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Relationship between allopregnanolone and negative mood in postmenopausal women taking sequential hormone replacement therapy with vaginal progesterone

Lotta Andréen, Inger Sundström-Poromaa, Marie Bixo, Agneta Andersson, Sigrid Nyberg, Torbjörn Bäckström*

Umeå Neurosteroid Research Center, Department of Clinical Science, Obstetrics and Gynecology, Norrlands University Hospital, Umeå SE-901 85, Sweden

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Summary *Objective*: To compare severity of negative mood and physical symptoms between women with different progesterone, allopregnanolone, and pregnanolone plasma concentrations during sequential Hormone Replacement Therapy (HRT) with vaginal progesterone suppositories.

Design: A randomized, placebo-controlled, double-blind, crossover study.

Method: Postmenopausal women ($n=36$) with climacteric symptoms were treated with 2 mg estradiol daily during three 28-day cycles. Vaginal progesterone suppositories with 400, 800 mg/day or placebo were added sequentially for 14 days per cycle. Daily symptom ratings using a validated rating scale were kept. Blood samples for progesterone, allopregnanolone, and pregnanolone radioimmunoassays were collected during each treatment cycle.

Results: Women were divided into three groups (low, medium, and high) based on plasma allopregnanolone concentration during progesterone treatment. The concentration of allopregnanolone in the medium group corresponds to the concentration seen during the mid luteal phase of the menstrual cycle. Within women with medium allopregnanolone concentration significantly more negative mood and physical symptoms were rated during progesterone treatment compared to treatment with unopposed estrogen or placebo. Between women significantly more negative mood symptoms were seen during progesterone treatment cycles with medium allopregnanolone concentration compared to cycles with low concentration. Plasma progesterone, allopregnanolone, and pregnanolone concentrations increased with increasing progesterone dose. Progesterone and allopregnanolone plasma concentrations increased 2 h after vaginal administration of progesterone at 400 and 800 mg/day.

* Corresponding author. Tel.: +46-90-785-2144; fax: +46-90-776-006.
E-mail address: torbjorn.backstrom@obgyn.umu.se (T. Bäckström).

Conclusion: Vaginal progesterone in sequential HRT causes negative mood, most likely mediated via allopregnanolone.

1. Introduction

Sequential hormone replacement therapy (HRT) causes cyclicity in mood and physical symptoms in many women taking HRT. The symptoms are similar to symptoms seen during the luteal phase in ovulatory cycles and in premenstrual dysphoric disorder (PMDD). It appears that the addition of progestagens (Hammarback et al., 1985; Magos et al., 1986; Bjorn et al., 2000), and vaginal progesterone (Andreen et al., 2003) in sequential HRT provokes these negative mood symptoms. These progestagen- and progesterone-induced adverse mood effects seem to be dose-dependent but surprisingly, more accentuated negative mood effects are reported during treatment with 10 mg Medroxyprogesteronacetet (MPA) compared with 20 mg (Bjorn et al., 2002) and during treatment with 400 mg vaginal progesterone compared with 800 mg (Andreen et al., 2003). Similar influence on negative mood has been noted in women using oral contraceptives (OC) (Cullberg, 1972). In concordance with HRT studies, women on OC tends to feel worse on lower compared to higher dosages of progestagens (Cullberg, 1972).

Progesterone is metabolized to allopregnanolone (3α-OH-5α-pregnan-20-one) and pregnanolone (3α-OH-5β-pregnan-20-one) (de Lignieres et al., 1995). Both are neurosteroids and active agonists on the $GABA_A$ receptor (Majewska et al., 1986). In humans a high dose of pregnanolone is hypnotic and anesthetic (Carl et al., 1990; Sundstrom et al., 1998). Allopregnanolone has been reported to have anxiolytic effects in animals, although these effects never have been documented in humans (Wieland et al., 1991). However, a number of recent reports have indicated that allopregnanolone might not be as beneficial as previously thought, particularly not in low doses. In animal studies allopregnanolone has been shown to inhibit learning and memory (Johansson et al., 2002) and increase appetite (Chen et al., 1996), while short-term treatment has been reported to induce anxiety (Gulinello et al., 2001).

In fact, several $GABA_A$ receptor agonists, including allopregnanolone, have bimodal effects. In low doses or concentrations they induce loss of impulse control, negative mood, and aggression/irritability (Miczek et al., 1993, 1997, 2003; Ferrari et al., 1997; Beauchamp et al., 2000; Masia et al., 2000; Fish et al., 2001), whereas high doses induce sedation, hypnosis, and anxiolysis, and are antiepileptic (Herzog, 1991; Wieland et al., 1991; Sundstrom et al., 1998).

In the case of progesterone-induced adverse mood effects during HRT, there are also other factors to take into account. As the effects of progesterone are dependent on the metabolism to 5-α, 3-α hydroxy metabolites, the concentration of the $GABA_A$ receptor active metabolite will not only be dependent on the dose of progesterone given, but also influenced by the route of administration. Vaginal bacteria and mucosa seem not to have 5α- and 5β-reductases and 3α- and 20α-hydroxylases, and vaginally administered progesterone is absorbed without significant metabolic changes in contrast to orally administered progesterone, which is metabolized in the gut, intestinal wall, and liver (de Lignieres et al., 1995). In premenopausal women, plasma concentrations of progesterone are similar after oral and vaginal administration, while allopregnanolone plasma concentrations are significantly lower and pregnanolone plasma concentrations are not even significantly increased after vaginal administration (de Lignieres et al., 1995).

Given the reported findings of possible adverse mood effects of allopregnanolone, as well as the evidence of bimodal actions of a number of $GABA_A$ receptor agonists, the objective of this randomized, double-blind, placebo-controlled, crossover study was to investigate if the severity of mood and physical symptoms is related to progesterone, allopregnanolone, or pregnanolone plasma concentrations following vaginally administered progesterone. Furthermore, the study aimed at evaluating the differences in plasma concentrations of progesterone, allopregnanolone, and pregnanolone between two different doses of vaginally administered progesterone.

2. Methods

2.1. Subjects

Thirty-six women with climacteric symptoms were recruited and randomly assigned to treatment in a double-blind, cross-over study. The mean age was

52 years, range 44–60 years. Most subjects, 97%, were working and 65% were married. All subjects had climacteric symptoms with hot flushes or sweatings and all were more than 6 months postmenopausal. All women had an intact uterus and ovaries and had not used any HRT for the three months prior to inclusion in the study. They had no contraindications to HRT and were considered physically healthy. The women were not receiving any steroid treatment; had no history of psychiatric illness; and had not been treated with psychopharmacological drugs within the past 6 months. Subjects with ongoing psychiatric illness were excluded by use of PRIME-MD. PRIME-MD has been developed to help primary care physicians to screen, evaluate, and diagnose mental disorder. This diagnostic tool is constructed to conform to DSM-IV criteria and has been validated for use in a primary care setting (Spitzer et al., 1994). The agreement between PRIME-MD and independent psychiatric diagnoses guided by a structured interview is generally excellent across modules, with an overall accuracy of 88% (Spitzer et al., 1994). Before inclusion, subjects gave written informed consent and agreed to keep a daily record of symptoms. Before taking part, the patients underwent a physical and gynecological examination including routine vaginal ultrasonography. The Umeå University Ethical Committee and the National Medical Products Agency approved the design of the study.

2.2. Sample description

Twenty-nine women completed the study and were used in the further analysis. Of the 36 women who were originally included for the study, two dropped out during the study course (one due to heavy withdrawal bleeding and breast tenderness, one due to nausea). Five women were excluded (two due to a major life event during the study period, one due to failure in giving blood samples, and two due to failure in medicine intake).

2.3. Study design

The effect of progesterone 400 and 800 mg/day, given as vaginal suppositories, on mood, physical symptoms, and plasma concentrations of progesterone, allopregnanolone, and pregnanolone was evaluated in a randomized, placebo-controlled, double-blind, crossover design.

The study started with a 28-day run-in cycle where the patients were treated with 2 mg of estradiol valerat PO (Schering AG, Germany) daily and 10 mg of MPA PO (Leo Pharma, Sweden) on days 15–28 of the cycle. Because estrogen is known to decrease climacteric symptoms which can interfere with the well-being during the first month of treatment, this run-in cycle was included to avoid interference with mainly estrogen-dependent effects on climacteric symptoms in the subsequent analyses (Holst et al., 1989). The drawback to this procedure is that all cycles following a progestagen treatment will have a period of 3–4 days in the beginning of the next cycle where the symptoms from the previous cycle decline (Bjorn et al., 2000).

In the following three 28-day cycles, the women were treated with estradiol valerat PO in a dose of 2 mg daily throughout the study period. Progesterone or placebo vaginal suppositories were randomly added on days 15–28 of each cycle. One group started the sequential treatment with progesterone 800 mg/day, the second group with progesterone 400 mg/day, and the third group with placebo vaginal suppositories. All suppositories were given as 400, 200 mg, or placebo twice a day. A crossover to a new treatment was carried out after each cycle. The two progesterone doses chosen are the two pharmaceutical preparations available in Sweden at present and it has been shown that there is a significant increase in progesterone plasma concentrations after administration of the suppositories (de Lignieres et al., 1995). The vaginal formulation was a waxy suppository containing progesterone in a base of semi-synthetic glycerides produced from hydrogenated vegetable oil by interesterification.

The vaginal suppositories were prepared by Apoteket AB, Malmö, Sweden (the national pharmacy company), Production and Laboratories. They were made to appear identical. Packing and randomization were done by the pharmacy at Umeå University Hospital.

The primary outcome measure was the daily symptom ratings made by the patients throughout the study. We used a modified form of the Cyclicity Diagnoser (CD), an instrument for diagnosing cyclic symptoms that has been validated for the diagnosis of PMDD (Sanders et al., 1983; Sundstrom et al., 1999) but also used to evaluate HRT-related symptom changes in postmenopausal women (Sundstrom et al., 1999; Bjorn et al., 2000; Andreen et al., 2003). The CD included four physical symptoms (breast tenderness, hot flushes, abdominal bloating, and withdrawal bleeding) and seven psychological symptoms (cheerfulness, friendliness, libido, anxiety/tension, irritability, fatigue, and depression). The effects on daily life

caused by symptoms were graded. The CD is a Likert scale, graded from 0 to 8, where 0 indicates complete absence of a particular symptom and eight represents the maximum severity of the symptom. The patients can detect one scale step as a difference in mood experience, as shown in a study of symptom severity in women with PMDD (Sundstrom et al., 1999).

A gynecologist saw the patients twice, at inclusion and at termination of the study (16 weeks). During the study, patients made scheduled visits to a research nurse at 4, 8, and 12 weeks. Weight, blood pressure, and gynecological examination, including a routine vaginal ultrasound, were followed up after 16 weeks. The Umeå University Ethical Committee and the National Medical Products Agency approved the design of the study.

2.4. Steroid assays

Two blood samples for progesterone, allopregnanolone, and pregnanolone analyses were collected every cycle, during the last week of progesterone treatment. The first blood sample was taken immediately before the vaginal administration of progesterone in the morning (nadir sample). Two hours later the second blood sample for the same steroid analyses was taken (2 h sample).

Measurements of plasma progesterone were made by Delfia progesterone kits (Wallac Oy, Turku, Finland), a fluoroimmunoassay, according to the manufacturer's instructions.

2.4.1. Extraction and celite chromatography

Allopregnanolone and pregnanolone were measured with radioimmunoassay (RIA) after pre-assay diethylether extraction and celite chromatography purification. The 0.2 ml serum sample was extracted using 3.0 ml diethylether (Merck, Pro Analysi), evaporated under nitrogen and dissolved in 1.0 ml isooctane (Merck, Pro-analysi) saturated with ethyleneglycol (J.T. Baker, for analysis), before application to the column. The celite column chromatography was performed as follows. Glass columns (50 mm $\times$ 5 mm i.d.) were tightly packed with a mixture of celite (Mansville, Denver CO, USA, heated to 600 °C over night), and propylene glycol (Merck, Pro-analysi), weight: volume = 1:1. Isooctane (10 ml) was percolated through the columns before sample applications. The elution pattern was: first sample was applied. Thereafter 1.0 ml isooctane wash, followed by 1.5 ml isooctane to obtain 5α- and 5β-pregnan-3, 20-dione, additional 4.0 ml isooctane to obtain progesterone, additional 3.0 ml isooctane to obtain allopregnanolone and in the next 4.0 ml 4-pregnen-3α-ol-20-one and pregnanolone was eluted but 5α-pregnan-20β-ol-3-one is not eluted with isooctane. Thereafter a mixture (60:40) isooctane: toluene (Fisher chemicals, for analysis) was percolated. Pregnanolone was eluted and with the first 4 ml of the mixture and 5β-pregnan-3α, 21-diol-20-one and 5α-pregnan-3α, 21-diol-20-one were eluted with further 5.0 ml isooctane: toluene (60:40). The allopregnanolone and pregnanolone containing fractions were evaporated under nitrogen. Recovery was determined for each assay using 300–500 cpm of tritium-labeled allopregnanolone or pregnanolone (New England Nuclear, Boston, USA) added to a plasma sample before extraction and measuring the amount recovered after chromatography. The recovery of allopregnanolone was 78% and for pregnanolone was 85%.

2.4.2. RIA

The allopregnanolone antiserum, was raised against 3α-hydroxy-20-oxo-5α-pregnan-11-yl carboxymethyl ether coupled with bovine serum albumin (Purdy et al., 1990) and the pregnanolone antiserum against 3α, 21-dihydroxy-5β-pregnan-20-one 21-hemisuccinate coupled with bovine serum albumin (Sundstrom et al., 1998). Both antisera have low cross reactivity against its 5-reduced isomer. The antiserums were kind gifts from Dr. Robert H. Purdy, Department of Psychiatry, College of Medicine, University of California, San Diego, CA, USA. The standard curve was established by preparing duplicate tubes containing eight concentrations of unlabeled allopregnanolone or pregnanolone to give a range from 0 to 5000 pg. Antibody solution was prepared using tritium labeled steroid, 3 million cpm/30 ml ready made solution, 65 mM boric acid (Merck) buffer, pH = 8.0, bovine serum albumin solution 100 mg/ml (Sigma, St Louise, USA), human gamma globulin solution 20 mg/ml (Octapharma, Sweden) and antiserum in ratio 30:1:1:0.0002. The solution was allowed to equilibrate overnight at 8 °C. Antibody solution (200 μl) was added to all standard and sample tubes and the mixture allowed to stand overnight at 8 °C. After the addition of 200 μl saturated ammonium-sulfate each tube was again mixed and centrifuged at 20,000 rpm for 20 min. Thereafter the supernatant was aliquoted into a counting vial and diluted with 3.0 ml Optiphase scintillation medium (Wallac, Finland). The samples were counted in a RackBeta (Wallac, Finland) scintillation counter. The sensitivity of the assays was 25 pg, with an intraassay coefficient of variation

for allopregnanolone and pregnanolone of 6.5% and interassay coefficient of variation of 8.5%.

2.5. Statistics

Symptoms were analyzed separately and in clusters of related symptoms based on an earlier principal component analysis (Sanders et al., 1983). Related symptoms were grouped together as summarized symptom scores: 'negative mood symptoms'; tension, irritability, depression, and fatigue, 'positive mood symptoms'; cheerfulness and friendliness, and 'physical symptoms'; breast tenderness and bloating.

Differences in symptom scores during the treatment cycles within individuals, and the effects of the different plasma steroid concentrations on summarized negative-, positive-, and physical symptoms, were analyzed by 2-way analysis of variance (ANOVA) with repeated measures, and one-way ANOVA when suitable. Least significant difference test was used as post hoc method when applicable. In cases of missing values, the last value brought forward was used in an intention to treat manner in the repeated measure analysis. Mean (SEM) was given as measure of central tendency and variance. When squid distributions were found, median and inter quartile range (IQR, 25–75 percentiles) were given and non-parametric statistics were used. The SPSS statistical package was used for the analyses. $P<0.05$ was considered significant.

3. Results

3.1. Progesterone

Progesterone plasma concentrations were significantly increased 2 h after the application (2 h sample) compared to the concentration before the application (nadir sample) during treatment with progesterone at 400 mg/day p ($P<0.001$) and 800 mg/day ($P<0.001$). During treatment with progesterone 800 mg/day, plasma concentrations of progesterone were significantly higher in nadir sample ($P<0.001$) and 2 h sample ($P<0.001$) compared to 400 mg/day. Plasma progesterone concentrations are shown in Table 1.

3.2. Allopregnanolone

Allopregnanolone plasma concentrations were also significantly increased in 2 h sample compared to nadir sample during treatment with progesterone at 400 mg/day ($P<0.01$) and 800 mg/day ($P<0.01$). During treatment with progesterone 800 mg/day, plasma concentrations of allopregnanolone were significantly higher in nadir sample ($P<0.001$) and 2 h sample ($P<0.001$) compared to 400 mg/day (Table 1).

3.3. Pregnanolone

No difference in pregnanolone plasma concentrations between nadir sample and 2 h sample was

Table 1 Plasma progesterone, allopregnanolone, and pregnanolone concentrations are given as median and IQR (25 and 75 percentile) in the nadir sample and 2 h sample during treatment with placebo, progesterone (P.) 400 and 800 mg/day.

	Plasma concentration		*P* value (Nadir vs. 2 h sample)
	Nadir sample (nmol/l) median (IQR)	2 h sample (nmol/l) median (IQR)	
Progesterone			
Placebo	1.0 (0.5-1.2)	0.5 (0.3-1.0)	<0.01
P. 400 mg/d	16 (6.9-24)[a]	26 (18-34)[b]	<0.001
P. 800 mg/d	27 (10-43)[a]	34 (24-56)[b]	<0.001
Allopregnanolone			
Placebo	0.4 (0.3-0.5)	0.4 (0.3-0.5)	N.S.
P. 400 mg/d	4.5 (2.5-5.3)[c]	5.0 (3.7-6.0)[d]	<0.01
P. 800 mg/d	5.2 (4.0-8.3)[c]	6.1 (4.5-8.1)[d]	<0.01
Pregnanolone			
Placebo	0.5 (0.4-0.6)	0.5 (0.4-0.5)	N.S.
P. 400 mg/d	1.0 (0.8-1.1)[e]	1.0 (0.9-1.1)[f]	N.S.
P. 800 mg/d	1.1 (0.9-1.4)[e]	1.2 (1.0-1.3)[f]	N.S.

Analysis method Wilcoxon's matched pair signed rank test. P. 400 vs. 800 mg/day. [a]$P<0.001$; [b]$P<0.001$; [c]$P<0.001$; [d]$P<0.001$; [e]$P<0.025$; [f]$P<0.001$.

seen with any of the used progesterone doses. During treatment with progesterone 800 mg/day, plasma concentrations of pregnanolone were significantly higher in nadir sample ($P<0.025$) and 2 h sample ($P<0.001$) compared to 400 mg/day. Results in Table 1.

During the placebo treatment progesterone decreased in the 2 h sample but no other differences in steroid concentrations were seen.

3.4. Groups based on steroid concentrations

When the plasma concentrations of the progesterone metabolites were obtained it became obvious that there was a great intra- and inter-individual variation of allopregnanolone concentration. The total range of allopregnanolone during progesterone treatment (0.37–10.7 nmol/l) corresponds to values from low follicular to above luteal phase concentration in the menstrual cycle. Intra- and inter-individual variations in vaginal steroid resorption and metabolism are known to be major (de Lignieres et al., 1995). Therefore, plasma concentration of allopregnanolone (nadir sample) was used to divide the progesterone treatment cycles ($n=58$) into three groups with one third of the treatment cycles in each group. The groups were formed without accounting for the dose of progesterone administered. The three groups, based on median allopregnanolone concentration, were low concentration group (2.3 (1.4–3.5, IQR) nmol/l, $n=19$), medium concentration group (4.8 (4.5–5.3) nmol/l, $n=19$), and high concentration group (7.4 (5.9–9.1) nmol/l, $n=20$). The concentrations of progesterone, allopregnanolone and pregnanolone were significantly different between the three allopregnanolone concentration groups, F (2,57) = 8.1–92.0; $P<0.001$. The concentration of allopregnanolone in the low group was in the follicular phase range, in the medium group in the luteal phase range and in the high group above what is normally seen in the luteal phase of the menstrual cycle (Schmidt et al., 1994; Wang et al., 1996). The effect of plasma allopregnanolone concentration on mood and physical symptoms was evaluated within subjects using double-blind, crossover design and with repeated measures ANOVA and between subjects in the different allopregnanolone groups using the same statistical methods. Groups were also formed according to their plasma progesterone and pregnanolone concentration (one-third of the treatment cycles in each group as described above) and results are shown below.

3.5. Allopregnanolone and symptoms within subjects

3.5.1. Symptom cyclicity: differences between unopposed estradiol and progesterone treatment

The best period of the estrogen phase, when positive mood was at its highest and negative mood and physical symptoms at their lowest, occurred during the five last days of the estrogen phase (days 11–15). The worst period peaked during the late progesterone phase (days 25–1). In treatment cycles with medium concentration of allopregnanolone, the women scored significantly more negative mood and physical symptoms during the progesterone treatment period (days 25–1) compared to the estradiol only period (days 11–15), F (1,18) = 5.02; $P<0.05$, and F (1,18) = 16.4; $P<0.01$, respectively (Fig. 1). In treatment cycles with low allopregnanolone concentration, a cyclicity in physical symptoms was seen, F (1,18) = 10.4; $P<0.01$ (Fig. 1). Furthermore, a cyclicity in summarized positive symptom scores was evident in treatment cycles with high allopregnanolone concentration, F (1,19) = 5.75; $P<0.05$. Results including mean ± SEM of summarized symptom scores are shown in Table 2.

3.5.2. Differences between progesterone and placebo treatment

During treatment cycles with medium concentration of allopregnanolone (days 25–1), the women scored significantly more negative mood and physical symptoms during treatment with progesterone compared to placebo, F (1,18) = 7.63; $P<0.05$ and F (1,18) = 21.7; $P<0.001$, respectively. Furthermore, women in treatment cycles with high allopregnanolone concentration scored significantly more summarized positive mood symptoms during placebo compared to progesterone treatment, F (1,19) = 5.20; $P<0.05$. Results in Table 2.

3.6. Groups based on progesterone and pregnanolone concentration; symptoms within subjects

When groups were formed according to their progesterone or pregnanolone levels (as described above) no significant effect on mood and physical symptoms was obtained within the subjects.

3.7. Allopregnanolone and symptoms between subjects

3.7.1. Mood

During the end of the progesterone treatment (days 25–1), women in treatment cycles with

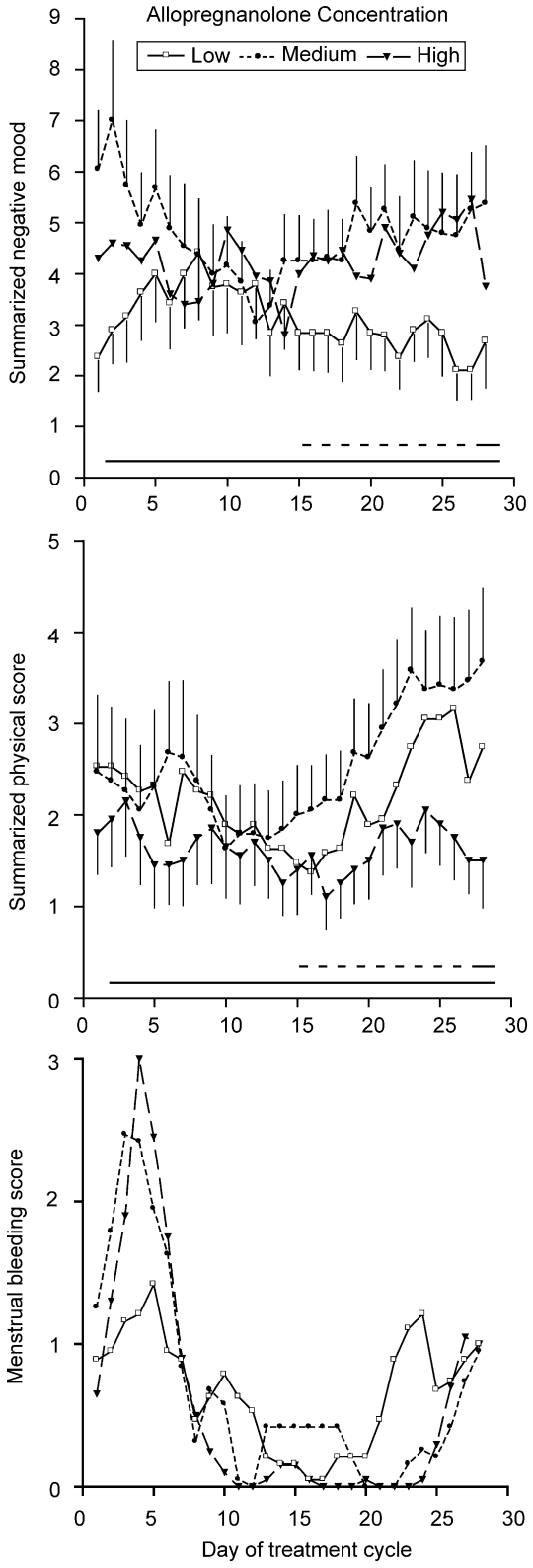

Figure 1 Mean summarized negative mood, physical, and bleeding scores in postmenopausal women on continuous estrogen (filled line, cycle days 1-28)+ sequential vaginal progesterone (dotted line, cycle days 15-28) treatment. Progesterone treatment cycles were divided into allopregnanolone (median, IQR) plasma concentration groups with low (2.3, 1.4-3.5 nmol/l), medium (4.8, 4.5-5.3 nmol/l), and high (7.4, 5.9-9.1 nmol/l) concentration. Women with medium allopregnanolone concentration scored significantly more negative mood symptoms compared to women with low concentration, F (1,36)=8.16; $P<0.01$, and significantly more physical symptoms compared to women with high concentration, F (1,37)=4.96; $P<0.05$. A significant cyclicity in negative mood and physical symptoms was seen, F (1,18)=5.02; $P<0.05$ and F (1,18)=16.4; $P<0.01$ respectively, in women with medium allopregnanolone concentration. A cyclicity in physical symptoms was also seen in women with low allopregnanolone concentration, F (1,18)=10.4; $P<0.01$. The bleeding scores indicates onset of bleeding the last days of progesterone-phase with maximal bleeding cycle days 3-5 next treatment cycle. 0, absence of bleeding.

medium allopregnanolone concentration had significantly more summarized negative mood symptoms (tension, irritability, depression, and fatigue), F (1,36)=8.16; $P<0.01$, compared to women in treatment cycles with low concentration (Fig. 1). In fact, separate investigation of each negative mood symptom showed that women in the medium allopregnanolone concentration group rated significantly more irritability (F (1,36)=5.84; $P<0.05$) and anxiety/tension (F (1,36)=6.10; $P<0.05$) compared to the low concentration group. In addition, more depression and fatigue were rated in the medium and high allopregnanolone concentration groups compared to the low concentration group, although not significant. Results in Table 3.

Similar results were obtained when groups were formed according to their 2 h sample for allopregnanolone plasma concentration (as described above). A significant difference in summarized negative mood scores between women in treatment cycles with low 2 h and medium 2 h plasma concentrations of allopregnanolone was evident, F (1,36)=5.35; $P<0.05$.

A correlation analysis was made between the allopregnanolone concentration and the summarized negative mood scores of the same day as the blood sample was taken. The analysis was made separately in the low, medium, and high allopregnanolone concentration groups. A significant negative correlation was evident in the medium concentration group Rs=−0.526; P=0.021, but no significance was found in the low concentration group (Rs=0.017; n.s.) or the high concentration group (Rs=0.067; n.s.). In Fig. 2, the median mood score is plotted related to the allopregnanolone concentration. There is a significant increase in negative mood score between <3 and 4 nmol/l groups ($P<0.05$, Fig. 2). In fact, the above results indicate

Table 2 Summarized symptom scores during treatment with unopposed estrogen (treatment days 11–15), estrogen + progesterone, and estrogen + placebo (treatment days 25–1), in postmenopausal women divided into groups with low ($n=19$), medium ($n=19$), and high ($n=20$) allopregnanolone (allo.) concentrations (conc.).

	Mean ± SEM			F (1,18–19); P		
	Estrogen	Progesterone	Placebo	Estrogen vs. progesterone	Placebo vs. progesterone	Estrogen vs. placebo
Low allo. conc.						
Negative symptoms	3.3 ± 0.8	2.7 ± 0.6	3.6 ± 0.7	N.S.	N.S.	N.S.
Positive symptoms	9.3 ± 1.0	9.3 ± 0.9	9.5 ± 1.0	N.S.	N.S.	N.S.
Physical symptoms	1.7 ± 0.6	3.0 ± 0.4	3.3 ± 1.1	10.4; <0.01	N.S.	N.S.
Medium allo. conc.						
Negative symptoms	3.8 ± 0.7	5.9 ± 0.9	3.2 ± 0.6	5.02; <0.05	7.63; <0.05	N.S.
Positive symptoms	10. ± 0.7	9.5 ± 0.7	10. ± 0.5	N.S.	N.S.	N.S.
Physical symptoms	1.8 ± 0.5	3.9 ± 0.7	1.0 ± 0.4	16.4; <0.01	21.7; <0.001	N.S.
High allo. conc.						
Negative symptoms	3.8 ± 1.1	5.1 ± 1.4	3.5 ± 0.9	N.S.	N.S.	N.S.
Positive symptoms	10. ± 0.8	8.9 ± 0.8	9.9 ± 0.9	5.75; <0.05	5.20; <0.05	N.S.
Physical symptoms	1.6 ± 0.4	2.1 ± 0.4	1.4 ± 0.5	N.S.	N.S.	N.S.

Analysis method was 2-way analysis of variance (ANOVA) with repeated measures (independent factors were treatment days and cycle phase, i.e. estrogen-, progesterone- or placebo phase).

a bimodal pattern between negative mood and allopregnanolone concentration as shown in Fig. 2.

Women displayed no relationship between positive mood symptoms and allopregnanolone concentration.

3.7.2. Physical symptoms

During the end of the progesterone treatment (days 25–1), women displayed significantly more physical symptoms in treatment cycles with medium allopregnanolone concentration compared to high concentration, F (1,37) = 4.96; $P<0.05$ (Fig. 1). In particular, symptom scores of breast tenderness were accentuated during cycles with medium compared to high concentration, F (1,37) = 6.43; $P<0.05$. Results in Table 3.

3.7.3. Symptoms and steroid plasma concentrations during unopposed estradiol treatment

During estrogen treatment (days 11–15), no significant difference in steroid plasma concentrations or summarized symptom scores were seen between women in treatment cycles with low, medium, or high allopregnanolone plasma concentrations. Results in Table 3.

3.8. Groups based on progesterone and pregnanolone concentration: symptoms between subjects

When groups were formed according to their progesterone or pregnanolone levels (as described above) no significant effect on mood and physical symptoms was obtained except during treatment cycles with medium pregnanolone plasma concentration (nadir sample), where significantly more negative mood symptoms were seen (5.55 ± .79) compared to the high pregnanolone concentration cycles (2.54 ± .40), F (1,37) = 4.98; $P<0.05$.

4. Discussion

The main finding of this study is that postmenopausal women in treatment cycles with medium allopregnanolone concentration display significantly more negative mood symptoms during progesterone treatment than during treatment with placebo and unopposed estradiol. In addition, women with medium allopregnanolone plasma concentration report during progesterone treatment a significant deterioration in mood symptoms compared to women in cycles with low levels of allopregnanolone.

During treatment cycles with high allopregnanolone plasma concentration, positive mood symptoms show a significant deterioration while the cyclical negative mood changes display a greater variability and no difference in negative mood symptoms during the progesterone treatment compared to placebo was found. Thus, our study indicates that mood-deteriorating effects are associated with allopregnanolone concentration during the progesterone phase of HRT and that this effect is absent during the unopposed estrogen

Table 3 Plasma steroid concentrations during estrogen + placebo, and estrogen + progesterone treatment, and symptom scores during unopposed estrogen phase (days 11–15) and estrogen + progesterone treatment phase (days 25–1) in postmenopausal women divided into groups with low ($n=19$), medium ($n=19$), and high ($n=20$), allopregnanolone (allo.) concentrations (conc.).

	Allopregnanolone groups					
	Low conc. allo.	Medium conc.allo.	High conc. allo.	Low conc. allo.	Medium conc.allo.	High conc. allo.
Plasma concentration median (IQR) (nmol/l)	Estrogen + placebo			Estrogen + progesterone		
Progesterone	0.8 (0.5-1.7)	0.5 (0.2-0.7)	0.9 (0.5-1.3)	5.9 (1.5-13)	21 (12-31)	30 (19-52)
Allopregnanolone	0.4 (0.3-0.5)	0.4 (0.3-0.4)	0.4 (0.3-0.4)	2.3 (1.4-3.5)	4.8 (4.5-5.3)	7.4 (5.9-9.1)
Pregnanolone	0.5 (0.4-0.6)	0.5 (0.4-0.6)	0.5 (0.4-0.5)	0.8 (0.6-1.1)	1.0 (0.9-1.2)	1.2 (1.0-1.5)
Symptom scores mean ± SEM	Estrogen only phase			Estrogen + progesterone phase		
Summarized negative symptoms	3.2 ± 0.4	3.9 ± 0.3	3.8 ± 0.6	**2.4 ± 0.4**[a]	**5.0 ± 0.6**[a]	4.9 ± 0.8
Irritability	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	**0.7 ± 0.1**[b]	**1.4 ± 0.2**[b]	1.2 ± 0.2
Anxiety/tension	0.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	**0.6 ± 0.1**[c]	**1.1 ± 0.2**[c]	0.9 ± 0.2
Depression	0.4 ± 0.1	0.5 ± 0.1	1.0 ± 0.2	0.4 ± 0.1	0.9 ± 0.1	1.2 ± 0.2
Fatigue	1.2 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	0.8 ± 0.2	1.6 ± 0.2	1.6 ± 0.2
Summarized positive symptoms	9.4 ± 0.4	10. ± 0.3	9.8 ± 0.4	9.6 ± 0.4	10. ± 0.4	9.4 ± 0.4
Cheerfulness	4.7 ± 0.2	5.0 ± 0.2	5.3 ± 0.2	4.9 ± 0.2	5.1 ± 0.2	4.9 ± 0.3
Friendliness	4.7 ± 0.2	5.1 ± 0.2	4.5 ± 0.2	4.7 ± 0.2	5.2 ± 0.2	4.6 ± 0.3
Summarized physical symptoms	1.8 ± 0.2	1.8 ± 0.2	1.5 ± 0.2	2.8 ± 0.3	**3.5 ± 0.4**[d]	**1.7 ± 0.2**[d]
Abdominal bloating	1.2 ± 0.2	0.6 ± 0.1	1.0 ± 0.1	1.5 ± 0.2	1.2 ± 0.2	0.9 ± 0.1
Breast tenderness	0.5 ± 0.1	1.1 ± 0.2	0.5 ± 0.1	1.3 ± 0.2	**2.2 ± 0.3**[e]	**0.8 ± 0.1**[e]
Hot flushes	0.5 ± 0.2	0.4 ± 0.2	0.3 ± 0.2	0.6 ± 0.2	0.8 ± 0.2	0.7 ± 0.2
Bleeding	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.8 ± 0.2	0.6 ± 0.2	0.8 ± 0.2
Effects on daily life	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1

Analysis method for symptom scores was 2-way analysis of variance (ANOVA) with repeated measures (independent factors were treatment days and cycle phase). Median, IQR and non-parametric methods were used for steroid concentrations. Medium vs. low allo. conc. Medium vs. high allo. conc. [a]$F(1,36)=8.16$, $P<0.01$; [b]$F(1,36)=5.84$, $P<0.05$; [c]$F(1,36)=6.10$, $P<0.05$; [d]$F(1,37)=4.96$, $P<0.05$; [e]$F(1,37)=6.43$, $P<0.05$.

phase and during placebo treatment. No relationship between symptom severity and plasma progesterone concentration was found. Finally, a significant increase in progesterone and allopregnanolone plasma concentration was seen 2 h after vaginal administration of progesterone 400 and 800 mg/day.

In our study particularly women with medium allopregnanolone concentration reported negative mood effects during the progesterone treatment phase. Importantly, their level of allopregnanolone was similar to levels seen in the luteal phase of the menstrual cycle (Table 3) (Wang et al., 1996; Schmidt et al., 1994).

Women with low allopregnanolone concentration, on the contrary, reported no mood-deteriorating effect during the progesterone phase. The concentration of allopregnanolone seen in this group is lower than the physiological level seen during the luteal phase of the menstrual cycle. Actually, several treatment cycles had allopregnanolone concentrations similar to what is seen during the follicular phase of the menstrual cycle (Wang et al., 1996; Schmidt et al., 1994). Earlier studies have indicated that plasma concentration of progesterone and its metabolites are correlated to the concentration in the brain, which is why we can assume that the allopregnanolone concentration in the brain is low in this group (Bixo et al., 1997). With plasma concentrations of allopregnanolone in the follicular phase range, no effect on central nervous system (CNS) can be expected as the follicular phase of the menstrual cycle is related to presence of positive symptoms and absence of negative symptoms (Backstrom et al., 1983; Wang et al., 1996). Adverse mood

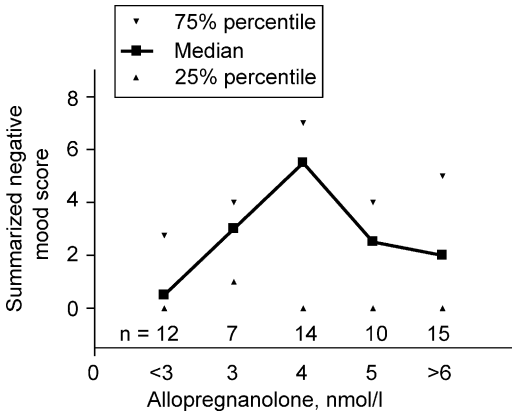

Figure 2 Median, inter quartile range (75 and 25% percentile) were used for measurement central tendency and variance of summarized negative mood scores in postmenopausal women treated with estrogen + progesterone. The mood scores were from the same day as and the plasma allopregnanolone concentrations were analyzed. The women were divided by their allopregnanolone concentrations in groups less than 3 ≤ 3, 3-4 = 3, 4-5 = 4, 5-6 = 5, and above 6 nmol/l.

during the menstrual cycle develops shortly after ovulation when the plasma progesterone and allopregnanolone concentrations start to increase (Backstrom et al., 1983; Wang et al., 1996). In agreement with the present observation are results from a controlled randomized study of antidepressant treatment for PMDD where post-treatment allopregnanolone levels were significantly lower in the improved subjects compared to the unimproved subjects (Freeman et al., 2002). Furthermore, Girdler et al has shown that PMDD is associated with higher allopregnanolone levels and lower stress induced elevations in allopregnanolone and also that PMDD women with greater levels of premenstrual anxiety and irritability had significantly reduced allopregnanolone levels in the luteal phase relative to less symptomatic PMDD women (Girdler et al., 2001).

Women with high allopregnanolone concentration had plasma allopregnanolone concentrations above what is maximally seen during the luteal phase (Schmidt et al., 1994; Wang et al., 1996). In fact, their allopregnanolone levels corresponded to concentrations seen during early pregnancy (Luisi et al., 2000). These women scored significantly less positive, but not more, or less, negative mood symptoms compared to women with low concentrations and compared to placebo treatment. The mean symptom severity in this group is similar to the symptom severity in the group with medium allopregnanolone levels, but due to a larger variability in symptom severity and few subjects in the high concentration group, the lack of significance is possibly caused by lack of power in comparison between the concentration groups.

The findings in the high allopregnanolone concentration group may be interpreted as though negative mood effects decline at high, supra-physiological plasma allopregnanolone concentrations. This interpretation is supported by the bimodial relationship between allopregnanolone concentration and the negative mood symptoms of the same day as allopregnanolone was measured. In other studies, no mood effects except symptoms of sedation have been related to high allopregnanolone concentrations. In a study with intramuscular progesterone treatment resulting in concentrations of allopregnanolone well beyond those seen during normal menstrual cycles, sedation and decrease in ratings of vigor and friendliness were noted (de Wit et al., 2001). With oral progesterone treatment, plasma allopregnanolone was significantly correlated to measures of fatigue, confusion, delayed verbal recall, and decreased symbol copying in subjects who achieved high levels (Freeman et al., 1993). In a controlled treatment study, using micronized oral progesterone, producing supra-physiological concentrations of allopregnanolone; no effect on mood symptoms compared to placebo was noted in women with PMDD (Vanselow et al., 1996). In addition, high allopregnanolone concentrations during the luteal phase can be related to decreased symptom severity as indicated in a study of allopregnanolone-symptom relationship between two cycles of women with PMS. In that study higher levels of allopregnanolone during the luteal phase were associated with slightly improved symptom ratings (Wang et al., 1996). The results are similar to them found by Girdler et al 2001.

There is however a paradox in the mood-allopregnanolone relationship. If a dose response effect between allopregnanolone levels and negative mood symptoms exists, the women should feel worse when a higher concentration is achieved. Such dose-response relationship is seen between the groups with low and medium allopregnanolone concentrations, whereas among women with high concentration no further mood deterioration is seen. Actually, no significant differences in mood scores between women with low and high concentrations of allopregnanolone were present. If allopregnanolone has a bimodial effect on mood in humans, as in rats (Beauchamp et al., 2000; Fish et al., 2001), an inverse relationship between concentration and effect is expected when the maximum negative mood effect is passed. This was also seen in the median group. One can

assume that the level of allopregnanolone corresponding to when the maximum negative mood effect is achieved varies between individuals as the sensitivity for pregnanolone can vary between individuals (Sundstrom et al., 1998). It is conceivable that certain women in the high concentration group might not have reached the maximum negative mood effect while others have passed the peak and are less negatively influenced by allopregnanolone and others still might even have reached an anxiolytic effect. Subsequently, a larger variability in symptom severity is seen in the high concentration group and this might also affect the chances to reach significance in the statistical tests.

The finding of a bimodal effect of allopregnanolone confirm the findings of previous studies in which several $GABA_A$ receptor positive allosteric modulators (i.e. allopregnanolone, benzodiazepines, barbiturates, and ethanol) have been reported to have a bimodial effect in the CNS. In low doses or concentrations, allopregnanolone (Beauchamp et al., 2000; Fish et al., 2001), benzodiazepines (Miczek et al., 1993; Ferrari et al., 1997), barbiturates (Masia et al., 2000), and ethanol (Miczek et al., 1997) induce negative reactions such as anxiety and aggression/irritability both in humans and animals. On the other hand, high doses or concentrations induce anxiolytic, sedative, and antiepileptic effects, some effects shown in both humans and animals and others only in animals (Carl et al., 1990; Wieland et al., 1991; Sundstrom et al., 1998; de Wit et al., 2001). Bimodial changes in negative mood have also been noted in postmenopausal women during HRT. Postmenopausal women report more progestagen/progesterone-induced adverse mood effects with a lower dose, compared to a higher dose, of both MPA (Bjorn et al., 2002) and vaginal progesterone (Andreen et al., 2003).

The GABA transmitter system is the major inhibitory system in the CNS and the previously mentioned substances enhance the effect of GABA on the $GABA_A$ receptor. A low dose of allopregnanolone injected into the cerebral ventricular of rats produces a conditioned place aversion, whereas a higher dose has no effect (Beauchamp et al., 2000). Allopregnanolone increases aggression in rats and has similarities to alcohol in alcohol-heightened aggression up to a certain level, whereas further increase in allopregnanolone and alcohol instead decreased aggressive behavior (Fish et al., 2001). Moreover, low-dose diazepam given to humans elicits more aggression than placebo under experimental conditions (Ben-Porath and Taylor, 2002), while in higher doses diazepam is sedative and anxiolytic. Induction of severe negative mood reactions by barbiturates has been found in 2.5–6% of patients undergoing amobarbital injections as a workup for epilepsy surgery (Lee et al., 1988; Kurthen et al., 1991; Masia et al., 2000). Emotional reactions seem common after amobarbital injections, while severe emotional outbursts are rare and they seem to be related to earlier negative psychological experiences like traumatic events (Masia et al., 2000).

Progesterone treatment with 400 mg/d and 800 mg/d are represented in all three allopregnanolone concentration groups. Possible explanations are the variability in resorption and metabolism between individuals. In addition it is known that the thickness of the vaginal mucosa increase over time during estrogen therapy and the thickness of the mucosa influence the resorption. The finding that plasma concentrations of progesterone and allopregnanolone did raise after application shows that progesterone is rapidly metabolized to allopregnanolone after vaginal administration. Interestingly, pregnanolone plasma concentrations did not increase 2 h after application of the vaginal suppository, indicating that the metabolism of progesterone to pregnanolone after vaginal administration is either slow or absent. However, as a significant difference in pregnanolone plasma concentration was present between treatment with 400 and 800 mg/day, metabolism at a slow rate must occur.

In conclusion, the present study demonstrated that post menopausal women treated vaginally with progesterone and showing medium allopregnanolone plasma concentration rated significantly more negative mood symptoms during progesterone treatment compared to treatment with unopposed estrogen and placebo. In addition, significantly more negative mood symptoms were seen in cycles with medium allopregnanolone plasma concentration compared to cycles with low concentration. The findings indicate that the relationship between allopregnanolone and negative mood symptoms is bimodal as medium plasma concentrations of allopregnanolone are related to more negative mood reactions compared to lower and higher concentrations.

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