DiVA – Digitala Vetenskapliga Arkivet <http://umu.diva-portal.org>

This is an article published in **Gynecological endocrinology**

Citation for the published paper:

Erika Timby, Helena Hedström, Torbjörn Bäckström, Inger Sundström-Poromaa, Sigrid Nyberg

Allopregnanolone, a GABA-A receptor agonist, decreases gonadotropin levels in women. A preliminary study.

Gynecological endocrinology, 2011, Vol. 27, Issue: 12, pp. 1087-1093]

URL: <http://dx.doi.org/10.3109/09513590.2010.540603>

Allopregnanolone, a GABA_A receptor agonist, decreases gonadotropin levels in women. A preliminary study.

Erika Timby MD^{1*}, Helena Hedström MD^{1*}, Torbjörn Bäckström MD, PhD¹, Inger Sundström-Poromaa MD, PhD², Sigrid Nyberg PhD¹, Marie Bixo MD, PhD¹

¹Umeå Neurosteroid Research Centre, Department of Clinical Science, Obstetrics and Gynaecology, Umeå University, SE-901 85 Umeå, Sweden

²Department of Women's and Children's Health, Uppsala University, SE-751 05 Uppsala, Sweden

*These two authors have contributed equally to the paper.

Correspondence to:

Marie Bixo MD, PhD

Department of Clinical Science, Obstetrics and Gynaecology

Umeå University

SE-901 85 Umeå, Sweden

E-mail: marie.bixo@obgyn.umu.se

Phone: +46-90-785 34 61

Short title: Allopregnanolone decreases gonadotropins

Key words: FSH, LH, GABA, allopregnanolone, isoallopregnanolone

Abstract

Animal studies suggest regulatory effects on the hypothalamic-pituitary-gonad axis by allopregnanolone, an endogenous GABA_A receptor agonist. Elevated levels of allopregnanolone in women with hypothalamic amenorrhea have been seen.

Isoallopregnanolone is an isomer to allopregnanolone, but without GABA_A receptor effects.

The purpose of this study was to investigate effects of allopregnanolone and isoallopregnanolone on gonadotropin levels in healthy women of fertile age. Ten women were given allopregnanolone and five women isoallopregnanolone intravenously in follicular phase. Repeated blood samples were drawn during the test day. Main outcomes were changes in serum levels of FSH, LH, oestradiol and progesterone.

Serum-FSH decreased between 5 and 105 minutes after the allopregnanolone injection ($F(16,144) = 2.18, p = 0.008$). Serum-LH were reduced between 5 and 35 minutes following the allopregnanolone injection ($F(16,144) = 2.63, p = 0.001$). Serum-oestradiol and -progesterone were not significantly changed after allopregnanolone injections. No effect on gonadotropin levels were seen after administration of isoallopregnanolone. Allopregnanolone reduces FSH and LH levels in women and the effect might be mediated via a specific GABA_A receptor activation since isoallopregnanolone lacked this effect. Although the number of women was small, the results suggest a regulatory mechanism on the hypothalamic-pituitary-gonadal axis by allopregnanolone.

Introduction

The pulsatile secretion of follicle-stimulating hormone (FSH) and luteinising hormone (LH) from the pituitary reflects the pulsatile secretion of GnRH (Gonadotropin-Releasing Hormone) from the medial basal hypothalamus. Abnormalities of GnRH secretion, both regarding frequency and amplitude of pulses, are discernible in women with hypogonadotropic hypogonadism [1]. Animal and *in vitro* studies suggest that the effect is mediated by neuroactive steroids [2-5]. Allopregnanolone (3 α -hydroxy-5 α -pregnan-20-one) is an endogenous metabolite from progesterone but also a neuroactive steroid and a potent modulator of the gamma-aminobutyric acid A (GABA_A) receptor [6]. It potentiates the effect of GABA by binding to the GABA_A receptor complex, thus enhancing inhibitory neurotransmission. In humans, injections of allopregnanolone cause sedation, objectively measured by a reduced saccadic eye velocity [7]. Isoallopregnanolone (3 β -hydroxy-5 α -pregnan-20-one) is also a progesterone metabolite and an isomer to allopregnanolone. As far as we know today, isoallopregnanolone lacks hormonal or GABA_A receptor effects [8]. Allopregnanolone and isoallopregnanolone serum concentrations vary during the menstrual cycle and in pregnancy in parallel with progesterone [9-11], as a reflection of steroid synthesis in the ovaries [9, 12]. In addition, both steroids are secreted from the adrenal cortex and can be synthesised *de novo* in the central nervous system from cholesterol [9]. Allopregnanolone secretion from the adrenal cortex increases during stress as shown in both animals and humans [13-14].

There are animal studies suggesting regulatory neuroendocrine effects of allopregnanolone via the GABA_A receptor. In female rats, ovulation was suppressed by allopregnanolone injections into the cerebral ventricles (icv), evidenced by a decreased number of oocytes collected at estrus. When endogenous allopregnanolone was blocked, the number of oocytes was subsequently increased [15]. In ovariectomised rats, primed with oestrogen and

progesterone, LH serum levels decreased following icv allopregnanolone, suggesting involvement of the GABA_A receptor and the dopamine system [16]. Barbiturates, also well known GABA_A agonists, have been shown to inhibit ovulation in rats through the GABA_A receptor [17].

Inhibition of hypothalamic GnRH secretion is the most common explanation behind anovulation in women of fertile age. However, the condition known as hypothalamic amenorrhea has so far been little understood and diagnosed by exclusion of other causes for anovulation. Although hypothalamic amenorrhea brings about major problems like infertility and osteoporosis, the mechanisms are poorly understood and further studies are warranted. In the present study, we aimed to investigate if the GABA active steroid allopregnanolone could affect the secretion of FSH, LH, oestradiol and progesterone when administered in the follicular phase in healthy, menstruating women.

Methods

Subjects

Twelve women were included in the allopregnanolone (ALLO) group and seven women in the isoallopregnanolone (ISOALLO) group. The women were recruited to each arm separately through advertisement in the local newspaper. Pharmacokinetics and sedative effects of allopregnanolone have been described previously for the ALLO group [7], as well as pharmacokinetics and sedative effects of isoallopregnanolone in the ISOALLO group [18]. Inclusion criteria for both groups were healthy women aged 18-40 with regular menstrual cycles. Exclusion criteria were treatment with any steroid compound (including oral contraceptives and hormonal intrauterine devices) for at least six months prior to enrolment in the study, treatment with benzodiazepines or other psychotropic drugs within the last three months, and treatment with any daily over-the-counter drug during the last four weeks prior to

inclusion. Further exclusion criteria were any current or previous significant somatic disease, any mental disorder, including premenstrual dysphoric disorder, during the last six months, or a history of drug abuse or excessive alcohol use (more than six glasses of wine or beer during one day within the last four weeks). The presence of psychiatric disorders at inclusion was evaluated using a structured psychiatric interview, Primary Care Evaluation of Mental Disorders (PRIME-MD). Prior to inclusion, a physical and gynaecological examination was performed, as well as a routine blood chemistry screen (total blood cell count, plasma glucose, liver enzymes, creatinine, sodium and potassium). All subjects had negative pregnancy tests and normal blood chemistry screens. No night work or jet-lag travels were allowed during the week preceding the study day. The women were informed in detail, both orally and in writing, about the purpose and performance of the study and gave their oral and written informed consent prior to inclusion. The study procedures were in accordance with ethical standards for human experimentation, established by the Declaration of Helsinki 1975, revised in 1983. The Regional Ethical Review Board, Umeå University and the Medical Products Agency of Sweden approved the study.

Experimental substances

The Umeå University Hospital Pharmacy prepared both the allopregnanolone and isoallopregnanolone experimental substances. The allopregnanolone solution for intravenous administration was formulated with purified allopregnanolone (3 α -hydroxy-5 α -pregnan-20-one, Umecrine AB, Umeå, Sweden), 15 mg dissolved in 100 ml albumin solution (Albumin, 200 mg/ml) using an ultrasound bath. The final solution contained 0.126 ± 0.003 mg/ml (mean $\pm$ SEM) allopregnanolone ($n = 9$). The isoallopregnanolone solution for intravenous administration was formulated with purified isoallopregnanolone (3 β -hydroxy-5 α -pregnan-20-one, Umecrine AB, Umeå, Sweden), 8 mg dissolved in 100 ml albumin solution (Albumin,

200 mg/ml) using an ultrasound bath. The solution was then filtered through two sterile filters. The final solution contained 0.0736 ± 0.00807 mg/ml (mean $\pm$ SD) isoallopregnanolone ($n = 6$). Concentrations of allopregnanolone and isoallopregnanolone, in each batch, were determined using high performance liquid chromatography (HPLC) and UV absorbance, respectively, as described earlier [19].

Study protocol

Administration of allopregnanolone or isoallopregnanolone was performed in the follicular phase in both study groups. In the ALLO group, the experiment was performed on cycle day 5-10, and in the ISOALLO group, on cycle day 6-12. To avoid influence of diurnal variations, all study patients were tested during the same time of the day with the first injection at 08.30 AM. No subjects had consumed alcohol during 24 hours prior to testing. Caffeine and tobacco use was restricted throughout the study day. Prior to the experiment, an intravenous cannula was inserted in each forearm, one for administration of the experimental substance, and one for drawing blood samples.

In the ALLO group, baseline serum levels of allopregnanolone, FSH, LH, progesterone and estradiol were drawn 15 minutes before the first allopregnanolone injection. Three intravenous injections of allopregnanolone were given with 30-minute intervals, using doses of 0.015, 0.03 and 0.045 mg/kg, producing a cumulative dose of 0.09 mg/kg. The reason for repeated dosing was that in this study, allopregnanolone was given for the first time to humans. This cumulative dose produced a sedative effect measured by a reduced saccadic eye movement, confirming that the dose affected the GABA-system [7]. Each injection was given over 30 seconds. After the first allopregnanolone injection, blood samples for serum concentrations of allopregnanolone, FSH, LH, progesterone and oestradiol were drawn at 5, 13, 21, 35, 43, 51, 65, 73, 81, 95, 105, 115, 150, 330, 600 and 780 minutes.

In the ISOALLO group, baseline serum levels of isoallopregnanolone, allopregnanolone, FSH, LH, progesterone and oestradiol were drawn 5 min before the first isoallopregnanolone injection. Three intravenous injections of isoallopregnanolone were given with 30-minute intervals, using doses of 0.04, 0.06 and 0.10 mg/kg, producing a cumulative dose of 0.20 mg/kg. The reason for repeated dosing was that in this study, isoallopregnanolone was given for the first time to humans. This cumulative dose did not affect the GABA-system as confirmed with saccadic eye movement measurements [18]. Each injection was given with an infusion rate of 2 mg/min. Blood samples for serum concentrations of isoallopregnanolone, allopregnanolone, FSH, LH, progesterone and oestradiol were drawn at 5, 13, 18, 35, 43, 48, 65, 73, 88, 95, 105, 115, 150, 330, 600 and 780 minutes after the first isoallopregnanolone injection.

Extraction and separation of cross reacting steroids

All samples were extracted with diethyl ether. Plasma (0.4 ml) was pipetted into a cylindrical flat bottom glass vial, where after water (0.5 ml) and diethyl ether (3.0 ml) were added. The samples were then allowed to stand on an orbital shaker for ten minutes. Following the liquid-liquid extraction, the water phase was frozen, and the ether phase was decanted and evaporated under nitrogen. In the ALLO group, allopregnanolone was separated from cross reacting steroids with celite chromatography. The method has been described in detail earlier [7]. In the ISOALLO group, separation of allopregnanolone and isoallopregnanolone from cross reacting steroids was made with HPLC, and in addition, allopregnanolone was eluted from the HPLC. The fractions were symmetrically collected around the peak retention time for isoallopregnanolone or allopregnanolone for further analysis with radioimmunoassay (RIA). No cross-reacting steroids had retention times close to the collected fractions. The recovery of the extraction and HPLC procedure was for isoallopregnanolone 95%. The

recovery of allopregnanolone was 98% and the results were compensated for recovery. The method has been described in detail earlier [18].

Radioimmunoassays of allopregnanolone and isoallopregnanolone

Allopregnanolone was measured by RIA using a polyclonal rabbit antiserum raised against 3 α -hydroxy-20-oxo-5 α -pregnan-11-yl carboxymethyl ether coupled to bovine serum albumin, made by R. H. Purdy, (The Scripps Research Institute, La Jolla, CA, USA; 20). The method has been described in detail earlier [7]. The sensitivity of the assay was 25 pg with an intra-assay coefficient of variation for allopregnanolone of 6.5% and an inter-assay coefficient of variation of 8.5%. For the isoallopregnanolone (3 β -hydroxy-5 α -pregnan-20-one) analysis, an antibody against pregnenolone (pregnenolone-3-monohemisuccinate-HSA; ICN Pharmaceuticals, Inc Orangeburg, NY, USA) was used as it also binds to isoallopregnanolone. Cross-reactivity with isoallopregnanolone was 26.6%, allopregnanolone 13%, 5 α -pregnan-3,20-dione 7%, 5 β -pregnane-3 β -ol-20-one <1%, 5 β -pregnan-3 α -ol-20-one <1%, 5 α -pregnan-3 α ,20 α -diol <1%, pregnenolone 100%. The sensitivity of the assay (2 SD above the blank mean) was 0.11 pmol or 35.8 pg, with an intra-assay coefficient of variation of 8.5% and an inter-assay coefficient of variation of 10.7%. The method has been described in detail earlier [18].

Analyses of FSH, LH, progesterone and oestradiol

Concentrations of FSH and LH were analyzed with a solid phase, two-site chemiluminescent immunometric assay (Immulite®). Progesterone levels were measured with a sequential competitive immunoassay and oestradiol with a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite®). All analyse kits were purchased from Diagnostic Products Corporation, Corporate Offices, Los Angeles, CA, USA.

Statistics

Serum concentrations of FSH, LH, progesterone and oestradiol are described as mean (SEM). Differences at baseline between the ALLO and the ISOALLO groups, concerning concentrations of allopregnanolone, FSH, LH, progesterone and oestradiol, as well as demographic data, were explored by the Mann-Whitney U-test. In each group, changes in serum concentrations from baseline and throughout the experiment were analysed by one-way ANOVA (analysis of variance) with repeated measures and least significant difference (LSD) as post hoc test. In the ANOVA, time-point was used as the independent factor and hormone concentration as the dependent factor. Maucley's test was performed for correction of degrees of freedom. The Spearman Rank Correlation test was used to explore the relationship between concentrations of allopregnanolone and gonadotropins. The SPSS statistical package (version 13.0) was used for all statistical analyses. P values less than 0.05 were considered to be statistically significant.

Results

Demographic data of the study groups are presented in Table I. Out of twelve included subjects, ten women completed the allopregnanolone (ALLO) study; two never performed the experiment because of changes of residency. Out of seven included subjects, five women completed the isoallopregnanolone (ISOALLO) study. Of the two drop-outs, one was excluded because of a vasovagal reaction on the test day at insertion of the cannulas and was never given any test injection. The second one was excluded to avoid interference with the preovulatory LH peak which was ongoing at the planned test day. The serum concentrations of allopregnanolone for one woman in the ALLO group were missing at 150, 600 and 780 minutes. In the ISOALLO group there were missing values for serum concentrations of

allopregnanolone for one woman at 5 and 88 minutes, and for another woman at 150 minutes. Missing values for serum concentrations of isoallopregnanolone in the ISOALLO group were for one woman at 105 minutes, for another woman at 330 and 600 minutes, and for a third woman at 330 minutes.

Figure 1 shows the serum concentrations of FSH and LH following allopregnanolone and isoallopregnanolone injections. FSH concentrations were significantly reduced between 5 and 105 minutes after the first allopregnanolone injection ($F(16,144) = 2.18$, $p = 0.008$, results from post hoc analyses displayed in Figure 1A, top panel). Likewise, serum concentrations of LH were significantly reduced between 5 and 81 minutes following the first allopregnanolone injection, with exception for 43 and 51 minutes ($F(16,144) = 2.63$, $p = 0.001$, results from post hoc analyses displayed in Figure 1A, middle panel). In the ALLO group, post hoc analyses also indicated decreased serum concentrations of FSH at 330 minutes ($p < 0.05$) and for LH at 330 minutes ($p < 0.01$), 600 and 780 minutes ($p < 0.05$, data not shown). Furthermore, a small but significant negative correlation between concentrations of allopregnanolone and FSH was found ($r_s = -0.17$, $p = 0.031$), however not between concentrations of allopregnanolone and LH. In the ISOALLO group, no significant changes were seen in serum concentrations of FSH and LH during the first 150 minutes until 780 minutes (Figure 1B, top and middle panel).

Serum concentrations of progesterone and oestradiol following allopregnanolone and isoallopregnanolone injections are shown in Figure 2. Progesterone concentrations decreased in the ALLO-group ($F(16,144) = 6.15$, $p < 0.001$). Post hoc analyses revealed a significant change at 5 minutes ($p < 0.05$, see Figure 2A, top panel) and there was also a significant decrease at 780 min ($p < 0.05$, data not shown). In the ISOALLO group, concentrations of progesterone were however increased ($F(16,64) = 4.54$, $p < 0.001$). Post hoc analyses indicated a significant increase in progesterone concentration between 35 and 73 minutes and

again at 95 and 115 minutes (Figure 2B, top panel). There was also a significant increase in serum concentrations of progesterone at 780 min ($p < 0.05$) in the ISOALLO group (data not shown). Oestradiol concentrations were not affected by injections of allopregnanolone or isoallopregnanolone (Figure 2, bottom panel).

The serum concentrations of allopregnanolone after each injection in the ALLO group are shown in Figure 1A, bottom panel. The serum concentrations of isoallopregnanolone and allopregnanolone after each injection in the ISOALLO group are shown in Figure 1B, bottom panel. In parallel to increasing concentrations of isoallopregnanolone there was also a rise in allopregnanolone concentrations (Figure 1B). Baseline concentrations of allopregnanolone differed between the ALLO group and the ISOALLO group (0.46 ± 0.05 vs 0.77 ± 0.06 nmol/l; $p = 0.01$). Baseline concentrations of oestradiol were also different (100.3 ± 36.6 vs 133.2 ± 31.0 pmol/l; $p = 0.043$), and for FSH a significant difference was approached (6.20 ± 0.25 vs 4.34 ± 0.70 mIU/ml; $p = 0.05$). Baseline serum concentrations for LH and progesterone did not differ between the two groups.

Discussion

The main finding of the present study is that allopregnanolone, a GABA_A-active steroid, decreases the serum levels of FSH and LH when given as intravenous injections in the follicular phase. In addition, the negative correlation noted between allopregnanolone and FSH levels points to the possibility of a direct mechanism.

However, there are several limitations to our study. Firstly, the number of participants was small. Secondly, the two groups were recruited and tested at different occasions. Thirdly, the concentrations of allopregnanolone were supra-physiological. The possible consequences of the methodological limitations to our study will be further discussed below.

There was a slight difference in baseline levels of oestradiol and FSH between the two groups. A plausible explanation to the higher baseline levels of oestradiol in the ISOALLO group is that they had higher body mass index. Forty percent of the subjects in the ALLO group were tobacco smokers but none in the ISOALLO group. Whether this fact influenced the hormone levels and/or metabolism is not clear. Other factors that could possibly interfere with baseline concentrations of oestradiol or FSH, e.g. age, menstrual cycle length and actual cycle day at injection, were not significantly different between the two groups.

An important question is if the effect seen here with supraphysiological concentrations of allopregnanolone could be applied to the physiological situation. The decrease in gonadotropins in the ALLO group was present 5 minutes after the first injection of allopregnanolone, when serum levels of allopregnanolone were approximately 20 nmol/l. Mean physiological levels of allopregnanolone in healthy women are 0.3-1.9 nmol/l in the follicular phase and 2.1-3.7 nmol/l in the luteal phase [10, 21-22]. In humans suffering from acute stress, endogenous allopregnanolone levels have been shown to rise from 1.0 to 1.3 nmol/l and the change was statistically significant [14]. Although the increase is discrete, the time point in relation to menstrual cycle phase as well as if the rise is constant or not might be of importance for effects on the ovulatory mechanism. The highest physiological allopregnanolone levels are reached in late pregnancy when maternal mean levels in plasma are 45-75 nmol/l with an inter-individual variation between 20 and more than 150 nmol/l [11, 23]. Still, as the changes in gonadotropin levels were detectable at the first time point after injection and effects of lower concentrations between baseline and the 5 minute value remain unstudied, an effect on gonadotropin levels of allopregnanolone in the physiological interval can not be excluded.

The results presented here are in accordance with earlier *in vitro* and animal studies where GnRH secretion, decreased gonadotropin levels and suppression of ovulation have been noted

following allopregnanolone administration [2-4, 15-16]. In addition, other GABA_A agonists have been found to inhibit ovulation in rats [17], however not in primates [24]. Several of the above mentioned studies displayed no effect of isoallopregnanolone on neuroendocrine function and it was thus concluded that the allopregnanolone effect is an exclusive GABA_A receptor mediated mechanism [2-4]. A ~~limited~~ number of studies have shown ~~absence of, or opposite~~ effects of allopregnanolone on neuroendocrine function *in vitro* and *in vivo* [25-28]. One explanation for these discrepancies might be that the sub-composition of the GABA_A receptor is different during different endocrine conditions [29-30]. Another plausible explanation is that the effect of allopregnanolone displays a bimodal pattern under certain circumstances [31].

There is a limited body of evidence indicating a relationship between levels of endogenous allopregnanolone and anovulation in humans. Higher baseline serum levels of allopregnanolone have been reported in subjects suffering from hypothalamic amenorrhea compared to healthy controls in the follicular phase of the menstrual cycle [32]. Moreover, elevated serum levels of allopregnanolone were found in patients with premature ovarian failure compared to both postmenopausal and fertile women [33]. Apart from pregnancy, the only other situation where elevated levels of allopregnanolone have been noted in humans is during stress [14].

As expected, both concentrations of allopregnanolone in the ALLO group and concentrations of isoallopregnanolone in the ISOALLO group increased in a dose-dependent manner following the injections. However, in parallel with the rise in isoallopregnanolone levels in the ISOALLO group there was a slightly delayed increase in allopregnanolone levels. In an earlier study, a correlation between allopregnanolone and isoallopregnanolone levels was noted after administration of isoallopregnanolone [18]. The reason for this relationship is probably that a metabolism of isoallopregnanolone into allopregnanolone could occur, a

phenomenon observed earlier [34]. The lack of effect on gonadotropins in the ISOALLO group supports the theory that the allopregnanolone decreasing effect on gonadotropin secretion is mediated through a specific GABA_A receptor activation and not an unspecific steroidal effect [35].

In the ALLO group, there was no increase in progesterone or oestradiol levels. Thus, the decrease in FSH and LH was not caused by negative feed-back of progesterone and oestradiol on the hypothalamic-pituitary-gonadal axis. In the ISOALLO group, on the other hand, there was an increase in progesterone levels starting at 35 min, a finding that is difficult to interpret. This increase of progesterone was however modest and did obviously not affect the gonadotropin levels. Nevertheless, the result is in accordance with an earlier study where pregnanolone (an isomer to allopregnanolone with similar but less potent effects on the GABA_A receptor) injections were given to women in the follicular phase and an increase in progesterone levels was found but no effect on gonadotropin secretion or oestradiol levels [36].

In conclusion, the present study shows that allopregnanolone reduces serum levels of FSH and LH in healthy women in the follicular phase, but does not influence serum concentrations of oestradiol and progesterone. Isoallopregnanolone did not affect levels of gonadotropins, indicating that the effect is mediated via the GABA_A receptor. Whether the decreased levels of gonadotropins are due to inhibited synthesis, decreased secretion, altered pulsatility or increased metabolism is unclear. Further studies are warranted to explore if a physiological rise in allopregnanolone, for instance in response to stress, could interfere with gonadotropin effects on the pituitary, and ultimately reproductive function in women.

Acknowledgements

The antiserum used in the allopregnanolone RIA was a kind gift from Professor R. D. Purdy Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA, USA. Mrs Elisabeth Zingmark and Ms Agneta Andersson are recognized for excellent laboratory work.

Declaration of Interest

This study was supported by grants from the Swedish Research Council, medicine proj. 4X-11198, EU structural fund objective 1 program, Umeå University Foundations, Västerbottens läns landsting, Umeå kommun, the Astrid Karlsson Foundation, and the Tore Nilsson Foundation. The authors declare no other financial interests relevant to the present study.

Table I

Demographic data for the ALLO group and the ISOALLO group.

	ALLO group n=10	ISOALLO group n=5	P-values*
Age, years, median (range)	26 (19-30)	30 (23-39)	NS
Body mass index, median (range)	22 (20-25)	24 (22-32)	p=0.01
Menstrual cycle length, days, median (range)	30 (28-36)	29 (27-31)	NS
Married, %	20.0	60.0	
Parity, median (range)	0 (0-2)	0 (0-2)	
Tobacco users, %	40.0	0	

* Mann-Whitney U-test

References

1. Santoro N, Filicori M, Crowley WF Jr. Hypogonadotropic disorders in men and women: diagnosis and therapy with pulsatile gonadotropin-releasing hormone. *Endocr Rev* 1986;7(1):11-23.
2. Calogero AE, Palumbo MA, Bosboom AMJ, Burrello N, Ferrara E, Palumbo G, Petraglia F, D'Agata R. The neuroactive steroid allopregnanolone suppresses hypothalamic gonadotropin-releasing hormone release through a mechanism mediated by the gamma-aminobutyric acid_A receptor. *J Endocrinol* 1998; 158: 121-125.
3. Sim JA, Skynner MJ, Herbison AE. Direct regulation of postnatal GnRH neurons by the progesterone derivative allopregnanolone in the mouse. *Endocrinology* 2001; 42(10): 4448-4453.
4. Sullivan SD, Moenter SM. Neurosteroids alter γ -aminobutyric acid postsynaptic currents in gonadotropin-releasing hormone neurons: a possible mechanism for direct steroidal control. *Endocrinology* 2003; 144(10): 4366-4375.
5. Yin C, Ishii H, Tanaka N, Sakuma Y, Kato M. Activation of A-type γ -amino butyric acid receptors excites gonadotropin-releasing hormone neurons isolated from adult rats. *J Neuroendocrinol* 2008; 20(5): 566-575.
6. Majewska MD, Harrison NL, Schwartz RD, Baker JL, Paul SM. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science* 1986; 232: 1004-1007.

7. Timby E, Balgard M, Nyberg S, Spigset O, Andersson A, Porankiewicz-Asplund J, Purdy RH, Zhu D, Backstrom T, Sundstrom-Poromaa I. Pharmacokinetic and behavioral effects of allopregnanolone in healthy women. *Psychopharmacology (Berl)* 2006; 186: 414-424.
8. Gee KW, Chang WC, Briton RE, McEwen BS. GABA-dependent modulation of the Cl⁻ ionophore by steroids in rat brain. *Eur J Pharmacol* 1987; 136: 419-423.
9. Corpechot C, Young J, Calvel M, Wehrey C, Veltz JN, Touyer G, Mouren M, Prasad VV, Banner C, Sjoval J. Neurosteroids: 3 alpha-hydroxy-5 alpha-pregnan-20-one and its precursors in the brain, plasma, and steroidogenic glands of male and female rats. *Endocrinology* 1993; 133: 1003-1009.
10. Genazzani AR, Petraglia F, Bernardi F, Casarosa E, Salvestrone C, Tonetti A, Nappi RE, Luisi S, Palumbo M, Purdy RH, Luisi M. Circulating levels of allopregnanolone in humans, gender, age, and endocrine influences. *J Clin Endocrinol Metab* 1998; 83(6): 2099-2103.
11. Parizek A, Hill M, Kancheva R, Havlikova H, Kancheva L, Cindr J, Paskova A, Pouzar V, Cerny I, Drbohlav P, Hájek Z, Stárka L. Neuroactive pregnanolone isomers during pregnancy. *J Clin Endocrinol Metab* 2005; 90: 395-403.
12. Ottander U, Poromaa IS, Bjurulf E, Skytt A, Backstrom T, Olofsson JI. Allopregnanolone and pregnanolone are produced by the human corpus luteum. *Mol Cell Endocrinol* 2005; 239: 37-44.

13. Purdy RH, Morrow AL, Moore Jr PH, Paul SM. Stress-induced elevations of γ -aminobutyric acid type A receptor-active steroids in the rat brain. *Proc Natl Acad Sci USA* 1991; 88: 4553-4557.

14. Droogleever Fortuyn Hal A, van Broekhoven F, Span PN, Backstrom T, Zitman FG, Verkes RJ. Effects of PhD examination stress on allopregnanolone and cortisol plasma levels and peripheral benzodiazepine receptor density. *Psychoneuroendocrinology* 2004; 29: 1341-1344.

15. Genazzani AR, Palumbo MA, de Micheroux AA, Artini PG, Criscuolo M, Ficarra G, Gou AL, Benelli A, Bertolini A, Petraglia F, Purdy RH. Evidence for a role for the neurosteroid allopregnanolone in the modulation of reproductive function in female rats. *Eur J Endocrinol* 1995; 133(3): 375-380.

16. Laconi MR, Cabrera RJ. Effect of centrally injected allopregnanolone on sexual receptivity, luteinizing hormone release, hypothalamic dopamine turnover, and release in female rats. *Endocrine* 2002; 17(2): 77-83.

17. Hagino N, Ramaley JA, Gorski RA. Inhibition of estrogen-induced precocious ovulation by pentobarbital in the rat. *Endocrinology* 1966; 78: 451-454.

18. Hedstrom H, Bixo M, Nyberg S, Spigset O, Zingmark E, Backstrom T. Studies of pharmacokinetic and pharmacodynamic properties of isoallopregnanolone in healthy women. *Psychopharmacology (Berl)* 2009; 203: 85-98.

19. Turkmen S, Lundgren P, Birzniece V, Zingmark E, Backstrom T, Johansson IM. 3beta-20beta-dihydroxy-5alpha-pregnane (UC1011) antagonism of the GABA potentiation and the learning impairment induced in rats by allopregnanolone. *Eur J Neurosci* 2004; 20: 1604-1612.
20. Purdy RH, Moore PH Jr, Rao PN, Hagino N, Yamaguchi T, Schmidt P, Rubinow DR, Morrow AL, Paul SM. Radioimmunoassay of 3alpha-hydroxy-5alpha-pregnan-20-one in rat and human plasma. *Steroids* 1990; 55: 290–296.
21. Wang M, Seippel L, Purdy RH, Backstrom T. Relationship between symptom severity and steroid variation in women with premenstrual syndrome: Study on serum pregnenolone, pregnenolone sulfate, 5α- pregnane- 3,20-dione and 3α-hydroxy-5α-pregnan-20-one. *J Clin Endocrinol Metab* 1996; 81: 1076-1082.
22. Havlikova H, Hill M, Kancheva L, Vrbikova J, Pouzar V, Cerny I, Kancheva R, Starka L. Serum profiles of free and conjugated neuroactive pregnanolone isomers in nonpregnant women of fertile age. *J Clin Endocrinol Metab* 2006; 91: 3092-3099.
23. Hill M, Cibula D, Havlikova H, Kancheva L, Fait T, Kancheva R, Parizek A, Starka L. Circulating levels of pregnanolone isomers during the third trimester of human pregnancy. *J Steroid Biochem Mol Biol* 2007; 105: 166-175.
24. Hagino N. Effects of Nembutal on LH release in baboons. *Horm Metab Res* 1979; 11: 296-300.

25. Murphy LL, Mahesh VB. Selective release of luteinizing hormone by 3 alpha-hydroxy-5 alpha-pregnan-20-one in immature ovariectomized estrogen-primed rats. *Biol Reprod* 1984; 30(4): 795-803.
26. Brann DW, Putnam CD, Mahesh VB. Gamma-aminobutyric acidA receptors mediate 3 alpha-hydroxy-5 alpha-pregnan-20-one-induced gonadotropin secretion. *Endocrinology* 1990; 126(4): 1854-1859.
27. El-Etr M, Akwa Y, Fiddes RJ, Robel P, Baulieu EE. A progesterone metabolite stimulates the release of gonadotropin-releasing hormone from GT1-1 hypothalamic neurons via the γ -aminobutyric acid type A receptor. *Proc Natl Acad Sci USA* 1995; 92(9): 3769-3773.
28. Wiebe JP. Nongenomic actions of steroids on gonadotropin release. *Rec Progr Horm Res* 1997; 52: 71-101.
29. Han SK, Abraham IM, Herbison AE. Effect of GABA on GnRH neurons switches from depolarization to hyperpolarization at puberty in the female mouse. *Endocrinology* 2002; 143: 1459-1466.
30. Shen H, Gong QH, Aoki C, Yuan M, Ruderman Y, Dattilo M, Williams K, Smith SS. Reversal of neurosteroid effects at $\alpha 4\beta 2\delta$ GABAA receptors triggers anxiety at puberty. *Nat Neurosci* 2007; 10(4): 469-477.

31. Andréén L, Sundstrom-Poromaa I, Bixo M, Nyberg S, Backstrom T. Allopregnanolone concentration and mood – a bimodal association in postmenopausal women treated with oral progesterone. *Psychopharmacology* 2006; 187: 209-221.
32. Genazzani AD, Luisi M, Malavasi B, Strucchi C, Luisi S, Casarosa E, Bernardi F, Genazzani AR, Petraglia F. Pulsatile secretory characteristics of allopregnanolone, a neuroactive steroid, during the menstrual cycle and in amenorrheic subjects. *Eur J Endocrinol* 2002; 146: 347-356.
33. Bernardi F, Hartmann B, Casarosa E, Luisi S, Stomati M, Fadalti M, Florio P, Santuz M, Luisi M, Petraglia F, Genazzani AR. High levels of serum allopregnanolone in women with premature ovarian failure. *Gynecol Endocrinol* 1998; 12(5): 339-345.
34. Stromstedt M, Warner M, Bannor CD, MacDonald PC, Gustafsson JA. Role of brain cytochrome P450 in regulation of the level of anaesthetic steroids in the brain. *Mol Pharmacol* 1993; 44(5): 1077-1083.
35. Stromberg J, Haage D, Taube M, Backstrom T, Lundgren P. Neurosteroid modulation of allopregnanolone and GABA effects on the GABA-A receptor. *Neuroscience* 2006; 142: 73-81.
36. Sundstrom I, Backstrom T. Endocrine response to pregnanolone. *Gynecol Endocrinol* 1999; 13: 352-360.

Figure legends

Figure 1

Serum concentrations (mean $\pm$ SEM) of FSH (top panel), LH (middle panel), allopregnanolone and isoallopregnanolone (bottom panel), following allopregnanolone (1A) or isoallopregnanolone (1B) injections. Concentrations are shown at baseline (-15 minutes in the ALLO group, -5 minutes in the ISOALLO group) and until 150 minutes after the first injection of allopregnanolone and isoallopregnanolone, respectively. The steroid injections are indicated by dotted lines. Significant differences from baseline are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Serum concentrations (mean $\pm$ SEM) of progesterone (top panel) and oestradiol (bottom panel) from baseline until 150 minutes after the first injection of allopregnanolone in the ALLO group (2A) and isoallopregnanolone in the ISOALLO group (2B). The steroid injections are indicated by dotted lines. Significant differences from baseline are indicated: * $p < 0.05$, ** $p < 0.01$.

Figure 1

Figure 2