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NEUROSTEROIDS: BIOSYNTHESIS, METABOLISM AND FUNCTION OF PREGNENOLONE AND DEHYDROEPIANDROSTERONE IN THE BRAIN

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Summary—Pregnenolone (P) and dehydroepiandrosterone (D) accumulate in the brain as unconjugated steroids and their sulfate (S) and fatty acid (L) esters. The microsomal acyl-transferase activity is highest in immature (1–3 weeks old) male rats. The immunocytochemical and biochemical evidence for P biosynthesis by differentiated oligodendrocytes is reviewed. The importance of P synthesis for its brain accumulation is assessed by the intracysternal injection of the inhibitor aminoglutethimide. Primary glial cell cultures convert P to 20-OH-P, PL, progesterone, 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnane-20-one (Polone). Astroglial cell cultures also produce these metabolites, whereas neurons from 17-day mouse embryos only form 20-OH-P. P and D are converted to the corresponding 7α-hydroxylated metabolites by a very active *P*-450 enzyme from rat brain microsomes. Several functions of neurosteroids are documented. P decreases in olfactory bulb of intact male rats exposed to the scent of estrous females. D inhibits the aggressive behavior of castrated male mice towards lactating female intruders. The D analog 3β-methyl-androst-5-en-17-one, which cannot be metabolized into sex steroids and is not demonstrably androgenic or estrogenic is at least as efficient as D. Both compounds elicit a marked decrease of PS in rat brain. The Cl^- conductance of γ-aminobutyric ($GABA_A$) receptor is stimulated by GABA agonists, an effect which is enhanced by Polone and antagonized by PS. Thus, P metabolites in brain as well as steroids of extraencephalic sources may be involved physiologically in $GABA_A$ receptor function. The neurosteroids accumulated in brain may be precursors of sex steroid hormones and progesterone receptors have been localized in glial cells. P and D do not bind to any known intracellular receptor. A heat stable P binding protein has been found in brain cytosol with distinct ligand specificity. A binding component specific for steroids sulfates, including Polone S, DS and PS, in the order of decreasing affinity is localized in adult rat brain synaptosomal membranes. Its relationship to the $GABA_A$ receptor is under current investigation.

1. INTRODUCTION

It is well-established that local target tissue metabolism is an important factor in the mechanisms of action of circulating gonadal steroids. Not only may such a metabolism be involved in the regulation of intracellular hormone levels, but it may also provide an essential contribution to the cellular response. The brain is a site of extensive steroid metabolism. Aromatization and 5α-reduction represent major routes of androgen metabolism. Rat brain tissues also metabolize progesterone to several 5α-reduced products. The importance of these two pathways lies in the fact that they give rise to metabolites with considerable biological activity, and thus are involved in the mechanism by which circulating androgens and progestins influence neuroendocrine function and behavior [1, 2].

The characterization of pregnenolone (P), dehydroepiandrosterone (D), their sulfate esters (S) and their fatty acid esters (lipoidal derivatives, L) in the rat brain, and the observation of their large cerebral accumulation in other mammalian species (mouse, pig, guinea pig, monkey and human) [3–9] have led us to reconsider the steroid–brain interrelationships. We initially discovered the presence of D and DS in the rat brain [3] to our surprise, since rodent adrenals do not produce sizable amounts of this steroid, contrary to primates. Moreover, we observed that, besides the large difference between brain and blood concentrations, the cerebral concentrations of D and DS were apparently independent of adrenal and gonadal sources. This contrasted with testosterone and corticosterone,

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the concentrations of which are lower in brain than in plasma, and which readily disappear after removal of the corresponding endocrine glands. Therefore, we started to measure P, the immediate biochemical precursor of D in steroidogenic cells of peripheral glands, and the results confirmed those obtained with D [5]. Moreover, P and PS concentrations in brain were about 10-fold larger than those of D and DS, a situation often found in precursor–product relationships.

2. P AND D IN THE RAT BRAIN

The measurements of pregnenolone (as P, PS and PL) and dehydroepiandrosterone (as DS and DL) were made in the whole brain of young adult male and female rats 10 h after lights on. This schedule was chosen because brain neurosteroids undergo large circadian (and also infradian) variations, with the largest values around the time of lights off under a 12 h/12 h light regimen (Fig. 1) [10]. Therefore, the values for male rats reported in Table 1 are larger than those of our earlier publications, where the rats were killed 2–3 h after lights on [3, 5]. The levels of both neurosteroids are in the same range in males and females. The acyl-transferase responsible for the formation of PL and DL was studied further [11]. Subcellular fractionation of the rat brain indicated that the synthetic activity was mainly enriched in the microsomal fraction.

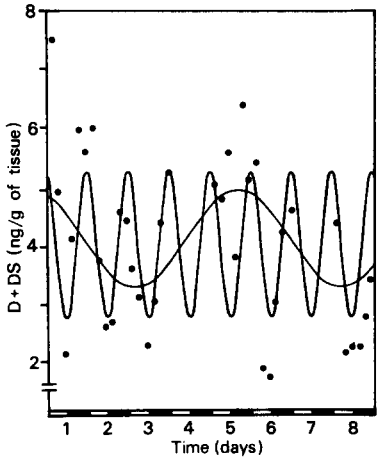

Fig. 1. Circadian and infradian rhythms of D in brain of F Holtzman rats. The experiment began at the start of a 12 h light span, indicated by open bars. Measurements included unconjugated D and its sulfate ester DS. For the sake of clarity, the cosine curves representing the circadian and infradian variations have been represented separately. The overall probability of significance for the concomitant fit of cosine functions with an about 5-day and a 1-day period is <0.001.

Table 1. Neurosteroids in the rat brain

	Neurosteroid (ng/g tissue)				
Rats	P	PS	PL	DS	DL
M	25 ± 8	19 ± 6	46 ± 14	2.1 ± 0.5	0.59 ± 0.30
F	32 ± 15	19 ± 6	46 ± 19	1.7 ± 0.4	0.34 ± 0.12

Rats of the Sprague–Dawley Ofa strain (Iffa-Credo, L'Arbresles, France) were killed when 11 weeks old, 10 h after lights on. Measurements were made on whole brain and are expressed in ng/g of tissue (mean ± SD, $n = 9$ or 10). Values for D were <0.5 ng/g, below the limit of detection in the experimental conditions used. Data are taken from Ref. [34], however, the values for DL have been corrected and are lower than those previously reported.

The acyl-transferase utilized endogenous fatty acids for the esterification of steroids. The enzymatic activity had a pH optimum of 4.2 in acetate buffer. The apparent K_m was 3.3×10^{-5} M for P and $V_{max} = 4.9$ nmol/h/mg/of microsomal protein. A high level of synthetic activity was found in the brain of young male rats (1–3 weeks old) which rapidly decreased with further increase in age. Saponification of the purified product yielded P and a mixture of myristate (C14: 0.9%), palmitate (C16: 0.26%), stearate (C18: 0.11%), oleate (C18: 1.21%) and linoleate (C18: 2.5%) as predominant fatty acid esters.

The rates of esterification of several radioactive steroids by a rat brain microsomal preparation have been determined (Table 2). Among 5-ene-3β-OH and 17β-OH steroids, the velocity of the reactions increased with the polarity of the substrate. Steroids with a hydroxyl group at positions C-17α, C-21 or C-11β were not substrates under our experimental conditions. Rat brain microsomes contained also an esterase activity [12] that, under the optimal incubation conditions used for the assay, hydrolyzed [^{3}H]D stearate and [^{3}H]P stearate at the rates of

Table 2. Rates of esterification of cholesterol and steroids by rat brain microsomes (pmol of radioactive steroid/mg protein/h)

5-Ene-3β-hydroxy steroids	17β-Hydroxy steroids	Other steroids
Cholesterol <1		
	DHT 181	
P24	T 188	T-17α[a]
D 120	DIOL 200	B[a]
17-OH P 102		17-OH PRO[a]
	E_2 310	Estrone 5

The standard incubation mixture contained 200 μl of acetate buffer (0.25 M, pH 4.6), 100 μl of Tween 20 (2 mg/ml of 0.32 M sucrose), 100 μl of EDTA–Na (50 nM), 0.8 μM ^{14}C- or ^{3}H-labeled substrate, 150 μl of rat brain microsomal suspension (1 mg protein/ml) and 0.32 M sucrose, in a total volume of 550 μl. After incubation in a shaking water bath at 37°C for 1 h under air, extraction and TLC in diisopropyl ether–isooctane (1:1, v/v) followed by diisopropylether–diethylether (9:1, v/v), the areas corresponding to the substrate and to its fatty acid esters were scraped off and counted.

[a]Not measurable.

8 and 3 pmol/h/mg of microsomal protein, respectively. Incubations of several tissues of the rat (including the brain) with corticosterone and estradiol (E_2), result in the formation of the corresponding fatty acid esters [13]. The enzyme which produces the C-17 fatty acid esters of the estrogens may be different from that which synthesizes the C-21 esters of the corticoids, thus supporting our suggestion of multiple acyl-transferases. However, steroids such as T and androst-5-ene-3β-diol (DIOL), but also D, act as competitive inhibitors of E_2-17β acyl-transferase in bovine placental microsomes [14]. Further work is needed before drawing definitive conclusions about the number of distinct steroid acyl-transferases.

3. P FORMATION FROM STEROL PRECURSORS

Since at least a fraction of brain P and D seemed independent of the steroidogenic activity of endocrine glands, and since no evidence was obtained either for extraglandular sources or for release from known derivatives stored in the brain, the hypothesis of *de novo* synthesis from cholesterol, following the same pathway as in steroidogenic glandular cells, was considered.

We decided to look for the presence of specific enzymes involved in cholesterol side-chain cleavage by immunohistochemistry. Cytochrome P-450_{scc} (for side-chain cleavage) is found in mitochondria of all steroidogenic endocrine cells as part of a three-enzyme hydroxylase system with adrenodoxin reductase and adrenodoxin. The presence of the latter protein has been demonstrated in bovine brain mitochondria, together with undefined P-450 detected spectrophotometrically [15]. P-450_{scc} has been purified from bovine adrenal mitochondria, and specific antisera were generated [16]. We have used the corresponding IgGs (kindly provided by M. Waterman) to set up an immunohistochemical technique for the detection of cytochrome P-450_{scc} in rat tissues [16]. We have verified that the bovine IgGs were able to stain the specific cytochrome in rat adrenal cortex and ovaries [17, 18]. The optimal conditions thus defined were applied to the adult male rat brain. Specific immune staining was detected in the white matter throughout the brain.

Although the immunohistochemical results fulfilled all the criteria of specificity, and were later on confirmed by the use of antibodies to the rat P-450_{scc}, kindly provided by J. Orly, and were consistent with the detection of an antigen with the expected molecular size of P-450_{scc} on Western blots [19, 20], it was nevertheless mandatory to obtain the biochemical demonstration of side-chain cleavage activity. Since the myelin of the white matter is made by a particular type of glial cells, the oligodendrocytes—we have isolated oligodendrocyte mitochondria, incubated them with [^{3}H]cholesterol, and obtained [^{3}H]P and its reduced derivative [^{3}H]20-OH-P ([^{3}H]pregn-5-en-3β,20α-diol) [21]. P and 20-OH P were characterized by chromatography, formation of acetates, reverse-phase HPLC and recrystallization after reverse isotopic dilution.

The combination of immunohistochemical and biochemical evidence thus allowed us to conclude that *de novo* steroid biosynthesis from cholesterol can be performed by brain cells. The term "neurosteroids" applied to brain P and D was therefore justified. The previous failure to demonstrate P-450_{scc} activity in the rat brain was probably due to the relatively low number of oligodendrocytes in whole brain, and even after the successful experiment with oligodendrocyte mitochondria, we again did not succeed to obtain [^{3}H]P from [^{3}H]cholesterol with whole brain mitochondria. Conversely, the presence of P throughout the brain, with somewhat larger amounts in the olfactory bulbs and hypothalamus, can be explained by the generalized distribution of oligodendrocytes in the CNS.

The biosynthesis of P from sterol precursors was confirmed by the incubation of newborn rat glial cell cultures in the presence of [^{3}H]mevalonolactone (MVA) [22]. The endogenous supply of mevalonate was restricted by the use of mevinolin (20 μM), a specific inhibitor of 3β-hydroxy 3β-methyl glutaryl CoA reductase. Trilostane, a specific inhibitor of 3β-hydroxysteroid dehydrogenase-isomerase, was also added to prevent, in part, further metabolism of P (Fig. 2).

Cells dissociated from cerebral hemispheres of newborn rats and maintained in long-term culture undergo progressive differentiation to mature oligodendrocytes and astrocytes. Therefore, we have investigated the ontogenesis of steroidogenic activity in cultured glial cells. In order to follow oligodendrocyte differentiation, we measured the activity of the enzyme 2′-3′ cyclic nucleotide 3′-phosphodiesterase (CNPase) [23]. After day 10 in our culture conditions, CNPase activity and the biosynthesis of P increased in parallel, reaching their highest

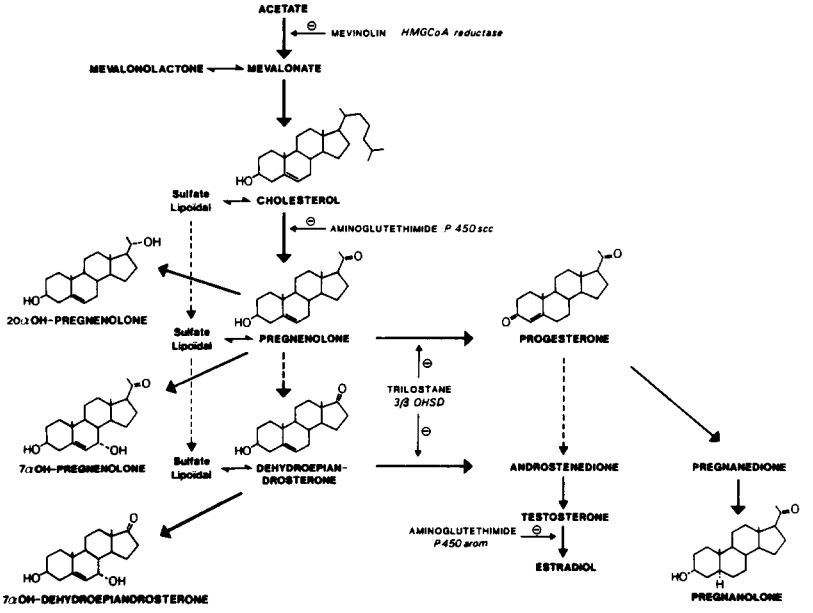

Fig. 2. Biosynthesis of steroids in rat brain (dotted lines indicate possible pathways, not yet demonstrated).

levels at day 21 (Fig. 3). This result suggested that the onset of steroidogenic activity was related to oligodendroglial differentiation. After 3 weeks of culture, oligodendrocytes covered a layer of polygonal astrocytes, and showed highly branched processes specific of differentiated cells. Both cell types were characterized by indirect immunofluorescence with monoclonal antibodies to galactocerebroside (Gal C) and to glial fibrillary acidic protein (GFAP), respectively. About 60% of the cells were mature oligodendrocytes. Their cytoplasm was specifically and intensely immunostained with anti-P-450_{scc} IgGs [22]. Double-labeling experiments were performed. As a rule, the cells immunostained with the antibody to Gal C also reacted with anti-P-450_{scc} IgGs [24]. Astrocytes were only slightly immunoreactive with anti-P-450_{scc} IgGs. It was, therefore, unlikely that they could directly contribute significantly to the production of steroids, even it is possible that astrocytes exert a positive influence on the differentiation [25] and on the steroidogenic activity of oligodendrocytes.

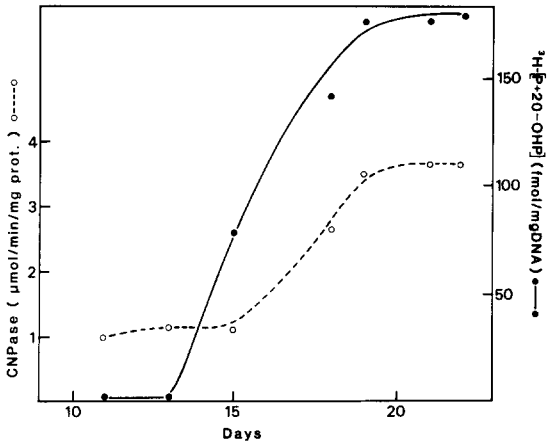

Fig. 3. Ontogenesis of oligodendrocyte differentiation and steroid biosynthesis. Glial cells from the same primary culture were incubated with [^{3}H]MVA (1 μCi/ml), after different culture intervals from 10 to 23 days. After a 16 h incubation with the radioactive precursor, cells were recovered, homogenized and aliquots taken for DNA, protein and CNPase measurements. The remaining homogenates and the incubation media were extracted and the extracts analyzed on TLC. The mean values of triplicate dishes are indicated.

Aminoglutethimide (AG) is a potent inhibitor of P-450_{scc}. When adrenal cells are incubated with AG, cholesterol accumulates in mitochondria, and is available for side-chain cleavage when the inhibitor is removed [26]. We have obtained similar results with newborn rat glial cells after 3 weeks of culture. During the preincubation of primary cultures with [^{3}H]MVA for 48 h in the presence of AG, glial cells accumulated [^{3}H]cholesterol [27]. The culture medium was removed and the cells were further incubated for 16 h in the presence of dibutyryl (db) cAMP and trilostane, but with neither mevinolin nor [^{3}H]MVA. After the release of AG blockade, [^{3}H]20-OH P was formed exclusively in the culture medium, whereas [^{3}H]cholesterol was formed in the cells and represented $\sim$10% of the radioactivity in cell extracts. The formation of [^{3}H]20-OH P was

time-dependent, and in the 1–16 h interval, the [^{3}H]20-OH P/[^{3}H]cholesterol ratio increased from 0.5 to 10.4%. Addition of dexamethasone to the culture medium for 64 h resulted in a 30% increased rate of [^{3}H]20-OH P synthesis, contrasting with a large inhibition of intracellular [^{3}H]cholesterol. Thus, the 20-OH P/cholesterol ratio was increased 2-fold by dexamethasone. Glucocorticosteroids were previously reported to enhance cAMP production and steroid synthesis by ovine adrenocortical cells in primary culture [28].

AG was also given *in vivo* (20 mg/kg) to 11-week-old male rats of the Sprague–Dawley strain. At the same time, rats received dexamethasone to prevent endogenous ACTH release. The degree of adrenal *P*-450 inhibition was monitored at frequent intervals by measuring plasma corticosterone 30 min after a bolus of 1–24 ACTH. The concentrations of P were measured in the brain of control animals (under dexamethasone + ACTH) and in AG treated rats [Fig. 4(a)]. Simultaneous inhibition of peripheral and brain P production was rapidly followed by a 5-fold decrease of P in brain, thus indicating that brain P undergoes a rapid turnover.

To confirm that the P lowering effect was related to the inhibition of $P\text{-}450_{scc}$ in brain, AG was also injected intracisternally to male CD rats (20 mg/kg in 10 μl of saline). Vehicle injected controls and AG treated rats were killed after 5 h, then P and D were measured in brain [Fig. 4(b)]. The concentrations of both steroids were about 4-fold lower in AG treated rats than in controls.

Although they need confirmation, the experiments with AG show that brain P undergoes a rapid turnover and that $P\text{-}450_{scc}$ activity in the brain seems necessary to maintain the cerebral accumulation of neurosteroids.

The concentrations of P and D in human brain removed at autopsy 5–24 h post-mortem were reported previously [7, 8]. The values for P, D and their sulfate esters were much higher than those of the sex steroid hormones in all areas of the brain and in the same range as those reported in the rat. Hence, it is tempting to speculate that human brain is capable of *de novo* biosynthesis of steroids. We have detected, with the immunoperoxidase technique, $P\text{-}450_{scc}$, adrenodoxin and adrenodoxin reductase in human brain. They were co-localized in the white matter of the cerebellum, removed at autopsy from a male cadaver, 7 h post-mortem [29].

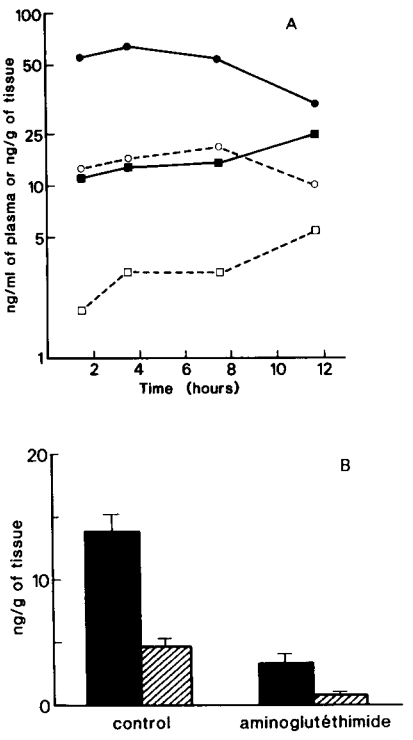

Fig. 4. Effect of AG on P concentrations in brain and plasma. *(A) Subcutaneous injection.* Dexamethasone and AG (AG+, 10 mg/kg) or DMSO (AG−) were injected at time 0. AG+: □ plasma; ■ brain. AG−: ○ plasma; ● brain. *(B) Intracisternal injection.* AG was injected intracisternally (10 μg in 10 μl saline.). P and D concentrations were measured 5 h after the injection, controls received 10 μl of saline by the same route.

4. P METABOLISM IN THE BRAIN

Preliminary experiments where rat brain minces had been incubated with [^{3}H]P (0.6 μM) yielded [^{3}H]progesterone ([^{3}H]PRO) in accordance with previously published results [30]. However, the cell types containing the 5-ene-3β-hydroxysteroid dehydrogenase-isomerase enzyme responsible for the conversion of P to PRO were unknown.

Therefore, we have incubated primary glial cell cultures (containing a mixed population of astrocytes and oligodendrocytes) (see Section 3), with [^{3}H]P in a concentration (65 pmol/ml) corresponding to the average amount of P found in adult rat brain, for 24 h in the presence of db cAMP but without trilostane. After the incubation, extracts from cell and media were analyzed by TLC [22]. Among the radioactive metabolites observed we found a radioactive compound with the R_f of PRO, representing about 3% of the total radioactivity. [^{3}H]PRO was identified by reverse-phase HPLC

and by crystallization after reverse isotopic dilution.

Further metabolism to 5α-pregnane-3,20-dione (pregnanedione) and to 3α-hydroxy-5α-pregnane-20-dione (pregnanolone), was observed. They were identified by HPLC and by crystallization after reverse isotopic dilution. Other polar and nonpolar metabolites were also found. The nonpolar fraction was analyzed after saponification: about 2/3 of its radioactivity migrated as authentic P on TLC. The polar fraction included [^{3}H]20-OH P and an abundant more polar metabolite of P.

P metabolism by astroglial cells has also been investigated. Cells were mechanically dissociated from the cerebral cortex or striatum of 17-day rat embryos. Selective culture conditions were used. They allow the growth of astroglial cells (mainly type 1 astrocytes) and contain no neurons, no fibroblasts, no oligodendrocytes and few microglia. After 3 weeks of culture, the cells were incubated with 100 nM [^{3}H]P for 24 h, without or with 5α-reductase and 5-ene-3β hydroxysteroid dehydrogenase inhibitors. The steroids were extracted and separated by TLC. Further characterization of radioactive PRO, pregnanedione, pregnanolone and 20-OH P was performed by reverse-phase HPLC followed by crystallization after reverse isotopic dilution. Astroglial cells from cerebral cortex and from striatum metabolized [^{3}H]P to [^{3}H]PRO, pregnanedione and [^{3}H]pregnanolone. Cells from cerebral cortex tended to be more active than those from striatum. The amounts of PRO and its metabolites formed were about 4-fold smaller than in newborn rat glial cell cultures. The 5α-reductase inhibitor completely suppressed pregnanedione and pregnanolone.

Dissociated brain cells from 17-day mouse embryos were also cultured in media that only allow the growth of neurons (MEM/FR medium supplemented with insulin, transferrin, PRO, putrescine and selenium). On the 5th day of culture, they were incubated with [^{14}C]P for 24 h. The metabolites were separated by two-dimensional TLC (benzene–methanol 19:1, followed by cyclohexane-ethyl acetate 4:6). The TLC were then submitted to autoradiography. The extracts of cells and media contained no radioactive compound with the migration of PRO or its 5α-reduced metabolites. The only compound identified was 20-OH P that represented 3.2% of the incubated [^{14}C]P, almost exclusively in the medium.

The elusive 17,20-lyase

We have devoted major efforts to the elucidation of D formation in the brain, since indirect evidence, reviewed in Section 2, indicated that the accumulation of D in mammalian brain appeared independent of peripheral steroidogenic glands.

However, incubations of [^{3}H]P with brain slices, homogenates and microsomes, or with primary cultures of mixed glial cells or astrocytes and neurons of rat or mouse embryos, never produced a radioactive metabolite with the chromatographic behavior of [^{3}H]D. As we were aware of a report indicating that [^{3}H]PRO was converted to androgens by selected brain areas of the adult male cat [31], we decided to use [^{14}C]PRO as substrate for the investigation of 17α-hydroxylase, 17,20-lyase activity (P-$450_{17\alpha}$) in rat brain microsomes. As boiled microsomes, or microsomes where P-450 cytochromes had been inactivated by carbon monoxide, or intact microsomes incubated without cofactors, contrary to incubations in the absence of microsomes, gave similar yields of [^{14}C](A) androstenedione from [^{14}C]P, with a large increase at pH 10.4 vs pH 7.4, we concluded that no evidence for P-$450_{17\alpha}$ activity was available in the rodent brain. A similar artifactual conversion of 17α-OH PRO to A has been discussed by Karavolas *et al.* [32]. Moreover, mixed cultures of newborn rat glial cells were incubated with [^{14}C]PRO, for 24 h in the presence of 5α-reductase inhibitor. No metabolite with the chromatographic properties of A was observed. Finally, all attempts to demonstrate the P-$450_{17\alpha}$ antigen immunohistochemically in rat brain with antibodies to the enzyme purified from pig testis (kindly donated by I. Mason), and in guinea pig brain with specific antibodies to the enzyme from guinea pig adrenal (kindly donated by S. Takemori) [33], were unsuccessful.

Other metabolic transformation of steroids in the brain

Several hydroxysteroid dehydrogenase activities have been described in brain. We have observed a prominent 20α-hydroxysteroid dehydrogenase activity in newborn rat glial cell cultures, with P or PRO as substrates [22]. The 17β-hydroxysteroid dehydrogenase activity is of particular interest. It may convert D to its metabolite DIOL, which has weak estrogenic potency [34, 35]. However, our attempts to measure DIOL concentrations in male rat brain

with a specific RIA have been unsuccessful so far. Also, no DIOL formation was observed in our incubations of brain slices or homogenates with radioactive D, in the absence of added cofactors.

D is converted to a very polar metabolite by rat brain microsomes. Incubation of microsomes with [4-^{14}C, 7β ^{2}H]D or [4-^{14}C, 16^{2}H]P yielded the corresponding 7α-hydroxylated metabolites, that were characterized by TLC, HPLC and reverse isotopic dilution, and identified by GC–MS (R. Morfin and Y. Akwa, unpublished data). When 0.5 μM substrates were incubated in phosphate buffer at pH 7.4 with 0.5 mM NADPH as cofactor, the yield of 7α-OH DHA was 260 pmol/mg protein/h and the yield of 7α-OH P was 90 pmol/mg protein/h. No formation of these metabolites was observed with heat inactivated microsomes, microsomes flushed with carbon monoxide or in the absence of cofactor. Primary cultures of newborn rat glial cells, and astroglial cell cultures from 18-day embryonic cerebral hemispheres converted D and P to large amounts of the respective 7α-OH metabolites. Neurons cultured from 16 day embryonic cerebral hemispheres also formed small amounts of tentatively identified 7α-metabolites. The formation of 7α-OH metabolites of D and P was abolished when the cultures were flushed with carbon monoxide or incubated with 5 μM estradiol. These results are in accordance with the presence of a major species of cytochromes *P*-450 in rat brain microsomes, previously identified as 5α-androstane-3β,17β-diol hydroxylase [36].

5. FUNCTION OF NEUROSTEROIDS

P decreases in olfactory bulb (OB) of male (M) rats exposed to female (F) siblings

Young adult Sprague–Dawley M rats were exposed for 7 days to the scent of a group of cycling F rats, in the absence of visual or tactile communication (M/F). Controls were M rats similarly exposed to other M rats (M/M). They were killed at 2 p.m. and the OB, amygdala (AMY) and hypothalami (HYP) were collected, as well as the remaining brain, the plasma, spleen and adrenals. P and D were measured by the combined solvolytic procedure, and T in the neutral ethyl acetate extract. Heterosexual exposure resulted in a significant decrease of P concentrations in OB and to a lesser extent in HYP, but not in any other tissue sample [37], thus confirming previously published results [38, 39]. The concentrations of T in the brain structures, plasma, spleen and adrenals were either slightly increased or unchanged, as were those of D.

In M rats castrated 7 days before sacrifice (ORX), T decreased to undetectable levels in plasma and brain. The P concentration in brain decreased to 50% of controls. Exposure to the scent of F rats (ORX/F) produced no further decrease in P concentrations in any tissue investigated. D was unaffected. Injection of T to ORX males, so as to bring about supranormal concentrations of T in plasma and brain, reestablished concentrations of P in OB, AMY and HYP in excess of those of intact M rats of the M/M groups, and the decrease of P in the OB of the ORXT/F group was observed as in intact M rats. Finally, the pheromonal message of F rats was related to estrus and was not perceived after the F rats were spayed.

Therefore, changes in the local concentrations of P in a specific area related to olfaction obey signals such as pheromones, and could be somehow related to the normal mechanisms of heterosexual behavior.

D inhibits the aggressive behavior of castrated M mice

Group-housed triads of castrated M mice attack lactating F intruders. Previous reports indicated that T, E_2 and D inhibit this aggressive behavior [40, 41]. The tranformation of injected D into brain T is very small but statistically significant. To completely eliminate the possibility that the activity of D was related to its conversion into the more active molecules T and E_2, behavioral experiments were repeated with the D analog 3β-methyl-androst-5-en-17-one (CH_3-D). This molecule cannot be metabolized into sex steroids and is not demonstrably estrogenic in rodents. Nevertheless, it inhibited the aggressive behavior of castrated mice at least as efficiently as D itself [9]. The effects of D and CH_3-D injections on the levels of neurosteroids in mouse brain have been investigated (Fig. 5). Unexpectedly, the only significant change common to both is a marked decrease of PS. How this change relates to the inhibition of aggression requires further investigation.

Neurosteroids and GABA$_A$ receptors

The $GABA_A$ receptor is an oligomeric protein complex which, when activated by agonists,

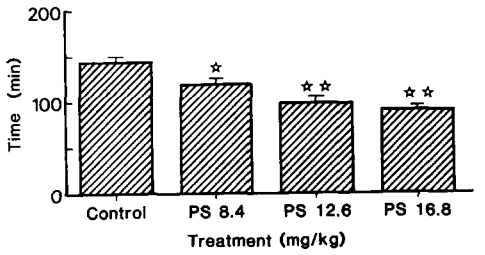

Fig. 5. Effect of i.p.-injected PS on pentobarbital-induced sleeping time. Fischer-344 rats received pentobarbital (45 mg/kg, i.p.). 8.4, 12.6 and 16.8 mg/kg of PS were injected in 5 ml of 0.9% saline, pH 7.0 (for each group $n = 5$). Control rats received 5 ml of 0.9% saline ($n = 8$). Values represent mean ± SEM, significantly different from control by $P < 0.05$ (☆) and $P < 0.01$ (☆☆) by one-way ANOVA and Dunnett's test.

produces an increase in neuronal membrane conductance to Cl^- ions, resulting in membrane hyperpolarization and reduced neuronal excitability. A number of centrally active drugs, including convulsants, anticonvulsants, anesthetics and anxyolitics, bind to distinct, but interacting domains of this receptor complex, to modulate Cl^- conductance [42, 43]. There are inhibitory and excitatory steroids interacting with the $GABA_A$ receptor complex in nerve cell membranes, at distinct sites.

The inhibitory steroid metabolites, such as tetrahydrodeoxycorticosterone and pregnanolone, both mimic and enhance the effects of GABA. These steroids potentiate both benzodiazepine and muscimol binding, whereas they inhibit the binding of the convulsant *t*-butylbicyclophosphorothionate (TBPS), in a manner similar to barbiturates [44, 45]. Such actions of reduced metabolites of PRO and deoxycorticosterone correspond with their reported hypnotic/anesthetic properties.

The potentiating and direct actions of both steroids were expressed in a human cell line transfected with the $\beta 1$, $\alpha 1$ and $\alpha 1\beta 1\gamma 2$ combinations of human $GABA_A$ receptor subunits [46]. Although the minimal structural requirement for expressing steroid and barbiturate actions are the same, the mechanism of $GABA_A$ receptor modulation by pregnane steroids may differ from that of barbiturates.

PS, at micromolar concentrations, interacts with the $GABA_A$ receptor complex as a picrotoxin-like antagonist [47]. PS binds to a convulsant picrotoxin-TBPS recognition site and inhibits pentobarbital-enhanced benzodiazepine binding and GABA agonist-stimulated Cl^- uptake into synaptoneurosomes. DS, PS and D (in order of decreasing activity), also counteract GABA-activated Cl^- conductance in cultures of neonatal rat cortical neurons [48].

The convulsant properties of PS are consistent with the results of *in vivo* experiments. PS stereotaxically injected into the lateral ventricle of pentobarbital anesthetized rats, significantly shortened the sleep-time [49a] (Fig. 5).

6. THE MECHANISM(S) OF ACTION OF NEUROSTEROIDS

The 3β-hydroxy-5-ene steroids accumulated in brain may be precursors of corresponding 4-ene-3 keto-steroid "classical" hormones. We have shown the transformation of P into PRO and observed the one of D into A. The cell culture experiments have indicated that such a conversion occurs in mixed glial cell cultures and in astroglial cell cultures, but not in cultures of neurons. Locally formed PRO, if it has a physiological role, may act paracrinally or autocrinally. Indeed, glial cells contain a PRO receptor (PR), inducible by estrogens [49b] (I. Jung-Testas and M. Renoir, in preparation). The PR has already been detected in the HYP, cortex and meningiomas. The concentration of PRO in the brain of young adult male rats is not negligible (about 1–2 ng/g of tissue) [39]. However, the contribution of brain synthesis to it is yet unknown, and there is no report of PRO distribution in castrated-adrenalectomized animals.

P and D do not bind to any known *intracellular receptor*. Low affinity P binding proteins have been described in other tissues of the rat and the guinea pig [50, 51]. The concentration of P in brain is quite compatible with binding to such protein(s). A heat stable P binding protein has been found in brain cytosol (D. Zucman, unpublished). The K_d for P is ~80 nM. The relative binding affinities of the heated protein are: P (100%), 3β-hydroxy-5α-pregnane-20-one (68%), pregn-5-ene-3β,20α-diol (5%), PRO (42%), D (20%) and pregnanedione (10%). Neither DS nor cortisol, corticosterone, T and E_2 bind. P binding sites have also been described in the rat OB [52]. The concentration of binding sites measured in total brain cytosol is larger than that expected for a receptor. The relevance of this protein to the mechanims of accumulation of P and D in brain is under current investigation. Its properties are different of those previously described for P, oxysterol and fatty acid binding proteins.

Neurosteroids may bind to a *membrane receptor*. The only well-documented case for such an interaction of steroid hormones at the

cell surface is the initiation of meiosis of *Xenopus laevis* oocytes by PRO [53]. Photoaffinity labeling experiments have demonstrated a specific ~30 kDa binder [54].

Adult rat brain synaptosomal membranes have been prepared according to a modification of the method of Towle and Sze [55]. A glass fiber filter binding assay was used (M. J. Sancho, unpublished results). The optimal binding conditions with [^{3}H]DS as ligands were obtained at pH 5.2, 23°C for 8 h. The dissociation constants of DS and PS were 0.8 and 2.0 μM, respectively. The binding component was destroyed by heat and by proteinase K. Binding was specific for steroid sulfates, with a relatively narrow structural requirement. Several ligands of the $GABA_A$ receptor did not displace [^{3}H]DS from its binding site.

The $GABA_A$ receptor complex may be the synaptosomal target for naturally occurring neurosteroids. P is synthetized in oligodendrocytes and brain sulfokinase activity has been reported, even the type of cells involved is not yet established. The conversion of P to PRO was observed in mixed glial and astroglial cell cultures, and PRO can in turn be converted to its tetrahydrogenated derivative pregnanolone, consistent with the preferential localization of 5α-reductase activity in white matter [56]. PS and pregnanolone, as indicated above, have opposite effects on $GABA_A$ receptors, and seem to interact with distinct binding sites on the receptor complex (Fig. 6). Therefore, P metabolites in brain as well as steroids of extraencephalic sources may be involved physiologically in $GABA_A$ receptor function.

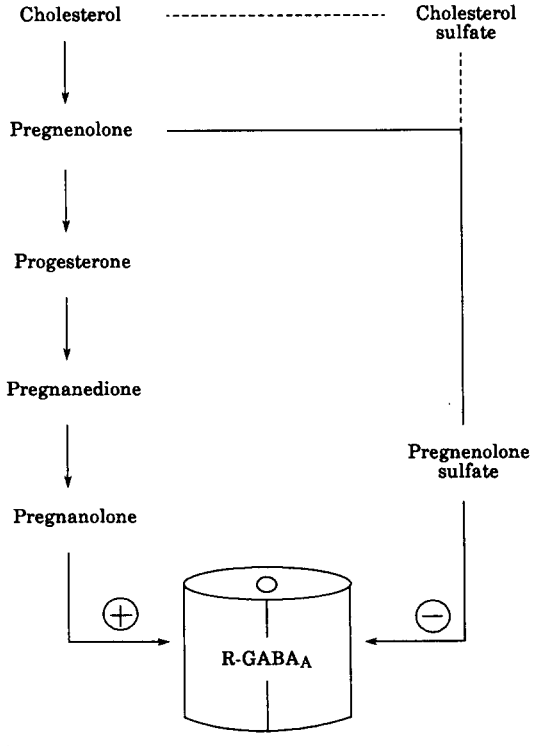

Fig. 6. Schematic representation of the modulation of $GABA_A$ receptor by neurosteroids.

7. CONCLUSIONS

P, D and their sulfate and fatty acid esters accumulate in the rat brain (and in the brain of several other mammalian species including the human). Endocrine manipulations and behavioral experiments have indicated that the accumulation of these steroids in brain is, at least for a large part, independent of steroidogenic gland contribution. Their concentrations appear to be controlled by mechanisms proper to the brain, which in turn may depend, in part, upon gonadal steroid hormones. Immunocytochemical and biochemical evidence has accrued for the steroidogenic cytochrome $P\text{-}450_{scc}$ complex in rat brain oligodendrocytes, thus accounting for P formation. The origin of D, however, remains obscure. The functional significance of these observations needs further investigations, although the data at hand suggest an autocrine and/or paracrine function of neurosteroids.

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