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Progesterone-transforming enzyme activity in the hypothalamus of the male rat

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Abstract

The aim of the present study was to assess the activities of the progesterone (Pr) transforming enzyme systems 3α-oxidoreductase (3α-OR), 5α-reductase (5α-R) and 20α-oxidoreductase (20α-OR) in the hypothalamus of the male rat, at different stages of sexual maturation and following castration and adrenalectomy. Special attention was paid to transformation to 3α-reduced compounds previously shown to inhibit FSH synthesis and secretion. Homogenates of hypothalamic tissue were incubated with ^{14}C-progesterone. Pr-metabolites were isolated, identified by gas chromatography/mass-spectrometry (GC/MS) and measured by liquid scintillation counting (LSC).

In adult rats a ratio of 6:2.5:1 for 5α-R:3α-OR:20α-OR enzyme- activities was found. The hypothalamic 5α-R and particularly 3α-OR activities were considerably higher before puberty (10–20 day old rats) than in adulthood. Adrenalectomy in adult rats resulted in an increased activity of the three enzyme systems. No significant changes were seen following castration.

Among the isolated metabolites, 3α-hydroxy-pregn-4-en-20-one (3α-Pr) and 3α-hydroxy-5α-pregnane-20-one (5α,3α-Pr) were identified. Conversion to both these neurosteroids was considerably higher during prepuberty than in adulthood. The finding that before puberty the hypothalamus has a markedly increased capacity to convert Pr to 3α-reduced compounds, such as 3α-Pr, known to effectively inhibit FSH release, warrants further research into the mechanisms regulating the hypothalamic formation of biologically active Pr derivatives and their role in the regulation of gonadotropin secretion.

Keywords: Hypothalamus; Progesterone; Enzyme-activity

1. Introduction

Investigations in the seventies indicated that mammalian brain tissue is able to transform testosterone (T) or Pr to 3α- and 5α-reduced metabolites [1–5], the conversion capacity being somewhat higher in males than in females. More recent studies [6–14] have confirmed these findings and shown that brain tissue, and more particularly the hypothalamus, contains different enzyme-systems: aromatase, 5α-reductase (5α-R), 3α-oxidoreductase (3α-OR) and 20α-oxido-reductase (20α-OR). The brain localisation, cellular and subcellular distribution, and biochemical characteristics of these enzymes have been established [8,15–19].

Although our knowledge of the physiological role of 5α- and 3α-reduction of Δ^4-3-keto-steroids in the hypothalamus is still incomplete, there are indications that some neurosteroids produced through the action of these enzymes are biologically active and might be involved in the regulation of such basic physiological functions as sleep, memory, response to stress and reproduction [20–25]. The fact that 5α- and/or 3α-reduced derivatives of T inhibit LH secretion more than T itself does indicate that brain 5α- and 3α-re-

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duction of steroids might be involved in the mechanisms that regulate gonadotropin secretion by the pituitary gland [4,20–21].

Studies by Wiebe et al. [26–32] have shown that some Pr metabolites, produced in the hypothalamus or pituitary gland may be implicated in the regulation of gonadotropin secretion. In both in vivo and in vitro experiments in the rat, low concentrations (1 nM) of 3α-hydroxy- pregn-4-en-20-one (3α-Pr) especially suppressed FSH synthesis and secretion. These inhibitory effects of 3α-Pr involved genomic and non- genomic mechanisms of action [23,29,30].

These observations suggest that the extent of 3α-Pr formation in brain tissue — and more particularly in hypothalamic tissue exhibiting specific 3α-Pr receptor binding [23,29] — might modulate, at least in part FSH secretion in the rat. The extent of the conversion of Pr to 3α-Pr in the hypothalamus depends mainly on both the concentration of substrate and the relative activity of Pr and 3α-Pr transforming enzyme systems in brain tissue.

It is well known that the secretion of gonadotropins changes with age and is affected by castration and adrenalectomy. We therefore investigated whether they might affect the activity of the Pr transforming enzymes (5α-R, 3α-OR, 20α-OR) in the hypothalamus of the male rat and thus result in changes in the formation of 5α- and/or 3α-reduced metabolites, and particularly of 3α-Pr.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade: dichloromethane (Carlo Erba, Milano, Italy); methylalcohol, petroleum ether (60°–80°C), ethanol, silicagel-60, sodium sulfate, primary and secondary sodium phosphate (Merck, Darmstadt, Germany); nicotinamide-adenine-dinucleotide phosphate (NADPH) (Boehringer-Pharma, Mannheim, Germany); methoxime-hydrochloride (Sigma, Missouri, USA); trimethylsilyl-imidazole (TSIM) and a 100-5 C18 AB column, 250 mm × 4.6 mm, were purchased from Macherey and Nagel, Duren, Germany.

The following steroids were obtained from Steraloids (Wilton, NH, USA): progesterone(Pr): pregn-4-ene-3,20-dione; 5α-pregnane-3,20-dione (5α-Pr); 3α-hydroxy-5α-pregnan-20-one (5α,3α-Pr); 20α-dihydroprogesterone (20α-Pr): 20α-hydroxy-pregn-4-en-3-one; 5α-pregnane-3α, 20α-diol (5α,3α, 20α-Pr); 6β-hydroxy-Pr:6β-hydroxy-pregn-4-ene-3,20-dione; 6α-hydroxy- Pr: 6α-hydroxy-pregn-4-ene-3,20-dione; testosterone (T): 17β-hydroxy-androst-4-en-3-one; epitestosterone: 17α-hydroxy-androst-4-en-3-one; estradiol-17β (E2): estra-1,3,5(10)-triene-3,17β-diol; estrone(E1):3-hydroxy-estra-1,3,5(10)-trien-17-one.

3α-Hydroxy-pregn-4-en-20-one (3α-Pr) was synthezised (see Acknowledgements) from Pr according to the method described by Wiebe et al. [32]. Identification was based on mass-spectrometry and on ^{1}H and ^{13}C NMR analysis.

4-^{14}C-Progesterone (SA = 56.4 mCi/mmol) was obtained from NEN Inc. (Boston, Mass, USA).

2.2. Methods

2.2.1. Incubation experiment

The activity of 5α-R, 3α-OR or 20α-OR was estimated from the sum of the metabolites produced — through their specific enzyme — from 4-^{14}C- progesterone during incubation in vitro of an homogenate of hypothalamic tissue. The experiments were performed on male Wistar rats. Rats of 10, 15, 21, 30, 60 or 90 days were used to study the effect of age. The effect of adrenalectomy and castration was studied in mature rats of 90 days which were castrated or adrenalectomized 28 days before the incubation experiment. The rats were sacrificed by decapitation, blood was collected, the brains were removed and the hypothalamus (mediobasal hypothalamus + preoptic area) was dissected out. Hypothalami from 5 to 6 rats were pooled, weighed (between 442 and 578 mg) and homogenized in 5 ml phosphate-buffer (0.1 M; pH = 7.2) using a Potter-Elvejhem homogenizer. The tissue homogenate was centrifuged at 800 g for 10 min and the supernatant was incubated for 4 h in a perfusion-flask with 12,600 ng 4-^{14}C-progesterone and in the presence of 1 μM NADPH. The flask was continuously shaken and perfused with a stream of oxygen. A 100 μl volume of the supernatant was taken for measuring the protein content according to Lowry [33].

Blood from the corresponding animals was pooled. Pr, T and estrogens (E2 + E1) were extracted from plasma with ether and separated by HPLC before estimation by radioimmunoassay (RIA).

2.2.2. Analytical method

Immediately after the incubation, the metabolites and unmetabolized Pr were extracted twice with a 20 times higher volume of dichloromethane, preliminary experiments having shown that the ^{14}C-labeled compounds are transferred quantitatively (i.e. more than 98%) into the organic phase. The extracts were pooled and evaporated to dryness under a stream of nitrogen. The residue was dissolved with 1.5% ethanol in dichloromethane (200 μl) and applied on a silicagel-column (ID: 2.7 cm; height: 32 cm; 70 g silicagel); the metabolites were eluted with 1.5% ethanol in dichloromethane.

Six fractions were collected: fraction A: (120th–

180th ml); fraction B: (181st–240th ml); fraction C: (241st–360th ml); fraction D: (361st–540th ml); fraction E: (541st–760th ml); fraction F: (761st–1150th ml). Each fraction was evaporated to dryness under a stream of nitrogen, dissolved in methanol/water (65:35) containing a known amount of epitestosterone (external standard for correction for experimental losses during HPLC-chromatography) and filtered through a Teflon-filter (FH 0.5 μm, Nihon Millipore Kogyo KK, Yonezwaw, Japan). Methanol/water eluates and washings from the filter-system were evaporated to dryness and concentrated at the bottom of a small tube. The residue was dissolved in 120 μl methanol/water (65:35) and 100 μl were injected into the Rheodyne 100 μl loop injector of the HPLC-instrument (SP8600, Thermo Separation Products, Riviera Beach, Florida, USA). Isocratic elution of the metabolites from the Nucleosil 100-5 C18 AB column was performed with methanol/water (65:35). The radioactivity of the eluate was followed (Berthold RadioMonitor LB 504 HS, Berthold, Wildbad, Germany). The eluates corresponding to the radioactive peaks were collected, evaporated to dryness and dissolved in 2 ml methanol.

Pr, T and estrogens (E2 + E1) in the ether extracts were separated by HPLC-chromatography using the same Nucleosil 100-5 C18 AB column. Isocratic elution of the steroids was performed with methanol/water (60:40), giving three fractions containing Pr, T and E2 + E1, respectively. The steroid content of each fraction was estimated using a RIA- kit (Biomerieux, Lyon, France) with a highly specific antiserum against either Pr, or T or E2 (cross-reactivity of E1 was 0.64), respectively.

2.2.3. Quantification of metabolites and enzyme activities

A known portion of each fraction was measured by LSC-spectrometry (Tri-carb-1500 spectrometer, Camberra-Packard, Meriden, CT, USA) using a solution of 300 mg POPOP + 5 g PPO/l toluene as scintillator. The amount of each metabolite was calculated from the radioactivity measured in the corresponding fraction and from the specific activity (56.4 mCi/mmol) of the incubated ^{14}C-progesterone measured in the same conditions, and expressed as ng/100 mg protein. The remaining portion was used for identification studies.

Enzyme activity was calculated from the sum of the metabolites produced through a specific enzyme and expressed as ng/100 mg protein:

- 3α-OR activity: sum of 3α-Pr, 5α,3α-Pr and 5α,3α,20α-Pr
- 5α-R activity: sum of 5α-Pr, 5α,3α-Pr and 5α,3α,20α-Pr
- 20α-OR activity: sum of 20α-Pr and 5α,3α,20α-Pr

The metabolites 6α-OHPr and 6β-OHPr could not be quantified since they were only isolated and identified at the end of the study.

2.2.4. Identification of metabolites

The identification of the steroid metabolites was based on:

1. The chromatographic pattern of the metabolites during chromatography on a silicagel-column, a HPLC-column and on paper (in the Bush system: petroleum ether/methanol/water (100:70:30); and
2. The gas chromatographic/mass-spectrometric (GC/MS) characteristics of the methoxime-trimethylsilyl-ether (MO-TMS) derivative of the metabolites.

GC/MS was performed with a gas-chromatograph/ION trap Saturn II mass spectrometer (Varian Ass., Walnut Creek, CA, USA). The MO-TMS derivatives were prepared according to the procedure described by Leunissen and Thyssen [34]. The MO-TMS derivative was dissolved in 50 μl of n-hexane.

The Varian STAR 3400 capillary gas chromatograph was fitted with a J&W Scientific (Folsom, CA, USA) 30 m × 0.25 mm I.D. fused-silica capillary column (DB-5MS, 0.25 μm film thickness) directly coupled to the ion trap detector. Injections were performed on a Varian 1077 split/splitless capillary injector equipped with a straight tube glass insert.

Helium was used as carrier gas at a linear flow of 30 cm/s. The GC program was as follows: the initial temperature of 150°C was maintained for 1 min, programmed up to 250°C at 20°C/min and then up to 300°C at 2.4°C/min and maintained for 2 min. The temperatures of the injector and transferline were 270°C and 285° C, respectively.

To determine the retention indices (RI), the steroids were injected as a mixture with alkanes (C_{24}–C_{30}) in a linear GC temperature program, starting from 150°C up to 290°C at 4°C/min.

The manifold temperature of the MS was kept at 220°C, the filament current was 40 μA.

2.3. Synthesis of 3α-hydroxy-pregn-4-en-3-one (3α-Pr)

3α-Hydroxy-progesterone was synthesized by selective reduction of progesterone as described by Wiebe et al. [32]. Purification of the reaction mixture by medium pressure liquid chromatography, thin layer chromatography and recrystallisation from hexane yielded a compound sufficiently pure to be analysed by ^{1}H and ^{13}C nuclear magnetic resonance spectrometry (NMR). The nuclear magnetic resonance spectra were obtained on a Jeol EX-270 FT-NMR spectrometer. The steroid

was dissolved in deuterated trichloromethane with tetramethylsilane as internal standard. ^{1}H NMR spectra were recorded at 270 Mhz; the ^{13}C NMR spectra at 67.5 Mhz.

Mass spectrometric analysis was performed after derivatisation of the steroid to its MO-TMS derivative according to the procedure described.

3. Results

3.1. Identification studies

3.1.1. Identification of synthesized 3α-hydroxy-pregn-4-en-3-one (3α-Pr)

The data from the NMR analyses were in perfect agreement with those described by Wiebe et al. [32] for 3α-Pr. Further evidence of the identity of the synthesised steroid was obtained by mass spectrometry of the MO-TMS derivative (see Fig. 1). The ions at m/z 142, 143 and 388 (M^+-29) are characteristic for steroids with a 3-OH-TMS-3-ene structure [35,36]. Confirmation of the stereochemical 3α-position of the 3-hydroxy function follows from the calculation of the ratios of intensities of the ions M^+-15, M^+-90 and M^+-105 [33,34].

The spectrum of the 3-hydroxy-pregn-4-en-3-one shows a ratio >1 for the fragment-ions M^+ to M^+-15 and <1 for the fragment-ions M^+-90 to M^+-105, which is in agreement with the data from Houghton et al. [37] and from de Boer et al. [38] for a 3α-position of the OH-group.

3.1.2. Identification of the isolated metabolites

Identification of the isolated ^{14}C-metabolites (Table 1) was based on:

1. A complete agreement of their chromatographic pattern with that of the authentic steroid during chromatography on the silicagel-column, the Nucleosil 100-5 C18 HPLC column and on paper in the system petroleum-ether/methanol/water (100:70:30).
2. A perfect agreement between the mass-spectrum of the MO-TMS derivative of the isolated ^{14}C-labeled metabolite with that of the authentic steroid (see Table 1), the fragment-ions of the ^{14}C-labeled metabolite being two atom-mass-units (amu) higher than those of the authentic unlabeled compound.

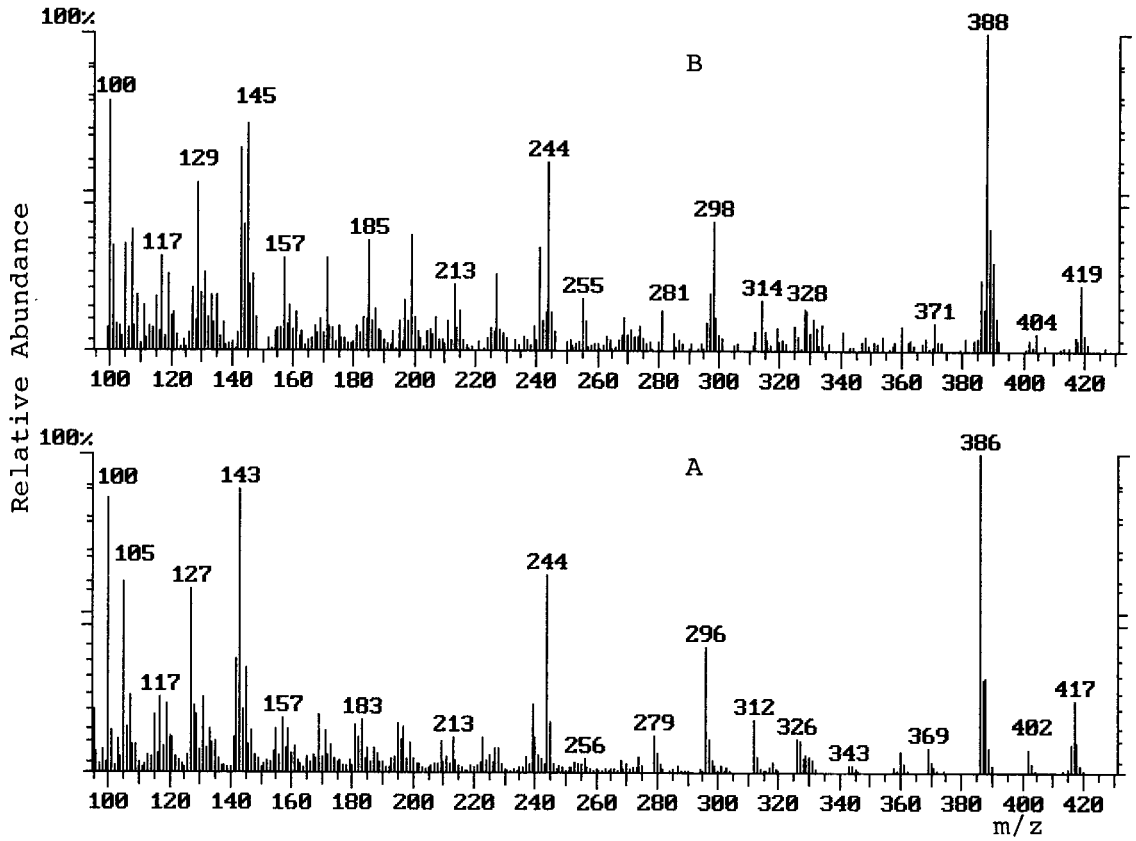

Fig. 1. Electron impact mass spectra of the MO-TMS derivatives of synthetised 3α-hydroxy-pregn-4-en-3-one (A) and of 3α-hydroxy-^{14}C-pregn-4-en-3-one isolated from incubated hypothalamic tissue (B).

Table 1
Mass spectrometric characteristics and retention indices of 4-^{14}C-labeled progesterone and isolated metabolites

Compound	RI[a]	Spectrometric characteristics[b]		
		M m/z	Base peak m/z	Fragment-ions m/z
S	2907 2913	374(27)	343	359(10), 311(11), 288(34), 275(16), 155(17)
A	2868 2881	376(< 1)	345	361(4), 290(45), 277(19), 244(6), 100(10)
B	2737	419(23)	388	404(6), 390(43), 298(46), 244(66), 145(80), 144(45), 129(59), 100(80)
C	2741	421(< 1)	390	406(5), 300(9), 283(8), 243(9), 173(10), 100(14)
D	2933 2938	419(48)	117	404(4), 388(8), 314(6), 303(24), 298(25), 288(37), 155(27), 127(20)
X1	2990 3019	462(2)	447	431(16), 415(82), 403(3), 374(6), 360(5), 316(5)
X2	2923 2937	462(2)	447	431(12), 415(82), 403(3), 374(4), 360(5), 316(5)

[a] Retention index corresponding to syn and anti isomers of the MO-TMS derivatives.
[b] Values in parentheses refer to the relative intensity in terms of % base peak for each amu of fragments; S: progesterone; A: 5α-pregnane-3,20-dione; B: 3α-hydroxy-pregn-4-en-20-one; C: 3α-hydroxy-5α-pregnan-20-one; D: 20α-hydroxy-pregn-4-en-3-one; X1: 6α-hydroxy-pregn-4-ene-3,20-dione; X2: 6β-hydroxy-pregn-4-ene-3,20-dione.

The amounts of the isolated 5α,3α,20α-Pr were too low to permit a valid mass-spectrometric identification. The identity was based on the similarity of the chromatographic properties of the ^{14}C-labeled metabolite with those of authentic 5α,3α,20α-Pr.

3.2. Effect of age, castration and adrenalectomy on hypothalamic progesterone metabolism

The extent of Pr metabolism by hypothalamic tissue was rather low. After 4 h incubation, an average of 88.2% (between 86.1% and 90.4%) of labeled substrate was recovered as unmetabolized 4-^{14}C-progesterone.

3.2.1. Effect of age

The metabolite levels produced by hypothalamic tissue as a function of age are summarized in Table 2.

Hypothalami from adult male rats (90 days) transformed Pr mainly to 5α-Pr and 5α, 3α-Pr; the conversion to 20α-Pr was low and to 3α-Pr even lower; only very small amounts of 5α,3α,20α-Pr were formed. A similar qualitative metabolisation pattern was observed in hypothalami from young (10–60 days) rats. In fact, at all ages, Pr was transformed mainly to 5α-Pr and 5α,3α-Pr; the conversion to 20α-Pr and 5α,3α,20α-Pr was low to very low. However, the relative conversion rate to 3α-Pr was higher for rats of 15–21 days than for adult rats.

The extent of the conversion to each of these metabolites (5α-Pr, 5α,3α-Pr and 3α-Pr) was considerably ($P < 0.01$) higher in young (10–30 days) than in adult rats and especially high in rats of 15–21 days. Maximal values were reached between the 15th and 21st day (3α-Pr and 5α,3α-Pr) or between the 21st and 30th day (5α-Pr). Thereafter, the levels decreased progressively to reach, after about 30 days, values comparable to those for adult (90 days) rats.

A summary of the enzyme activities as a function of age (Table 3) shows that: (1) the 5α-R/3α-R/20α-R ac-

Table 2
Metabolisation of ^{14}C-progesterone by hypothalami as a function of age[a]

age (days)	*n*	Metabolites produced (ng/100 mg protein)				
		5α-Pr	3α-Pr	5α,3α-Pr	20α-Pr	5α,3α,20α-Pr
10	3	1147** (942–1360)	176** (153–202)	1606** (1518–1684)	68** (47–89)	26 (19–32)
15	5	999* (623–1525)	590** (390–809)	3484** (1344–4294)	85 (58–112)	22 (18–31)
21	5	1603** (1225–1991)	446** (341–542)	3939** (3035–4770)	181 (124–216)	28 (19–41)
30	4	1788** (1099–2623)	101 (72–167)	896* (696–1128)	143 (112–178)	31 (14–43)
60	4	862* (738–1393)	55 (34–76)	431 (254–662)	152 (120–203)	34 (28–44)
90	7	704 (394–921)	87 (31–113)	489 (219–751)	178 (118–252)	42 (28–74)

[a] Abbreviations: Significant difference with respect to values for rats of 90 days; *: $P < 0.05$; **: $P < 0.01$ (Mann-Whitney test); *n*: number of incubations (5–6 hypothalami/incubation).

Table 3
Effect of age on hypothalamic enzyme activities[a]

age	n	activity (ng metabolites/100 mg protein)		
		5α-R	3α-OR	20α-OR
10	3	2780** (2648–2906)	1810** (1699–1908)	94** (76–111)
15	5	4505** (3811–5386)	4096** (3224–5079)	107** (72–130)
21	5	5571** (4279–6386)	4414** (3459–5133)	209 (146–248)
30	4	2715** (1927–3624)	1028* (816–1309)	175 (126–221)
60	4	1327 (1095–1926)	520 (340–766)	186 (148–247)
90	7	1235 (877–1488)	618 (327–916)	219 (166–304)

[a] Abbreviations: 5α-R: 5α-reductase; 3α-OR: 3α-oxidoreductase; 20α-OR: 20α-oxidoreductase; *n*: number of incubations; significant difference with respect to values for rats of 90 days: *: $P < 0.05$; **: $P < 0.01$ (Mann-Whitney test).

tivity ratio in mature rats was about 6:2.5:1, (2) the hypothalamic 20α-OR activity was significantly ($P < 0.01$) lower before puberty (first two weeks of life) than in adulthood, and (3) the hypothalamic 5α-R and 3α-OR activities in young rats of 10–21 days were clearly higher ($P < 0.01$) than in adult rats: the 3α-OR activity was enhanced by a factor 6, the 5α-R activity by a factor 4.

3.2.2. Effects of castration and adrenalectomy

No qualitative difference in Pr metabolisation pattern was seen after castration or adrenalectomy (Table 4). However, an important increase ($P < 0.01$) in conversion to all metabolites except 5α,3α,20α-Pr was noted after adrenalectomy (Table 5) while after castration only the conversion to 3α-Pr was somewhat increased ($P < 0.05$). After castration, the hypothalamic enzyme activities did not differ significantly from those of the control animals (Table 5). In adrenalectomized rats, however, the activity of the three enzymes 5α-R, 3α-OR and 20α-OR was significantly ($P < 0.01$) increased.

3.3. Progesterone, testosterone and estrogen plasma levels

Plasma steroid levels are summarized in Table 6. Plasma from male rats younger than 21 days or older than 40 days contained comparable amounts of Pr. Significantly higher ($P < 0.01$) Pr levels were recorded between days 21 to 30. Plasma Pr concentration in adult male rats was not affected by castration, but, as expected, was considerably ($P < 0.01$) decreased after adrenalectomy.

Before 30 days of age, the plasma contained low amounts of T. Thereafter, T levels increased to reach, after about 60 days, values comparable to those in adult (90 days) rats. Castration of adult rats resulted in the expected fall in plasma T while, after adrenalectomy, only a small but significant ($P < 0.05$) increase in plasma T was observed.

The reported estrogen levels concern the sum of E1 and E2, both being eluted in the same fraction during HPLC and estimated by RIA (see Section 2). Additional experiments, in which E1 and E2 were separated before estimation by RIA, showed that plasma from young rats (age <30 days) contained about 5–6 times more E1 than E2 (data not shown). A consider-

Table 4
Metabolisation of ^{14}C-progesterone by hypothalami from castrated or adrenalectomized rats[a]

Identity	Metabolites produced (ng/100 mg protein)				
	5α-Pr	3α-Pr	5α,3α-Pr	20α-Pr	5α,3α,20α-Pr
Control rats ($n = 7$)	704 (394–921)	87 (31–113)	489 (219–751)	178 (118–252)	42 (28–74)
Castrated rats ($n = 8$)	759 (362–950)	124* (89–155)	552 (434–762)	199 (164–222)	53 (38–77)
Adrenalectomized rats ($n = 8$)	1371** (856–2318)	143* (81–306)	698** (428–1024)	404** (289–518)	55 (37–82)

[a] Abbreviations: Significant difference with respect to control values; *: $P < 0.05$; **: $P < 0.01$ (Mann-Whitney test); *n*: number of incubations.

Table 5
Effect of castration and adrenalectomy on hypothalamic enzyme activities[a]

Identity	Activity (ng metabolites/100 mg protein)		
	5α-R	3α-OR	20α-OR
Control rats ($n = 7$)	1235 (877–1613)	618 (327–916)	219 (166–304)
Castrated rats ($n = 8$)	1364 (995–1577)	729 (575–847)	261 (223–304)
Adrenalectomized rats ($n = 8$)	2124** (1322–3403)	896** (617–1391)	459** (326–579)

[a] Abbreviations: 5α-R: 5α-reductase; 3α-OR: 3α-oxido-reductase; 20α-OR: 20α-oxidoreductase; *n*: number of incubations; significant difference with respect to control values; *: $P < 0.05$; **: $P < 0.01$.

Table 6
Plasma steroid levels in male rats[a]

Age (days)	n	Plasma level (°) of		
		progesterone (pg/ml)	testosterone (pg/ml)	estrogens (#)
10	2	1424 (1208–1641)	538** (420–647)	334** (311–356)
15	5	1730 (1164–2118)	599** (495–788)	396** (343–448)
21	5	1741 (1390–2332)	413** (336–512)	233** (187–267)
30	4	3170** (2814–3856)	447** (354–539)	57** (48–76)
40	2	3687** (3132–4112)	1442* (1261–1622)	30** (26–33)
60	4	1753 (1293–2512)	1944 (1476–2142)	10 (8–14)
90	7	1633 (1304–1994)	2191 (1863–2744)	11 (8–16)
90 Ca	8	1265 (896–1430)	145** (124–183)	12 (9–19)
90 Ad	8	224** (161–274)	2694* (2252–3422)	13 (11–22)

[a] Abbreviations: (°): Plasma steroid levels are given as mean values with ranges (in parentheses); *n*: number of analysed plasma-pools (each pool from 5–6 rats); (#): Mainly estrone (see text); Ca: rats castrated three weeks before incubation; Ad: rats adrenalectomized four weeks before incubation. Significant difference with respect to values for rats of 90 days; *: $P < 0.05$; **: $P < 0.01$ (Mann-Whitney test).

ably higher ($P < 0.01$) estrogen level was found in plasma from young (less than 21 days old) adult male rats. This level was about ten times higher than in mature female rats (42 pg/ml; $n = 6$). From 21 days onwards, it decreased gradually to reach the normal adult range by 60 days. Plasma from castrated or adrenalectomized adult male rats contained very low amounts of estrogens, which were statistically not different ($P > 0.05$) from those in intact adult males.

4. Discussion

Data from the literature suggest that 5α-reduction and 3α-oxidoreduction of T and Pr in the hypothalamus and pituitary, with local production of 5α- and 3α-reduced metabolites, are implicated in the modulation and/or expression of the feedback regulation of gonadotropin secretion by these steroids (4,24–26,31). The results of the present study (1) confirm that transformation of Pr in the hypothalamus of the male rat results in the formation of 3α-Pr, a metabolite with inhibitory effects on FSH synthesis and secretion (23,25–26); and (2) show that in prepuberty, characterized by a low gonadotropic activity, the pattern of enzymatic activity favours the formation of 3α-reduced compounds.

The intensity of a steroid-mediated biological effect generally depends on the extent of steroid-receptor binding, i.e. on the concentration of both steroid and receptor. A possible regulatory action on gonadotropin secretion by 5α- or 3α-reduced derivatives of Pr, i.e. 3α-Pr and 5α,3α-Pr, including either genomic or non-genomic mechanisms of action, can thus be expected to be related to the concentration of these steroids achieved locally in the target tissues. The concentration of such Pr derivatives in the target tissues (i.e. hypothalamus and pituitary) may be influenced by a direct external supply via the blood, but is also determined by the in situ transformation of Pr. The local production of 3α-Pr or 5α,3α-Pr will depend (1) on the availability of Pr substrate, both through blood supply and in situ synthesis [14,39]; and (2) on the activity of the enzymes (5α-R, 3α-OR, 20α-OR) that transform Pr and intermediate substrates in brain tissue.

In the present study the activities of the three enzymes in the hypothalamus of the male rat were estimated simultaneously, using Pr as substrate, in order to assess hypothalamic capacity to convert Pr into 3α-Pr and/or 5α,3α-Pr at the moment of sacrificing the animal. Enzyme activities were estimated on the basis of an in vitro experiment in which the availability of substrate was not limited, so that the enzymes could function at a maximal rate, more than 86% of the Pr substrate remaining unchanged at the end of the incubation. Preliminary investigations had moreover shown that higher substrate concentrations did not result in a substantial increase in the production of metabolites. However, it is impossible to say to what extent the estimated relative enzyme activities reflect the real in vivo situation.

In adult male rats the hypothalamus displayed a high 5α-R activity, a lower but substantial 3α-OR activity and a low 20α-OR activity (ratio: 6:2.5:1). Both 5α-R and 3α-OR activities were considerably higher before puberty (10–20 days old rats), confirming published data [1,5,7]. Moreover, before puberty 3α-OR activity increased more than 5α-R activity, favouring the formation of 3α-reduced rather than 5α- reduced derivatives. If this also applies in vivo, then more 5α,3α-Pr and particularly 3α-Pr may be formed in the hypothalamus of prepubertal than of adult rats, on condition that the Pr substrate concentration is not a limiting factor. The hypothalamus synthesizes Pr [39,14]. In male rats, Pr concentrations in the hypothalamus are even higher than in plasma [14]. We have confirmed previous observations [40] that the plasma of young rats, at an age marked by a high Pr transforming capacity in the hypothalamus, contains substantial amounts of Pr. Between 10 and 20 days of age, plasma Pr levels were as high as in adult rats and between 30 and 40 days of age they were significantly ($P < 0.05$) higher.

The higher prepubertal 5α-R and 3α-OR activities are not only observed in the hypothalamus of the male rat, but also in testicular tissue. Both testicular enzyme activities are high up to the age of about 21 days; then decrease with age. On the other hand, 7α-hydroxylase activity, which is low before puberty, increases [41].

Whether the high hypothalamic 5α-R and 3α-OR ac-

tivities during prepuberty, might be related to high plasma estrone levels during this period is a moot point. The observation that treatment of ovariectomized rats with estradiol (1 μg/day for 5 days) can enhance the hypothalamic conversion of Pr to 5α-Pr [3] indicates that estrogens might stimulate 5α-R ans 3α-OR activities, but no causal link can be made.

The enhanced capacity of the prepubertal hypothalamus of the male rat to produce 3α-reduced Pr metabolites, including 3α-Pr, coincides with a period of low FSH secretion; FSH levels increase from about the 40th day on [43–46]. However, our experiments cannot establish a causal relationship between changes in hypothalamic Pr metabolism and FSH secretion.

The hypothalamic activities of 5α-R, 3α-OR and 20α-OR were unchanged after castration but enhanced after adrenalectomy. This suggests that adrenocorticosteroids may directly or indirectly influence the activity of these enzymes. Unlike us, Cheng et al. [16] found no effect of adrenalectomy on 3α-OR activity of brain tissue of the rat. This discrepancy may be due to a different time-interval between adrenalectomy and measurement of enzyme activity or to their estimation of 3α-OR by the conversion of 5α-dihydrotestosterone to androsterone. Recently, 11β-oxidoreductase activity in the pituitary of the male rat was found to increase after adrenalectomy, with a return to normal levels after replacement treatment of the adrenalectomized rats with corticosterone or dexamethasone [42].

In summary, the results of this study confirm the findings of Wiebe [23], showing that the reversible 3α-oxidoreduction of Pr resulting in the formation of 3α-Pr, occurs in the hypothalamus as in many other tissues. They also show (1) that the capacity of the hypothalamus to transform Pr into 5α- and/or 3α-reduced derivatives is higher during prepuberty than in adulthood; and (2) that the enzymatic pattern of the hypothalamus at prepuberty is modulated in favour of the formation of 3α-reduced compounds, including 3α-Pr. These findings warrant further research into the mechanisms regulating the hypothalamic production of biologically active 3α-reduced Pr derivatives and their possible role in the regulation of gonadotropin secretion.

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References

[1] C. Denef, C. Magnus, B.S. McEwen, Sex differences and hormonal control of testosterone metabolism in rat pituitary and brain, J. Endocrin. 59 (1973) 606–621.
[2] H.J. Karavolas, S.M. Herf, Conversion of progesterone by rat medial basal hypothalamic tissue to 5α-pregnane-3,20-dione, Endocrinology 89 (1971) 940–942.
[3] Y.J. Cheng, H.J. Karavolas, Conversion of progesterone to 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnan-20-one by rat medial basal hypothalami and the effects of estradiol and stage of estrous cycle on the conversion, Endocrinology 93 (1973) 1157–1162.
[4] L. Martini, F. Celotti, M. Serio, 5α-Reductase deficiency in humans: support to the theory that 5α-reduction of testosterone is an essential step in the control of LH secretion, J. Endocrinol. Invest. 2 (1979) 463–464.
[5] L. Martini, The 5α-reduction of testosterone in the neuroendocrine structures, Endocrine Rev. 3 (1982) 1–25.
[6] C. Corpechot, J. Young, M. Calvel, C. Wehrey, J.N. Veltz, G. Touyer, J. Mouren, V.V. Prasad, C. Banner, J. Sjoval, E.E. Baulieu, P. Robel, Neurosteroids: 3α-hydroxy-5α-pregnan-20-one and its precursors in the brain, plasma and steroidogenic glands of male and female rats, Endocrinology 133 (1993) 1003–1009.
[7] R.C. Melcangi, F. Celotti, M. Ballabio, P. Castano, A. Poletti, S. Milani, S. Martini, Ontogenetic development of the 5α-reductase in the rat brain: cerebral cortex, hypothalamus, purified myelin and isolated oligodendrocyte, Dev. Brain Res. 44 (1988) 181–188.
[8] R.C. Melcangi, F. Celotti, M. Ballabio, P. Castano, L. Martini, Testosterone 5α-reductase activity in the rat brain is highly concentrated in white matter structures and in purified myelin sheats of axons, Steroid Biochem. 31 (1988) 173–180.
[9] R.C. Melcangi, F. Celotti, M. Ballabio, P. Castano, R. Massarelli, A. Poletti, L. Martini, 5α-reductase activity in isolated and cultured neuronal and glial cells of the rat, Brain Res. 516 (1990) 229–236.
[10] R.C. Melcangi, F. Celotti, M. Ballabio, A. Poletti, L. Martini, Testosterone metabolism in peripheral nerves: presence of the 5α-reductase 3α-hydroxysteroid-dehydrogenase enzymatic system in the sciatic nerve of adult and aged rats, J. Steroid Biochem. 35 (1990) 145–148.
[11] F. Celotti, R.C. Melcangi, P. Negri-Cesi, A. Poletti, Testosterone metabolism in brain cells and membranes, J. Steroid Biochem. Mol. Biol. 40 (1991) 675–678.
[12] L. Martini, R.C. Melcangi, R. Maggi, Androgen and progesterone metabolism in the central and the peripheral nervous system, J. Steroid Biochem. Mol. Biol. 47 (1993) 195–205.
[13] M.P. Carey, C.A. Aniszewski, J.P. Fry, Metabolism of progesterone in mouse brain, J. Steroid Biochem. Mol. Biol. 50 (1994) 213–217.
[14] P. Robel, J. Young, C. Corpechot, W. Mayo, F. Perche, M. Haug, H. Simon, E.E. Beaulieu, Biosynthesis and assay of neurosteroids in rats and mice functional correlates, J. Steroid Biochem. Mol. Biol. 53 (1995) 355–360.
[15] F. Celotti, R.C. Melcangi, P. Negri-Cesi, M. Ballabio, L. Martini, Differential distribution of the 5alpha-reductase in the central nervous system of the rat and the mouse: are the white matter structures of the brain target tissues for testosterone action?, J. Steroid Biochem. 26 (1987) 125–129.
[16] K.C. Cheng, J. Lee, M. Khanna, K.N. Qin, Distribution and ontogeny of 3-alpha-hydroxysteroid dehydrogenase in the rat brain, J. Steroid Biochem. Mol. Biol. 50 (1994) 85–89.
[17] X. Li, P.J. Bertics, H.J. Karavolas, Regional distribution of cytosolic and particulate 5α-dihydroprogesterone 3α-hydroxy-

steroid oxidoreductases in female rat brain, J. Steroid Biochem. Mol. Biol. 60 (1997) 311–318.
[18] F. Celotti, P. Negri-Cesi, A. Poletti, Steroid metabolism in the mammalian brain: 4alpha-reduction and aromatization, Brain Res. Bull. 44 (1997) 365–375.
[19] L. Martini, F. Celotti, R.C. Melcangi, Testosterone and progesterone metabolism in the central nervous system: cellular localization and mechanism of control of enzymes involved, Cell. Mol. Neuroendocrinology 16 (1996) 271–282.
[20] E.E. Baulieu, Neurosteroids: of the nervous system, by the nervous system, for the nervous system, Recent Prog. Horm. Res. 52 (1997) 1–32.
[21] S.H. Mellon, Neurosteroids: biochemistry, mode of action and clinical relevance, J. Clin. Endocrinol. Metab. 78 (1994) 1003–1008.
[22] F. Celotti, R.C. Melcangi, L. Martini, The 5α-reductase in the brain: molecular aspects and relation to brain function, Front. Neuroendocrinol. Metabol. 13 (1992) 163–215.
[23] J.P. Wiebe, Nongenomic actions of steroids on gonadotropin release, Recent Progr. Horm. Res. 52 (1997) 71–101.
[24] M. Zanisi, M. Motta, L. Martini, Inhibitory effect of the 5α-reduced metabolites of testosterone on gonadotrophin secretion, J. Steroid Biochem. 56 (1973) 315–316.
[25] S. Zoppi, M. Lechuga, M. Motta, Selective inhibition of the 5α-reductase of the rat epididymis, J. Steroid Biochem. 31 (1992) 509–514.
[26] J.P. Wiebe, P.H. Wood, Selective suppression of follicle-stimulating hormone by 3α-hydroxy-4-pregnen-20-one, a steroid found in Sertoli cells, Endocrinology 120 (1987) 2259–2264.
[27] P.H. Wood, J.P. Wiebe, Selective suppression of follicle-stimulating hormone secretion in anterior pituitary cells by the gonadal steroid 3α-hydroxy-4-pregnen-20-one, Endocrinoloy 125 (1989) 41–48.
[28] J.P. Wiebe, D. Boushy, K.J. Barr, FSH regulating steroid produced by hypothalamus and pituitary, Biol. Reprod. 48 (1993) 30 (Suppl).
[29] S. Dhavantari, J.P. Wiebe, Suppression of follicle stimulating hormone by the gonadal- and neurosteroid 3α-hydroxy-4-pregnen-20-one involves actions at the level of the gonadotrope membrane/calcium channel, Endocrinology 134 (1994) 371–376.
[30] J.P. Wiebe, S. Dhavantari, P.H. Watson, Y.U. Huang, Suppression in gonadotropes of gonadotropin-releasing hormone-stimulated follicle-stimulating hormone release by the gonadal- and neurosteroid 3α-hydroxy-4-pregnen-20-one involves cytosolic calcium, Endocrinology 134 (1994) 377–382.
[31] J.P. Wiebe, P.H. Wood, M. Temple, S. Dhavantari, Action of 3α-hydroxy-4-pregnen-20-one on the hypothalamo-pituitary level, J. Steroid Biochem. 36 (1990) 269 (Suppl).
[32] J.P. Wiebe, C. Deline, K.D. Buchingham, V. Dave, J.B. Stothers, Synthesis of the allylic gonadal steroids, 3α-hydroxy-4-pregnen-20-one and 3α-hydroxy-4-androsten-17-one and of 3α-hydroxy-5α-pregnan-20-one, Steroids 45 (1985) 39–51.
[33] O.H. Lowry, N.J. Rosebrough, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 151 (1951) 265–275.
[34] W.J.J. Leunissen, J.H.H. Thyssen, Quantitative analysis of steroid profiles from urine by capillary gas chromatography: I. accuracy and reproductibility of the sample preparation, J. of Chromat. 146 (1978) 365–380.
[35] D.J. Harvey, P. Vouros, Influence of the 6-trimethylsilyl group on the fragmentation of trimethylsilyl derivatives of some 6-hydroxy- and 3,6-dihydroxy-steroids and related compounds, Biomed. Mass. Spectrom. 6 (1979) 135–143.
[36] C.H.L. Shackleton, Mass Spectrometry: application to steroid and peptide research, Endocrin. Rev. 6 (1985) 441–486.
[37] E. Houghton, A. Ginn, P. Teale, C. Minoo, J. Copsey, Comparison of the use of mass spectrometry and methylene unit values in the determination of the stereochemistry of estranediol, the major urinary metabolite of 19-nortestosterone in the horse, J. Chromatogr. 479 (1989) 73–83.
[38] D. de Boer, E.G. de Jong, R.A.A. Maes, J.M. van Rossum, The methyl-5a-dihydrotestosterones mesterolone and drostanolone; gas chromatographic mass spectrometric characterisation of urinary metabolites, J. Steroid Biochem. Molec. Biol. 42 (1992) 411–419.
[39] I. Jung-Testas, Z.Y. Hu, E.E. Beaulieu, P. Robel, Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of glial cells, Endocrinology 125 (1989) 2083–2091.
[40] K.D. Dohler, W. Wuttke, Serum LH, FSH, prolactin and progesterone from birth to puberty in female and male rats, Endocrinology 94 (1974) 1003–1008.
[41] E. Lacroix, W. Eechaute, I. Leusen, Influence of age on the formation of 5α-androstanediol and 7α-hydroxytestosterone by incubated rat testes, Steroids 25 (1975) 649–661.
[42] R.B. Idrus, N.B. Mohamad, P.B. Morat, A. Saim, Kadir K.B. Abdul, Differential effect of adrenocorticosteroids on 11β-hydroxysteroid-dehydrogenase bioactivity at the anterior pituitary and hypothalamus in rats, Steroids 61 (1996) 448–452.
[43] A.C. Dalkin, G.A. Bourne, D.R. Pieper, S. Regiana, J.C. Marshall, Pituitary and gonadal gonadotropin-releasing hormone receptors during sexual maturation in the rat, Endocrinology 108 (1981) 1658–1664.
[44] V. Chan, R.N. Clayton, G. Knox, K.J. Catt, Ontogeny of pituitary GnRH receptors in the rat, Endocrinology 65 (1981) 2086–2092.
[45] A. Negro-Vilar, L. Krulich, S.M. McCann, Changes in serum prolactin and gonadotropins during sexual development of the male rat, Endocrinology 93 (1978) 660–664.
[46] J.M. Ketelslegers, W.D. Hetzel, R.J. Sherins, K.J. Catt, Developmental changes in testicular gonadotropin receptors: plasma gonadotropins and testosterone in the rat, Endocrinology 103 (1978) 212–222.