHEA</i>	4	6.66	<u>387</u>
<i>7β-Hydroxy-DHEA</i>	4	7.82	<u>387</u>

was heated at 60 °C for 1 hour. Then, the mixture was evaporated under nitrogen, 50 μ l of Sylon BFT (99 % BTSFA + 1 % TMCS) was added and the mixture was heated at 90 °C for 45 min. Further, the mixture was evaporated again, 20 μ l of isooctane was added with brief mixing, and 4 μ l of the mixture was injected into GC-MS system.

GC-MS analysis

GC separation was carried out using a ZEBRON ZB-50, 15 m \times 0.25 mm column, and 0.15 μ m film thickness (cat No 7EG-G004-05). The temperature of the injection port and interface was 300 °C and 320 °C, respectively.

To identify and simultaneously analyze twelve steroids at maximum sensitivity, the protocol consisted of four sample or standard injections with identical temperature and pressure gradient were recorded but with different sets of the effective masses. The gradient was as follows:

Splitless high-pressure injection for 1 min at 100 kPa, 1 min delay at 120 °C and 30 kPa, then a steep increase 40 °C/min and 8.5 kPa/min to 220 °C and 51 kPa, followed by a gradual increase 2.9 °C/min and 0.5 kPa/min to 240 °C and 54.5 kPa and then by a rapid increase 40 °C/min and 8.5 kPa/min to 310 and 70 °C, where a two-minute plateau was maintained. The duration of the analysis/ per one injection was 14.19 min.

The detector voltage was 1.2 kV and the

sampling rate was 0.25 s. The temperature of the interface was 310 °C. The first of the pair of effective masses (m/z) was used for the calculation of concentration, while the second value was measured to ensure the correct identification of the substance. Besides agreement in the retention times, the identity of substances with the standards was confirmed using the effective masses characteristic of TMS derivatives of pregnanolone isomers (m/z = 285, 300 and 375) and for the TMS derivative of pregnenolone (m/z = 298, 241 and 288).

The ketones and diketones usually showed two or more peaks corresponding to individual stereo-isomers of the methoxy-derivatives. The substances monitored at various effective masses (the effective masses used and the peaks used for calculations are underlined) are shown in Table 1.

Standard mixtures, used for calibration were derivatized in the same manner as the samples. They contained the steroid investigated in three concentrations: 1000 pg/ μ l, 100 pg/ μ l and 10 pg/ μ l and trideuterated DHEA in concentration 1000 pg/ μ l as an internal standard. To eliminate the effect of losses during sample processing, the internal standard (20 ng) was also added to each sample prior the diethyl-ether extraction.

Statistical data analysis

For evaluation of differences before and after the trial, a robust Wilcoxon's paired test was applied. Statgraphics Plus v. 5.1 statistical software from Manugistics

Table 2: Steroid levels before and after ejaculation in young men

Substance	BEFORE TRIAL						AFTER TRIAL					
	n	Mean	SD	Median	Lower	Upper	n	Mean	SD	Median	Lower	Upper
					quartile	quartile					quartile	quartile
<i>Pregnenolone</i>	14	4.29	3.93	2.60	1.37	5.38	14	5.90	11.00	2.30	1.35	4.44
<i>17-Hydroxy-pregnenolone</i>	14	4.14	2.02	4.31	2.29	5.16	14	6.49	3.11	5.18	4.12	9.55
<i>DHEA</i>	14	21.9	4.2	20.8	18.8	26.0	14	24.5	8.7	23.6	18.5	30.0
<i>androst-5-ene-3β,17β-diol</i>	14	4.23	2.94	3.35	2.17	3.99	14	5.22	5.81	2.89	2.03	6.89
<i>Androstenedione</i>	14	2.37	1.86	1.75	1.17	2.52	14	2.77	3.12	1.54	0.84	3.18
<i>Testosterone</i>	14	23.2	13.0	18.2	12.2	32.9	14	24.4	17.4	21.2	12.8	27.0
<i>Allopregnanolone</i>	14	0.043	0.044	0.027	0.016	0.045	14	0.033	0.032	0.027	0.009	0.038
<i>Androsterone</i>	14	1.89	1.23	1.51	1.07	2.12	14	2.17	1.71	1.54	1.08	2.36
<i>3α-Hydroxy-5β-androstan-17-one</i>	14	0.554	0.439	0.421	0.307	0.471	14	0.611	0.497	0.402	0.279	0.690
<i>3β,17β-dihydroxy-5α-androstane</i>	14	0.431	0.231	0.380	0.274	0.591	14	0.544	0.548	0.348	0.195	0.763
<i>7α-Hydroxy-DHEA</i>	14	1.34	0.73	1.20	0.84	1.56	14	1.37	0.56	1.28	1.09	1.71
<i>7β-Hydroxy-DHEA</i>	14	0.637	0.322	0.541	0.367	0.813	14	0.646	0.265	0.542	0.478	0.843

Correlation matrix of relations between the levels of endogenous 3 α -hydroxy-5 β -androstane derivatives and their precursors in young men. DHEA...dehydroepiandrosterone; Adiol...5-androstene-3 β ,17 β -diol; Adion...androstenedione (5-androstene-3,17-dione); T...testosterone; A3a5a17o...androsterone (3 α -hydroxy-5 β -androstan-17-one); A3a5a17b...androstadiol (3 β ,17 β -dihydroxy-5 α -androstane); the numbers in upper and lower parts of the cells demonstrate Pearson's correlation coefficients and their statistical significance; the cells above and below the diagonal show simple pair and partial correlations (adjusted to constant levels of all the steroids except the pair investigated), respectively.

(Rockville, MA, USA) was used for the calculations. Relationships between steroid levels were evaluated using Pearson's correlations and partial correlations with adjustment of all the variables to constant values except for the pair studied.

Due to non-Gaussian data distribution and heteroscedasticity, the data were treated by power transformations of the individual variables. The transformation parameters were found with the use of normal probability plots reaching a minimum mean square error to regression line for the optimum transformation parameters. Statgraphics Plus v.5.1 statistical software was used to find the best transformation, as well as for the correlation analysis. To detect multivariate outliers the F-distributed Mahalanobis distance was used; NCSS 2002 statistical software from Number Cruncher Statistical Systems (Kayville, UT, USA) was used for the latter analysis. Plots of the theoretical vs. experimental fractiles and symmetry plots of the multivariate data were created using QC-Expert statistical software from Trilobyte (Pardubice, Czech Republic) to check the multivariate normal distribution in the transformed-data set.

Results

Steroid concentrations in the serum before and after ejaculation

Table 2 summarizes the basic statistical data (means, medians, upper and lower quartiles) on the concentrations of studied steroids in the blood serum before and after ejaculation from 14 healthy men. The concentrations of twelve neuroactive and neuroprotective steroids, 3 α ,17 β -dihydroxy-5 α -androstane, 3 α -hydroxy-5 α -androstan-17-one, 3 α -hydroxy-5 β -androstan-17-one, androst-5-ene-3 β ,17 β -diol, 17-hydroxy-pregnenolone, DHEA, testosterone, androstenedione, 3 α -hydroxy-5 α -pregnan-20-one (allopregnanolone), pregnenolone, 7 α -hydroxy-DHEA, and 7 β -hydroxy-DHEA are presented. The only significant difference between control before masturbation and after ejaculation was found in the concentration of 17 α -hydroxypregnenolone ($p < 0.03$).

Correlations between steroids

Table 3 shows simple pair correlations between the steroids as well as partial correlations with adjustment to a constant value of all steroids included in the table except for the pair under investigation. From the

Table 3: Correlation matrix of relations between the levels of endogenous 3 α -hydroxy-5 α -androstane derivatives and their precursors in 14 young men before and after ejaculation.

	DHEA	Adiol	Adion	T	A3 α 5 α 17 α	A3 α 5 α 17 β
DHEA		0.440 0.019	0.310 0.109	0.219 0.264	0.030 0.880	0.140 0.477
Adiol	0.555 0.005		0.607 0.001	0.814 0.000	0.613 0.001	0.749 0.000
Adion	0.308 0.143	-0.066 0.759		0.514 0.005	0.660 0.000	0.647 0.000
Testosterone	-0.334 0.111	0.736 0.000	0.149 0.487		0.517 0.005	0.564 0.002
A3 α 5 α 17 α	-0.242 0.255	0.050 0.818	0.357 0.087	0.034 0.875		0.788 0.000
A3 α 5 α 17 β	-0.250 0.238	0.528 0.008	0.188 0.380	-0.257 0.226	0.472 0.020	

DHEA...dehydroepiandrosterone; Adiol...5-androstene-3 β ,17 β -diol; Adion...androstenedione (5-androstene-3,17-dione); T...testosterone; A3 α 5 α 17 α ...androsterone (3 α -hydroxy-5 α -androstane-17-one); A3 α 5 α 17 β ...androstenediol (3 α ,17 β -dihydroxy-5 α -androstane); the numbers in upper and lower parts of the cells demonstrate Pearson's correlation coefficients and their statistical significance; the cells above and below the diagonal show simple pair and partial correlations (adjusted to constant levels of all the steroids except the pair investigated) respectively.

androgens and androgen precursors, the 3 α -androstane metabolites correlated strongly with 3 β ,17 β -androstenediol and less pronouncedly with testosterone when using simple pair correlations without adjustment to any other steroid. In contrast to the simple pair correlations, the partial correlations with adjustment to constant levels of the remaining steroids included in the table were found only between 3 α ,17 β -dihydroxy-5 α -androstane and androst-5-ene-3 β ,17 β -diol and between androstenedione and 3 α -hydroxy-5 α -androstane-17-one. Alternatively, no partial correlation was found between 3 α -hydroxy-androstane metabolites and testosterone. Neither did the 3 α -androstane metabolite correlate with DHEA using both the pair and partial correlation.

Discussion

Our study shows for the first time the levels of

endogenous pregnane and androstane neuroactive derivatives in young men before and after ejaculation. The results show that the allopregnanolone levels in young men are considerably lower than those reported previously in premenopausal women (Genazzani *et al.* 1998, Hill *et al.* 2005). Alternatively, 5-reduced androgen metabolites, as expected, reach higher levels than in women due to the higher production of testosterone and its precursors. The levels of pregnenolone, 17 α -hydroxypregnenolone, androstenedione, and androstenediol are in the nmol/l range. Allopregnanolone and androstane metabolites (5 α -androstane-3 α ,17 β -diol, 3 α -hydroxy-5 α -androstane-17-one and 3 α -hydroxy-5 β -androstane-17-one as well as 7-hydroxylated isomers of dehydroepiandrosterone are circulating in lower concentrations (by 1-2 orders of magnitude). They can be considered – from a quantitative point of view – as minor constituents of steroid hormone content in the serum (Table 2).

The samples taken 5-10 min after ejaculation do not differ significantly from those taken before, except for 17 α -hydroxypregnenolone, the direct precursor of 17 α -hydroxyprogesterone and of major androgens. Some insignificant tendency of a decrease after ejaculation can be seen for allopregnanolone. Therefore, it seems improbable that a decrease of allopregnanolone concentration could be a factor for triggering oxytocin secretion as postulated by Leng and Russell (Leng and Russell 1999, Russell *et al.* 2003).

Neuroactive steroids are potent endogenous neuromodulators with rapid actions in the central nervous system. There is considerable information about the modulatory activity of 5-reduced derivatives of progesterone. However, the significance of the testosterone-derived neuroactive steroid such as 3 α -androstenediol is not well understood (Reddy 2003, 2004a, 2004b, Frye and Edinger 2004, Frye *et al.* 2004).

5 α - and 5 β -dihydrotestosterone, androsterone or androstenediol exhibited a concentration-response inhibition of spontaneous contractile myometrial activity and their relaxing effect was also observed on the contractions induced by a high potassium solution. The blockade of L-type calcium channels seems to be involved in the non-genomic relaxing action of androgens (Perusquia *et al.* 2005).

Table 3 shows a correlation between the androstane metabolites and testosterone. Analgesic, anxiolytic and cognitive-enhancing effects of testosterone are mediated in part through actions of its 5 α -reduced,

nonaromatizable metabolite dihydrotestosterone (DHT) and/or 3α -androstenediol (3α -diol) in the hippocampus (Edinger and Frye 2004, Edinger *et al.* 2004, Frye and Edinger 2004, Frye *et al.* 2004). Similarly, testosterone-derived neurosteroid 5α -androstane- $3\alpha,17\beta$ -diol could contribute to the net cellular actions of testosterone on neural excitability and seizure susceptibility (Reddy 2004a,b). The experiments with rats in the conditioned fear paradigm (Edinger *et al.* 2004) indicate that the androgen effects to enhance learning may be mediated in part by actions of 5α -reduced metabolites in the hippocampus. It could thus be expected that higher levels of 5α -reduced androstanes could interfere with ejaculation.

Neuroactive progesterone and testosterone saturated derivatives with 5α -configuration modulate sexual receptivity in rodents. Through actions in the hypothalamus that are independent of intracellular androgen receptors but involve GABA and/or GABA_A/benzodiazepine receptor complexes, 3α -androstenediol inhibits lordosis. Frye and Vongher (2001) suggested that allopregnanolone and 3α -androstenediol, can influence the lordosis, i.e. *via* non-classical actions at intracellular steroid receptors.

Neuroactive steroids are known to modulate GABA receptors in the brain. GABA receptors are, however, also present in several non-neural tissues, including endocrine organs e.g. the pituitary, pancreas and testis. In the case of the rat testis, GABA appears to be linked to the regulation of steroid synthesis by Leydig cells *via* GABA_A receptors, but neither testicular sources of GABA, nor the precise nature of testicular GABA receptors are fully known (Geigerseder *et al.* 2003). The mere presence of GABA receptors in human testes raises the question whether circulating neuroactive steroids might also be able to modulate testicular function.

In our study testosterone levels remained

unaltered during sexual arousal and orgasm induced by masturbation in agreement with the findings of Kruger *et al.* (2003). Against our expectations, neither allopregnanolone nor androstenediols levels changed after orgasm and ejaculation. The only significantly increased steroid was 17α -hydroxy-pregnenolone. In respect to its role as an intermediate compound on the start of the biosynthetic pathway of other active steroids, it could be speculated that ejaculation stimulates the 5-ene-pathway of androgen production of active androgens or that 17α -pregnanolone itself plays a certain role in the endocrine regulation.

Concerning the metabolism of androgens and androgen precursors to 3α , 5α -androstane-metabolites the correlations (Table 3) indicate that the conversion of testosterone to dihydrotestosterone is not a critical step in the formation of 3α -hydroxy- 5α -androstane metabolites. On the other hand, androstenedione and 3β , 17β -androstenediol show significant partial correlations with the corresponding 17-oxo- and 17β -hydroxy- 3α -hydroxy- 5α -androstane metabolites. The correlations show that the formation of 3α , 5α -androstane metabolites are relatively independent of DHEA and testosterone levels and depends more on androstenedione and 3β , 17β -androstenediol. This finding could be ascribed to the rapid metabolism of testosterone to DHT. However, in this case higher values of pair correlations between testosterone and 3α , 5α -androstane metabolites could be expected than those we have found. In the case of androstenedione, the second possibility is a direct reduction of 3-oxo-group catalyzed by 3α -hydroxysteroid oxidoreductase.

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