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Peripheral neuroactive steroids may be as good as the steroids in the cerebrospinal fluid for the diagnostics of CNS disturbances

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ABSTRACT

To compare the predictivity of the neuroactive steroids in the cerebrospinal fluid and peripheral blood for the diagnostics of CNS disturbances, eighteen unconjugated steroids were quantified in the cerebrospinal fluid (CSF) from the 3rd ventricle and 18 unconjugated steroids and 7 steroid polar conjugates were measured in the serum using GC–MS and RIA. Eight postmenopausal women (56–78 years of age) and 7 men (22–88 years of age) with hydrocephalus were enrolled in the study. The sensitivity of the method ranged from low femtogram to low picogram levels depending on the steroid fragmentation pattern. Using multivariate regression, a model for simultaneous prediction of the CSF steroids from the serum steroids was completed. Then, the penetrability of the individual steroids across the blood-brain-barrier was evaluated and the sources of various brain steroids were estimated. Our data show that a part of the steroids may be synthesized de novo in the CNS. However, substantial part of the steroid metabolites may be synthesized in the CNS from the steroid precursors or directly transported from the periphery. The CNS in situ synthesis and transport from periphery might be complementary in some cases, i.e. brain synthesis might provide minimum level of steroids, which are indispensable for the CNS functions.

1. Introduction

Although several authors [1–4] have analyzed the steroid transport between the periphery and the brain, the respective data is still incomplete. Hence, we evaluated the correlations of the steroids between the periphery and the cerebrospinal fluid (CSF), considering also the effect of the steroid conjugation in the periphery. In addition, we have followed the correlations between the steroids within the CSF. The aim of the study was to estimate to what extent the peripheral steroids may contribute to the steroid metabolome in the CNS and how important may be the contribution of the *in situ* brain synthesis. To our concept the steroid brain *in situ* synthesis provides minimum amount of steroids, which is indispensable for the CNS functions in the situations when the peripheral sources misfire. However, usually in physiological situations the penetration of peripheral steroids across the blood-brain-barrier provides the majority of the CNS steroids, at least in the form of the parent substances, which are further metabolized in the CNS. We also believe that changes in the peripheral steroid production are promptly reflected in the CNS steroid metabolome. Therefore, the predictive value of the peripheral steroids and particularly the neuroactive steroids could be comparable with the predictivity of the CSF steroids. Hence, the substantially less invasive collection of the peripheral steroids may be as good as the collection of the CSF ones for the diagnostics of CNS diseases, which are most probably connected with an imbalance in peripheral steroidogenesis (like postpartum depressions, catamenial epilepsy, premenstrual syndrome and possibly the affective disorders) and could also reflect the sex differences and various physiological changes like pregnancy, parturition or aging.

For the verification of these assumptions we evaluated the correlations between peripheral and CSF steroids using a wide spectrum of bioactive steroids, their precursors and metabolites. The variety of steroids measured in the present study is unique.

2. Materials and methods

2.1. Subjects

Eight postmenopausal women (56–78 years) and 7 men (22–88 years) underwent an endoscopic 3rd ventriculostomy because of obstructive hydrocephalus.

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Table 1
List of steroids under investigation and the methods used for quantification.

1	A2. . .androstenedione (GC–MS)
2	A3α5α. . .5α-androstane-3α-hydroxy-17-one; androsterone (GC–MS)
3	A3β5α. . .5α-androstane-3β-hydroxy-17-one; epiandrosterone (GC–MS)
4	AT7α. . .5-androstene-3β,7α,17β-triol (GC–MS)
5	AT7β. . .5-androstene-3β,7β,17β-triol (GC–MS)
6	Cort. . .cortisol (RIA)
7	DHEA. . .dehydroepiandrosterone (GC–MS)
8	DHEA16α. . .3β,16α-dihydroxy-5-androstene-17-one; 16α-hydroxy-DHEA (GC–MS)
9	DHEA7α. . .3β,7α-dihydroxy-5-androstene-17-one; 7α-hydroxy-DHEA (GC–MS)
10	DHEA7β. . .3β,7β-dihydroxy-5-androstene-17-one; 7β-hydroxy-DHEA (GC–MS)
11	P3α5α. . .3α-hydroxy-5α-pregnane-20-one; allopregnanolone (GC–MS)
12	P3β5α. . .3β-hydroxy-5α-pregnane-20-one; isopregnanolone; epiallopregnanolone (GC–MS)
13	Preg. . .pregnenolone (GC–MS)
14	Preg16α. . .3β,16α-dihydroxy-5-pregnene-20-one; 16α-hydroxy-pregnenolone (GC–MS)
15	Prog. . .progesterone (RIA)
16	Prog16α. . .16α-hydroxy-4-pregnene-3,20-dione; 16α-hydroxy-progesterone (GC–MS)
17	Prog17. . .17-hydroxy-progesterone (RIA)
18	T. . .testosterone (GC–MS)

C at the end of the abbreviation, polar conjugates of the steroid; GC–MS, gas chromatography–mass spectrometry; RIA, radioimmunoassay.

2.2. Sample collection

The surgeries were performed under a general endotracheal anesthesia in patients who were treated in the Department of Neurosurgery of the Faculty Hospital St. Anne's in Brno. The patients were operated for either tumorous or non-tumorous lesions. Neuroendoscopic system Wolf or Storz was used for the surgery. Neuroendoscopic access to the third ventricle [5,6] was as follows: At the beginning of the neuroendoscopic procedure samples of the cerebrospinal fluid (CSF) were collected from 3rd ventricle through the foramen of Monro and from the lateral ventricle afterwards for cytological analysis, for tumor markers and steroid analysis. Particular attention was paid not to dilute the samples and biopsy catheter of own construction was used for the sampling. Before the surgery a peripheral blood sample (10 mL) was taken from the cubital vein. The blood components were separated and the serum and CSF samples were subsequently stored in deep freeze at −80 °C until analyzed.

2.3. Steroid analyses

Eighteen unconjugated steroids were quantified in the cerebrospinal fluid from the 3rd ventricle (CSF) and 18 unconjugated steroids and 7 steroid polar conjugates were measured in the serum using GC–MS and RIA (Table 1).

2.4. Chemicals and reagents

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

2.5. Instruments

The GC–MS system was supplied by Shimadzu (Kyoto, Japan). The GCMS-QP2010 Plus system consisted of a gas chromatograph equipped with automatic flow control, AOC-20s and an autosampler. Quadrupole detector with electron-impact ionization was used for the analyses. Adjustable electron voltage of 10–195 V was set to 70 V. Emission current was set to 160 μA. A capillary column with a medium polarity RESTEK Rxi (diameter 0.25 mm, length 15 m, film thickness 0.1 μm was used for analyses. The temperature of the injection port, ion source and interface was maintained at 220, 300, 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 up to 3 mL/min. The samples were injected using the high-pressure mode which was applied at 200 kPa and this pressure was maintained for 1 min. The detector voltage was set to 1.4 kV.

2.6. Sample preparation for GC–MS analysis

When using the GC–MS platform, the unconjugated steroids were extracted from 1 mL of plasma or CSF with diethyl-ether (3 mL). The diethyl-ether extract was dried in the block heater at 37 °C. The lipids in the dry residue of the diethyl-ether extract were separated by partitioning between a mixture of methanol-water 4:1 (1 mL) and pentane (1 mL). The pentane phase was discarded and the polar phase was dried in the vacuum centrifuge at 60 °C (2 h). The dry residue from the polar phase was derivatized first with 50 μL methoxylamine-hydrochloride solution in pyridine (2%) on oxo-groups (60 °C, 1 h). The mixture after the first derivatization was dried in the flow of nitrogen and the dry residue was treated with 50 μL reagent Sylon B (99% of bis(trimethylsilyl)-trifluoroacetamide and 1% of trimethylchlorosilane) forming trimethylsilyl derivatives on hydroxy-groups (TMS-MOX derivatives) (90 °C, 1 h). Finally, the mixture after the second derivatization step was dried in the flow of nitrogen, the dry residue was dissolved in 20 μL of isooctane and 1 μL of the solution was used for GC–MS analysis.

The steroid conjugates remaining in the polar residues after diethyl-ether extraction were analyzed as follows: the polar residues were dried in the vacuum centrifuge at 37 °C (5 h) and the dry residues containing steroid polar conjugates (mostly sulfates and glucuronides) were hydrolyzed as described elsewhere [7]. The hydrolyzed samples were again dried in the vacuum centrifuge at 37 °C (5 h). The dried residues were reconstituted with 1 mL of chromatographic water and then extracted from 1 mL of water solution into 3 mL diethyl-ether. The diethyl-ether extract was further processed in the same way as the free steroids. In contrast to the sample preparation of free steroids, the dry residue after the second derivatization step was dissolved in 200 μL of isooctane instead of the 20 μL of isooctane. Prior to further processing, the original samples and the polar phases after diethyl-ether extraction (which were used for the quantification of the steroid conjugates) were spiked with 17α-estradiol (as an internal standard) to attain a concentration of 1 and 10 ng/mL, respectively. The internal standard was recorded at effective masses m/z = 231, 285 and 416. The addition of the internal standard to the body fluid before sample preparation (free steroids) and to polar phase after diethyl-ether extraction (conjugated steroids) assured that the losses during the sample processing were not critical for the steroid quantification.

2.7. Temperature and pressure gradients for the GC–MS analysis of trimethylsilyl derivatives and the retention times of the steroids

To effectively utilize the biological material and to suitably separate the isomers with similar fragmentation, the individual samples were applied in independent courses, in each case employing a part of the steroids under investigation. The choices of the steroids measured within the individual courses, the temperature and pressure gradients, and the effective masses used for the measurement in selected ion monitoring mode were all optimized to attain a min-

Table 2
Temperature gradients used for steroid analysis at constant linear velocity 60 cm s^{-1}.

Method	Step				Initial pressure [kPa]	Injection temp. [°C]	Overall time [min]
	Initial conditions (final temperature, temperature gradient, hold time) [°C, °C min^{-1}, min]						
	1	2	3	4			
G1	80 (–, 1)	190 (40, 0)	210 (4, 0)	300 (20, 5)	34	220	18.25
G2	80 (–, 0)	190 (40, 0)	205 (1.6, 0)	300 (40, 5)	34	240	19.50
G3	80 (–, 1)	200 (40, 0)	240 (8, 0)	300 (40, 5)	34	220	15.50

Table 3
Schedule for selected ion monitoring of 16 steroids in the human cerebrospinal fluid and serum.

Method	Start time (min)	End time (min)	Channel, *m/z*						
			1	2	3	4	5	6	7
G1	8.20	9.20	208	254	327	344	432		
	9.20	9.85	208	270	327	360	432		
	9.85	10.60	268	270	285	358	360	416	
	10.60	11.00	268	358	389				
	11.00	12.50	288	312	313	344	384	386	474
G2	11.50	12.80	231	285	416				
	12.80	14.00	241	298	388				
	14.30	14.85	273	286	341	372			
G3	7.70	8.40	266	285	356	387	416		
	8.40	9.00	266	356	387	446			
	10.30	10.80	156	188	429				

imum limit of detection at sufficient selectivity. The temperatures and pressure gradients for the detection of steroids are shown in Table 2 while the schemes for fragment recording are displayed in Table 3. The effective masses, retention times of the chromatographic peaks, sequence number of injection for the steroid groups and gradients that were used for quantification of the individual steroids are shown in Table 4.

2.8. Calibration curve

In all cases the mixtures of authentic standards were processed in the same way as samples. The mixtures were specific for each of the independent courses as mentioned above. The standards were injected in three different amounts for each steroid (10, 100 and 1000 pg).

2.9. Validation procedure

For the evaluation of linearity of the test procedure, the calibration curves (consisting of mixtures of standards in isooctane serially diluted in concentrations covering the concentration range of each steroid in CSF and circulation) were followed considering also the levels of the corresponding circulating polar conjugates. At least 6 concentration levels were measured for each steroid. Two-parameter linear regression was used for data processing. Respecting the excellent linearity for all substances

Table 4
Effective masses and retention times for the investigated steroids.

Gradient	Steroid	*m/z*	Retention time [min]		σ[b]
			Peak 1	Peak 2	
G1	5-Androstene-3β,7α,17β-triol	208, 327, 432[a]	8.52	–	0.016
G1	Androsterone	270, 360	9.61	–	0.013
G1	5-Androstene-3β,7β,17β-triol	208, 327, 432	9.80	–	0.012
G1	17α-Estradiol (internal standard)	285, 416	10.03	–	0.011
G1	Epiandrosterone	270, 360	10.35	–	0.012
G1	Dehydroepiandrosterone	268, 358	10.36	–	0.011
G1	Testosterone	268, 358, 389	10.76	10.96	0.010
G1	Pregnenolone	288, 312, 386	11.27	–	0.009
G1	16α-Hydroxy-pregnenolone	384, 474	11.50	–	0.009
G1	Androstenedione	313, 344	11.77	11.82	0.009
G2	17α-Estradiol (internal standard)	231, 285, 416	8.04	–	0.031
G2	Allopregnanolone	241,298, 388	13.23	–	0.010
G2	Isopregnanolone (epiallopregnanolone)	241,298, 388	13.72	–	0.008
G2	Progesterone	273, 286, 372, 341	14.60	14.67	0.007
G3	7α-Hydroxy-dehydroepiandrosterone	266, 356, 387	8.01	–	0.012
G3	17α-Estradiol (internal standard)	285, 416	8.04	–	0.012
G3	16α-Hydroxy-dehydroepiandrosterone	266, 356, 446	8.65	8.84	0.012
G3	7β-Hydroxy-dehydroepiandrosterone	266, 356, 387	8.88	–	0.012
G3	16α-Hydroxy-progesterone	156, 188, 429	10.49	10.54	0.006

[a] Peaks and effective masses used for quantification are underlined.
[b] σ, standard deviation of retention time for quantitation peak.

Table 5
Analytical criteria for multi-component steroid analysis.

Steroid	Linearity of a test procedure		Repeatability CV [%], $n = 6$	Limit of detection	
	Slope	R		[pmol/L]	[fg]
Pregnenolone	1.33	0.9952	3.3	2.13	33.6
Dehydroepiandrosterone	0.96	0.9986	2.3	2.2	31.7
Progesterone	0.98	0.9968	4.5	7.7	121
Androstenedione	1.02	0.9980	2.7	11.3	162
Testosterone	1.29	0.9982	3.6	4.77	68.7
Allopregnanolone	1.24	0.9967	2.2	1.53	24.4
Isopregnanolone (epiallopregnanolone)	1.28	0.9955	1.7	1.17	18.6
Androsterone	1.19	0.9997	4.1	0.39	5.66
Epiandrosterone	1.16	0.9997	2.8	0.49	7.11
7α-Hydroxy-dehydroepiandrosterone	1.00	0.9999	1.3	0.585	8.83
7β-Hydroxy-dehydroepiandrosterone	1.01	0.9992	1.0	0.434	6.55
5-Androstene-3β,7α,17β-triol	1.01	0.9999	3.3	0.0563	0.861
5-Androstene-3β,7β,17β-triol	1.00	0.9985	5.1	0.0404	0.618
16α-Hydroxy-pregnenolone	1.01	0.9962	2.6	0.154	2.56
16α-Hydroxy-dehydroepiandrosterone	0.99	0.9993	1.2	0.388	5.85
16α-Hydroxy-progesterone	1.17	0.9983	1.0	2.05	33.8

investigated (Table 5); a mean response factor method was used for the steroid quantification. Repeatability was checked using isooctane solution containing 100 pg of each steroid after derivatization procedure. The limit of detection for the individual steroids was calculated using the triple of the respective signal-to-noise ratio.

The criterion of accuracy was based on the relationship between the amounts of the added steroids and the amounts detected by the GC–MS assay. This validation step was done in quadruplicate. The individual body fluids (CSF and serum) were spiked with the steroid measured in amounts about the triple of median values found for the individual steroids and the accuracy values were expressed as percentage of the assayed concentration relative to the calculated concentration (recovery). The recoveries ranged from 75 to 104%.

2.10. Steroid analysis by radioimmunoassay

Due to unfavorable fragmentation and insufficient sensitivity for CSF levels, the cortisol and Prog17 concentrations in the CSF and circulation were measured by RIA. The cortisol was measured using the method of Bicikova et al. [8] (intra-assay and inter-assay CV was 5.2% and 9.8%, respectively). Prog17 was determined using commercial kit from Immunotech (Marseilles, France) (intra-assay and inter-assay CV was 5.2% and 6.5%, respectively). The only modification of the procedure suggested by the producer was the use of 2 mL of CSF being evaporated in the vacuum centrifuge and reconstituted to a volume recommended by the producer to avoid problems with sensitivity.

2.11. Statistical data analysis

Pearson's correlations were used for the evaluation of the relationships between the steroids. To eliminate the effect of confounders, partial correlations were applied as well. To attain symmetry and homoscedasticity in the data distribution, the original data was transformed to a Gaussian distribution before further processing using a power transformation. Statistical software Statgraphics Centurion, version XV from Statpoint Inc. (Herndon, Virginia, USA) was used for data processing.

3. Results and discussion

The analytical criteria concerning selectivity, linearity of a test procedure, repeatability and sensitivity for each steroid are shown in Tables 4 and 5. The accuracy values were expressed as recovery (for details see "Section 2.9"). The recovery values ranged from 75 to 104%. The linearity was satisfactory for all steroids. The correlation coefficients of two-parameter linear regression ranged from 0.9952

Table 6
Levels of steroids in the cerebrospinal fluid (CSF) and serum (nmol/L).

Steroid	CSF, free steroids			Serum, free steroids			Serum, polar conjugates		
	Quartile			Quartile			Quartile		
	Median	Lower	Upper	Median	Lower	Upper	Median	Lower	Upper
Pregnenolone	0.0604	0.0448	0.0659	0.417	0.245	0.786	35.3	17.0	54.5
Dehydroepiandrosterone	0.078	0.058	0.154	2.28	1.11	4.47	1544	726	2095
Progesterone	0.235	0.194	0.306	1.68	1.50	2.21			
17-Hydroxy-progesterone	0.0167	0.0077	0.0217	0.77	0.56	1.97			
Androstenedione	0.208	0.123	0.273	1.26	0.67	1.89			
Testosterone	0.231	0.186	0.428	6.8	4.2	12.8			
Allopregnanolone	0.0078	0.0058	0.0110	0.125	0.075	0.149	3.65	1.88	6.57
Isopregnanolone	0.0399	0.0244	0.0584	0.189	0.155	0.210	7.0	4.8	13.0
Androsterone	0.0047	0.0033	0.0135	0.111	0.080	0.215	331	213	1043
Epiandrosterone	0.0044	0.0021	0.0080	0.128	0.067	0.215	109	76	295
7α-Hydroxy-dehydroepiandrosterone	0.300	0.178	0.375	0.79	0.52	1.28			
7β-Hydroxy-dehydroepiandrosterone	0.0369	0.0107	0.0538	0.314	0.171	0.442			
5-Androstene-3β,7α,17β-triol	0.0068	0.0038	0.0177	0.095	0.040	0.167			
5-Androstene-3β,7β,17β-triol	0.0119	0.0035	0.0205	0.0504	0.0339	0.0829			
16α-Hydroxy-pregnenolone	0.0014	0.0012	0.0041	0.071	0.052	0.223			
16α-Hydroxy-dehydroepiandrosterone	0.0059	0.0044	0.0115	0.180	0.103	0.216	71.8	34.9	94.1
16α-Hydroxy-progesterone	0.072	0.047	0.158	2.51	1.69	3.11			
Cortisol	5.59	2.24	9.15	412	155	607			

Table 7
Comparison of CSF steroid levels in nmol/L with the results of other authors.

Steroid				Preg	DHEA	Prog	A2	T	P3α5α	DHEA7α	DHEA7β	DHEA16α	Cort
Our results	GC-MS[a] RIA[b]	Postmenopausal women and men with hydrocephalus	median (quartiles)	[a]M: 0.0604 (0.0503, 0.0649) F: 0.0548 (0.0431, 0.0677)	[a]M: 0.129 (0.079, 0.293) F: 0.0656 (0.0493, 0.1028)	[b]M: 0.269 (0.251, 0.399) F: 0.194 (0.148, 0.23)*	[a]M: 0.26 (0.225, 0.31) F: 0.123 (0.08, 0.169)*	[a]M: 0.425 (0.299, 0.432) F: 0.186 (0.141, 0.211)*	[a]M: 0.00717 (0.00576, 0.00963) F: 0.00862 (0.00617, 0.01191)	[a]M: 0.3 (0.224, 0.472) F: 0.254 (0.152, 0.356)	[a]M: 0.0369 (0.0206, 0.0925) F: 0.0247 (0.0094, 0.0503)	[a]M: 0.00591 (0.00408, 0.02315) F: 0.00649 (0.00439, 0.00934)	[b]M: 6.85 (5.45, 12.06) F: 2.76 (0.36, 6.65)
Murphy [9]	CPB	M+F, pathology? , age ?	mean										16
			SD										7
Backstrom [10]	RIA[a] CPB[b]	F, 19-65 years	mean			0.14[a]		0.049[b]					
			SD			0.03		0.01					
		M, 19-65 years	mean			0.12		0.39					
			SD			0.02		0.04					
Carroll [11]	CPB	Non-depressed psychiatric	mean										14
			SD										
Schwarz [12]	RIA	M (age?)	mean		1.7	0.32	1.1	0.74					26
			SD		2.1	0.45	0.45	1.2					37
		F (age ?)	mean		1.7	0.32	1.1	0.2					26
			SD		2.1	0.45	0.45	0.24					37
George [13]	RIA	M (age ?)	mean	0.95		0.32							
			SD	0.7		0.13							
		F (age ?)	mean	0.67		0.35							
			SD	0.41		0.35							
Guazzo [14]	EIA RIA	Sex ? 3-85 years old	mean		0.8								18
			SD										
Molnar [15]	RIA	Postmenopausal F	mean			0.39		0.15					
			SD			0.24		0.15					
Uzunova [16]	GC-MS	Unipolar major dep. M 37-51 years	mean	0.053		0.03			0.014				
			SD	0.024		0.17			0.006				
		Unipolar major dep., F 23-45 years	mean	0.063		0.26			0.016				
			SD	0.024		0.17			0.006				
Murakami [17]	HPLC +RIA	Postmenopausal F	mean		0.76								9.1
			SD										
Kim [18]	RIA[a] GC-MS[b]	M 56-86 years (mean=63)	mean	2.0[a]	0.25[a]					0.26[a]	0.26[a]	0.74[b]	
			SD	1.1	0.05					0.24	0.24	0.36	
		F 19-69 years (mean=45)	mean	5.5	0.32					0.24	0.16	0.92	
			SD	2.1	0.2					0.14	0.05	0.43	
Rasmusson [19]	GC-MS	F, follicular phase	mean		0.22	0.39			0.036				
			SD		0.11	0.34			0.021				

M: males, F: females, CPB: competitive protein binding assay, RIA: radioimmunoassay, GC–MS: gas chromatography–mass spectrometry; EIA, enzyme immunoassay, HPLC: high performance liquid chromatography.

to 0.9999 (Table 5). The levels of free and conjugated steroids are shown in Table 6.

Table 7 shows a comparison of our results with the data of other authors [9–19]. Our data from GC–MS analysis are in fair agreement with the GC–MS results of other studies, which is most apparent in Preg showing very close values to the data of Uzunova et al. [16]. However, the results of the present study and the results of Uzunova et al. [16] show lower values than the corresponding data from immunoassays, i.e. the data of George et al. [13] and our previous data obtained by RIA [18]. This is also valid for the DHEA CSF levels while compared with the GC–MS data of Rasmusson et al. [19] and to our previous data obtained using RIA [18]. Progesterone CSF levels are mostly in a good agreement with the data reported elsewhere [10,12,13,15,16,19]. Our CSF androstenedione data (obtained using GC–MS) are substantially lower than those of Schwarz and Pohl [12] obtained by RIA. This is possibly due to the greater selectivity of GC–MS when compared with RIA. Testosterone, as expected, shows significantly higher CSF levels in male subjects as has been already proven by Backström et al. [10] and Molnár and Kassai-Bazsa [15]. Allopreg-

Table 8
Ratios of serum steroid levels to median levels in the cerebrospinal fluid (CSF).

Steroid	CSF, free steroids			Serum, free steroids			Serum, polar conjugates		
	Quartile			Quartile			Quartile		
	Median	Lower	Upper	Median	Lower	Upper	Median	Lower	Upper
Pregnenolone	1.0	0.7	1.1	6.9	4.1	13.0	584	281	903
Dehydroepiandrosterone	1.0	0.7	2.0	29.3	14.2	57.5	19,849	9,332	26,932
Progesterone	1.0	0.8	1.3	7.1	6.4	9.4			
17-Hydroxy-progesterone	1.0	0.5	1.3	46.4	33.6	118.0			
Androstenedione	1.0	0.6	1.3	6.0	3.2	9.1			
Testosterone	1.0	0.8	1.9	29.7	18.1	55.7			
Allopregnanolone	1.0	0.7	1.4	16.1	9.6	19.1	469	241	844
Isopregnanolone	1.0	0.6	1.5	4.7	3.9	5.3	175	120	327
Androsterone	1.0	0.7	2.9	23.6	17.1	45.5	70,129	45,114	221,196
Epiandrosterone	1.0	0.5	1.8	28.8	15.1	48.5	24,597	17,075	66,532
7α-Hydroxy-dehydroepiandrosterone	1.0	0.6	1.2	2.6	1.7	4.3			
7β-Hydroxy-dehydroepiandrosterone	1.0	0.3	1.5	8.5	4.6	12.0			
5-Androstene-3β,7α,17β-triol	1.0	0.6	2.6	14.0	5.9	24.7			
5-Androstene-3β,7β,17β-triol	1.0	0.3	1.7	4.2	2.8	6.9			
16α-Hydroxy-pregnenolone	1.0	0.8	2.9	50.3	36.8	157.3			
16α-Hydroxy-dehydroepiandrosterone	1.0	0.7	1.9	30.5	17.4	36.5	12,149	5,901	15,934
16α-Hydroxy-progesterone	1.0	0.7	2.2	34.8	23.4	42.9			
Cortisol	1.0	0.4	1.6	73.7	27.7	108.6			

nanolone levels are lower than the GC–MS data of Uzunova et al. [16] possibly due to the age distribution of our subjects. Concerning the 7-hydroxy-metabolites of DHEA our present GC–MS data for DHEA7α shows almost the same results like our previously published data obtained by RIA [18] but lower values for DHEA7β, which may be a consequence of the insufficient selectivity of the RIA method.

The correct identification of the substances in the present study was guaranteed by a congruence of the fragmentation pattern and retention time with the standard (at least 2 fragments + retention time(s)). In addition, the correlations were checked between the precursors and the products considering the known steroid metabolic pathways (data not shown). Further, the steroids containing oxo-group mostly produced two-peak response, thus we also checked the ratios of peak 1 to peak 2 for the individual fragments.

Table 8 depicted the ratios of steroid levels to median levels in the CSF and documented pronouncedly lower levels of the CSF steroids in comparison with the levels in circulation. The ratios of circulating steroids/CSF steroids showed values from 2.6 for DHEA7α to 77 for cortisol. The ratios of circulating steroid polar conjugates to unconjugated steroids in the CSF showed values from 175 for P3β5α to 70,000 for A3α5α.

The correlations between the steroids in the CSF and serum are demonstrated in Table 9. In general, C19-3β-hydroxy-5-ene steroids showed significant correlations between circulation and CSF, particularly the 7α/β-hydroxy-metabolites of DHEA and androstenediol (Fig. 1). Borderline but relatively strong correlations of pregnenolone and DHEA serum conjugates, being primarily of adrenal origin, with the free steroids in the CSF may reflect the differences in the activity of the adrenal cortex between the subjects.

After adjustment to constant serum PregC, the partial correlation between CSF Preg and serum Preg was insignificantly negative ($r = -0.390$, $p = 0.2$) but the corresponding correlation between CSF Preg and serum PregC was significantly positive ($r = 0.645$, $p = 0.05$, for constant serum Preg). These relationships indicated minor influence of circulating Preg on the levels of Preg in the CSF possibly due to the negligible concentration of the serum Preg when compared with the serum PregC and due to the independent synthesis of Preg from cholesterol in the CNS.

Concerning the possibility of Preg conversion to DHEA in the CNS, we found no significant correlation between Preg and DHEA in

Table 9
Pearson's correlations between circulating steroids and steroids in the cerebrospinal fluid.

Steroid	CSF vs. serum free steroids		CSF vs. serum conjugates		Serum free steroids vs. serum conjugates	
	r	*p*	*r*	*p*	*r*	*p*
Pregnenolone	0.225	0.459	0.588	0.057	0.742	0.009
Dehydroepiandrosterone	0.820	0.001	0.532	0.092	0.620	0.042
Progesterone	0.087	0.778	–	–	–	–
17-Hydroxy-progesterone	0.586	0.097	–	–	–	–
Androstenedione	0.558	0.047	–	–	–	–
Testosterone	0.320	0.287	–	–	–	–
Allopregnanolone	0.727	0.005	0.139	0.667	0.155	0.630
Isopregnanolone	0.091	0.768	0.177	0.582	0.429	0.164
Androsterone	0.505	0.078	0.168	0.621	0.406	0.215
Epiandrosterone	0.820	0.001	0.450	0.165	0.477	0.138
7α-Hydroxy-dehydroepiandrosterone	0.917	0.000	–	–	–	–
7β-Hydroxy-dehydroepiandrosterone	0.941	0.000	–	–	–	–
5-Androstene-3β,7α,17β-triol	0.867	0.000	–	–	–	–
5-Androstene-3β,7β,17β-triol	0.890	0.000	–	–	–	–
16α-Hydroxy-pregnenolone	0.843	0.000	–	–	–	–
16α-Hydroxy-dehydroepiandrosterone	0.735	0.004	-0.048	0.882	0.303	0.338
16α-Hydroxy-progesterone	0.486	0.092	–	–	–	–
Cortisol	0.889	0.000	–	–	–	–

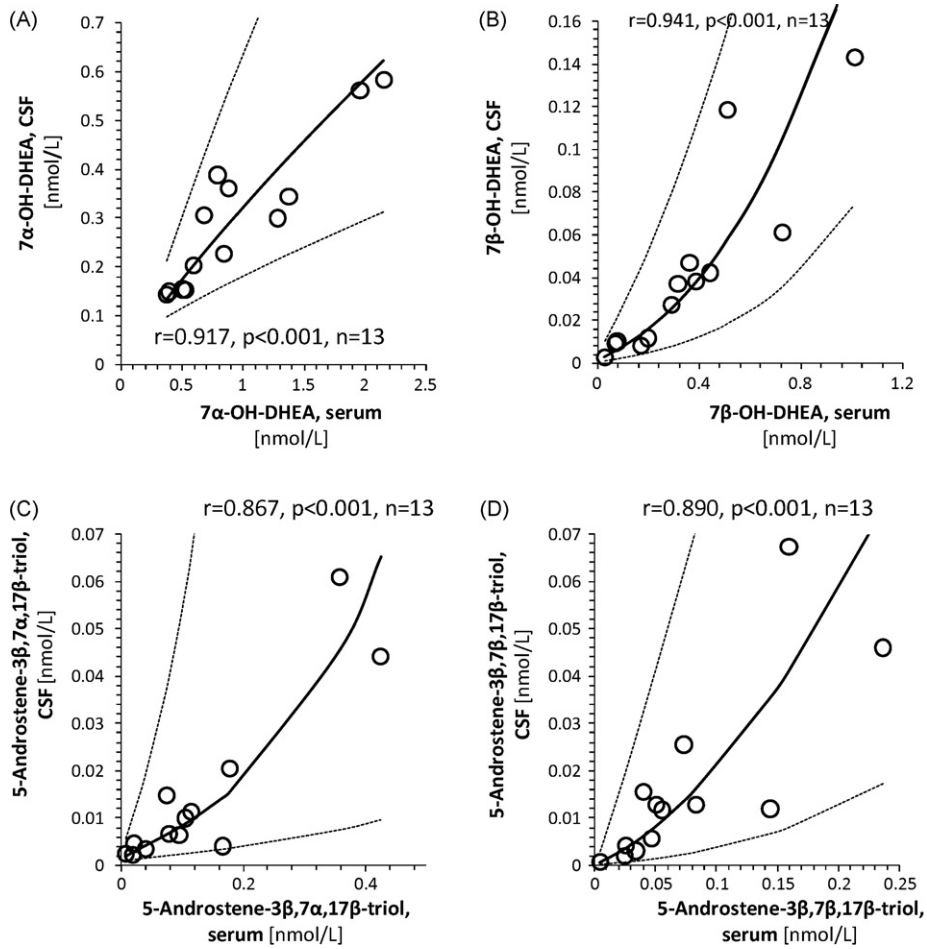

Fig. 1. Relationships between the concentrations of steroids in the cerebrospinal fluid (CSF) from the 3rd brain ventricle and serum levels. (A) 7α-hydroxy-dehydroepiandrosterone, (B) 7β-hydroxy-dehydroepiandrosterone, (C) 5-androstene-3β,7α,17β-triol and (D) 5-androstene-3β,7β,17β-triol. The bold full curve represents the principal axis after retransformation to the original scale, while the thin dashed line is the retransformed 95% confidence ellipsoid.

the CSF ($r = 0.383$, $p = 0.2$) but we observed significant ones between circulating DHEA and DHEA in the CSF ($r = 0.820$, $p = 0.001$). This data indicated the key role of peripheral C17-hydroxylase-C17, 20-lyase activity for the ratios between DHEA and Preg in the CSF. The results also outlined that DHEA, in contrast to Preg, is exclusively of peripheral origin, which is in accordance with the concept of negligible or even missing activity of C17-hydroxylase-C17, 20-lyase in the CNS.

In the CSF, we observed significant correlations between T and A2 (Fig. 2), and between serum and CSF A2 (Table 9) but insignificant correlation between serum and CSF testosterone (Table 9). These relationships might indicate that T levels in the CSF depend on the transport of A2 from the periphery to the CNS and on the conversion of A2 to T within the CNS and might be only partly dependent of serum T levels.

In the CSF, DHEA correlated with DHEA7α ($r = 0.791$, $p < 0.001$), DHEA7α correlated with DHEA7β ($r = 0.957$, $p < 0.001$) and AT7α correlated with AT7β ($r = 0.946$, $p < 0.001$) (Fig. 3). These relationships pointed to a brain *in situ* 7α-hydroxylation.

7α/β-Hydroxy-metabolites of DHEA and androstendiol showed tight correlations both between and within the body fluids. As expected, the respective correlations in the serum were also highly significant (Table 9, Fig. 1 and Fig. 3B and C). The correlations between DHEA7α and DHEA7β in the CSF (adjusted to constant DHEA7α and DHEA7β in the serum, $r = 0.718$, $p = 0.006$), as well as between AT7α and AT7β in the CSF ($r = 0.952$, $p < 0.001$, adjusted to constant AT7α and AT7β in the serum) indicated uncompli-

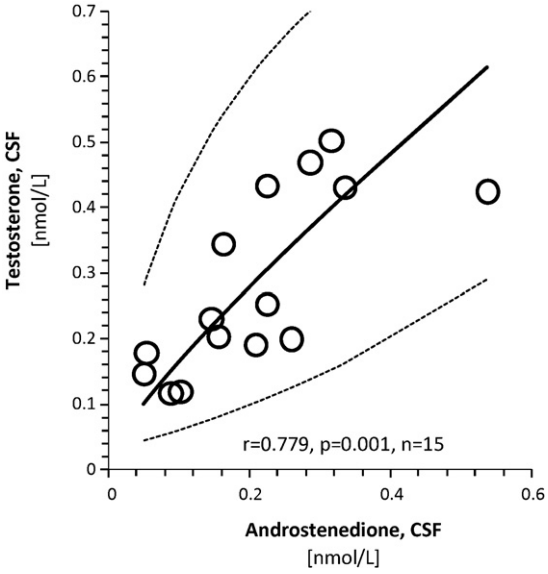

Fig. 2. Relationships between the concentrations of testosterone and androstenedione in the cerebrospinal fluid (CSF) from the 3rd brain ventricle. The drawings and symbols are the same as for Fig. 1.

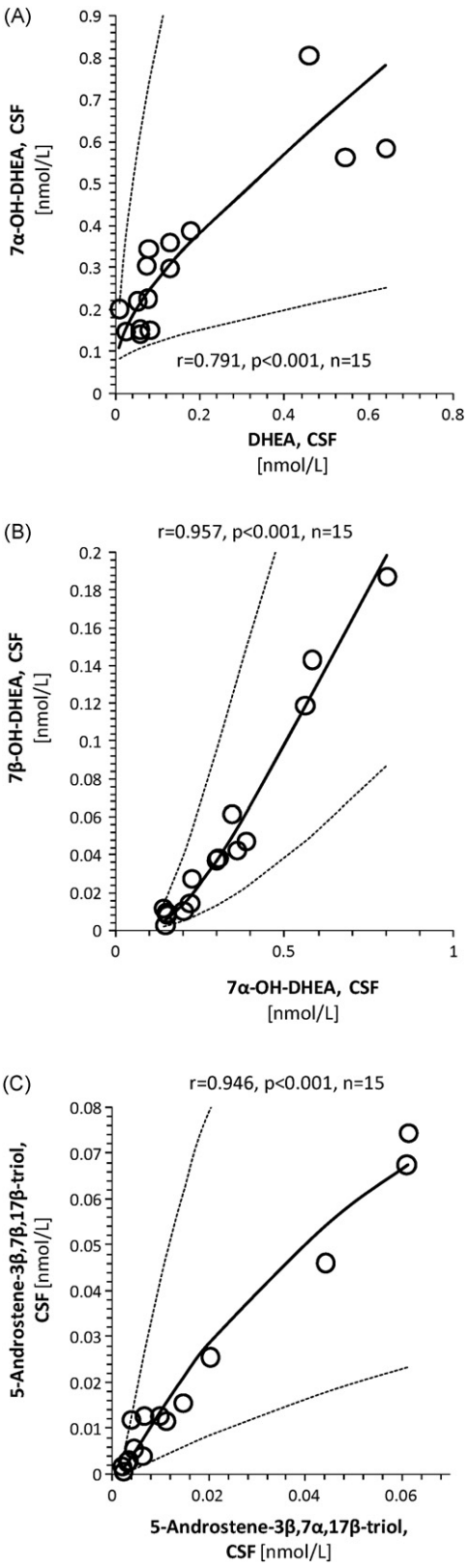

Fig. 3. Relationships between concentrations of steroids in the cerebrospinal fluid (CSF) from the 3rd brain ventricle. (A) DHEA and 7α-hydroxy-dehydroepiandrosterone, (B) 7α-hydroxy-DHEA and 7β-hydroxy-dehydroepiandrosterone, (C) 5-androstene-3β,7α,17β-triol and 5-androstene-3β,7β,17β-triol. The drawings and symbols are the same as for Fig. 1.

cated interconversion between 7α- and 7β-hydroxy-metabolites (via 7-oxo-intermediate) in the CNS independently of their transport from the periphery. The inter-conversion between 7α- and 7β-hydroxy-metabolites may be of an importance considering that this metabolic step is catalyzed by the same enzyme system, which provides interconversion between cortisol and cortisone [20,21]. The excess of cortisol in the CNS may exert an excitotoxic effect [22]. 7α/β-Hydroxy-metabolites of C19 steroids are possible competitors with the excitotoxic glucocorticoids for the active sites of 11β-hydroxysteroid dehydrogenase and the formers are known as immunomodulatory and anti-glucocorticoid substances [21,23,24].

The relatively close concentrations of the aforementioned steroids in the CSF and serum (Tables 6 and 8) as well as the tight correlations between CSF and serum 7-hydroxy-steroids (Table 9) demonstrated relatively uncomplicated transport of these substances between CSF and peripheral circulation.

In brief, the 7α-hydroxy-steroids may originate in the brain, may be further converted to 7β-hydroxy-metabolites but all of the already mentioned steroids may also penetrate from the periphery in the CNS.

While the proportion between *in situ* synthesized steroids and the steroids penetrating across the blood-brain-barrier from the periphery did not show pronounced differences for the 7-hydroxy-steroids, this was not the case for 16α-hydroxy-metabolites. Certain amount of Preg16α and DHEA16α in the CSF appeared to be of a peripheral origin and a part of these steroids may be also synthesized in the CNS from DHEA as documented by the significant correlations between Preg16α and Preg in the CSF ($r = 0.514$, $p = 0.05$), DHEA16α and DHEA in the CSF ($r = 0.614$, $p = 0.015$), and by significant correlations of Preg16α between CSF and serum ($r = 0.843$, $p < 0.001$) and of DHEA16α between CSF and serum ($r = 0.735$, $p = 0.004$).

Like the Preg16α and DHEA16α, Prog16α significantly correlated with the parent steroid in the CSF ($r = 0.602$, $p = 0.02$) but Prog16α in the CSF showed only borderline correlation with the serum Prog16α ($r = 0.486$, $p = 0.09$). The data indicated that Prog16α synthesis in the CSF may be more important than the transport of the steroid from the periphery.

The 3α- and 3β-hydroxy-5α-reduced metabolites of C21 and C19 steroids significantly correlated between the CSF and the serum (Table 9, Fig. 4) and showed significant inter-correlations within the CSF (Fig. 5). The above-mentioned relationships indicated mutual interconversion via 3-oxo intermediates in CNS and periphery as well as transport of the substances from periphery to the CNS. Concerning the importance of the steroid transport from the periphery for the levels in the CSF, the ratios of the CNS steroids to serum steroids are more favorable for the 5α-pregnanes in comparison with the 5α-androstanes (Tables 6 and 8).

In conclusion, we found pronouncedly lower levels of the CSF steroids in comparison with the levels in circulation. However, major differences were found between the individual steroids. The dependence of Preg in the CSF on the peripheral PregC is in accordance with the findings of Wang et al. [3], who extensively studied the transport of pregnenolone across the blood-brain-barrier (BBB). The authors applied peripherally supraphysiological concentrations of pregnenolone sulfate and followed the concentrations of pregnenolone and pregnenolone sulfate in various regions in the brain and periphery. Their study yielded clear evidence that pregnenolone sulfate injected *i.v.* can cross the BBB without being hydrolyzed to the more lipophilic Preg, and can thus be taken up by the brain. Our concept that in humans, not the peripheral Preg but the PregC is of importance for Preg concentrations in the CNS is further supported by a great excess of PregC levels over Preg levels in the human circulation (see Tables 6 and 8). To Wang et al. [3] the ratio of penetrability for free pregnenolone to that for pregnenolone sulfate did not exceed ten, however the ratio of PregC to Preg in our

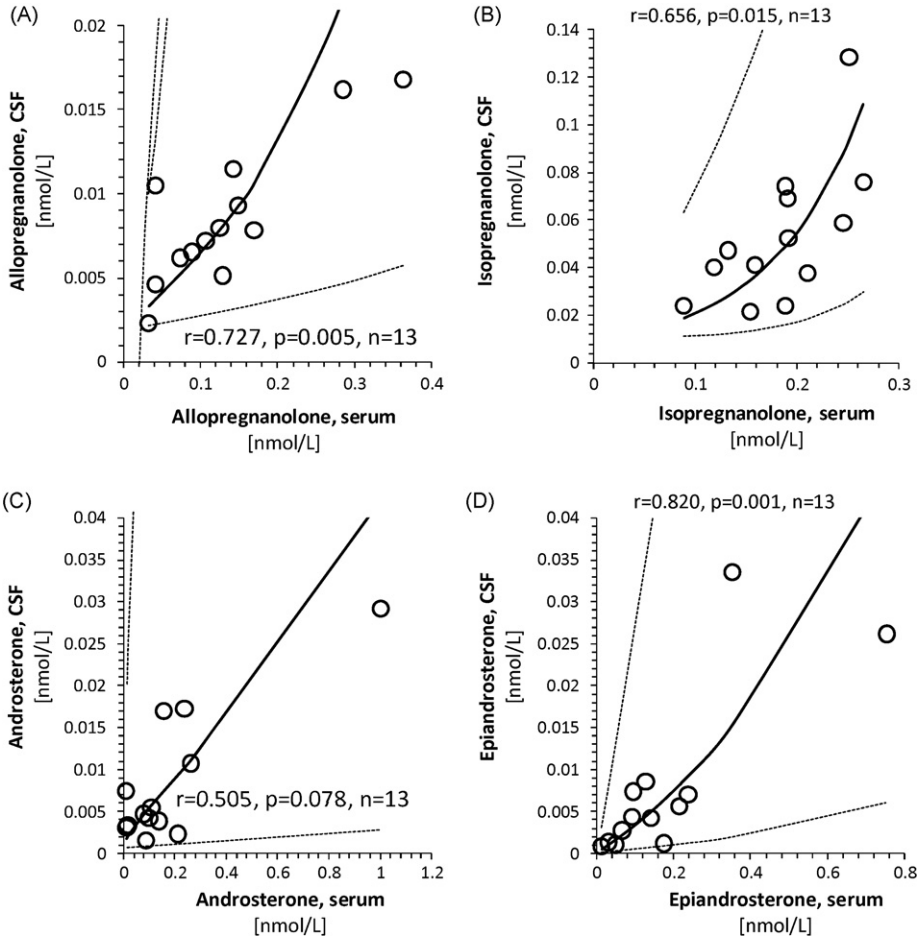

Fig. 4. Relationships between the concentrations steroids in the cerebrospinal fluid (CSF) from the 3rd brain ventricle and serum. (A) allopregnanolone, (B) isopregnanolone, (C) androsterone and (D) epiandrosterone. The drawings and symbols are the same as for Fig. 1.

serum samples was almost 100. In addition, the correlation analysis indicated minor influence of the circulating unconjugated Preg on the levels of Preg in the CSF probably also due to the important contribution of an independent synthesis of Preg from cholesterol, which was formerly demonstrated in the CNS [25].

The situation appears to be different for DHEA. The results of Asaba et al. [26] also indicated the possibility of transport of DHEAS, however from the CNS, rather than into the CNS. Nevertheless, the authors found only about ten times lower DHEAS influx compared to DHEAS efflux. This means that considering about 700 times higher serum DHEAC levels in comparison with those found for DHEA in our serum samples as well as almost 20,000-fold higher DHEAC concentrations when compared to CSF we may also expect significant influx of DHEAS following this huge concentration gradient. However, in contrast to the situation of Preg in the CSF most likely depending primarily on circulating PregC, our respective correlations indicated that DHEA in the CSF depends more on the serum unconjugated DHEA. In contrast to Preg our data showed

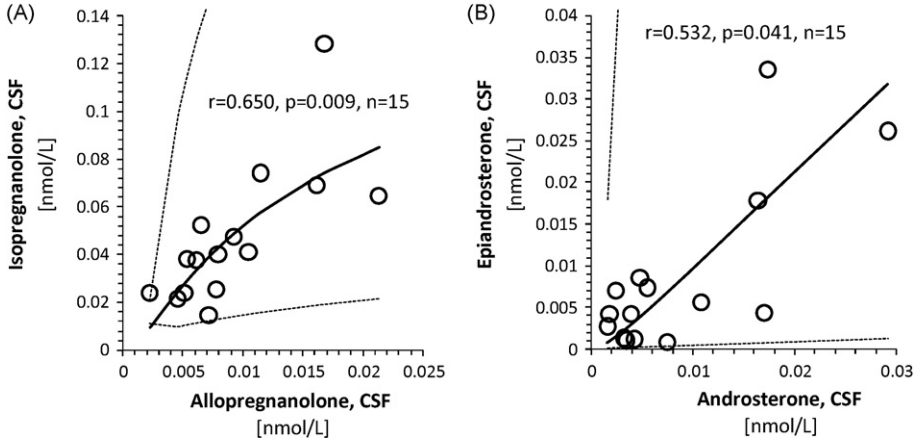

Fig. 5. Relationships between steroid concentrations in the cerebrospinal fluid (CSF) from the 3rd brain ventricle. (A) Allopregnanolone and isopregnanolone and (B) androsterone and epiandrosterone. The drawings and symbols are the same as for Fig. 1.

that DHEA in the CSF is exclusively of peripheral origin, which is in accordance with the concept of negligible or even missing activity of C17-hydroxylase-C17, 20-lyase in the CNS. Borderline but relatively strong correlations of pregnenolone and DHEA serum conjugates, being primarily of adrenal origin, with the free steroids in the CSF may reflect the differences in the activity of the adrenal cortex between the subjects.

In general, our data are compatible with the concept that a part of the steroids may be synthesized *de novo* in the CNS. However, substantial part of the steroid metabolites may be synthesized in the CNS from the steroid precursors or directly transported from the periphery. The CNS synthesis and transport from periphery might be complementary in some cases, i.e. brain synthesis might provide minimum level of steroids, which are indispensable for the CNS functions. However, brain steroids of peripheral origin may reflect various physiological situations like sex differences, menstrual cycle, pregnancy and parturition or pathologies like hormonal disorders, premenstrual syndrome and postpartum depression. These situations might be manifested not only by alterations in their levels by also by changes in the CNS functioning.

Acknowledgement

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