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OVARIAN HORMONES AND EFFECTS IN THE BRAIN

Studies of Neurosteroid Sensitivity, Serotonin Transporter and Serotonin$_{2A}$ Receptor Binding in Reproductive and Postmenopausal Women

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Cover picture:
"The brain" by Frida N-Wihlbäck

To Ulf, Petter, Joakim and Frida, with love

ABSTRACT

Background: Estrogen has been reported to enhance well-being and quality of life during the climacteric phase. In women with an intact uterus estrogen treatment is always combined with progestins in order to protect the endometrium from hyperplasia and malignancies. However, in certain women the addition of progestins causes cyclicity in negative mood symptoms and physical symptoms similar to those encountered during ovulatory cycles in women with premenstrual dysphoric disorder (PMDD). The ovarian hormones estradiol and progesterone have profound effects on a number of neurotransmitter systems in the brain, such as the gamma aminobutyric acid (GABA) system and the serotonergic system. Progesterone metabolites, such as allopregnanolone and pregnanolone (also referred to as neurosteroids) modify the $GABA_A$ receptor in the central nervous system (CNS) and enhance GABAergic inhibitory transmission. Neurosteroid sensitivity in human studies can be studied by saccadic eye movement measurements using pharmacodynamic challenges with pregnanolone. Altered neurosteroid sensitivity has been suggested as a possible contributory factor to the progesterone/progestin-induced adverse mood effects of hormone replacement therapy (HRT). There is also evidence of estrogen treatment affecting the serotonergic system in postmenopausal women, although progestin addition has been less well studied.

Aims and method: The aim was to investigate whether the negative mood symptoms experienced during the progestin or progesterone phase of HRT were associated with changes in neurosteroid sensitivity, or changes in platelet serotonin uptake site (transporter) and serotonin$_{2A}$ (5-HT$_{2A}$) receptor binding. The intention was also to investigate whether hormonal changes during the normal menstrual cycle affect these peripheral serotonergic parameters. Postmenopausal women with climacteric symptoms were given HRT in two randomized, double-blinded, placebo-controlled crossover studies. The women received 2 mg estradiol (E$_2$) continuously during 28-day cycles. Synthetic progestins or natural progesterone were added sequentially during the last 14 days, and compared to a placebo addition. Before treatment, as well as during the last week of each treatment cycle the pharmacodynamic response to pregnanolone was assessed using saccadic eye movement measurements. Throughout the studies daily symptom ratings were made. In the study regarding synthetic progestins, platelet serotonin transporter and 5-HT$_{2A}$ receptor binding were assayed before entering the study, as well as during the last week of each treatment cycle. In the study on reproductive women, blood samples were collected for analysis of platelet serotonin transporter and 5-HT$_{2A}$ receptor binding at six different points in time during the menstrual cycle.

Results and conclusion: The addition of synthetic progestins to estrogen treatment increased negative mood symptoms and physical symptoms, whereas positive symptoms decreased. The addition of progestins also increased the sensitivity to pregnanolone. The addition of natural progesterone to estrogen treatment increased the sensitivity to pregnanolone. However, in this study the pregnanolone sensitivity was enhanced also during estrogen treatment. Women expressing cyclicity in negative

mood symptoms were more sensitive to pregnanolone than women without symptom cyclicity. Thus, it is evident that mood deterioration during HRT is associated with altered neurosteroid sensitivity. Platelet serotonin transporter and 5-HT$_{2A}$ receptor binding did not change during the different treatment conditions in HRT. Thus, we were unable to explain the negative mood changes of HRT by use of these peripheral serotonergic parameters. In the study on reproductive women however, it was clear that the serotonergic variables did change during the menstrual cycle. Binding to the serotonin transporter was higher in the late follicular phase than in the ovulatory, early luteal or mid-luteal phases. Binding to the 5-HT$_{2A}$ receptor was higher in the early follicular phase and the early luteal phase than in the mid-luteal phase. These findings may provide a link between the ovarian steroids, and the GABAergic and serotonergic neurotransmitter systems, which in turn, could explain part of the specific vulnerability that women have for the development of adverse mood effects during HRT, mood and anxiety disorders and for the deterioration of mood so frequently seen during the luteal phase.

SVENSK SAMMANFATTNING

Östrogenbehandling under klimakteriet ger kvinnor ökat välbefinnande och förbättrad livskvalité. För att förhindra cellförändringar i livmoderns slemhinna måste dock östrogenbehandlingen hos kvinnor med intakt livmoder alltid kombineras med gulkroppshormon (naturligt progesteron eller syntetiska gestagener) kontinuerligt eller i perioder (sekventiellt). Sekventiell kombinationsbehandling (hormone replacement therapy, HRT) medför, hos vissa kvinnor, cykliska negativa humörförändringar samt fysiska biverkningar liknande de symtom som kvinnor med premenstruellt syndrom upplever efter ägglossning i menstruationscykeln. Äggstockshormonerna östrogen och progesteron utövar effekt på en rad signalsubstans-system i hjärnan, som t.ex. gamma amino smörsyra (GABA) systemet och serotonin systemet. GABA-systemet har generellt hämmande effekter i hjärnan så att stimulering av GABA-systemet bl.a. har lugnande, sömninducerande och kramplösande effekter. Serotonin systemet är inblandat i en rad olika funktioner, som t.ex. depression, ångest och sexuellt beteende. Nedbrytningsprodukter av progesteron, som allopregnanolon och pregnanolon (även kallade neurosteroider), binder till $GABA_A$-receptorn i hjärnan och stimulerar GABA-systemets hämmande funktion. Känslighet för neurosteroider kan hos människa studeras genom mätning av ögonrörelsehastigheten, den s.k. saccadrörelsen. En saccad är den snabba ögonrörelse som görs vid skiftning av blickfokus, d.v.s. när vi flyttar blicken från ett föremål till ett annat. Den hastighet som rörelsen har står utanför viljans kontroll och anses allmänt som ett objektivt mått på trötthet. I dessa studier har saccadmätningar kombinerade med farmakodynamiska belastningar med pregnanolon (d.v.s. injektion av pregnanolon som i sin tur stimulerar $GABA_A$-receptorn och förlångsammar saccadhastigheten) använts som mått på neurosteroidkänslighet. Förändrad neurosteroidkänslighet har föreslagits som en möjlig bidragande orsak till de negativa humörbiverkningar, orsakade av gestagen/progesteron, som ses hos kvinnor med HRT. Det finns även fynd talande för att östrogenbehandling påverkar serotoninsystemet hos postmenopausala kvinnor, men däremot är tillägg av gestagen mindre väl studerat. Hos människa anses återupptaget av serotonin i blodplättar (i perifert blod) och till nervterminaler i hjärnan vara liknande aktiva processer. Både i blodplättar och i hjärnan kodas transportproteinerna av samma gen och har identiska aminosyreuppsättningar. På samma sätt anses serotonin$_{2A}$ (5-HT$_{2A}$) receptorn i blodplättar och i hjärnan likna varandra. Blodplättemetoden används därför i stor utsträckning vid studier av serotonineffekter hos människa och kan vara ett mått även på effekt i CNS.

Målet för våra studier var att undersöka om de negativa humörbiverkningar uppvisade under progesteron/gestagen-tillägget av HRT var associerade till förändrad neurosteroidkänslighet, eller förändrade mått vad gäller serotoninsystemet. Ett annat mål var att undersöka om hormonvariationer under den normala menscykeln påverkar serotoninsystemet. Friska kvinnor i klimakteriet (med klimateriebesvär) erhöll HRT i två randomiserade (slumpmässigt blandade), dubbel-blindade (varken patient eller undersökare visste vilken behandling patienten hade), placebokontrollerade (även inaktiv substans gavs), cross over (alla patienter erhöll samtliga studieläkemedel)

studier. Kvinnorna fick 2 mg östradiol kontinuerligt under 28-dagars cykler. Syntetiska gestagener eller naturligt progesteron gavs i tillägg de sista 14 dagarna och jämfördes mot placebo. Innan behandlingens inledning, såväl som under den sista behandlingsveckan i varje cykel gjordes pregnanolon-belastningar kombinerade med mätning av saccadrörelser. Dagliga symtomskattningar gjordes under hela studiernas gång. I studien avseende syntetiska gestagener togs serotoninprover innan studiestart samt under den sista veckan i varje behandlingscykel. I studien av reproduktiva kvinnor under menscykeln togs serotoninprover vid sex olika tillfällen under menscykeln.

Resultaten av våra studier visade att tillägget av syntetiska gestagener till östrogenbehandling medförde ökade negativa humörsymtom samt fysiska symtom samtidigt som positiva symtom minskade. Tillägget av gestagener ökade även känsligheten för pregnanolon (neurosteroidkänsligheten). Tillägget av naturligt progesteron till östrogenbehandling ökade också neurosteroidkänsligheten, men i den studien var känsligheten för pregnanolon ökad även under enbart östrogenbehandlingen. Kvinnor som uppvisade en cyklisk variation av negativa humörbiverkningar var mer känsliga för pregnanolon än kvinnor utan cykliska svängningar. Vi anser det därför klart att humörförändringar under HRT är associerade till förändrad neurosteroidkänslighet. Varken återupptaget av serotonin eller bindning till 5-HT$_{2A}$ receptorn i blodplättar förändrades under HRT-studiens gång. Vi kan därför inte förklara de negativa humörbiverkningarna med förändringar i perifera mätresultat avseende serotoninsystemet. I studien där reproduktiva kvinnor undersöktes var det emellertid uppenbart att serotoninmarkörerna förändrades under menscykelns gång. Antalet återupptagsställen var högre i sen follikelfas (före ägglossning) än i ovulationsfas (ägglossning), tidig lutealfas (efter ägglossning) eller veckan före mens. Bindning till 5-HT$_{2A}$ receptorn var högre i tidig follikel- och tidig lutealfas jämfört med veckan före mens. Fynden kan innebära en länk mellan äggstockshormoner och GABA- samt serotonin systemet. Dessa kan, i sin tur, bidra till en förklaring till den specifika sårbarhet kvinnor har för utveckling av negativa humörsymtom av HRT, depressions- och ångesttillstånd samt för de negativa humöreffekter som så ofta framträder veckorna innan mens.

CONTENTS

ABBREVIATIONS

ANOVA	analysis of variance
B_{max}	maximum number of binding sites/density of receptors
CD	cyclicity diagnoser
CEE	conjugated equine estrogen
CNS	central nervous system
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
EOG	electro-oculography
E_2	estradiol
FSH	follicle-stimulating hormone
GABA	gamma aminobutyric acid
$GABA_A$ receptor	gamma aminobutyric acid type A receptor
GnRH	gonadotropin-releasing hormone
5-HIAA	5-hydroxyindole acetic acid
HPG-axis	hypothalamic-pituitary-gonadal axis
HRT	hormone replacement therapy
5-HT	serotonin
5-HT_{2A} receptor	serotonin type 2_A receptor
$[^3H]LSD$	radioactively labeled lysergic acid diethylamide (radioligand)
$[^3H]paroxetine$	radioactively labeled paroxetine (radioligand)
K_d	dissociation constant/affinity of ligand to receptor
LEDs	light-emitting diodes
LH	luteinizing hormone
MPA	medroxyprogesterone acetate
mRNA	messenger ribonucleic acid
NETA	norethisterone acetate
PET	positron emission tomography
PMDD	premenstrual dysphoric disorder
PMS	premenstrual syndrome
Prime-MD	Primary Care Evaluation of Mental Disorders
SEM	standard error of mean
SEV	saccadic eye velocity
SHBG	sex-hormone-binding globulin
SSRI	selective serotonin re-uptake inhibitor
VAS	visual analog scale

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

   I Wihlbäck A-C, Sundström-Poromaa I, Nyberg S, Bäckström T. *Sensitivity to a Neurosteroid is Increased during the Progestagen Addition of Postmenopausal Hormone Replacement Therapy.* Neuroendocrinology 2001; 73: 397-407.

  II Wihlbäck A-C, Nyberg S, Bäckström T, Bixo M, Sundström-Poromaa I. *Estradiol and the Addition of Progesterone Increase the Sensitivity to a Neurosteroid in Postmenopausal Women.* Psychoneuroendocrinology 2005; 30(1): 38-50.

 III Wihlbäck A-C, Sundström-Poromaa I, Allard P, Mjörndal T, Spigset O, Bäckström T. *Influence of Postmenopausal Hormone Replacement Therapy on Platelet Serotonin Uptake Site and 5-HT$_{2A}$ Receptor Binding.* Obstetrics and Gynecology 2001; 98: 450-457.

 IV Wihlbäck A-C, Sundström-Poromaa I, Bixo M, Allard P, Mjörndal T, Spigset O. *Influence of Menstrual Cycle on Platelet Serotonin Uptake Site and Serotonin$_{2A}$ Receptor Binding.* Psychoneuroendocrinology 2004; 29(6): 757-66.

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INTRODUCTION

THE MENOPAUSE

Definition and symptoms

As the ovaries age and gradually loose their activity a woman will eventually reach menopause. The menopause is defined as that point in time when permanent cessation of menstruation occurs, i.e. the last menstrual bleeding. The ovaries, due to loss of follicular activity, are no longer able to produce enough estrogen to proliferate the endometrium and thus signal the permanent end of fertility. In normal, non-surgical menopause the definite diagnosis cannot be set until 12 months have passed without any menstrual bleeding. In the industrialized world the menopause occurs at approximately 51 years of age, and nearly two years earlier in smoking women (Midgette 1990, McKinlay 1992). Burger and co-workers propose that the term "perimenopause" should be used to describe the period that commences when the first features of approaching menopause begin until at least one year after the final menstrual bleeding (Burger 2002). The climacteric is a wider, less defined term, including a longer period of time during which the woman passes through a change from the reproductive part of life to the postmenopausal years.

The population of the earth is continuously growing and the age distribution is changing. The United Nations estimates that the population aged 60 or more will increase from 605 million in 2000, to 1.2 billion in 2025 (United Nations 1999). In Sweden there are 1.7 million women aged 50 or over, representing 19% of the population (Nilsson 2001), a proportion that will increase in the future. In the Western world women live more than one third of their lives after menopause. Bearing this in mind, the effect of sexual hormones on female health after menopause warrants increasing interest.

Historically multiple physical and mental conditions have been attributed to the climacteric syndrome. The only symptoms definitely proven to be so, apart from osteoporosis, are vasomotor symptoms and genitourinary atrophy (Oldenhave 1993). The first sign of entering the perimenopausal period is usually a disturbance of the menstrual pattern resulting in irregular bleeding. The disturbance is secondary to shorter ovulatory cycles and an increased number of anovulatory cycles (Rannevik 1995). In the Massachusetts Women's Health Study McKinlay and co-workers found that 10% reached menopause abruptly, whereas most women experienced an irregular bleeding pattern approximately four years before menopause (McKinlay 1992).

The most commonly described symptoms of the perimenopause are vasomotor symptoms (attacks of hot flushes and sweating). Approximately 75% of menopausal women will for some time during the climacteric stage suffer from such vasomotor symptoms (Hammar 1984, Hagstad 1986, Berg 1988, McKinlay 1992). Most women are affected during the initial years after menopause and usually the symptoms diminish after 4-5 years. As many as 15-20% are, however, affected by attacks of hot

flushes and sweating up to 15 years after menopause (Berg 1988). A smaller group of women (10%) report vasomotor symptoms although they still have a regular menstrual pattern (McKinlay 1992). The pathogenesis of the vasomotor symptoms is still obscure. The most plausible explanation is a change of thermoregulation initiated centrally within the hypothalamus, modulated by fluctuations in levels of sex steroids and mediated by certain neurotransmitters such as opioid peptides (Kronenberg 1994, Hammar 1997).

Apart from vasomotor symptoms and genitourinary atrophy other common symptoms reported by patients in association with perimenopause are depressive mood swings and insomnia (Stadberg 1997, Dennerstein 2000). Whether these symptoms are directly attributable to the menopausal transition or if they are the result of vasomotor symptoms has been debated over the years. Findings by Schmidt and co-workers indicate an efficacy of estrogen in relieving depression not solely mediated by altering either hot flushes or disturbed sleep (Schmidt 2000). Other studies, however, favor the "symptom hypothesis", that any association found between menopause and depression is most likely to be explained by vasomotor symptoms. In a longitudinal study Avis and co-workers showed that depression was not significantly associated with menopause status or with annual change in estradiol. The results of their study indicated that hot flushes/night sweats and trouble sleeping were positively correlated to depression scores (Avis 2001). Dennerstein and co-workers obtained similar results (Dennerstein 2000). In an earlier longitudinal study by Avis it was stated that prior depression was the variable most predictive of subsequent depression in the menopause and that onset of natural menopause was not (Avis 1994). However, in the same study it was evident that experiencing a long perimenopausal period (at least 27 months) was associated with an increased risk of depression (Avis 1994). In a Swedish study depressive symptoms were found to be positively correlated to the severity of the vasomotor symptoms (Hammar 1984). How far a woman has come in the climacteric process might be of importance as a significant relationship between vasomotor symptoms and depression was found only in perimenopausal women but not in postmenopausal or older premenopausal women (Joffe 2002). This could be explained by great variations in hormonal levels. In the perimenopause hormonal levels vary more than they do when a woman is postmenopausal. Several studies suggest that periods of heightened hormonal variability, i.e. menarche (Angold 1999), premenstrual periods (Soares 2001b), post-partum (Kendell 1981, Chaudron 2001) and perimenopause (Freeman 2004) increase the vulnerability to depression among subgroups of women. Neuroendocrine factors are likely to contribute to the overall increased risk of developing mood disorders in women. Perhaps the most obvious interaction between ovarian steroids and mood is found in women with premenstrual dysphoric disorder (PMDD). Patients with PMDD only suffer from depressed mood and anxiety during ovulatory cycles. If anovulation occurs, either spontaneously or induced by gonadotropin-releasing hormone (GnRH) agonists, a marked reduction in symptoms is perceived (Hammarbäck 1988, 1991). A history of prior depression and post-partum depression is common among women with PMDD (Pearlstein 1990) and some researchers argue that PMDD itself could be a risk factor for future major depressive disorder (Graze 1990, Hartlage 2001).
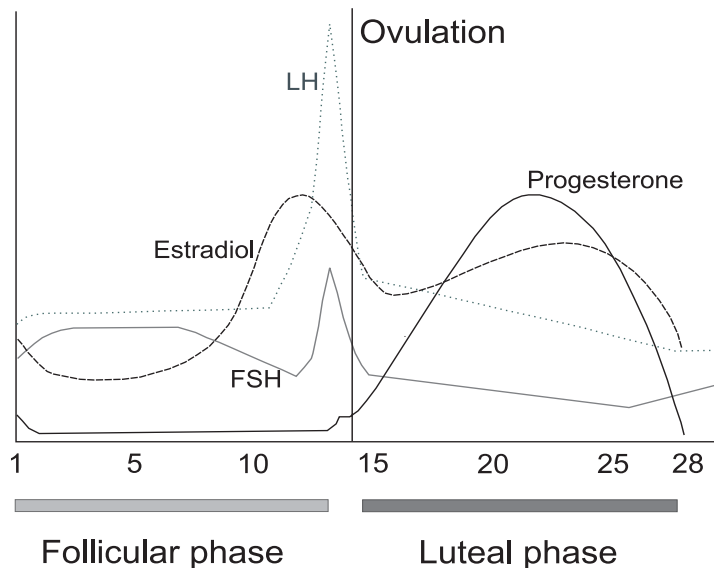
Figure 1. Schematic illustration of the hormonal variations during the menstrual cycle.

Hormonal changes from the reproductive years to the menopause

The variations of estradiol, progesterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) during a normal menstrual cycle have long been well established. The hormonal fluctuations during a menstrual cycle are presented in Figure 1. Other hormones, such as inhibins, involved in the cycle have been elucidated during the past ten years (Groome 1996). Inhibins are peptides and capable of inhibiting FSH secretion (Klein 1996). Inhibin A is mainly derived from the dominant follicle and the subsequent corpus luteum (Roberts 1993, Groome 1996) in analogy with estradiol. The curve showing the mean serum concentration of inhibin A is therefore parallel to that of estradiol during the menstrual cycle (Klein 1996). The source of inhibin B is the pool of small antral follicles (Roberts 1993, Groome 1996) and is primarily secreted in the follicular phase of the menstrual cycle. The generally accepted theory of reproductive aging in women is that the pool of small antral follicles continuously diminishes and accordingly secrete less inhibin B, which allows for a subsequent and gradual increase in FSH over the years (Klein 1996, Burger 2000). This gradual increase in FSH produces an increased effect on the dominant follicle, which, accordingly, is able to maintain an adequate production of estradiol and inhibin A until late perimenopause (Burger 1995 and 2000, Shifren 2000). Age-related hypothalamic changes, as well as a changing ability of estradiol to influence the secretion of GnRH are probably also involved. These changes may contribute to the onset of irregular cycles which ultimately lead to acyclicity (for review see (Wise 2002)). The perimenopause is a time of markedly fluctuating hormone levels as demonstrated in a number of studies (Sherman 1975, Metcalf 1979 and 1981, Hee 1993). The serum concentrations of estradiol start to decline in late perimenopause, about two years before menopause, as the dominant follicle is no longer capable of maintaining estradiol production (Burger 1999, Shifren 2000). Studies have shown that

already three months after menopause estradiol concentrations are significantly lower and reach their nadir at 12 months after menopause (Guthrie 1996).

The production of progesterone takes place in the corpus luteum, i.e. after ovulation has occurred. In the reproductively aging woman the frequency of anovulatory menstrual cycles increases. Rannevik and co-workers have shown that the frequency of cycles with progesterone concentrations indicating ovulation decreased from 60% to less than 10% during the six years preceding menopause. In their study all women had serum concentrations of progesterone of less than 2 nmol/L postmenopausally (Rannevik 1995).

Treatment – effects and side effects, with particular reference to mood

The effects of estradiol (E_2) and conjugated equine estrogen (CEE) as treatments for vasomotor symptoms (MacLennan 2001) and atrophic vaginitis have been widely documented (Campbell 1977, Wiklund 1993). There is also evidence of a beneficial effect on mood but, as discussed earlier, this effect may be primary or in fact secondary to the abolishment of vasomotor symptoms.

Estrogen treatment and psychological well-being

There is evidence of improvement in well-being in healthy female subjects. For instance, estradiol treatment has been shown to be mood enhancing in healthy, naturally postmenopausal women without climacteric symptoms (Ditkoff 1991). Studies performed by Campbell and Whitehead also suggest an independent beneficial psychological effect of estrogen in postmenopausal women with minor climacteric symptoms (Campbell 1977). Sherwin and co-workers have found evidence that mood co-varies with circulating estradiol levels in generally healthy, surgically menopausal, nondepressed women (Sherwin 1988). Finally, there may also be a dose response in estrogen effects on mood. When naturally menopausal, healthy women were given different doses of estrogen, a higher energy level and a more enhanced sense of well-being were reported with the higher estrogen dose (Sherwin 1989).

Estrogen treatment and depression

In postmenopausal women reporting depressive mood, treatment with CEE was superior to placebo in reducing depressive mood symptoms (Carranza-Lira 1999). Regarding major depressive disorder during the climacteric phase there is also evidence of a positive effect of estrogen supplementation. Studies seem to indicate a superior effect of estrogen treatment on major depression in perimenopausal women than in menopausal women. Transdermal estradiol replacement was effective as treatment of depression for perimenopausal women, and the patients also sustained the antidepressant effect after a four-week washout period, although somatic complaints (such as vasomotor symptoms) increased in frequency (Soares 2001a). In a study by Cohen and co-workers investigating short-term use of estradiol as depression treatment in perimenopausal and postmenopausal women the perimenopausal women showed better treatment results. The same study suggested that antidepressant benefit might be independent of improvement in vasomotor symptoms (Cohen 2003). As mentioned earlier, findings by Schmidt and co-workers indicate an efficacy of estrogen

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on depression not solely mediated by altering either hot flushes or disturbed sleep (Schmidt 2000). Estrogen is, however, not considered to be adequate as a single drug when it comes to major depression but has been suggested as a promising supplementary treatment to enhance the antidepressant effect of, for example, selective serotonin re-uptake inhibitors (SSRI) (Derman 1995).

Estrogen treatment combined with progestins, and effects on mood
For women with an intact uterus, estrogen therapy is always combined with progestin or progesterone treatment in order to avoid endometrial hyperplasia and a subsequent augmented risk of endometrial carcinoma (Whitehead 1978, Voigt 1991). This is, however, a problem as a sequential addition of progestins induces cyclical negative mood changes similar to premenstrual syndrome (PMS) symptoms (Hammarbäck 1985, Björn 2000). These changes affect compliance. Just over a third (35%) of the women who discontinued their hormone replacement therapy (HRT) did so because of negative mood symptoms (Björn 1999). A few studies suggest that these adverse effects may be related to the type of progestin used. For instance, norethisterone acetate (NETA) caused more negative mood symptoms than medroxyprogesterone acetate (MPA) in postmenopausal women (Björn 2000).

So, whereas estrogen treatment has been shown to lower the scores for depressed mood, progesterone given in combination with estrogen counteracts the effect of estrogen on mood (Zweifel 1997). This might also be true for major depressive disorder, although the combination of estrogen and progestin has not been so widely studied as estrogen-only therapy with regard to major depression. In a review article Grigoriadis argues that the addition of progestin abolishes the positive additive antidepressant effect of estrogen during treatment with SSRI (Grigoriadis 2002). The results obtained from the pre-terminated arm of the WHI study (Women's Health Initiative) where HRT was administered in a continuous combined regimen (progestagens added every day to the estrogen treatment) do not show any beneficiary long-term results concerning quality of life. These patients were, however, older postmenopausal women without menopausal symptoms (Hays 2003).

OVARIAN STEROIDS AND THE BRAIN

Sex steroid hormones play fundamental roles in the development and function of the central nervous system (CNS). Differences are found in the structure and function of the brains of male and female animals, and humans (Panzica 2004). The most obvious task of sex steroid hormones is to govern the fertility process by influencing the hypothalamic-pituitary-gonadal (HPG) axis. The CNS acts both as a source and a target of sex steroids and of their metabolites. Memory and learning (Sherwin 1997), balance (Hammar 1996) and pain perception (Dawson-Basoa 1996, Wise 2000) are some of the several CNS functions that have been implied to be modulated by the ovarian steroids. Several conditions and symptoms show menstrual cycle linked patterns such as PMDD or PMS (Bäckström 1983, Sanders 1983, Hammarbäck 1989a), catamenial epilepsy (Laidlaw 1956, Bäckström 1976, Herzog 1997) and

menstrual migraine (MacGregor 1996). From animal experiments we know that sex steroids influence sexual behavior (Pfaff 1999, Mong 2003).

Steroid hormones are believed to act by several different mechanisms in the CNS. Firstly, there is the classical genomic mechanism in which the steroids bind to intracellular receptors to modulate transcription and protein synthesis (McEwen 1994). Secondly, during the past decade it has been shown that steroids can also produce rapid effects on excitability and synaptic function through direct membrane mechanisms, such as ligand-gated ion channels and neurotransmitter transporters (Wong 1996). The classical genomic mechanism has a response time on the order of several minutes, hours or days, whereas the membrane mechanism is faster; seconds to minutes. From studies on the rhesus monkey it was early established that the classical hormonal nuclear receptors for estradiol and progesterone are present in neurons and uniquely distributed in certain regions of the brain (Pfaff 1976). Estrogen plays important neurotrophic and neuroprotective roles during adulthood (Wise 1999). For estrogen both α and β receptors have been identified in the human brain (Österlund 2000a, 2000b) and from animal studies locations throughout the brain have been established, especially regions involved in memory, learning and reproductive behavior (Shughrue 1997).
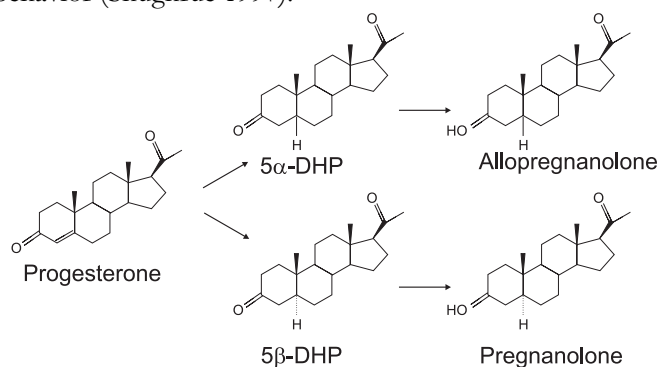

Figure 2. Synthesis of progesterone metabolites 5α-dihydro-progesterone (5α-pregnane-3,20-dione), 3α-hydroxy-5α-pregnane-20-one = allopregnanolone and the 5β-stereo-isomer of allopregnanolone: pregnanolone (3α-hydroxy-5β-pregnane-20-one).

In the light of the various effects of sex steroids on both the developing and adult nervous system, it was a significant finding that some steroids, called *neurosteroids*, are synthesized within the brain and peripheral nerves by glial cells (Baulieu 1991). The term neurosteroids refers to the site of synthesis – the nervous system. These potent steroidal compounds are synthesized from cholesterol or blood-borne precursors (Baulieu 1981) and examples are progesterone as well as its metabolites: 5α-dihydro-progesterone (5α-pregnane-3,20-dione), 3α-hydroxy-5α-pregnane-20-one=allo-pregnanolone and the 5β-stereo-isomer of allopregnanolone; pregnanolone (3α–hydroxy-5β-pregnane-20-one) (Majewska 1992, Mellon 1994). The synthesis of progesterone metabolites is explained in Figure 2.

Post-mortem studies in reproductive and postmenopausal women have indicated that allopregnanolone is accumulated in the brain. The highest levels of allopregnanonlone were found in the substantia nigra and basal hypothalamus (Bixo 1997). Although neurosteroids only represent one aspect of ovarian steroid interactions within the central nervous system, an increasing number of reports demonstrate their profound effects on various CNS functions. From animal experiments allopregnanolone and pregnanolone have been proposed to be involved in neuroendocrine functions such as those governing GnRH release from the hypothalamus (Vincens 1994, el-Etr 1995), LH and FSH pituitary release (Brann 1990), ovulation inhibition (Genazzani 1995), modulating female sexual behavior (McCarthy 1995) and increasing in the brain in response to stress (Purdy 1991).
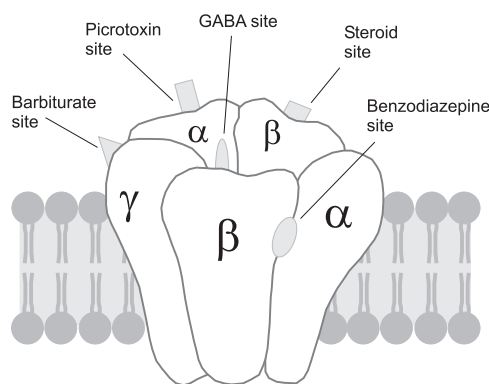

Figure 3. Theoretical model of the GABA$_A$ receptor complex. The GABA$_A$ receptor is composed of five subunits and contains binding sites for GABA, neurosteroids, benzo-diazepines, barbiturates, alcohol and most anesthetic agents.

Allopregnanolone and pregnanolone have anxiolytic and anesthetic properties and were proved, as early as in the 1980s, to exert this action by enhancing gamma aminobutyric acid (GABA)-stimulated chloride conductance in the rat brain (Harrison 1984). The GABA transmitter system is the major inhibitory system in the mammalian CNS (Rang 1995). The GABA$_A$ receptor consists of five subunits forming a ligand-gated chloride channel, see Figure 3 (Luddens 1991). There are a number of different subunits which are represented by different structural classes (for example α, β, γ, δ and ε) (Mehta 1999). Most functional receptors contain assemblies of α/β/γ or α/β/δ subunits (Davies 1997). When GABA binds to its receptor the influx of chloride ions increases, thereby hyperpolarizing the post-synaptic membrane. This consequently renders the post-synaptic cell less prone to excitation. The GABA$_A$ receptors are the site of action of various pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, steroids, anesthetics, ethanol and anticonvulsants. These drugs modulate the GABA-induced chloride ion flux by interacting with separate and distinct allosteric binding sites (Sieghart 1995). As mentioned earlier certain endogenous metabolites of progesterone bind to the GABA$_A$ receptor with high affinity in a similar way to barbiturates (Majewska 1986). The most potent of the

progesterone metabolites is allopregnanolone, followed by its 5β-stereo-isomer, pregnanolone, which was found to be about 60% as active as the 5α-compound (Paul 1992). Neurosteroids have also been shown to increase the affinity of benzodiazepines to the GABA$_A$ receptor *in vitro* (Majewska 1986, McAuley 1993).

Estrogen effects on the GABA system

Observations from animal experiments indicate that estrogen may regulate the GABA$_A$ receptor messenger ribonucleic acid (mRNA) expression at a transcriptional level, and that this is only likely to occur within regions of the rat brain possessing estrogen receptors (Herbison 1995). Effects on levels of GABA$_A$ active steroids as well as on response to GABAergic drugs after estradiol supplementation have been reported in animal experiments. In ovariectomized rats allopregnanolone levels decreased centrally and peripherally, but were restored by estrogen supplementation (Genazzani 2000, Stomati 2002). Estradiol potentiated the antinociceptive effect of allopregnanolone in ovariectomized rats (Frye 1996), and there is also evidence that both estradiol and estradiol+progesterone treatment in ovariectomized hamsters increase the number of GABA$_A$ receptors in the brain, although in different regions (Canonaco 1989). Prior results obtained by our group show that estradiol pretreatment increases the allopregnanolone inhibition in rat and guinea-pig hippocampus (Landgren 1995, Wang 1997). Furthermore, there is evidence of estrogen influencing the subunit composition of the GABA$_A$ receptor (Smith 2000).

Measuring neurosteroid sensitivity in humans

Most studies on neurosteroids and the GABA$_A$ receptor have for practical reasons naturally been performed on animals. In human studies many results rely on measuring peripheral concentrations of GABA or neurosteroids in plasma, serum or cerebrospinal fluid. A method of investigating the functional GABA$_A$ receptor sensitivity has evolved, measuring saccadic eye velocity (SEV) in response to sedative drugs effective on the GABA$_A$ receptor (Hommer 1986). A saccade is a rapid, jump-like movement of the eye from one fixation point to another, used by the eye in order to change the focus of the fovea. Maximal SEV has a large variation of 350-600 degrees per second between subjects (Bollen 1993), but is stable within subjects, both within a testing period and between testing (Gentles 1971, Mercer 1990, Roy-Byrne 1990, Glue 1991). Once a saccade has started, it is generally regarded to be outside conscious control and not subjected to motivational influences (Gentles 1971), and therefore SEV is considered to provide an objective measure of sedation. Neurophysiological data on saccadic eye movements show that they are controlled by the frontal eye fields, substantia nigra, superior colliculus, pontine reticular formation and cerebellum (Becker 1989). Injections of GABA agonists into the area of the superior colliculus reduce the velocity of the saccade, while injections of these substances into the substantia nigra result in irrepressible saccades. This is explained by neurons from the substantia nigra exerting inhibitory actions on saccade-related cells of the superior colliculus (Hikosaka 1985a, 1985b).

SEV has been proposed as an objective and sensitive measure of CNS depression, benzodiazepine effect, and consequently of GABA$_A$ receptor sensitivity in humans

(Hommer 1986, Ball 1991). Several studies have shown that SEV is reliably reduced in a dose-dependent manner by benzodiazepines (Hommer 1986) and that this reduction is reversed by the benzodiazepine antagonist flumazenil (Ball 1991). The effect of pregnanolone can also be measured with saccadic eye movement measurements and this compound has also been found to reduce SEV in a dose-dependent fashion (Sundström 1998a). Furthermore, benzodiazepine- or pregnanolone-induced increase in self-ratings of sedation is highly correlated with reduction in SEV (Hommer 1986, Sundström 1998a). After 30-60 minutes of testing a certain tiring effect has been detected and drugs that increase arousal, such as amphetamine can abolish this. These drugs, however, do not increase SEV above the baseline (Tedeschi 1983). So far no drug has been found to have a stimulatory effect on SEV and it is therefore considered that the saccade-generating system operates at near to maximum activity and that it is not possible to increase SEV above baseline values pharmacologically (Glue 1991). Although increased self-rated sedation is highly correlated to a reduction in SEV, studies have nonetheless been able to show that SEV is not merely a measure of sedation. Thyrotropin-releasing hormone has been shown not to reverse benzodiazepine-induced slowing of SEV, whereas the increased self-ratings of sedation were almost completely reversed (Glue 1992).

Neurosteroids and GABA$_A$ receptors in reproductive women

In normal reproductive women, circulating levels of allopregnanolone and pregnanolone during the menstrual cycle vary like that of progesterone. During the luteal phase the mean concentration of allopregnanolone is higher, 4–12 nmol/L (Wang 1996) than in the follicular phase, 1 nmol/L (Purdy 1990, Mellon 1994, Bicikova 1995, Wang 1996, Genazzani 1998). The same is true for pregnanolone with higher mean values in the luteal phase (2.3 nmol/L) than in the follicular phase (<1 nmol/L) (Sundström 1998c). Plasma concentrations of pregnanolone ranging from 80 to 160 nmol/L cause sedation (Sundström 1999b) and 530–1700 nmol/L anesthesia (Carl 1990). Changes in concentrations of progesterone and allopregnanolone have been seen in the brain (Purdy 1991, Bixo 1995). In a human post-mortem study concentrations of allopregnanolone were measured in brain areas and in serum in reproductive and postmenopausal women (Bixo 1997). Regional differences were evident implying different local mechanisms for steroid uptake and binding. CNS levels of allopregnanolone were roughly 14–21 ng/g, depending on the brain region examined. There was also evidence that the secretion pattern during the menstrual cycle is reflected in the brain as women in the luteal phase had significantly higher brain concentrations of allopregnanolone than postmenopausal controls (Bixo 1997).

In an attempt to study suspected changes on the GABA$_A$ receptor complex due to ovarian steroids in normal menstruating women de Wit and Rukstalis performed benzodiazepine challenges during different phases of the menstrual cycle (de Wit 1997). Baseline measures of arousal showed that higher levels of allopregnanolone during the luteal phase were associated with lower arousal scores, which is consistent with the theory that allopregnanolone acts on the GABA$_A$ receptor complex. A puzzling result was, however, that subjects with the highest levels of allopregnanolone reported increases in scorings of arousal after administration of the benzodiazepine

triazolam (de Wit 1997). The authors discussed a possible cross-tolerance to benzodiazepines in these patients since benzodiazepines and allopregnanolone are both $GABA_A$ receptor agonists. Our group has performed studies on normal menstruating women using SEV and sedation scores combined with pharmacological challenges as an instrument to evaluate neurosteroid sensitivity and effects of ovarian steroids on the $GABA_A$ receptor complex during normal menstrual cycles. The results show that neurosteroid sensitivity in healthy female subjects is increased during the luteal phase compared to the follicular phase (Sundström 1997a, 1998a). We interpret these results as being secondary to higher endogenous concentrations of neuroactive steroids, such as pregnanolone and allopregnanolone, during the luteal phase than in the follicular phase.

During pregnancy in healthy women maternal serum concentrations of progesterone and allopregnanolone rise significantly throughout gestation (Luisi 2000). During the third trimester of pregnancy plasma levels of pregnanolone and allopregnanolone are about 12-30 nmol/L (Hill 2001). Already one hour after parturition maternal serum allopregnanolone decreases significantly, whereas pregnanolone serum concentration does not decrease until one day after parturition (Hill 2001).

Neurosteroids and $GABA_A$ receptors after menopause
No significant differences were found, by Genazzani and co-workers, regarding circulating levels of allopregnanolone between reproductive women during the follicular phase and postmenopausal women (Genazzani 1998). Postmenopausal women do, however, have significantly lower concentrations of progesterone than reproductive women (Genazzani 1998). This suggests that ovarian progesterone is not the major determinant of circulating allopregnanolone in postmenopausal women. As mentioned earlier, a human post-mortem study in which concentrations of allopregnanolone in brain areas and in serum in reproductive and postmenopausal women were measured showed that the secretion pattern during the menstrual cycle was reflected in the brain (Bixo 1997). Reproductive women in the luteal phase had significantly higher brain concentrations of allopregnanolone than postmenopausal controls.

Neurosteroids, $GABA_A$ receptors and mood
One of the most clear-cut examples of the interaction between mood, neurosteroids and the GABA system is PMDD. This syndrome is defined in the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) as a cluster of certain negative mood symptoms as well as physical symptoms occurring during the luteal phase and disappearing a few days after onset of menstruation (Endicott 1999). As many as 75% of all women experience some sort of negative mood symptoms or physical symptoms premenstrually (Andersch 1986) but only 2–6% of all women actually fulfill the criteria for PMDD (Ramcharan 1992, Sveindottir 2000). Ovulation, and the subsequent formation of a corpus luteum, is required for the development of symptoms. During anovulatory cycles, either spontaneous or induced by GnRH agonists, these specific symptom clusters do not appear (Hammarbäck 1988, 1991).

Even though the relationship between symptom development and the progesterone peak in the luteal phase is obvious, most scientists still agree on the absence of peripheral markers of HPG axis dysfunction or significantly different absolute levels of progesterone or estradiol in PMDD patients (Rubinow 1988, Halbreich 1993a, Wang 2001). There are, however, findings suggesting that PMDD women differ from healthy reproductive women in that they exhibit an altered GABA$_A$ receptor sensitivity. Dysfunction of other neurotransmitter systems has also been proposed as possible modulator of PMDD, but in this thesis the focus is on ovarian steroids and especially neurosteroids, and therefore other neurotransmitter systems (apart from the serotonin system) will not be discussed further.

The possible explanation that premenstrual symptoms in PMDD patients are caused by an altered response to progesterone itself has been questioned based on the results of studies employing the intracellular progesterone receptor blocker, RU486. Treatment with low doses of this compound does not ameliorate premenstrual symptoms (Chan 1994), and interest has thus been focused on metabolites of progesterone acting as neurosteroids, such as pregnanolone and allopregnanolone. No simple relationship appears to exist between peripheral concentrations of neurosteroids and PMDD, and studies have yielded contradictory results. There are results indicating no difference in allopregnanolone concentrations between PMDD patients and controls (Schmidt 1994, Wang 1996, Sundström 1998c), significantly lower concentrations in PMDD patients (Rapkin 1997, Bicikova 1998, Monteleone 2000) and significantly higher levels among PMDD patients (Girdler 2001). Regarding peripheral levels of pregnanolone, no differences have been found between PMDD patients and controls (Wang 1996, Sundström 1998c). So, as far as the above studies are concerned no definite proof exists of finding an explanation in a deficiency or excess of peripheral concentrations of neurosteroids in PMDD. There are, however, findings supporting the theory that peripheral concentrations of allopregnanolone are associated with the severity of symptoms in PMDD patients. Earlier studies performed by our group have shown that greater luteal phase concentrations of allopregnanolone were associated with improved symptom ratings in PMDD patients (Wang 1996). This has further been substantiated by Girdler and co-workers. The PMDD patients with higher anxiety and irritability scores had lower concentrations of allopregnanolone during the luteal phase (Girdler 2001). However, in PMDD patients treated with SSRIs, allopregnanolone levels were not affected by treatment and, seemingly contradictory, improvement as such, whether induced by SSRI treatment or placebo, was associated with lower levels of allopregnanolone, rather than increased levels (Freeman 2002).

Our group has also conducted a series of experiments to examine GABA$_A$ receptor sensitivity using saccadic eye movements when performing a series of pharmacological challenges with GABAergic drugs in healthy females and compared the results to those of PMDD patients. These experiments clearly demonstrate that PMDD patients have reduced functional GABA$_A$ receptor sensitivity throughout the menstrual cycle in comparison with control subjects (Sundström 1997a, 1997b, 1998a). Furthermore this reduced sensitivity to GABAergic substances appears to be influenced by the symptom severity experienced by the PMDD patients as those with severe symptoms

were even less sensitive (Sundström 1997a). With regard to mental stress PMDD patients have also been shown to have an altered neurosteroid response. In healthy control subjects 83% responded by exhibiting the expected stress-induced increase in allopregnanolone, whereas only 42% of PMDD patients did so (Girdler 2001). There is also evidence of reduced GABA levels during the menstrual cycle in PMDD patients compared to healthy controls who instead show a gradual increase in GABA levels from the follicular to the luteal phase (Halbreich 1996). Proton magnetic resonance spectroscopic studies measuring cortical GABA levels, on the other hand, show that healthy controls exhibit a decrease during the menstrual cycle. The PMDD patients instead showed an increase in their cortical GABA levels from the follicular to the luteal phase (Epperson 2002). In the above study it was also clear that cortical GABA levels were reduced in PMDD patients during the follicular phase compared to healthy controls. These seemingly contradictory results of cortical, versus plasma levels of GABA, suggest that peripheral measures of GABA function may not accurately reflect central function.

Neurosteroids, GABA$_A$ receptors and hormonal treatment

Previous studies conducted by our group have shown that HRT causes cyclical mood changes similar to, but milder than, those experienced during the luteal phase in PMDD women (Hammarbäck 1985, Björn 2000). This suggests that these exogenous hormones have a similar effect on the CNS to the endogenous ones. A recent study has demonstrated that allopregnanolone concentrations in the peripheral blood increase during HRT (Bernardi 2003). The most prominent increase was seen in patients who received HRT with progestin molecules different from 19-nor derivatives (such as MPA, nomegestrol, dihydrogesterone or cyproterone acetate). Furthermore, higher allopregnanolone concentrations were seen in patients receiving the estrogen compound transdermally than orally. Women with a PMS history during their reproductive life (which is not necessarily equivalent to the diagnosis of PMDD) have been shown to react by exhibiting more negative mood effects due to progestin addition in HRT (Björn 2000, Ödmark in press).

As progesterone metabolites such as pregnanolone and allopregnanolone have been shown to have anxiolytic properties in animal experiments (Wieland 1991, Bitran 1993 and 1995), great hope has historically been placed on progesterone as a form of treatment for PMDD and other anxiety disorders. In healthy, non-anxious subjects on low-dose oral contraceptives, oral micronized progesterone produced levels of allopregnanolone and pregnanolone correlated to the plasma progesterone level (Freeman 1993). In subjects who exhibited high levels of these anxiolytic metabolites significant changes in fatigue as well as impairment in psychomotor tests were found to be positively correlated to the plasma concentration of the metabolites (Freeman 1993). Oral micronized progesterone results in high levels of allopregnanolone, but is unable to relieve symptoms of anxiety and depression in PMDD patients (Freeman 1995, Vanselow 1996, Wyatt 2001). The same results, i.e. no significant improvement in PMDD women, were obtained in a study using progesterone suppositories (Freeman 1990).

Le Mellédo and co-workers tested female patients diagnosed with panic disorder in the early follicular phase when endogenous concentrations of estrogen and progesterone are at their nadir. The patients were pretreated with MPA 10 mg orally or placebo for three consecutive days before the panicogenic pharmacological agent pentagastrin was administered intravenously. The panic response was significantly decreased following MPA pretreatment compared to placebo (Le Melledo 2001). The authors argued that this antipanic/anxiolytic effect of MPA most probably was achieved through modulation of the $GABA_A$ receptor complex.

Given the fact that most adverse mood symptoms during HRT are induced during the progestin phase, greatly reminiscent of PMDD symptoms, and that the pathophysiology of PMDD is argued to be partially explained by neurosteroids, the menopause, HRT and neurosteroids have attracted increasing interest. So far few studies have been performed on functional $GABA_A$ receptor sensitivity, menopause and the influence of HRT.

THE SEROTONIN SYSTEM

As discussed above, most adverse mood symptoms during HRT occur during the progestin phase and largely resemble PMDD symptoms. As dysfunction of the serotonergic system has been proposed as a significant contributor to the pathophysiology of PMDD, it is obvious that the serotonin system is an interesting area of scientific investigation regarding adverse mood symptoms during HRT.

Already in the 1950s Brodie and co-workers identified serotonin (5-hydroxytryptamine, 5-HT) as a neurotransmitter (Brodie 1955). The precursor to serotonin is tryptophan, an essential amino acid (Wurtman 1983) that is taken up in the brain and by certain enzymatic processes converted to serotonin (Spigset 1990). Physiological functions or symptoms in which serotonin is involved are numerous and include aggression, impulse control, anxiety, sexual behavior, pain, sleep and appetite (Spigset 1997a). Dysfunction of serotonergic transmission has been regarded as an important mechanism in several psychiatric disorders, particularly major depression and anxiety disorders (Meltzer 1989, Charney 1990). There are a number of different serotonin receptors and subtypes. Of these, the serotonin$_{2A}$ (5-HT$_{2A}$) receptor has been shown to be involved in depression and generalized anxiety among certain other psychiatric disorders (Spigset 1997a). The effect of serotonin in the synapse is terminated partly by the re-uptake mechanism of a specific protein localized presynaptically, also referred to as the serotonin transporter. Pharmacological agents able to block this mechanism (SSRIs) are effective in treating depression (Meltzer 1989), panic disorder (Rosenbaum 1995), obsessive compulsive disorder (Piccinelli 1995), bulimia (Brambilla 1995) and PMDD (Steiner 1995).

Evaluating the serotonin system
In humans, the uptake of serotonin in platelets and in brain transmitter terminals follows similar active transport processes (Marcusson 1990). Also, the transporter

proteins in platelets and in the CNS are encoded by the same gene, and their amino acid sequences are identical (Lesch 1993). Ligands commonly used to label the platelet serotonin transporter are [³H]paroxetine and [³H]imipramine. Of these [³H]paroxetine has the higher affinity, and higher level of specific binding to the transporter (Mellerup 1983, Bäckström I. 1989).

As for the serotonin transporter the 5-HT$_{2A}$ receptor in platelets is similar to that in the human brain (Andres 1993). In some earlier studies of the 5-HT$_{2A}$ receptor [³H]ketanserin was used as the radioligand. However, nowadays [³H]lysergic acid diethylamide ([³H]LSD) is considered a more appropriate ligand for the 5-HT$_{2A}$ receptor in platelets (Steckler 1993). In receptor binding studies the maximum number of binding sites, i.e. the density or number of receptors (B$_{max}$) and also the affinity of the ligand to the receptor or the dissociation constant (K$_d$) are calculated. Efforts have also been made to utilize peripheral serotonin markers, including platelet 5-HT$_{2A}$ receptors and serotonin transporters, as biological correlates to antidepressant drug response. Studies have, however, yielded mixed results precluding any definite conclusions. Positron emission tomography (PET) studies can be performed using specific radioligands for the 5-HT$_{2A}$ receptor, to investigate cortical distribution of the receptor in human subjects (Smith G. 1998).

The serotonin system and ovarian hormones

Ovarian steroids have been shown to profoundly influence the activity of the serotonergic system. However, although there is a great amount of literature on the subject it is difficult to draw any unambiguous conclusions on how the brain serotonergic system is influenced by hormones. This is due to the way in which the hormone (usually estradiol) is administered, i.e. single bolus injection or long-term treatment, and the dosage of the hormone (often very high, non-physiological doses). Also, effects in the brain are region specific, and as many different animal species have been used, it is difficult to extrapolate the results to effects in the human brain. In animal experiments, there is evidence of CNS region-specific effects on serotonin synthesis, turnover, uptake, release, and receptors by estradiol and progesterone (Bethea 1998, Birzniece 2001 and 2002). Estrogen induces the diurnal fluctuation in serotonin in the hypothalamus (Cohen 1988) and progesterone increases the turnover rate of serotonin (Ladisich 1977). Moreover, estradiol-inducible progesterone receptors have been found to be co-localized on serotonergic neurons of the raphe nuclei in non-human primates (Bethea 1993). In ovariectomized rats, single-dose estradiol increases 5-HT$_{2A}$ receptor binding in areas essential for cognition, emotion, mood and mental state (Summer 1995, Fink 1996, Cyr 1998). Furthermore, at the time of the estradiol-induced LH surge, 5-HT$_{2A}$ receptor binding was up-regulated in these areas compared to other phases of the estrus cycle (Sumner 1997). In ovariectomized rats long-term estradiol in combination with progesterone increased the 5-HT$_{2A}$ mRNA expression in the ventral hippocampus in a region-specific manner (Birzniece 2002). Treating rats with antidepressants causes downregulation of 5-HT$_2$ receptors in the brain, an effect that is abolished by ovariectomy (Kendall 1982). Adding estrogen to the antidepressant treatment, however, reinstates the downregulating mechanism.

Estradiol and progesterone have also been shown to exert effects on the CNS serotonin re-uptake transporter. In non-human primates, long-term treatment with estradiol or combined estradiol and progesterone reduced the expression of mRNA for the serotonin transporter in the dorsal raphe nuclei (Bethea 1998). Likewise, estradiol-treated rats displayed a decrease in [^{3}H]paroxetine binding in the hippocampus (Mendelson 1993).

The serotonin system in reproductive women

There is also evidence of ovarian steroids profoundly influencing the activity of the serotonergic system in human studies. Women are more prone to experience mood-deteriorating effects of tryptophan depletion than men (Ellenbogen 1996) and serotonin synthesis, measured by *in vivo* PET scanning has been shown to differ between men and women (Nishizawa 1997, Chugani 1998).

Results of changes in peripheral measures of serotonergic transmission during the human menstrual cycle are often variable and sometimes contradictory. Measures of whole-blood concentrations of serotonin have been shown to be unaltered during the menstrual cycle in healthy female subjects (Rapkin 1987). However, one study indicated an increase at the time of ovulation as well as prior to the onset of menstrual bleeding (Hindberg 1992). Binding to the serotonin re-uptake transporter (by use of imipramine) has also been studied throughout the menstrual cycle. Most results indicate no alterations (Malmgren 1987, Ashby 1988, Steege 1992). There are, however, also studies indicating significant increases in platelet serotonin transporter binding in the late luteal phase (Tam 1985) as well as contradictory findings of significant decreases in the late luteal phase (Rojansky 1991).

Binding to the 5-HT$_{2A}$ receptor (B$_{max}$) is increased during the second week of the menstrual cycle in regularly cycling women compared to women using oral contraceptives (Spigset 1997c). In this same study no differences were found between men and women. The lack of gender differences is supported by several studies (Pandey 1990, 1993 and 1995, Andres 1993), but contradicted by others (Arora 1989) with findings of higher B$_{max}$ in males than in females. Burnet and co-workers measured 5-HT$_{2A}$ mRNA in post-mortem human brain and showed that the receptor mRNA was present in the neocortex, but to a lesser extent in the hippocamus (Burnet 1995). No 5-HT$_{2A}$ mRNA was detected in the cerebellum, substantia nigra or striatum.

To our knowledge no studies have so far been conducted regarding 5-HT$_{2A}$ receptor binding during different phases of a more thoroughly monitored menstrual cycle. Studies using the more specific compound paroxetine to label the serotonin re-uptake transporter during the menstrual cycle are also lacking.

The serotonin system after menopause

Studies on the influence of aging on serotonergic measures have yielded mixed results. There are studies indicating no differences (Arora 1989 and 1993, Pandey 1995,

Spigset 1997c), but also contrary findings. Regarding binding to the 5-HT_{2A} receptor there are reports of a decrease in K_d (Norman 1990) as well as a decrease in both K_d and B_{max} (McBride 1994, Sheline 2002) with increasing age. In postmenopausal women a positive correlation has been found between total plasma estrogen concentration and free plasma levels of the serotonin precursor tryptophan (Aylward 1976). In peri- and postmenopausal women, platelet serotonin content was found to be positively correlated to plasma E_2 concentrations (Guicheney 1988).

Most studies on sex steroid treatment during menopause and its relation to peripheral serotonergic measures have focused on estrogen treatment only. E_2 treatment was found to increase urinary excretion of the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) (Lippert 1996), but this increase was abolished by the addition of NETA (Mueck 1997). Data from a PET study on postmenopausal women demonstrated that E_2 plus progesterone administration significantly increased cerebral cortical 5-HT_{2A} binding potential. The study was, however, based on only five patients. Furthermore, the investigators were unable to determine whether E_2 administration alone increased 5-HT_{2A} receptors to a plateau level and that progesterone administration did not further elevate the binding, or whether progesterone addition did in fact elevate the binding potential (Moses 2000). In a more recent study ten postmenopausal women were examined before and after estrogen replacement therapy. In this study it was shown that estrogen treatment increased 5-HT_{2A} receptor binding in human prefrontal regions as demonstrated by PET (Kugaya 2003).

The relationship between sex hormones and the serotonin system also seems obvious regarding CNS-effects. Halbreich and co-workers found that postmenopausal women treated with a serotonin agonist had a blunted response (in prolactin and cortisol production) compared to reproductive women. Estrogen replacement increased the response (Halbreich 1995). The authors suggested that decreased serotonergic activity in postmenopausal women might contribute to their vulnerability to affective disorders and that estrogen replacement therapy could possibly decrease this vulnerability and add to the efficacy of serotonergic antidepressants when warranted. This has further been evaluated showing estrogen to be a promising supplementary treatment to enhance the antidepressant effect of SSRIs (Derman 1995, Schneider 1997 and 2001). A beneficial side effect of SSRI treatment of depression in women during the climacteric period is that some of these pharmacological agents have been suggested to alleviate hot flushes (Loprinzi 2000 and 2002, Stearns 2000). The positive effect of estrogen on depression, as mentioned earlier, is to a large extent abolished by progestin addition and it has been speculated whether this in fact is mediated by an effect on the serotonergic parameters (Grigoriadis 2002). PMDD women do not experience negative mood symptoms during anovulatory cycles (Hammarbäck 1988, 1991) or after menopause (Casson 1990), but the symptoms reappear in menopausal women with a PMS history when HRT is administered (Björn 2000). Bearing this, as well as the fact that women with PMDD benefit from treatment with SSRIs, in mind, adds further support to the theory that negative mood symptoms provoked by progestins could be mediated by an influence on the serotonergic system. As already stated, most studies on sex steroid treatment during menopause and its relation to

peripheral serotonergic measures have been focused on estrogen treatment only, and well-conducted randomized studies on the addition of progestins are lacking.

The serotonin system and mood disorders

Given the positive results of serotonergic drugs in the treatment of depression a logical interpretation would be that depressed patients have reduced levels of serotonin. Some studies do in fact support this theory, indicating reduced concentrations of 5-HT and its metabolite 5-HIAA in the cerebrospinal fluid of depressed patients as well as decreased tryptophan concentrations and serotonin transporter binding sites (for review see (Nemeroff 1998)). However, the situation is more complex, as exemplified by studies of monoamine (serotonin, norepinephrine, and dopamine) peripheral concentrations indicating that a reduction in these does not necessarily entail depressive symptoms in nondepressed subjects, nor worsen the symptoms in depressed ones (Delgado 2000). Moreover, approximately 30% of depressed patients do not in fact respond to SSRIs (Nemeroff 1998). Depression is a set of behavioral symptoms manifested by interactions of hormonal and neurotransmitter systems in numerous brain regions. It is unlikely that any one molecule will be solely responsible for such a complex phenomenon as depression.

Low platelet [^{3}H]paroxetine binding has been found in studies on patients with major depression (Nemeroff 1994, Alvarez 1999), whereas others have not found any differences between healthy controls and patients with major depression (Lawrence 1994, Hrdina 1995) or dysthymia (Ravindran 1994). In seasonal affective disorder, one study failed to reveal any differences in platelet [^{3}H]paroxetine binding between patients and healthy controls (Ozaki 1994), whereas the number of [^{3}H]paroxetine binding sites significantly decreased following light therapy in other studies (Mellerup 1993, Smedh 1999).

In several psychiatric disorders, including depression, specific changes in platelet 5-HT$_{2A}$ characteristics have been reported (Biegon 1987). Treatment of depressed patients with the SSRI sertraline decreased B$_{max}$ (Butler 1988). Depressed patients, in particular those with suicidal tendencies exhibit higher B$_{max}$ values for the 5-HT$_{2A}$ receptor than controls (Hrdina 1997). In that particular study, the higher levels did not change with antidepressant treatment and clinical improvement and was therefore suggested more as a trait than a state phenomenon (Hrdina 1997).

As discussed above one of the most clear-cut examples of interaction between mood and ovarian hormones is PMDD. No definite proof exists of HPG-axis dysfunction in PMDD women, but the primary pathophysiology of the syndrome is now instead thought to be symptoms of an exaggerated neuroendocrine reaction to normal hormonal fluctuations (Roca 1996, Steiner 2000). Increasing evidence suggests that serotonin is pivotal in the pathogenesis of PMDD. Not only has altered serotonergic neurotransmission been associated with depression and many other symptoms characteristic of PMDD, including poor impulse control, irritability and carbohydrate craving (Eriksson 1990), but there is also conclusive proof of SSRI treatment being effective in treating PMS and PMDD (Wyatt 2002). Studies on PMDD patients

indicate a blunted neuroendocrine response to serotonergic compounds indicating abnormal post-synaptic serotonergic response in these patients (Halbreich 1993b). Whole-blood serotonin concentration is reduced in PMDD women (Rapkin 1987) and depletion of tryptophan aggravates the syndrome (Menkes 1994).

Earlier studies have revealed a change in serotonin function in PMDD patients with reduced platelet [^{3}H]imipramine binding sites in the follicular phase (Steege 1992). However, a more recent study by our group, using [^{3}H]paroxetine to label the platelet serotonin transporter, indicates that women with PMDD have a higher number of binding sites than controls in the follicular phase (Bixo 2001). Furthermore, a low dose of buserelin (a GnRH agonist) was given and during this treatment the PMDD patients did not differ from controls regarding the number of [^{3}H]paroxetine binding sites, while a simultaneous reduction in depression scores was evident (Sundström 1999a, Bixo 2001). This is supported by earlier studies using [^{3}H]imipramine in which platelet binding sites in depressed patients were normalized following antidepressant treatment (Suranyi-Cadotte 1982, Langer 1986).

A PET study using a radioactive labeled serotonin precursor ([^{11}C]5-HTP) investigating PMDD women suggested a lower uptake of the precursor in the luteal phase compared to the follicular phase. There was also an association between menstrual-phase worsening of irritability and lower uptake of brain 5-HTP in women with disabling PMDD (Eriksson 2002).

NEUROSTEROIDS, GABA$_A$ RECEPTORS AND SEROTONIN

There is much proof that the serotonergic system is in close and complex reciprocal relationship with ovarian hormones. Already in the 1970s it was established, from animal experiments, that progesterone increases the turnover rate of 5-HT (Ladisich 1977). Most studies have involved the investigation of the effect of serotonergic drugs on neurosteroids or on GABA receptor sensitivity.

In vitro studies indicate that SSRIs modulate the activity of neurosteroidogenic enzymes increasing the production of allopregnanolone (Griffin 1999). These findings are supported by animal experiments where a SSRI induced an increase in allopregnanolone levels in rat brain, suggesting a modulating effect on the same neurosteroidogenic enzymes (Uzunov 1996). In animal experiments it has also been shown that SSRIs influence GABA$_A$ receptor function with enhancement at low SSRI concentrations and inhibition at high SSRI concentrations (Tunnicliff 1999). A binding site for the SSRI fluoxetine on the GABA$_A$ receptor with stimulatory properties has even been suggested (Robinson 2003). Moreover, a direct interaction between the GABA and serotonin system is evident in the hippocampus, where serotonin neurons often end at inhibitory GABAergic interneurons (Gulyas 1999). Human experiments also indicate neurosteroid alterations by SSRIs as successful antidepressant treatment increases the cerebrospinal fluid levels of allopregnanolone, whereas unsuccessful treatment does not (Uzunova 1998).

The delay of SSRI treatment in improving mood symptoms in depressed patients has been debated. Animal experiments performed by Nechmad and co-workers indicate a rise in brain, but not serum, concentrations of allopregnanolone after three weeks of SSRI treatment, but not earlier (Nechmad 2003). SSRI are effective in treating PMDD with an onset of action much more rapidly than when SSRIs are effective in treating depression. This suggests that a different mechanism of action is involved. As SSRI treatment increases $GABA_A$ receptor sensitivity to pregnanolone in PMDD patients (Sundström 1998b) it is also likely that neurosteroids are involved in this rapid action of SSRI on PMDD symptoms.

Thus, it is evident that the serotonergic system is intimately linked to, and influenced by, neurosteroids. As altered neurosteroid sensitivity has been suggested as a possible contributory factor to the progesterone/progestin-induced adverse mood effects of HRT, and there is also evidence of estrogen treatment affecting the serotonergic system in postmenopausal women (although progestin addition has been less well studied) it seemed logical to investigate neurosteroid sensitivity and changes in serotonergic parameters in postmenopausal women on HRT. Moreover, in accordance with the discussion on hormonal influence on the serotonergic system, it would be interesting to find out whether normal hormonal levels during the menstrual cycle have any influence on serotonergic parameters in healthy females.

AIMS

1. To investigate the sensitivity of the CNS to pregnanolone in postmenopausal women before and during sequential hormone replacement therapy involving synthetic progestins.

2. To investigate the sensitivity of the CNS to pregnanolone in postmenopausal women before and during sequential hormone replacement therapy involving natural progesterone.

3. To investigate platelet serotonin transporter and 5-HT$_{2A}$ receptor binding in postmenopausal women before and during sequential hormone replacement therapy.

4. To investigate platelet serotonin transporter and 5-HT$_{2A}$ receptor binding in reproductive women during six different stages of the menstrual cycle.

SUBJECTS AND METHODS

SUBJECTS

In all 53 postmenopausal women and 28 reproductive women participated in these studies. The patients were recruited through an advertisement in the local newspaper. None of the women was included in more than one of the clinical trials, and the trials were all performed at the Department of Obstetrics and Gynecology at Umeå University Hospital. The study procedures were in accordance with the ethical standards for human experimentation established by the Declaration of Helsinki of 1975, revised in 1983. The patients all gave written informed consent and the Ethics Committee of Umeå University, Sweden approved the studies.

Table 1. Demographic data of the study groups for papers I-III. The results presented in papers I and III are based on the same study population, except for one woman who refrained from the blood sampling required for the analyses in paper III.

	Papers I and III n = 24*	Paper II n = 26
Mean age, years (range)	51,3 (46-56)	52,4 (42-60)
Mean time after last menstruation, months ± SEM	25,0 ± 4,0	18,5 ± 3,4
Parity, mean ± SEM	2,2 ± 0,2	2,0 ± 0,2
Mean weight, kg ± SEM	66,4 ± 2,2	69,4 ± 2,0
Education (university/college), n	11 (45,8%)	14 (53,8%)
Employed, n	24 (100%)	23 (88,5%)
Married, n	21 (87,5%)	23 (88,5%)

* n = 23 in paper III

Postmenopausal women

The results presented in Papers I and III are based on the same study population. Thirty patients were included, but three patients fulfilled the criteria for major depressive disorder and were thus excluded from the analysis in Paper I and analysed separately from the rest of the study population in Paper III. One woman did not want to take part in the blood sampling required for the analysis presented in Paper III, and the number of participating patients in Paper III is thus one less than in Paper I. The results presented in Paper II are based on a study on 28 patients. The demographic data for the patients are presented in Table 1. Subjects were more than six months past their last menstrual period and had not used any other hormone replacement therapy for at least the past three months prior to taking part in the study. They all had vasomotor symptoms and FSH above 30 IU/L. The participating women had no contraindications towards HRT and were considered physically healthy.

Exclusion criteria were treatment with any steroid compound, benzodiazepines or other psychotropic drugs for at least six months prior to enrolment in the study. Patients with ongoing mood disorders, anxiety disorders, other psychiatric disorders or drug abuse were excluded. Before inclusion, physical and gynecological examination, including transvaginal ultrasound, were performed, as well as determination of FSH. The Medical Products Agency of Sweden approved the clinical trials.

Reproductive women
Twenty-eight healthy women between the ages of 25 and 40 with regular menstrual cycles (28 ± 2 days) were included. The exclusion criteria were treatment with any steroid compound (including oral contraceptives), benzodiazepines or other psychotropic drugs for at least six months prior to enrolment in the study. Premenstrual dysphoric disorder was excluded by retrospective reports of no mood symptom deterioration in the premenstrual phase. Patients with ongoing mood disorders, anxiety disorders, other psychiatric disorders or drug abuse were excluded. Before inclusion, physical and gynecological examinations were performed, as well as determination of serum concentrations of FSH, LH, thyroid-stimulating hormone, routine blood chemistry tests (red and white blood cell count, platelets, liver enzymes, c-reactive protein, creatinase) and routine urine chemistry tests (glucose and protein). All subjects showed negative pregnancy test results and normal blood and urine chemistry test results. Throughout the month of study, no drugs, including acetylsalicylic acid or other over-the-counter medications or herbal remedies, were allowed.

= pregnanolone challenge and blood sampling for serotonin transporter and $5HT_{2A}$ receptor binding
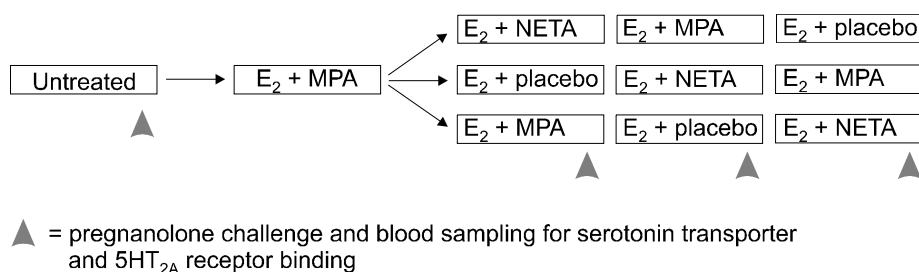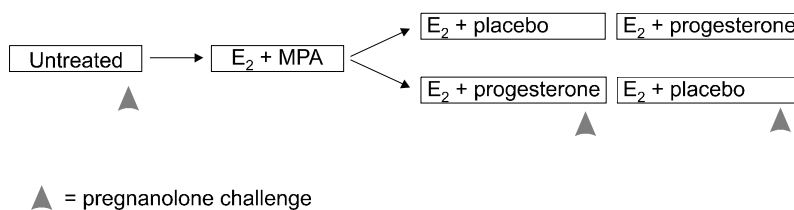
Figure 4. Design of the study presented in papers I and III. Hormone replacement therapy (HRT) was administered in a randomized, double-blinded, placebo-controlled crossover design. The women were treated for four 28-day cycles. Estradiol valerate (E_2) at a dose of 2 mg was given continuously throughout the study period. The first treatment cycle was a run-in cycle. During this cycle medroxyprogesterone acetate (MPA) was added on days 15-28. After the run-in cycle, the following three cycles were randomized regarding the last 14 days of E_2 treatment. During these last two weeks, E_2 treatment was combined either with 10 mg MPA, 1 mg norethisterone acetate (NETA) or placebo. A pregnanolone challenge was administered on four occasions, indicated by the arrows: before treatment and during the last week of each randomized treatment cycle. Blood samples were collected on the same occasions for analysis of platelet [³H]paroxetine and [³H]LSD binding.

EXPERIMENTAL DESIGN

Papers I-III
In both studies on postmenopausal women (Papers I-III) the HRT was administered in a randomized, double blinded, placebo-controlled crossover design. The first treatment cycle was, in both studies, a run-in cycle that gave the women time to familiarize themselves with HRT treatment. During this cycle estradiol valerate (E_2) at a dose of 2 mg was given continuously and MPA was added on days 15-28. In the first study (Papers I and III) the women were treated for four 28-day cycles. E_2 at a dose of 2 mg was given continuously throughout the study period. After the run-in cycle the following three cycles were randomized regarding the last 14 days of E_2 treatment. During these two weeks E_2 treatment was combined either with 10 mg MPA, 1 mg NETA or placebo. The study design is shown in Figure 4. The National Pharmacy Company/Production & Laboratories in Umeå prepared the capsules so that their appearance was identical. Blood samples for platelet [^{3}H]paroxetine binding and platelet [^{3}H]LSD binding were collected on four occasions: before entering the study and during the last week of each randomized treatment cycle. The results regarding platelet [^{3}H]paroxetine binding and platelet [^{3}H]LSD binding are presented in Paper III. In the second study (Paper II) the women were treated for three 28-day cycles. E_2 at a dose of 2 mg was given continuously throughout the study period. After the run-in cycle the following two cycles were randomized regarding the last 14 days of E_2 treatment. During these two weeks E_2 treatment was combined with vaginal suppositories twice daily containing either 400 mg progesterone (Progesterone MIC 400 mg, The National Pharmacy Company/Production, Malmö, Sweden), or placebo. The study design is illustrated in Figure 5.

= pregnanolone challenge

Figure 5. Design of the second study (Paper II). Hormone replacement therapy (HRT) was administered in a randomized, double-blinded, placebo-controlled crossover design. The women were treated for three 28-day cycles. Estradiol valerate (E_2) at a dose of 2 mg was given continuously throughout the study period. The first treatment cycle was a run-in cycle. During this cycle medroxyprogesterone acetate (MPA) was added on days 15-28. After the run-in cycle, the following two cycles were randomized regarding the last 14 days of E_2 treatment. During these last two weeks, E_2 treatment was combined with vaginal suppositories twice daily containing either 400 mg progesterone or placebo. A pregnanolone challenge was administered on three occasions, indicated by the arrows: before treatment and during the last week of each randomized treatment cycle.

The suppositories had identical appearance. The choice to administer progesterone vaginally was made in order to avoid first passage metabolism in the liver and the

formation of progesterone metabolites, which are more abundant after oral administration (de Lignieres 1995). Also, through vaginal administration the serum concentrations of progesterone and its metabolites would better mimic values seen during the normal menstrual cycle (Bäckström 1986). Packing and randomization were done by the pharmacy at Umeå University Hospital. Compliance was assessed by counting the remaining capsules and measuring serum concentrations of sex hormone-binding globulin (SHBG) and FSH at each visit. After completing the study, patients underwent a new physical and gynecological examination and were offered continued HRT by prescription.

Cyclicity Diagnoser (Papers I-III)
Starting one month before entering the study and subsequently throughout the study the postmenopausal patients performed daily symptom ratings using a modified form of the Cyclicity Diagnoser (CD) scale. The CD scale is an instrument designed for diagnosing cyclical symptoms. It has been validated for diagnosis of premenstrual syndrome (Sundström 1999a) and also used, in the same modified form as in this study, during studies of sequential HRT in postmenopausal women (Björn 2000, 2002, 2003). The modified CD scale included four physical symptoms: breast tenderness, hot flushes, abdominal bloating and withdrawal bleeding, and seven psychological symptoms: cheerfulness, friendliness, libido, anxiety/tension, irritability, fatigue and depression (see Appendix 1 where one page and envelope of the CD scale is exemplified). The effects on daily life caused by symptoms were graded. The CD scale is a Likert scale, well suited for repeated measurement analysis. As are all scales for subjectively reported symptoms, the CD scale is mainly used for intra-individual analysis and not for comparisons between subjects. The crossover design of the studies made it, however, possible to compare the changes in reported symptoms between subjects. The CD scale is powerful enough to detect a difference in mood of one scale step (Seippel 1998). In Papers I and III the scale was graded from 1 to 9, where 1 indicated complete absence of a particular symptom, and 9 represented the maximal severity of the symptom. In Paper II we changed the grading to 0-8, with the same endpoints as the previous scale grading. This change was due to input from the participants of the first study who thought it seemed more logical to grade complete absence of a symptom as 0 rather than 1.

Primary care evaluation of mental disorders (Papers I-IV)
The presence of psychiatric disorders and/or drug abuse was evaluated in all women, using a structured psychiatric interview; the primary care evaluation of mental disorders, Prime-MD. This tool has been validated for use in primary care settings and conforms to diagnostic criteria in DSM-IV (Spitzer 1994). The screening questionnaire (patient form) is presented in Appendix 2. If the patient indicated a positive response to any of the key questions regarding affective disorders, a structured psychiatric interview was carried out by the doctor, using a specific instruction form for deeper evaluation.

Paper IV
Blood samples for the determination of platelet [3H]paroxetine binding, platelet [3H]LSD binding, estradiol and progesterone serum concentrations were taken on six

occasions during one menstrual cycle. Blood sampling was scheduled to coincide with the early follicular phase (postmenstrual day 4 ± 1), and late follicular phase (postmenstrual day 12 ± 1), as measured from the day of onset of menstrual bleeding. Ovulation was confirmed in each subject using urinary testing with Clearplan (Unipath, Bedford, UK) which predicts the pre-ovulatory rise in LH concentration. The ovulatory sample was taken on the day of, or the day after, a positive urinary LH assay. Luteal phase blood sampling was scheduled according to the positive LH assay to coincide with the early luteal phase (postovulatory day 4 ± 1), mid-luteal phase (postovulatory day 8 ± 1), and late luteal phase (postovulatory day 12 ± 1). Blood was sampled from the antecubital vein with a 20-gauge needle and collected into polyethylene tubes containing 1.6 mg ethylenediaminetetra-acetic acid per milliliter of blood for the platelet binding assays. All blood samples were taken between 07:30 and 10:00.

PREGNANOLONE CHALLENGE

A pregnanolone challenge was administered on four or three occasions during the studies on postmenopausal women, Papers I and II, respectively. In the study presented in Paper I: before treatment (pretreatment), during estradiol treatment combined with placebo (estradiol-only, E_2), during estradiol treatment combined with MPA (E_2+MPA) and during estradiol treatment combined with NETA (E_2+NETA). In the study presented in Paper II: before treatment (pretreatment), during estradiol treatment combined with placebo (estradiol-only, E_2) and during estradiol treatment combined with progesterone (E_2+progesterone). The pregnanolone challenge was always performed during the last week of each treatment. Testing was carried out at a gynecological outpatients' department. An intravenous cannula was inserted in each forearm and blood samples were taken for analysis of the basal level of pregnanolone in serum. To establish the baseline, three sets of saccadic eye movement measurements and visual analog scale (VAS) ratings of sedation were made, with 5 minutes' delay between each test. Thereafter, an intravenous injection of 0.10 mg/kg pregnanolone was given. All injections were given over 60 seconds. After the pregnanolone injection, saccadic eye movement recordings and visual analog ratings for sedation were made at 5, 13, 18, 25, 30, 45 and 60 minutes (Paper I) or at 5, 13, 18, 25 and 30 minutes (Paper II). The experimental medications were prepared by the pharmacy at Umeå University Hospital. Intravenous pregnanolone was formulated as pregnanolone (CoCensys Inc, Irvine, CA, USA) 15 mg dissolved in albumin solution (Kabi Pharmacia, Stockholm, Sweden, 200 mg/mL) using an ultrasound bath and brought to a volume of 50 mL. The solution contained 0.3 mg/mL of pregnanolone. At the exact end of the saccadic eye movement measurement at the 5-minute postinjection time-point, blood was drawn from the arm contralateral to that used for drug administration in order to measure the pregnanolone plasma level.

MEASUREMENTS OF EYE MOVEMENTS

Pharmacodynamic response to pregnanolone was assessed by measuring the effect of the pregnanolone injection on saccadic eye movement parameters and VAS ratings of sedation. Some of the neurophysiological background to saccadic eye movements has already been described in the Introduction ("Measuring neurosteroids sensitivity in humans"). From a single saccadic eye movement measurement it is possible to derive several parameters that can be used to determine the degree of pharmacological influence (Figure 6). The reaction time, or saccade latency, usually varies from 150 to 300 ms and is measured as the time between target movement and saccade onset. This parameter has been regarded as a measure of higher central nervous system function, i.e. the time taken to make the decision to produce a saccade (Glue 1991). Saccade accuracy, or precision is measured as the difference between attempted target and actual target at the end of the saccade. Usually there is a small undershoot which is corrected by producing a compensatory saccade to reach the attempted target. This parameter has also been shown to be decreased by benzodiazepines. The saccadic eye movement parameters used in our studies were SEV, saccade acceleration/deceleration and saccade latency.
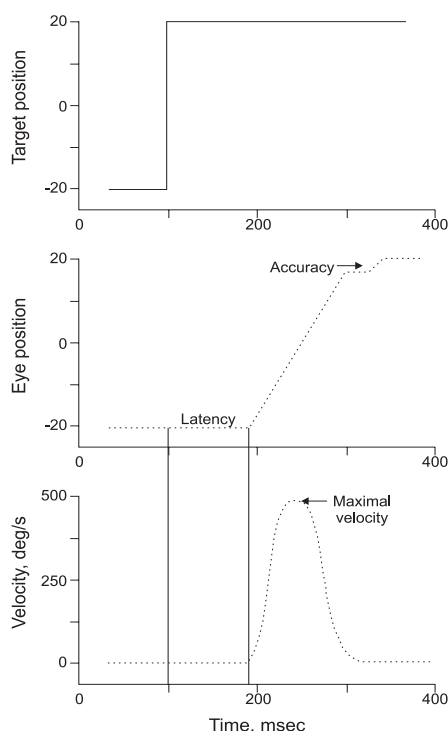
Figure 6. Description of saccadic parameters. This figure is a diagrammatic (but not authentic) representation of a single horizontal saccade. The change in eye position elicits a change in potential between the electro-oculography recording electrodes which is proportional to the displacement of the eyes. From this change the saccadic velocity can be derived.

Saccadic eye velocity was measured using electro-oculography (EOG) with the CSGAAS5 system, fully documented elsewhere (Marshall 1985). A diagram of the experimental arrangements is shown in Figure 7. The test was performed in a quiet, semi-lit room with the patient sitting in a comfortable chair. Head movement was prevented by supporting the subject's head with a pillow. EEG cup electrodes (Synetics AB, Stockholm, Sweden) with a small amount of electrode gel (Elefix, Nihon Kohden) were used. After the skin had been exfoliated with Skinpure cream (Nihon Kohden), the electrodes were placed one cm lateral of the outer canthus of both eyes, with one common electrode in the center of the forehead. Electrode impedance was measured and confirmed to be less than 5 kΩ. The subject was instructed to watch an array of light-emitting diodes (LEDs) placed at eye-level, 67 cm from the glabella. The target for the eye movements was an illuminated LED. The subject was asked to look at the illuminated LED and to move her eyes to the next target (the next illuminated LED) as that LED was turned off and the next one in the array was lit. Subjects were instructed not to anticipate targets.
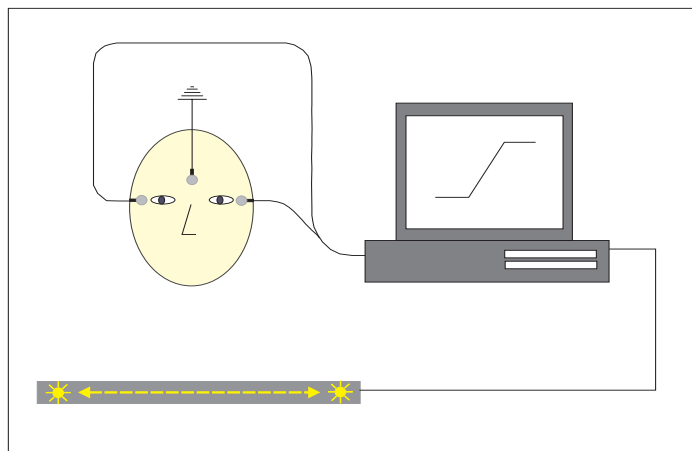
Figure 7. Diagram of the experimental arrangement for saccadic eye movement measurements.

The target movements took place at 1.5-second intervals. A fixed, non-random sequence of 4 x 24 targets producing target steps of 10, 20, 30, and 40 degrees, was displayed with a brief rest in between. The first four of these 24 target steps of each session were not included in the subsequent analyses in order to allow the subject to adjust to the procedure. The EOG was DC amplified and low-pass filtered (-3dB at 50 Hz) before being digitized to 12-bit resolution at a sampling frequency of 250 Hz. A personal computer controlled the target movements and digitized the waveform using an analogue-digital converter. The 80 individual EOGs, resulting from the 4 x 20 target steps were stored and analysed off-line according to the method of Marshall and Richens (Marshall 1985). First, the digitized data from each target displacement was processed to locate saccades. To avoid preemptive saccades and blinking artifacts only saccades initiated 50 to 400 milliseconds after target movements were included. Also, to be considered a saccade, the recorded eye movement had to display a velocity

of more than 100 degrees/second. Second, each saccade was analysed to determine the size of the saccade in degrees, the peak saccadic velocity and latency from target movement to onset of saccade. Saccade accuracy was determined by comparing the actual eye position at the end of the saccade with the attempted target. All saccadic parameters were further processed by plotting a velocity-saccade size curve, known as the main sequence (Baloh 1975). The relationship between saccade size and peak velocity is important, since it remains constant even when voluntary control of saccades is attempted. The main sequence was fitted by a quadratic equation to the peak velocity data using the calculated saccade angle as the independent variable. The influence of outliers in the data was minimized by carrying out the fitting procedure twice and weighing the second fit with the inverse of the square of the residuals from the first fit. The values of peak velocity for 10-, 20-, 30- and 40-degree saccades were then calculated by interpolation. Similar main sequences were plotted for peak saccade deceleration/acceleration and saccade latency. Saccades with an amplitude of 30 degrees were chosen for further analyses as SEV reaches a maximum at approximately 30-35 degrees of angular movement (Baloh 1975).

VISUAL ANALOGUE RATINGS OF SEDATION

A VAS scale was used to rate sedation during the pregnanolone challenge. The scale extended from 0 to 100 mm, where 0 indicates complete absence of sleepiness and 100 represents falling asleep. The patients were asked to rate their sedation using the VAS scale after every set of saccadic eye movement measurements.

MEASUREMENTS OF PLATELET [3H]LSD AND [3H]PAROXETINE BINDING

Platelet [3H]LSD binding

The method used for the platelet [3H]LSD binding assay has been described in detail previously (Spigset 1997c). In brief, platelet-rich plasma was obtained by centrifugation at 180 g for 15 minutes at 20°C. A platelet pellet was then obtained by centrifugation at 1200 g for 10 minutes at 10°C and stored frozen at -70°C until use. On the day of the analysis, the platelet pellet was re-suspended in hypotonic Trisbuffer, homogenized, centrifuged at 30000 g for 15 minutes, washed, homogenized, centrifuged once more, and suspended in the incubation buffer. Thereafter, aliquots of the preparation were incubated in triplicate for four hours at 37°C with seven concentrations of [3H]LSD (76.7 Ci/mmol, DuPont NEN., Boston, MA, USA) ranging from 0.25 to 2.5 nM. Non-specific binding was assessed in the presence of 300 nM spiperone. The binding was terminated by cell harvester filtration through Whatman GF/C filters, prewashed in a 0.3% solution of polyethylenimine. The radioactivity trapped by the filters was determined by liquid scintillation spectroscopy. The total bound [3H]LSD did not exceed 2% of the total radioactivity.

Platelet [^{3}H]paroxetine binding

The method of platelet [^{3}H]paroxetine binding has been presented in detail previously (Andersson 1990). In brief, platelet-rich plasma was obtained by centrifugation at 180 g for 15 minutes at 20°C, and the same amount of assay buffer (50 mM Tris hydrochloride, 120 mM sodium chloride, pH 7.4) was added (1:1). After centrifugation at 3000 g for 20 minutes at 4°C, the pellet was re-suspended in assay buffer, re-centrifuged at 3000 g for 10 minutes at 4°C and homogenized in 10+10 mL of the buffer, using a Kinematica Polytron homogenizer (Lucerne, Switzerland) at setting 6 for 7 seconds. After a final centrifugation at 15000 g for 10 minutes at 4°C, the pellets were stored at -70°C until assayed. On the day of the analysis, the platelet pellet was re-suspended in assay buffer to a final volume of 35 mL. The homogenates were incubated for 60 minutes at 25°C in a total volume of 1600 µL, consisting of 750 µL of the tissue homogenate, 750 µL of radioligand and 100 µL of buffer or drug. Nine concentrations of [^{3}H] paroxetine (19.7 Ci/mmol, DuPont NEN., Boston, MA, USA) ranging from 0.01 to 1.2 nM were used. Specific binding was defined as the difference between total binding and binding in the presence of 10 µM of citalopram (Andersson 1990). The binding experiments were performed in duplicate. After the addition of 6 mL ice-cold buffer, the homogenates were rapidly filtered through Whatman GF/C filters (Whatman, Maidstone, UK) using a 24-channel cell harvester (Brandel, Gaithersburg, MD, USA). Finally, the filters were washed with three 6 mL rinses of the buffer. The radioactivity trapped by the filters was determined by liquid scintillation spectroscopy. For the highest paroxetine concentrations, the total bound [^{3}H]paroxetine did not exceed 5% of the total radioactivity. For both radioligand binding methods, final protein concentrations were measured according to Lowry (Lowry 1951) with modifications suggested by Markwell (Markwell 1978).

STEROID ASSAYS

Pregnanolone and allopregnanolone were measured with radioimmune assay (RIA) after pre-assay diethylether extraction and celite chromatography, as described previously (Sundström 1998a). The pregnanolone antiserum was raised against 3α,21-dihydroxy-5β-pregnan-20-one 21-hemisuccinate coupled to bovine serum albumin. [11,12]^{3}H-pregnanolone (New England Nuclear, Boston, MA, USA) was used for tracer and for internal recovery estimates in the assays. The level of quantification of the assay was 150 pmol/L. The intra-assay coefficient of variation was 5.2% and the interassay coefficient of variation was 7.8% at 50 nmol/L. Allopregnanolone antiserum was raised against 3α-hydroxy-20-oxo-5α-pregnan-11α-yl carboxymethyl ether coupled to BSA, with intra- and interassay coefficients of variation of 6.5% and 8.5%, respectively. The antisera for pregnanolone and allopregnanolone were kind gifts of Dr. R.H. Purdy.

For the analysis of FSH, LH, SHBG, estradiol and progesterone the methods used by the laboratory at Umeå University Hospital changed between the different studies, for details see each paper.

STATISTICS

The statistical procedures are described in detail in the Papers (I-IV). Some general remarks on the statistical methods used are, however, necessary. As the first treatment cycle was used as a run-in cycle (Papers I-III), only the randomized cycles are included in the analyses. Saccade parameters and self-ratings of sedation were calculated as Δ scores (difference from baseline at every point in time). Analysis of variance (ANOVA) with repeated measures was used to evaluate the saccadic parameters, sedation change scores and the serotonergic parameters. All values are displayed as means ± SEM. The SPSS statistical package was used for all analyses. A p-value < 0.05 was considered significant.

RESULTS

The rates of, as well as the reasons for patient discontinuation in the different studies have been described in the separate papers.

HORMONAL CHANGES IN POSTMENOPAUSAL WOMEN

Estradiol, FSH, SHBG and progesterone

In the first study on postmenopausal women, described in Papers I and III, E_2, FSH and SHBG were analysed before and throughout the study. These values were used as a crude measure of compliance and changed according to our expectations. FSH levels decreased throughout the course of the study ($p < 0.01$) and SHBG levels increased ($p < 0.01$). There was also a significant increase in the concentrations of circulating E_2 over the study period ($p < 0.01$). In the study presented in Paper II, E_2 also increased significantly ($p < 0.01$) during treatment, whereas progesterone only rose during treatment with E_2+progesterone ($p < 0.001$).

Pregnanolone

In the study described in Paper I the baseline concentration of pregnanolone was lower during E_2-only cycles than before treatment ($p < 0.05$), but the 5-minute postinjection concentrations of pregnanolone were the same for the different treatment cycles. In the study described in Paper II the baseline concentrations of pregnanolone ($p < 0.001$) and allopregnanolone ($p < 0.001$) were higher during E_2+progesterone cycles, as expected. The 5-minute postinjection concentration of pregnanolone was also higher during E_2+progesterone cycles ($p < 0.05$) than during pretreatment or E_2. However, the 5-minute postinjection concentrations of pregnanolone during pretreatment and E_2 did not differ.

NEUROSTEROID SENSITIVITY AND EFFECTS OF HRT ON MOOD IN POSTMENOPAUSAL WOMEN

Treatment with synthetic progestins
Mood effects

The average scores for summed physical, negative and positive symptoms during the last 14 days of each treatment cycle are shown in Figure 8. There was a significant difference between estrogen-only cycles and cycles with both estrogen and progestagen indicating an overall discomfort for the women during the progestin phases (summed negative symptoms: $p < 0.001$, summed positive symptoms: $p < 0.001$ and summed physical symptoms: $p < 0.001$).
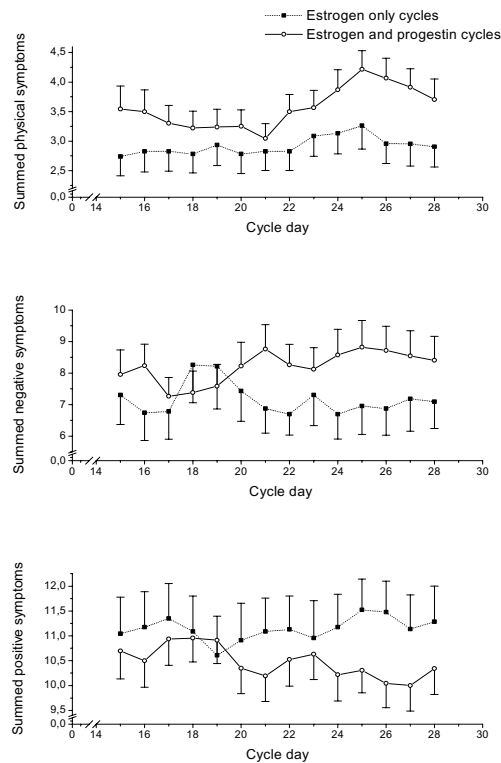
Figure 8. Daily symptom ratings on a 9-point Cyclicity Diagnoser scale of summed physical, negative and positive mood symptoms during the last 14 days of sequential estradiol (E_2)/progestin treatment in 24 postmenopausal women. Each point represents the mean±SEM of the summed symptom scores for E_2 only, and for E_2+progestin cycles. During cycles with progestin addition patients displayed significantly higher scores for summed physical symptoms $F_{1,22} = 32.70$, $p < 0.001$ and for summed negative symptoms $F_{1,22} = 30.17$, $p < 0.001$. Summed positive symptom scores were significantly lower during cycles with progestin $F_{1,22} = 36.57$, $p < 0.001$.

Pregnanolone challenge

The average area under the effect–time curve for saccadic parameters and sedation scores during the pregnanolone challenge of each treatment is shown in Figure 9. Compared to pretreatment conditions, estrogen therapy alone did not change any of the saccadic parameters or the sedation response to pregnanolone. Adding progestins to the estrogen treatment did, however, increase the responsiveness to pregnanolone. Treatment with MPA or NETA increased the area under the effect–time curve for sedation scores compared to pretreatment: $p < 0.01$ and $p < 0.05$, respectively. Also, the post-pregnanolone effect on saccadic deceleration was increased by progestin addition compared to only E_2 treatment. The response was most evident during E_2+MPA treatment ($p < 0.05$), but was also observable during E_2+NETA treatment, however, not reaching significance ($p = 0.051$). Furthermore, the SEV response to pregnanolone challenge was increased during E_2+NETA treatment compared to E_2

treatment (p < 0.05). A trend towards a significantly enhanced effect of pregnanolone during E_2+MPA treatment compared to E_2 was also evident (p = 0.070). Comparing the two progestins to each other in regard to any of the saccadic parameters or in sedation scores revealed that there were no detectable differences in post-pregnanolone responses between E_2+MPA and E_2+NETA treatment.
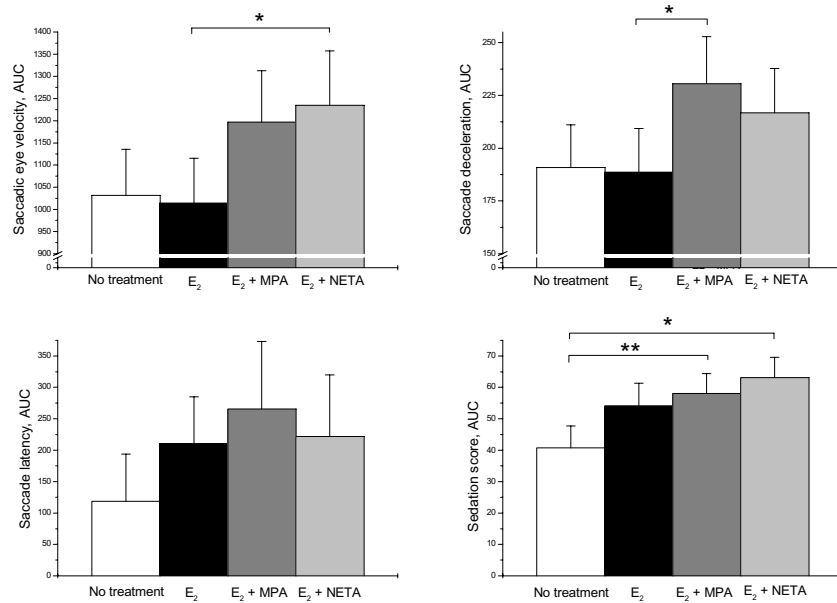
Figure 9. Mean±SEM area under the effect-time curves (AUC) for saccadic eye velocity (SEV), saccade deceleration, saccade latency and sedation scores during pregnanolone challenge. The effects of pregnanolone on saccadic parameters and sedation scores were evaluated in 24 postmenopausal women before treatment, during estradiol (E_2) treatment, during E_2 treatment combined with medroxyprogesterone acetate (MPA) and E_2 treatment combined with norethisterone acetate (NETA). An increased AUC represents an greater reduction in SEV and saccade deceleration. Treatment with MPA as well as NETA increased the AUC for sedation scores: pretreatment vs. E_2+MPA $F_{1,21}$ = 10.48, p < 0.01, and pretreatment vs. E_2+NETA $F_{1,21}$ = 7.32, p < 0.05. The saccade deceleration response to pregnanolone increased during E_2+MPA treatment, E_2 vs. E_2+MPA $F_{1,21}$ = 7.74, p < 0.05. Furthermore, the SEV response to pregnanolone challenge was increased during E_2+NETA treatment compared to E_2 treatment $F_{1,21}$ = 4.87, p < 0.05.

Treatment with natural progesterone
Pregnanolone challenge
The pregnanolone-induced changes in saccadic parameters and sedation scores at the first three postinjection times during the different treatments are shown in Figure 10. Compared to pretreatment values, the pregnanolone-induced reduction in saccadic eye velocity and saccade acceleration was more pronounced during E_2 cycles (p < 0.01 and p < 0.001, respectively) and during E_2+progesterone cycles (p < 0.05 and p < 0.01, respectively). Saccade latency increased significantly after the pregnanolone injection during E_2+progesterone treatment (p < 0.05) compared to pretreatment

testings, whereas during E_2 the results did not reach significance (p = 0.054). Also, the pregnanolone-induced change in sedation scores was increased during E_2 (p < 0.001) and during E_2+progesterone cycles (p < 0.001) compared to pretreatment.
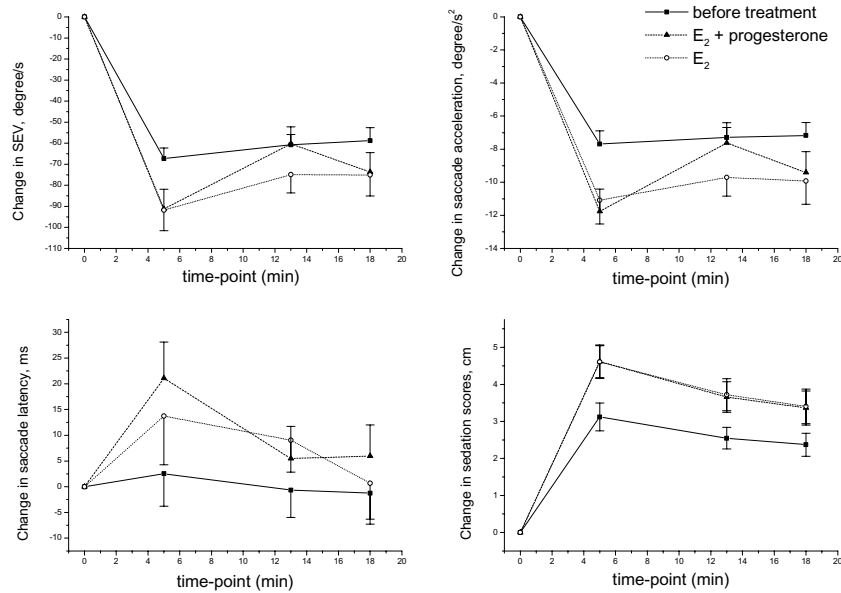
Figure 10. Effect of pregnanolone on saccadic parameters and sedation scores were evaluated in 26 postmenopausal women before treatment, during estrogen treatment and during estrogen treatment combined with progesterone. Mean ± SEM of the change in saccadic eye velocity, saccade acceleration, saccade latency and sedation scores during pregnanolone challenge at the first three postinjection times during the different treatments. Treatment with estradiol (E_2) alone or E_2+progesterone induced a significantly enhanced response to pregnanolone compared to pre-treatment in saccadic eye velocity ($F_{2,25}$ = 6.46, p < 0.01), saccade latency ($F_{2,25}$ = 3.31, p < 0.05), saccade acceleration ($F_{2,25}$ = 7.72, p < 0.01) and sedation scores ($F_{2,25}$ = 11.30, p < 0.001).

Mood symptom cyclicity
Mean scores for summed negative symptoms in women expressing symptom cyclicity and women with no cyclicity in negative mood symptoms during the randomized cycles are shown in Figure 11. The pregnanolone-induced changes in saccadic parameters and sedation scores at the first three postinjection times during the two treatment cycles (E_2 and E_2+progesterone) for the eight women expressing symptom cyclicity and the 18 women with no cyclicity in negative mood symptoms are shown in Figure 12. After pregnanolone injection, women expressing symptom cyclicity responded by exhibiting a more pronounced reduction in saccadic eye velocity (p < 0.05) and a more pronounced increase in saccade acceleration (p < 0.05), and sedation scores (p < 0.05) compared to women with no cyclical changes in negative mood during HRT.
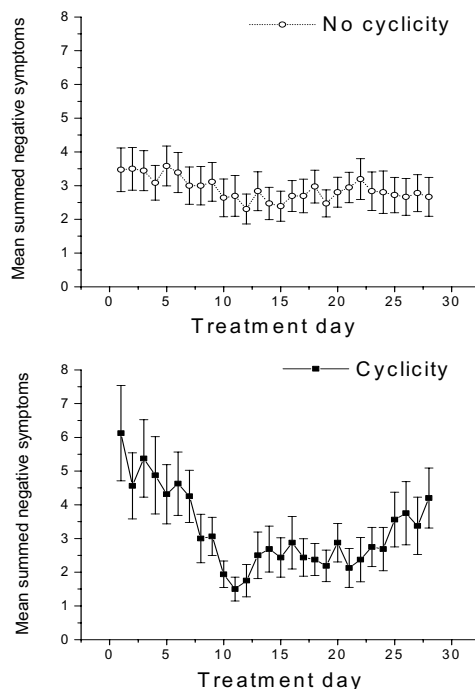
Figure 11. Twenty-six postmenopausal women kept daily symptom ratings (0-8) on a Cyclicity Diagnoser scale during sequential estrogen/progesterone treatment. By comparing the best seven days to the worst seven days of each treatment cycle in each subject (Wilcoxon rank sum test) women were grouped into those who displayed a significant cyclical increase in negative mood symptoms and those who did not. Eighteen women were characterized as expressing no cyclicity and 8 women as expressing a significant cyclical increase in negative mood symptoms during treatment. Each point represents the mean ± SEM of summed negative symptoms of two treatment cycles.

PLATELET SEROTONIN TRANSPORTER AND 5-HT$_{2A}$ RECEPTOR BINDING

Postmenopausal women before and during HRT

There were no significant changes in the values of B_{max} or K_d for [^{3}H]paroxetine binding or [^{3}H]LSD binding between pretreatment conditions and E_2, E_2+MPA or E_2+NETA treatment. Likewise, there were no significant differences between E_2+MPA and E_2+NETA in [^{3}H]paroxetine or [^{3}H]LSD binding.

Reproductive women

Mean (± SEM) values for B_{max} and K_d for [^{3}H]LSD binding at six different stages of the menstrual cycle are presented in Figure 13. B_{max} for [^{3}H]LSD binding was significantly higher in the early follicular phase than in the mid-luteal phase (p <0.001).
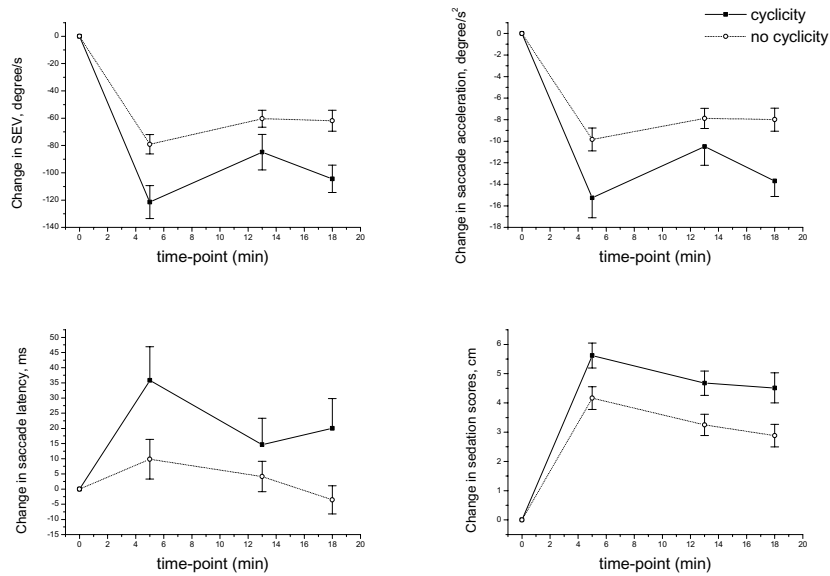
Figure 12. Pregnanolone effect on saccadic parameters and sedation scores were evaluated in 26 postmenopausal women characterized as expressing cyclicity in negative mood symptoms (n=8) or not (n=18). The mean±SEM change in saccadic parameters and sedation scores at the first three postinjection times during the two treatment cycles estradiol (E_2) and E_2+progesterone are shown. After pregnanolone injection, women expressing symptom cyclicity responded by expressing a more pronounced reduction in saccadic eye velocity ($F_{1,24}$ = 6.91, $p < 0.05$) and saccade acceleration ($F_{1,24}$ = 5.15, $p < 0.05$), and furthermore, a more pronounced increase in sedation scores ($F_{1,24}$ = 5.27, $p < 0.05$) compared to women with no cyclical changes in negative mood during HRT. Saccade latency in response to pregnanolone did not differ between the groups ($F_{1,24}$ = 3.72, $p = 0.07$).

Also, there was a significant reduction in B_{max} for [^{3}H]LSD binding between early luteal and mid-luteal phases ($p < 0.05$).

Mean (± SEM) values for B_{max} and K_d for [^{3}H]paroxetine binding at six different stages of the menstrual cycle are presented in Figure 14. In the late follicular phase, B_{max} for [^{3}H]paroxetine binding was significantly higher than in the ovulatory ($p < 0.01$), early luteal ($p < 0.05$) and mid-luteal phase ($p < 0.01$).

In the early follicular phase, as well as during the ovulatory phase, there was a positive correlation between estradiol serum concentrations and K_d for [^{3}H]paroxetine binding (both $p<0.001$). During the mid-luteal phase there was an inverse correlation between estradiol serum concentrations and K_d for [^{3}H]LSD binding ($p<0.05$), and an inverse correlation between progesterone serum concentrations and B_{max} as well as K_d for [^{3}H]LSD binding (both $p<0.05$).

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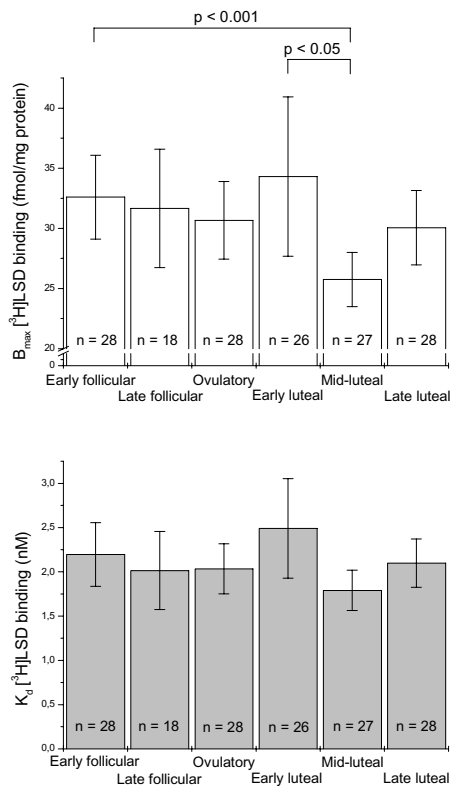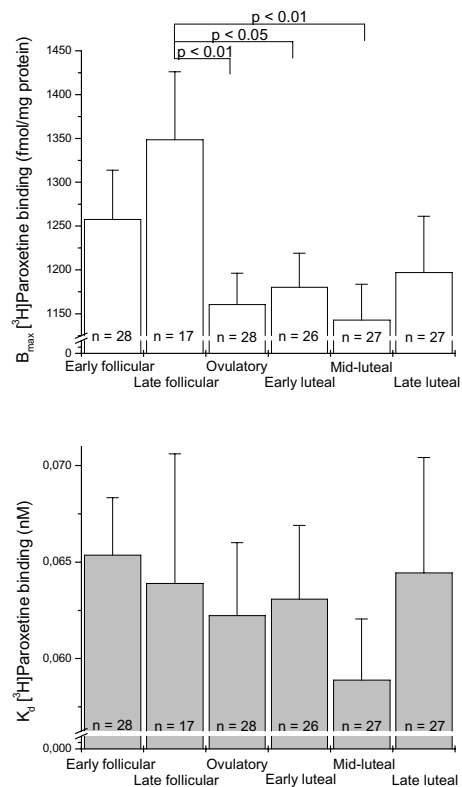
Figure 13

Figure 14

Figure 13. Changes in B_{max} and K_d for platelet [^{3}H]LSD binding in 28 healthy women throughout the menstrual cycle. Bars represent the mean±SEM of the change in B_{max} and K_d during six stages of the menstrual cycle. B_{max} for [^{3}H]LSD binding was significantly higher in the early follicular phase than in the mid-luteal phase ($p < 0.001$). Also, there was a significant reduction in B_{max} for [^{3}H]LSD binding between the early luteal and mid-luteal phases ($p < 0.05$). K_d for [^{3}H]LSD binding did not change significantly during the six menstrual cycle stages.

Figure 14. Changes in B_{max} and K_d for platelet [^{3}H]paroxetine binding in 28 healthy women throughout the menstrual cycle. Bars represent the mean±SEM of the change in B_{max} and K_d during six stages of the menstrual cycle. In the late follicular phase, B_{max} for [^{3}H]paroxetine binding was significantly higher than in the ovulatory ($p < 0.01$), early luteal ($p < 0.05$) and mid-luteal phase ($p < 0.01$). K_d for [^{3}H]paroxetine binding did not change significantly during the six menstrual cycle stages.

DISCUSSION

The results show that the addition of synthetic progestins to estrogen treatment increased negative mood symptoms and physical symptoms, whereas positive symptoms were decreased. The addition of progestins or natural progesterone to estrogen treatment increased the sensitivity to pregnanolone. However, during estrogen-only treatment the two clinical trials yielded different results in terms of pregnanolone sensitivity. In the first study, estrogen treatment did not increase pregnanolone sensitivity, whereas in the second study the pregnanolone sensitivity was enhanced during estrogen treatment. Women expressing cyclicity in negative mood symptoms were more sensitive to pregnanolone than women without symptom cyclicity. Thus, it is evident that mood deterioration during HRT is associated with altered neurosteroid sensitivity. Neither platelet serotonin transporter or 5-HT_{2A} receptor binding changed during the different treatment conditions in HRT. Thus, we were unable to explain the negative mood changes of HRT in terms of these peripheral serotonergic parameters. In the study on reproductive women, however, it was clear that the serotonergic variables did change during the menstrual cycle. The density of platelet serotonin uptake sites was higher in the late follicular phase than in the ovulatory, early luteal or mid-luteal phases. Binding to the 5-HT_{2A} receptor was higher in the early follicular phase and the early luteal phase than in the mid-luteal phase. These findings may provide a link between the ovarian steroids, neurosteroids and the serotonergic neurotransmitter systems which, in turn, could explain part of the specific vulnerability that women have for the development of adverse mood effects during HRT, mood and anxiety disorders and for the deterioration of mood so frequently seen during the luteal phase.

There are, however, some methodological considerations which may bias these conclusions and these considerations are discussed below.

METHODOLOGICAL CONSIDERATIONS

As stated in the Introduction the menopause is defined as that point in time when permanent cessation of menstruation occurs, i.e. the last menstrual bleeding. Furthermore, Burger and co-workers propose that the term "perimenopause" should be used to describe the period of time that commences when the first indications of approaching menopause appear until at least one year after the final menstrual period (Burger 2002). Using this definition a woman would not be referred to as "postmenopausal" until at least one year has passed since her last menstrual bleeding. In our studies we chose to regard the women as postmenopausal already six months after their last menstrual bleeding. This was done for practical reasons; we feared that it would be difficult to gather enough patients by asking women, sometimes with severe climacteric symptoms, to wait at least one year until they could start HRT. One appropriate question is thus whether we have examined perimenopausal women, or women in fact not even close to menopause, only experiencing an irregular bleeding

pattern. By making sure that the women all had vasomotor symptoms, FSH above 30 IU/L and by ascertaining the actual number of months since last menstrual bleeding (mean±SEM) which were 25.0±4.0 and 18.5±3.4 for the two studies, we feel confident that the majority of these women had passed their absolute last menstrual bleeding. In the first study, described in Papers I and III, 17 out of 24 patients had in fact had their last menstrual bleeding more than one year ago, i.e. were postmenopausal by definition. In the second study, presented in Paper II, the corresponding number was 16 out of 26. Thus, even though approximately one third of the women should be considered perimenopausal by definition, it is likely that only a small number, if any, of them would have further menstrual bleeding.

The use of Prime-MD, which has been evaluated for screening and diagnosing of depressive and anxiety disorders in primary care settings, is a weakness of the studies. A more thorough research tool for evaluating and diagnosing previous and on-going psychiatric diseases, especially those that may influence the GABA system, i.e. depression, anxiety and drug abuse, would have been preferable. However, given its utility and ease of use, along with positive experience from earlier studies, Prime-MD was considered to be an adequate tool for assessing the prevalence of psychiatric disorders in this gynecologic outpatient setting. The agreement between Prime-MD diagnoses and independent psychiatric diagnoses derived by a structured interview is generally excellent across diagnostic modules, with an overall accuracy of 88% (Spitzer 1994).

No washout phase was included between the different treatment cycles in the two studies on postmenopausal women, nor were the women kept on E_2 after the final treatment cycle. As our main measurements (neurosteroid sensitivity) were performed during the last week of each treatment cycle to determine possible effects during progestin/progesterone phases, the E_2-only phase of each treatment cycle can be regarded as a washout phase. Still, had longer washout periods been included, as well as an E_2 cycle after the last treatment cycle, we would have had the advantage of a more clear-cut progestin/progesterone phase in terms of daily symptom ratings, allowing for more firm diagnosis of symptom cyclicity. The study design, however, has not interfered with the pregnanolone challenges as it can be assumed that any influence of progestin/progesterone has disappeared when the next pregnanolone challenge was performed during the last week of the subsequent treatment cycle. According to prior pharmacokinetic studies of vaginal progesterone, elimination half-time is 8-14 hours, depending on the emulsion used for the vaginal suppositories (de Lignieres 1995, Mircioiu 1998) and pharmacodynamic studies have suggested that the impact on negative mood disappears within five days (Andreen 2003). In previous studies performed by our group the impact on negative mood symptoms produced by synthetic progestins in one treatment cycle (of the same length as the studies in this thesis) was shown to disappear well before the progestin phase in the next treatment cycle (Björn 2000, 2002). By increasing washout periods we would have extended the E_2-only periods and thereby increased the risk of bleeding disturbances, which would probably have influenced the drop-out rate negatively.

The reproductive women did not complete any CD-scale ratings of mood and physical symptoms before entering the study, or during the study. If this had been done we would have been able to definitely rule out subjects with PMDD. However, since the women in our study did not consider themselves to suffer from premenstrual symptoms, it can be assumed that the ratings would not have indicated any such problems. On the contrary, it is more common for women seeking help for premenstrual complaints to fail to display a cyclical pattern in typical symptoms on the CD scale during two consecutive menstrual cycles. Performing ratings during the study would have given us the opportunity of correlating findings of altered peripheral serotonergic parameters to changes in mood. This would have added strength to our findings.

NEUROSTEROID SENSITIVITY IN POSTMENOPAUSAL WOMEN

The results are unambiguous, demonstrating increased neurosteroid sensitivity during sequential addition of oral synthetic progestins, as well as vaginal natural progesterone, to estradiol treatment in postmenopausal women. The increased pregnanolone sensitivity was substantiated by findings in both the saccadic parameters and in the sedation response. We interpret these findings as an augmented supply of $GABA_A$-active compounds by the progestin/progesterone treatment. Increased sensitivity to pregnanolone during progestin/progesterone treatment is in agreement with results from previous studies on the interaction between ovarian steroids and GABAergic drugs. MPA has been proven to be CNS-active as it has anesthetic properties (Meyerson 1967) and NETA metabolites are active at the $GABA_A$ receptor. For instance, in animal experiments 3α-5α-NET is able to induce a dose-dependent decrease in anxiety, although its effect is of lower potency than that of allopregnanolone (Picazo 1998). Orally administered progesterone has previously been shown to increase benzodiazepine sensitivity in postmenopausal women (McAuley 1995). Animal experiments also indicate a correlation between increased $GABA_A$ receptor sensitivity and high concentrations of progesterone (Majewska 1989, Schumacher 1989, Bitran 1991 and 1993, Westerling 1991).

HRT provides a reproducible model for studies of interactions between ovarian steroids and psychomotor measures of CNS depression. Our findings are in agreement with studies on responsiveness to GABAergic drugs during the menstrual cycle in healthy reproductive women. Earlier results obtained by our group have indicated increased sensitivity to diazepam and pregnanolone among healthy females during the luteal phase of the menstrual cycle (Sundström 1997a, 1998a). These results are interpreted as being secondary to higher endogenous concentrations of neuroactive steroids, such as pregnanolone and allopregnanolone, during the luteal phase than in the follicular phase. However, using less sensitive measures, others have found no change in psychomotor performance after triazolam or alprazolam challenges during the menstrual cycle (de Wit 1997, McAuley 1999, Rukstalis 1999). Clearly, compared to HRT, the menstrual cycle leads to more complex hormonal changes with rapidly decreasing progesterone levels in the mid- to late luteal phase. Given the findings of decreased benzodiazepine sensitivity during progesterone

withdrawal (Moran 1998, Smith S. 1998b), it is conceivable that changes in sensitivity to GABAergic drugs during the menstrual cycle are easily disguised by alterations in endogenous progesterone levels and consequent alterations in GABA$_A$ receptor function.

However, even though the interpretation of the present results is that the increased sensitivity to pregnanolone during progestin or progesterone addition is an effect mediated through the metabolites of MPA, NETA or progesterone and their subsequent effect on the GABA$_A$ receptor, the possibility still remains that it might be an effect mediated through the binding of MPA, NETA, progesterone or their metabolites to the progesterone receptor, or through interaction with different neurotransmitter systems. This is, however, a speculation beyond the scope of the present studies. Since it still cannot be assumed that neurosteroids bind only to the GABA$_A$ receptor, inferences about functional GABA$_A$ receptor sensitivity cannot easily be made.

HRT and mood

The results showed that the addition of synthetic progestins to estrogen treatment increased negative mood symptoms and physical symptoms, whereas positive symptoms were decreased. The effects on mood of HRT were discussed extensively in the Introduction. A few studies suggest that the adverse effects may be related to the type of progestin used, or the administered dose. For instance, NETA has been found to cause more negative mood symptoms than MPA in postmenopausal women (Björn 2000). The present results did not reveal any differences between the two progestins (NETA and MPA) with regard to neurosteroid sensitivity. The study was, however, not powered to detect any differences in adverse mood effects between MPA and NETA, so the finding of no difference regarding neurosteroid sensitivity between the two progestins cannot be related to the aspect of mood. There are other findings regarding the neuroendocrine effects of different variations of HRT. Different HRT regimens have been evaluated in a study using the change in plasma endorphin (after naloxone and clonidine administration) as a marker of neuroendocrine effect. Estradiol was administered continuously and synthetic progestins (nomegestrol acetate or cyproterone acetate) or natural progesterone were added sequentially. No differences were found between the progestins/progesterone used in that study, supporting our results (Stomati 1997). A recent study, however, indicates that the type of progestin used does make a difference. It was demonstrated that allopregnanolone concentrations in the peripheral blood increased during HRT and that the most prominent increase was seen in patients who received HRT with progestin molecules different from 19-nor derivatives (such as MPA, nomegestrol, dihydrogesterone or cyproterone acetate). Furthermore, higher allopregnanolone concentrations were seen in patients receiving the estrogen compound transdermally than in those receiving them orally (Bernardi 2003).

Regarding progestin dose, it has been suggested that the addition of 20 mg MPA to estradiol treatment is more beneficial than 10 mg of MPA (Björn 2002). It also seems that the level of estrogen is involved in symptom provocation. A higher dose of

estrogen during the progestin phase of HRT, (3 mg E_2 as opposed to 2 mg E_2) induced more severe negative mood symptoms (Björn 2003). The latter finding is supported by studies on PMDD women, indicating that high concentrations of luteal-phase estradiol or LH are related to the severity of negative premenstrual symptoms (Hammarbäck 1989b, Seippel 1998).

HRT, mood and neurosteroid sensitivity

A major overall finding was that the increased neurosteroid sensitivity evident in our studies was connected to mood deterioration. In the first study (Paper I) the patients displayed significantly higher scores for summed negative mood symptoms and significantly lower scores for summed positive mood symptoms while receiving the progestin addition. In the second study, based on their daily symptom ratings, women who experienced a cyclical pattern in negative mood symptoms during sequential HRT displayed an enhanced response to pregnanolone compared to women with no cyclical changes in negative mood during treatment (Paper II). Thus, the results indicate that increased pregnanolone sensitivity might predispose women to cyclical negative mood changes during HRT. However, it must be emphasized that reporting cyclical negative mood symptoms during HRT does not, by definition, indicate that these women are more affected by treatment. In this study, there were other women who actually had higher scores of negative mood symptoms than women with symptom cyclicity, but their scores for negative mood were high throughout all the treatment cycles. According to the definition of symptom cyclicity, these women, although worse off in terms of negative symptoms, were defined as non-cyclical.

It is tempting to relate problems of cyclical negative mood symptoms provoked by progestin/progesterone addition during HRT to the negative mood symptoms experienced by PMDD women in the luteal phase, but the question is whether the two conditions are comparable at all. In a previous study by our group healthy postmenopausal women on HRT with progesterone were found to display a cyclical pattern in negative mood symptoms. This cyclical pattern was only seen when the patients received 400 mg progesterone daily, but not at a dose of 800 mg (Andreen 2003). However, women with prior PMS, although suffering from cyclical symptoms during their menstrual cycles (while still reproductive) and also suffering from more severe negative mood symptoms during HRT (Björn 2000), did not display a progesterone-induced cyclicity in negative mood during HRT (Andreen 2003).

Given the results of the present studies the retrospective reports of our patients cannot be used to evaluate prior PMDD, as any such diagnosis would result in an overestimation of the disorder. Previous findings presented by our group indicate reduced responsiveness to benzodiazepines and pregnanolone of patients with PMDD (Sundström 1997a, 1997b, 1998a). In healthy controls pregnanolone sensitivity increased in the luteal phase of the menstrual cycle, whereas PMDD patients exhibited reduced sensitivity to pregnanolone in the luteal phase (Sundström 1998a). The symptom severity within the PMDD group also had an impact on the response to GABAergic drugs. Patients with severe PMDD symptoms displayed less reduction in SEV and sedation response to benzodiazepine as well as to pregnanolone

than patients with less severe PMDD symptoms (Sundström 1997b, 1998a). Clearly, pregnanolone sensitivity in PMDD patients and pregnanolone sensitivity during progestin addition in healthy postmenopausal women are not similar. PMDD patients have been shown to have a decreased sensitivity to pregnanolone, whereas healthy postmenopausal women displayed increased sensitivity to this particular neurosteroid during progestin or progesterone addition. Furthermore, whereas patients with severe PMDD were even less sensitive to pregnanolone, postmenopausal women with symptom cyclicity during HRT were even more sensitive to pregnanolone. Hence, our interpretation is that the increased pregnanolone sensitivity during HRT in healthy postmenopausal women is not mediated through the same mechanisms as the decrease in pregnanolone sensitivity induced during the menstrual cycle of PMDD patients.

Another possible explanation of why neurosteroid sensitivity during progestin-induced adverse mood effects in postmenopausal women differs from findings among PMDD women in the luteal phase could be the process of aging. It is most probable that our postmenopausal women had already developed some changes in the CNS due to the aging process. As extensively discussed in the Introduction, the CNS is most important in regulating the reproductive process, and reciprocally the CNS is greatly influenced by hormonal variations. The fact that aging of the brain makes a difference to the reproductive process has been illustrated in several studies. For example, ovaries of young rodents transplanted into old hosts fail to cycle normally, whereas ovaries of old rodents transplanted into young hosts respond to neuroendocrine signals and reproductive cyclicity is restored (Peng 1972, Felicio 1983). Also, in light of the changes induced by oophorectomy on the behavioral response to allopregnanolone, with "a need" for estrogen in order for an anxiolytic effect to be achieved by allopregnanolone (Laconi 2001) and $GABA_A$ receptor binding (Akinci 1997), findings in postmenopausal and reproductive women are difficult to compare. In a study examining the acute effects of single intramuscular progesterone injections differences were seen between reproductive women during the follicular phase and postmenopausal women (de Wit 2001). The ratio of allopregnanolone to progesterone was smaller in postmenopausal women at certain points in time. The authors discussed whether this indicated that postmenopausal women have a slower conversion of progesterone to allopregnanolone at high concentrations. Even though levels of progesterone similar to luteal-phase values were reached, these had little effect on mood or behavior, only modestly increasing ratings of fatigue in both groups. The small mood effects were, however, more evident, and also seemingly contrary to those in normally menstruating women (decreasing feelings of vigor and friendliness) than in the postmenopausal women (increasing positive mood). The authors presented different explanations, such as a brief progesterone increase is not enough to influence mood, or that progesterone with its metabolites is only able to produce mood effects during another phase of the menstrual cycle (the luteal phase). Further explanations could be that estrogen is required to enable the progesterone effect (as in the luteal phase or in HRT in postmenopausal women), or that mood effects induced by progesterone will only be seen in certain vulnerable individuals (de Wit 2001).

Thus, even though our studies cannot shed any light upon the question of why women with PMDD experience negative symptoms during the luteal phase, it does seem evident that neurosteroids are involved in both PMDD as well as in negative mood effects caused by the addition of progestin or progesterone in HRT.

Estrogen and neurosteroids

The findings of increased neurosteroid sensitivity during progestin/progesterone addition to estradiol treatment in postmenopausal women were evident in both studies. In the second study, described in Paper II there was, however, a divergent result, namely of augmented neurosteroid sensitivity also during the E_2-only treatment cycle. The increased sensitivity to pregnanolone was evident in the saccadic parameters as well as in the sedation response. During E_2-only cycles, neither baseline, nor postinjection levels of neurosteroids differed from pretreatment levels and the increased pregnanolone sensitivity seen during E_2-only treatment is, hence, not readily explained by increased availability of neurosteroids. This finding indicates that progesterone addition might not be needed to enhance pregnanolone sensitivity. There are several possible explanations for these divergent findings. Firstly, the findings presented in Paper II might merely be due to chance. However, the unchanged pregnanolone sensitivity during E_2-only treatment (Paper I) was true for the saccadic eye movement parameters, while regarding the sedation response there was a trend towards increased sensitivity also during E_2-only treatment. Secondly, the divergent results might be due to the fact that a different study group was used, possibly including more patients with prior severe premenstrual symptoms during their reproductive years. As adequate methods for retrospective reports of prior PMDD in postmenopausal women are lacking we were unable to assess this. However, human studies on PMDD patients have indicated that these patients seem to be equally susceptible to negative mood changes caused by estradiol or progesterone. In a study by Schmidt and co-workers women with PMDD and controls were evaluated in terms of mood response to different hormonal settings. A GnRH analog was administered to obtain ovarian suppression and this resulted in improved symptom ratings for the PMDD patients. When either estradiol or progesterone was added to the GnRH treatment the negative symptoms recurred in the PMDD patients, but not in the controls. This was interpreted as a general abnormal response to normal hormonal fluctuations in PMDD patients (Schmidt 1998). All the patients in the study presented in Paper II had positive retrospective reports of PMS symptoms during the reproductive part of their lives, but only a few had more abundant complaints. As mentioned above there is at present no reliable tool to definitely diagnose PMDD retrospectively and it was considered that the retrospective reports of the patients could not be used to evaluate prior PMDD. However, given the enhanced pregnanolone sensitivity during E_2-only cycles, it is possible to speculate that the present results were obtained by including more patients with prior PMDD in the study described in Paper II. Thirdly, the finding of increased pregnanolone sensitivity during E_2-only treatment is in agreement with the results of a number of animal experiments where augmented response to GABAergic drugs after estradiol supplementation has been reported. In ovariectomized rats, estradiol potentiated the antinociceptive effect of allopregnanolone (Frye 1996), and there is

also evidence that both estradiol and estradiol+progesterone treatment in ovariectomized hamsters increases the number of $GABA_A$ receptors in the brain, although in different regions (Canonaco 1989). Prior results obtained by our group show that estradiol pretreatment increases the allopregnanolone inhibition in rat and guinea-pig hippocampus (Landgren 1995, Wang 1997). Finally, the divergent results of the two studies might be the result of a clinical difference (although not statistically significant) in the time since the last menstrual bleeding between the two patients samples. In one group (Paper I) the time since the last menstrual bleeding was 25.0 ± 4.0 (mean±SEM) months and in the other (Paper II) 18.5 ± 3.4 months, thus indicating that a longer time had elapsed since menopause in the patients in our first study. Other studies have indicated changes in terms of plausible $GABA_A$ receptor effects with time since menopause. McAuley and co-workers found that oral progesterone treatment exacerbated the sedative, as well as the memory and psychomotor impairing effects of intravenous administration of benzodiazepine (triazolam) in postmenopausal women (McAuley 1995). Data from the same research group indicate that women with a longer elapsed time since menopause exhibit higher progesterone concentrations after administration as well as increased response to benzodiazepines (McAuley 1996), hence a longer time since menopause seems to increase the sensitivity to $GABA_A$-active drugs. In our study, on the other hand, the group of women with a shorter time since menopause expressed an increased sensitivity to pregnanolone during E_2-only cycles.

SEROTONIN TRANSPORTER AND 5-HT$_{2A}$ RECEPTOR BINDING

In the study on postmenopausal women the different treatment regimes did not induce any significant changes in any of the peripheral serotonin parameters investigated in the study. Likewise, there were no significant differences between E_2+MPA and E_2+NETA treatment with regard to [³H]paroxetine or [³H]LSD platelet binding. However, in the study on reproductive women, the peripheral serotonergic parameters showed dramatic changes during the menstrual cycle. This was interpreted as being due to the fact that the relatively low hormonal levels of estradiol and synthetic progestins attained during HRT are insufficient to affect the [³H]paroxetine and [³H]LSD platelet binding, whereas the high and fluctuating endogenous concentrations of steroid hormones in reproductive women greatly affect these serotonergic measures.

The lack of difference between the various forms of treatment in the postmenopausal study is not due to failure of the treatments to affect mood. Even though the hormonal levels during HRT are considered to be low, they are still able to induce an improvement in mood symptoms during E_2-only cycles which is abolished by progestin addition.

Major depression is approximately two to three times more common in women than in men (Weissman 1977) and several studies suggest that periods of heightened hormonal variability, i.e. menarche (Angold 1999), premenstrual periods (Soares 2001b), post-partum (Kendell 1981, Chaudron 2001) and perimenopause (Freeman

2004) increase the vulnerability to depression in some groups of women. Bearing this in mind, and considering the considerable effects of the fluctuating endogenous concentrations of steroid hormones during the menstrual cycle on the peripheral serotonergic measurements it seems obvious that neuroendocrine factors probably contribute to the overall increased risk of developing mood disorders in women.

To the best of the author's knowledge, there are no prior reports on the effects of progestin addition to estrogen therapy on platelet [^{3}H]paroxetine or [^{3}H]LSD binding in postmenopausal women. There have, however, been some investigations of postmenopausal estrogen treatment and its interaction with the serotonin transporter and the 5-HT$_{2A}$ receptor, although these are scarce and have yieldied inconsistent results. There are positive findings in surgically menopausal women treated with estrogen, showing an upregulation of platelet [^{3}H]imipramine binding sites (Sherwin 1990). However, in naturally postmenopausal women treated with an E$_2$ implant no significant changes were found in platelet [^{3}H]imipramine or [^{125}I]LSD binding compared to the pretreatment condition (Best 1989), which supports the results of the current studies.

This seeming lack of influence of treatment on the serotonergic parameters in postmenopausal women must be discussed in terms of the platelet method. The anucleate platelet has a lifespan of 10-14 days and its characteristics are determined by events that affected the formation of the megakaryocyte weeks earlier. Assuming a lifespan of ten days for the platelet means approximately 10% renewal of the platelet population each day. By this reasoning a certain steady influence on the megakaryocyte will be evident when the whole platelet population has been completely renewed, i.e. after approximately ten days. Taking this into account, the results might have been different if blood sampling had been performed at a later stage, for instance during the first week of the subsequent treatment cycle. In studies using platelet serotonergic measurements there is a tendency towards significant changes appearing 1-2 weeks after the period of detectable mood changes. For instance, in studies on patients with PMDD, lower platelet [^{3}H]imipramine binding than in controls has been detected during the follicular phase, approximately 1-2 weeks after the peak of premenstrual symptoms in the luteal phase (Steege 1992, Veeninga 1992). Also, in a more recent study on PMDD patients performed by our group using [^{3}H]paroxetine to label the platelet serotonin transporter, a change in the amount of binding sites was evident in the follicular phase. In this study the women suffering from PMDD exhibited a higher amount of binding sites than controls, in contrast to the above cited studies (Bixo 2001). Nevertheless, regarding the effect of E$_2$-only treatment on the serotonergic parameters studied in postmenopausal women in the present work, the length of the treatment period should be sufficient to detect any estrogen-induced changes. Consequently, only progestin-induced changes could have been concealed by the turn-over time of platelets. Another possibility discussed in Paper III, namely the absence of an effect of E$_2$-only treatment on the serotonin measures, was that even longer treatment times could be required to detect a change in platelet [^{3}H]paroxetine and [^{3}H]LSD binding. However, in the light of the profound changes seen during the study on reproductive women this possibility seems less realistic.

In the study on healthy reproductive women the lowest values of both the [³H]paroxetine and the [³H]LSD binding were obtained in the mid-luteal phase, at the same time as progesterone concentrations reach their peak. Binding to the platelet serotonin transporter was significantly higher in the late follicular phase than in the ovulatory, early luteal and mid-luteal phases. Binding to the 5-HT$_{2A}$ receptor, on the other hand, decreased between the early luteal and mid-luteal phases, and there was also a significant difference between the early follicular and mid-luteal phases. In the early follicular phase, estradiol and progesterone concentrations are at their nadir, in contrast to the mid-luteal phase when both progesterone and estradiol concentrations are high. Thus, the most obvious interpretation would be that progesterone, or the synergistic effect of estradiol and progesterone, has a more profound impact on [³H]LSD binding than estradiol on its own. This is further supported by the finding of significant inverse correlations between progesterone serum concentrations and B$_{max}$ as well as K$_d$ for [³H]LSD, and a significant inverse correlation between estradiol serum concentrations and Kd for [³H]LSD binding in the mid-luteal phase. B$_{max}$ for [³H]paroxetine binding, on the other hand, reached its maximum during the late follicular phase during the pre-ovulatory estradiol peak and while progesterone serum concentrations are still low. Although no changes were observed in K$_d$ for [³H]paroxetine binding during the follicular phase, there was significant correlation between estradiol serum concentrations and K$_d$ for [³H]paroxetine binding at this stage of the menstrual cycle.

In analogy with the discussion above on the lifespan of platelets, the possibility naturally exists that the results of the reproductive women might in fact mirror hormonal effects from an earlier phase of the menstrual cycle. This theory, however, demands a certain steady influence on the megakaryocyte which might not be the case in studies on the menstrual cycle where hormonal changes are faster than during HRT. If the influence is shorter and vanishes rapidly the effect on the platelet population is at its peak on the same day as the influence disappears. It has also been suggested that the 5-HT$_{2A}$ receptor configuration might undergo relatively rapid changes in order to compensate for intracellular (platelet) and/or extracellular (plasma) alterations (Spigset 1997b). In theory it might be possible that a substance such as progesterone, through unknown mechanisms, could change the structure of the 5-HT$_{2A}$ receptor or the serotonin transporter and thereby affect affinity (K$_d$) and/or the number of receptors available for binding (B$_{max}$). Studies employing more frequent blood sampling (e.g. every day) throughout the menstrual cycle are thus warranted.

Data on healthy human control subjects regarding ovarian steroid interactions with the serotonergic system are scarce, often variable, and sometimes contradictory. These studies are discussed in the Introduction and will therefore only be mentioned briefly here. [³H]Imipramine binding in platelets has been reported to be unaltered throughout the menstrual cycle in most instances in healthy controls (Malmgren 1987, Ashby 1988, Steege 1992). However, some studies have indicated significant increases in platelet [³H]imipramine binding in the late luteal phase (Tam 1985), and others significant decreases in the late luteal phase (Rojansky 1991, Veeninga 1992). These studies should be interpreted with caution as [³H]imipramine is not considered a

sufficiently selective ligand for the serotonin re-uptake transporter (Mellerup 1983, Bäckström I. 1989). An earlier study has indicated that 5-HT$_{2A}$ receptor binding is increased during the second week of the menstrual cycle in regularly cycling women compared to women using oral contraceptives (Spigset 1997b). One reason for the discrepancies between the studies referred to above could be the unconfirmed monitoring of menstrual cycle phases. A more proper scheduling of the menstrual cycle in future studies, in order to obtain reproducible results between studies, need to be advocated. In most cases, blood sampling was scheduled according to the onset of menses, and confirmation of ovulation, if any, was retrospective, based solely upon increased progesterone concentrations in the luteal phase. Due to the variability in follicular phase length, such scheduling is often inadequate and late follicular, ovulatory and early luteal phase events may be confused and data consequently misinterpreted. The present study on reproductive women and changes of serotonergic parameters during the menstrual cycle was better monitored hormonally than any of the studies referred to above.

Our findings are partly in agreement with studies of the effect of estradiol and progesterone on serotonin re-uptake transporter mRNA expression in nonhuman primates. Compared to ovariectomized animals, animals treated with long-term estradiol or estradiol+progesterone showed lower expression of serotonin re-uptake transporter mRNA (Bethea 1998). Short-term administration or single injections of estradiol have, on the other hand, been shown to increase serotonin re-uptake transporter mRNA expression in the amygdala, hypothalamus and dorsal raphe in rodents (McQueen 1997).

Since the reproductive women did not keep daily records of mood symptom changes during their menstrual cycle it was impossible to definitely rule out PMDD (as discussed under "Methodological considerations"), but most unfortunately it is also impossible to relate any of the changes in serotonergic parameters to possible (if any) changes of mood. At inclusion, these women certified that they had no negative mood symptoms during the luteal phase, and the results from this group cannot thus be directly compared to studies on depressed patients or to patients with PMDD; however, a short comment on earlier results is in order. Studies on the changes in the variables of the serotonin transporter or the 5-HT$_{2A}$ receptor during different psychiatric conditions and their treatment have yielded conflicting results. In this study a decrease in B$_{max}$ was observed for both the serotonin transporter and the 5-HT$_{2A}$ receptor during the luteal phase, a period of time during which, at least, women with PMDD experience negative mood changes. According to previous studies this change in serotonergic parameters indicates a positive treatment effect (Butler 1988, Biegon 1990), but the lifespan of platelets must be taken into account. It might actually be that the "positive treatment effect" seen during the luteal phase actually mirrors effects during the follicular phase. Moreover, it is not known whether the patients had any mood changes during the luteal phase. As already mentioned, a more recent study by our group, using [^{3}H]paroxetine to label the platelet serotonin transporter, indicates that women with PMDD have a higher number of binding sites than controls in the follicular phase (Bixo 2001). Furthermore, a low dose of buserelin (a GnRH agonist) was given and during this treatment the PMDD patients did not

differ from controls regarding the number of [³H]paroxetine binding sites, while a simultaneous reduction in depression scores was evident (Sundström 1999a, Bixo 2001). Clearly, the mood effects were related to the serotonergic parameters, as well as to the hormonal changes induced by the GnRH agonist.

The results must be considered bearing in mind the question of whether changes seen in peripheral measures are applicable to effects in the CNS and direct outcome measures by means of effects on mood. Results from studies on correlations between central and peripheral serotonergic measurements have been performed by comparing PET studies with platelet serotonin parameters. The results so far are negative. In two separate studies, 12 and 10 healthy volunteers (6 men + 6 women and 3 men + 7 women) were examined by PET scan with [¹⁸F]setoperone (binding to cortical 5-HT$_{2A}$ receptors) and [³H]LSD platelet binding. No significant correlations were found (Cho 1999, Yatham 2000). Results are similar regarding the serotonin transporter. Malison and co-workers examined 15 drug-free depressed patients as well as 15 healthy controls (7 men and 8 women in each group) and compared [³H]paroxetine platelet binding with injection of [¹²³I]β-CIT (binding to the serotonin transporter) using brainstem single photon emission computed tomography (SPECT). The results showed that there was a significant reduction in brainstem binding in depressed patients compared to healthy controls. Platelet [³H]paroxetine binding was, however, not altered and was not significantly correlated with brainstem binding (Malison 1998). As expected, most of these scientists discourage efforts to use the platelets and peripheral blood cells to learn more about neurotransmission abnormalities in the brains of psychiatric patients. However, it has been suggested that these peripheral measurements could be used to detect hormonal effects that may be relevant to the brain (Mann 1998). Furthermore, given the small patient groups used in the above cited studies of central and peripheral markers of the serotonin system, the absence of correlations could merely be due to a type-II error. The methods are also not easily accessible in clinical practice and moreover quite expensive, and a suitable "peripheral" method would therefore be most valuable.

WHY DO CERTAIN WOMEN SUFFER FROM ADVERSE MOOD EFFECTS DUE TO PROGESTIN/PROGESTERONE?

Even though the underlying mechanisms behind progestin/progesterone-induced negative mood symptoms in postmenopausal women have been evaluated in a number of clinical trials, they are still not completely understood. Based on the results of the present studies, the concept of neurosteroids and to a certain extent the serotonin system can be discussed.

The major overall finding of these studies was that increased neurosteroid sensitivity was associated with mood deterioration. It can therefore be argued that increased neurosteroid sensitivity is an indicator for generally higher sensitivity to progestins/progesterone and that neurosteroid sensitivity could be a valuable method in the search for and development of more "mood-sparing" progestins.

Since several GABA$_A$ receptor agonists such as benzodiazepines, barbiturates, alcohol and allopregnanolone have been shown to exert biphasic effects on mood and behavior, this phenomenon must be addressed. At high concentrations, these agonists are able to induce a general enhancement of many GABA$_A$ receptors in several regions of the CNS, thus leading to a general overall effect. At high concentrations pregnanolone and allopregnanolone are sedative, hypnotic and anesthetic, both in animals and in humans (Norberg 1987, Carl 1990, Sundström 1998a). At low serum concentrations, however, benzodiazepines (Wenzel 2002), barbiturates (Masia 2000), alcohol (Miczek 1997) and allopregnanolone (Beauchamp 2000) induce loss of impulse control, negative mood and aggression/irritability in certain individuals. It has been hypothetically argued that this seemingly contradictory effect (called disinhibition) is secondary to inhibition of other inhibitory neurons demanding higher concentrations of the GABA$_A$ agonist for a GABA enhancing effect, or are localized in a CNS region with lower accumulation of the GABA agonist and are not yet affected by the substance. It has also been speculated that the explanation lies in the subunit composition of the GABA$_A$ receptor, and that the GABA$_A$ agonist changes the subunit composition thereby affecting the sensitivity of the receptor. Through animal experiments it has been shown that the rise of allopregnanolone induces changes in expression of the α4 subunit of the GABA$_A$ receptor in the hippocampus concomitantly with the induction of anxiety and development of benzodiazepine insensitivity (Gulinello 2001). A biphasic effect on negative mood has also been noted following different dosages of MPA and progesterone in postmenopausal women. Postmenopausal women taking sequential HRT feel worse on 10 mg MPA than 20 mg MPA (Björn 2002), and worse on 400 mg/day of vaginal progesterone than 800 mg/day (Andreen 2003). Since the patients in the present study remained on one dose of progestin/progesterone during the whole period of the studies it is not possible to discuss our results in terms of dose-dependent biphasic effects.

Besides the biphasic effects discussed above, the concepts of tolerance and abstinence must be mentioned. Continuous and long exposure to pregnanolone and allopregnanolone induces changes in GABA$_A$ receptor subunit composition, downregulation of GABA$_A$ receptors and decreased GABA function (Yu 1995, Yu 1996). This could theoretically render women with regular menstrual cycles less sensitive to pregnanolone or allopregnanolone compared to postmenopausal women because of regular exposure of the receptors to progesterone (and its metabolites) during the luteal phase of the menstrual cycle. Other sources of GABA$_A$-active steroids are the adrenal glands during stress. Allopregnanolone and tetrahydrodesoxycorticosterone (THDOC) are known to be produced during stress (Purdy 1991, Barbaccia 1998 and 2001). Chronic stress would lead to continuous production of these stress-related steroids, creating a situation where the GABA$_A$ receptors downregulate and tolerance subsequently develops. This may be a reason why women with PMDD differed from controls and from the postmenopausal women in these studies. However, no tool was used to interpret the level of chronic stress, and the results cannot be discussed in this regard. Furthermore, there are not enough previous results to determine whether pregnanolone or allopregnanolone does in fact produce different effects in regularly cycling women compared to postmenopausal ones. Whether the increased sensitivity to benzodiazepines in older

women (McAuley 1996) is caused by changes in the GABA$_A$ receptor or if the explanation lies in pharmacokinetic factors is not clear. In an attempt to study suspected changing effects on the GABA$_A$ receptor complex by ovarian steroids in normal menstruating women de Wit and Rukstalis performed bensodiazepine challenges (triazolam) during different phases of the menstrual cycle. Baseline measures of arousal showed that higher levels of allopregnanolone during the luteal phase were associated with lower arousal scores, which is consistent with the theory that allopregnanolone acts on the GABA$_A$ receptor complex. A puzzling result was, however, that women with the highest levels of allopregnanolone reported increased in scores of arousal after administration of triazolam (de Wit 1997). The authors discussed a possible cross-tolerance to bensodiazepines in these patients since bensodiazepines and allopregnanolone are both GABA$_A$ receptor agonists. In PMDD women reduced benzodiazepine, alcohol and pregnanolone sensitivity have been found during the luteal phase of the menstrual cycle (Sundström 1997a and 1998a, Nyberg 2004). It can be speculated that women with PMDD, and not those without the disorder, develop tolerance to progesterone metabolites during the luteal phase. This tolerance leaves them with a heightened feeling of anxiety, especially by the end of the luteal phase when progesterone starts to decline and they start to suffer from abstinence. The development of tolerance and abstinence in women suffering from PMDD could theoretically be the result of a change in the subunit composition of the GABA$_A$ receptor in these women. Abstinence from progesterone metabolites and their effect on the GABAergic system has been studied in animal experiments. Withdrawal of allopregnanolone in rats induced anxiety concomitant with an increase in the α4 subunit of the GABA$_A$ receptor in the hippocampus and a subsequent decrease in sensitivity to benzodiazepines (Smith S. 1998a). Further studies showed that suppression of the GABA$_A$ receptor α4 subunit prevented these allopregnanolone withdrawal symptoms (Smith S. 1998b). No human studies have been conducted in the area of withdrawal of progesterone.

Since no signs of decreased sensitivity were found during the course of the present studies (indicating an absence of carry-over effects) it might be that the doses used, or the duration of the treatment were not sufficient to induce tolerance. However, it was clear from this work and also from other studies carried out by our group mentioned earlier (Björn 2000 and 2002, Andreen 2003) that the negative mood symptoms observed during the progestin/progesterone phase continued for a few days after the progestin/progesterone treatment had been terminated, i.e. during the first few days of the next cycle when the women only received estradiol. It is possible that this phenomenon is in fact due to tolerance development and subsequent abstinence. Another relevant question is whether a longer duration of hormonal therapy than that used here would have resulted in a different, or a more pronounced result. Bernardi and co-workers found that allopregnanolone concentrations continued to rise during different forms of HRT up to one year after commencing treatment (Bernardi 2003). Also, long-term, low-dose dehydroepiandrosterone oral supplementation showed similar results (Genazzani 2003). In an earlier study by our group it has been shown that the negative mood symptoms observed during the progestin phase were most pronounced during the first treatment cycle, and diminished thereafter in subsequent cycles (Björn 2000). One explanation could be that the continued treatment resulted in

higher allopregnanolone concentrations and that this rise had a positive effect on mood. Bearing in mind the discussion on tolerance above it is possible that a different HRT regimen and especially a longer time course would have produced different results in the present studies. However, the aim was to illuminate the cause of negative mood symptoms during progestin/progesterone addition to HRT, which is the reason for choosing the most common kind of HRT: sequential combined HRT treatment with a monthly addition of progestin/progesterone.

No definite conclusions can be drawn from the present experiments regarding the provocation of negative mood symptoms by progestin/progesterone addition during HRT in terms of serotonergic parameters. Hopefully, further PET studies with progestin/progesterone additions will shed light upon the issue. It does, however, appear that neurosteroids interact with the serotonergic system as the reduced pregnanolone sensitivity in PMDD patients was normalized during successful treatment with the SSRI citalopram (Sundström 1998b).

FUTURE INVESTIGATIONS

It is clear from the above that investigations of the issue of tolerance to, and abstinence from, progesterone metabolites as a possible explanation of the occurrence of negative mood symptoms in postmenopausal women on HRT would be interesting.   In accordance to this a study on neurosteroids sensitivity in postmenopausal women on HRT when the progesterone addition is withdrawn has been initiated.

In the future it would also be interesting to investigate postmenopausal depressed women before and during HRT in terms of serotonergic measurements. In the study on postmenopausal women three patients were excluded due to depression. We analysed their data separately, and although no reliable conclusions can be drawn from such a small sample, it was obvious that they responded differently to the study group. B_{max} for [^{3}H]LSD binding decreased significantly in this group compared to the study group during E_2-only treatment, as well as during E_2+progestin treatment. Decreased binding to the platelet 5-HT$_{2A}$ receptor has been demonstrated previously in depressed patients treated with SSRIs (Butler 1988, Biegon 1990). As estrogen has been shown to have a beneficiary effect on mood in postmenopausal women the relationship between postmenopausal depression, HRT and the serotonergic system deserves a closer look.

As mentioned above, findings by our group indicate reduced pregnanolone sensitivity in PMDD patients and subsequent normalization of the sensitivity during successful treatment with the SSRI citalopram (Sundström 1998b). Thus, an interesting topic for future investigation is whether the negative mood symptoms and the increased sensitivity to pregnanolone exhibited by women during the progestin/progesterone phase of HRT, are influenced by SSRIs. Also, the relationship between former PMDD and climacteric symptoms should be investigated further. Studies have shown that perimenopausal complaints and the severity of vasomotor symptoms seem to be

correlated to a history of premenstrual symptoms (Skarsgard 1996, Morse 1998). It would therefore be interesting to perform a study on prospectively diagnosed PMDD women postmenopausally in terms of neurosteroid sensitivity before and during HRT. The best way to clearly elucidate this, but difficult and very time-consuming, would be to investigate these PMDD women in terms of neurosteroid sensitivity while still reproductive and then follow them into their postmenopausal years.

GENERAL CONCLUSIONS

- In postmenopausal women treated with estrogen the addition of synthetic progestins increases negative mood symptoms and physical symptoms, whereas positive symptoms decrease.

- The addition of progestins or natural progesterone to estrogen treatment in postmenopausal women increases the sensitivity to pregnanolone.

- In terms of pregnanolone sensitivity and synthetic progestins there are no differences between the two progestins MPA and NETA.

- During estrogen-only treatment the two clinical trials yielded different results in terms of pregnanolone sensitivity. The first study showed that estrogen treatment did not increase pregnanolone sensitivity, whereas the second study showed that pregnanolone sensitivity was enhanced during estrogen treatment.

- Postmenopausal women expressing cyclicity in negative mood symptoms during HRT are more sensitive to pregnanolone than women without symptom cyclicity. Thus, it is evident that mood deterioration during HRT is associated with altered neurosteroid sensitivity.

- Platelet serotonin transporter and 5-HT$_{2A}$ receptor binding do not change during different treatment conditions in sequential HRT involving synthetic progestins. Thus, it was not possible to explain the negative mood changes of sequential HRT by these peripheral serotonergic parameters.

- In reproductive women, a significant variation in platelet serotonin transporter and 5-HT$_{2A}$ receptor binding is evident during the menstrual cycle.

- These findings may provide a link between the ovarian steroids, neurosteroids and the serotonergic neurotransmitter systems, which in turn, could explain some of the specific vulnerability that women show for the development of adverse mood effects during HRT, mood and anxiety disorders and for the deterioration of mood so frequently seen during the luteal phase.

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Appendix 1

Värmevallningar	Svullnadskänsla	Glad	Spänd/Orolig

34

1

Day 36
Day 35
Day 34
Day 33
Day 32
Day 31
Day 30
Day 29
Day 28
Day 27
Day 26
Day 25
Day 24
Day 23
Day 22
Day 21
Day 20
Day 19
Day 18
Day 17
Day 16
Day 15
Day 14
Day 13
Day 12
Day 11
Day 10
Day 9
Day 8
Day 7
Day 6
Day 5
Day 4
Day 3
Day 2
Day 1

Namn:_____

Födelsenummer:_____

Nummer:_____

Senaste mens

Start_____/_____

Start datum [   ]

34	1

PATIENTINFORMATION "Cyklicitets-diagnoser":

Till skattningsskala för daglig skattning av hormon- relaterade symptom.

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.

1. Skattningsskalan för de olika symptomen fyller du i **varje kväll** strax före sänggåendet. Det är inte meningen att Du skall göra en djupdykning i dig själv, utan Du fyller i skattningsformuläret på relativt kort tid och "så som du känner att det är". Börja med skattningarna i samband med att mensen startar och gör skattningarna i ett svep även om du skattar under flera månader.

2. Skattningsskalorna (3 per omgång) ligger i förslutna kuvert där dagens skala är synligt i ett smalt fönster på mitten av kuväret. Markering dag 1 syns då till vänster på det nedre bladet. 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. Skriv skattningsdagens datum i fönstret ovanför det smala fönstret. Skalan kan sedan dras fram så att nästa dags skattningar blir synliga i det smala fönstret. Kuvertet öppnas av din läkare vid återbesöket.

3. Skattningsskalan omfattar 4 negativa och 3 positiva **psykiska symptom**, 3 allmänna **fysiska symptom**, notering av ev. **blödningar** inklusive mens 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 symptom.** Du förväntas använda **hela skalan** under en menscykel. Om Du t.ex. skattar ett negativt symptom som nedstämdhet maximalt (dvs 8) betyder detta att du är så nedstämd som du brukar vara när du mår som sämst under en menscykel. Avsaknad av symptom som nedstämdhet (dvs 0) betyder att du inte alls känner dig nedstämd just då. Detsamma gäller för positiva symptom dvs att en 8:a på symptomet glad betyder att Du är så glad som du är när Du är som gladast under en menscykel och en 0:a betyder avsaknad av glädje.

Skattningsdagens datum

Namn:......................................

Födelsedatum:....................................

Datum för senaste menstruations start............./............/..........

Startdatum för skattningar.............................
      Årtal.............

"Cyklicitets-diagnoser" av Professor Torbjörm Bäckström
Skattningsskala nummer 1.

Värmevallning

Svullnad

Glad

Spänd och orolig

Day 1

Start datum

34   1

2/1

Appendix 2

Namn: _____ Pers.nr: _____ Datum: ___ / ___ 20____

Kön: man ☐   kvinna ☐

Detta formulär är till för att Din läkare bättre skall förstå Dina eventuella fysiska eller psykiska problem.
Läkaren kommer eventuellt att ställa flera frågor i samband med enskilda punkter.

VÄGLEDNING: Det är viktigt att Du fyller i JA *eller* NEJ på *samtliga* 28 frågor.

Har Du under den *senaste månaden ofta* haft...						Under *senaste månaden*		
	JA	NEJ		JA	NEJ		JA	NEJ
1 Magont	☐	☐	14 Trötthet eller bristande energi	☐	☐	24 Har Du haft skuldkänslor eller känt oro för Din alkoholkonsumtion?	☐	☐
2 Ryggont	☐	☐	15 Sömnproblem	☐	☐			
3 Smärta i armar, ben, leder (höfter, knän etc)	☐	☐	16 Tappat kontrollen över ätandet	☐	☐	25 Har Du under någon enskild dag druckit 5 eller fler glas öl, vin eller sprit?	☐	☐
4 Smärtor eller problem vid menstruation	☐	☐	17 Minskad lust att göra saker som Du vanligen tycker om	☐	☐	26 Har Du återkommande idéer, fantasier eller impulser som verkar dumma, konstiga, frånstötande eller hemska?	☐	☐
5 Smärtor eller problem vid samlag	☐	☐	18 Att Du har känt Dig illa till mods, deprimerad eller känt att framtiden ser hopplös ut	☐	☐			
6 Huvudvärk	☐	☐				27 Finns det saker som Du gör om och om igen, eller tankar Du måste tänka upprepade gånger för att känna Dig lugn?	☐	☐
7 Smärtor i bröstet eller tryck över bröstet	☐	☐	19 Nervositet, ångest eller oro	☐	☐			
8 Yrsel	☐	☐	20 Ängslan över många olika saker	☐	☐	28 Är Du rädd för att bli generad eller förödmjukad i sociala situationer, eller undviker Du sådana situationer?	☐	☐
9 Svimningar	☐	☐	**Under *senaste månaden***					
10 Hjärtklappning	☐	☐	21 Har Du haft en plötslig känsla av ångest eller panik?	☐	☐	I det stora hela skulle Du beskriva Din hälsa som:		
11 Andnöd	☐	☐				Perfekt ☐		
12 Förstoppning, lös avföring eller diarré	☐	☐	22 Har Du tänkt på att minska Din alkohol-konsumtion?	☐	☐	Utmärkt ☐		
						God ☐		
13 Illamående, gasbildning eller matsmältnings-problem	☐	☐	23 Har någon klagat på att Du dricker för mycket?	☐	☐	Mindre god ☐ Dålig ☐		

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