

# The neuroactive steroid allopregnanolone suppresses hypothalamic gonadotropin-releasing hormone release through a mechanism mediated by the gamma-aminobutyric acid<sub>A</sub> receptor

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## Abstract

The central nervous system (CNS) is able to synthesize and/or metabolize steroid hormones. These neuroactive steroids are capable of modulating several brain functions and, among these, they seem to regulate the hypothalamic–pituitary–gonadal (HPG) axis. Indeed, recent observations have shown that 5 $\alpha$ -pregnane-3 $\alpha$ -ol-20-one (allopregnanolone), one of the most abundant naturally occurring neuroactive steroids, suppresses ovulation and sexual behaviour when administered within the CNS. The present study was undertaken to evaluate the effects of allopregnanolone and its inactive stereoisomer, 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one, upon the release of gonadotropin-releasing hormone (GnRH) from individually-incubated hemihypothalami. Allopregnanolone suppressed GnRH release in a concentration-dependent manner with maximal activity in the nanomolar range, a range at which this neurosteroid is capable of playing a biological action. The specificity of allopregnanolone suppression of GnRH release was provided by the lack of effect of its known inactive stereoisomer. To evaluate the involvement of

gamma-aminobutyric acid<sub>A</sub> (GABA<sub>A</sub>) receptor, we examined the effects of two neurosteroids with GABA-antagonistic properties, pregnanolone sulfate (PREG-S) and dehydroepiandrosterone sulfate (DHEAS), and of bicuculline, a selective antagonist of the GABA binding site on the GABA<sub>A</sub> receptor, on allopregnanolone (10 nM)-suppressed GnRH release. Both PREG-S and bicuculline overcame the inhibitory effects of allopregnanolone on GnRH release, whereas DHEAS did not. To substantiate the involvement of the GABA<sub>A</sub> receptor further, we tested the effects of muscimol, a selective agonist for this receptor, which suppressed GnRH release.

In conclusion, allopregnanolone suppressed hypothalamic GnRH release *in vitro* and this effect appeared to be mediated by an interaction with the GABA<sub>A</sub> receptor. We speculate that the inhibitory effect of allopregnanolone on the HPG axis may also be caused by its ability to suppress hypothalamic GnRH release.

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## Introduction

An increasing body of literature has shown that the central nervous system (CNS) is capable of metabolizing and/or synthesizing steroid hormones. These steroids of central origin have been termed neurosteroids or neuroactive steroids (Baulieu *et al.* 1987). They regulate the activity of the gamma-aminobutyric acid<sub>A</sub> (GABA<sub>A</sub>) receptor in a bimodal fashion: some neuroactive steroids behave as allosteric GABA<sub>A</sub> receptor agonists (Majewska *et al.* 1986), whereas others act as antagonists of this receptor (Majewska & Schwartz 1987). One of the most potent naturally occurring neuroactive steroids with allosteric GABA-agonistic properties is 5 $\alpha$ -pregnane-3 $\alpha$ -ol-20-one (allopregnanolone) (Majewska *et al.* 1986).

Neuroactive steroids constitute a group of multimodal neuromodulators involved in stress (Purdy *et al.* 1990, Guo *et al.* 1995), depression and anxiety (Bertholini *et al.* 1986, Schwartz-Giblin & Pfaff 1987), and cognitive functions (Flood & Roberts 1988). The evidence that the estrous cycle and some sexual behavioral changes are associated with modified neurosteroid activity in the CNS has suggested that they may also play an important role in reproductive function. A pituitary site of action has been suggested by the finding that 3 $\alpha$ -hydroxy-4-pregnene-20-one suppresses basal and gonadotropin-releasing hormone (GnRH)-stimulated follicle-stimulating hormone (FSH) secretion in primary cultures of rat anterior pituitary cells (Wiebe & Wood 1987, Wiebe *et al.* 1994). On the other hand, the proestrous-related changes of hippocampal

(Palumbo *et al.* 1995) and hypothalamic (Genazzani *et al.* 1995) allopregnanolone contents and the evidence that its passive immunoneutralization suppresses ovulation and sexual behaviour (Genazzani *et al.* 1995), suggests that allopregnanolone also acts centrally. The present study was undertaken to evaluate the effects of allopregnanolone on hypothalamic GnRH release *in vitro*. A possible involvement of the GABA<sub>A</sub> receptor was evaluated by testing the effects of pregnanolone sulfate (PREG-S) and dehydroepiandrosterone sulfate (DHEAS), two neurosteroids with GABA-antagonistic properties (Majewska & Schwartz 1987, 1990), and of bicuculline, a selective antagonist of the GABA binding site on the GABA<sub>A</sub> receptor (Bowery *et al.* 1984), on allopregnanolone-suppressed GnRH release.

## Materials and Methods

Experiments were performed as previously reported (Calogero *et al.* 1993). Briefly, intact male Sprague-Dawley rats weighing 200–225 g (Charles River, Calco, Co, Italy) were killed by decapitation, and hypothalamic blocks were rapidly removed. The hypothalami were sectioned longitudinally and placed in a water-jacketed incubator at 37 °C, under an atmosphere of 5% CO<sub>2</sub> and 95% air. Preincubation and incubation were carried out using medium 199 with modified Earle's salt (Life Technology, Paisley, Strathclyde, UK) containing 0.1% bovine serum albumin (Sigma Chemical Co., St Louis, MO, USA) and 10 µM bacitracin (zinc salt, Aldrich, Milwaukee, WI, USA) (M199).

The hemi-hypothalami were pre-incubated for 30 min before the experiment started. The experimental design consisted of four passages of the hemi-hypothalami through different wells (one tissue/0.5 ml) every 45 min (total length of the experiment 180 min). Tissue transfer from one well to another was carried out with a 3 × 3 mm nylon mesh grid (nylon monofilament 44 µ, Small Parts, Miami, FL, USA). In the fourth well each hemi-hypothalamus employed in this study was exposed to 60 mM KCl to test tissue viability by identifying the response of GnRH. Two different protocols were used in this study.

Protocol 1 was designed to evaluate the effects of allopregnanolone, its inactive stereoisomer 5α-pregnane-3β-ol-20-one, PREG-S, DHEAS, bicuculline and muscimol (Sigma Chemical Co.) upon unstimulated GnRH release. Therefore, each hemi-hypothalamus was incubated in M199 without test substance in the first 2 wells (only the GnRH concentration in the first well was used as the basal release for a given tissue) and exposed to graded concentrations of the above-mentioned compounds in the third well (treated GnRH release). The effect of the zero concentration (control) was evaluated by incubating the hemi-hypothalami allocated to this treatment with M199 alone in the third well.

Protocol 2 was designed to evaluate the effects of PREG-S, DHEAS or bicuculline on allopregnanolone-suppressed GnRH release. The hemi-hypothalami were incubated in M199 in the first well (basal GnRH release), in the second and third wells they were incubated in graded concentrations of the above-mentioned substances. A maximally effective concentration of allopregnanolone (10 nM) was added in the third well (treated GnRH release). The effects of allopregnanolone were determined by incubating the hemi-hypothalami allocated to this treatment in M199 containing allopregnanolone alone in the third well.

The concentration of GnRH in the incubation medium was measured by RIA as previously reported (Calogero *et al.* 1993). Total binding of <sup>125</sup>I-labeled GnRH to the antiserum (AEC-12) was 43.4 ± 2.1% and non-specific binding was 2.2 ± 0.2%. The sensitivity (ED<sub>90</sub>) of the assay was 0.7 ± 0.004 pg/well. The intra-assay and inter-assay coefficients of variation were 2.8 ± 0.1% and 17.1% respectively.

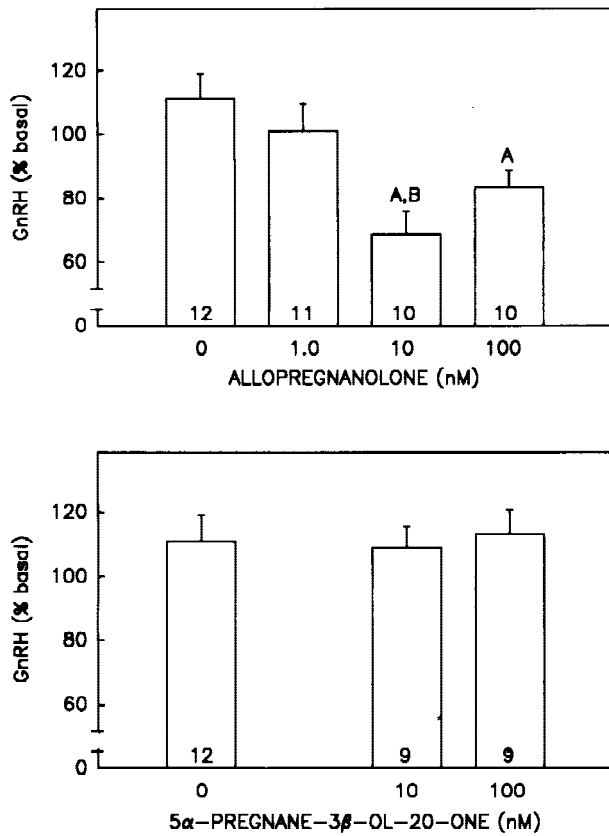
The results (means ± s.e.m.) were calculated as a percentage of the basal GnRH release for each hemi-hypothalamus, applying the following formula: GnRH (%) = treated GnRH release divided by basal GnRH release multiplied by 100. Statistical evaluation was carried out employing one-way analysis of variance (ANOVA) followed by the Duncan's multiple range test or unpaired Student's *t*-test, as appropriate. Significance was accepted at a *P* value lower than 0.05.

## Results

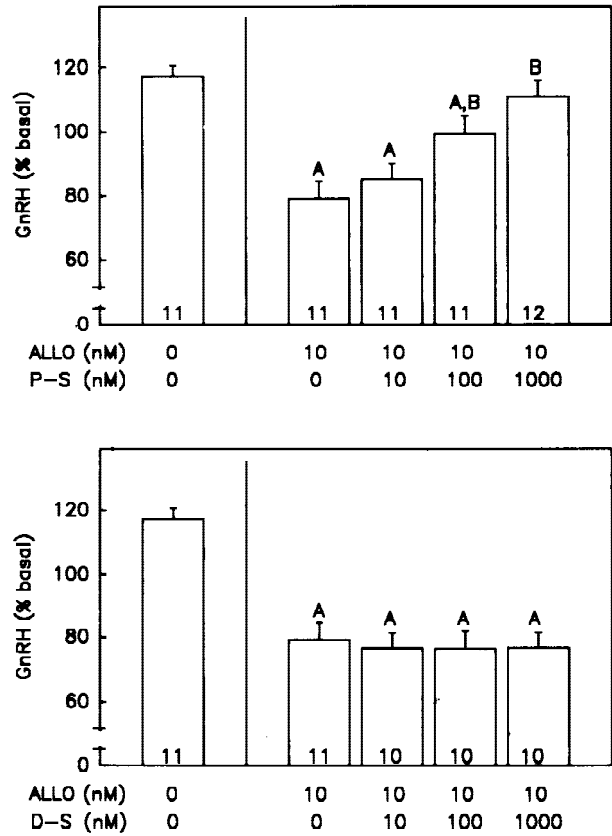
The mean GnRH release was 8.9 ± 0.7 pg/well in M199 alone (well 1) and it increased by about threefold following exposure to KCl (well 4). The slightly higher release of GnRH in M199 alone found in well 3 explains why the zero columns of each figure have a value greater than 100%.

### *Effects of allopregnanolone on GnRH release*

The effect of increasing concentrations of allopregnanolone on GnRH release is shown in Fig. 1 (upper panel). Allopregnanolone suppressed GnRH release in a concentration-dependent fashion (*P* < 0.005, ANOVA). The maximally effective concentration tested was 10 nM which suppressed GnRH release by about 28%. At higher concentrations, allopregnanolone was less effective. To test the specificity of this effect, we evaluated whether 5α-pregnane-3β-ol-20-one, an inactive stereoisomer of allopregnanolone, had any effect on GnRH release. In contrast to allopregnanolone, this neurosteroid did not have any effect upon unstimulated GnRH release (Fig. 1, lower panel).



**Figure 1** Effects of allopregnanolone (upper panel) and of the allopregnanolone inactive stereoisomer 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one (lower panel) on hypothalamic GnRH release *in vitro*. The number of hemi-hypothalami tested for each concentration is shown at the base of each bar. A,  $P < 0.05$  vs zero; B,  $P < 0.05$  vs 1.0 nM.



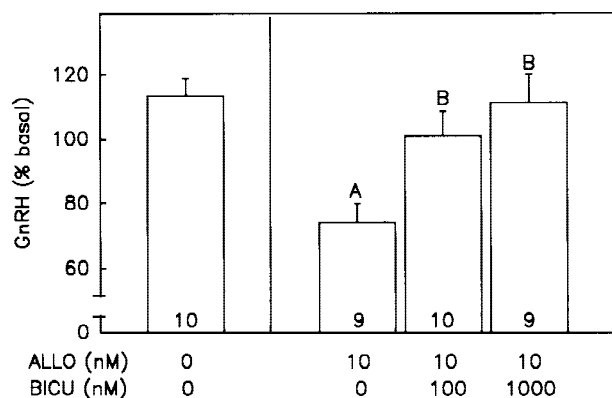
**Figure 2** Effects of pregnanolone-sulfate (P-S) (upper panel) or dehydroepiandrosterone-sulfate (D-S) (lower panel) on hypothalamic GnRH release suppressed by a maximally effective concentration of 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one (ALLO) *in vitro*. The number of hemi-hypothalami tested for each concentration is shown at the base of each bar. A,  $P < 0.05$  vs control (left hand bar); B,  $P < 0.05$  vs ALLO 10 nM.

#### Involvement of the GABA<sub>A</sub> receptor

The suppressive effect of 10 nM allopregnanolone was counteracted by PREG-S in a concentration-dependent fashion (Fig. 2, upper panel). At concentrations 100 times higher, PREG-S overcame completely the inhibitory effect of allopregnanolone. In contrast, DHEAS, another steroid with GABA<sub>A</sub> receptor antagonistic properties, did not have any effect on GnRH release suppressed by allopregnanolone (Fig. 2, lower panel). To evaluate further the involvement of the GABA<sub>A</sub> receptor, we tested the effect of allopregnanolone on GnRH release in the presence of bicuculline, an antagonist of the GABA binding site on the GABA<sub>A</sub> receptor. Bicuculline overcame the suppressive effects of allopregnanolone in a concentration-dependent fashion ( $P < 0.005$ , ANOVA) (Fig. 3). PREG-S, DHEAS and bicuculline did not have any effect upon non-stimulated GnRH release (Table 1). The involvement of the GABA<sub>A</sub> receptor was further substantiated by the finding that muscimol, a selective agonist for this receptor, suppressed GnRH release (Table 1).

#### Discussion

The present study shows that allopregnanolone suppresses the release of hypothalamic GnRH *in vitro*. This effect appears to be specific since an inactive stereoisomer of allopregnanolone had no effect. This finding supports recent data showing an inhibitory role for this neuroactive steroid on the ovulation process. Indeed, the central administration of allopregnanolone inhibits ovulation in rats, while an increased ovulatory rate was obtained when the activity of endogenous allopregnanolone was blocked by injecting an anti-pregnanolone serum into the cerebral ventricles (Genazzani *et al.* 1995). Further support for a central site of allopregnanolone action comes from a study showing a decrease in its hypothalamic content on the afternoon of proestrus, with a subsequent attenuation of the inhibition of GnRH release (Genazzani *et al.* 1995). The lack of changes in brain cortex concentration adds specificity to the changes seen at the hypothalamic level.



**Figure 3** Effects of the GABA<sub>A</sub> receptor antagonist bicuculline (BICU) on hypothalamic GnRH release suppressed by a maximally effective concentration of 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one (ALLO) *in vitro*. The number of hemi-hypothalami tested for each concentration is shown at the base of each bar. A,  $P<0.05$  vs control (left hand bar); B,  $P<0.05$  vs ALLO 10 nM.

Neuroactive steroids interact with the ionotropic GABA<sub>A</sub> receptor whose activation produces an influx of chloride ions through the associated chloride channel, resulting in hyperpolarization of the neuronal membrane. The activity of the GABA<sub>A</sub> receptor is modulated by benzodiazepines and barbiturates, which potentiate the effects of GABA, and by convulsants, such as picrotoxin, which inhibit receptor activity. Allopregnanolone is a potent activator of the GABA<sub>A</sub> receptor (Majewska *et al.* 1986, Harrison *et al.* 1987, Lambert *et al.* 1990). Similarly to other GABA-agonistic neurosteroids, allopregnanolone (a) enhances GABA and benzodiazepine binding to brain membranes (Majewska *et al.* 1986, Harrison *et al.* 1987, Majewska *et al.* 1988, Peters *et al.* 1988, Turner *et al.* 1989), (b) inhibits the binding of convulsant agents to the GABA<sub>A</sub> receptor-operated chloride channel (Majewska *et al.* 1986, Gee *et al.* 1988, Turner *et al.* 1989), (c) enhances the chloride ion transport induced by GABA (Majewska *et al.* 1986, Turner *et al.* 1989, Im *et al.* 1990), and (d) potentiates the GABA<sub>A</sub> receptor-mediated current in neurons (Majewska *et al.* 1986, Harrison *et al.* 1987,

Peters *et al.* 1988). GABA-enhancing effects are obtained at nanomolar concentrations (threshold concentration is 20–30 nM), but a slightly higher concentration is required to directly open the chloride channel in neurons (Majewska *et al.* 1986, Harrison *et al.* 1987, Lambert *et al.* 1990).

Our finding that allopregnanolone was fully effective in suppressing GnRH release at a concentration of 10 nM, suggests that its effect is mediated by interaction with the GABA<sub>A</sub> receptor. Further support for this hypothesis came from the finding that the allopregnanolone inactive stereoisomer 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one, a compound lacking the 3 $\alpha$ -hydroxyl group which is vital for the GABA-agonistic properties (Im *et al.* 1990), was devoid of effect. Furthermore, the GABA<sub>A</sub> antagonistic neuroactive steroid, PREG-S, and the antagonist of the GABA binding site on the GABA<sub>A</sub> receptor, bicuculline, were able completely to counteract the suppressive effects of allopregnanolone on GnRH release *in vitro*. However, DHEAS, another steroid with GABA<sub>A</sub> antagonistic features and capable of inhibiting the GABA-induced currents in neurons (Majewska *et al.* 1988, 1990), was devoid of effects. Although we do not have a definitive explanation for this discrepancy, it may relate to the different mode and/or site of action of these steroids. For example, while PREG-S inhibits the binding of several chloride channel ligands, DHEAS is devoid of this capability (Majewska & Schwartz 1987, Majewska *et al.* 1990). Moreover, DHEAS, unlike PREG-S, does not enhance the benzodiazepine binding on the GABA<sub>A</sub> receptor, but reduces it (Demirgoren *et al.* 1991). An involvement of the GABA<sub>A</sub> receptor in the regulation of GnRH release is also supported by studies showing that the infusion of GABA<sub>A</sub>, but not GABA<sub>B</sub>, receptor antagonists into the infundibular–median eminence induces GnRH release (Mitsushima *et al.* 1994) and that the chronic administration of the selective GABA<sub>A</sub> receptor agonist muscimol causes a decrease in GnRH mRNA levels in the hypothalamus (Li & Pelletier 1993). Accordingly, we found that it is able to suppress the release of GnRH *in vitro*. The lack of PREG-S, DHEAS or bicuculline effects on unstimulated GnRH *in vitro* is not at variance with these *in vivo* findings, but may be ascribed to a lack of tonic GABAergic inhibition in the explanted tissue.

**Table 1** Effects of pregnenolone-sulfate (PREG-S), dehydroepiandrosterone-sulfate (DHEAS), bicuculline and muscimol upon unstimulated hypothalamic GnRH release. Results are expressed as percentage of basal GnRH release. The number of hemi-hypothalami tested for each concentration is shown in parentheses

	Concentration ( $\mu$ M)			
	0	0.01	0.1	1.0
PREG-S	107.6 $\pm$ 4.2% (9)	104.3 $\pm$ 8.3% (11)	104.3 $\pm$ 9.1% (8)	109.7 $\pm$ 5.5% (9)
DHEAS	107.6 $\pm$ 4.2% (9)	101.9 $\pm$ 7.1% (10)	109.9 $\pm$ 9.2% (9)	107.5 $\pm$ 6.6% (8)
Bicuculline	113.4 $\pm$ 5.5% (10)	NT	96.5 $\pm$ 8.1% (10)	112.5 $\pm$ 13.0% (9)
Muscimol	115.8 $\pm$ 4.3% (7)	87.1 $\pm$ 6.5%* (7)	71.7 $\pm$ 5.3%* (7)	NT

NT, not tested.

\* $P<0.05$  vs zero concentration (ANOVA followed by Duncan's test).

In conclusion, the present study showed that the neuroactive steroid allopregnanone is able to suppress the release of GnRH from the rat hypothalamus through a mechanism involving the GABA<sub>A</sub> receptor. We speculate that the suppressive effects of allopregnanone on the reproductive function and sexual activity may also be mediated by its ability to inhibit hypothalamic GnRH release.

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