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# Pregnenolone sulfate antagonizes dizocilpine amnesia: role for allopregnanolone

**D. L. Cheney, D. Uzunov and A. Guidotti[CA]**

Nathan S. Kline Institute for Psychiatric Research, Center for Neuropharmacology, 140 Old Orangeburg Rd, Orangeburg, NYU, NY 10962, USA

PREGNENOLONE (PREG) is metabolized in brain to progesterone (PROG), 5α-dihydroprogesterone (5α-DHP) and allopregnanolone (ALLO). Infusion of adrenalectomized/castrated rats with PREG sulfate prevented the cognition deficit elicited by the ionotropic glutamate receptor antagonists, dizocilpine and CPPene. Using a new gas chromatographic/mass-spectrometric method, we demonstrated that PREG sulfate infusion markedly increased the PREG, PROG, 5α-DHP and ALLO brain content. The increase in 5α-DHP and ALLO, but not PREG or PROG content and the antagonism of dizocilpine amnesia observed by injecting rats with PREG sulfate was reversed by inhibiting the conversion of PROG to 5α-DHP with the 5α-reductase blocker SKF 105111. We and others have shown that ALLO potently modulate $GABA_A$ receptor function whereas 5α-DHP fails to induce rapid changes in neurotransmitter receptor function. Thus it is possible to suggest that the increase in the brain content of ALLO, rather than 5α-DHP, mediates the effect of PREG sulfate on dizocilpine- or CPPene-induced cognition deficit.

**Key words:** Neurosteroids; Glutamate; GABA; Cognition deficit; Dizocilpine; CPPene; 5α-reductase blockers; SKF-105111; Allopregnanolone

[CA]Corresponding Author

## Introduction

Studies in animals and humans receiving systemic 5-pregnen-3 β-ol-20-one (pregnenolone, PREG) make it possible to attribute a number of pharmacological actions to this steroid.[1] Among these actions, the effects of PREG and its more water-soluble derivative, PREG sulfate, on cognition in rodents are of particular interest. Administration of PREG sulfate facilitates memory consolidation in mice[2] and in rats prevents the cognition deficit elicited by dizocilpine, a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist[3] and CPPene, a competitive NMDA receptor antagonist.[4]

PREG, synthesized in brain or taken up into the brain from plasma, is the precursor of all known neurosteroids.[1] Moreover, accumulating literature on the pharmacological actions of PREG sulfate, 5α-pregnan-3 α-ol-20-one (allopregnanolone, ALLO) and 5-androstene-3 β-ol-17-one (dehydroepiandrosterone, DHEA) indicates that these neurosteroids are modulators of γ-aminobutyric $acid_A$ ($GABA_A$)[5–7] and/or glutamate receptor function.[8,9] Because 5α-reductase inhibitors were reported to attenuate the antagonism of dizocilpine behavioral action elicited by an increase in brain PREG sulfate content following administration of PREG sulfate to rats,[3] it is tempting to speculate that PREG itself may be inactive but may function as a precursor of neurosteroids which act as modulators of GABAergic and glutamatergic inotropic receptors operative in memory and learning processes.

In the rat brain, PREG is used to synthesize pharmacologically relevant amounts of 4-pregnen-3,20-dione (progesterone, PROG), 5α-pregnan-3,20-dione (5α-dihydropropgesterone, 5α-DHP) and ALLO.[10] Thus, an adequate supply of locally synthesized PREG, or externally administered PREG, could optimize GABA or glutamate receptor function in brain areas possessing enzymatic competence to convert PREG to neuroactive steroids.[10]

This study aimed to identify the structure of the brain PREG metabolites produced following the systemic administration of PREG sulfate to rats which can mediate the antagonism of dizocilpine induced cognition deficit.

## Materials and Methods

*Animals:* Sprague–Dawley rats (220–240 g body weight; Zivic-Miller, Zelionople, PA) were adrenalectomized (ADX) and castrated (CX) 7 days before the experiment. Neurosteroids were measured following decapitation by guillotine; the brain was quickly removed and stored at −20°C. All animal procedures were in strict accordance with the NIH guide for the care and use of laboratory animals and were approved by the Animal Care Committee.

*Reagents:* Dizocilpine maleate ((+)-MK-801 hydrogen maleate) was purchased from RBI (Natick, MA); D-CPPene (D(−)-(E)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid) was obtained from Sandoz Research Institute, Bern, Switzerland; hydroxypropyl-$\beta$-cyclodextrin was obtained from Aldrich, Milwaukee, WI; dimethyl sulfoxide (DMSO) and PREG sulfate were purchased from SIGMA (St. Louis, MO); PREG, PROG, 5$\alpha$-DHP and ALLO were from Steraloids (Wilton, NH) and SKF 105111 ((17 $\beta$)17[[bis(1-methyl)amino]-carbonyl]androstane-3,5-diene-3-carboxylic acid) was a gift from Smith Kline Beecham Pharmaceuticals (King of Prussia, PA).

*Drug preparation:* PREG sulfate was dissolved in saline containing DMSO (1% v/w) and 15% hydroxypropyl-$\beta$-cyclodextrin (5% v/w). Vehicle was prepared in the same way. PREG sulfate or vehicle was infused via tail vein at the rate of 0.34 ml min$^{-1}$. Drugs or vehicle were administered in a volume of 1 ml kg$^{-1}$, i.v., and 2.0 ml kg$^{-1}$, i.p.

*Behavioral testing:* The test chamber for passive avoidance testing consisted of two compartments (30 cm wide×30 cm long×40 cm high) separated by a door which could be raised. One compartment was lighted; the other was dark. An electric shock of graded intensity was delivered through the steel grid floor. The acquisition trial began by placing a rat in the lighted compartment 25 min following the injection of either vehicle or a test solution. After 5 s the door was raised. When the rat entered the dark compartment, the door was closed and the rat received inescapable foot-shock (1 mA for 2.0 s). Twenty-four hours after this acquisition the avoidance retention was estimated by placing the rat again in the lighted compartment. The latency to enter the dark compartment was recorded during 3 min.

*Dizocilpine- or CPPene-induced cognition deficit:* In preliminary experiments, dizocilpine (0.15 mg kg$^{-1}$, 30 min, i.p.) or CPPene (2.5 mg kg$^{-1}$, 30 min, i.p.) disrupted avoidance retention in absence of either ataxia or stereotypies. Moreover, rats receiving either dizocilpine, CPPene or vehicle 30 min before the test entered the dark compartment with virtually identical latency time. Twenty-four hours after the footshock, dizocilpine or CPPene-treated rats entered the dark compartment without delay, whereas vehicle-treated rats entered only after a 2–3 min delay.

*Gas chromatography-Mass fragmentography (GC-MF) neurosteroid analysis:* The procedures used to measure neurosteroids have been detailed elsewhere.[10] Briefly, rat brains were homogenized in 5 vol. of 0.4 N formic acid and were extracted three times with 15 ml of ethyl acetate. The supernatants were collected and lyophilized. In these extracts, steroids were separated by high performance liquid chromatography (HPLC) with 10 $\mu$m Lichrosorb 100 Diol, 4×250 mm column (EM Science, Gibbston, NJ) equilibrated with hexane at a flow rate of 1 ml min$^{-1}$ and the steroids were eluted with tetrahydrofuran-acetate gradient using a linear increase from 0 to 12.5% in 6 min, a second linear increase from 12.5 to 20% in 30 min and a final linear increase from 20 to 80% in 10 min. The eluate was collected at 1 min intervals and the specific fractions containing PROG, ALLO and PREG after addition of appropriate standards were evaporated to dryness under a stream of nitrogen. These residues were dissolved in 100 $\mu$l of acetone. The derivatizing reagent heptafluorobutyric acid anhydride (HFBAA, 25 $\mu$l) was added and the samples kept at room temperature for 1 hr. Thereafter the derivatizing agents were evaporated to dryness under a stream of nitrogen, the HFBAA derivatives were dissolved in 7–15 $\mu$l hexane and an aliquot of this final solution was injected onto the gas chromatograph. The specific HPLC fractions containing 5$\alpha$-DHP were evaporated to dryness under a stream of nitrogen, 50 $\mu$l of methoxyamine HCl (2%) in pyridine were added and the samples were reacted for 1 h at 65°C. The derivatizing reagents were evaporated to dryness under a stream of nitrogen. The residue was taken up in 1 ml chloroform:hexane (1:1) and passed through a short column (~3 cm) of Sephadex LH-20. The column was washed with 1 ml chloroform:hexane (1:1). The void volume (2 ml) containing methoxime (MO) derivatives was evaporated to dryness, the MO derivatives were dissolved in 10–15 $\mu$l hexane and an aliquot injected onto the gas chromatograph.

HFBAA and MO derivatives of the neurosteroids were quantified by GC-MF using an HP 5971 Mass Selective Detector coupled to an HP 5890A gas chromatograph as previously described.[10] Each of the derivatized steroids had a unique retention time as it eluted from the gas chromatographic column and a unique fragmentographic pattern which further enhanced the specificity of the analysis. Sensitivity and selectivity were optimized using the mass spectrometer in the selective ion monitoring mode. For routine analyses an even greater sensitivity was possible by focusing on only one specific m/z value for each derivatized steroid: PREG, 298 m/z; PROG, 510 m/z; 5$\alpha$-DHP 343 m/z; and ALLO, 496 m/z).

*Statistical analysis:* All results are presented as mean±s.e.m. Data was subjected to Student's *t*-test or an analysis of variance followed by Newman–Keul's test.

## Results

*Passive avoidance test:* ADX/CX male rats were used to avoid the possibility that peripheral synthesis of steroids may influence the brain neurosteroid content. Rats receiving varying doses of PREG sulfate, 0.15 mg kg$^{-1}$ dizocilpine, 5 mg kg$^{-1}$ of the 5$\alpha$ reductase inhibitor SKF 105111 or a combination of the above drugs 30 min before the acquisition trial entered the dark compartment with the same time latency (10–15 s) as vehicle-treated rats. Twenty-four hours later, rats treated with vehicle or with SKF 105111 entered the dark compartment with a latency of 167±11 s or 150±7.0 s respectively (Fig. 1), whereas rats treated with dizocilpine entered the dark compartment within 31±11 s (Fig. 1) demonstrating disruption of passive avoidance retention response.

As shown in Figure 1, 5 min i.v. infusion of increasing concentrations of PREG sulfate 25 min before the acquisition trial antagonized the dizocilpine inhibition of the passive avoidance retention measured 24 h later, in a dose-dependent manner. The 20 mg kg$^{-1}$ dose was chosen for the remainder of the behavioral and biochemical experiments to ensure maximal antagonism of the dizocilpine-induced inhibition of the passive avoidance by high levels of PREG serving as precursor or PROG, 5$\alpha$-DHP and ALLO brain synthesis. Notably, the reversal of the amnesic effect of dizocilpine with 20 mg kg$^{-1}$ of PREG sulfate was abated by pretreating the animals with SKF 105111 (see Fig. 1). In another study, we reported that SKF 105111 does not attenuate the antagonism of dizocilpine action caused by either ALLO or THDOC.[3]

To determine whether or not this same effect could

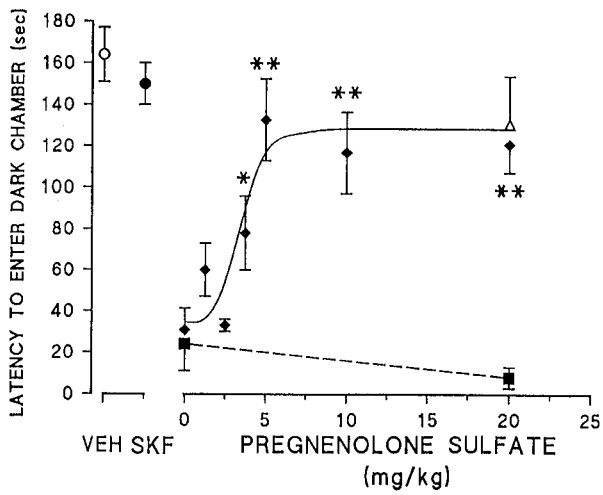


FIG. 1. Dose-dependent antagonism by PREG sulfate (5 min i.v. infusion, 25 min before acquisition trial) of the disruption of the behavioral performance elicited by dizocilpine in the passive avoidance test. Data represent mean±s.e.m. of 5–12 determinations. □, vehicle treated animals; ■, SKF 105111 (5 mg kg$^{-1}$, i.v., 30 min before the acquisition trial) treated animals; , dizocilpine (0.15 mg kg$^{-1}$, i.p., 30 min before the acquisition trial) plus SKF 105111; ▲, dizocilpine; △, PREG sulfate alone. *$p$<0.01.

be observed with a different class of NMDA receptor antagonists, CPPene, a competitive NMDA receptor antagonist was used. Rats receiving CPPene (2.5 mg kg$^{-1}$, i.p.) 30 min before the acquisition trail entered the dark compartment with the same latency as vehicle-treated rats (data not shown). Twenty-four hours later (in the retention trial) rats treated with CPPene alone entered the dark compartment with a delay of 57±13 s ($n$=5), whereas rats treated with CPPene and infused with PREG sulfate entered after a delay of 133±15 s ($n$=5; $p$<0.01).

*GC-MF analysis of brain neurosteroid content:* Brain levels of PROG, 5$\alpha$-DHP and ALLO were significantly reduced in ADX/CX rats compared with normal controls (Table 1). In ADX/CX rats dizocilpine had no significant effect on brain content of PREG, PROG or ALLO. Infusion of 20 mg kg$^{-1}$ PREG sulfate in ADX/CX rats increased the whole brain content of PREG by almost 50-fold measured 25 min following infusion. The whole brain content of PROG and 5$\alpha$-DHP increased by 5 fold and that of ALLO by >6-fold (Table 1). However, the increase in 5$\alpha$-DHP and ALLO, but not that of PREG or PROG, content following PREG sulfate infusion was reversed by inhibiting the conversion of PROG to 5$\alpha$-DHP using the 5$\alpha$-reductase inhibitor, SKF 105111 (Table 1).

## Discussion

These experiments demonstrate that PREG sulfate administered systemically to rats antagonized the disruption of passive avoidance retention response caused by both dizocilpine, a non-competitive antagonist, and CPPene, a competitive antagonist of the NMDA receptor. Furthermore, pretreatment with the 5$\alpha$-reductase inhibitor SKF 105111 blocked the antagonism of dizocilpine behavioral actions by PREG sulfate, suggesting a possible role of 5$\alpha$-reduced PROG metabolites in the modulation of the disruption of passive avoidance elicited by dizocilpine.

To establish whether a correlation exists between the antagonism of the dizocilpine action and the brain content of PREG sulfate or one of its brain metabolites, PREG and its metabolites, PROG, 5$\alpha$-DHP and ALLO were measured 25 min after infusion of PREG sulfate in rats with or without pretreatment with SKF 105111. When administered systemically PREG sulfate does not readily enter the brain, but presumably it is desulfated in peripheral tissues (i.e. liver) by the action of a microsomal sulfatase[11] and the resulting PREG is then taken up by the brain where it can be metabolized into PREG sulfate by the very active brain sulfotransferases.[12] Alternatively PREG may be metabolized by brain enzymes into PROG,

**Table 1.** SKF 105111 blocks the PREG sulfate (Sulf)-induced increase in whole brain 5α-DHP and ALLO, but not PREG or PROG, content in adrenalectomized/castrated (ADX/CX) rats

| Treatment | ADX/CX | PREG (nmol $g^{-1}$) | PROG (pmol $g^{-1}$) | 5α-DHP (pmol $g^{-1}$) | ALLO (pmol $g^{-1}$) |
|---|---|---|---|---|---|
| Vehicle | Yes | 0.61±0.066 | 5.7±0.92* | 0.85±0.23* | 2.2±0.20* |
| Dizocilpine (Diz) | Yes | 0.43±0.11 | 6.6±2.9 | N.D. | 2.1±0.75 |
| SKF 105111 | Yes | N.D. | N.D. | N.D. | 1.5±0.14 |
| Diz+PREG Sulf | Yes | 28±3.2† | 28±6.7† | 4.2±0.80† | 14±1.2† |
| Diz+PREG Sulf+SKF 105111 | Yes | 20±2.2† | 27±7.9† | 1.9±3.5‡ | 3.7±1.2‡ |
| Sham-operated+vehicle | No | 1.2±0.47 | 36±8.0 | 3.2±0.92 | 3.9±0.65 |

Data represent mean±s.e.m. of 5–7 samples. *$p<0.05$ compared with sham-operated+vehicle rats. †$p<0.01$ compared with ADX/CX vehicle-treated rats. ‡$p<0.01$ compared with dizocilpine plus PREG Sulf-treated rats. N.D.=Not Determined. Dizocilpine (0.15 mg $kg^{-1}$, i.p.); SKF (5 mg $kg^{-1}$, i.p.) or vehicle was administered 30 min before sacrifice. Pregnenolone sulfate (20 mg $kg^{-1}$, i.v.; 5 min infusion) or vehicle were administered 25 min before sacrifice.

5α-DHP and ALLO. The enzyme 3β-hydroxysteroid dehydrogenase, which is the rate limiting step for the formation of PROG from PREG, is present in brain[12] but is not found in most peripheral tissues with the exception of adrenal and testis which were removed for these experiments. We have shown that in ADX/CX rats infused with PREG sulfate, the PROG levels in liver are virtually undetectable.[10] In the absence of appropriate PROG concentrations the 5α-reductases and 3α-hydroxysteroid oxidoreductases of liver are not able to synthesize 5α-DHP and ALLO. Therefore, we might infer that the large increase in PROG, 5α-DHP and ALLO (Table 1) found in the brains of ADX/CX rats infused with PREG sulfate reflects a local synthesis of these steroids from PREG.

However, after PREG sulfate treatment, the brain content of PREG, PROG and 5α-DHP (Table 1) and PREG sulfate,[3] failed to reach the high micromolar concentrations required to affect directly glutamate and GABA gating of NMDA or $GABA_A$ receptors, respectively,[13–17] or to affect the binding of dizocilpine to crude synaptic membranes.[3] In contrast, after PREG sulfate administration the brain content of ALLO increased from 2.2±0.20 pmol $g^{-1}$ to 14±1.2 pmol $g^{-1}$, a concentration that based on pharmacological experiments should be sufficient to positively modulate GABA action at $GABA_A$ receptors[18,19] suggesting that ALLO and not 5α-DHP, PROG, PREG or PREG sulfate mediates the antagonism of the dizocilpine disruption of passive avoidance retention.

To firmly establish that an increase of 5α-reduced metabolites of PROG in brain participates in the attenuation of dizocilpine-induced cognition deficit, rats receiving PREG sulfate were pretreated with SKF-105111, a potent inhibitor of 5α-reductase type 1 and 2.[20] SKF-105111, in doses that attenuate the conversion of PROG to 5α-DHP and ALLO (Table 1), eliminated the protective action of PREG sulfate on the passive avoidance retention disruption elicited by dizocilpine. These results allow one to infer that PREG sulfate prevents dizocilpine-induced cognition deficit via an increase of brain 5α-DHP and ALLO synthesis. Interestingly, in a behavioral study we have reported[3] that ALLO pretreatment antagonizes dizocilpine-induced amnesia and that the effect of ALLO unlike that of PREG sulfate is independent from 5α-reductase inhibition.

Pharmacological studies indicate that 5α-DHP in nM concentrations can alter gene expression via an interaction with PROG receptors, but fails to induce rapid changes in neurotransmitter receptor function.[21] Thus, it is likely that the antagonism of PREG sulfate on dizocilpine-induced cognition deficit is mediated via ALLO. Although SKF 105111 does not reduce brain content of ALLO (2.7±0.67 pmol $g^{-1}$) in ADX/CX rats following PREG sulfate administration to control values (2.2±0.20 pmol $g^{-1}$), the content of ALLO is reduced to the level found in sham-operated rats (3.9±0.65 pmol $g^{-1}$). We know that this level of ALLO in the brains of sham-operated rats is not sufficient to modify the dizocilpine-induced passive avoidance disruption.

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