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PROGESTERONE METABOLISM IN THE PINEAL, BRAIN STEM, THALAMUS AND CORPUS CALLOSUM OF THE FEMALE RAT

ISRAEL HANUKOGLU, HARRY J. KARAVOLAS* and ROBERT W. GOY

Department of Physiological Chemistry, The Waisman Center for Mental Retardation, and The Regional Primate Research Center, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

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SUMMARY

Specific brain regions, namely, thalamus, tectum, tegmentum, cerebellum, medulla and pineal, from five proestrous rats were incubated for 30 min with [^{3}H]-progesterone. After reverse isotopic dilution analysis, the following metabolites were identified in all incubations by purification to constant specific activity, derivative formation and/or gas liquid chromatography trapping: [^{3}H]5α-pregnane-3, 20-dione (10–20% of the starting substrate except pineal — 0.7%), [^{3}H]3α-hydroxy-5α-pregnan-20-one (1.6–3.8% except for pineal — 0.5%) and [^{3}H]20α-hydroxy-4-pregnen-3-one (0.05–0.11%). Preliminary results from the corpus collosum incubation indicated the presence of the same metabolites. Although some apparent constant specific activities were obtained for 20α-hydroxy-5α-pregnan-3-one and 5β-pregnane-3, 20-dione, the low levels of ^{3}H associated with these steroids did not permit a definitive identification. The results indicate the presence of at least Δ^4-steroid 5α-reductase, 3α-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase activities with progesterone as substrate in the brain regions examined.

INTRODUCTION

The hypothalamus and anterior pituitary are well established loci of action for some neuroendocrine effects of progesterone[1,**]. Recently other discrete regions of the central nervous system, e.g., thalamus, mesencephalon, cerebellum, medulla

* Please address reprint requests to Dr. H. J. Karavolas, 707A Waisman Center, 2605 Marsh Lane, Madison, Wisc. 53706, U.S.A.

** Trivial steroid names and abbreviations used are: progesterone, 4-pregnene-3, 20-dione; 5α-dihydroprogesterone (5α-DHP), 5α-pregnane-3, 20-dione; 5β-dihydroprogesterone (5β-DHP), 5β-pregnane-3, 20-dione; testosterone, 17β-hydroxy-4-androsten-3-one.

and pineal, also have been suggested as target sites for progesterone on the basis of several methodological approaches: (a) observations of central nervous system (CNS) related variables (lordosis, luteinizing hormone-releasing factor (LHRF) stores) in the presence of progesterone implants in the brain[25,30]; (b) observations of variables (electrical activity, HIOMT, serotonin metabolism) within specific regions of the CNS, following progesterone treatment either in vitro or in vivo[9,13,20]; (c) correlations of variables (MAO, HIOMT) from specific regions of the CNS with different reproductive stages of females that are modulated by progesterone[12,44]; and (d) analyses of regional radioactivity uptake after [^{3}H]progesterone treatment[9,14,22,38]. Furthermore, progesterone appears to affect certain CNS related general parameters, e.g., various behavior patterns[10,18,19,42], CNS differentiation[31], I.Q.[5], EEG[9,18], brain excitability[18,43].

A knowledge of the metabolism of progesterone in these brain regions may be necessary for elucidating the mechanism(s) of action of progesterone. Previous in vitro studies have shown that rat hypothalamus and anterior pituitary can metabolize progesterone to 5α-dihydroprogesterone (5α-DHP), 3α-hydroxy-5α-pregnan-20-one and to trace amounts of 20α-hydroxy-4-pregnen-3-one (for review, see ref. 15). Further, in recent in vivo uptake studies, accumulation of [^{3}H]5α-DHP in these neuroendocrine centers and cerebral cortex was demonstrated with either [^{3}H]-progesterone or [^{3}H]5α-DHP[14]. Additionally, 5α-DHP was shown to have some progesterone-like effects on neuroendocrine processes such as gonadotropin regulation[15] and lordosis behavior[4,23,40]. These observations support a hypothesis that metabolism of progesterone in these neural centers may be a requisite step for some or all of its actions.

The present in vitro studies were undertaken to determine and compare the pattern of progesterone metabolism in other regions of the female rat CNS that may be additional target sites for progesterone. This information may be helpful for understanding the molecular mechanism(s) underlying the diverse effects of progesterone on the CNS.

METHODS

Materials

[1,2-^{3}H]progesterone (specific activity, 57.4 Ci/mmole) was purchased from the New England Nuclear Corp. Its radiochemical purity was 97–98% after thin-layer chromatography (TLC) with system A.

The following TLC systems were used and are referred to by the following alphabetical notations: (A) benzene–methanol (19:1); (B) cyclohexane–*n*-butyl acetate (1:2); (C) benzene–ethyl acetate, (8:2); (D) chloroform–cyclohexane–*n*-butyl acetate (1:1:1); (E) ethyl ether–cyclohexane–*n*-butyl acetate (1:2:4); (F) ethyl ether–cyclohexane–*n*-butyl acetate (1:4:9); (G) ethyl ether–*n*-hexane–methanol (4:5:1); (H) ethyl ether–*n*-hexane–isopropanol (6:3:1); (I) ethyl ether–*n*-hexane–isopropanol (3:6:1); (J) chloroform–ethyl acetate (13:1); (K) *n*-hexane–ethyl acetate (3:1); (T) two-dimensional TLC (system A followed by system B)[14]. The systems were chosen on the basis

of their ability to separate the metabolites and substrate from one another and from their epimers.

The column packing for gas liquid chromatography (GLC) was 3% OV-1 on 100–120 mesh Gas Chrom Q. The areas under the GLC peak tracings were obtained with an Autolab System IV computing integrator for chromatography. Unless otherwise indicated, all other materials and instruments used have been previously described[3,14].

Animals and tissues

Five proestrous Holtzman rats weighing 180–200 g were sacrificed between 12:00–13:00 h by CO_2 asphyxiation[32]. After decapitation, pineal and the following brain regions were dissected according to the demarcation lines below. Thalamus: the area within the vertical and horizontal planes of the anterior commissure and the vertical and horizontal planes of the posterior end of the stria medullaris thalami. Tectum: corpus quadrigeminus with dorsal part of the central grey as the ventral border. Tegmentum: the rest of the mesencephalon with the anterior portion of the pons as the posterior border. Cerebellum: a 3–4 mm transverse slice of the dorsal cerebellum including parts of the vermis and the two hemispheres. Medulla: a 2–3 mm slice of medulla oblongata. Corpus callosum: 3–4 mm to either side of the median sagittal plane as the sagittal borders. The tissues were placed in Petri dishes containing Medium 199 which were kept on ice. The pieces of tissue were cut into 2–4 smaller pieces and each pool of tissue placed in the appropriate incubation flask.

Incubations

The procedures were the same for all the pools of tissue. [1,2-^{3}H]Progesterone in ethanol (2.2×10^6 disint/min in experiment 1 and 2.70×10^6 disint/min in experiment 2) was put into a 10 ml Erlenmeyer flask. After evaporation of the ethanol, 1 ml Medium 199 was added and the flask was incubated for 30 min in a Dubnoff metabolic shaker at 37 °C under an atmosphere of 5% CO_2 and 95% O_2. The pool of tissue was then placed into the flask and incubated for 30 min under the previous conditions. The incubation was stopped with the addition of 4 ml of absolute ethanol and placement of the flask into a dry ice-acetone bath.

Extraction and identification of the radioactive steroids

In the first experiment (exp. 1), the contents of each flask were homogenized in a McShan homogenizer. Each homogenate was mixed on a Vortex mixer and centrifuged at $1000 \times g$ for 10 min (all subsequent centrifugations were also done at $1000 \times g$ for 10 min). The supernatant was taken as the first extract. The pellet was extracted with 3 ml of 80% ethanol and then with 3 ml of ethyl acetate. The 3 extracts were combined and dried under N_2 at room temperature (all subsequent solvent evaporations were done under the same conditions). The residue was suspended in 1 ml H_2O and extracted 5 times with 3 ml of H_2O equilibrated ethyl acetate. Less than 1% of the total incubated ^{3}H remained in the water phase. The 5 extracts were combined and 150 μg of each of the following carrier steroids were added: proges-

terone, 5α-DHP, 5β-DHP, 3α-hydroxy-5α-pregnan-20 one, 20α-hydroxy-4-pregnen-3-one, 20α-hydroxy-5α-pregnan-3-one and 5α-pregnane-3α, 20α-diol. The solvents were evaporated and the residue redissolved in 300 μl ethyl acetate and approximately half was subjected to two-dimensional TLC in system T.

The carrier steroids were visualized under UV light and by exposing the plate to iodine vapors. The steroid zones were scraped and eluted twice with 1.5 ml absolute ethanol and once with 1.5 ml ethyl acetate. The combined extracts were dried and the residue (eluate) redissolved in approximately 100 μl ethanol.

To determine specific activity (SA), portions of each eluate were taken for determination of mass by GLC (0.3–3 μg of steroid in 1–5 μl ethanol) and for measurement of radioactivity (0.5–6 μg of steroid). The remaining eluate was subjected to successive TLC purifications and SA determinations until 3 or 4 successive SAs were constant within 5% (approximately) of their mean. As further evidence of radiochemical purity, the purified radiolabeled steroids from the last TLC were acetylated by liquid phase acetylation[27] for further purifications and SA determinations and/or subjected to GLC and trapping the eluates to determine whether mass and radioactivity were congruent.

In the second experiment (exp. 2) the carrier steroids were added to the incubation flask immediately after the incubation was stopped for quantitative assessment of the radioactive metabolites and remaining substrate ([^{3}H]progesterone). All other procedures were the same as those in exp. 1, except that only two SAs were determined for progesterone, 5α-DHP, 3α-hydroxy-5α-pregnan-20-one and 20α-hydroxy-4-pregnen-3-one. These metabolites had been already identified in exp. 1 and the validity of the purification procedures for these steroids established. Corpus callosum incubations were done only in exp. 2.

Control incubations

Two incubations were done with tissues denatured by boiling to ascertain the enzymatic nature of the reductions. Pineal and cerebellum were collected as described above. The two pools of tissue were placed in tubes with Medium 199 and immersed in a boiling water bath for 1 h. A third control incubation was done without any tissue. The pineal pool and tissueless incubations were included in exp. 1, and cerebellum pool incubation was included in exp. 2.

Preliminary TLC scans

Portions of each tissue extract containing 20,000–50,000 counts/min were subjected to TLC system A to determine the extent of metabolism of [^{3}H]progesterone and to provide preliminary information for subsequent analyses. After chromatography, 1 cm long zones of the tracks were successively removed and placed into scintillation counting vials containing 200 μl of H_2O and 10 ml of a scintillation counting solution for aqueous samples (2 vol. of toluene containing 0.4% PPO and 0.01% dimethyl POPOP and one volume of Triton X-100)[28].

In order to determine whether the two largest peaks of ^{3}H observed in the initial TLC scans represented a single steroid or more, the two zones were scraped and eluted, and eluate chromatographed in system B for TLC scanning as described above.

RESULTS

Preliminary TLC scans

The TLC scans of all tissue extracts showed similar patterns of ^{3}H distribution (Fig. 1). In each case, the TLC zone with the highest percentage of radioactivity (68–90%) had the same mobility as progesterone, and the zone with the second highest percentage of radioactivity (10–20% except pineal — 1%) had a mobility similar to that of 5α-DHP or 5β-DHP (Fig. 1). The remaining zones had low levels of radioactivity (less than 3%). Further TLC analysis showed that 90–99% of the radioactivity in the two largest peaks represented [^{3}H]progesterone and [^{3}H]5α-DHP.

Reverse isotopic dilution analysis

On the basis of the TLC scans (Fig. 1) and previous studies of progesterone and

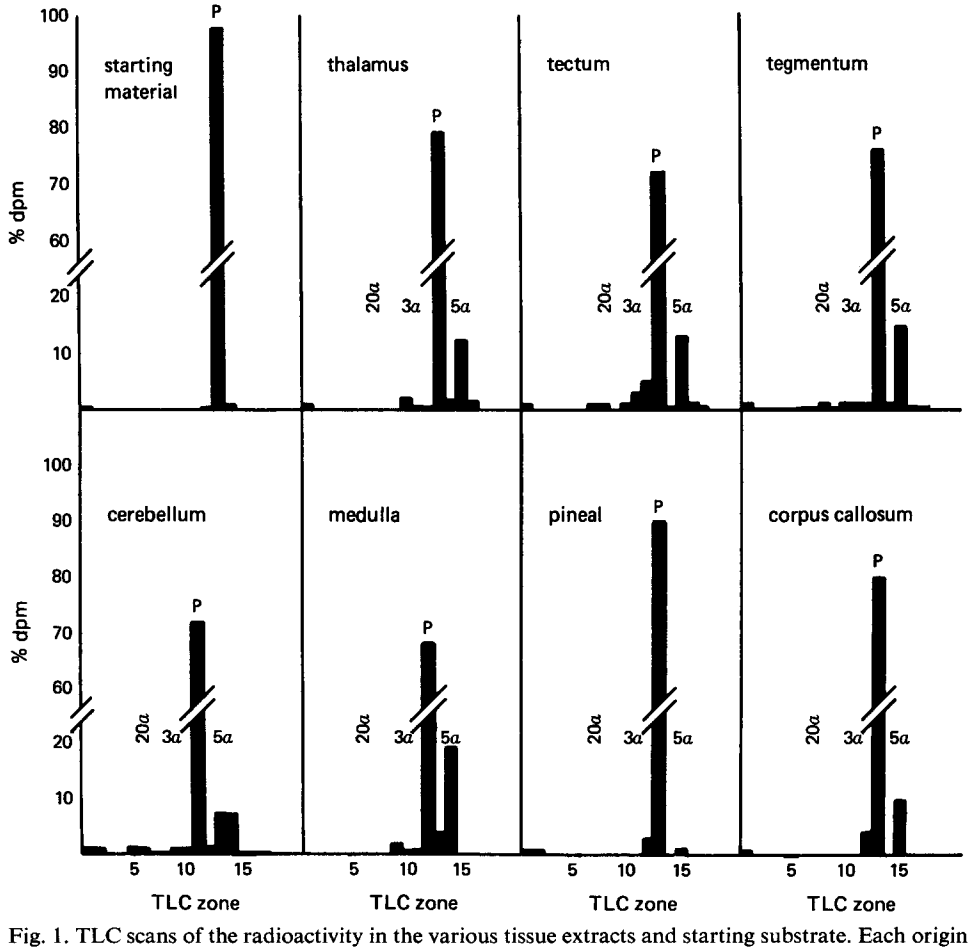

Fig. 1. TLC scans of the radioactivity in the various tissue extracts and starting substrate. Each origin is designated as zone 1. The bar height represents the percentage of radioactivity in a 1 cm zone while the sum of all the bars for one brain region equals 100%. Abbreviations used are: P, progesterone; 5α, 5α-DHP; 3α, 3α-hydroxy-5α-pregnan-20-one; 20α, 20α-hydroxy-4-pregnen-3-one; and indicate the mobility of the respective reference steroid. All other details are described in the text.

TABLE I

Evidence for the identification of [^{3}H]steroids isolated from incubations of [^{3}H]progesterone with specific brain regions by purification to constant specific activity (exp. 1)

Steroid	*Purification step: TLC system*	*Brain region specific activity (disint/min/μg steroid)*												*Control (boiled tissue or no tissue)*
		Thalamus		*Tectum*		*Tegmentum*		*Cerebellum*		*Medulla*		*Pineal*		
Progesterone	1:T	5060		7840		8300		11700		6030		10000		—§
	2:C	4710		8680		8490		12300		6070		10900		—
	3:D	4940		9340		8880		12100		5720		11200		—
	4:E	4980		9280		8220		11500		5630		10600		—
5α-Dihydro-progesterone	1:T	1150		1160		1220		1270		1100		64.1		—
	2:C	1010		1290		1370		1250		1070		60.7		—
	3:D	1050		1230		1280		1270		1150		67.0		—
	4:E	990		1150		1310		1200		1050		59.9		bkg
3α-Hydroxy-5α-pregnan-20-one[a]	2:C+	258		225		170		404		145		29.7		—
	3:D	252		230		163		382		159		32.4		—
	4:F	249		223		148		419		163		26.8		—
	5:C*	228		218		168		425		157		27.1		bkg
20α-Hydroxy-4-pregnen-3-one[b]	6:I+	9.4		10.3		4.5		21.0		6.4		21.3		—
	7:C	9.3		10.3		5.0		21.3		6.1		19.9		—
	8:J	10.9		10.1		4.8		20.1		6.4		19.6		
	9:C*	10.2		9.6		4.4		21.7		6.1		23.6		bkg
			exp. 2		exp. 2		exp. 2		exp. 2		exp. 2		exp. 2	
20α-Hydroxy-5α-pregnan-3-one[c]	5:J+,*	—	4.1	—	5.7	—	2.5	—	8.4	—	6.4	—	—	bkg
	6:D	—	4.0	—	5.7	—	2.4	—	6.6	—	6.9	—	—	—
	7:G	—	4.3	—	5.7	—	2.3	—	6.8	—	7.2	—	—	
5β-Dihydro-progesterone[d]	7:K+	bkg	2.8	bkg	4.4	bkg	6.0	bkg	3.5	bkg	4.5	bkg	bkg	bkg
	8:D	—	4.0	—	5.0	—	5.6	—	2.8	—	4.9	—	—	—
	9:C*	—	2.7	—	1.7	—	3.5	—	3.4	—	3.8	—	—	—
5α-Pregnane-3α, 20α-diol[e]	5:G+,*	—	bkg	—	bkg	—	bkg	—	bkg	—	bkg	—	—	bkg

§ Not determined.

+ The TLC systems used prior to this are: a, T; b, T, G, H, F, E; c, T, C, C, E; d, T, B, C, D, K, B; e, T, C, H, F.

* As the acetates except for d, i.e., the eluates obtained from the preceding step were subjected to an acetylation reaction to form acetates of a, b, c, and e and to see if ^{3}H could be dissociated from d.

TABLE II

Comparison of the accumulated amounts of the 4 major [^3H]steroids previously isolated and identified from incubations of [^3H]progesterone with specific brain regions

Steroid	*Brain regions*** *(disint/min × 10^2)*						
	*Thalamus (127)**	*Tectum (166)*	*Tegmentum (214)*	*Cerebellum (286)*	*Medulla (302)*	*Pineal (10)*	*Control (266)*
Progesterone	19300 (69.3)**	19600 (70.5)	18400 (66.3)	17900 (64.4)	15100 (64.4)	25600 (92.1)	27100 (97.6)
5α-Dihydro-progesterone	2770 (10.0)	3080 (11.1)	3100 (11.2)	3150 (11.3)	5040 (18.1)	206 (0.74)	bkg
3α-Hydroxy-5α-pregnan-20-one	528 (1.9)	447 (1.6)	480 (1.7)	1050 (3.8)	578 (2.1)	44 (0.51)	bkg
20α-Hydroxy-4-pregnen-3-one	18 (0.06)	21 (0.08)	14 (0.05)	32 (0.11)	18 (0.06)	30 (0.11)	bkg

* Tissue pool weight in mg.

** Percentage of incubated radioactivity associated with the steroid after reverse isotopic dilution.

*** These results are those of exp. 2. The respective values for corpus collosum (39 mg) were: progesterone, 227 × 10^4 disint/min (81.5%); 5α-DHP, 217,000 disint/min (7.8%); 3α-hydroxy-5α-pregnan-20-one, 18,300 disint/min (0.7%); 20α-hydroxy-4-pregnen-3-one, 5300 disint/min (0.19%); 20α-hydroxy-5α-pregnan-3-one, 3900 disint/min (0.14%); and 5β-DHP, 1100 disint/min (0.04%), but these values are the means of two SA determinations and further purification to achieve constant SA was not performed.

20α-hydroxy-4-pregnen-3-one metabolism in the rat CNS the following metabolites were possible: [^{3}H]5α-DHP, [^{3}H]3α-hydroxy-5α-pregnan-20-one, [^{3}H]20α-hydroxy-4-pregnen-3-one, [^{3}H]20α-hydroxy-5α-pregnan-3-one, [^{3}H]5α-pregnane-3α, 20α-diol, and [^{3}H]5β-DHP.

Purifications to a constant specific activity were obtained in both experiments for [^{3}H]progesterone, [^{3}H]5α-DHP, [^{3}H]3α-hydroxy-5α-pregnan-20-one and [^{3}H]20α-hydroxy-4-pregnen-3-one (Tables I and II). Additional evidence for the identity of these compounds was provided by derivative formation and/or GLC trapping. The low levels of radioactivity associated with 20α-hydroxy-5α-pregnan-3-one, 5α-pregnane-3α, 20α-diol and 5β-DHP precluded a definitive identification although some apparent constant SAs were obtained in exp. 2 for [^{3}H]20α-hydroxy-5α-pregnan-3-one (after derivative formation) and [^{3}H]5β-DHP.

In exp. 2, where a quantitative assessment of the amount of [^{3}H]steroid present was possible, the percentage conversion of [^{3}H]progesterone to the 5α-reduced metabolites generally correlated with the weight of tissue pool present in the incubations. Brain regions weighing 127–302 mg (thalamus, tectum, tegmentum, cerebellum, medulla)converted 10–20% of [^{3}H]progesterone to [^{3}H]5α-DHP, 1.6–3.8% to [^{3}H]-3α-hydroxy-5α-pregnan-20-one and less than 0.2% to other identified metabolites (Table II). The 10 mg pineal pool produced low amounts of [^{3}H]5α-DHP (0.7%) and [^{3}H]3α-hydroxy-5α-pregnan-20-one (0.2%), but equal or greater amounts of 20α-hydroxy-4-pregnan-3-one than those of the above brain regions (Table III).

The radioactivity associated with the identified metabolites and [^{3}H]progesterone accounted for 75–95% of the radioactivity initially present (Table II). On the basis of the TLC scans (Fig. 1) and reverse isotopic dilution analysis, it appears that if there were unidentified metabolites they would represent at a maximum only a few percent of the total radioactivity.

DISCUSSION

This study demonstrated the conversion of [^{3}H]progesterone to [^{3}H]5α-DHP, [^{3}H]3α-hydroxy-5α-pregnan-20-one and [^{3}H]20α-hydroxy-4-pregnen-3-one in incubations of thalamus, tectum, tegmentum, cerebellum, medulla and pineal with [^{3}H]-progesterone at a concentration similar to that of progesterone in plasma[8]. Preliminary results suggest the presence of the same metabolites in the corpus callosum incubation (Fig. 1, Table II). The order of the amount of the metabolites accumulated was [^{3}H]5α-DHP > [^{3}H]3α-hydroxy-5α-pregnan-20-one > [^{3}H]20α-hydroxy-4-pregnen-3-one for all regions examined (Table II). Small amounts of ^{3}H were associated with 20α-hydroxy-5α-pregnan-3-one, 5β-DHP and 5α-pregnane-3α, 20α-diol but the low levels of ^{3}H did not permit a definitive identification (Table I). Nonetheless, if these [^{3}H]steroids were bona fide metabolites they would appear to be present only in trace amounts.

The results above indicate the presence of at least Δ^4-steroid reductase, 3α-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase activities with progesterone as substrate within all the brain regions examined. In female rats, 5α-

reductase, 3α-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase activities were reported previously in incubations of radiolabeled progesterone or 20α-hydroxy-4-pregnen-3-one with anterior pituitary, hypothalamus and cerebral cortex (for review, see ref. 15). 5α-Reductase and 3α-hydroxysteroid dehydrogenase activities were also shown in incubations of various regions of female rat brain with testosterone[6,7,17,34,38]. Presently, however, it is not known whether the enzymes for testosterone and progesterone as substrates are the same. Further, the enzymatic activities observed in this study are not unique to the rat brain[15,16,21,24,29,34–36,41].

Although several investigators have searched for 5β-reduced metabolites in incubations of rat brain with radiolabeled progesterone or testosterone[6,32,34,37] only Sholiton and Werk have reported evidence for a 5β-reduced metabolite and that only in trace amounts[34]. In the present study the levels of ^3H associated with 5β-DHP were too low for a definitive identification and if [^3H]5β-DHP was present in these amounts, it would represent less than 0.04% of the starting substrate. Nevertheless, these low levels of [^3H]5β-DHP, if present, should not be a priori considered as unimportant. For example, the very low conversion of androgens to estrogens in brain appears to be of prime importance in some of the CNS effects of androgens[26].

The pathway of testosterone metabolism in male rat brain[6] appears to be similar to progesterone metabolism in female rat brain except for the aromatization to estrogens. Furthermore, the order of the amount of analogous testosterone metabolites formed[6] is also similar.

The effects of these metabolites of progesterone on various CNS functions, their loci of action and the underlying molecular mechanisms are largely unknown and remain to be elucidated. However, the progesterone-like effects of 5α-DHP on some neuroendocrine processes, e.g., gonadotropin regulation[15], and lordosis behavior[4,23,40] and the selective accumulation of 5α-DHP in rat hypothalamus and anterior pituitary[14] provide support for the hypothesis that this metabolite of progesterone may be an intermediary in some neural effects of progesterone. Additionally, 3α-hydroxy-5α-pregnan-20-one appears to be a more potent anesthetic than progesterone[11]. The conversion of progesterone to this potent anesthetic steroid within the CNS may be important if the anesthetic effects of progestins reflect a mechanism of physiological significance. Thus, these CNS conversions of progesterone may result in the formation of intermediaries for some of the above specific neural effects of progesterone or other progesterone related effects on general CNS parameters such as CNS differentiation[31], various behavior patterns[18,19,42] and EEG[9,18]. Alternatively, these metabolites may be products of the inactivation of the active steroidal compound.

As to the possible underlying molecular mechanisms, the rapid and slow onsets of progesterone's CNS effects suggest that extranuclear sites of action and mechanisms not involving transcriptional changes should also be considered for progesterone and/or its metabolites (e.g., the rapid anesthetic effect of progesterone[23] is probably a membrane action[33]). Indeed, the recent demonstration of a specific effect of progesterone on the release of LHRF from hypothalamic synaptosomes[2] provides support for the notion of extranuclear loci of action for progesterone and/or its metabolites.

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