Allopregnanolone and Mood

Studies of Postmenopausal Women during Treatment with Progesterone

Lotta Andréen

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From the Department of Clinical Science, Obstetrics and Gynecology, Umeå University, Umeå, Sweden

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To My Family

A small step towards understanding the mysteries of the female brain
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Abstract

Introduction. The addition of progestagens in sequential hormone therapy (HT) provokes negative mood in certain women. This action is supposed to be mediated through the gamma aminobutyric acid A (GABA_A) system, which is the major inhibitory system in the mammalian CNS. Allopregnanolone and pregnanolone, both neuroactive metabolites of progesterone, as well as benzodiazepines, barbiturates and alcohol act as positive modulators of the GABA_A receptor. Contradictory results from studies on the effect of GABA_A receptor modulators are reported. Beneficial properties such as anaesthesia, sedation, anticonvulsion and anxiolysis are reported in human and animal studies. However, recent reports have indicated occurrence of adverse, anxiogenic and aggressive effects. It has actually been suggested that several GABA_A receptor agonists, including allopregnanolone, have biphasic effects. Low concentrations increase an adverse, anxiogenic effect, whereas higher concentrations decrease this effect and show beneficial, calming properties.

Aims. To investigate if progesterone treatment induces adverse mood in postmenopausal women and if the severity in mood symptoms is related to progesterone, allopregnanolone or pregnanolone serum concentrations. Furthermore, the studies aimed at evaluating differences in serum progesterone, allopregnanolone and pregnanolone concentrations induced by different doses and routes of administration of progesterone.

Methods. Two randomised, placebo-controlled, double-blind crossover studies of postmenopausal women with climacteric symptoms were performed. In these studies postmenopausal women were used as a model to investigate adverse mood effects of progesterone treatment. Subjects were treated with estradiol continuously. Different doses of progesterone, given either vaginally or orally, were added sequentially during the last 14 days of each treatment cycle. Daily symptom ratings were kept using a validated rating scale. Blood samples for progesterone, allopregnanolone and pregnanolone analyses were collected during each treatment cycle. In addition, a study regarding the pharmacokinetics after ingestion of low-dose oral micronised progesterone (20 mg/40 mg) was conducted with postmenopausal women. Blood samples for the analyses of progesterone, allopregnanolone and pregnanolone were collected and pharmacokinetic parameters were calculated.

Results. Postmenopausal women on sequential HT with vaginal and oral progesterone experience significant mood deterioration during the progesterone phase while on a low dose of progesterone but not on higher doses or the placebo. Negative mood symptoms occurred when the serum concentration of allopregnanolone was similar to endogenous luteal phase levels, whereas lower and higher concentrations had no significant effect on mood. Mood deterioration during progesterone treatment resembles symptoms seen in women with premenstrual dysphoric disorder (PMDD) and, as earlier reported for PMDD, it was evident that only certain postmenopausal women experience adverse mood during progesterone treatment. In addition, pharmacokinetic analyses show that low-dose oral progesterone can be used as a prodrug to allopregnanolone when the aim is to achieve physiological concentrations of allopregnanolone in humans.

Conclusions. A bimodal association, which resembles an inverted U-shaped curve, between serum allopregnanolone concentration and adverse mood is observed in postmenopausal women treated with progesterone. Furthermore, the addition of low-dose progesterone to estradiol induces adverse mood in postmenopausal women, whereas higher doses and placebo have no mood-deteriorating effect.

Key words. Progesterone, allopregnanolone, GABA, mood, bimodal
Abbreviations

3-PBC  3-propyloxy-beta-carboline
5 HT  5-hydroxytryptamine (serotonin)
ANOVA  analyses of variance
β-CCT  beta-carboline-3-carboxylate-t-butyl ester
C₀  concentration of steroid produced endogenously
Cₘₐₓ  maximum concentration
Cₜₜ  concentration at steady state
CEE  conjugated equine estrogen
CNS  central nervous system
CD  Cyclicity Diagnocer
DSM-IV  Diagnostic and Statistical Manual of Mental Disorders, 4th edition
E₂  estradiol
ER  estrogen receptor
FSH  follicle-stimulating hormone
GABAₐ  gamma aminobutyric acid A
GnRH  gonadotropin-releasing hormone
HT  hormone therapy
IQR  inter quartile range
MPA  medroxyprogesterone acetate
PET  positron emission tomography
PMDD  premenstrual dysphoric disorder
PMS  premenstrual syndrome
PR  progesterone receptor
Prime-MD  Primary Care Evaluation of Mental Disorders
RIA  radio immunoassay
SEM  standard error of mean
SERT  serotonin transporter
SSRI  selective serotonin reuptake inhibitor
VAS  Visual Analogue Scale

Definition

Biphasic/Bimodal  the words are used synonymously for an inverted U-shaped association between symptom severity and steroid concentration
Original Papers

The thesis is based on the following original articles, which will be referred to in the text by their roman numerals:


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Introduction

Hormone-induced negative mood

Mood disorders are common health problems affecting women, especially during the reproductive years. Women are approximately two times as likely as men to report a lifetime history of major depression or anxiety disorder. The sex difference begins in the early adolescence and persists through the mid 50s (Kessler et al., 1993; Wittchen et al., 1994). Periods of hormonal variability, that is, menarche (Angold et al., 1999), premenstrual periods (Soares et al., 2001), postpartum (Chaudron et al., 2001) and perimenopause (Freeman et al., 2004) have been suggested to increase the risk of mood disorders in certain women. Therefore, it seems likely that sex steroid hormones can provide one possible explanation for the differences in mood disorders observed between the genders. The central nervous system (CNS) is both a producer and a target of sex steroids, and two conditions present evidence of the interaction between mood, steroids and CNS: premenstrual dysphoric disorder (PMDD) and negative mood symptoms encountered during sequential addition of progestagens to estrogen treatment in postmenopausal women.

Premenstrual dysphoric disorder (PMDD)

The menstrual cycle is a most remarkable system of hormonal changes along the hypothalamic-pituitary-gonadal axis affecting morphological and endocrine events in the ovaries and endometrium. The menstrual cycle is divided into the follicular phase, the ovulation, which results in the formation of a corpus luteum, and the luteal phase. Figure 1 shows changes in hormone concentrations during the menstrual cycle.

PMDD is a menstrual cycle–linked syndrome defined by the cyclical recurrence of mental as well as physical symptoms occurring during the luteal phase and disappearing a few days after the onset of menstruation. The syndrome is defined by the American Psychiatric Association in the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV, American Psychiatric Association, 1994). Symptoms include depressed mood, anxiety, emotional lability, irritability, decreased interest
in usual activities, difficulty in concentrating, lack of energy, and eating and sleeping disturbances, as well as bloating and breast tenderness. In the premenstrual week, mood deterioration or physical symptoms are reported by 50% to 75% of fertile women, but only 2% to 6% of all women fulfill the criteria for PMDD (Andersch et al., 1986; Rivera-Tovar and Frank, 1990; Sveindottir and Backstrom, 2000). In an ovulatory menstrual cycle, a corpus luteum is present in the ovary, and a subsequent rise in progesterone concentration is seen. This rise in progesterone and allopregnanolone is required for the development of PMDD symptoms. In anovulatory cycles, either spontaneous or induced by gonadotropin-releasing hormone (GnRH) agonists, the cyclicity in symptoms disappears (Hammarback and Backstrom, 1988; Hammarback et al., 1991). Nevertheless, other research suggests that the classical endocrine nuclear progesterone receptors are not involved in the pathophysiology of premenstrual syndrome (PMS, a mild form of PMDD), as evidenced by
the failure of a progesterone receptor antagonist (mifepristone [RU 486]) to reduce the physical or behavioural manifestations of PMS (Chan et al., 1994). Therefore, interest has been focused on metabolites of progesterone such as allopregnanolone and pregnanolone, which are both neurosteroids acting in the CNS (Baulieu, 1991).

A relationship between PMS/PMDD development and variation in steroid hormone concentration exists. The symptoms seem to gradually increase in parallel with the rise in serum levels of progesterone and allopregnanolone, but with a delay of 3 to 5 days between the hormone and symptom peaks (Backstrom et al., 1983; Redei and Freeman, 1995; Wang et al., 1996). However, no simple relationship appears to exist between peripheral concentrations of steroid hormones and either the diagnosis of PMDD or the severity in symptoms. Previous studies have yielded contradictory results. Some studies indicate no difference in allopregnanolone concentrations between women with PMDD and controls (Schmidt et al., 1994; Sundstrom and Backstrom, 1998b; Wang et al., 1996), and others show either significantly higher levels (Girdler et al., 2001) or significantly lower levels (Rapkin et al., 1997) in allopregnanolone concentrations among PMDD patients. A disparity among the study results is also found with regard to the severity in PMDD symptoms and its relationship to peripheral hormone steroid concentrations. In an earlier study performed by our group higher luteal phase allopregnanolone concentrations were associated with improved symptom ratings in PMDD patients (Wang et al., 1996). Conversely, Girdler et al. have reported that PMDD patients with greater levels of premenstrual anxiety and irritability had significantly reduced levels of allopregnanolone in the luteal phase (Girdler et al., 2001). Results from a controlled, randomised study of antidepressant treatment for PMDD indicated that improvement was associated with significantly reduced allopregnanolone levels (Freeman et al., 2002).

**Menopause**

Menopause is the final sign of the end of fertility. It is defined as the time when a permanent stop of menstruation occurs, and the mean age for natural menopause in the industrialised world is approximately 51 years of age (McKinlay et al., 1992). Menopause is preceded by a gradual ageing
of the ovaries where the first sign of ageing is a malfunction of the corpus luteum with reduced hormone production as a result. The next step is a reduction in the follicular activity resulting in reduced estrogen production, followed by the occurrence of anovulatory cycles. The reduction in estrogen will eventually halt the proliferation of the endometrium and thus stop the menstrual bleeding.

Perimenopause is estimated to last for nearly four years (McKinlay et al., 1992). This period is distinguished by irregularity in menstrual bleedings in most women, rising levels of pituitary follicle-stimulating hormone (FSH) and eventually declining levels of estrogen (Burger et al., 2002). Burger and co-workers have suggested that the perimenopausal period describes the time from when signs of approaching menopause begin until at least one year after the last menstrual bleeding (Burger et al., 2002). The climacteric period is a less defined period when women pass from the reproductive part of life to postmenopausal years. Approximately 75% of women will suffer from different degrees of vasomotor symptoms (attacks of hot flushes and sweating) during that period (Hammar et al., 1984; McKinlay et al., 1992). The vasomotor symptoms are most intense during the first year after menopause and the symptoms usually diminish 4 to 5 years later. Yet as many as 10% of women may still have attacks of hot flushes and sweating more than 15 years after menopause (Berg et al., 1988).

Apart from vasomotor symptoms and genitourinary atrophy, other common reported symptoms associated with perimenopause are insomnia and depressive mood swings (Dennerstein et al., 2000; Stadberg et al., 1997). Whether these symptoms are explained by the vasomotor symptoms or whether they are, in fact, caused by the menopausal transition is debatable. Findings by Schmidt et al. indicate that estradiol (E2) can effectively treat perimenopausal depression independently of its beneficial effects on vasomotor symptoms (Schmidt et al., 2000). In another study, depressive symptoms were found to be positively correlated to the severity of the vasomotor symptoms (Hammar et al., 1984). Avis et al. added to the body of literature favour for the “symptom hypothesis” when they showed that depression was not associated with menopausal status or changes in estradiol but is most likely explained by vasomotor
symptoms and sleep problems (Avis et al., 2001). A study by Dennerstein and et al. also obtained similar results (Dennerstein et al., 2000).

**Estrogen treatment and effects on mood**

The effectiveness of the treatment of vasomotor symptoms (MacLennan et al., 2001; Rebar et al., 2000) and atrophic vaginitis (Wiklund et al., 1993) with estradiol or conjugated equine estrogen (CEE) is well documented. Evidence for the improved quality of life of women with climacteric symptoms treated with estrogens has also been reported (Rebar et al., 2000; Wiklund et al., 1993). As mentioned earlier, whether the depressed mood swings experienced by certain women during perimenopause are primary or in fact secondary to the vasomotor symptoms and sleep disturbances is not clear. Whether estrogen treatment has an effect on depressed mood has also been debated. Some authors claim that peri- and postmenopausal women with depressive moods reported beneficial effects of estrogen supplementation (Carranza-Lira and Valentino-Figueroa, 1999; Cohen et al., 2003; Schmidt et al., 2000). Conversely, other authors report no mood improvement with estrogen therapy (Greendale et al., 1998). In addition, postmenopausal women without vasomotor symptoms did not report increased well-being with unopposed estrogen treatment (Girdler et al., 1999; Hays et al., 2003). However, estrogen treatment has been shown to enhance the effect of antidepressant treatment with selective serotonin reuptake inhibitors (SSRIs) (Schneider et al., 2001; Schneider et al., 1997), but the positive effect of estrogen on depression is to a large extent abolished by the addition of progestagens (Grigoriadis and Kennedy, 2002).

**Progestagen treatment and effects on mood**

Ever since reports of an increased risk of endometrial cancer during unopposed estrogen therapy were published in the mid-70s, estrogen has been combined with either progestagen or progesterone treatment to avoid endometrial hyperplasia and cancer (Whitehead, 1978). However, the sequential addition of progestagens induces cyclical negative mood symptoms in certain women, similar to symptoms encountered in PMDD, including depression, anxiety and irritability (Bjorn et al., 2000; Hammarback et al., 1985; Magos et al., 1986). The progestagen-induced
adverse mood effects seem to be dose-dependent but, surprisingly, more accentuated negative mood effects are reported during treatment with 10 mg compared with 20 mg of medroxyprogesterone acetate (MPA) (Bjorn et al., 2002). In an earlier retrospective study, approximately 30% of women stated occurrence of negative mood during hormone therapy (HT) with progestagens, and 35% stated negative side-effects as a reason for discontinuing HT (Bjorn and Backstrom, 1999). Moreover, a higher estrogen dose increases negative mood when administered with progestagens, whereas the estrogen dose did not affect mood deterioration when estrogen was used alone (Bjorn et al., 2003). As mentioned earlier, it appears that there is some evidence for increased well-being during estrogen therapy in peri- and postmenopausal women with climacteric symptoms, whereas the necessary addition of progestagens in women with an intact uterus might counteract the effect of estrogen on mood (Grigoriadis and Kennedy, 2002; Zweifel and O'Brien, 1997). Apart from progestagens, natural progesterone can also be added to estradiol in order to protect the endometrium. Natural progesterone is used for that purpose in certain western countries, but not in Sweden. Some research has suggested that natural progesterone should provoke less negative mood compared with synthetic progestagens (Martorano et al., 1998). To our knowledge, different doses of natural progesterone have not previously been investigated with respect to its possible mood-improving or deteriorating effects in postmenopausal women on sequential HT.

Steroid biosynthesis and metabolism

As mentioned earlier, estrogen and progesterone are the major female sex hormones. Estrogen is required for the development of female phenotype, sexual maturation, female genital function and skeletal maintenance. Progesterone is necessary for conception and the maintenance of pregnancy. The precursor of all steroids is cholesterol, which is obtained mainly from the diet but can also be synthesised de novo or can be derived in many cells of the nervous system from low-density lipoproteins (Jung-Testas et al., 1992; Jurevics and Morell, 1995). In adult women, the main sources of estradiol are the granulose cells of the developing follicle and the corpus luteum in the ovary. The adrenal gland can produce androstenedione, which can be converted to estradiol or testosterone. Testosterone is then converted to estradiol in fat, placenta, endometrium,
liver, intestines, skin, muscle and brain tissue. Progesterone is synthesised mainly in the granulose cells of the corpus luteum, but certain synthesis is also seen in the placenta and the adrenals (Speroff, 2005). The enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase are needed for the synthesis of allopregnanolone from progesterone, whereas its 5β-stereoisomer, pregnanolone, is produced by enzymatic activity of 5β-reductase and 3α-hydroxysteroid dehydrogenase. Allopregnanolone and pregnanolone are neuroactive steroids with high affinity to the gamma aminobutyric acid A (GABA<sub>A</sub>) receptor complex, the major inhibitory system in the mammalian CNS (Majewska et al., 1986). Allopregnanolone has been found to be the most potent of the progesterone metabolites, followed by pregnanolone (Paul and Purdy, 1992; Timby et al., 2005; Zhu et al., 2001). Figure 2 shows the main pathway of steroid hormone synthesis.

**Figure 2.** Steroid biosynthesis. Adapted from Compagnone and Mellon (2000).

The serum concentrations of progesterone and allopregnanolone vary throughout the reproductive years. The circulating levels of allopregnanolone and pregnanolone follow that of progesterone in fertile
women, with higher concentrations during the luteal phase compared with the follicular phase (Genazzani et al., 1998; Wang et al., 1996). In the follicular phase of the menstrual cycle, allopregnanolone level is about 1 nmol/l (Bicikova et al., 1995; Genazzani et al., 1998). It rises in the luteal phase and peaks at approximately 4 nmol/l (Genazzani et al., 1998; Wang et al., 1996). Serum levels of allopregnanolone have been found to be two- to threefold higher than pregnanolone concentrations throughout the luteal phase (Ottander et al., 2005). During the late stages of pregnancy, progesterone and allopregnanolone reach the highest levels, sometimes more than 100 nmol/l (Hill et al., 2000; Luisi et al., 2000; Parizek et al., 2005). In postmenopausal women the allopregnanolone concentration is low, less than 1 nmol/l (Genazzani et al., 1998). Rannevik et al. have shown that the frequency of menstrual cycles with progesterone concentrations indicating ovulation decreases from 60% to less than 10% during the six years preceding menopause. In their material all women had progesterone concentrations of less than 2 nmol/l postmenopausally (Rannevik et al., 1995). Table 1 shows steroid concentrations throughout women’s reproductive and postmenopausal years.

Table 1. Serum concentrations of progesterone, allopregnanolone and pregnanolone during the follicular and luteal phases in reproductive women, and during the postmenopausal period. Concentrations are given as mean ± SEM.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Follicular phase</th>
<th>Luteal phase</th>
<th>Postmenopausal period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone (nmol/l)</td>
<td>5.3 ± 2.1 a</td>
<td>34.4 ± 7.8 a</td>
<td>1.6 ± 1.1 a</td>
</tr>
<tr>
<td></td>
<td>5.0 ± 0.5 b</td>
<td>34.7 ± 2.4 b</td>
<td>1.2 ± 0.1 c</td>
</tr>
<tr>
<td>Allopregnanolone (nmol/l)</td>
<td>0.8 ± 0.3 a</td>
<td>3.7 ± 1.0 a</td>
<td>0.7 ± 0.3 a</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 0.2 d</td>
<td>3.6 ± 0.2 b</td>
<td>0.7 ± 0.1 c</td>
</tr>
<tr>
<td>Pregnanolone (nmol/l)</td>
<td>0.6 ± 0.0 e</td>
<td>1.1 ± 0.5 e</td>
<td>0.7 ± 0.1 e</td>
</tr>
</tbody>
</table>

Data are cited from following references:

a (Genazzani et al., 1998); b (Wang et al., 1996); c (Andreen et al., 2006); d (Timby et al., 2005); e (Sundstrom et al., 1998)

A human post-mortem study revealed that the steroid concentrations during the menstrual cycle are reflected in the brain. Thus, women in the luteal phase had significantly higher brain concentrations of allopregnanolone than postmenopausal controls (Bixo et al., 1997). In that study regional differences in steroid concentrations were also evident,
indicating differences in steroid uptake and binding. However, studies have shown that increases in brain neurosteroid concentrations can occur without changes in peripheral concentrations (Inai et al., 2003; Uzunov et al., 1996).

Absorption of progesterone

When women are treated with progesterone, the mood effects are dependent on the metabolism of allopregnanolone and pregnanolone, which will be discussed in detail later. The concentration of these GABA<sub>A</sub> receptor active metabolites will not only be dependent on the dose of progesterone given but also influenced by the route of administration. Progesterone can be administered either orally, as micronised progesterone, or as vaginal suppositories. As vaginal bacteria and mucosa appear to lack 5α- and 5β-reductases and 3α- and 20α-hydroxylases, vaginally administered progesterone is absorbed without significant metabolic changes in contrast to orally administered progesterone, which is metabolised in the gut, intestinal wall and liver (de Lignieres et al., 1995). In premenopausal women, plasma concentration of progesterone is similar after oral and vaginal administration of single-dose progesterone, while allopregnanolone plasma concentration is significantly lower and pregnanolone plasma concentration does not increase after vaginal administration (de Lignieres et al., 1995). No studies have so far investigated the pharmacokinetics of progesterone, allopregnanolone and pregnanolone after repeated administration of low doses of progesterone, assumed to cause allopregnanolone and pregnanolone concentrations close to or within the physiological ranges.

Pathophysiology of hormone induced mood changes

As described above, ovarian steroids play important roles in the modulation of mood and anxiety. The mechanism behind these effects is not fully understood, but the major effects of the ovarian steroids and its metabolites are thought to be mediated through actions in the CNS. Alternatively, the ovarian steroids themselves through the classical genomic mechanism might cause the effect. The latter mechanism has been described earlier and is distinguished by steroids binding to intracellular receptors and thereby modulating transcription and protein
synthesis (McDonnell et al., 1992; McEwen and Woolley, 1994). A similar mode of action is observed with intracellular estrogen and progesterone receptors in the brain. The response time to this action is several minutes, hours or even days. Steroids can also produce more rapid (non-genomic) effects through direct membrane mechanism, for example by modifying ligand-gated ion channels and neurotransmitter transporters (McEwen, 2001; Wong et al., 1996). This effect is mediated within seconds to minutes by the progesterone metabolites.

A number of important neurotransmitter systems in the brain exist, but since detailed discussion of all these mechanisms is far beyond the scope of this thesis, this discussion will concentrate on the neurotransmitter system that is obviously involved in adverse mood effects, the GABA system. In addition, the serotonin system will also be mentioned since abnormal serotonergic neurotransmission is thought to be one of the factors in the development of depression. Furthermore, anxiety disorders and the introduction of SSRIs represent an important landmark in the pharmacological treatment of depression and many other psychiatric disorders, including PMDD. Although the genomic and non-genomic mechanisms will be described individually, it is important to bear in mind that an undoubtedly complex interaction exists between these systems and that the mechanisms of steroid actions might operate within the same neuron and even through interaction with the same molecular target (McEwen, 1991; Schumacher, 1990).

Steroid hormone receptors

Both estrogen and progesterone are highly lipid soluble and therefore easily cross the blood–brain barrier. Researchers have known for quite a long time that both estrogen receptors (ER) and progesterone receptors (PR) are found in the brain (Pfaff and McEwen, 1983) and that besides the brain many peripheral organs are important targets of these steroid hormones. In 1997 a second ER, ER-β, was discovered (Enmark et al., 1997). Both ERs have properties in the brain, but ER-α is found mainly in the amygdala and hypothalamus (Osterlund et al., 2000a), whereas ER-β is found in the hippocampus and cerebral cortex (Osterlundet et al., 2000b). These regions are involved in emotional processing and cognition (Alves et al., 1998; Osterlund et al., 1998; Sherwin and Tulandi, 1996). Two
progesterone receptors (PR) are also known, PRA and PRB. These receptors are likewise distributed in many peripheral tissues and parts of the brain (Alves et al., 1998; Bethea, 1993; Kato et al., 1994). Interestingly, estradiol supplementation decreases ERs but induces PRs (Alves et al., 1998; Greco et al., 2001), and a co-expression of ER-\(\alpha\), ER-\(\beta\) and PR is found in several areas of the brain (Greco et al., 2001).

*The serotonin system*

Negative mood encountered during sequential HT occurs during the progestagen phase and presents with symptoms that resemble those of PMDD, as discussed earlier. A dysfunction of the serotonin (5HT) neurotransmitter system has been suggested to influence the pathophysiology of PMDD, and treatment with SSRIs has been shown to be effective in certain women (Cohen et al., 2002; Dimmock et al., 2000). Decreased plasma and cerebrospinal fluid concentrations of allopregnanolone in depressed patients increase to normal levels after successful treatment with SSRIs (Romeo et al., 1998; Strohle et al., 2000; Uzunov et al., 1996). Therefore, the serotonergic system is also of interest with regard to negative mood effects during progestagen treatment.

Serotonin was identified as a neurotransmitter as early as the 1950s by Brodie and colleagues (Brodie et al., 1955), and it was found to be synthesised from the essential amino acid tryptophan (Wurtman, 1983) by enzymatic processes in different tissues, including the brain. Serotonin acts through at least 18 different serotonin receptor subtypes in the brain (Barnes and Sharp, 1999). The serotonergic system is involved in numerous physiological and behavioural functions such as aggression, impulse control, anxiety, sexual behaviour, stress response, sleep and appetite. Abnormal serotonergic neurotransmission is suggested to be one of the factors in development of psychiatric disorders, particularly depression and anxiety disorders. Important progress in the pharmacological treatment of depression (Meltzer, 1989) and other psychiatric disorders such as panic disorders, obsessive compulsive disorders and eating disorders (Goodnick and Goldstein, 1998; Masand and Gupta, 1999; Vaswani et al., 2003) was made with the development of SSRIs. SSRIs inhibit serotonin transporter (SERT), which is responsible for the reuptake of serotonin into the presynaptic nerve terminal. These
drugs are thought to increase the serotonin concentration in the synaptic cleft and prolong its activity at the postsynaptic receptor sites. After two to three weeks of treatment, a decreased sensitivity of the presynaptic receptors occurs, thus enhancing the neurotransmission of serotonin (Elena Castro et al., 2003; Hensler, 2003). However, SSRIs have a different effect in PMDD. The benefit of SSRI treatment is observed rapidly in PMDD, during the first or the second treatment day (Landen and Eriksson, 2003). This indicates that the mechanism of the SSRI in hormone-induced negative mood changes is different from the mechanism in depression. Another indication of a different mechanism is that continuous treatment with SSRI in PMDD causes tolerance, which is not observed in antidepressive therapy (Wikander et al., 1998).

General conclusions about the interaction between ovarian steroids and serotonergic function are difficult to draw since a wide variety of results have been obtained from animal and human studies on this subject. Results from animal studies are not included in this brief summary. With regard to human studies, an increase in 5HT2a receptor bindings (one subtype of the 5HT receptor) in the cortex has been detected with positron emission tomography (PET) after combined estrogen and progesterone treatments (Moses et al., 2000; Moses-Kolko et al., 2003). However, this study was based on only five subjects and it was not clearly determined if the addition of progesterone, in fact, increased the binding potential. In another study, 10 postmenopausal women showed increased 5HT2a receptor bindings in prefrontal regions during treatment with estrogen replacement therapy (Kugaya et al., 2003). Furthermore, a direct connection between the serotonin and GABA systems has been demonstrated. The GABA_A receptor subunit composition was found to be changed in knockout mice lacking the 5HT1a receptor (Sibille et al., 2000), and when PMDD patients were treated with SSRIs, the decreased sensitivity towards pregnanolone normalised in parallel with an improvement in symptoms (Sundstrom and Backstrom, 1998a). As well, estradiol also has antidepressive effects, which can be abolished by progestagens (Grigoriadis and Kennedy, 2002). Bearing the last two points in mind, and the fact that women with PMDD benefit from treatment with SSRIs, one can assume that the serotonin system is involved in progesterone/progestagen-induced negative mood symptoms although the mechanism behind their interaction is unknown.
The gamma aminobutyric acid (GABA) system

The GABA transmitter system is the major inhibitory system in the mammalian CNS. GABA is formed from the amino acid glutamate in GABAergic neurons by enzymatic reaction. When GABA binds to the GABA<sub>A</sub> receptor, the influx of chloride ions increases, hyperpolarising the post-synaptic membrane and making the postsynaptic cell less prone to excitation. Apart from GABA, neurosteroids, benzodiazepines, barbiturates, alcohol and most anaesthetic agents bind to the GABA<sub>A</sub> receptor. These drugs are active agonists and modulate the GABA-induced chloride ion influx by interacting with allosteric binding sites (Sieghart, 1995).

The GABA<sub>A</sub> receptor is composed of five subunits, which form a ligand gated chloride channel (Luddens and Wisden, 1991). At least 18 subunits have been described (6 α, 3 β, 3 γ, δ, ε, π, 3 ρ) (Mehta and Ticku, 1999; Rudolph et al., 2001). Figure 3 illustrates a model of the GABA<sub>A</sub> receptor.

Figure 3. The GABA<sub>A</sub> receptor complex. The GABA<sub>A</sub> receptor consists of five subunits with binding sites for GABA, neurosteroids, benzodiazepines, barbiturates and alcohol.
The distribution of different subunits varies throughout the brain in a heterogeneous way, and different combinations of subunits contribute to distinct pharmacological properties of the GABA$_A$ receptor. The most functional receptors consist of combinations of $\alpha/\beta/\gamma$ or $\alpha/\beta/\delta$ subunits (Davies et al., 1997). The function of each subunit is not fully understood, but several studies indicate that certain subunits have particular importance. For example, the sedative effect of benzodiazepines was found to be mediated via the $\alpha1$ subunit (McKernan et al., 2000; Rudolph et al., 1999) and the benzodiazepine-induced anxiolytic effects are mediated via modulation of the $\alpha2$ subunit of the GABA$_A$ receptor (Low et al., 2000). In a study by Gulinello et al., the $\alpha4$ subunit seemed to be implicated in the regulation of anxiety (Gulinello et al., 2001). It has been shown that GABA$_A$ receptors that contain the $\alpha4$ subunit (Benke et al., 1997; Hevers and Luddens, 1998) or $\delta$ subunits instead of $\gamma$ subunits (Benke et al., 1996) are insensitive to modulation of benzodiazepines. It is tempting to simply attribute the different effects of the GABA$_A$ receptor to different subunits, but recent research with knockout mice and subunit specific agonists indicates a more complex and divergent relationship (Paronis et al., 2001). The behavioural as well as adverse effects of GABA$_A$ receptor modulators will be discussed in the next chapter.

Steroids and the central nervous system (CNS)

As mentioned earlier, sex steroid hormones play fundamental roles in the development and function of CNS. In general, estradiol practices excitatory actions and progesterone inhibitory effects on CNS. Apart from reproductive functions, ovarian steroids may be involved in memory and learning (Sherwin, 1997) and balance (Hammar et al., 1996). It has been proven that certain effects of the steroids are not merely mediated by the classical genomic action but rather by a direct membrane action. The GABA system and the effects of GABA$_A$ receptor modulators, especially allopregnanolone, represent one of the most obvious non-genomic actions of ovarian steroid metabolites in the brain and will therefore be discussed in more detail.
**Neurosteroids, neuroactive steroids and GABA-steroids**

In the 1980s and 1990s Baulieu and co-workers made some remarkable findings. Some steroids, called neurosteroids, are synthesised de novo in the central and peripheral nervous system by glial cells, astrocytes and neurons (Baulieu, 1991; Baulieu and Robel, 1990; Compagnone and Mellon, 2000). As the term *neurosteroids* suggests, these are a group of steroids synthesised in the nervous system. The precursor is mainly cholesterol, and examples of neurosteroids are progesterone as well as its neuroactive metabolites \(3\alpha\)-hydroxy-5\(\alpha\)-pregnane-20-one (allopregnanolone) and \(3\alpha\)-hydroxy-5\(\beta\)-pregnane-20-one (pregnanolone). Precursors to progesterone, pregnenolone and pregnanolone sulfate as well as estrogen, can also be classified as neurosteroids since the definition includes all steroids being synthesised in various regions of the nervous system. Later the term *neuroactive steroid* was introduced, referring to steroid hormones that are active on neuronal tissues (Paul and Purdy, 1992). The neuroactive steroids may be synthesised either endogenously in the brain or by peripheral endocrine organs, but act on neuronal tissues and represent one aspect of steroid interaction within the CNS. The term *GABA-steroids* refers to the steroids active as modulators of the GABA\(_A\) receptor irrespective of synthesis origin or status as endogenous or exogenous. Examples of GABA-steroids are \(3\alpha\)-hydroxy-5\(\alpha/\beta\) metabolites of the sex hormones progesterone (allopregnanolone and pregnanolone), testosterone and stress hormone desoxycorticosterone (tetra-hydro-desoxycorticosterone [THDOC]). GABA-steroids modulate the effect of GABA on the GABA\(_A\) receptor. The concentration of GABA steroids in blood and tissue varies with the production activity in the adrenals, ovaries and testicles (Backstrom et al., 2003).

As early as 1942, Selye reported the sedative and anaesthetic properties of progesterone and some of its metabolites (Selye, 1942). Later, it was shown that very high concentrations of progesterone as well as allopregnanolone and pregnanolone are needed to induce sedation and anaesthesia. Progesterone given intravenously in doses of 400 mg to 600 mg induces sleep/anaesthesia in humans (Merryman et al., 1954). Plasma concentrations of pregnanolone ranging from 80 nmol/l to 160 nmol/l cause sedation (Sundstrom et al., 1999a) and from 530 nmol/l to 1700 nmol/l cause anaesthesia (Carl et al., 1990). Neurosteroids accumulate in
the brain. In rats the concentration of progesterone in the brain varies in parallel with its cyclical production by the ovaries, the highest concentrations being found in the striatum and hypothalamus (Bixo and Backstrom, 1990). When rats were injected intravenously with anaesthetic doses of progesterone, the ratio of 5α-pregnane-3,20-dione (5α-DHP) to progesterone was about 100 times higher in the brain tissue than in the plasma (Bixo and Backstrom, 1990). The synthesis and metabolism of neurosteroids are also region-dependent in the human brain. In the earlier mentioned post mortem study by Bixo et al. the highest concentrations of progesterone were found in the amygdala, cerebellum, hypothalamus and nucleus accumbens, whereas the highest levels of allopregnanolone were detected in the substantia nigra, hypothalamus and amygdala (Bixo et al., 1997).

The GABA<sub>A</sub> receptor modulators, behaviour and mood

Allopregnanolone and pregnanolone, like benzodiazepines, barbiturates and alcohol, are neuroactive modulators of the GABA<sub>A</sub> receptor. It seems plausible to assume that all GABA allosteric modulators have similar behavioural as well as adverse effects. An increasing number of reports during the past two decades have described numerous beneficial and adverse effects derived from the GABA<sub>A</sub> complex in the brain.

Neurosteroids and GABA<sub>A</sub> receptors

As mentioned earlier, high doses of progesterone as well as allopregnanolone and pregnanolone have anti-epileptic, hypnotic and anaesthetic effects in humans (Backstrom et al., 1984; Carl et al., 1990; Sundstrom and Backstrom, 1998b). In addition, allopregnanolone has been reported to have anxiolytic effects in animals, although these effects have never been documented in humans (Bitran et al., 1991; Wieland et al., 1991). As early as the 1980s, the anxiolytic and anaesthetic properties were proved to exert their action by enhancing GABA-stimulated chloride conductance in the rat brain (Harrison and Simmonds, 1984; Majewska et al., 1986). Therefore, great hope has historically been placed on progesterone as a treatment for PMDD, but several studies have reported that treatment with progesterone is unable to relieve symptoms of anxiety and depression in PMDD patients (Freeman et al., 1995; Vanselow et al.,
With respect to postmenopausal women, our group has previously shown through use of saccadic eye movement parameters as objective measures of sedation that women who display significant cyclicity in mood symptoms during HT with vaginal progesterone are more sensitive to the sedative effects of an intravenous pregnanolone injection compared with women without cyclical symptoms (Wihlback et al., 2005).

A number of recent reports have actually indicated that allopregnanolone might not be as beneficial as previously thought. In animal studies, allopregnanolone has been shown to induce aggression (Fish et al., 2001; Miczek et al., 1997) and short-term treatment has been reported to induce anxiety (Gulinello et al., 2001). In a study by our group using the Morrison water mice for measuring learning, allopregnanolone was shown to inhibit learning and memory (Johansson et al., 2002) and in another study, carried out on rats, allopregnanolone increased appetite (Chen et al., 1996). It has even been suggested that allopregnanolone may mediate the effect of benzodiazepines and alcohol in humans and laboratory animals (Fish et al., 2001; Morrow et al., 1999; Torres and Ortega, 2003).

Benzodiazepines and GABA<sub>\Lambda</sub> receptors

Benzodiazepines exert their behavioural effects through allosteric binding to the GABA<sub>\Lambda</sub> receptor complex (Miczek et al., 2003). The main effects of benzodiazepines are sedation (Gottesmann, 2002), anxiolysis (Ballenger, 2001), muscle relaxation and anti-convulsion (Treiman, 2001). However, unexpected and paradoxical reactions toward benzodiazepines are reported in both humans and experimental animals. Certain patients react to benzodiazepines with aggression, confusion, violent behaviour and loss of impulse control (Ben-Porath and Taylor, 2002; Hall and Zisook, 1981; Honan, 1994). Weinbroum et al. reported a 10.2% incidence of paradoxical events to midazolam in patients who underwent surgery during a three-month period and showed that the treatment with flumazenil (a benzodiazepine receptor antagonist) effectively reversed the midazolam-induced paradoxical behaviours (Weinbroum et al., 2001). Groups at risk for this type of reaction include children, the elderly, alcoholics and patients with personality or psychotic disorders (Mancuso et al., 2004).
Several reports from animal studies focus on benzodiazepine-heightened aggression similar to the paradoxical increases in aggressive outbursts observed in humans (Ferrari et al., 1997; Gourley et al., 2005; Miczek, 1974). The use of benzodiazepine antagonists seems to counteract the benzodiazepine-heightened aggression in laboratory animals in a similar way as seen in humans (Gourley et al., 2005; Weerts et al., 1993a).

Alcohol and GABA_A receptors

The effect of alcohol is similar to many of the reported actions of neurosteroids as well as benzodiazepines. For instance sedative, anxiolytic, anticonvulsant and anaesthetic properties are reported (Eckardt et al., 1998). Alcohol alters the function of a number of neurotransmitters, including the dopaminergic, serotonergic and GABAergic systems. The basis for the alcohol–GABA interaction is not well documented although some evidence exists that the GABA_A receptor complex is one mechanism through which alcohol mediates many of its behavioural effects (Grant, 1994). Many case reports on alcohol-heightened aggression in humans have been published. Likewise, a number of human experimental studies have reported increased aggression after alcohol consumption (Cherek et al., 1992; Dougherty et al., 1996). However, the study by Dougherty et al. showed that in a small subset of individuals, the greatest increase in aggressive behaviour occurred after consumption of the lowest dose of alcohol in comparison with higher doses (Dougherty et al., 1996).

In addition, alcohol is the drug that is consistently associated with increased aggressive and violent behaviour in certain laboratory animals (Miczek, 1974). Among many actions of alcohol, the positive modulation of the GABA_A receptor is reported to be of particular significance with regard to aggressive behaviour in animals (de Almeida et al., 2004; Fish et al., 2001; Miczek et al., 1997). Studies have shown that pretreatment with flumazenil (a benzodiazepine antagonist) and β-CCt (a subunit specific GABA_A receptor antagonists) prevents alcohol-heightened aggressive behaviour (de Almeida et al., 2004; Weerts et al., 1993b). An earlier study performed by our group showed that alcohol enhance pregnanolone induced anaesthesia, indicating an interaction between neurosteroids and alcohol (Wang et al., 2001).
As described above, there is an obvious contradiction in effects mediated by GABA-active modulators. In an attempt to explain this paradox, it has been suggested that several GABA_A receptor agonists, including allopregnanolone, have biphasic effects, with low doses or concentrations increasing an adverse, anxiogenic effect, and high doses or concentrations decreasing this effect and having more beneficial, calming properties. The exact mechanism of this phenomenon is not known, but it is often referred to as a biphasic or bimodal effect.

In a study by Miczek and co-workers, low doses of allopregnanolone, alphaxalone (a synthetic GABA-steroid) and alcohol increased aggression in mice, whereas high doses reduced the aggressive behaviour (Miczek et al., 1997). Figure 4 shows the results from their study.

![Figure 4. A biphasic effect of three positive GABA_A receptor modulators, allopregnanolone (filled circles), alphaxalone (filled squares) and alcohol (filled triangles), on the frequency of attack bites expressed as percentage of baseline (dashed horizontal line) by male resident mice confronting an intruder. Adapted from (Miczek et al., 1997; Miczek et al., 2003). Reprinted with permission from the author and copyright holder.](image)

Similar findings are reported from other animal studies showing that allopregnanolone and other GABA_A receptor modulators induce irritability/aggression (Fish et al., 2001; Gourley et al., 2005; Yoshimura and Ogawa, 1989) and anxiety (Beauchamp et al., 2000) in a bimodal...
pattern. In the study by Gourley and co-workers (2005), the biphasic benzodiazepine-heightened aggressive behaviour seen in rats treated with different doses of midazolam and triazolam was antagonised by flumazenil (a broad spectrum benzodiazepine antagonist) and with β-CCt and 3-PBC (both GABA<sub>A</sub> receptor antagonists with preferential action at the α1 subunit). These findings further support the evidence that the GABA<sub>A</sub> receptor complex is the relevant site for both heightening and reducing effects of benzodiazepines as well as neurosteroids and alcohol, although the exact molecular mechanism is not known.

Interestingly, reports from human studies indicate that a bimodal effect of the GABA<sub>A</sub> receptor modulators might also be replicated in humans. Aggressive and depressive behaviours occur in a significant proportion of patients with cardiac conditions undergoing transesophageal echocardiography after intravenous administration of midazolam (Wenzel et al., 2002). Low doses of diazepam elicit more aggression than a placebo during experimental conditions (Ben-Porath and Taylor, 2002), while at higher doses diazepam acts as a sedative and anxiolytic. In agreement with these findings are reports of negative emotional reactions in certain individuals during intracarotid barbiturate supply (Kurthen et al., 1991; Lee et al., 1988; Masia et al., 2000). Furthermore, a subset of individuals reacted with greatest increase in aggressive responses after consuming low doses of alcohol, compared with higher doses (Dougherty et al., 1996). Similar findings concerning alcohol and aggression are reported by Cherek et al. (1992). Moreover, oral progesterone treatment in women caused significant changes in fatigue as well as impairment in psychomotor tests in subjects achieving high levels of allopregnanolone and pregnanolone, while those with lower metabolite levels reported no negative effects (Freeman et al., 1992). In a study with intra-muscular progesterone treatment resulting in concentrations of allopregnanolone well beyond those seen during normal menstrual cycles, sedation and decrease in ratings of vigour and friendliness were noted (de Wit et al., 2001). These findings indicate that a bimodal action of positive GABA<sub>A</sub> receptor modulators could provide a possible explanation for the reported discrepancies in the effects of neurosteroids. However, the possible relationship between allopregnanolone concentrations and adverse mood effects in humans remains to be elucidated.
Aims of the Thesis

The overall goal of the present work was to study the relationship between allopregnanolone concentration and negative mood symptoms in postmenopausal women following orally and vaginally administered progesterone.

The specific aims of the different papers were:

I. To investigate if negative mood symptoms are induced by vaginal progesterone in postmenopausal women.

To investigate whether or not the potential adverse mood effects during progesterone treatment are dose-dependent.

II. To investigate if the severity of negative mood is related to progesterone, allopregnanolone or pregnanolone serum concentration following vaginally administered progesterone.

III. To investigate the pharmacokinetics of progesterone, allopregnanolone and pregnanolone in postmenopausal women treated with a low dose of oral micronised progesterone.

To investigate if a low dose of oral micronised progesterone can be used as a prodrug when the treatment goal is to achieve physiological premenopausal serum concentrations of allopregnanolone in postmenopausal women.

IV. To investigate if the severity of negative mood is related to allopregnanolone concentration in a bimodal fashion after administration of oral micronised progesterone.

To investigate if only certain postmenopausal women experience mood deterioration during the addition of oral progesterone in HT.
Material and Methods

Subjects

Eighty-seven postmenopausal women were recruited to these studies, and 75 women completed the clinical trials and were included in the analyses. Most participants were recruited through advertisements in local newspapers and several were recruited from an outpatient department for climacteric complaints. None of the women were included in more than one trial. The study population in Papers I and II was recruited at the Department of Obstetrics and Gynecology at Umeå University Hospital and the Department of Women’s Health, Sundsvalls Hospital. Likewise, the study population in Paper IV was recruited at the same departments and, in addition, at the Department of Women’s and Children’s Health, Uppsala University Hospital. The results in Paper III were based on a trial performed at the Department of Obstetrics and Gynecology at Umeå University Hospital. The study procedures were performed in accordance with the ethical standards for human experimentation established by the Declaration of Helsinki of 1975, revised in 1983. The Umeå University Ethical Committee and the National Medical Products Agency approved the design of the studies.

All subjects were more than 6 months postmenopausal, had intact uterus and ovaries and had not been on HT for the 3 months prior to inclusion in the studies. They were considered physically healthy and had no contraindications to HT. Subjects were not receiving any steroid treatment, had no history of psychiatric illness and had not been treated with psychopharmacological drugs for at least 6 months prior to enrolment in the studies. All subjects in Papers I, II and IV had climacteric symptoms including hot flushes and/or sweating. Occurrence of vasomotor symptoms in subjects was not recorded in Paper III.

Papers I and II are based on the same study population except for two women who were not included in the analyses in Paper II. Of the 36 women who were originally included for the study, two dropped out during the study (one due to heavy withdrawal bleeding and breast tenderness, the other due to nausea) and three women were excluded from Paper I (one due to a major life event during the study period, two due to
In the analyses for Paper II, two further women were excluded (one due to refusal to give blood samples and one due to major life event). The major life event in the woman excluded in Paper II, unfortunately, was not discovered until the analyses in Paper II were performed and, therefore, she was not excluded from Paper I. Nevertheless, the results and the interpretation of the results in Paper I did not change when the statistical analyses were controlled after she was excluded from the data. Erratum has been sent to the European Journal of Endocrinology.

Paper III is based on seven women. Eight women were originally included but one dropped out during the study course due to palpitations and breast pain. The first nine blood samples drawn from that participant were included in the analyses.

Paper IV includes results from 37 women. Of the 43 women who were originally included in the study, six dropped out during the course of the study (one due to depression, one due to abdominal bloating and fatigue, two due to fear of side effects). Two women were excluded (one due to a major life event and one due to protocol violation). The demographic data of the subjects who completed the clinical trials and were included in the analyses are presented in Table 1.

### Table II. Demographic data of the study populations in Papers I–IV. Papers I and II are based on the same study population except for two women who were not included in the analyses in Paper II.

<table>
<thead>
<tr>
<th></th>
<th>Papers I and II n = 31¹</th>
<th>Paper III n = 7</th>
<th>Paper IV n = 37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y, mean and range)</td>
<td>52 (44–60)</td>
<td>54 (44–65)</td>
<td>53 (43–63)</td>
</tr>
<tr>
<td>Weight (kg, mean and range)</td>
<td>69 (50–94)</td>
<td>69 (60–80)</td>
<td>72 (56–104)</td>
</tr>
<tr>
<td>BMI ² (mean and range)</td>
<td>-</td>
<td>26 (21–30)</td>
<td>26 (21–33)</td>
</tr>
<tr>
<td>Having partner (%)</td>
<td>81%</td>
<td>-</td>
<td>89 %</td>
</tr>
<tr>
<td>Education, college or university (%)</td>
<td>58%</td>
<td>-</td>
<td>84 %</td>
</tr>
<tr>
<td>Parity (%)</td>
<td>90%</td>
<td>-</td>
<td>87%</td>
</tr>
<tr>
<td>Years after menopause (y, mean and range)</td>
<td>2 (1–11)</td>
<td>4 (1–10)</td>
<td>3 (0.5–9)</td>
</tr>
<tr>
<td>Previous HT ³ (%)</td>
<td>39%</td>
<td>63%</td>
<td>70%</td>
</tr>
<tr>
<td>(y, mean and range)</td>
<td>2 (0.3–14)</td>
<td>5(3–8)</td>
<td>4 (0.1–14)</td>
</tr>
</tbody>
</table>

¹ n = 29 in Paper II, ² BMI = Body mass index, ³ HT = Hormone therapy
The presence of a psychiatric disorder and/or drug abuse was evaluated in all women before inclusion in the studies presented in Papers I, II and IV. Subjects with ongoing psychiatric illness were excluded through use of the Primary Care Evaluation of Mental Disorders (PRIME-MD) questionnaire. The screening questionnaire is presented in the appendix 1. PRIME-MD has been developed to help primary care physicians screen, evaluate and diagnose mental disorders. This diagnostic tool conforms to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criteria and has been validated for use in a primary care setting (Spitzer et al., 1994). A deeper evaluation was made with a structured interview by the doctor using a specific form for follow up with women who gave a positive response to questions regarding affective disorders or drug abuse.

Study design

The studies evaluated the effect on mood, physical symptoms and/or steroid serum concentrations of either vaginally (Papers I and II) or orally (Papers III and IV) administered progesterone.

In Papers I, II and IV, sequential HT with estradiol and progesterone was administered in a randomised, placebo-controlled, double-blind, crossover design. Subjects were treated with estradiol valerate (Schering AG, Germany) orally at a dose of 2 mg daily throughout the study periods. Progesterone or placebo was randomly added in 2 doses (morning and evening) during the last 14 days of each treatment cycle. A crossover to a new treatment was carried out after each cycle. The studies began with a run-in cycle during which the patients were treated with 2 mg/day of estradiol valerate orally and 10 mg of MPA (Leo Pharma, Sweden) orally during the last 14 days of the treatment cycle. Given the positive effects of estrogen on well-being due to the reduction of vasomotor symptoms during the first month of treatment, this run-in cycle was included to avoid interference with mainly estrogen-dependant 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 to 4 days in the beginning of the next cycle where the symptoms from the previous cycle decline (Bjorn et al., 2000).
Papers I and II
After the run-in cycle, vaginal suppositories containing 200 mg or 400 mg of progesterone or the placebo were administered twice daily on cycle days 15 to 28 for the following three cycles. The waxy vaginal suppositories contained progesterone in a base of semi-synthetic glycerides produced from hydrogenated vegetable oil by interesterification. The suppositories were identical in appearance, and were prepared by Apoteket AB, Production and Laboratories (Malmö, Sweden). Packing and randomisation were done by the pharmacy at Umeå University Hospital. The two progesterone doses chosen were the two pharmaceutical preparations available in Sweden at that time.
Progesterone was administered vaginally in order to avoid metabolism in the gut and intestinal wall and first passage in the liver. Thus, the serum concentrations of progesterone and its metabolites were expected to better resemble the concentrations seen during the normal menstrual cycle (de Lignieres et al., 1995). Two blood samples for progesterone, allopregnanolone, pregnanolone and estradiol analyses were collected for 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). The second blood sample for the same steroid analyses was taken 2 hours later (2 h sample). The study design is illustrated in Figure 5.

Paper IV
In Paper IV, progesterone or placebo was added twice daily on cycle days 20 to 33 during the treatment cycles following the run-in cycle. Progesterone was administered orally as soft gelatine capsules containing 15 mg, 30 mg and 100 mg of micronised progesterone or placebo in monohydrous lactose. The capsules were made to appear identical, and were prepared, packed and randomised by Apoteket AB, Production and Laboratories (Stockholm, Sweden). Progesterone was administered orally in order to assure metabolism to neuroactive steroids. The three progesterone doses were chosen because earlier studies indicated that they provide serum concentrations of progesterone and allopregnanolone in the physiological (Andreen et al., 2006) and supraphysiological ranges (de Lignieres et al., 1995). In addition, the study in Paper IV ended with a run-out cycle during which the patients were treated with 2 mg of estradiol
Figure 5. Design of the first study (Papers I and II). Sequential HT, with oral estradiol (E2) at the dose of 2 mg daily and vaginal progesterone (P) at 400 mg/day, 800 mg/day or placebo added during the last 14 days of each treatment cycle (days 15–28) were administered in a randomised, placebo-controlled, double-blind, crossover design. The first cycle was a “run-in cycle”, with 2 mg of estradiol daily and 10 mg of medroxyprogesterone acetate (MPA) on days 15–28. Blood samples for the analyses of progesterone, allopregnanolone and pregnanolone were collected twice every treatment cycle during the last week of progesterone treatment. The first blood sample was drawn immediately before the vaginal administration of progesterone in the morning (nadir sample) and the second 2 hours later (2 h sample).

Valerate daily and 10 mg of MPA during the last 14 days of the treatment cycle. This run-out cycle was included to secure endometrial shedding and continued daily symptom scoring following the last treatment cycle (when symptoms from the previous cycle decline). Two blood samples for progesterone, allopregnanolone and estradiol analyses were collected for each cycle on 2 different days during the last week of progesterone treatment. The blood samples were drawn immediately before administration of progesterone in the morning. The study design is illustrated in Figure 6.

Paper III
Paper III is based on our second study and describes the pharmacokinetics of oral micronised progesterone. On the morning of the first day of the study, 20 mg of micronised progesterone was administered orally. Blood samples for the analysis of progesterone, allopregnanolone and
Figure 6. Design of the study in Paper IV. Sequential HT with oral estradiol (E2) at 2 mg daily and oral progesterone (P) at 30 mg/day, 60 mg/day, 200 mg/day or placebo during the last 14 days of each treatment cycle (days 20–33) was administered in a randomised, placebo-controlled, double-blind, crossover design. The first cycle was a “run-in cycle” and the last a “run-out cycle” with 2 mg of estradiol daily and 10 mg of medroxyprogesterone acetate (MPA) on days 20–33. Two blood samples for progesterone and allopregnanolone analyses were collected for each treatment cycle, on 2 different days, during the last week of progesterone treatment. The blood samples were drawn immediately before administration of progesterone in the morning.
pregnanolone in the serum were collected immediately before the initial progesterone dose ($C_0$ for determination of the baseline hormone levels produced endogenously) and 1, 2, 3, 4, 6, 8, 12 and 24 hours after the first dosage. On the following 6 days (days 2–7), 20 mg of micronised progesterone was administered orally twice a day (at 8 a.m. and 8 p.m.).

Blood samples were drawn once daily, immediately before the morning dose. On day 7, blood samples were also collected 2, 4, 6, 8 and 12 hours after the dosage. Finally, a sample was collected 60 hours after the last dose. The preparation of the oral formulation was identical to the one described in Paper IV.

Measurements of mood (Papers I, II and IV)

Subjects rated their symptoms daily throughout the studies using a modified form of the Cyclicity Diagnoser (CD) scale. Subjects were familiarised with the CD scale during the run-in cycle, but these rating scores were not used in the analyses. The CD scale was designed for diagnosing cyclical symptoms and it has been validated for the diagnosis of premenstrual syndrome (Sanders et al., 1983; Sundstrom et al., 1999b). The modified form of the CD scale used in these studies has been used in earlier studies of sequential HT and continuous combined HT in postmenopausal women (Bjorn et al., 2000; Bjorn et al., 2002; Bjorn et al., 2003; Odmark et al., 2004; Wihlback et al., 2001; Wihlback et al., 2005). The envelope and the pages of the CD scale are presented in the appendix 2. The modified CD scale included four physical symptoms (breast tenderness, hot flushes, abdominal bloating and withdrawal bleeding), seven psychological symptoms (cheerfulness, friendliness, libido, anxiety/tension, irritability, fatigue and depression) and rating of daily life impairment. The CD is a Likert scale, graded from 0 to 8, where 0 indicates complete absence of a particular symptom and 8 represents the maximum severity of the symptom. The subjects can detect one scale step as a difference in mood experience, as shown in a study of symptom severity in women with PMDD (Seippel and Backstrom, 1998). Analyses of symptoms were performed separately and in clusters of related symptoms based on an earlier principal component analysis (Sanders et al., 1983). Related symptoms were grouped together as summarised symptom scores: ‘negative mood symptoms’, including tension, irritability, depression and fatigue; ‘positive mood symptoms’, including
cheerfulness and friendliness; and ‘physical symptoms’, such as breast tenderness and bloating. The rating scale used is thought to be of minor importance as long as the key symptoms for the purpose are included (Halbreich et al., 1993).

All rating scales for subjectively reported symptoms were mainly made for the comparison within the same individual and not between different individuals. For example, depression rated 6 by one woman is not necessarily worse than depression rated 5 by another woman. Therefore, the CD scale is well suitable for repeated measurement analyses of cyclicity within individuals. Whether this type of ordinal scale, as well as the ordinary Visual Analogue Scale (VAS), could be used to compare symptom scores between individuals has been debated. However, it is well known that VAS is frequently used in clinical practice as an absolute measure to compare severity in symptoms between subjects (Aitken, 1969; Callahan and Pincus, 1990; Maxwell, 1978). In addition, the crossover design of these studies made it possible to compare changes in rated symptoms between individuals.

Steroid assays

The procedures for the steroid assays are described in detail in Paper II (Andreen et al., 2005). Nevertheless, some general remarks on the methods for the analyses are important. Analyses of serum progesterone and estradiol were made by commercial fluoroimmunoassay kits (Delfia) according to the manufacturer’s instructions.

Allopregnanolone and pregnanolone were measured with radio immunoassay (RIA) after pre-assay diethylether extraction and celite chromatography purification of samples. Recovery was determined for each assay using 300 to 500 cpm of tritium-labelled allopregnanolone or pregnanolone (New England Nuclear, Boston, MA, USA) added to a plasma samples before extraction and by measuring the amount recovered after chromatography. The recovery for allopregnanolone averaged 78% and for pregnanolone 85%. The results were compensated according to the recovery.

RIA was performed using polyclonal rabbit antiserum. 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., 1998c). Both antisera were kind gifts
from Dr. R. H. Purdy, Department of Neuropharmacology, The Scripps
Research Institute (La Jolla, CA, USA). The antiserum has a low cross
reactivity against its 5-reduced isomer. The sensitivity of the assays was
25 pg, with an intra-assay coefficient of variation for allopregnanolone
and pregnanolone of 6.5% and inter-assay coefficient of variation of 8.5%.

Statistics

The statistical methods are described in detail in Papers I to IV. Two-way
analysis of variance (ANOVA) with repeated measures, and one-way
ANOVA, when suitable, were used to test differences in symptom scores
during the treatment cycles within, as well as between, the individuals and
the effects of the different serum steroid concentrations on summarised
negative, positive and physical symptoms. Values of symptom scores and
steroid concentrations with normal distribution are displayed as means ±
standard error of the mean (SEM). Nevertheless, some steroid
concentrations and symptom scores displayed skewed distributions and the
measures of central tendency are therefore given as median and inter-
quartile range in Papers II and IV. The SPSS statistical package was used
for the analyses. The pharmacokinetic parameters in Paper III were
calculated using the software package Kinetica Version 4.3 (InnaPhase
Corporation, Philadelphia, PA, USA). Pharmacokinetic parameters were
compared with one-way ANOVA with repeated measures. For
comparisons between the two groups of hormone levels, Wilcoxon
matched pairs signed ranks tests were applied. $P < 0.05$ was considered
significant.
Results

Steroid concentrations during treatment with vaginal and oral progesterone (Papers II–IV)

The concentrations of progesterone, allopregnanolone and pregnanolone following progesterone treatment are dependent on the route of administration. In these studies we treated women with vaginal and oral micronised progesterone at different doses in order to achieve physiological as well as supraphysiological steroid concentrations. Differences in steroid concentrations during the treatments are shown in Table III. Serum pregnanolone was not analysed after oral progesterone treatment in Paper IV because the laboratory was not able to perform the analyses due to the lack of radioactive pregnanolone tracer at that time.

Pharmacokinetics of steroids after treatment with a low dose of oral progesterone (Paper III)

Pharmacokinetic parameters were obtained after administration of 20 mg of oral progesterone twice daily. The serum concentrations of progesterone, allopregnanolone and pregnanolone reached their steady state ($C_{ss}$) after the first day with this dosage. Median inter-quartile range (IQR) $C_{ss}$ values are shown in Table III. The $C_{ss}$ values were all significantly higher than the steroid hormone levels produced endogenously immediately before the beginning of the study ($P < 0.05$). Furthermore, a significant difference between the $C_{ss}$ values for progesterone, allopregnanolone and pregnanolone was observed ($F[2,12] = 11.40; P < 0.01$), with concentrations of pregnanolone significantly lower than those of progesterone and allopregnanolone ($P < 0.01$ and $P < 0.05$, respectively). The serum concentrations of the steroids are shown in Figure 7.

The key pharmacokinetic variables for progesterone, allopregnanolone and pregnanolone are summarised in Table IV. Serum concentrations of allopregnanolone and pregnanolone 60 hours after the last dose of progesterone were significantly higher compared with the endogenous steroid levels ($P < 0.05$ and $P < 0.05$, respectively), but a corresponding difference was not observed with respect to progesterone.
Table III. Serum progesterone, allopregnanolone and pregnanolone concentrations given as median and inter-quartile range in postmenopausal women during treatment with (1) vaginal progesterone (P.) at 400 mg/day, 800 mg/day and placebo (n = 29) (Paper II), (2) oral micronised progesterone (P.) at 30 mg/day, 60 mg/day, 200 mg/day, and placebo (n = 37) (Paper IV), and (3) oral micronised progesterone (P.) at 40 mg/day (n = 7) (Paper III). All blood samples were drawn immediately before progesterone administration in the morning.

<table>
<thead>
<tr>
<th></th>
<th>Paper II n = 29</th>
<th>Paper IV n = 37</th>
<th>Paper III n = 7</th>
</tr>
</thead>
</table>
| **Median serum concentration (IQR)
Progesterone nmol/L** |                 |                 |                 |
| Vaginal placebo P. 400 mg/d | 1.0 (0.5–1.2)    | 16 (6.9–24)    | 1.6 (1.1–2.1)   |
| Vaginal P. 800 mg/d | 16 (10–43)       | 27 (1.8–3.3)   | 2.4 (3.0–5.1)   |
| Oral placebo | 1.6 (1.8–3.3)   | 3.9 (3.0–5.1)  | 8.4 (6.3–12.)   |
| Oral P. 30mg/d | 2.4 (1.8–3.3)   | 3.9 (3.0–5.1)  | 8.4 (6.3–12.)   |
| Oral P. 60mg/d | 2.4 (1.8–3.3)   | 3.9 (3.0–5.1)  | 8.4 (6.3–12.)   |
| Oral P. 200mg/d | 2.4 (1.8–3.3)   | 3.9 (3.0–5.1)  | 8.4 (6.3–12.)   |
| Oral P. 40 mg/d | 1.9 (1.7–2.3)   | 1.5 (1.4–2.0)  |                 |
| **Allopregnanolone nmol/L** |                 |                 |                 |
| Vaginal placebo P. 400 mg/d | 0.4 (0.3–0.5)    | 4.5 (2.5–3.3)  | 0.2 (0.1–0.2)   |
| Vaginal P. 800 mg/d | 0.5 (4.0–8.3)    | 5.2 (0.6–1.1)  | 0.9 (1.0–2.1)   |
| Oral placebo | 0.2 (0.6–1.1)   | 1.5 (1.0–2.1)  | 4.2 (2.9–5.5)   |
| Oral P. 30mg/d | 0.2 (0.6–1.1)   | 1.5 (1.0–2.1)  | 4.2 (2.9–5.5)   |
| Oral P. 60mg/d | 0.2 (0.6–1.1)   | 1.5 (1.0–2.1)  | 4.2 (2.9–5.5)   |
| Oral P. 200mg/d | 0.2 (0.6–1.1)   | 1.5 (1.0–2.1)  | 4.2 (2.9–5.5)   |
| Oral P. 40 mg/d | 0.2 (0.6–1.1)   | 1.5 (1.0–2.1)  |                 |
| **Pregnanolone nmol/L** |                 |                 |                 |
| Vaginal placebo P. 400 mg/d | 0.5 (0.4–0.6)    | 1.0 (0.8–1.1)  | -               |
| Vaginal P. 800 mg/d | 0.5 (0.9–1.4)    | 1.1 (0.9–1.4)  | -               |
| Oral placebo | -               | -               | -               |
| Oral P. 30mg/d | -               | -               | -               |
| Oral P. 60mg/d | -               | -               | -               |
| Oral P. 200mg/d | -               | -               | -               |
| Oral P. 40 mg/d | 1.3 (1.0–1.5)   |                 |                 |

1IQR = Inter-quartile range
Figure 7. Mean ± SEM serum concentrations of progesterone, allopregnanolone and pregnanolone in postmenopausal women (n = 7). Base = endogenous steroid hormone levels. Day 2 = 24 hours after ingestion of a single oral dose of 20 mg progesterone. From day 3, the samples were obtained as trough samples 12 h after ingestion of 20 mg of progesterone twice daily.

Table IV. Serum progesterone, allopregnanolone and pregnanolone concentrations given as median and inter-quartile range in postmenopausal during treatment with a single dose of 20 mg micronised progesterone on day 1 and 20 mg twice daily on days 2–7. C₀ = endogenous steroid hormone level, C_max = maximum concentration after 20 mg of micronised progesterone on day 1, C_day7 = steroid concentration immediately before the last dose of progesterone in the morning on day 7, C_60h = steroid concentration 60h after the last progesterone dose.

<table>
<thead>
<tr>
<th></th>
<th>C₀</th>
<th>C_max</th>
<th>C_day7</th>
<th>C_60h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone nmol/L</td>
<td>1.1 (1.0–1.3)</td>
<td>8.9 (4.8–15)</td>
<td>2.2 (1.3–2.7)</td>
<td>1.2 (0.8–1.3)</td>
</tr>
<tr>
<td>Allopregnanolone nmol/L</td>
<td>0.7 (0.7–0.8)</td>
<td>6.4 (5.3–9.8)</td>
<td>1.9 (1.7–2.3)</td>
<td>1.0 (1.0–1.3)</td>
</tr>
<tr>
<td>Pregnanolone nmol/L</td>
<td>0.7 (0.6–0.8)</td>
<td>4.4 (3.5–6.2)</td>
<td>1.5 (1.4–1.5)</td>
<td>1.3 (0.9–1.3)</td>
</tr>
</tbody>
</table>

¹IQR = Inter-quartile range
Differential negative mood occurrence in women (Papers I and IV)

Earlier studies have indicated that only certain women experience cyclicity with worsening of negative mood and physical symptoms during progestagen treatment in sequential HT (Björn and Backstrom, 1999; Greendale et al., 1998; Kirkham et al., 1991). The question is how to identify these women in order to study adverse mood effects. Since not all women suffer from PMDD (Sveindottir and Backstrom, 2000), and findings by Björn and co-workers suggest that women with a history of PMS are more likely to develop negative mood while on progestagens (Björn and Backstrom, 1999), we identified women with (n = 13) and without (n = 18) a history of PMS in Paper I. We defined PMS during fertile life by a retrospective report of mood deterioration prior to menstruation, which decreased and disappeared within 4 days after the onset of menstrual bleeding. The influence of PMS symptoms on daily life was graded, and women whose family relations, social activities or work were negatively affected by the symptoms were considered to have a history of PMS. In Paper I only women without prior PMS displayed significant symptom cyclicity with deterioration in negative mood during treatment with vaginal progesterone at 400 mg/day (Andreen et al., 2003).

A reported history of PMS is known to be an unreliable method of defining PMS, and according to DSM-IV criteria, daily prospective symptom ratings for two or more menstrual cycles are needed to diagnose PMDD. Therefore, we abandoned the retrospective reports of PMS used in Paper I to define women with mood deterioration during the progesterone phase in sequential HT. Instead, we identified women with symptom cyclicity during sequential HT by using the prospective daily symptom ratings from the CD scale in Paper IV. In that paper, we defined symptom cyclicity as an increase in negative mood symptoms with 2 scale steps or more during progesterone treatment compared with estrogen-only treatment. Furthermore, our criteria required that negative mood symptoms were not merely an exacerbation of high negative mood scores throughout the estrogen period and that mood deterioration during placebo treatment was absent. These criteria are similar to the criteria for PMDD in DSM-IV. Sixteen women (43%) fulfilled the criteria for symptom cyclicity and were therefore analysed separately in Paper IV.
Women defined to have symptom cyclicity scored significantly more negative mood symptoms during treatment with 30 mg/day of progesterone compared with the estradiol-only period ($F_{[1,15]} = 8.73; P < 0.01$), while women without symptom cyclicity scored significantly higher negative mood during the estradiol-only period compared with women who experienced cyclicity ($F_{[1,35]} = 5.13; P < 0.05$). The results are shown in Figure 8.

![Figure 8](image-url)

**Figure 8.** Mean ± SEM of summarised negative mood in postmenopausal women treated with 2 mg of estradiol continuously (dotted line) and with progesterone at 30 mg/day on treatment days 20–33 (filled line). Women were divided into two groups: one group with cyclicity in mood ($n = 16$) and the other group without cyclicity ($n = 21$). Significant cyclicity with worsening of negative mood during the progesterone phase was seen in the group of women with cyclicity ($F_{[1,15]} = 8.37, P < 0.01$). Women without cyclicity had higher negative mood scores during the estrogen phase compared with women with cyclicity ($F_{[1,35]} = 5.13; P < 0.05$).
Relationship between progesterone dose and adverse mood

Vaginal progesterone (Paper I)
Women without a history of PMS during fertile life reported significant symptom cyclicity with adverse mood during the progesterone phase while receiving 400 mg/day of progesterone ($F[1,17] = 5.92; p < 0.05$). No symptom cyclicity was reported during treatment with the higher dose (800 mg/day) of progesterone or with the placebo. 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 last 5 days of the estrogen phase (days 11–15). The worst period peaked during the late progesterone phase (days 24–28). Therefore, these periods are compared in the analyses.

Oral micronised progesterone (Paper IV)
During randomised sequential HT with daily estradiol and oral progesterone at 30 mg/day, 60 mg/day, 200 mg/day or the placebo added during the last 14 days of each treatment cycle, women experienced mood deterioration during treatment with the lowest dose of oral progesterone. Thus, similar results were obtained during vaginal and oral progesterone treatments. The group of women who fulfilled the criteria for symptom cyclicity ($n = 16$) in Paper IV scored significantly more negative mood symptoms during treatment with 30 mg/day of progesterone compared with the estradiol-only period ($F[1,15] = 8.73; P < 0.01$). 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 last 7 days of the estrogen phase (days 13–19). The worst period peaked during the late progesterone phase (days 30–3). Therefore, these periods are compared in the analyses. The results are shown in Figure 10.

In addition, women with symptom cyclicity rated experienced increased daily life impairment during the progesterone treatment period compared with the estradiol-only period ($F[1,15] = 5.31; P < 0.05$) in treatment cycles with progesterone at 30 mg/day. Moreover, these women reported significantly more negative mood symptoms during the progesterone phase when treated with progesterone 30 mg/day compared with the higher doses of progesterone and the placebo ($F[3,45] = 4.38; P < 0.01$).
Figure 10. Mean ± SEM of summarised negative mood scores in postmenopausal women (n = 16) with symptom cyclicity. Women were treated with 2 mg of estradiol continuously (dotted line) and with progesterone at 30 mg/day or placebo treatment days 20–33 (filled line). During progesterone treatment, a significant cyclicity with worsening of negative mood during the progesterone phase was observed (F[1,15] = 8.37; P < 0.01).

In the post hoc test, negative mood scores during treatment with 30 mg/day of progesterone were higher compared with the negative mood scores during 60 mg/day (P < 0.05), 200 mg/day (P < 0.05) and placebo (P < 0.05). The results are shown in Figure 11.
Figure 11. Mean ± SEM of summarised negative mood in postmenopausal women (n = 16) with symptom cyclicity. Women were treated with 2 mg of estradiol continuously and with progesterone at 30 mg/day, 60 mg/day, 200 mg/day, or placebo on days 20–33. Negative mood symptoms were analysed during the maximal severity period (days 30–3). During that period women had significantly higher negative mood scores when treated with progesterone at 30 mg/day compared with the other treatments (F[3,45] = 4.38; \( P < 0.01 \)).

Relationship between allopregnanolone concentration and adverse mood

Vaginal progesterone (Paper II)
The total range of allopregnanolone concentrations during vaginal progesterone treatments in Paper II was 0.37 to 10.7 nmol/L, which corresponds to the values from below follicular to above luteal phase concentration in the menstrual cycle. The median (IQR) serum 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 were: low concentration group (2.3 (1.4–3.5) 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). Treatment with both progesterone doses was represented in all three groups. The concentration of allopregnanolone in the low concentration group was in the follicular phase range, in the medium concentration group in the luteal phase range and in the high concentration group above what is normally seen in the luteal phase of the menstrual cycle (Wang et al., 1996).

When the concentration of allopregnanolone was in the luteal phase range (medium concentration group), women expressed a significant symptom cyclicity with mood deterioration during the progesterone treatment period compared with estradiol-only period (F[1,18] = 5.02; P <0.05). 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 last 5 days of the estrogen phase (days 11–15). The worst period peaked during the late progesterone phase (days 25–1). Therefore, these periods are compared in the analyses. Moreover, women in treatment cycles with allopregnanolone concentration in the luteal phase range reported significantly more negative mood symptoms during the progesterone phase compared with women in treatment cycles with low allopregnanolone concentration (F[1,36] = 8.16; P < 0.01). Corresponding results were not evident in the high allopregnanolone concentration group. Results are shown in Figure 12.

A correlation analysis between the allopregnanolone concentration groups and negative mood scores reveal that 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 or high concentration groups. The described correlation between negative mood and allopregnanolone concentration indicates a bimodal pattern.
Figure 12. Mean summarised negative mood in postmenopausal women (n = 29) treated with continuous estrogen (filled line, cycle days 1–28) + sequential vaginal progesterone (dotted line, cycle days 15–28). Progesterone treatment cycles (n = 58) were divided into allopregnanolone (median, IQR) serum 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. Significant cyclicity in negative mood was seen in women with medium allopregnanolone concentration (F[1,18] = 5.02; P < 0.05). Women with medium allopregnanolone concentration scored significantly more negative mood symptoms compared with women receiving low concentration (F[1,36] = 8.16; p < 0.01) during the progesterone phase.

Oral micronised progesterone (Paper IV)
The range of allopregnanolone concentration during oral progesterone treatment in Paper IV was 0.19–19.6 nmol/L, which is a considerably wider range compared with the range observed during vaginal treatment in Paper II. Therefore, the women with oral progesterone treatment were divided into groups based on a range of allopregnanolone concentration obtained from all blood samples collected (n = 215). The groups were 0–0.5 nmol/L (n = 8), 0.5–1.0 nmol/L (n = 55), 1.0–1.5 nmol/L (n = 41), 1.5–2 nmol/L (n = 22), 2–3 nmol/L (n = 31), 3–4 nmol/L (n = 16), 4–5
nmol/L (n = 18) and > 5 nmol/L (n = 24). Women were represented in 2 to 5 groups each, with a mean ± SEM of 3.7 ± 0.1 groups/subject.

A significant difference in summarised negative mood scores between the eight different allopregnanolone concentration groups was noted (F[7, 207] = 2.90; P < 0.01). The women scored significantly higher on the negative mood scale when allopregnanolone concentration was in the range of 1.5–2 nmol/L compared with lower and higher concentrations. Analyses of the relationship between positive mood symptoms and allopregnanolone concentration show similar results, and the summarised positive mood scores were likewise related to serum allopregnanolone concentration but in the opposite direction (F[7, 207] = 5.23; P < 0.001). The results show a bimodal association between allopregnanolone concentration and mood and are shown in Figure 13. A detailed description of the differences between the groups appears in the legend of Figure 4 in Paper IV.
Figure 13. Mean ± SEM of summarised negative mood (top panel) and positive mood (bottom panel) in postmenopausal women (n = 37) treated with estrogen and progesterone. The mood scores were from the same day as the serum allopregnanolone analysis. Blood samples from the women were grouped by their allopregnanolone concentrations: 0–0.5 nmol/L (n = 8), 0.5–1 nmol/L (n = 55), 1–1.5 nmol/L (n = 41), 1.5–2 nmol/L (n = 22), 2–3 nmol/L (n = 31), 3–4 nmol/L (n = 16), 4–5 nmol/L (n = 18), and > 5 nmol/L (n = 24). *significant difference in mood from 0–0.5 nmol/L; * P < 0.05, ** P < 0.01, *** P < 0.001. All statistics were calculated by one-way ANOVA.
Discussion

The work in this thesis concerns the relationship between the sex steroid progesterone, its neuroactive metabolite allopregnanolone and negative mood symptoms in postmenopausal women treated with progesterone. We used postmenopausal women with vasomotor symptoms as a model to investigate this issue. The main findings from our studies are that the addition of a low dose of progesterone to estradiol causes mood deterioration, an effect not seen with higher doses or placebo. Our results indicate that allopregnanolone causes mood deterioration and that the effect is dependent on the allopregnanolone concentration in a bimodal fashion. Furthermore, it was evident that certain women experience adverse mood during progesterone treatment while others do not. These findings give rise to some considerations that are discussed below.

Is adverse mood really an effect of allopregnanolone?

The results show an association between allopregnanolone concentration in the luteal phase range and adverse mood during vaginal progesterone treatment in postmenopausal women (Paper II). No association between progesterone or pregnanolone concentration and mood was evident in that study. Similar results were obtained during oral progesterone treatment (Paper IV). Based on this, we hypothesise that allopregnanolone causes negative mood in a concentration-dependent fashion. A crucial point is whether the scored negative mood symptoms during progesterone treatment in our studies really reflect an effect mediated by allopregnanolone at the GABA<sub>A</sub> receptor complex.

The relationship of allopregnanolone and fatigue is unambiguous (de Wit et al., 2001; Freeman et al., 1993; Timby et al., 2005). Evidence from animal models of anxiety (Gulinello et al., 2001) and irritability/aggression (de Almeida et al., 2004; Fish et al., 2001; Gourley et al., 2005) indicates the importance of allopregnanolone and the GABA<sub>A</sub> receptor in the etiology of progesterone-induced mood symptoms. In a series of studies, the group of Dr. Sheryl Smith has shown that the progesterone effects are mediated via the GABA<sub>A</sub> receptor modulator allopregnanolone (Gulinello et al., 2001; Smith et al., 1998a; Sundstrom Poromaa et al., 2003). In addition, evidence exists for the interaction
between allopregnanolone and other symptoms related to progesterone treatment, such as increased appetite (Chen et al., 1996) and disturbance in learning and memory (Johansson et al., 2002; Matthews et al., 2002; Shumaker et al., 2003). On the contrary, a study of an animal model of depression demonstrated that administration of allopregnanolone produced antidepressant-like effects, which were potentiated by serotonergic agents (Khisti and Chopde, 2000; Khisti et al., 2000).

The anaesthetic properties of progesterone were shown to be mediated by allopregnanolone through effective potentiation of GABA-stimulated chloride conductance in the rat brain as early as 1986 (Majewska et al., 1986; Mok and Krieger, 1990). Prior studies in laboratory animals have shown that several other effects such as aggression (Fish et al., 2001; Miczek et al., 2003), anxiolysis (Bitran et al., 1995; Wieland et al., 1991) and anticonvulsant effects (Kokate et al., 1999) are mediated through allopregnanolone binding to the GABA<sub>A</sub> receptor complex. In a recent study by our group, three increasing doses of allopregnanolone were administered intravenously to women in the follicular phase, and objective (saccadic eye movement measurement) and subjective measurements of sedation correlated to increased serum allopregnanolone concentrations (Timby et al., 2005).

The addition of the broad-spectrum benzodiazepine antagonist flumazenil as well as β-CCT and 3-PBC (both GABA<sub>A</sub> receptor antagonists with preferential action at the α<sub>1</sub>-subunit) antagonised a benzodiazepine-heightened aggression in rats (Gourley et al., 2005). Likewise, β-CCT was found to decrease alcohol-heightened aggressive behaviour in mice in a study by de Almeida et al. (2004). The allopregnanolone antagonist UC1011 (3β-20β-dihydroxy-5α-pregnane) has been shown to reduce negative allopregnanolone effects on learning observed in Morris water maze by modulating the GABA<sub>A</sub> receptor (Turkmen et al., 2004).

These findings are in agreement with results from other studies on the effect of ovarian steroids and other GABA<sub>A</sub> receptor active substances. Chan et al. (1994) reported that symptoms in women with severe PMS during the luteal phase were not affected by treatment with RU 486 (a nuclear progesterone receptor antagonist) in a crossover design. They concluded that blocking the progesterone effects does not reduce the physical or behavioural manifestations of PMS. Moreover, it seems
unlikely that the rapid effect of progesterone on CNS excitability, with sedation and anaesthesia induced within seconds to minutes, is mediated by its classical intracellular receptor.

Negative moods included in the daily symptom ratings with the CD scale are anxiety/tension, irritability, fatigue and depression. The mood symptoms measured are all included in the diagnostic criteria for PMDD according to DSM-IV. At least one symptom of depression, anxiety/tension or irritability must be present for the PMDD diagnosis. Ovulatory menstrual cycles with a subsequent rise in progesterone and allopregnanolone concentration are required for premenstrual symptom development. The symptom development is thought to be mediated by allopregnanolone, although no simple relationship between peripheral allopregnanolone levels and mood severity has been proven so far.

These findings suggest that the mood deterioration encountered by postmenopausal women during the influence of progesterone can at least in part be explained by an allosteric binding of allopregnanolone to the GABA_A receptor complex.

Is it reasonable that only low doses of progesterone cause adverse mood?

The progesterone-induced adverse mood observed in postmenopausal women appears to be dose-dependent. In our studies, negative mood symptoms were only reported during the addition of the lowest dose of vaginal progesterone (Paper I) and oral micronised progesterone (Paper IV). It may seem intuitive that higher a dosage would cause more side-effects. The opposite scenario occurs with regard to mood symptoms and progesterone as well as progestagen dosage. In a crossover study by Björn and co-workers 10 and 20 mg of MPA were compared in postmenopausal women during sequential HT. Women in that study responded with more negative mood symptoms to the lower dose of MPA compared with those receiving the higher dose (Bjorn et al., 2002). MPA is also metabolised into GABA-active metabolites, although the anaesthetic effect of MPA is less potent than that of progesterone (Belelli and Herd, 2003; Meyerson, 1967). Moreover, oral contraceptives with lower progestagen content have been reported to cause more negative mood changes compared with a compound containing a higher progestagen content (Cullberg, 1972). In
our studies (Papers I and IV), increased dosage of progesterone corresponds to a significant increase in progesterone, allopregnanolone and pregnanolone serum concentration. We hypothesise that the paradoxical finding of mood deterioration during treatment with low dose progesterone is caused by a biphasic effect of allopregnanolone on the GABA\textsubscript{A} receptor, which will be discussed in detail in the next part of this chapter.

Is there a bimodal association between mood deterioration and allopregnanolone concentration?

Our findings indicate a bimodal association between allopregnanolone concentration and negative mood during vaginal progesterone treatment (Paper II). The bimodal association appeared to be even more evident when oral progesterone was used (Paper IV), probably due to a wider range in allopregnanolone concentration and higher number of analysed blood samples in that study. A biphasic dose response curve may explain the contradictory effects mediated by positive GABA\textsubscript{A} receptor modulators. In certain individuals, low doses or concentrations of positive GABA\textsubscript{A} receptor modulators cause increased levels of anxiety and aggression whereas the effect of higher doses of these compounds generally shift from heightening aggressive behaviour to being sedative and anti-aggressive. A hypothetical model of the biphasic action is shown in Figure 14.

![Figure 14. Hypothetical model of the biphasic action mediated by GABA\textsubscript{A} receptor agonists in certain individuals. + increased negative mood, - decreased negative mood.](image-url)
In addition, several animal studies have confirmed this hypothesis. A biphasic action of GABA_A receptor active substances, including allopregnanolone, has been reported in laboratory animals (Beauchamp et al., 2000; Fish et al., 2001; Miczek et al., 1997). In a recent study by Gourley et al. (Gourley et al., 2005), a biphasic benzodiazepine-heightened irritability/aggressive behaviour was evident in rats. Midazolam significantly increased the duration of irritability/aggressive behaviour at 1.0 and 1.7 mg/kg but not at 0.3 and 3.0 mg/kg. The aggressiveness increased with triazolam at 0.03 mg/kg but not with lower or higher doses. In addition, the benzodiazepine-heightened irritability/aggression was antagonised by flumazenil (a benzodiazepine antagonist) and with β-CCt and 3-PBC (both subunit-specific GABA_A receptor antagonists) in that study.

Research concerning the relationship between adverse effects and dosages/concentrations of GABA_A receptor active substances in humans is limited. However, reports from human studies indicate that GABA_A receptor modulators in certain situations induce adverse effects such as anxiety, irritability, and aggression in a bimodal fashion. Human studies have reported paradoxical reactions with benzodiazepine-induced aggression following administration of low-dose benzodiazepines (Ben-Porath and Taylor, 2002; Wenzel et al., 2002). Paradoxical reactions to midazolam such as aggression/irritability and disorientation were reported in 10.2% of patients who underwent lower body surgery under spinal or epidural anaesthesia, and the paradoxical behaviour was effectively reversed by flumazenil (Weinbroum et al., 2001). Aggressive outbursts following administration of barbiturates (Kurthen et al., 1991; Lee et al., 1988; Masia et al., 2000) and alcohol (Dougherty et al., 1996) have also been reported in certain individuals. However, high doses of benzodiazepines, barbiturates and alcohol are known to induce the opposite effects—anxiolysis, sedation and even anaesthesia.

Our studies show that the allopregnanolone concentration, which corresponds to the most severe negative mood symptoms, was higher when progesterone was administered vaginally (Paper II) than orally (Paper IV). This finding seems plausible taking into consideration that oral progesterone is metabolised to both allopregnanolone and, to a higher extent, the GABA_A active modulator pregnanolone compared with vaginal
administration as shown in Paper II and III. Pregnanolone was not measured in Paper IV.

Given these findings and the clear-cut relationship between allopregnanolone concentration and adverse mood demonstrated in our studies (Papers II and IV), we conclude that a bimodal association between allopregnanolone and adverse mood in postmenopausal women on progesterone exists.

What is the mechanism for the biphasic action? Disinhibition – a hypothesis

The biphasic action appears both paradoxical and contradictory, and the exact mechanism for the biphasic phenomena is not known. It has been suggested that the effect is linked to the \( \text{GABA}_A \) receptor complex by a mechanism called disinhibition (Miczek et al., 2003). It has been hypothetically argued that suppression of inhibition leads to increased excitation. The failed inhibition might be caused by a change in the sensitivity of the \( \text{GABA}_A \) receptor, which may be influenced by several factors, including the subunit composition of the receptor and environmental factors. As described earlier, the specific combination of subunits determines the receptor sensitivity to different \( \text{GABA}_A \) receptor modulators, which may influence the function of the receptor (Belelli et al., 2002; Miczek et al., 2003). Furthermore, it has been reported that the receptor sensitivity to \( \text{GABA} \) agonists can be modulated by environmental factors such as stress (Biggio et al., 1990; Concas et al., 1996) or hormonal therapy, as shown in studies by Dr. Sheryl Smith and co-workers. In their studies, withdrawal after progesterone exposure increased anxiety and produced benzodiazepine insensitivity in female rats. These events were linked to up regulation of the \( \alpha_4 \) subunit of the \( \text{GABA}_A \) receptor, an effect that was attributed to the conversion of progesterone to allopregnanolone (Smith et al., 1998a; Smith et al., 1998b). An increase in anxiety and modulation of the \( \text{GABA}_A \) receptor subunits were reported after short time exposure to neuroactive steroids by Gulinello et al. (2001).

Theoretical models of disinhibition of \( \text{GABA}_A \) receptors suggest suppression of the inhibitory effect mediated by \( \text{GABA} \) neurons. For example, a \( \text{GABA}_A \) receptor with high sensitivity for neurosteroids will
enhance the inhibitory action of GABA at low neurosteroid concentrations. On the other hand, receptors with low sensitivity to neurosteroids require higher concentrations of neurosteroids for activation.

Thus, an interneuron originating from a highly sensitive GABAergic neuron may inhibit the less sensitive GABA$_A$ receptor in a neuron that inhibits negative mood. The receptor with low affinity to neurosteroids is thus prevented from exerting its own inhibitory effect, resulting in disinhibition. This theoretical model is suggested to explain alcohol- (Fish et al., 2001) and benzodiazepine-heightened (Gourley et al., 2005) aggressive behaviour in mice and rats. An illustration of the disinhibition hypothesis is shown in Figure 15.

Figure 15. Hypothetical model of the disinhibition hypothesis. Illustration by Viktor Andréen.
Why do only certain women experience adverse mood during progesterone treatment?

Our studies (Papers I and IV) show that only certain women experience mood deterioration during progesterone treatment. Similar findings are reported in several HT studies, where some women developed negative mood while on progestagens but others did not (Bjorn and Backstrom, 1999; Bjorn et al., 2000; Greendale et al., 1998; Kirkham et al., 1991). As mentioned earlier, the negative mood symptoms encountered during progesterone treatment in postmenopausal women are similar to symptoms of PMDD. Only a small percentage of women (2–6%) fulfil the criteria for PMDD (Sveindottir and Backstrom, 2000), while about 30% of women report moderate to severe PMS with mood deterioration and physical symptoms in the premenstrual week. It has been reported that adverse mood effects of oral contraceptives were mainly found in women with PMS (Cullberg, 1972). Treatment of women with PMS with GnRH-agonist caused recurrence of symptoms during “add-back” with estradiol or progesterone, while placebo treatment and hormone treatment in controls had no adverse effects (Schmidt et al., 1998). Why do only certain women experience mood deterioration during influence of steroid hormones? The answer to that question is still unknown. However, it is evident that the brain steroid sensitivity differs between women with PMDD and controls. Earlier studies conducted by our group have shown reduced pregnanolone sensitivity (Sundstrom et al., 1998c) as well as reduced sensitivity to other GABA$_A$ receptor modulators like benzodiazepines (Sundstrom et al., 1997) and alcohol (Nyberg et al., 2004) in the luteal phase of women with PMDD. Stress is a cause of rapid allopregnanolone increase in plasma and the brain (Barbaccia et al., 2001; Purdy et al., 1991), and the sensitivity to stress is different between women with PMDD and controls (Deuster et al., 1999; Girdler et al., 1998; Woods et al., 1998).

One can speculate that women with PMDD might develop tolerance to progesterone metabolites during the luteal phase and will, therefore, experience adverse mood effects at the end of the luteal phase. Tolerance development has been described earlier as a result of prolonged exposure to GABA$_A$ agonists, as for example in cases of an endogenous rise in allopregnanolone concentration during stress, pregnancy and exogenous treatment with progestagens (Czlonkowska et al., 2001; Palmer et al.,
It has even been suggested that at the end of the luteal phase, when the levels of progesterone and its metabolites start to decline, women who develop tolerance might even experience a reinforced feeling of anxiety when they begin to suffer from the withdrawal effect. Development of tolerance and withdrawal effects of allopregnanolone have been studied in several animal experiments. In an animal model of PMS/PMDD, Gulinello et al. (2001) showed that short term exposure to allopregnanolone in rats induced benzodiazepine insensitivity and increased anxiety in association with the upregulation of the α4 subunit of the GABA_A receptor. Previous work from the same laboratory demonstrated increase in anxious behaviour during withdrawal of progesterone and allopregnanolone (Smith et al., 1998a; Smith et al., 1998b). Thus, it has been argued that the explanation to this issue lies in the GABA_A receptor subunit composition, and that exposure to GABA-agonists may change the subunit composition. Whether changes in GABA_A subunit composition are actually seen in PMDD women with reduced sensitivity to GABA-agonists is not known.

With regard to the connection between PMDD during fertile life and adverse mood during progesterone treatment in the postmenopausal period it is interesting but difficult to investigate properly. Daily prospective symptom ratings are needed to diagnose PMDD according to DSM-IV criteria and therefore it is not possible to diagnose after menopause. Interestingly, Wihlback and co-workers have reported increased sensitivity to pregnanolone during the addition of progestagens and progesterone to estrogen treatment in postmenopausal women. Postmenopausal women expressing cyclicity in negative mood were more sensitive to pregnanolone than women without cyclicity (Wihlbek et al., 2001; Wihlbak et al., 2005). Apparently, the group of normal postmenopausal women with symptom cyclicity was more sensitive to the effects of allopregnanolone and its 5β-stereoisomer pregnanolone. These findings are in line with the results in Papers I and IV, where only certain women reported mood deterioration during progesterone addition to estrogen treatment. It seems plausible that postmenopausal women with low endogenous levels of allopregnanolone and pregnanolone have increased neurosteroid sensitivity, and therefore more vulnerable to mood deterioration while on progesterone. On the contrary, other studies by our group demonstrated that postmenopausal women with a reported history of PMS responded with more negative mood symptoms while on
progestagens compared to women without prior PMS (Björn et al., 2000; Odmark et al., 2004). Whether this difference can be referred to the retrospective reports of PMS, differences in personality, stress, depression, differences in progesterone/progestagen concentrations or, in fact, differences in GABA_\text{A} receptor sensitivity is beyond to the scope of investigation and discussion of this thesis.
General Conclusions

1. Negative mood symptoms are induced during the addition of vaginal progesterone to estradiol treatment in postmenopausal women.

2. Progesterone-induced mood deterioration is dose dependent in postmenopausal women. Only low doses of vaginal and oral micronised progesterone are related to negative mood, whereas higher doses and placebo have no effect on mood.

3. Association was observed between allopregnanolone concentration in the luteal phase range and adverse mood during vaginal progesterone treatment in postmenopausal women.

4. Pharmacokinetic analyses of low-dose oral micronised progesterone show that serum concentrations of allopregnanolone and pregnanolone are significantly heightened 60 h after the last dose compared with endogenous steroid levels.

5. Low-dose oral micronised progesterone can be used when the aim is to achieve physiological premenopausal serum concentrations of allopregnanolone in postmenopausal women.

6. A bimodal association between allopregnanolone concentration and negative mood is evident in postmenopausal women treated with progesterone.

7. Only certain postmenopausal women experience mood deterioration during the addition of vaginal as well as oral progesterone to estradiol.
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Appendix 1
The Primary Care Evaluation of Mental Disorders (PRIME-MD)

**PATIENTFORMULÄR (PF)**

<table>
<thead>
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<th>Har Du under den senaste månaden ofta haft...</th>
<th>Under senaste månaden</th>
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<td><strong>JA</strong></td>
<td><strong>NEJ</strong></td>
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<td><strong>1</strong> Magont</td>
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<td><strong>2</strong> Ryggont</td>
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<tr>
<td><strong>3</strong> Smärtor i armar, ben, ledar (höfter, knän etc)</td>
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<tr>
<td><strong>4</strong> Smärtor eller problem vid minnesträning</td>
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<td><strong>5</strong> Smärtor eller problem vid samlag</td>
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<td><strong>13</strong> Utmanande, gasbildning eller matsmältningars problem</td>
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**Under senaste månaden**

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<td><strong>21</strong> Har Du haft en plötslig känslo av ängst eller panik?</td>
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<tr>
<td><strong>22</strong> Har Du tänkt på att minska Din alkoholkonsumtion?</td>
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</tr>
<tr>
<td><strong>23</strong> Har någon klagat på att Du dricker för mycket?</td>
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© Pfizer 1996
PATIENTINFORMATION till skattningsskala för
daglig skattning av hormon-relaterade symptomer.

Du markerar i skalan genom att fylla ovalen ( ).

Använd inte röd penna. Gör inte ett kryss eller annan markering. Gör bara en markering per symptom och dag.


3. Skattningsskalan omfattar 4 negativa och 2 positiva psykiska symptom, 3 allmänna fysiska symptom, sexuell lust, notering av ev. blödningar inklusive menstruering samt en värdering av hur mycket symptomen påverkar dig själv, familjen, det sociala livet och arbetet.

4. Varje symptom skattas på en skala från 0 till 8 där 0 betyder avsaknad av symptom och 8 betyder maximala symtom. Du förväntas använda båda skalan under skattningsperioden. Om Du t.ex. skattar ett negativt symptom som nedsättning maximalt (dvs 8) betyder detta att Du är så nedsatt som du brukar vara när du måste som sämst. Avsaknad av symptom som nedsättning (dvs 0) betyder att Du inte alls känner dig nedsatt. Dessutom gäller för positiva symptom dvs att en 8a på symtomet glad betyder att Du är så glad som du är när Du är så glad och en 0a betyder avsaknad av glädje.

5. Kom ihåg att fylla i startdatum för varje skattningsomgång!
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### The CD scale

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<th>Sexuell lust</th>
<th>Irritable</th>
<th>Trött/Orkeslös</th>
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<td>2</td>
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Appendix 2
The CD scale page 3