The Steroid Metabolome in Men With Mood and Anxiety Disorders

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Summary

The mood and behavior of individuals result from an orchestra of many factors. Among them steroids play an important role; however, only several common hormones have been investigated in this respect. It has been demonstrated that some steroid metabolites long considered merely the products of steroid hormone metabolism in fact possess considerable activity in the CNS. For this reason we studied the steroid metabolome including 50 analytes in 20 men with depression, 20 men with anxiety and 30 healthy controls. Significant differences were found not only between controls and men with either depression or anxiety, but also between men with depression and anxiety. Particularly striking were those steroids until now not generally associated with depression or anxiety, namely conjugated steroid forms, especially sulfates.

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Introduction

In affective disorders, it is generally accepted that a crucial role for neurotransmission is played by the serotoninergic (Steiner 2011) and noradrenergic systems with concomitant activation of the axis hypothalamus – pituitary – adrenals (HPA) (Horstmann and Binder 2011, Detka *et al.* 2013, Herbert 2013, Belvederi *et al.* 2014, Crowly and Gridler 2014, Jacobson 2014) However,

many other hormones and various factors have been identified as modulators of mood and behavior in such disorders. Recent studies have described the role of brain-derived neurotrophic factor (BDNF) (Pluchino *et al.* 2013, Numakawa *et al.* 2014), thyroid hormones (Duntas and Mails 2013), inflammation (Halaris 2013), immunity (Pitychoutis and Papadopoulou-Daifoti 2010), melatonin (Boyce and Hopwood 2013), oxytocin and vasopressin (Scantamburlo *et al.* 2009, Matsuzaki *et al.* 2012), the renin-angiotensin-aldosterone system (Franklin *et al.* 2012, Murck *et al.* 2012), the cannabinoid system (Martykánová 2010, Gorzalka and Hill 2011, Smaga *et al.* 2014), ghrelin (Steiger *et al.* 2011) and nutritional factors. Many other associations of various factors with depression are given in the paper by Lang and Borgwardt (2013).

Despite this variety of data, only some hormones and factors have successfully been used for biochemical characterisations of affective disorders. Among the most repeatedly validated biomarkers of depression are a decrease in platelets and lymphocytes binding the serotonin transporter, hypercortisolemia, hypocholesteremia, a decrease in brain-derived neurotrophic factor, a decrease in CREB phosphorylation, and an increase in interleukin 6 (Caruncho and Rivera-Baltanás 2010).

Neuroactive steroids occupy a particular position, acting on GABA$_A$ receptors (GABA$_A$Rs) to potentiate the effects of GABA on these receptors. There is considerable evidence for the plasticity of GABA$_A$Rs associated with altered levels of neurosteroids. These effects have been observed for ovarian steroids (Estrada-Camarena *et al.* 2010, Soares 2013, MacKenzie and Maguire 2014, Schiller *et al.* 2014, Pařízek *et al.* 2014),

testosterone (Amore *et al.* 2012, Khera 2013, McHenry *et al.* 2014), dehydroepiandrosterone (DHEA) (Veronese *et al.* 2015) and other neurosteroids (Reddy 2010, Patte-Mensah *et al.* 2014).

For this reason we analyzed the steroid metabolome in order to discover differences that could potentially lead to affective depression or anxiety.

Materials and Methods

Subjects

For this study we used three groups of subjects: 1) twenty patients with depressive disorder (unipolar or bipolar) according to the International Classification of Diseases, 10th Revision (ICD-10) (WHO, 2004), 2) twenty patients with specific anxiety disorder (Phobia, Panic Disorder, Obsessive-compulsive Disorder, Generalized Anxiety Disorder, Mixed Anxiety Depressive Disorder, Acute Reaction to Stress, Adjustment Disorder, Post-traumatic Stress Disorder) according to the ICD-10, and 3) thirty healthy matching controls. The subjects were 18-45 years old (men with depression: mean age 33.8±8.09, median 35 years of age; men with anxiety: mean age 30.25±7.63, median 27.5; controls: mean age 28.56±7.64, median 26.5). Their BMI was at the border of normal and over-weight, with a BMI of 24.1 for men with depression (22.6, 26.6) (median and 95 % continence interval), 25.2 (22.8, 28.9) for men with anxiety and 24.6 (23, 27.1) for controls.

The diagnosis of psychiatric disorder was confirmed using The Mini-International Neuropsychiatric Interview (M.I.N.I.) (Sheehan *et al.* 1998). The interview was performed by an independent clinical psychiatrist. Patients were required to have a minimum symptom severity (depressive or anxiety) of 4 on the Clinical Global Impressions Scale (CGI) (Guy 1976), suggesting at least moderately severe illness.

Exclusion criteria were: another psychiatric diagnosis on Axis I (especially organic disorder of the CNS, abuse and/or dependence on psychoactive substances), incapacity to sign an informed consent form, severe metabolic or endocrine illness, treated or untreated, and administration of drugs known to change steroid levels. In addition, healthy controls had to be free of any psychiatric disorder (both current and past), including organic impairment of the CNS, abuse and/or dependence on psychoactive substances. All controls were non-smokers. The Ethical Committee of the Institute of Endocrinology in Prague approved the

protocol of the study, and written informed consent was obtained from all participants.

Blood sampling

Blood samples were drawn at 7 a.m. following overnight fasting from the cubital or forearm vein. Each sample was collected into a cooled plastic tube containing 100 µl of 5 % EDTA. Plasma was obtained by centrifugation for 5 min at 2000 g at 4 °C, separated and frozen within half an hour of being drawn from the subject, and stored at –80 °C until analyzed.

Analytical methods
Analyses measured by RIA and IRMA

17-hydroxy-pregnenolone was measured by the RIA method, and 17-hydroxy-pregnenolone sulfate by the same method after hydrolysis (Hill *et al.* 1999). Estradiol was measured using an RIA kit from Orion (Finland) and 17-hydroxy-progesterone using a kit from Immunotech (France). Cortisol was measured using an RIA kit from Orion (Finland). Sex hormones bounding globulin (SHBG) were measured by the IRMA method (Orion, Finland). Kits from Immunotech (France) were used to measure LH and FSH (IRMA kit).

Steroids measured by GC/MS method

The levels of unconjugated steroids and steroid polar conjugates after hydrolysis were concomitantly measured in cubital vein blood using the GC-MS method (Hill *et al.* 2010).

In brief, free steroids were extracted from plasma by diethyl-ether; steroid conjugates were hydrolyzed and extracted. The resulting residues were derivatized by methoxyamine hydrochloride and analyzed by GC/MS as follows.

Steroids were purchased from Steraloids (Newport, RI, USA), Sylon B from Supelco (Bellefonte, PA, USA), methoxylamine hydrochloride from Sigma (St. Louis, MO, USA) and solvents from Merck (Darmstadt, Germany).

Instruments: Measurements of steroid levels were done on a GCMS-QP2010 Plus system by Shimadzu (Kyoto, Japan) consisting of a gas chromatograph equipped with automatic flow control, an AOC-20s autosampler, and a single quadrupole detector with an adjustable electron voltage of 10-195 V. A capillary column with a medium polarity RESTEK Rxi column (diameter 0.25 mm, length 15 m, film thickness 0.1 µm) was used for analyses. Electronimpact ionization

with electron voltage fixed at 70 V and emission current set to 160 µA was used. The temperatures of the injection port, ion source and interface were maintained at 220 °C, 300 °C, and 310 °C, respectively. Analyses were carried out in the splitless mode with a constant linear velocity of the carrier gas (He), which was maintained at 60 cm/s. The septum purge flow was set at 3 ml/min. The samples were injected using the high pressure mode (200 kPa), which was maintained for 1 min. The detector voltage was set to 1.4 kV.

Statistical data analysis

The changes of steroid levels were evaluated using a repeated measures ANOVA model consisting of a group factor, explaining differences between groups, and an age factor. Due to the non-Gaussian data distribution and non-constant variance, the original data was transformed by a power transformation to attain symmetric distribution of the data and residuals as well as homoscedasticity (Meloun *et al.* 2000). The homogeneity of the transformed data was checked by residual analysis as described elsewhere (Meloun *et al.* 2002).

Results

The results of analyses and statistical factors for age and group are given in Table 1.

Both depressive and anxious men differed significantly (p<0.05) from controls in a considerable number of hormonal parameters. Higher levels in controls compared to all patients were found for the free steroids progesterone, estrone, dihydrotestosterone, allopregnanolone, sulfates of pregnenolone, 20α-dihydropregnenolone, estrone, allopregnanolone, isopregnanolone, androsterone and epiandrosterone, and conjugates of pregnanolone, both 5β-pregnanediols, etiocholanolone and 5α-androstane 3β, 17β-diol. Lower levels in controls were only found for LH, androstenediol, pregnenolone and 16α-hydroxy-pregnenolone.

Men with anxiety disorders and depression mainly differed from controls (p<0.05) in having lower levels of estradiol and higher levels of 7β-hydroxy-DHEA and 5-androsten-3β,7β,17β-triol. Men with depression additionally had higher levels of DHEA, 16α-hydroxy-DHEA, epiandrosterone, cortisol and conjugated 5β-androstene-3α,17β-diol and lower levels of 17-hydroxypregnenolone sulfate and dehydroepiandrosterone sulfate (DHEAS).

There were significant differences between men with depression and men with anxiety for 20α-dihydropregnenolone sulfate, estradiol and cortisol, which were significantly higher in depressive patients, and for 17-hydroxypregnenolone sulfate, 5-androstene 3β,7β,17β-triol, progesterone and estrone sulfate, which were lower.

Discussion

Neurosteroids are positive allosteric modulators of GABA$_A$ receptors with powerful antiseizure, anxiolytic, analgesic and antidepressive activity in diverse animal models and in several clinical studies, and increase both synaptic and tonic inhibition. Sulfated neurosteroids such as pregnenolone sulfate, which are negative GABA$_A$ receptor modulators, act as memory-enhancing agents. Other ion channels such as AMPA, EAAT, NMDA- and metabotropic glutamate receptors (mGluR1 to mGluR8) have also recently received more attention in studies of depression (Hill *et al.* 2015).

Among the steroids analyzed in this study are compounds that have repeatedly been recognized as neuroactive steroids, e.g. allopregnanolone (Shule *et al.* 2014), testosterone (McHenry *et al.* 2014), pregnenolone (Reddy 2010), dehydroepiandrosterone (Kurita *et al.* 2013) and pregnanolone (Holubová *et al.* 2014). Differences in their levels between healthy controls and psychiatric patients with depression or anxiety were found for some but not all of them. For instance, practically no association with psychopathy was found for testosterone, though the literature frequently reports gender differences in the incidence of depression probably due to sexual hormones levels. Cortisol levels were found to be higher in men with depression than in controls, as reported in literature (Herbert 2013), but not in men with anxiety. Also, dehydroepiandrosterone and dehydroepiandrosterone sulfate levels differed from controls in patients with depression but not those with anxiety. The sulfated neurosteroids pregnenolone sulfate and DHEAS as well as DHEA have clear antidepressant effects in animals and humans (Kurita *et al.* 2013). Pregnenolone sulfate and DHEAS also enhance cognition in animals (Sabeti *et al.* 2007, Carta *et al.* 2012). DHEA, a precursor of DHEAS and a dietary supplement, has been widely investigated as a potential antidepressant (Peixoto *et al.* 2014). However, studies investigating DHEA and DHEAS concentrations in depression have yielded inconsistent results with both increases and decreases during major depression. Overall, there are

Table 1. The parameters for depressive men, men with anxiety and controls.

| Variable | Groups | | | ANOVA | | | | Multiple comparisons (p<0.05) |
	Depressive men (D)	Men with anxiety (A)	Controls (C)	Age F	Age p	Group F	Group p	
BMI [kg/m^2]	24.1 (22.6, 26.6)	25.2 (22.8, 28.9)	24.6 (23, 27.1)	18.5	0	2.7	0.073	
FSH [U/l]	3.2 (2.58, 4.44)	2.89 (2.44, 3.83)	3.11 (2.22, 4.31)	6.4	0.014	0	0.996	
LH [U/l]	4.72 (2.81, 6.21)	5.16 (3.12, 6.16)	3 (2.29, 4.4)	1.7	0.202	4.7	0.013	A>C, D>C
SHBG [nmol/l]	35.8 (25.2, 41.5)	33.1 (25.9, 39.7)	23.6 (18.8, 36.9)	1.2	0.276	1.3	0.287	
Pregnenolone [nmol/l]	2.45 (1.65, 3.27)	3.01 (2.14, 3.67)	1.83 (0.99, 2.46)	35.2	<0.001	8.6	0.001	A>C, D>C
Pregnenolone sulfate [nmol/l]	155 (114, 166)	196 (160, 223)	259 (209, 355)	13.1	0.001	9.6	0	A<C, D<C
17-Hydroxypregnenolone [nmol/l]	4.14 (2.2, 7.14)	3.11 (1.74, 5.46)	2.73 (1.32, 7.05)	8.3	0.006	1.7	0.198	
17-Hydroxypregnenolone sulfate [nmol/l]	13.9 (10.2, 18.5)	18.4 (13.9, 23.8)	18.6 (15.4, 22.8)	9.4	0.003	3.8	0.027	D<A, D<C
20α-Dihydropregnenolone [nmol/l]	2.06 (1.53, 2.71)	2.18 (1.71, 2.99)	1.77 (1.53, 2.24)	11.5	0.001	1.5	0.225	
20α-Dihydropregnenolone sulfate [nmol/l]	2080 (1120, 3000)	1350 (1040, 1840)	2740 (1810, 3970)	17.8	0	10.9	0	D>A, A<C, D<C
16α-Hydroxypregnenolone [nmol/l]	0.846 (0.48, 1.06)	0.55 (0.486, 0.676)	0.436 (0.277, 0.629)	9.3	0.003	7.6	0.001	A>C, D>C
Dehydroepiandrosterone (DHEA) [nmol/l]	30.7 (17, 42.2)	22.9 (18.6, 31.2)	18.3 (11.9, 29.9)	9.5	0.003	3.4	0.038	D>C
DHEA sulfate [nmol/l]	5580 (2960, 6630)	5440 (4220, 7470)	6930 (5900, 8750)	14.8	0	2.5	0.092	D<C
7α-Hydroxy-DHEA [nmol/l]	1.21 (0.738, 1.59)	1.2 (0.92, 1.61)	1.01 (0.724, 1.41)	6.9	0.011	1.7	0.196	
7β-Hydroxy-DHEA [nmol/l]	0.645 (0.388, 0.913)	0.726 (0.629, 0.822)	0.535 (0.413, 0.681)	17.7	0	5.8	0.005	A>C
16α-Hydroxy-DHEA [nmol/l]	0.326 (0.2, 0.523)	0.238 (0.185, 0.343)	0.163 (0.125, 0.274)	1.1	0.304	5.2	0.008	D>C
16α-Hydroxy-DHEA sulfate [nmol/l]	3.78 (2.67, 11.2)	7.18 (2.42, 13.4)	5.16 (3.05, 11.3)	0.6	0.46	0.5	0.627	
Androstenediol [nmol/l]	2.54 (1.12, 3.25)	2.83 (2.3, 3.77)	2.02 (1.51, 2.5)	8.8	0.004	5.8	0.005	A>C, D>C
Androstenediol sulfate [nmol/l]	1300 (687, 2330)	1300 (881, 1920)	2290 (1590, 3510)	4.2	0.045	5.4	0.007	A<C, D<C
5-Androstene-3β,7α,17β-triol [nmol/l]	0.228 (0.105, 0.262)	0.199 (0.181, 0.264)	0.187 (0.127, 0.22)	7	0.01	1.1	0.326	
5-Androstene-3β,7β,17β-triol [nmol/l]	0.0793 (0.0473, 0.12)	0.152 (0.136, 0.173)	0.0861 (0.0613, 0.136)	5.4	0.023	17.8	<0.001	D<A, A>C
Progesterone [nmol/l]	0.593 (0.471, 0.754)	0.761 (0.528, 0.954)	1.09 (0.906, 1.22)	1.9	0.173	17.3	<0.001	D<A, A<C, D<C
17-Hydroxyprogesterone [nmol/l]	5.14 (4.56, 5.77)	4.11 (3.58, 5.14)	4.69 (3.44, 6.48)	16.7	0	2.4	0.102	D>A
16α-Hydroxyprogesterone [nmol/l]	1.2 (1.06, 1.68)	0.991 (0.771, 1.26)	1.04 (0.769, 1.66)	2	0.163	1.3	0.282	
Androstenedione [nmol/l]	4.37 (2.99, 4.84)	3.78 (3.02, 4.5)	3.24 (2.78, 4.01)	0.4	0.542	2.6	0.085	D>C
Testosterone [nmol/l]	9.37 (7.76, 10.9)	9.66 (7.32, 11.8)	9.67 (7.33, 12)	2.4	0.126	0.1	0.936	

Table 1. (continued)

| Variable | Groups | | | ANOVA | | | | Multiple comparisons (p<0.05) |
| | Depressive men (D) | Men with anxiety (A) | Controls (C) | Age | | Group | | |
				F	p	F	p	
Estrone [nmol/l]	0.163 (0.137, 0.193)	0.145 (0.124, 0.179)	0.228 (0.173, 0.301)	0.4	0.513	4.7	0.012	A<C, D<C
Estrone sulfate [nmol/l]	1.18 (1.01, 1.66)	2.14 (1.6, 2.67)	4.44 (2.82, 8.64)	0	0.909	31.4	<0.001	D<A, A<C, D<C
Estradiol [nmol/l]	0.0643 (0.0561, 0.0824)	0.0473 (0.0384, 0.0597)	0.0625 (0.0481, 0.077)	0.4	0.543	5.1	0.009	D>A, A<C
5α-Dihydrotestosterone [nmol/l]	1.05 (0.914, 1.38)	1.08 (0.951, 1.46)	1.47 (1.19, 1.93)	3.6	0.064	7.1	0.002	A<C, D<C
Allopregnanolone [nmol/l]	0.126 (0.106, 0.162)	0.137 (0.0967, 0.174)	0.194 (0.137, 0.236)	4.5	0.038	6	0.004	A<C, D<C
Allopregnanolone sulfate [nmol/l]	6.36 (3.79, 9.17)	6.99 (5.4, 8.79)	15.1 (9.02, 20.5)	22.3	<0.001	18	<0.001	A<C, D<C
Isopregnanolone [nmol/l]	0.144 (0.1, 0.212)	0.156 (0.116, 0.182)	0.15 (0.0941, 0.215)	6.5	0.013	0.2	0.819	
Isopregnanolone sulfate [nmol/l]	8.76 (6.79, 11.2)	10.6 (8.04, 12.5)	17.8 (12.7, 26.4)	11.8	0.001	15.1	<0.001	A<C, D<C
Pregnanolone [nmol/l]	0.0547 (0.0348, 0.0767)	0.0551 (0.0361, 0.0738)	0.0688 (0.0522, 0.118)	0.8	0.369	2.6	0.08	D<C
Conjugated pregnanolone [nmol/l]	22.5 (16.7, 28.8)	21.3 (15.7, 26.7)	36.9 (22.6, 49.4)	3.1	0.085	8.7	0	A<C, D<C
Conjugated 5β-pregnane-3α,20α-diol [nmol/l]	15.8 (10.2, 22.1)	17.4 (12.8, 20.5)	31.5 (19.8, 50.8)	2.2	0.14	10.3	0	A<C, D<C
Conjugated 5β-pregnane-3β,20α-diol [nmol/l]	17.2 (12.1, 26)	19.1 (15, 25.9)	326 (157, 547)	9.6	0.003	135.8	<0.001	A<C, D<C
Androsterone [nmol/l]	0.605 (0.482, 0.769)	0.615 (0.494, 0.782)	0.701 (0.53, 0.906)	17.5	0	0.9	0.424	
Androsterone sulfate [nmol/l]	956 (502, 1890)	1370 (985, 1690)	2230 (1490, 3420)	17.4	0	7.4	0.001	A<C, D<C
Epiandrosterone [nmol/l]	1.62 (0.885, 1.95)	1.05 (0.934, 1.46)	0.977 (0.624, 1.28)	8.9	0.004	4.6	0.014	D>C
Epiandrosterone sulfate [nmol/l]	572 (366, 719)	579 (437, 825)	931 (703, 1330)	16.2	0	7.4	0.001	A<C, D<C
Etiocholanolone [nmol/l]	0.0948 (0.0544, 0.149)	0.133 (0.0933, 0.183)	0.128 (0.0834, 0.182)	0	0.855	2.1	0.137	D<A
Conjugated etiocholanolone [nmol/l]	52 (27, 71.6)	51.5 (37, 79.8)	81.9 (57.7, 115)	0.4	0.524	4.4	0.016	A<C, D<C
Conjugated epietiocholanolone [nmol/l]	30.5 (16.1, 56)	23.6 (16.4, 43.6)	53.2 (26.8, 84.7)	0.5	0.498	2.8	0.067	A<C
5α-Androstane-3α,17β-diol [nmol/l]	0.268 (0.146, 0.374)	0.229 (0.209, 0.274)	0.281 (0.223, 0.385)	4.3	0.043	2.9	0.062	A<C
Conjugated 5α-Androstane-3α,17β-diol [nmol/l]	112 (58.4, 138)	115 (64.7, 164)	81.5 (54.6, 241)	12.8	0.001	0.6	0.568	
5α-Androstane-3β,17β-diol [nmol/l]	0.0715 (0.0595, 0.0791)	0.0705 (0.0535, 0.0839)	0.0805 (0.0586, 0.106)	1.8	0.185	0.8	0.435	
Conjugated 5α-Androstane-3β,17β-diol [nmol/l]	120 (68.7, 162)	99.9 (58.2, 133)	273 (191, 358)	10.8	0.002	20.4	<0.001	A<C, D<C
Conjugated 5β-Androstane-3α,17β-diol [nmol/l]	19.6 (15.6, 31.9)	14 (11.8, 17.3)	7.62 (4.43, 30.1)	0.8	0.39	4.6	0.014	D>C
Cortisol [nmol/l]	567 (482, 682)	451 (384, 571)	408 (349, 538)	0.8	0.369	6.5	0.003	D>A, D>C

Statistical characteristic of steroid hormones and other hormones/substances using repeated measures ANOVA with the factors Age and Group. Age-F = age factor showing the differences according to the age of the subjects. Group-F = group factor showing the differences between controls (C), depressive men (D) and men with anxiety (A). p = significance. Values are given as medians and quartiles.

indications that adjunct DHEA could produce beneficial effects in patients with depression. Attention should be paid to the role of 7-hydroxylated derivatives of DHEA, found in this study to be characteristic for anxiety.

There is considerable evidence for the involvement of neurosteroids in the aetiology of anxiety disorders. Neurosteroids such as allopregnanolone and tetrahydrodeoxycortisone (THDOC) are potent anxiolytic agents (MacKenzie and Maguire 2013, Nuss 2015). Progesterone also has anxiolytic activity in animal models. Administration of progesterone produces similar sedative-anxiolytic effects in men and women (Bristot *et al.* 2014). In our hands men with depression and men with anxiety differed mainly in the levels of 20α-dihydro-pregnenolone sulfate, estradiol and cortisol, and in 17-hydroxypregnenolone sulfate, 5-androstene 3β,7β,17β-triol, progesterone and estrone sulfate.

The results of our study include several striking findings. The anticonvulsive and anxiolytic steroid allopregnanolone was less associated with changes of mood than its sulfate, and sulfates and conjugates were generally more frequently markers of such changes than free steroids. The greatest difference between controls and psychiatric patients was found for conjugated 5β-pregnane-3β, 20α-diol.

Considering the role of steroid sulfates in the brain it is necessary to take into account that we measured circulating steroid levels in blood, which do not necessarily correspond to levels in the brain or even in its specific regions. Sulfates have a limited ability to cross the blood-brain barrier, and the activity of sulfotransferase in the brain, which is responsible for the sulfatation of steroids, is very low (Kříž *et al.* 2005). Generally, sulfates and conjugates of steroids in are higher in plasma than free steroids. Despite this, the ratio of free and conjugated or sulfatated steroids is an important feature of the steroid metabolome, and it could be reflected in various target regions in the brain. In addition, levels of selected steroids in the plasma or serum could be helpful as markers of psychiatric disorders.

Conflict of Interest
There is no conflict of interest.

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