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Biosynthesis and Assay of Neurosteroids in Rats and Mice: Functional Correlates

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Pregnenolone (PREG), synthesized *de novo* in rodent brain, is the precursor of PREG sulfate (S) and progesterone (PROG). PROG is further converted to 5α-pregnane 3, 20-dione (DH PROG) and to 3α-hydroxy-5α-pregnan-20-one (TH PROG). PROG, DH PROG and TH PROG have been measured in the brain of male and female rats. Neither PROG nor DH PROG disappeared from brain, contrary to plasma, after combined adrenalectomy (ADX) and gonadectomy (CX). Trilostane decreased PROG and increased PREG in the brain of CX + ADX rats and mice, in accordance with a precursor to product relationship. As previously described in CX male mice, the neurosteroid DHEA and its analog 3β-methyl-androst-5-en-17-one (CH_3-DHEA) inhibited the aggressive behavior of female mice towards lactating female intruders. The decrease of biting attacks by DHEA was definitely more prominent in females neonatally imprinted with testosterone. The degree of inhibition of aggressive behavior was related to the decrease of PREG S concentrations in brain. The memory-enhancing effects of DHEA S and PREG S in male mice have been previously documented. Infusion of PREG S (12 fmol) into the nucleus basalis magnocellularis (NBM) of the rat after the acquisition trial enhanced memory performance in a two-trial recognition task (TTRT). Conversely, TH PROG (6 fmol), which potentiates GABAergic neurotransmission, disrupted performance when injected before the acquisition trial. Accordingly, we have found a positive correlation between the performances of 2-year-old rats in the TTRT and the concentrations of PREG S in the hippocampus, namely animals which performed best had the highest steroid levels.

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INTRODUCTION

The term neurosteroids applies to those steroids that are both synthesized in the nervous system, either *de novo* from cholesterol or from steroid hormone precursors, and that accumulate in the nervous system to levels that are at least in part independent of steroidogenic gland secretion rates [1]. Pregnenolone (PREG) is found in the brain of several mammalian species including rodents as the unconjugated steroid and its sulfate (S) and fatty acid (L) esters [2]. Evidence has been provided for *de novo* synthesis of PREG from cholesterol or mevalonate by oligodendrocytes, the glial cells involved in myelin synthesis. In turn, PREG can be converted to progesterone (PROG), and PROG to several reduced metabolites, among which 5α-pregnane-3,20-dione (5α-DH PROG) and 3α-hydroxy-5α-pregnan-20-one (3α,5α-TH PROG). The PREG → PROG conversion is performed by a family of enzyme isoforms, the Δ5-3β-hydroxysteroid dehydrogenases Δ5 → 4 isomerases (3β-HSD) [3]. Trilostane (4α,5-epoxy-17β-hydroxy-3-oxo-5α-androstane-2α-carbonitrile, TRIL) is a potent inhibitor of 3β-HSD [4]. Hence, it can prevent the PREG → PROG conversion.

We have extended to PROG and to PROG metabolites our early observations relating PREG accumulation in brain with its local biosynthesis. For that purpose, we have developed RIA procedures for the simultaneous measurement of PREG, PREG S, PREG L, PROG, 5α-DH PROG, and 3α,5α-TH PROG in plasma and tissues under several physiological and/or

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experimental conditions [5, 6]. Particularly, changes of PREG and PROG concentrations in brains of animals receiving TRIL might provide additional support for the existence of a physiologically relevant pathway of PROG synthesis in brain.

3α,5α-TH PROG AND ITS PRECURSORS IN THE BRAIN, PLASMA AND STEROIDOGENIC GLANDS OF MALE AND FEMALE RATS

Plasma and tissue samples were submitted to extraction, fractionation, solvolysis or saponification (as needed) and purification by chromatography on C18- and then on celite-microcolumns. These chromatographic steps were essential for ensuring the specificity of the ultimate radio-immunoassay procedures. Results were confirmed by GC/MS identification and quantification of 3α,5α-TH PROG.

Male rats (Sprague–Dawley, 200–220g body weight)

The concentrations of PREG, PREG S, PREG L, and to lesser extent those of PROG and TH PROG were greater in brain than in plasma (Fig. 1). TRIL produced significant increases of all the neurosteroids measured, because the blockade of corticosterone biosynthesis had relieved its negative feedback action on ACTH secretion. ACTH then stimulated cholesterol side chain cleavage, thus increasing the production of PREG and overcoming the inhibition of 3β-HSD activity. The final result was the maintenance of corticosterone production at the expense of a large increase of its precursors.

After combined castration and adrenalectomy, however, the brain extracts still contained measurable amounts of PREG, PREG S, PREG L, and PROG. The concentration of PREG S was unchanged compared to intact animals, whereas those of PREG and PROG were smaller, and TH PROG was undetectable; TRIL still produced a significant increase of PREG, while the concentration of PROG was significantly decreased, as expected from a precursor to product relationship.

Female rats

In the brain of cyclic females, PROG concentration was lower than in plasma, whereas those of 5α-DH PROG and of 3α,5α-TH PROG were markedly higher than the corresponding concentrations in plasma and in male brain. In OVX and ADX females, neither PROG, nor 5α-DH PROG, nor 3α,5α-TH PROG were detectable in plasma, whereas PROG and 5α-DH PROG were still present in brain. The concentrations of PROG and its reduced metabolites were much higher in the brain of 19 days pregnant females than in cyclic ones. Moreover, the ratios of both 5α-DH PROG and 3α,5α-TH PROG to PROG were much higher in brain than in plasma.

The persistence of PROG and of 5α-DH PROG in the brain of CX and ADX males and females is consistent with our previous reports about a steroidogenic pathway in the rat brain glial cells [2]. The direct formation of 3α,5α-TH PROG in brain was also suggested. Although a linear correlation was found between the concentrations of 3α,5α-TH PROG in plasma and brain and those of PROG in plasma, in

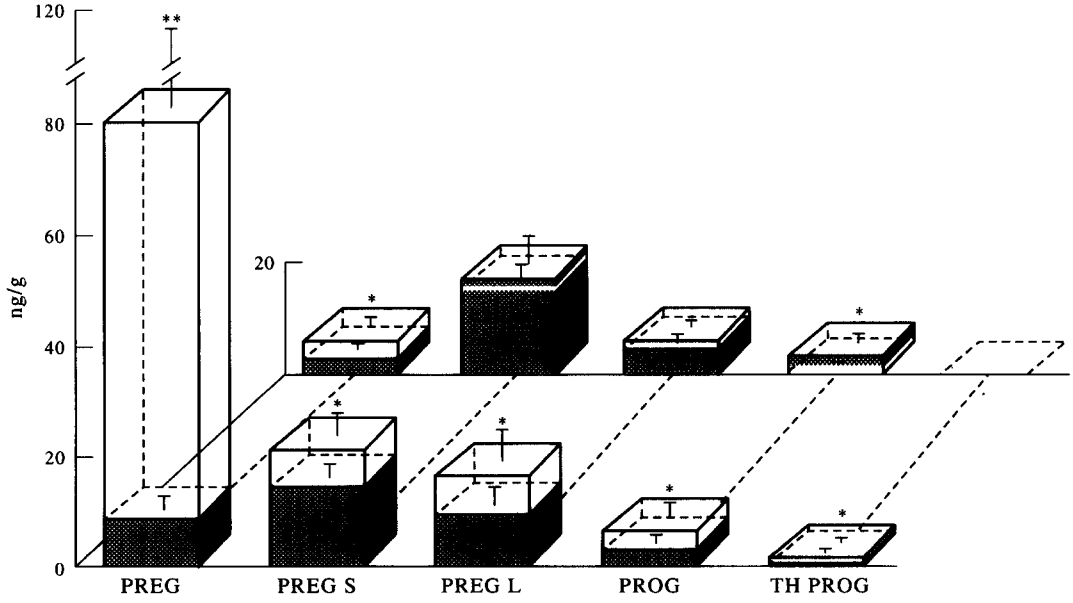

Fig. 1. Neurosteroids in brain of intact and operated males. Effects of Trilostane. Pregnenolone (PREG), its sulfate (S) and fatty acid esters (L), progesterone (PROG) and 3α-hydroxy-5α-pregnan-20-one (TH PROG), have been measured (mean ± SD). Front row: steroids in intact rats. Back row: steroids in rats castrated and adrenalectomized 2 weeks before sacrifice. Dotted columns: vehicle injected rats. Open columns: Trilostane treated rats. *Significant at $P < 0.05$. **Significant at $P < 0.02$, vs vehicle injected controls.

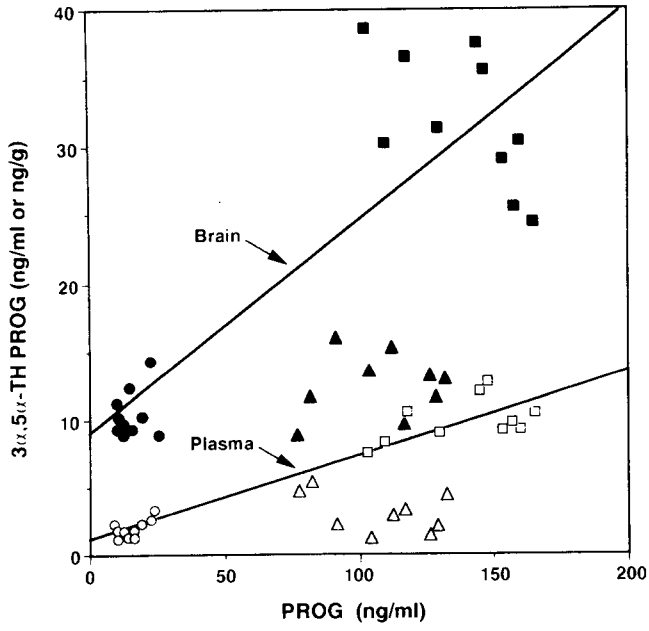

Fig. 2. Correlations of 3α,5α-TH PROG concentrations in plasma and brain with the concentrations of PROG in plasma of cyclic, pregnant and OVX-PROG-injected females. Open symbols, plasma 3α,5α-TH PROG vs PROG. Closed symbols, brain 3α,5α-TH PROG vs PROG. ○, ●, Random cyclic females. □, ■, Pregnant females. △, ▲, Spayed, PROG injected females. The regression lines were constructed for random cyclic and pregnant females. Values for spayed, PROG-treated females, fell below the corresponding regression lines. For 3α,5α-TH PROG in plasma: $y = 1.2 \pm 0.06x$. $r = 0.96$, $P < 0.01$. For 3α,5α-TH PROG in brain: $y = 9.2 \pm 0.16x$. $r = 0.85$, $P = 0.002$.

random cyclic and pregnant females, the slopes of the regression lines were strikingly different (0.06 vs 0.16) thus indicating that the site of formation of 3α,5α-TH PROG is predominantly the brain (Fig. 2).

PREG S, A NEUROACTIVE STEROID

The γ-aminobutyric acid (GABA) receptor type A ($GABA_A$-R) is an oligomeric protein complex that, when activated by agonists, produces an increase in neuronal membrane conductance to Cl^- ions and reduces neuronal excitability. A number of centrally active drugs, including convulsants, anticonvulsants, anesthetics and anxiolytics, bind to distinct but interacting domains of this receptor complex and modulate Cl^- conductance [7]. The inhibitory steroid metabolites such as 3α,5α-TH PROG both mimic and enhance the effects of GABA. PREG S, on the contrary, has a GABA antagonistic activity, at low micromolar concentrations *in vitro* [7]. Therefore, distinct sites for neurosteroids, mediating distinct allosteric modes of interaction, seem to exist on the $GABA_A$-R or in its membrane vicinity (Fig. 3). PREG S is also a positive modulator of NMDA receptors [8]. It may also influence opioid and sigma receptors. On the whole, PREG S behaves as a naturally excitatory neurosteroid.

We have obtained evidence for a modulatory role of PREG S in models of aggressiveness and memory.

The aggressive behavior of mice against lactating female intruders

DHEA and its hormonally inactive analog 3β-methyl-androst-5-en-17-one (CH_3-DHEA) inhibit the aggressive behavior of castrated male mice against lactating female intruders. Both molecules produce a marked and significant decrease of PREG S concentrations in the brain of treated castrated mice [9]. We have speculated that DHEA and CH_3-DHEA, by decreasing PREG S levels in the brain, might increase the GABAergic tone, which has repeatedly been implicated in the control of aggressiveness.

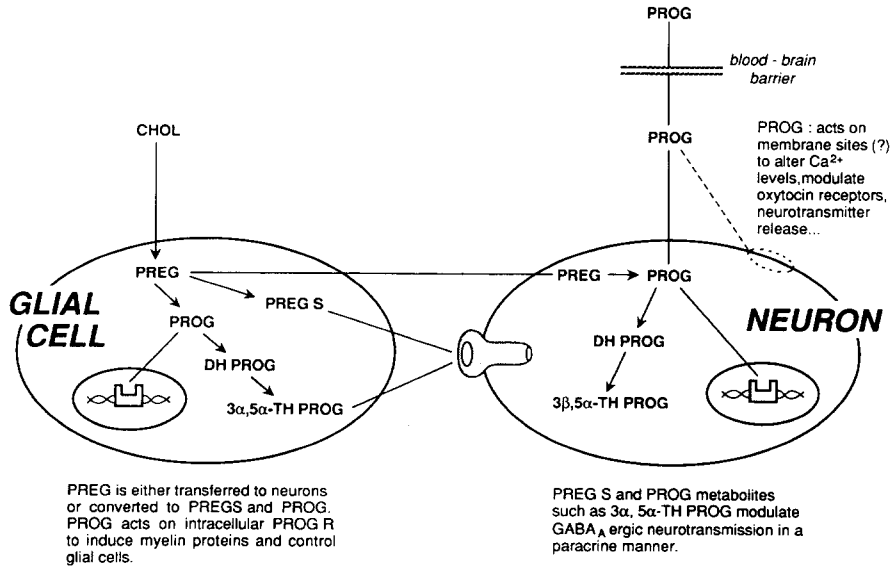

Fig. 3. A schematic view of the nongenomic and genomic mechanisms of neurosteroid action in neural tissue. The cross talk between glial cells and neurons implies the provision of steroid precursors and the modulation of $GABA_A$ receptors by steroid metabolites of glial origins.

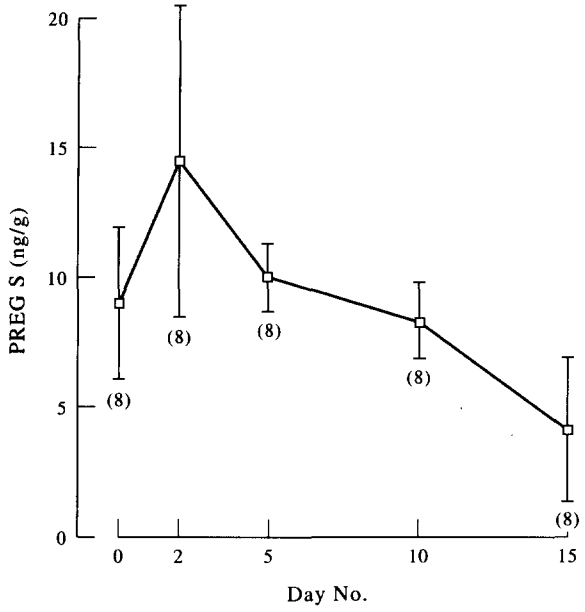

Fig. 4. Time-course of PREG S decrease in the brain of castrated male mice. Forty Swiss male mice were castrated when 7 weeks old. After a 4-week recovery, they received daily subcutaneous injections of 280 nmol of DHEA, except the control group that received only the oil vehicle. They were killed by decapitation 2 h after the last injection, and PREG S concentrations were measured in the whole brain as previously described [9].

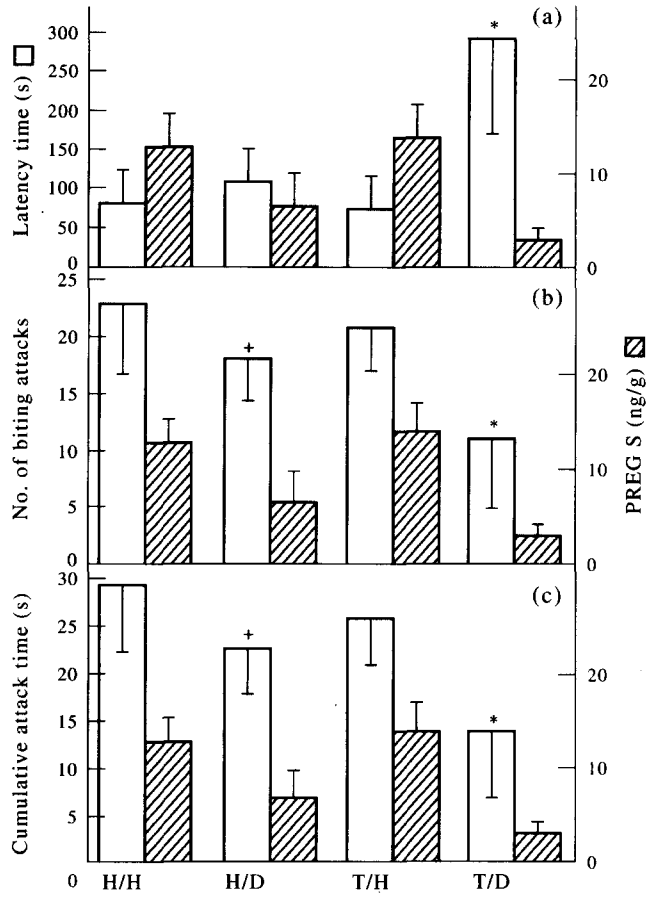

Fig. 5. Effects of DHEA on the aggressive behavior of female mice toward lactating intruders and on PREG S concentrations in brain. H, oil vehicle (10 μL s.c.); T, neonatal testosterone propionate (500 μg in oil); D, dehydroepiandrosterone (80 μg in oil daily for 15 days). The four experimental subgroups, made up of 30 10-week-old females each, were tested for attacks towards lactating intruders 2 h after the last injection. PREG S was measured in whole brain 2 h after sacrifice of similarly treated females. *Differs significantly from the other treatment groups +Differs significantly from oil controls.

The time-course of PREG S decrease in brain following DHEA administration supports this conclusion. Indeed, the castrated mice had to be treated for 2 weeks with DHEA before getting a clear-cut, significant inhibitory effect on aggressive behavior. Accordingly, the decrease of PREG S in brain was gradual, and became significant only 15 days after the onset of treatment (Fig. 4).

Adult female mice also display an aggressive behavior towards lactating intruders [10]. This aggressive behavior does not depend on ovarian hormones, since it persists after ovariectomy, and is not corrected by estradiol in spayed females. Since differences in hormonal control of aggressive behavior of males and females may be related to the neonatal imprinting, spontaneously provoked by testosterone in males, we have investigated the influence of DHEA treatment on spayed females and the modulation of this influence of DHEA by testosterone, injected to the newborn. Indeed, the females which had been androgenized at birth, then treated when adult with DHEA (T/D subgroup) were much less aggressive towards lactating intruders than mice from any other experimental subgroup (Fig. 5). In accordance with this observation, the decrease in the concentration of PREG S produced by DHEA was significantly larger in the T/D subgroup than in the H/D one.

The mechanisms by which DHEA or 3β-CH_3-DHEA decrease PREG S concentrations in brain are under current investigation. Preliminary results show that both compounds inhibit the formation of PREG S by the cytosol of female mouse liver (Table 1).

The memory-enhancing effects of PREG S

When administered intracerebroventricularly after training, DHEA S and PREG S have shown memory-enhancing effects in foot-shock avoidance training [11]. PREG S was the most potent. PREG S also blocks

Table 1. Inhibition of PREG sulfate ester synthesis by DHEA and 3β-CH_3-DHEA

Sulfokinase activity (pmol/h/mg protein)		
Control	DHEA	3β-CH_3-DHEA
11.8 (100%)	7.3 (62%)	2.7 (23%)

Cytosol of female mouse liver (~50 μg protein/100 μl) was incubated with 10 nM [^{3}H]PREG and 180 μM PAPS, without or with 140 μM competitor, at pH 5.5 and 37°C for 2 h.

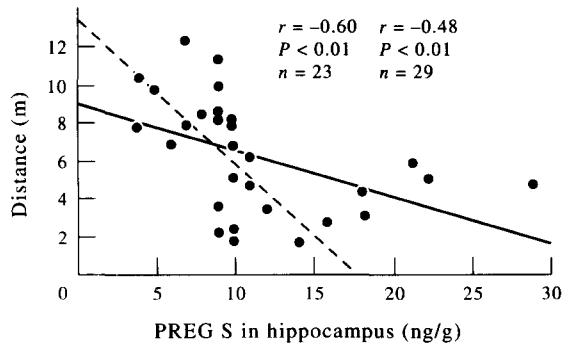

Fig. 6. Aging and memory. Correlations to neurosteroids. Memory performances of 29 aged rats (~2 years old) were measured in a water maze. At the completion of behavioral studies, animals were sacrificed and PREG S concentrations were measured in hippocampus. Linear regression indicated a significant positive correlation between memory performance (small distance covered) and PREG S concentrations ($r = -0.48$, $P < 0.01$).

NMDA-antagonist induced deficits in a passive avoidance memory task [12], and improves acquisition and retention of a food search task [13].

We have shown that infusion of PREG S (12 fmol) into the nucleus basalis magnocellularis (NBM) of the rat after the acquisition trial enhanced memory performance in a two-trial memory task [14]. A role for memory processes subserved by the NBM is of interest in view of the implication of this structure in neurodegenerative processes leading to memory loss. In order to obtain information about the physiopathological relevance of these observations, we have undertaken to evaluate the cognitive performances of aged rats. Aging is associated with impairment of cognitive functions and particularly with a decline of memory processes. However, there are considerable interindividual differences in the severity of age related impairments in both humans and animals. Some aged subjects are impaired whereas others perform as well as young ones. Memory performances of 29 aged rats (~24 months old) were measured in a water maze and in a two-trial recognition task. At the completion of the behavioral study, animals were sacrificed and brain were removed for analysis of PREG S concentrations in selected areas (Fig. 6). The results showed that there were considerable interindividual differences in memory performances of aged rats and that scores are correlated in the two tasks suggesting true memory evaluation. A striking observation was the significant positive correlation between the concentration of PREG S in the hippocampus and memory performance, namely the animals with better performances had greater levels of PREG S.

Although it remains to determine the cause–effect relationship between memory performance and neurosteroid concentrations in the hippocampus, these results support the possible neuroprotective role of PREG S (and likewise DHEA S) against neurodegenerative processes.

CONCLUSION

The effects of steroids on CNS neurotransmitter functions involve both genomic and non-genomic actions [1, 15]. Since several steroids are accumulated in the brain, independently (at least in part) of the contribution by steroidogenic glands, and since their presence can be related to steroid biosynthetic pathways in the brain, their designation as neurosteroids is justified. The levels of neurosteroids reached in the brain are compatible with their playing a physiologic neuromodulatory role in situations such as the estrous cycle, pregnancy and stress, and by influencing sexual behavior, mood, memory, developmental and aging processes.

Although firm correlations have been established between neurosteroid levels and some of these situations, further work is required to demonstrate the physiologic significance of neurosteroids and to define their sites of interaction in molecular terms.

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