J. steroid Biochem. Vol. 27, No. 4-6, pp. 649-655, 1987
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0022-4731/87 $3.00 + 0.00
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Proceedings of the VII International Congress on Hormonal Steroids (Madrid, Spain, 1986)

NEURO-STEROIDS: 3β-HYDROXY-Δ5-DERIVATIVES IN RAT AND MONKEY BRAIN

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Summary—The rat brain accumulates pregnenolone (P) as the unconjugated steroid, the sulfate ester (S) and fatty acid esters (L). P + PS do not disappear from rat brain after combined adrenalectomy (adx) and castration (orx). PL does not serve a source of P after adx + orx. P is metabolized by several rat brain regions to progesterone and to PL. Brain microsomes contain the acyl-transferase which converts P to PL using endogenous substrates.

Brain P and dehydroepiandrosterone (D) undergo a prominent circadian variation with their acrophases at the beginning of the dark span. The circadian variation of brain D persists after adx + orx.

The monkey brain (*Macaca fascicularis*) also accumulates P and D. Adrenal suppression with dexamethasone for 4 days does not decrease the concentrations of brain P and 3rd ventricle CSF P and D. The concentrations of brain D are decreased to a much smaller extent than plasma D.

D inhibits the aggressive behavior of castrated male mice exposed to lactating female intruders. This is not the case for DS or androst-5-ene-3β, 17β-diol. The D analog 3β-methyl-androst-5-en-17-one, which is not estrogenic and cannot be metabolized to testosterone or estradiol, is as active as D in inhibiting the aggressive behavior of castrated mice.

INTRODUCTION

We have obtained evidence for the occurrence and accumulation of pregnenolone (P) and of dehydroepiandrosterone (D) in the brain of several mammalian species (rat, mouse, monkey, and occasionally pig and human). P and D were identified as the unconjugated steroids, the sulfate esters (S), and the fatty acid esters (lipoidal derivatives, L). Definitive identification of the steroid moiety was made in rat brain extracts. After chromatography on celite and Lipidex columns, trimethylsilyl ethers were prepared and analyzed by GLC-MS. Compounds with the retention time and all diagnostically important m/z values of authentic trimethyl silyl ethers of P and D, respectively, were observed.

Measurements of brain P and D and of their sulfate esters were performed in the brain of adult male rats after the removal of testes and adrenals [1, 2]. As they persisted in brain after removal of steroidogenic organs, we proposed that their formation or accumulation in the rat brain depends on *in situ* mechanisms unrelated to the peripheral endocrine gland system. The 3β-hydroxy-Δ5-steroids do not interact with already described intracellular receptors, with the exception of 5-androstene-3β, 17β-diol, which binds to estrogen receptor with significant affinity, and may behave as a weak estrogen [3]. Mammalian brain contains all the enzymes that are necessary for the conversion of P(S, L) and D(S, L) into sex steroid hormones (review in Ref. [1]). The functional significance of such pathways remains to be established.

ANIMALS

Young adult male rats of the Sprague-Dawley strain were generally used. When indicated, they were castrated and adrenalectomized. Sham-operated rats were used as controls. They were kept in groups of 3-6 per cage at 18°C, usually under a 12 h light, 12 h darkness lighting schedule, and fed *ad libitum*. They were killed by decapitation. The brains were collected in ice-cold isotonic saline, weighed, and homogenized in 5 ml of isotonic saline. Trunk blood was collected on heparin for the measurement of plasma steroids.

Four adult female macaques (*Macaca fascicularis*) (age range 18-23 yr) had been castrated at least 6 months before the experiments. Two of them were used as controls. The two others received daily i.m. injections of dexamethasone (4 mg) for 3 days, the last injection was made about 18 h before death. One treated female and its control were killed the same day between 2.30 and 4.00 pm. They were anesthetized with an i.v. injection of penthobarbital, the carotid arteries were ligated, the skulls were opened, and the brain was removed after medullary transection. Thus, all the brain regions used for the measurement of steroids could be collected within 15 min of death. They were frozen on dry ice and kept in liquid nitrogen until assay. They were rapidly thawed and homogenized in 3 vol of isotonic saline. Blood was collected on heparin before treatment and immediately before the removal of brain, for the measurement of plasma steroids.

EXPERIMENTAL

Tracer D, DS, P, P stearate, corticosterone (B) or cortisol (F) were added to brain homogenates or plasma samples (diluted with 3 vol of isotonic saline) for the calculation of recovery, and unconjugated steroids were extracted with ethyl acetate (2 vol × 3 times). The water phase was brought to pH 1, and 20% sodium chloride (w/v) was added. Extraction with ethyl acetate was again performed and the extract, which contained steroid sulfates, was solvolysed at 37°C for 12–16 h. Whenever indicated, the first extraction was omitted, and the second extract thus contained both unconjugated steroids and steroid sulfate esters. The ethyl acetate extracts were washed with NaOH and water and taken to dryness. They were taken up in 2 ml of 70% methanol, left overnight at −20°C, and lipids were pelleted by centrifugation. These non-soluble residues contained about 95% of the lipoidal derivatives, and were saponified with methanolic KOH for analysis and measurement.

Partition chromatography on celite microcolumns allowed the stepwise separation of a non-polar fraction, and fractions containing P, D, and eventually B or F. Saponification of the non-polar fractions and of the non-soluble residues released steroids that were again submitted to celite chromatography. They corresponded to lipoidal conjugates—that is to say fatty acid esters [4].

The appropriate fractions of celite microcolumns containing P, D, B or F, were used for radioimmunoassay using specific antibodies having minimal cross-reaction with other steroids. The amounts of P or D were in agreement with the GLC–MS values, sometimes 10–20% higher.

We did not measure 5-androstene-3β,17β-diol, a reduced metabolite of D. We did not find measurable amounts of 17-hydroxy-pregnenolone (<0.2 mg/g of rat brain).

RESULTS

The quantitation of brain D and P appeared to depend on several environmental and methodological factors. Some have been documented, as age, lighting schedule, hour of sacrifice, housing conditions (number of males per cage, female rats in the same room), and condition after castration (orx) plus adrenalectomy (adx), or the corresponding sham operation (sham). The conditions of the removal and initial processing of brain tissue were also critical. Therefore, all experiments were conducted under strictly defined conditions, with appropriate internal controls, and their statistical significance evaluated.

It is recognized that the physiological conditions could not be standardized for the monkeys as well as they were for the rats.

The accumulation of brain pregnenolone and dehydroepiandrosterone is not related to peripheral endocrine glands

1. *Effects of combined adrenalectomy and castration on the brain steroids of adult male rats*. The rat adrenal secretes P, but not D, as reflected by the measurement of P(S) and D(S) in adrenals and in plasma. This led us to assume independent mechanisms for the formation and for accumulation of brain 3β-hydroxy-Δ5-steroids. The relevant findings have been reported previously [1, 2]. Neither D(S) nor P(S) disappeared or even decreased (compared to sham-operated controls) in brain 15 days after combined adx + orx. Brain D(S) was also unchanged after administration of corticotropin or dexamethasone for 3 days, or after an acute nociceptive stimulation. In contrast, conditions prevailing 2 days after adx + orx or the corresponding sham operation resulted in a significant increase of brain D(S) concentration. The results were less clearcut with P. Therefore, they were re-evaluated to include separate measurements of P, PS, and PL up to 1 month after operation (Fig. 1). Early after operation (3 and 5 days), the concentrations of P, PS, and PL exceeded the range determined for intact rats. The control values were reached <8 days after operation. Thereafter, they tended to decrease, but no difference was observed between sham-operated and adrenalectomized and castrated

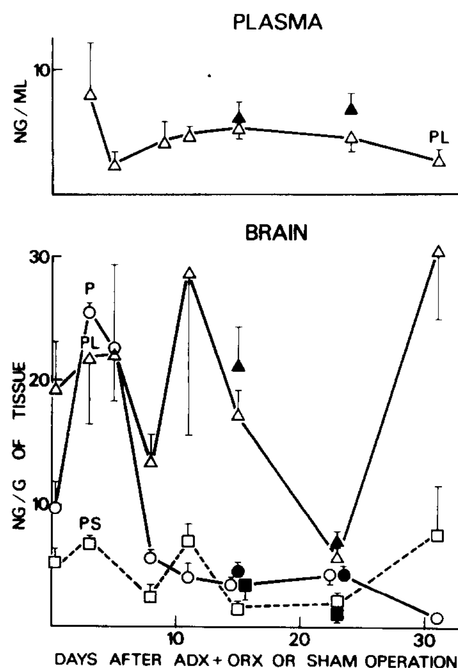

Fig. 1. P, PS, and PL in brain (ng/g) and PL in plasma (ng/ml) of male rats after combined castration and adrenalectomy. Young adult male Sprague–Dawley rats were either intact (day 0 controls), or castrated (orx) and adrenalectomized (adx), or sham-operated (sham). Brain P, PS, and PL were measured in whole brain 3, 8, 11, 15, 23 and 31 days after operation. ○, P; □, PS; △, PL; ●, ■, ▲, corresponding sham-operated controls.

animals. Unexpectedly, brain PL and to a lesser extent PS showed large infraction variation compatible with the occurrence of a circaseptan (about 7-day) rhythm. This possibility merits further investigation. Plasma P and PS were low, at the limit of detection in most samples. In contrast, higher concentration of PL were found and were not markedly decreased after removal of steroidogenic glands. The origin of plasma PL in operated animals is unknown. Circulating PL may serve as a source of brain P. However, the concentration of brain P, PS and PL were repeatedly much higher than the corresponding plasma values, thus confirming the existence of a mechanism responsible for P accumulation in the rat brain. Plasma corticosterone was measured in the plasma of adrenalectomized or sham operated rats. 15 days after sham operation it was 12.7 ± 1.4 ng/ml, whereas its concentration after operation was below 1 ng/ml in corresponding adrenalectomized rats. Similar values were obtained in 23 days. Therefore it seems unlikely that the regeneration of ectopic adrencortical tissue accounted for the maintenance of plasma PL and brain P, PS, and PL.

2. *Effects of adrenal suppression on* Macaca fascicularis *brain steroids*. Two control adult spayed females and 2 dexamethasone-treated females have been studied. The effectiveness of adrenal suppression was indicated by the decrease of plasma cortisol to undetectable levels 20 h after the last injection of DEX (Fig. 2). The plasma concentrations of D + DS were also much smaller after DEX treatment (91.5 and 93.5 ng/ml before DEX, 4.5 and 19.3 ng/ml after DEX, respectively). Since the plasma concentrations of P + PS and of PL were very low before treatment the effect of DEX could not be evaluated.

P(S, L) and D(S) were found in all brain regions investigated. Their concentrations tended to be higher in the hypothalamus, hippocampus, and rhinencephalon than in the cerebellar or cerebral cortex. The accumulation of P(S, L) in brain was not markedly modified by adrenal suppression, possibly with the exception of P + PS in hippocampus.

Conversely, the concentrations of D + DS in brain were much smaller than in plasma. This results from the impermeability of the blood–brain barrier to steroid sulfates [6]. Adrenal suppression resulted in markedly decreased brain D + DS concentrations. However, the fraction of D + DS remaining in brain after adrenal suppression was much larger than the corresponding fraction in plasma, suggesting that the accumulation of D(S) in monkey brain is partially independent of peripheral endocrine glands, as previously shown in the rat.

In another experiment, D. Van Vugt has collected cerebrospinal fluid (CSF) through an indwelling catether from the 3rd ventricle of 2 adult female monkeys (*Macaca fascicularis*) [7]. The concentrations of P + PS and of D + DS were measured in several pools of CSF taken before and after dexamethasone treatment (Table 1). Contrasting with the pronounced decrease of plasma steroids after adrenal suppression, the levels of D + DS in CSF were unchanged; the small number of P determinations did not allow to calculate the significance of the slight decrease observed.

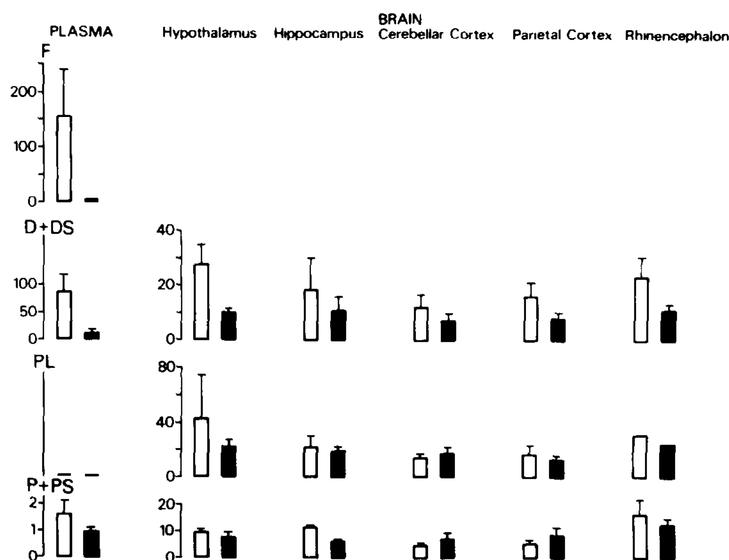

Fig. 2. Effect of dexamethasone on plasma (ng/ml) and brain (ng/g) steroids in *Macaca fasticularis*. F: Cortisol. P(S): Pregnenolone (Sulfate). PL: Pregnenolone fatty acid esters. D(S): dehydroepiandrosterone (Sulfate). Four castrated adult females were used. Two of them were kept as controls (open bars), two of them received 4 mg of dexamethasone daily for 3 days (closed bars). Plasma and brain samples were collected ~20 h after the last injection.

Table 1. Pregnenolone and dehydroepiandrosterone in the 3rd ventricle CSF of *Macaca fascicularis*

	Plasma (ng/ml)			CSF (ng/ml)	
	F + FS	P + PS	D + DS	P + PS	D + DS
Control					
Monkey No. 312	57	4.6	264	1.1 ± 0.2(2)	1.4 ± 0.3(6)
Monkey No. 594	109	5.4	241	1.0 ± 0.1(4)	1.4 ± 0.3(5)
Dexamethasone					
Monkey No. 312	18	0.6	113	0.5	1.4 ± 0.2(4)
Monkey No. 594	31	0.5	110	0.7 ± 0.2(5)	1.5 ± 0.3(6)

F: cortisol; P: pregnenolone; D: dehydroepiandrosterone. S: sulfate. The technique used measured the sum of unconjugated steroid and its sulfate ester. Dexamethasone (4 mg) was injected i.m. daily for 3 days. Monkeys Nos. 312 and 594 were normal adult females

Pregnenolone metabolism in the rat brain

The brains of 11-week-old male rats were used. Several brain regions were dissected and homogenized in 10 vol of CMRL medium. The 800 **g** supernatants were incubated in CMRL medium containing 11 μM glucose, 4.26 mM glucose-6-phosphate, 0.4 mM NADP, and 0.6 μM [^{3}H]pregnenolone (11.4 μCi/ml). Incubations were carried out under 5% CO_2 in O_2, at 37°C for 3 h. The incubations were extracted and processed as indicated in the Experimental section. Celite column chromatography yielded two non-polar fractions corresponding to [^{3}H]P fatty acid esters and [^{3}H]progesterone, respectively. [^{3}H]Progesterone was identified by HPLC, TLC before and after acetylation, and recrystallisation to constant specific activity. The yield of progesterone and of pregnenolone esters formed per 100 mg tissue was in the ~1% range in hypothalamus, amygdala, olfactory bulb, and other regions of the brain, with the exception of cerebellum and the frontal cortex, where very little metabolism was observed [8]. Since only about 5% of pregnenolone fatty acid esters are soluble in 70% methanol (corresponding to short-chain fatty acids), the overall conversion of [^{3}H]pregnenolone to [^{3}H]PL might be of the order of 20%.

The synthetic enzyme responsible for the formation of pregnenolone fatty acid esters was further investigated. Rat brain microsomes were prepared and incubated with [^{3}H]pregnenolone. P was converted to non-polar compounds with the chromatographic characteristics of endogenous brain PL or reference P stearate. The enzyme system had a pH optimum of 4.2 with acetate buffer in presence of Tween 20 (0.2 mg/ml). The apparent K_m was 3.3×10^{-5} M for P, and V_{max} was 4.9 nmol/h per mg of microsomal protein. A high level of synthetic activity was found in the brain of young male rats (1–3-week-old), which rapidly decreased with further increase in age. The synthetic enzyme has many characteristics in common with the testosterone acyl-transferase described by Kishimoto [9]. [^{3}H]Pregnenolone fatty acid esters were partially purified by chromatography on celite and silica gel columns, and by HPLC on a C18 column with acetonitrile as eluant. The resulting PL fractions were saponified, the released fatty acids were converted into their methyl esters, then separated and quantified by GC. They were tentatively identified according to the retention time of known reference fatty acid methyl esters. The major fatty esters found were myristate (C14:0, 9%), palmitate (C16:0, 26%) stearate (C18:0, 11%) oleate (C18:1, 21%) and linoleate (C18:2, 15%).

Circadian rhythms of 3β-hydroxy-Δ5-steroids and glucocorticosteroid in rat plasma and brain

Male adult rats (11–12-week-old) were housed in triads for 2 weeks. Lights were on between 7 a.m. and 8 p.m. Steroids were extracted from plasma and from brain by the combined extraction–solvolysis procedure, separated on celite columns and measured by RIA [2]. The rhythm characteristics were analysed by the cosinor method [10].

The levels of B + BS, P + PS, and D + DS were measured at 3-h intervals. They underwent circadian variations in the rat plasma and brain (Fig. 3). When the data were interpreted by the cosinor method, the acrophases of P in brain and of D in plasma significantly preceded the acrophase of B (Table 2) [11].

Plasma and brain B had been previously shown to undergo synchronous circadian rhythms [12]. We have extended the demonstration of circadian variations to P and D. The asynchrony of 3β-hydroxy-Δ5-steroid and glucocorticosteroid rhythms constitutes an additional argument in favor of partly separate coordinating mechanisms. In human beings, the acrophase of plasma cortisol precedes that of DS by about 6 h [13]. The species difference in internal timing of D vs B in the rat, and of DS vs cortisol in human beings, is not likely to depend on external factors such as the difference in activity–rest cycles of the two species being compared. The difference in internal timing of the two categories of steroids probably results from a modulatory effect of the central neuroendocrine system.

To determine the role of adrenal secretions, the experiment on circadian variations was repeated on groups of rats either intact or castrated and ad-

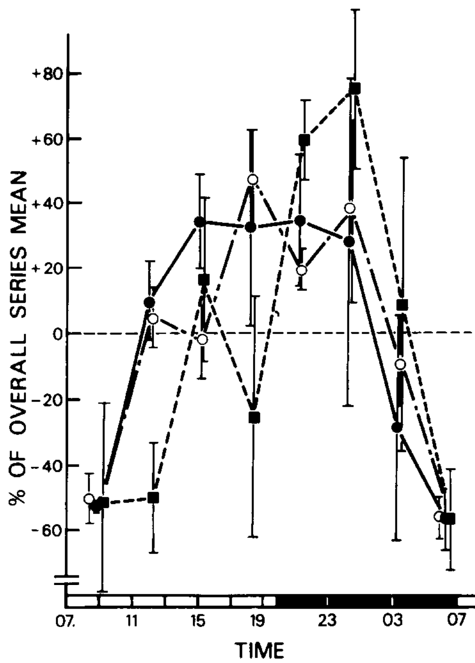

Fig. 3. Circadian variation of pregnenolone, dehydroepiandrosterone, and corticosterone in the rat brain. Measurements were performed every 3 h on groups of 3 rats. Results were expressed in per cent of overall series mean (mean ± SEM). The span of darkness is indicated by a black horizontal bar. ●, Pregnenolone; ○, Dehydroepiandrosterone; ■, Corticosterone.

renalectomized 15 days before killing. Measurements were done on groups of 6 rats killed 2, 5, 10, 13 and 16 h after lights on. The original data are expressed as percentages of the series mean, equated to 100%. These mean values are shown with their standard errors (Fig. 4). Measurements performed in intact rats confirmed the previously demonstrated circadian rhythm of brain B + BS, D + DS, and P + PS (Fig. 4a). Point estimates of circadian acrophases occurred late during the light and early during the dark span. Combined castration and adrenalectomy was followed by a dramatic decrease of plasma and brain corticosterone. As previously reported, brain P(S) and D(S) did not disappear from the rat brain 15 days after operation (Fig. 4b). Analysis of the characteristics of rhythms in operated animals by the cosinor method indicated a disappearance of cir-

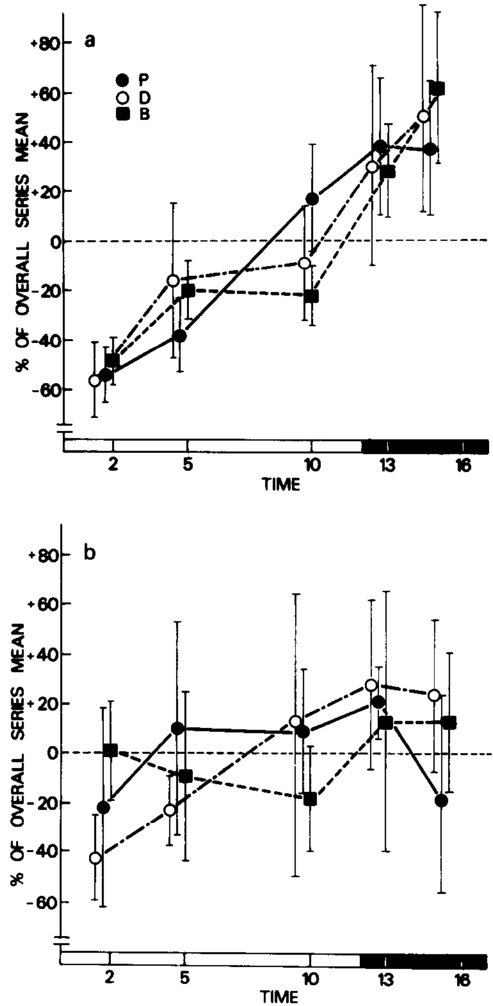

Fig. 4. Circadian variations of pregnenolone, dehydroepiandrosterone, and corticosterone in brain of intact and adrenalectomized rat. Measurements were performed 2, 5, 10, 13 and 16 h after "lights on" in groups of 5 rats. Results were expressed in per cent of overall series mean (mean ± SEM). The span of darkness is indicated by a black horizontal bar. 4(a) Intact rats; (b) rats adrenalectomized 15 days before killing. ●, pregnenolone; ○, dehydroepiandrosterone; ■, corticosterone.

cadian variation of B + BS in plasma and brain, and of D in plasma. By contrast, a statistically significant rhythm of brain D + DS persisted with an acrophase at the beginning of the dark span. A similar trend

Table 2. External circadian timing of P, D, and B in rat brain and plasma

Site	Variable	n	% Rhythm	P	Mesor mean ± SE	Amplitude mean ± SE	Acrophase 95% CL
			(light on from 7.00 to 20.00 h)				
Brain	D	24	59	<0.001	3.5 ± 0.2	1.5 ± 0.3	−297° (−275, −319)
(ng/g)	P	24	45	0.002	19.3 ± 1.7	9.6 ± 2.4	−280° (−250, −311)
	B	21	55	0.001	14.4 ± 1.4	8.7 ± 1.9	−326° (−299, −354)
Plasma	D	23	52	0.001	0.36 ± 0.02	0.15 ± 0.03	−278° (−250, −306)
(ng/ml)	P	21	29	0.047	1.01 ± 0.08	0.28 ± 0.11	−311° (−259, −3)
	B	20	69	<0.001	83.8 ± 6.6	57.15 ± 9.3	−325° (−305, −345)

Individual cosinor; n = number of rats. For acrophase, 360° ≡ 24 h, 0° ≡ 0.00 h.

Table 3. Effects of D, DS, ADIOL, or oil on aggression by castrated male mice towards lactating intruders

Treatment groups (triads)	Mean latency to first bite (sec) ± SD	Mean Number of biting attacks ± SD
Controls (9)	34.2 ± 49.7	36.1 ± 18.8
D (9)	189.4 ± 177.3°	15.5 ± 9.4†
DS (9)	115.3 ± 143.6	27.4 ± 15.0
ADIOL (9)	52.6 ± 49.9	29.1 ± 19.4

°Significantly different from controls ($P < 0.03$).
†Significantly different from controls ($P < 0.01$) (Kruskal–Wallis test).

was observed for P, although it was not statistically significant[5].

The persisting circadian rhythm of brain D + DS after the removal of steroidogenic peripheral glands confirmed the existence of autonomous brain mechanisms coordinating the accumulation of 3β-hydroxy-Δ5-steroids. This does not necessarily imply a local biosynthesis, since a rhythmic conversion from the fatty ester pool of brain steroids[9], and/or the circadian variation of a binding protein[14] could also account for our results.

Dehydroepiandrosterone inhibits agressive behavior of castrated male mice

Group-housed triads of castrated male mice attack lactating female intruders. Previous reports indicated that testosterone or estradiol inhibit this aggressive behavior[15]. Male mice of the Swiss strain were castrated or sham operated (intact males) when the animals were 7 weeks old. One month later, triads were randomly assigned to experimental groups injected each day subcutaneously with 280 nmol of D, DS, or Δ5-androstene-3β, 17β-diol (ADIOL) or vehicle alone. One lactating female was then introduced in each cage 2 h after the last injection, and the latency and number of attacks were recorded during 15 min. D had been reported to inhibit this aggressive behavior of castrated males in a dose-dependent manner[16]. Its activity has been confirmed, whereas no statistically significant differences between the control, DS, or ADIOL treated groups were evident (Table 3)[17]. The transformation of injected D into brain testosterone (T) was very small; the concentration of brain T in D injected castrated male mice was slightly but significantly ($P < 0.01$) increased, although it remained far below the level measured in intact males (Table 4). To completely eliminate the possibility that the activity of D was related to its conversion into the active molecules testosterone and estradiol, behavioral experiments were repeated with the D analog 3β-methyl-androst-5-en-17-one. This molecule cannot be metabolized into sex steroids and is not demonstrably estrogenic in rodents[18]. Nevertheless, it inhibited the aggressive behaviour of castrated mice at least as efficiently as D itself (Table 5).

CONCLUSION

Contrary to the classical steroid hormones, the 3β-hydroxy-Δ5-steroids pregnenolone and dehydroepiandrosterone are accumulated as the unconjugated steroid, the sulfate ester, and fatty acid esters, in rat brain, in concentrations far in excess of those found in plasma. The monkey brain also contains large amounts of pregnenolone and dehydroepiandrosterone, which persist after adrenal suppression with dexamethasone.

Investigation of circadian rhythms in the rat (including animals deprived of peripheral steroidogenic glands) brings further support for a modulatory effect of the central neuroendocrine system.

Although the origins and functions of brain 3β-

Table 4. D, DS, and testosterone (T) in brains of male swiss mice

	D	DS	T
Intact	1.8 ± 0.9(4)	2.9 ± 1.9(5)	2.8 ± 1.1(5)*
Castrated:			
untreated	2.3 ± 1.9(9)	4.9 ± 4.3(9)	0.04 ± 0.02(9)
treated with D (80 μg/d × 15 d)	45.5 ± 14.3(8)*	4.5 ± 4.3(7)	0.16 ± 0.06(9)*

Male mice of the Swiss strain, either intact or castrated at the age of 7 weeks, were thereafter housed in triads. After a 5-week interval, 27 castrated triads were treated with 80 μg D daily for 15 days, or with vehicle alone. They were killed by decapitation 2 h after the last injection. Groups of 9 brains were quickly removed, homogenized and processed for RIA of D(S) and T. Results are expressed in ng/g of tissue, mean ± SD (n).

*Significantly different from untreated control at $P < 0.01$.

Table 5. Effects of D and 3β-methyl-androst-5-en-17-one (D-CH_3) on agression by castrated male mice towards lactating intruders

Treatment groups (triads)	Mean latency to first bite (s) ±SD	Mean number of biting attacks ±SD
Controls (8)	58.6 ± 107.8	35.6 ± 10.9
D (17)	154.4 ± 217.7	20.7 ± 15.4°
D-CH_3 (19)	309.2 ± 339.7$^+$	18.0 ± 15.5$^+$

Controls were castrated, vehicle injected males. D and D-CH_3 were given at the daily dose of 80 μg. °Significantly different from controls ($P < 0.01$) (Anova and Newman–Keuls tests).

hydroxy-Δ5-steroids are still poorly understood, they may affect the brain by metabolism to sex steroid hormones, and they may be functionally related to sexual behavior [19] (possibly through direct modulation of the firing rates of neurons) [20]. This new chapter of steroid regulation of the brain may not be particular to rodent.

Acknowledgements—We thank R. Vihko (Oulu) for the provision of pregnenolone antibodies. Contributing experiments were performed by J. Sjövall and M. Axelson (Stockholm) for GLC–MS of steroids, J. Raulin and D. Lapous (Paris) for GLC of fatty acid methyl esters. This work was supported in part by Inserm contract PRC 120048, and by the MIR contract 83.C.0924.

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