



Neurosteroids: Oligodendrocyte mitochondria convert cholesterol to pregnenolone

(rat brain/glial cells/ Δ^5 - 3β -hydroxysteroids/cytochrome P-450_{sc})

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ABSTRACT Oligodendrocyte mitochondria from 21-day-old Sprague–Dawley male rats were incubated with 100 nM [3 H]cholesterol. It yielded [3 H]pregnenolone at a rate of 2.5 ± 0.7 and 5-[3 H]pregnene- 3β , 20α -diol at a rate of 2.5 ± 1.1 pmol per mg of protein per hr. Cultures of glial cells from 19- to 21-day-old fetuses (a mixed population of astrocytes and oligodendrocytes) were incubated for 24 hr with [3 H]mevalonolactone. [3 H]Cholesterol, [3 H]pregnenolone, and 5-[3 H]pregnene- 3β , 20α -diol were characterized in cellular extracts. The formation of the 3 H-labeled steroids was increased by dibutyl cAMP (0.2 mM) added to the culture medium. The active cholesterol side-chain cleavage mechanism, recently suggested immunohistochemically and already observed in cultures of C6 glioma cells, reinforces the concept of “neurosteroids” applied to Δ^5 - 3β -hydroxysteroids previously isolated from brain.

We have characterized two Δ^5 - 3β -hydroxysteroid metabolites of cholesterol, pregnenolone (3β -hydroxy-5-pregnen-20-one) and dehydroepiandrosterone (3β -hydroxy-5-androstene-17-one), in the brain of several mammalian species (rat, mouse, monkey, and occasionally pig and human). Definitive identification of the steroid moiety was made in rat brain extracts by gas/liquid chromatography–mass spectrometry (1–3). Pregnenolone has also been identified in acetone powder of rabbit brain by its mass spectrum (4). The Δ^5 - 3β -hydroxysteroids persisted in brain after removal of steroidogenic organs, and we therefore proposed that their formation or accumulation in the rat brain depends on *in situ* mechanisms unrelated to the peripheral endocrine gland system.

However, both S. Lieberman’s and our group were unable up to now to conclusively demonstrate the biosynthesis of pregnenolone in brain (3, 4). Pregnenolone is the key steroid synthesized from cholesterol in steroidogenic glands (5, 6). The oxidative side-chain cleavage of cholesterol is operated by a specific enzyme complex, which includes cytochrome P-450_{sc} (7). We have used specific antibodies to the bovine adrenal P-450 for the immunohistochemical localization of the enzyme in the adult rat brain, and we have found that the white matter was selectively immunostained throughout the brain (8). 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]pregnenolone and its reduced derivative 5-[3 H]pregnene- 3β , 20α -diol. In addition, cultures of fetal glial cells, incubated with [3 H]mevalonate, produced [3 H]cholesterol, [3 H]pregnenolone, and 5-[3 H]pregnene- 3β , 20α -diol.

MATERIALS AND METHODS

Preparation of Oligodendrocytes. Twenty-one-day-old male Sprague–Dawley rats (CD1 strain; Iffa Credo, L’Arbresle, France), kept under a 12-hr light/12-hr dark schedule, were killed by decapitation 1 or 2 hr after lights were turned on. Adrenals and the anterior part of the brain (excluding the cerebellum, the pons, and the olfactory bulbs) were quickly removed. Brains of 20 rats were collected in Hanks’ balanced salt solution (without Ca^{2+} and Mg^{2+}) containing penicillin and streptomycin, buffered with Hepes (1 mM), and adjusted to pH 7.4 with sodium bicarbonate. The method of Lisak *et al.* (9), as modified by Hiramaya *et al.* (10), was used. Briefly, brain minces were trypsinized (0.1% for 30 min at 37°C), followed by centrifugation at $200 \times g$ for 5 min at 4°C and washing with Hanks’ balanced salt solution. The final cell suspension was filtered through a nylon filter (mesh, 63 μm) to remove debris and blood vessels. A Percoll gradient centrifugation was then performed at 1500 rpm in a Sorvall SW 27 rotor for 45 min at 4°C. The upper layer containing myelin was carefully aspirated, while the lower layer and the pellet containing cell debris and erythrocytes were discarded. The intermediate layer, enriched in oligodendrocytes, was diluted with Hanks’ balanced salt solution and recentrifuged three times at $200 \times g$ for 5 min. Cells were counted in a hemocytometer. Under phase contrast, the oligodendrocytes appeared as small birefringent cells. The main contaminants were large cell nuclei.

Preparation of Mitochondria and Incubation with [3 H]Cholesterol. Oligodendrocytes or adrenals were homogenized in 0.25 M sucrose/0.5 mM EDTA (K^+)/10 mM Tris-HCl, pH 7.4, at 4°C. Mitochondria were prepared by differential centrifugation (11). The conditions described by Mason *et al.* (12) were used for side-chain cleavage of cholesterol. Mitochondria were resuspended in a solution containing 0.25 M sucrose, 20 mM KCl, 10 mM K_2HPO_4 , 5 mM MgCl_2 , 15 mM triethanolamine, and 19 μM trilostane (Sterling-Winthrop, Paris). The latter is a competitive inhibitor of 3β -hydroxysteroid dehydrogenase activity, which might decrease pregnenolone levels in our experiments. The final protein concentration was 0.8 mg/ml for brain (corresponding to $\approx 30 \times 10^6$ cells) and 1–2 mg/ml for adrenal mitochondria. [3 H]Cholesterol was added (5×10^6 cpm, 0.1 nmol) after suspension in 0.1 ml of Tris buffer (pH 7.4) containing 20 μg of Tween 80 (13). The reaction was initiated by the addition of 10 mM isocitrate to generate NADPH. Alternatively, a NADPH-generating system was used: 0.75 mg of NADP^+ , 2.5 mg of glucose 6-phosphate, 0.5 unit of glucose-6-phosphate dehydrogenase (from Bakers’ yeast; 260 units per mg of protein; Sigma), 95 μg of MgCl_2 in a total vol of 0.1 ml of phosphate buffer (0.1 M) (pH 7.4). The reaction was initiated by the

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Table 1. TLC of pregnenolone (I), pregnenolone acetate (Iac), 5-pregnene-3 β ,20 α -diol (IIa), 5-pregnene-3 β ,20 α -diol diacetate (IIa ac), 5-pregnene-3 β ,20 β -diol (IIb), and cholesterol (Ch^{ol})

	I	Iac	IIa	IIa ac	IIb	Ch ^{ol}
TLC1	0.38 \pm 0.03 (20)	ND	0.29 \pm 0.01 (20)	ND	0.28 \pm 0.01 (6)	0.59 \pm 0.04 (20)
TLC2	0.51 \pm 0.02 (6)	0.82 \pm 0.02 (7)	0.31 \pm 0.01 (6)	0.84 \pm 0.01 (7)	0.33 \pm 0.02 (6)	0.57 \pm 0.03 (6)
TLC3	0.10 \pm 0.01 (6)	0.37 \pm 0.07 (6)	0.05 \pm 0.04 (5)	0.48 \pm 0.02 (6)	0.06 \pm 0.01 (6)	0.23 \pm 0.02 (6)

Chromatograms were on silica gel F-254 plates. TLC1, cyclohexane/ethyl acetate (3:2); TLC2, benzene/ethanol (9:1); TLC3, cyclohexane/ethyl acetate (8:2). R_f , mean \pm SD (number of determinations). ND, not done.

addition of the generating system warmed at room temperature 5 min before use.

The final vol of the incubation mixture (adjusted to pH 7.4) was 1 ml, and 0.25-ml samples were removed at 0, 30, 60, and 120 min of incubation at 37°C under air. Each one was diluted to 2 ml with water at 4°C and extracted with ethyl acetate. The extract was taken to dryness under nitrogen and redissolved in 2 ml of benzene.

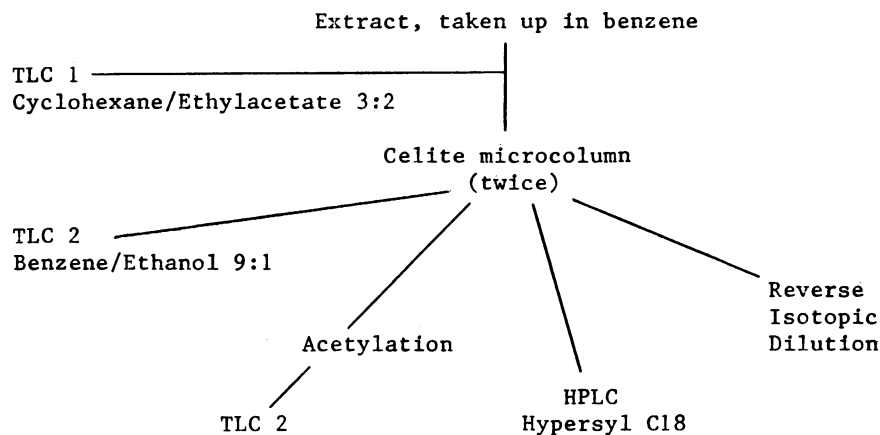
Cell Culture. Sprague-Dawley rats were killed between the 19th and the 21st day of pregnancy by decapitation. The fetuses of one or two females were removed under sterile conditions and placed in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM-FCS10), penicillin (100 units/ml), and streptomycin (100 μ g/ml). The cerebral hemispheres were dissected and the meninges and blood vessels were carefully removed. The brain tissue was collected in DMEM-FCS10, gently dispersed by repeated aspiration through a 10-ml disposable pipette, and passed through a nylon filter of 82- μ m mesh. The cells obtained from two hemispheres were seeded in 75-cm² culture flasks coated with poly-L-lysine hydrobromide (180 kDa; 29 μ g per ml of water; Sigma) (14). The cell viability was assessed by trypan blue exclusion at the time of plating and was \approx 95%. Cells were cultured in 15 ml of DMEM-FCS10 containing insulin (5 μ g/ml) in a humidified incubator at 37°C under 5% CO₂/95% air. The medium was replaced on days 1 and 6. On day 9, the cells were removed by trypsinization (0.25% trypsin and 3 mM EDTA in phosphate-buffered saline, PBS), pooled in DMEM-FCS10, and identical numbers of cells were plated in poly-L-lysine-coated Petri dishes (diameter, 100 mm). Each dish contained the cells corresponding to one cerebral hemisphere. The cultures were continued until day 20 in DMEM-FCS10, replacing culture medium every 3–4 days. Under these conditions, glial cells multiplied and reached confluency after \approx 3 days of secondary culture. The day before the experiments (day 21) FCS was replaced by 5% charcoal-treated calf serum (DMEM-CSCX) and this medium was used for incubating cells with the radioactive precursors.

Incubation of Cells with [³H]Mevalonolactone. This precursor of cellular cholesterol was used because it readily enters the cells. Each Petri dish, containing \approx 10⁶ glial cells, was

incubated for 24 hr with 5 ml of DMEM-CSCX containing 0.1, 1, or 10 μ Ci of [³H(N)]mevalonolactone per ml (New England Nuclear; 30 Ci/mmol; 1 Ci = 37 GBq), 20 μ M trilostane, and 20 μ M mevinolin (Merck Sharp & Dohme; an inhibitor of hydroxymethylglutaryl-CoA reductase, the key enzyme in mevalonate biosynthesis). When indicated, 0.2 mM dibutyryl cAMP was added. At the end of the incubation, the medium was decanted, the cells were washed twice with 0.5 ml of PBS, recovered by scraping with a rubber policeman, centrifuged, resuspended in 5 ml of PBS, and extracted with 5 ml of ethyl acetate three times. The extracts were taken to dryness under nitrogen and redissolved in 1 ml of benzene.

Characterization of [³H]Pregnenolone and 5-[³H]Pregnene-3 β ,20 α -diol. We have previously used celite microcolumn chromatography to isolate pregnenolone from brain (3). Propylene glycol (0.4 ml for 1 g of activated celite 535) was the stationary phase. Extracts were deposited onto the column in isooctane solution and eluted with isooctane/benzene (92:8) (vol/vol), pregnenolone passed in the 18- to 25-ml volume. Three thin-layer chromatography (TLC) systems were also used (Table 1), after addition of \approx 10 μ g of the corresponding reference compound. Pooled samples from TLC1 containing \approx 2500 dpm of either pregnenolone or 5-pregnene-3 β ,20 α -diol were acetylated with acetic anhydride/pyridine (1:1) at room temperature overnight. Pregnenolone acetate was rechromatographed with TLC2, whereas 5-pregnene-3 β ,20 α -diol diacetate was rechromatographed with TLC2 and -3. Further identification of pregnenolone was obtained by HPLC performed with a hypersyl C-18 column with a 50–80% gradient of methanol in water, and a flow rate of 1 ml/min. The retention time of the reference compound was 27.5 min. Finally, recrystallization after reverse isotopic dilution was performed to confirm the identity of pregnenolone and of 5-pregnene-3 β ,20 α -diol.

Other Methods. Proteins were measured by the method of Bradford using the Bio-Rad kit, and DNA was measured by the diphenylamine method of Burton. Adequate volumes of column chromatography fractions were taken to dryness, whereas TLC areas were scraped and directly counted in 5 ml

FIG. 1. Identification of [³H]pregnenolone.

of Pico-Fluor 15 (Packard) in a Tri-Carb 4660 spectrophotometer (Packard), with $\approx 50\%$ yield.

RESULTS

Characterization of Pregnenolone and of 5-Pregnene-3 β ,20 α -diol. Besides the partition chromatography on celite, the TLC with systems 1 and 2 and the reverse-phase HPLC, the tentatively identified [^3H]pregnenolone was acetylated and rechromatographed on TLC2 (Fig. 1). The R_f of the product was the same as that of authentic 3 β -acetoxy-5-pregnene-3-one. Finally, recrystallization after reverse isotopic dilution confirmed the identity of pregnenolone (Table 2), with an estimated degree of purity of 95%.

Due to the low amount of radioactive material and to the lack of radioactive reference compound, 5-[^3H]pregnene-3 β ,20 α -diol was tentatively identified by TLC1 and TLC2 (where it was slightly more polar than its 20 β -isomer 5-pregnene-3 β ,20 β -diol), and by acetylation and rechromatography on TLC3 (Fig. 2). The R_f of the product was the same as that of authentic 5-pregnene-3 β ,20 α -diol diacetate. Finally, recrystallization after reverse isotopic dilution confirmed the identity of 5-pregnene-3 β ,20 α -diol (Table 3) with an estimated degree of purity of 61%.

[^3H]Cholesterol Conversion to Pregnenolone by Oligodendrocyte Mitochondria. In three experiments, the conversion rate of [^3H]cholesterol to [^3H]pregnenolone by brain mitochondria was 2.5 ± 0.7 (mean \pm SD) pmol per mg of protein per hr. Cholesterol and pregnenolone represented 81–87% of total radioactivity. In two control experiments, the conversion rate by adrenal mitochondria was 14.5 and 15.2 pmol per mg of protein per hr, with cholesterol and pregnenolone representing 91–94% of total radioactivity. These results were not obtained under initial velocity conditions, the concentration of Tween 80 may need to be adjusted, and thus the 1–2% conversion of [^3H]cholesterol to [^3H]pregnenolone probably was suboptimal. Neither zero time controls nor boiled mitochondria formed any metabolite. A radioactive compound with the R_f of 5-pregnene-3 β ,20 α -diol was observed, with a conversion rate of 2.5 ± 1.2 pmol per mg of protein per hr. Radioactive metabolites more polar than pregnenolone and 5-pregnene-3 β ,20 α -diol were detected but not identified. Aminoglutethimide (500 μM), an inhibitor of cholesterol side-chain cleavage, completely counteracted the formation of pregnenolone, 5-pregnene-3 β ,20 α -diol, and of polar metabolites. When whole brain instead of oligodendrocytes was used to prepare mitochondria, no metabolite was found, even when the concentration of protein was increased to 5 mg/ml. Negative results were also obtained with kidney mitochondria. However, liver mitochondria metabolized [^3H]cholesterol to several compounds, including one with the R_f of pregnenolone on TLC1, with a conversion rate of 1.2 pmol per mg of protein per hr.

Table 2. Reverse isotopic dilution of [^3H]pregnenolone formed after incubation of oligodendrocyte mitochondria with [^3H]cholesterol

Solvents	Specific activities, dpm/mg		
	Crystals (C)	Mother liquors (M)	C/M ratio
Isooctane/benzene	1221	2526	0.49
Cyclohexane/ethylacetate	1139	1527	0.75
Methanol/water	1175	1138	1.03
Methanol/water	1113	1042	1.06
Theoretical	1174		

Pregnenolone (50 mg) was mixed with 58,710 dpm of putative [^3H]pregnenolone formed from [^3H]cholesterol.

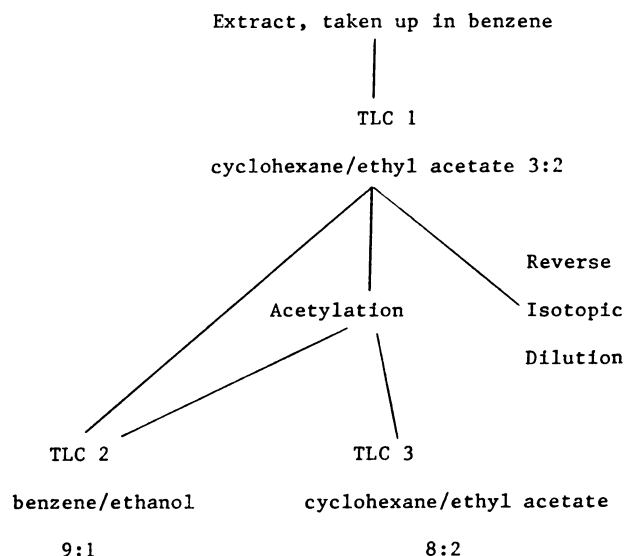


FIG. 2. Identification of 5-[^3H]pregnene-3 β ,20 α -diol.

[^3H]Cholesterol Conversion to 5-Pregnene-3 β ,20 α -diol by Oligodendrocyte mitochondria. To favor the formation of reduced metabolites by the mitochondrial preparations, a NADPH-generating system was used in two experiments. The conversion rate of [^3H]cholesterol to [^3H]pregnenolone was indeed decreased to 0.57 and 0.32 pmol per mg of protein per hr, whereas the conversion to 5-pregnene-3 β ,20 α -diol was 4.9 and 3.6 pmol per mg of protein per hr, respectively. Only traces of 5-pregnene-3 β ,20 β -diol were detected. Cholesterol, pregnenolone, and 5-pregnene-3 β ,20 α -diol represented 93–95% of total radioactivity. These results were not obtained under initial velocity conditions, and the incubation conditions may not have been optimal.

[^3H]Mevalonolactone Conversion to Pregnenolone and to 5-Pregnene-3 β ,20 α -diol by Glial Cell Cultures. Chromatography of cell extracts with TLC1 yielded several radioactive fractions (Fig. 2). The quantitatively major one migrated close to the solvent front. It did not release any pregnenolone or 5-pregnene-3 β ,20 α -diol after saponification. [^3H]Cholesterol was tentatively identified at R_f 0.58. Pregnenolone and 5-pregnene-3 β ,20 α -diol were characterized by their R_f with TLC1. Fractions of the eluates were acetylated, chromatographed with TLC systems 2 and 3, and migrated as authentic 3 β -acetoxy-5-pregnene-20-one and 5-pregnene-3 β ,20 α -diol diacetate. The percentage conversion of [^3H]mevalonolactone to combined pregnenolone and 5-pregnene-3 β ,20 α -diol was larger with the 0.1 $\mu\text{Ci/ml}$ than with the 1 $\mu\text{Ci/ml}$ concentration, but it was about the same when expressed in fmol per mg of DNA per 24 hr (Table 4). No steroids were detected with a 10 $\mu\text{Ci/ml}$ concentration of [^3H]mevalonolactone.

Table 3. Reverse isotopic dilution of 5-[^3H]pregnene-3 β ,20 α -diol formed after incubation of oligodendrocyte mitochondria with [^3H]cholesterol

Solvents	Specific activities, dpm/ng		
	Crystals (C)	Mother liquors (M)	C/M ratio
Ethylacetate/cyclohexane	3007	6850	0.44
Acetone/hexane	2255	3144	0.71
Ethylacetate/hexane	2393	2519	0.95
Acetone/cyclohexane	2313	2009	1.15
Theoretical	3792		

5-Pregnene-3 β ,20 α -diol (10 mg) was mixed with 37,920 dpm of putative 5-[^3H]pregnene-3 β ,20 α -diol formed from [^3H]cholesterol.

Table 4. [³H]Mevalonolactone (MVA) metabolism by glial cell cultures

	[³ H]MVA per incubation, pmol	Radioactivity in cell extract, % of [³ H]MVA in medium	Metabolites in cell extract			
			Ch ^{ol}	I	Ila	I + Ila, fmol per mg of DNA per 24 hr
			% total radioactivity			
Control	31	0.9	7.2	15.0	11.0	140
Bt ₂ cAMP	28	2.0	17.0	17.0	7.0	270
Control	168	0.9	9.4	1.9	2.7	130
Bt ₂ cAMP	175	1.0	6.8	6.7	0.7	250

The glial cells from 21-day-old rat fetal forebrains were cultured for 3 weeks as described in *Materials and Methods*. Fresh culture medium containing [³H]MVA at the concentrations indicated was then added for 48 hr and the cellular radioactivity was analyzed. Ch^{ol}, cholesterol; I, pregnenolone; Ila, 5-pregnene-3 β ,20 α -diol; Bt₂cAMP, dibutyl cAMP.

lactone. In cultures in which dibutyl cAMP was added to the culture medium, the conversion of [³H]mevalonolactone to combined pregnenolone and 5-pregnene-3 β ,20 α -diol was almost twice as large as in the control cultures. Compounds more polar than 5-pregnene-3 β ,20 α -diol were also observed, as in the experiments with oligodendrocyte mitochondria.

DISCUSSION

Pregnenolone has been previously detected and measured throughout the brain (2). Based on the results obtained after adrenalectomy and castration (2), and on the ontogenetic pattern of pregnenolone concentrations (3), we concluded that brain pregnenolone was apparently independent of peripheral sources. We attempted but failed to obtain the formation of radioactive pregnenolone after incubation of [³H]cholesterol or [³H]mevalonate with brain slices, homogenates, or mitochondria. The negative results were probably due to the relatively low number of oligodendrocytes in whole brain, and/or to the lack of access of radioactive precursor into mitochondria of cells embedded in myelin sheets. In fact, we had previously observed synthesis of pregnenolone by cultured rat C6 glioma cells incubated with mevalonate or cholesterol (3). The relevance of this finding to the physiological situation in normal glial cells, however, was uncertain. Incidentally, negative results were obtained with two neuroblastoma cell lines (neuro-2a and NOF) and with the mouse fibroblast L-919 cell line. Therefore, the immunohistochemical localization of P-450 and of adrenodoxin in the white matter was critical in designating oligodendrocytes as a cell type likely responsible for steroid production (8).

In this report, we show that preparations enriched in rat oligodendroglial cells can indeed perform the conversion of cholesterol to pregnenolone, to 5-pregnene-3 β ,20 α -diol, and to polar unidentified compound(s), likewise hydroxylated metabolite(s) of pregnenolone, thus showing that the side-chain cleavage mechanism is not restricted to steroidogenic endocrine glands and can occur in cells of the central nervous system. The characterization of the two identified steroids was based on chromatography in several systems, formation of acetate derivatives and rechromatography, and crystallization to constant specific activity after isotopic dilution. The presence of 20 α -hydroxysteroid dehydrogenase activity has been previously reported in rat and baboon brain (15).

The demonstration of *de novo* steroid biosynthesis by normal brain cells brings additional support to the term of "neurosteroids" applied to brain pregnenolone and dehydroepiandrosterone.

Pregnenolone is found in brain not only as the unconjugated steroid, but also as the sulfate ester and, quantitatively, mostly as fatty acid esters (3, 16). Since cholesterol sulfate and short-chain fatty acid esters can be substrates of the side-chain cleavage complexes (7), experiments will be necessary to evaluate their efficiency as precursors of pregnenolone in the brain.

In the adult male rat, the concentration of pregnenolone was of the order of 100 pmol per g of tissue (including the unconjugated, sulfate, and fatty acid ester forms), with larger amounts in the olfactory bulbs and hypothalamus, and smaller ones in the cerebral cortex, although within the same order of magnitude (3). Such generalized distribution can now be explained by a property of oligodendrocytes, whatever their localization in brain.

In oligodendrocytes, pregnenolone may undergo further metabolism to the C-19 steroid, side-chain cleaved dehydroepiandrosterone, and/or to progesterone and/or 5-pregnene-3 β ,20 α -diol, and/or to conjugates (sulfate and fatty acid esters). The demonstration of steroid formation is presently limited to the rat species. However, we know that pregnenolone persists in monkey brain after castration and adrenal suppression (16). Furthermore, immunoreactive cell bodies, likewise neurons, have been found in several rat brain areas related to olfactory pathways (8). Thus, steroidogenesis presumably may not be restricted to glial cells. How this observation relates to the variation of pregnenolone recorded in different parts of male rat brain exposed to the scent of either males or females (17) merits further investigation.

The physiological roles of pregnenolone as a neurosteroid and its mechanism(s) of action are still poorly understood. The conversion of pregnenolone to progesterone is already documented. Direct application of pregnenolone sulfate has been reported to change the firing rates of single neurons (3). Pregnenolone could bind to yet unsuspected receptor(s), intracellular or located at the membrane level. In favor of the last possibility, a recent report indicates that pregnenolone sulfate interacts with the γ -aminobutyric acid (GABA) receptor and behaves as an antagonist of GABAergic neurotransmission (18).

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