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NEUROSTEROIDS: A NEW BRAIN FUNCTION?

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Summary—The biosynthesis of neurosteroids proceeds through cholesterol side-chain cleavage, and gives rise to pregnenolone (P) and dehydroepiandrosterone (D). These steroids accumulate in the rat brain independently of the supply by peripheral endocrine glands. This led to the discovery of a steroid biosynthesis pathway in rat brain oligodendrocytes based on enzyme immunocytochemistry and conversion of radioactive precursors to C-21 steroids. Several biological functions have been proposed for P and D. They may serve as precursors of other steroids (such as progesterone and testosterone and their metabolites). They are implicated in the control of some behavioural activities. They have excitatory effects on neurons, and they modulate the function of $GABA_A$-receptors. These observations may apply to all mammalian species including the human, and the physiological significance of neurosteroid synthesis needs further investigation.

The relationship between steroids and cerebral function may be reconsidered in the light of a new fact: the existence of a biosynthetic pathway of these compounds from cholesterol, assured in the brain by the oligodendrocytes, glial cells which synthesize myelin.

INTRODUCTION

Steroid hormones are synthesized from cholesterol, in the adrenal gland for the gluco- and mineralocorticosteroids, and in the gonads and the placenta for those derivatives having a sexual influence [1]. Their lipophilicity explains their easy passage of the blood–brain barrier. At the cerebral level, steroid hormones influence the function of many nerve cells: the best known are the neurons which secrete "hypophysiotropic factors" stimulating the production of pituitary hormones such as ACTH and the gonadotropins. The neurons themselves are subject to a regulation (a feed-back control) by the corresponding steroid hormone. The mapping of intracellular steroid hormone receptors, obtained by autoradiography after administration of radioactive hormones or incubation of brain tissue slices and by immunohistochemistry using an anti-receptor antibody, has permitted a precise identification of the neuronal complexes implicated at the level of the hypothalamus (see Ref. [2] for a review). The mechanisms of steroid hormone action on mental, behavioural, and metabolic processes controlled by the brain remain little understood.

We have observed in the brain the presence of steroids and their synthesis from cholesterol, independent of endocrine gland function, and we have proposed the term *neurosteroids* to designate these compounds [3]. This brief review assembles the experimental arguments in favour of the synthesis of neurosteroids and the first observations of their activity.

Cerebral steroids unexplained by peripheral gland production

In the adrenal and genital glands, the first steroid formed from cholesterol is pregnenolone (P), whose 21-carbon structure results from the oxidative cleavage of cholesterol in the mitochondria. Cleavage of pregnenolone provokes the formation of dehydroepiandrosterone (D) (19-carbon). P and D conserve the $\Delta 5\text{-}3\beta$ hydroxylated structure of cholesterol (Fig. 1).

We have shown the presence of P and D in the rat brain, at concentrations superior to those of blood plasma, as opposed to corticosterone and testosterone whose plasma concentrations are greatly superior to cerebral concentrations [4, 5]. The concentrations of D are not affected by the administration of ACTH or adrenal inhibition by dexamethasone and cerebral P and D are subject to circadian variations shifted with respect to those of the adrenal steroids [6]. P is already present at a high concentration during the post-natal period, which in the rat is characterized by an almost complete inactivity of the adrenal glands. An argument (albeit an indirect one) which is particularly important in

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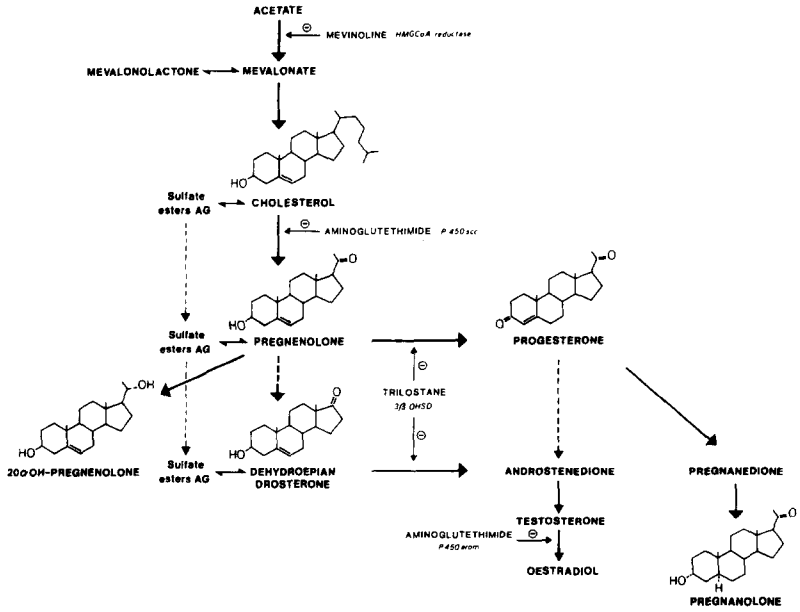


Fig. 1. Biosynthesis of cerebral steroids. The solid arrows indicate those metabolic conversions demonstrated. The role of 3β-hydroxy-3β-methyl glutaryl-coenzyme A reductase (HMG-Co-A reductase) in cholesterol synthesis by glial cells is documented elsewhere. HMG-Co-A reductase is inhibited by mevinoline, cytochrome $P450_{scc}$ by aminoglutethimide, and Δ5-3β-hydroxysteroid dehydrogenase-isomerase by trilostane. The conversion P → D is postulated but not demonstrated.

the support of the cerebral synthesis of P and D, is obtained after castration and adrenalectomy: P and D persist in the brain despite several weeks of peripheral hormone deficit, in contrast with testosterone of testicular origin which rapidly disappears. That it is not a question of cerebral storage seems indicated both by the administration of radioactive P and D whose rapid entry and exit of the cerebral compartment has been shown and by the administration of aminoglutethimide which, inhibiting the synthesis of pregnenolone, causes a decline in its concentration in the brain: the cerebral clearance of neurosteroids seems very rapid, implying a high renewal rate [6–8].

P and D are present in the brain in the forms of non-conjugated steroids, sulfate esters (S), and fatty acid esters (lipoidal derivatives, L). The concentrations of these different derivatives have been measured in young male and female adult rat brains (Table 1). The measurements were made 10 h after the beginning of illumination, close to the acrophase of circadian variations [9]. The values correspond to average concentrations to the order of 10^{-8} and even 10^{-7} M which, given the highly probable compartmentalization of the steroid, implies the existence of greatly elevated regional concentrations.

Table 1. Concentrations of neurosteroids pregnenolone (P), dehydroepiandrosterone (D) and their conjugates (S: sulfate ester, and L: lipoidic, fatty acid ester) in the rat brain

	P	PS	PL	DS
Males	25 ± 8	19 ± 6	46 ± 14	2.1 ± 0.5
Females				
Diestrus	32 ± 15	19 ± 6	46 ± 19	1.7 ± 0.4
Proestrus	27 ± 14	26 ± 6	50 ± 17	2.2 ± 0.4

The rats (Sprague–Dawley, strain Ofa (Iffa Credo, L'Arbresle) were sacrificed at the age of 11 weeks. The measures were carried out in whole brain and the results are expressed in ng/g (mean ± SD, $n = 9$ or 10). The Student's t-test showed significant differences for PS, DS, and DL depending on the stage of the estral cycle.

STEROIDOGENESIS BY OLIGODENDROCYTES AND NEUROSTEROID METABOLISM

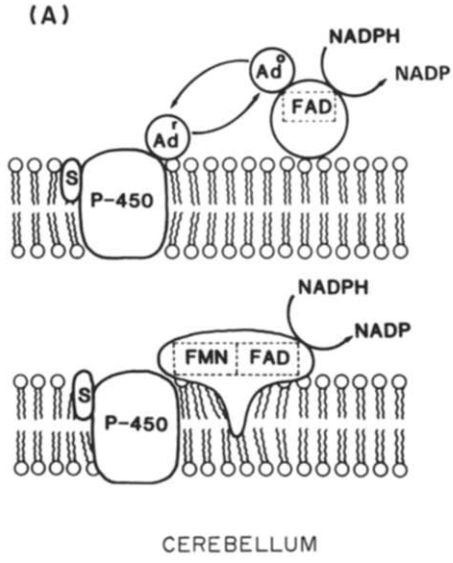
The demonstration of pregnonolone synthesized from cholesterol by a neural formation was very difficult. Experiments using tissue slices, homogenates, and subcellular fractions obtained from whole brain were negative for various reasons including cellular heterogeneity (supposing that the synthesis is limited to certain cells), the high level of endogenous cholesterol (diluting the radioactivity of the tracer-precursor intended to label P), and the difficult access to the enzymes of steroidogenesis (partition of tracer-precursor in the lipids).

Although the steroid biochemistry experiments failed, an immunohistochemical study to localize the enzymes implicated in brain cell steroidogenesis was successful. The enzymatic complex assuring the formation of P from cholesterol associates cytochrome $P450_{scc}$ (for

side-chain cleavage), adrenodoxine and adrenodoxine reductase (Fig. 2).

We have used serum immunoglobulins from rabbits immunized with cytochrome $P450_{scc}$ of bovine and rat adrenal glands. These antibodies recognize cytochrome $P450_{scc}$ in the steroidogenic cells of the adrenal gland, the ovary, and the testicle of the rat. In the brain of this animal, it was not easy to reunite the criteria of specificity of the immunohistochemical reaction. One must keep in mind the possibility of detecting one or several epitopes shared by the enzyme sought and other cerebral constituents. An immunological approach alone is in no way sufficient to prove the existence of an enzymatic activity. Nonetheless, this approach has indicated that, in a first approximation, $P450_{scc}$ is found in the white matter, wherever it is found [10].

The white matter is made up of nerve fibers wrapped in a myelin sheath, and we proposed that the cells which fabricate myelin, the oligodendrocytes, could also produce steroids, which is also suggested by immunohistochemistry [11]. There exist selective techniques to isolate oligodendrocytes, and



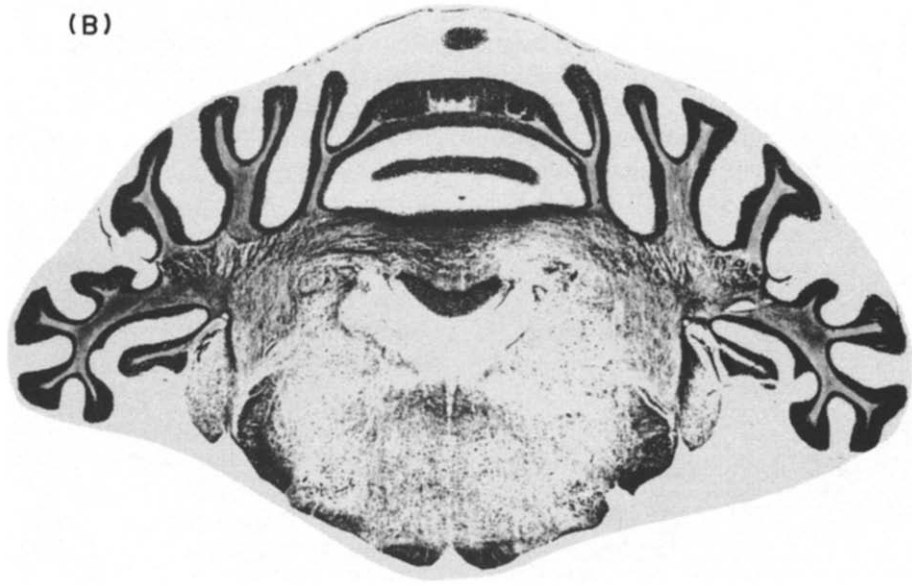


Fig. 2(A) and 2(B)

Fig. 2 continued overleaf.

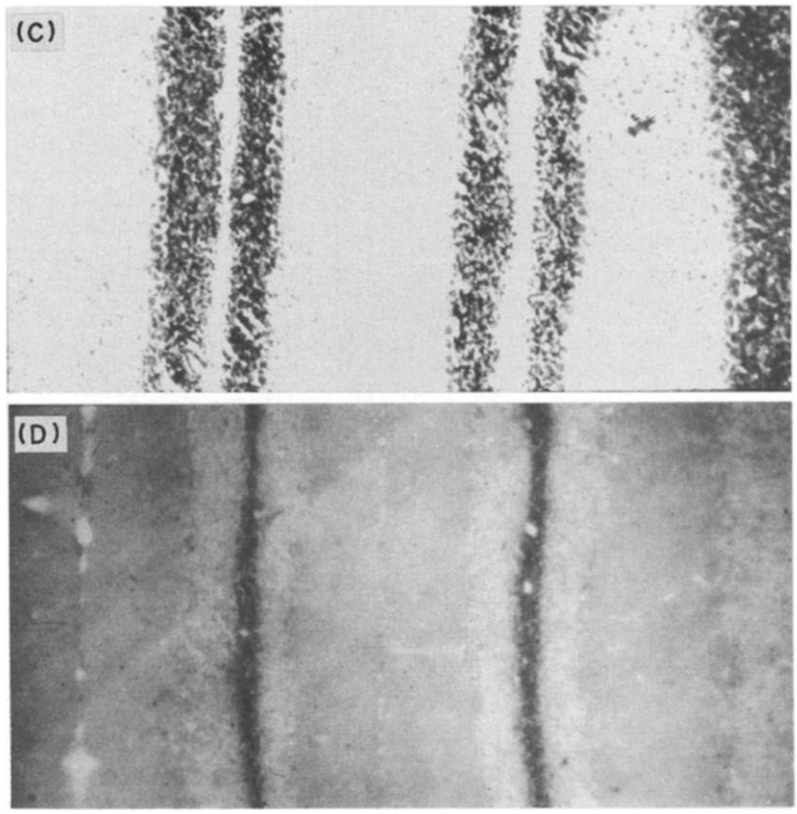


Fig. 2(C) and 2(D)

Fig. 2. Enzymatic cleavage of cholesterol. (A) Cytochrome $P450_{scc}$ is a mitochondrial membrane protein associated with adrenodoxine (Ad), reduced by FAD reductase. (B) Scheme of a transversal cut of rat cerebellum. (C) Rat cerebellum, after colouration with Masson's trichrome. (D) Immunohistochemical detection of $P450_{scc}$ by the peroxydase method in the rat cerebellum. Only the white matter, is coloured (courtesy of Dr C. Le Goascogne).

after having used one of these methods, we separated their mitochondria. Incubated according to conditions described to observe adrenal steroidogenesis, these mitochondria transformed [^{3}H]cholesterol into [^{3}H]P and a derivative of the latter, [^{3}H]20-OH P (tritiated Δ5-pregnene-3β,20α-diol) (Table 2, [12]). However, if in the same conditions one studies the metabolism of [^{3}H]cholesterol in the mitochondria of whole brain, there is no formation of [^{3}H]P, once again indicating the difficulty of demonstrating the reaction. Recently, other authors were able to show the existence of $P450_{scc}$ in cerebral mitochondria only in the pregnant rats [13].

Table 2. Conversion of [^{3}H]cholesterol to [^{3}H]P and [^{3}H]20-OHP by isolated mitochondria

	[^{3}H]P	[^{3}H]20-OHP
	(pmol/mg protein/h)	
Oligodendrocytes	2.6 ± 0.3	1.9 ± 0.5
	$n = 5$	$n = 4$
Adrenal cells	14.8	
	$n = 2$	

The demonstration was completed with glial cell cultures from newborn rats. These primary cultures contain precursors of two types of glial cells, astrocytes and oligodendrocytes. Experiments of double labelling with antibodies specific for constituents of oligodendrocytes (galactocerebroside) or of astrocytes (glial fibrillar acidic protein; GFAP) and anti-$P450_{scc}$ antibodies confirmed the presence of the steroidogenic enzyme in oligodendrocytes (Fig. 3) [14, 15]. Studies of biosynthesis in cell cultures were carried out using a radioactive cholesterol precursor, mevalonate. Moreover, we used mevinoline, an inhibitor of HMG-CoA-reductase, in order to favour the use of exogenous mevalonate and to obtain a higher specific radioactivity of products derived from the cholesterol synthesized. Further metabolism of pregnenolone was inhibited by trilostane, an inhibitor of 3β-hydroxysteroid dehydrogenase, an enzyme which transforms P into progesterone. The impact points of these inhibitors are indicated in Fig. 1. These experiments

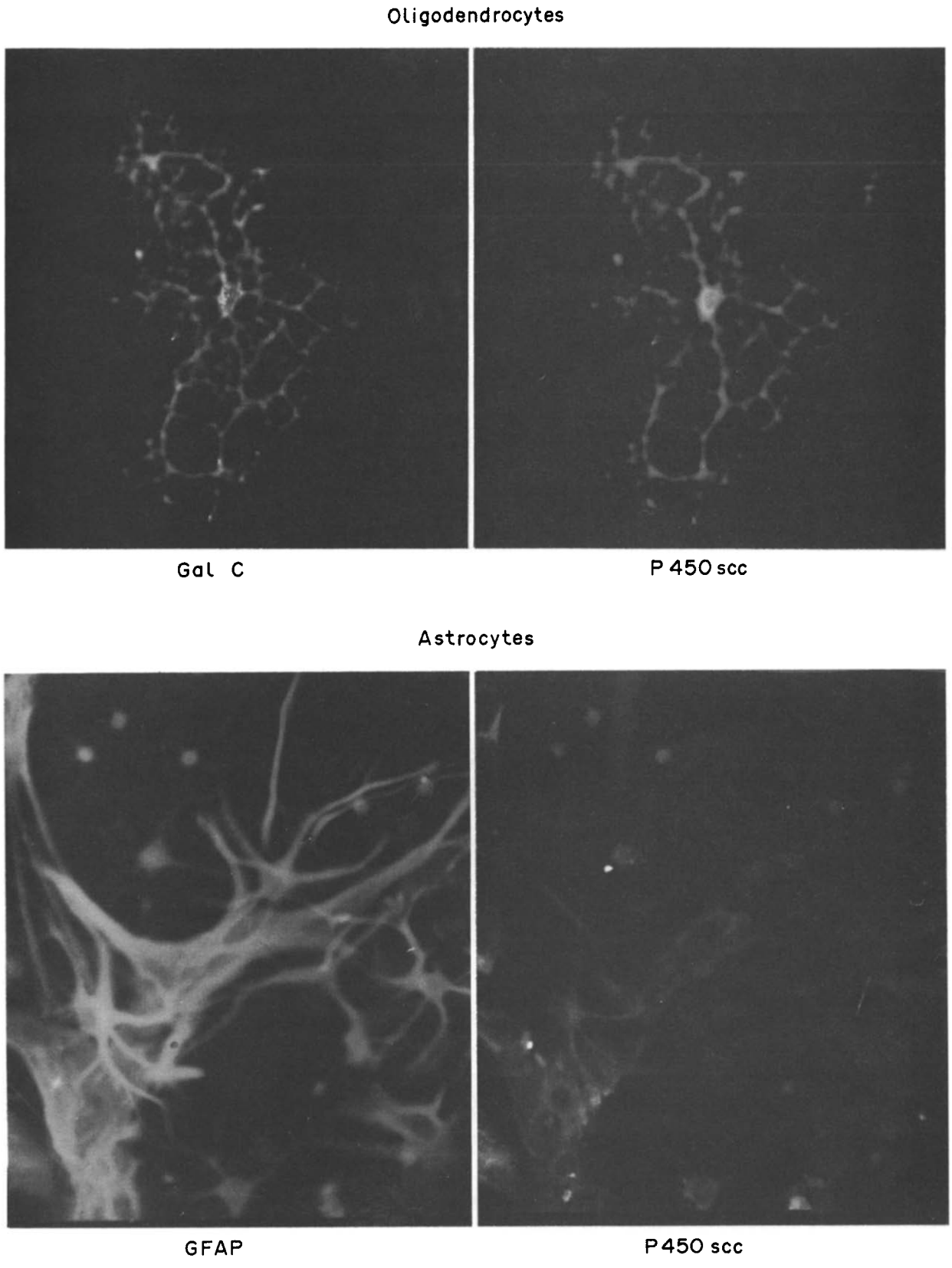


Fig. 3. Immunocytochemical localization of cytochrome $P450_{scc}$. Oligodendrocytes in culture. Double marking using a monoclonal antibody anti-Gal C labelled with fluorescein (left) and immunoglobulins anti-$P450_{scc}$ labelled with rhodamine (right). Astrocytes in culture. Double marking using an anti-GFAP antibody labelled with rhodamine (left) and anti-$P450_{scc}$ immunoglobulins, labelled with fluorescein (right) (courtesy of Dr I. Jung-Testas).

confirmed the synthesis of cholesterol, of P, and of 20-OH P by the glial cells. This synthesis, inhibited by aminoglutethimide, is increased by cyclic AMP analogues and by glucocorticosteroids. In these cultures, the appearance of steroidogenesis coincides with the differentiation of oligodendrocytes indicated by the increased activity of the marker enzyme 2′,3′-cyclic nucleotide phosphodiesterase (CNPase) (Fig. 4).

P can be transformed into progesterone in cultures of glial cells [6, 15]. In turn, progesterone can be partially converted into 5α-reduced metabolites, pregnanedione and pregnanolone (Fig. 1). It is not yet established whether the neurons themselves might carry out the same enzymatic conversions, using the pregnenolone from the oligodendrocytes.

Taking into account these *in vitro* experiments, one can consider that, as well as P and its esters, progesterone and its derivatives fall into the category of neurosteroids. The potential significance of such a definition will be seen later (see also Fig. 5). The concentration of progesterone in the male rat brain is to the order of 1–2 ng/g [16] and the concentrations of the 5α-reduced metabolites of progesterone have not yet been determined. It will be necessary to determine whether or not progesterone and its metabolites conform to the same criteria of independence in respect to peripheral sources as do P and D.

In the brain, P can be conjugated as a sulfate ester (PS) and as fatty acid esters. The activity of the acyl-transferase implicated is particularly abundant in the brain of young rats of 1–3 weeks, and the fatty acids mainly used are

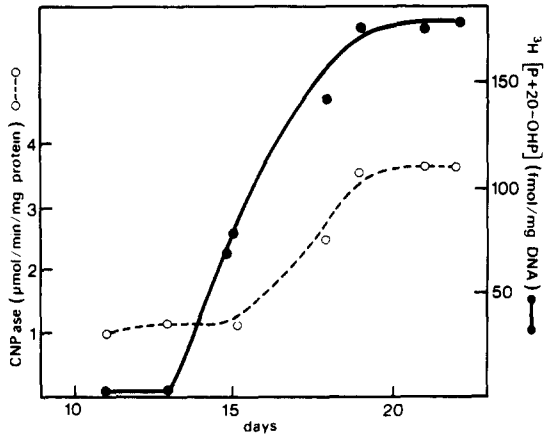


Fig. 4. Ontogenesis of oligodendrocyte differentiation in culture and steroid biosynthesis. Glial cells were incubated with [^{3}H]MVA. The conversion to [^{3}H]P and CNPase activity were measured at different days of culture.

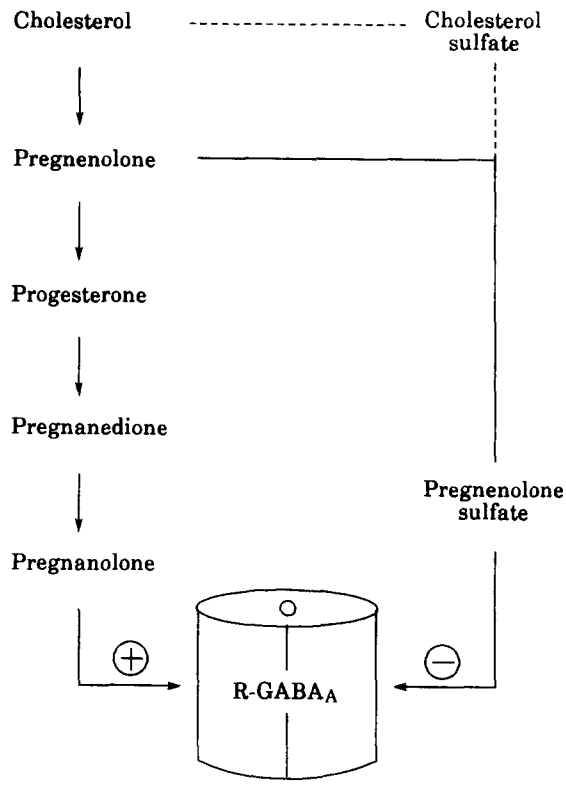


Fig. 5. Modulation of the $GABA_A$ receptor function by neurosteroids. Pregnenolone and PS have opposite effects (see text).

palmitic, oleic, linoleic, stearic, and myristic.

Like P, D can be esterified with fatty acids (same acyl-transferase activity) and sulfo-conjugated. The 3β-hydroxysteroid dehydrogenase which transforms P into progesterone can transform D into androstenedione, a testosterone precursor. Still, as we have seen, the greater part of the testosterone, if not all, found in the brain, is of peripheral origin, which does not exclude a limited production at certain precise sites not detected by the global measurements. It is also known that androstenedione and testosterone can be, in the hypothalamus, transformed into estrogens, another cytochrome *P*450 being responsible (cytochrome $P450_{Arom}$). Until now however, androgens of testicular origin seem to be the source of estrogens synthesized in the hypothalamus, and thus one cannot classify the estrogens as neurosteroids.

As for the synthesis of D, it remains conjectural. Until now, the biochemical studies we have pursued, with precursors such as [^{3}H]P and [^{3}H]progesterone, in order to demonstrate the formation of [^{3}H]D and [^{3}H]androstenedione, respectively, have been negative There is no biochemical indication available for the presence of a 17α-hydroxylase-17-20-desmolase per-

mitting the passage of 21-carbon steroids (P and progesterone) to 19-carbon steroids (D and androstenedione). We have even shown that an earlier result suggesting the formation of androstenedione from progesterone in the cat brain was probably an artefact. Moreover, antibodies anti-cytochrome $P450_{17\alpha}$ (of pig and guinea-pig), the enzyme implicated in the biochemical transformation sought after, gave no positive immunohistochemical result in the rat and guinea-pig, despite clear detection in the appropriate steroidogenic glands. If this microsomal enzyme is absent, it is possible that the origin of D passes by an alternative biochemical mechanism. Our studies continue in this respect.

NEUROSTEROIDS AND BEHAVIOUR

Two series of experiments indicated possible interventions of D and P in behavioural phenomena. An anti-aggressive effect of D was demonstrated in the particular model of castrated male mice becoming aggressive in the presence of a lactating female. This aggressiveness is absent in the intact male and is suppressed in the castrated male by the administration of testosterone or estradiol [18]. In collaboration [19], we have shown that D could, in small doses, make the aggressiveness of castrated males disappear. As D can be metabolically transformed into testosterone, even though the quantities found at the brain level were very small, we have used a derivative, 3β-methyl-5-androstene-17-one, lacking hormonal action and non-transformable into testosterone. This compound has an inhibitory action on the aggressiveness at least equal to that of D, and may thus be at the head of a new family of inhibitory steroids.

P present in the olfactory bulb of male rats seems to be involved in the chain of events linked to a heterosexual exposure of animals. In reference to values found in male rats exposed to the odour of other male rats, P levels selectively diminished in the olfactory bulbs of animals exposed to the odour of females in estrus [20]. The odouriferous signal of females seems to require ovarian function, since it disappears after castration and is re-established by estradiol. Moreover, the castration of males not only diminished the concentration of P in the olfactory bulb, but the pheromonal message became inoperative, while it was restored by administration of testosterone (which cannot be transformed into pregnenolone). It is rather remarkable that one can demonstrate a phenomenon, pheromonal and hormone-dependent, whose reception, at least that resulting in a change in concentration of P in a particular cerebral region, is equally hormone-dependent.

PS, whose cerebral presence and biosynthesis we have seen, is an inhibitor of the A receptor of GABA ($GABA_A$-R). To the biochemical evidences, one must add the recent demonstration of a reduction of the sleep period in the rat anaesthetized by a barbiturate [21]. One can ask oneself if this compound synthesized in the brain plays a physiological role in the modulation of the $GABA_A$-R (Fig. 5). Indeed, pharmacological experiments, already rather old, have shown the influence of certain steroids on the function of this same receptor: pregnanolone and other reduced metabolites of progesterone (and of deoxycorticosterone) are potentiators of the $GABA_A$ effect, and one of these compounds was used a long time ago as an adjuvant in general anaesthesia. This is probably the mechanism explaining the somnolence of pregnant women or patients treated with high doses of progesterone. The physiological production of such derivatives remains unshown at the present time.

Finally, it has been reported that D has a trophic effect on mouse neurons in culture and also reinforces long-term memory of an active avoidance behaviour [22].

MECHANISMS OF NEUROSTEROID ACTION

The intracellular receptors of steroid hormones are now well known. They are intranuclear proteins which can bind DNA. Presently, no result indicates the existence of nuclear receptors for D or P, nor for their esters. If one considers the progesterone synthesized by glial cells as a neurosteroid, the recent demonstration of progesterone receptor in these same cultures (I. Testas and J. M. Renoir, in preparation) indicates a classic mechanism of action for progesterone in a paracrine or autocrine rather than endocrine arrangement. The progesterone receptor was also found, besides an hypothalamic distribution, in the cerebral cortex and the meninges.

A mechanism of membrane action of neurosteroids cannot be excluded. There is a precedent for the existence of a membrane receptor in the case of the Xenopus oocyte, where progesterone, placed in the incubation medium, provokes

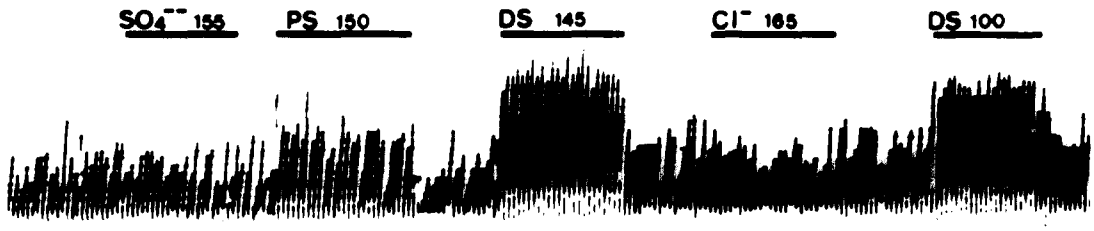


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Fig. 6. Excitatory effect of PS and DS on the electrical activity of a septopreoptic neuron in the guinea-pig. PS, ejected with a current of −150 nA for 30 s, and DS, ejected with a current of −100 nA for 25 s are active, as opposed to sulfate and chloride ions [26].

the re-initiation of meiosis [23]. Experiments of photoaffinity labelling permitted the characterization of a binding protein of ~30 kDa, with the properties of affinity and hormonal specificity of a receptor [24]. The effect of steroids on the adenylate cyclase of the whole cell has been reproduced *in vitro* on a membrane preparation [25]. For several years, electrophysiology experiments have shown that diverse steroids can stimulate the electrical activity of neurons when deposited at their contact, this was done for D, DS, and PS at the antero-septal region of guinea-pig brain (Fig. 6) [26].

The molecular mechanism of the anaesthetic effect of progesterone derivatives calls upon the $GABA_A$-R. This receptor is made up of several subunits, and it includes binding sites for GABA itself, barbiturates, and benzodiazepines. Pregnanolone and other reduced derivatives of progesterone potentialize the effect of GABA by binding to the receptor near to the barbiturate site [27]. This effect can be studied by examining the membrane potential or by measuring the entry of chloride in the GABAergic neurons (the $GABA_A$-R is a chloride channel which can be opened by GABA). On the contrary, PS acts as an antagonist, and, like picrotoxin, blocks the effects of GABA on the chloride channel [28]: PS reduces the frequency of chloride channel opening in neurons in culture [29]. DS is even more active. The $GABA_A$-R has become, during these last 2 yr, a family including more than 10 variants, and studies are underway to determine if the steroid effect is common to all. In any case, the action of neurosteroids on the $GABA_A$-R is a novel membrane effect of steroids.

The data concerning the biosynthesis of steroids in the brain are probably applicable to the human species. Neurosteroids have been measured at elevated concentrations in the brain of the cadaver [30, 31], and the presence of enzymes of the complex permitting side chain cleavage of cholesterol has been shown immunohistochemically [32]. From here to medical applications...

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