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Allopregnanolone effects in women

Clinical studies in relation to the menstrual cycle, premenstrual dysphoric disorder and oral contraceptive use

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”Morgon. Och *sakerna* förbi. Och HOTET som om det aldrig funnits. Hon var inte med barn och andra eftertankar behövdes inte.”

Ur Lifsens rot av Sara Lidman

Table of Contents

Table of Contents i

Abstract iii

Abbreviations v

Enkel sammanfattning på svenska vi

Original papers ix

Introduction 1

The menstrual cycle 1

Hormonal changes across the menstrual cycle 1

Brain plasticity across the menstrual cycle 2

Premenstrual symptoms and progesterone – a temporal relationship 3

Premenstrual symptoms in the clinic 3

Epidemiology of premenstrual symptoms/PMS/PMDD 3

The symptom diagnoses of PMDD and PMS 5

Comorbidity and risk factors in PMDD 6

Treatment options for PMDD 7

Trying to understand PMDD by *in vivo* and *in vitro* research 8

Etiological considerations in PMDD 8

Brain imaging in PMDD patients across the menstrual cycle 9

Connections between the GABA system and PMDD 10

Neurosteroids 12

In focus: allopregnanolone 13

Allopregnanolone effects 14

Isoallopregnanolone 15

The GABA transmitter system 16

The $GABA_A$ receptor 16

Tolerance development at the $GABA_A$ receptor – relevance for PMDD? 18

The GABA paradox – relevance for PMDD? 19

Measuring $GABA_A$ receptor sensitivity in humans 20

Saccadic eye velocity 20

Combined oral contraceptives 21

OCs and mood 22

OCs and neurosteroids 23

Allopregnanolone and the HPG axis 24

HPG axis 24

GnRH and gonadotropin secretion 25

The GABA transmitter system and gonadotropin secretion 26

Hypothalamic amenorrhea 26

Aims of the thesis **28**
Material and methods **29**
Subjects 29
Study Protocols 30
Preparation of study solutions 32
Dosage of allopregnanolone 32
Saccadic Eye Velocity 33
Visual analogue scale of sedation 35
Assays of allopregnanolone and isoallopregnanolone 35
Analyses of progesterone, estradiol, FSH and LH 37
Anxiety scales 37
Statistics 38
Results **40**
Demographics of the study groups 40
PMDD diagnosis 40
Progestogen components in the oral contraceptives 41
Timing of allopregnanolone challenge 41
Allopregnanolone concentrations at baseline 42
Pharmacokinetics of intravenous allopregnanolone 42
Serum concentrations of allopregnanolone after injections 43
Sedative effects of allopregnanolone in women 44
Allopregnanolone sensitivity over the menstrual cycle in healthy women 45
Allopregnanolone sensitivity in women on oral contraceptives 46
Allopregnanolone sensitivity in PMDD patients 46
Allopregnanolone sensitivity in PMDD patients compared to healthy subjects 47
Allopregnanolone and the HPG axis 47
Anxiety Scales 49
Side effects 49
Discussion **50**
Allopregnanolone sensitivity over the menstrual cycle 50
Allopregnanolone sensitivity in healthy women 50
Allopregnanolone sensitivity in PMDD patients 52
Allopregnanolone sensitivity in women on combined oral contraceptives 54
Adverse mood susceptibility 55
Allopregnanolone decreases gonadotropin levels 56
Methodological considerations 57
Concluding remarks and future perspectives 60
General Conclusions **62**
Acknowledgments **63**
References **65**
Appendix **1**

Abstract

Background: Premenstrual dysphoric disorder (PMDD) affects 3–8% of women in fertile ages. Combined oral contraceptives (OCs) are widely used and some users experience adverse mood effects. The cyclicity of PMDD symptoms coincides with increased endogenous levels of allopregnanolone after ovulation. Allopregnanolone enhances the effect of γ-aminobutyric acid (GABA) on the $GABA_A$ receptor, the principal inhibitory transmitter system in the brain. The sensitivity to other $GABA_A$ receptor agonists than allopregnanolone (i.e. benzodiazepines, alcohol and the 5β epimer to allopregnanolone, pregnanolone) has been reported to depend on menstrual cycle phase and/or PMDD diagnosis. Isoallopregnanolone, the 3β epimer to allopregnanolone, has previously been used to verify specific allopregnanolone $GABA_A$ receptor effects. Saccadic eye velocity (SEV) is a sensitive and objective measurement of $GABA_A$ receptor function.

Aims: To study the pharmacological effects, and any effect on gonadotropin release, of intravenous allopregnanolone in healthy women. A second aim was to explore whether allopregnanolone sensitivity differs over the menstrual cycle or during OC use in healthy women, and thirdly in PMDD patients.

Methods: Ten women were challenged with a cumulative dose of intravenous allopregnanolone in the follicular phase of the menstrual cycle. The effect on FSH and LH was compared to women exposed to isoallopregnanolone. A single dose of allopregnanolone was administered once in the follicular phase and once in the luteal phase in another ten healthy women and in ten PMDD patients, and additionally in ten women using OCs. Repeated measurements of SEV, subjectively rated sedation and serum concentrations after allopregnanolone injections were performed in all studies.

Results: Allopregnanolone dose-dependently reduced SEV and increased subjectively rated sedation. Healthy women had a decreased SEV response in the luteal phase compared to the follicular phase. By contrast, PMDD patients had a decreased SEV response and subjectively rated sedation response to allopregnanolone in the follicular phase compared to the luteal phase. There was no difference in the SEV response to allopregnanolone between women using oral contraceptives and healthy naturally cycling women. Allopregnanolone decreased serum levels of FSH and LH whereas isoallopregnanolone did not affect FSH and LH levels.

Conclusion: Intravenous allopregnanolone was safely given and produced a sedative response in terms of SEV and subjectively rated sedation in women. The sensitivity to allopregnanolone was associated with menstrual cycle phase, but in the opposite direction in healthy women compared to PMDD patients. The results suggest mechanisms of physiological tolerance to allopregnanolone across the menstrual cycle in healthy women and support that PMDD patients have a disturbed $GABA_A$ receptor function. In addition, one of our studies suggests that allopregnanolone might be involved in the mechanism behind hypothalamic amenorrhea.

Abbreviations

ACOG	American College of Obstetricians and Gynecologists
ACTH	adrenocorticotropic hormone
ALLO	allopregnanolone
APA	American Psychiatric Association
ASI	Anxiety Sensitivity Index
CRF	corticotropin-releasing factor
CSGAAS5	Cardiff Saccade Generation and Analysis System
DSM IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
EOG	electrooculography
fMRI	functional magnetic resonance imaging
FSH	follicle-stimulating hormone
GABA	γ aminobutyric acid
$GABA_A$ receptor	γ aminobutyric acid receptor type A
GnRH	gonadotropin-releasing hormone
HPA axis	hypothalamic-pituitary-adrenal axis
HPG axis	hypothalamic-pituitary-gonad axis
ISOALLO	isoallopregananolone
LH	luteinizing hormone
OCs	combined oral contraceptives
PMDD	premenstrual dysphoric disorder
PMDS	premenstruellt dysforiskt syndrom
PMS	premenstrual syndrome
PSS	Panic Symptoms scale
SADS	State Anxiety and Discomfort Scale
SEV	saccadic eye velocity
STAI	State-Trait Anxiety Inventory
TSH	thyroid stimulating hormone
VAS	visual analogue scale

Enkel sammanfattning på svenska

3–8% av kvinnor i barnafödande ålder uppfyller kriterierna för PMDD som på svenska heter premenstruellt dysforiskt syndrom (PMDS). Vid PMDS återkommer uttalade symptom som nedstämdhet, oro, humörsvängningar och irritation inför varje mens. Ytterligare symptom är sömnstörningar, orkeslöshet, koncentrationssvårigheter, en känsla av att vara okontrollerad, nedsatt intresse av dagliga aktiviteter, ändrad aptit och fysiska symptom som bröstömhet eller svullnad. För att få diagnosen PMDS måste symptomen påverka relationerna inom familj- eller arbetsliv, och symptomen ska försvinna inom några dagar från menstruationsstart. Orsaken till PMDS är oklar men det är tydligt att det krävs ägglossning med produktion av gulkroppshormon för att symptomen ska uppträda. En del kvinnor upplever humörbiverkningar av syntetiskt gulkroppshormon som används i p-piller och vid klimakteriebehandling. Verkningsmekanismerna bakom humörbiverkningar av p-piller vet vi nästan ingenting om. När det gäller PMDS vet vi att det inte är gulkroppshormonet i sig som orsakar humörsymptomen, men att det är något från gulkroppen, som bildas efter ägglossning, som utlöser symptombilden.

I den här avhandlingen har vi undersökt en nedbrytningsprodukt från gulkroppshormon, allopregnanolon, som bildas naturligt i kroppen efter ägglossning och under graviditet. Allopregnanolon bildas också i hjärnan och i binjurarna samt ökar vid stress hos både män och kvinnor. Allopregnanolon verkar via en receptor i hjärnan, $GABA_A$ receptorn, som hämmar aktiviteten i hjärncellerna. Hos djur vet man att allopregnanolon är ångestdämpande, sövande och kramplösande. Allopregnanolon påverkar $GABA_A$-receptorn i de låga koncentrationer som förekommer naturligt över menstruationscykeln. Den fysiologiska rollen för allopregnanolon hos människa är oklar men sannolikt har den stor betydelse för hjärnans utveckling under fosterstadiet. GABA-systemet är det största hämmande signalsystemet i hjärnan. Det finns djurmodeller av PMDS som talar för ett förändrat GABA-system. Studier av kvinnor har visat på en ändrad känslighet för lugnande mediciner (benzodiazepiner) och alkohol, vilka verkar via $GABA_A$-receptorn, vid PMDS. Kvinnor med PMDS har vidare visat sig ha en minskad känslighet för pregnanolon, en spegelsubstans till allopregnanolon som enbart skiljer sig i en enstaka molekylvinkel. Pregnanolon bildas också naturligt efter ägglossning och har samma effekter som allopregnanolon i djurförsök. Den minskade känsligheten för benzodiazepiner, alkohol och pregnanolon pekar mot att kvinnor med PMDS har en ändrad $GABA_A$-receptorfunktion. Känsligheten för just allopregnanolon har tidigare inte undersökts hos kvinnor. Med anledning av

fynden för andra $GABA_A$-receptor stimulerande substanser hos kvinnor med PMDS och i djurmodeller, så ville vi ge allopregnanolon till kvinnor och undersöka dess farmakologiska effekter. Ett sätt att mäta $GABA_A$-receptor-funktion är att mäta ögonsackadhastighet (SEV). SEV mäts genom att ögats rörelse efter en ljuspunkt som flyttar sig registreras på ett enkelt sätt via två elektroder i tinningen.

I studie I gav vi allopregnanoloninjektioner till 10 friska kvinnor i första delen av menstruationscykeln, då de kroppsegna nivåerna är mycket låga. Vi kunde mäta att SEV minskade och att kvinnorna kände sig tröttare ju högre nivån av allopregnanolon blev efter injektionerna. Inga allvarliga biverkningar noterades. Vi kunde också bestämma olika farmakologiska egenskaper som halveringstid. Utifrån den första studien kunde vi planera de följande studierna.

I studie II gavs en dos allopregnanolon till 10 friska kvinnor vid två tillfällen under menstruationscykeln, en gång efter och en gång innan mens. Samma dos gavs också vid ett tillfälle till 10 friska kvinnor som åt p-piller. Som tidigare sågs en minskning av SEV och en ökning av trötthetskänslan. Känsligheten för allopregnanolon hos kvinnor som åt p-piller skiljde sig inte tydligt från kvinnor utan p-piller. Hos kvinnor utan p-piller var minskningen av SEV mindre uttalad när allopregnanolon gavs innan mens jämfört med efter mens. Detta kan tala för att hjärnan anpassar sig till de kroppsegna allopregnanolonnivåerna efter ägglossning och blir mindre känslig för dess effekter.

I studie III gavs allopregnanolon till 10 kvinnor med PMDS vid två tillfällen under menstruationscykeln, en gång efter och en gång innan mens. Effekten på SEV och trötthetskänsla jämfördes mellan de två tillfällena och med de 10 friska kvinnorna som inte åt p-piller i studie II. Kvinnorna med PMDS var mindre känsliga för allopregnanolon när det gavs i början av menstruationscykeln, innan ägglossning, jämfört med när det gavs innan mens. Förändringen i SEV-känslighet för allopregnanolon över menstruationscykeln gick åt olika håll inom gruppen av PMDS-patienter jämfört med gruppen av friska kvinnor. Detta tyder på att GABA-systemet fungerar annorlunda hos PMDS patienter.

I studie IV undersöktes effekten av allopregnanolon på den hormonkedja som styr utvecklingen av äggblåsor mot ägglossning. Allopregnanolon sänkte nivåerna av hypofyshormonerna FSH och LH. Effekten av allopregnanolon jämfördes med effekten av en annan substans, isoallopregnanolon, som gavs till fem andra kvinnor i samma fas av menstruationscykeln. Isoallo-pregnanolon används i olika studier för att styrka en specifik effekt av allopregnanolon på $GABA_A$-receptorn. Isoallopregnanolon påverkade inte nivåerna av FSH och LH. Detta kan tyda på att allopregnanolon, som ökar

vid stress, kan ha betydelse för regleringen av ägglossningsfunktionen hos kvinnor.

Sammanfattningsvis så har vi gett den kroppsegna substansen allopregnanolon till kvinnor i doser som ökat allopregnanolon nivåerna i blodet till nivåer som normalt ses under sen graviditet. Vi har påvisat en sövande effekt i form av minskad SEV och ökad trötthetskänsla. Därutöver visar studierna att känsligheten för allopregnanolon ändras över menstruationscykeln, och riktningen på denna förändring skiljer sig mellan friska kvinnor och kvinnor med PMDS. I förlängningen kan ökad kunskap om de bakomliggande mekanismerna vid PMDS leda till utveckling av nya behandlingsmöjligheter.

Original papers

I. Timby E, Balgård M, Nyberg S, Spigset O, Andersson A, Porankiewicz-Asplund J, Purdy RH, Zhu D, Bäckström T, Sundström Poromaa I. **Pharmacokinetic and behavioral effects of allopregnanolone in healthy women.** Psychopharmacology (Berl) 2006 Jun; 186(3): 414-424

II. Timby E, Bäckström T, Nyberg S, Stenlund H, Wihlbäck AC, Bixo M. **Allopregnanolone sensitivity over the menstrual cycle and during oral contraceptives.** Manuscript

III. Timby E, Bäckström T, Nyberg S, Stenlund H, Wihlbäck AC, Bixo M. **Women with premenstrual dysphoric disorder have altered sensitivity to allopregnanolone over the menstrual cycle compared to controls.** Manuscript

IV. Timby E*, Hedström H*, Bäckström T, Sundström-Poromaa I, Nyberg, S, Bixo M. **Allopregnanolone, a $GABA_A$ receptor agonist, decreases gonadotropin levels in women. A preliminary study.** Gynecological Endocrinology 2011 Dec; 27(12): 1087-1093

**These authors have contributed equally in this work*

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Introduction

Women's lives are accompanied by fluctuations in sex steroid hormones from puberty to the end of the menopausal transition. There are physiological changes in sex steroid levels over the menstrual cycle, during pregnancy, puerperium, and breastfeeding. In addition to this, there are pharmacological variations for many women during hormonal contraception and postmenopausal hormonal replacement therapies. Women are at higher risk of affective disorders than men, and the impact of hormones could be a contributing factor. Many women experience cyclical negative mood over the menstrual cycle, and 3–8% of women suffer from premenstrual dysphoric disorder (PMDD). Negative mood symptoms are side effects of hormonal contraceptives and postmenopausal hormonal substitution. Apparently there is a need to explore the biological mechanisms of some women's susceptibility to negative mood associated with the variations of sex steroids and their metabolites. This thesis focuses on the $GABA_A$ receptor and the progesterone metabolite allopregnanolone, a potent $GABA_A$ receptor agonist. The $GABA_A$ receptor is part of the γ-aminobutyric acid (GABA) neurotransmitter system which is the main inhibitory transmitter system in the brain. The original studies are a set of small explorative studies on women in different states of sex steroid conditions including combined oral contraceptives (OCs) and PMDD.

The menstrual cycle

Hormonal changes across the menstrual cycle

The menstrual cycle is characterized by monthly fluctuations in the female sex steroid hormones, estradiol and progesterone, as illustrated in figure 1. The follicular phase (days 1–14) shows initially low levels of estradiol, later increasing, and positive feedback mechanisms on the pituitary secretion of luteinizing hormone (LH), elicits the LH surge that precedes ovulation. After ovulation, a corpus luteum is formed, producing progesterone in addition to estradiol. The presence of progesterone characterizes the luteal phase (day 15–28) that follows ovulation. If conception occurs, human choriogonadotropin (hCG) produced by the trophoblastic cells in the embryo maintains the steroid production in the corpus luteum, a function which is gradually replaced by the placenta. If conception does not occur, the corpus luteum regresses and the sex steroid levels rapidly decline, after which the menstrual bleeding occurs. The pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus is not included in figure 1 but regulates the release of follicle-stimulating hormone (FSH) and LH from the

pituitary. Hence, the endocrine control of reproduction is dependent on a functioning hypothalamus-pituitary-gonad (HPG) axis.

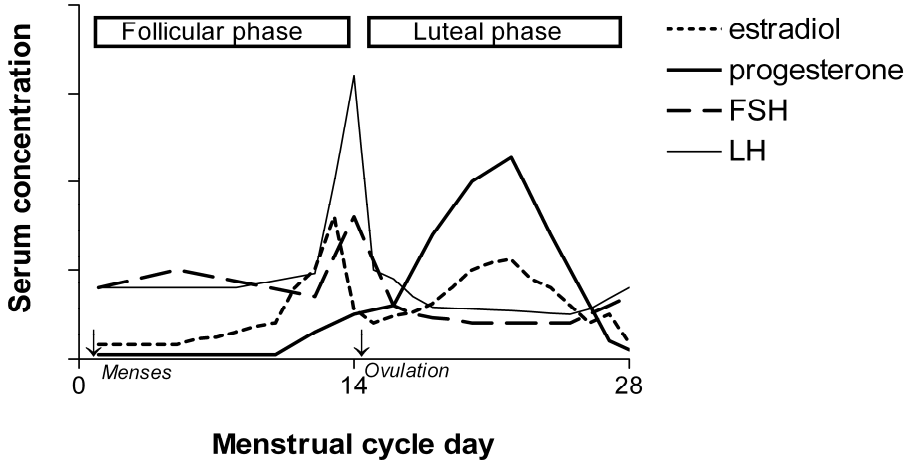

Figure 1. Schematic illustration of the principal hormonal fluctuations during the normal 28-day menstrual cycle. The sex steroids estradiol and progesterone are produced by the ovaries which in turn are controlled by the pituitary peptide hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The menstrual cycle is divided into the preovulatory follicular phase and the postovulatory luteal phase (Fritz and Speroff, 2011b).

Brain plasticity across the menstrual cycle

The hormonal fluctuations of the menstrual cycle are reflected in the brain. The emerging field of brain imaging techniques has demonstrated some examples of plasticity in brain functions across the menstrual cycle in healthy women without detecting differences in task performance (Fernandez *et al.*, 2003; Protopopescu *et al.*, 2005). Thus, there is a physiological adaptation in the brain to the variations of sex steroids. One functional magnetic resonance tomography (fMRI) study showed that neural recruitment was associated with menstrual cycle phase (menstruation vs. mid luteal) in a semantic decision language task (cognitive) in contrast to a letter-matching perceptual task (perceptual) (Fernandez *et al.*, 2003). Another fMRI study reported that orbitofrontal cortex activity in response to an emotional linguistic task (designed to provoke negative emotion), changed across the menstrual cycle (mid follicular vs late luteal) in subjects where premenstrual symptoms were ruled out by daily assessments (Protopopescu *et al.*, 2005). An enhanced reward-related neural response in the ventral striatum was found in the premenstrual phase compared to the follicular phase in 28 healthy women on fMRI scans (Ossewaarde *et al.*, 2010b). Exposure to exogenous sex steroids also influenced brain activity on

fMRI scans; a single intravenous injection of progesterone selectively increased the response to salient stimuli in the amygdala, a brain region important in emotional processing (van Wingen *et al.*, 2008).

Premenstrual symptoms and progesterone – a temporal relationship

Another association between the menstrual cycle and the brain, more established over the years, is the temporal relationship between the luteal phase of the menstrual cycle and premenstrual adverse mood in some women (Backstrom *et al.*, 1983; Hammarback *et al.*, 1991; Halbreich, 2003). Daily assessed ratings of premenstrual complaints mirror the appearance of the progesterone serum concentration curve in women with premenstrual dysphoric disorder (PMDD), see figure 2.

Premenstrual symptoms in the clinic

Epidemiology of premenstrual symptoms/PMS/PMDD

A majority of women report cyclical changes in at least one physical or psychological symptom across the menstrual cycle (Sveindottir and Backstrom, 2000; Wittchen *et al.*, 2002; Duenas *et al.*, 2011) but in most cases there is no interference with daily life. There are similarities in the reports of premenstrual symptoms worldwide (Dennerstein *et al.*, 2011). The temporal association with circulating ovarian steroids produced by the corpus luteum after ovulation and premenstrual symptoms is obvious (Backstrom *et al.*, 1983). In 3–8% of fertile women there is a cluster of cyclically recurrent severe and debilitating mood symptoms conforming to the diagnostic criteria of premenstrual dysphoric disorder, PMDD (Sveindottir and Backstrom, 2000; Wittchen *et al.*, 2002; Halbreich *et al.*, 2003). PMDD was defined in the fourth edition of the Statistic Manual of Mental Disorders (DSM IV), see the subheading below (APA, 1994). The pathophysiology behind PMDD is not fully known, and over the years there have been many speculations about its etiology, for a review see Halbreich (2003) and Cunningham *et al.* (2009), and later in this thesis. Many women seeking help for premenstrual complaints do not fulfill the diagnostic criteria of PMDD. There is a confusing terminology regarding the labeling of premenstrual symptoms. Premenstrual syndrome (PMS) has been the layman's term, but was also defined by the American College of Obstetricians and Gynecologists in 2000 (ACOG, 2000). According to different methodologies in assessing PMS and PMDD, the estimated prevalence of moderate or severe PMS varies from 8 to 32% across studies (Halbreich *et al.*, 2007). The DSM IV diagnostic criteria of PMDD have enabled clinical research towards conformity (O'Brien *et al.*, 2011). However, some

researchers fear that therapy could be withheld in individual cases who present severe, although not enough qualifying symptoms (O'Brien *et al.*, 2011). Some epidemiological studies presented "subthreshold PMDD" with a prevalence of 18.6% (Wittchen *et al.*, 2002), and this condition was the most dominant risk factor for developing PMDD over a 42-month follow-up with an odds ratio of 11.0 (95% CI 4.7–25.9) (Perkonigg *et al.*, 2004). Yet, whether PMDD is a clinical entity independent of PMS or not is still to be resolved (Halbreich *et al.*, 2007). This thesis focuses on prospectively rated PMDD.

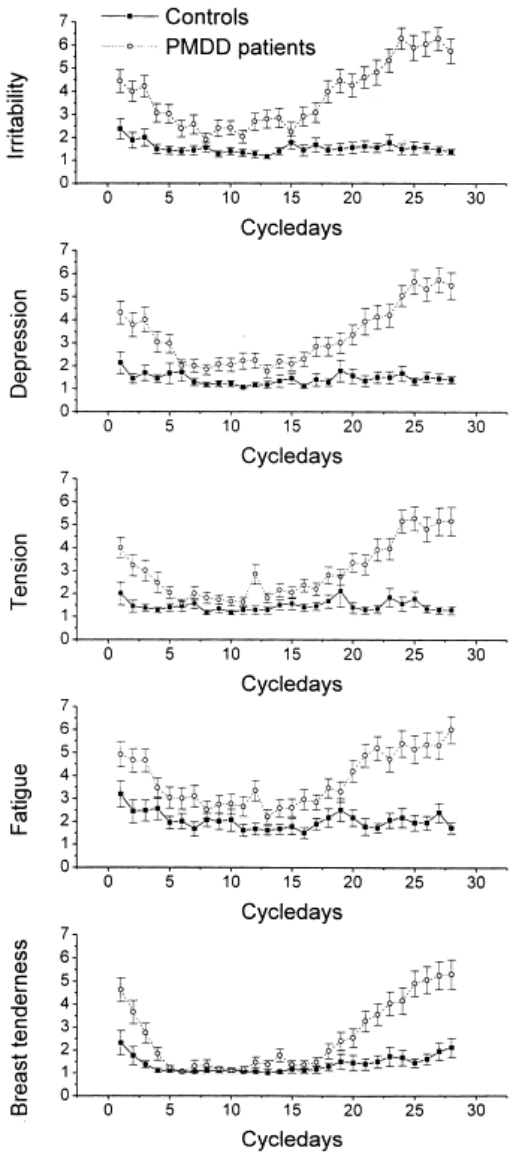

Figure 2. Cyclical emotional and physical symptoms related to the luteal phase of the menstrual cycle are displayed in PMDD patients (n=18). PMDD symptoms disappear within some days after the onset of menses. Control women (n=20) display no symptom cyclicity across the menstrual cycle. From Bixo *et al.* (2001). Reprinted with permission from Elsevier.

The symptom diagnoses of PMDD and PMS

PMDD is associated with a significant morbidity, and the burden of PMDD has been estimated to nearly equal the burden of major depression (Halbreich *et al.*, 2003). According to the DSM IV criteria for PMDD, five (of which at least one has to be among the first four affective symptoms) of eleven specified symptoms must be present during the week preceding menses and remit within a few days after the onset of menses. In addition, premenstrual symptoms must significantly interfere with social relationships or working performance and the symptoms should not be the cyclical worsening of an underlying affective disorder. The PMDD diagnosis requires daily prospective symptom ratings over two consecutive menstrual cycles (APA, 1994).

Table 1. DSM IV diagnostic criteria for PMDD.

A. Symptoms must be present for most of the time during the last week of the luteal phase, begin to remit within a few days after the onset of menses and be absent during the week after menses. Five (or more) of the listed symptoms must be present with at least one of the symptoms being (1), (2), (3) and (4):
   1. Markedly depressed mood, feelings of hopelessness, or self-deprecating thoughts
   2. Marked anxiety, tension, feelings of being "keyed up" or "on the edge"
   3. Marked affective lability (e.g., feeling suddenly sad or tearful or increased sensitivity to rejection)
   4. Persistent and marked anger or irritability or increased interpersonal conflicts
   5. Decreased interest in usual activities
   6. Concentration difficulties
   7. Marked lack of energy
   8. Marked change in appetite, overeating or food cravings
   9. Hypersomnia or insomnia
   10. Feeling out of control or overwhelmed
   11. Physical symptoms (e.g. breast tenderness, bloating)

B. Symptoms must interfere with work, school, usual activities or relationships

C. Symptoms are not merely an exacerbation of another disorder.

D. Criteria A, B and C must be confirmed by prospective daily ratings during at least two consecutive symptomatic cycles.

The ACOG definition of PMS is broader, requiring at least one symptom from a list of emotional and physical symptoms which must be present during the five days before menses and remit within four days of the onset of menstrual bleeding. Symptom(s) should not reoccur until day 13 of the menstrual cycle. The pattern must have been recognized in each of three

prior menstrual cycles and dysfunction in social or economic performance must be identified. In addition, PMS diagnosis requires prospective confirmation from two menstrual cycles (ACOG, 2000).

Comorbidity and risk factors in PMDD

Obviously, the requirement of daily prospective ratings to establish the PMDD diagnosis with the exclusion of other psychiatric disorders complicates the research area of comorbidity in this condition. However, when premenstrual symptoms were prospectively assessed, a 12–25% prevalence of co-morbid non-seasonal depressive disorder and PMDD was suggested (Hammarbäck *et al.*, 1989; Backstrom and Hammarback, 1991; Kim *et al.*, 2004). Electroencephalographic prefrontal brain asymmetry is a risk marker for major depression, a phenomenon which has also been described in PMDD patients, suggesting an overlap in risk profiles (Accortt *et al.*, 2011). In a study of seasonal affective disorder (SAD) performed during the summer remission period, with prospective ratings of premenstrual symptoms, the prevalence of PMDD was 46% among women with SAD compared to 2% in a healthy control group (Praschak-Rieder *et al.*, 2001). As with SAD patients, an increased sensitivity to bright light melatonin suppression in PMDD was reported (Parry *et al.*, 2010). In a summary of prospectively assessed PMDD data and the anxiety disorders spectrum, co-occurrence appeared, with panic disorder in 25%, social phobia in 19–23% and generalized anxiety disorder in 4–38% of study samples (Kim *et al.*, 2004). Several studies have reported common phenotypes in the panic response to various panic-inducing substances (e.g. CO_2 inhalation, sodium lactate infusion, cholecystokinin-tetrapeptide) among PMDD patients and patients with panic disorder: for a review see Vickers and McNally (2004). Also, sensitivity to stress has been more frequently reported by subjects with premenstrual symptoms, whereas a higher educational level seems to decrease the risk of reporting PMDD (Sadler *et al.*, 2010; Skrzypulec-Plinta *et al.*, 2010). Furthermore, an association between posttraumatic stress disorder (PTSD) and PMDD has been described in a 42-month longitudinal study, with an odds ratio (OR) of 4.2 (95% CI 1.2–12.0) for developing PMDD after traumatic events (Perkonigg *et al.*, 2004). A cross-sectional, secondary data analysis of 3968 female participants in a psychiatric epidemiology survey reported increased odds ratios for women with a history of trauma to report PMDD, both when the trauma was associated with PTSD (OR 8.14, 95% CI 3.56–18.58) or not associated with PTSD (OR 2.84, 95% CI 1.26–6.42) (Pilver *et al.*, 2011). A smaller cross-sectional study of 58 prospectively diagnosed PMDD patients found no significant increase in the experiences of physical, emotional, or sexual abuse (31%) compared to asymptomatic controls (21.3%) (Segebladh *et al.*, 2011). Notably, in the same

study, a third study group of care-seeking outpatients at a gynecological and obstetric department reported significantly increased experience of lifetime abuse (39.2%) (Segebladh *et al.*, 2011). However, the authors conclude that in the individual case, experience of abuse may be related to the development of PMDD. The findings of PMDD comorbidity is of clinical relevance to ensure optimal care and treatment of patients.

Treatment options for PMDD

The search for comorbidity associations are partly based on the common treatment options in major depression, anxiety disorders and PMDD. Currently, selective serotonin reuptake inhibitors (SSRI) are prescribed for PMDD, as evidenced by a Cochrane review (Brown *et al.*, 2009). In contrast to the delayed response to SSRI treatment in major depression, there is a rapid response to SSRI treatment in PMDD patients, and treatment can be limited to the luteal phase of the menstrual cycle (Eriksson *et al.*, 2008; Landen *et al.*, 2009). The anti-depressant venlafaxine, a selective inhibitor of norepinephrine and serotonin reuptake (SNRI), is less well studied, but decreased PMDD symptoms have been shown in a small randomized controlled trial (Freeman *et al.*, 2001). The effect of venlafaxine was replicated in intermittent treatment restricted to the luteal phase (Cohen *et al.*, 2004).

The obvious relation to ovarian cyclicity is the other current basis for PMDD treatment, namely to inhibit ovulation. Combined oral contraceptives (OCs) are the most frequently used treatment to induce anovulation, but the usefulness of OCs in PMDD patients may be limited by adverse mood events related to the progestogen compound (Cullberg, 1972; Bjorn *et al.*, 1999). However, when premenstrual symptoms were prospectively assessed in prior OC users, the prevalence of PMDD did not differ between prior OC users with adverse mood experience when using OCs, and prior OC users without such experience (Segebladh *et al.*, 2009b). OCs containing the spironolactone derivative progestogen drosperinone have been proven to relieve PMDD symptoms significantly (Lopez *et al.*, 2009), and seem beneficial when administered in a newly developed 24/4 regimen (Marr *et al.*, 2011a; Marr *et al.*, 2011b). Treatment with GnRH analogues to induce anovulation and subsequent symptom relief can also be successful, although not used as first-line therapy (Sundstrom *et al.*, 1999; Segebladh *et al.*, 2009a). In extremely severe cases, oophorectomy may be considered (Casson *et al.*, 1990). The two latter options require “add back” therapies with estradiol (counterbalanced with a progestogen to prevent endometrial hyperplasia) to prevent climacteric symptoms and the hypoestrogenic effects on bone mineralization.

The mineralocorticoid diuretic agent spironolactone has been used predominantly for physical symptoms, such as water retention, but has also been reported to improve psychological symptoms in a small double-blind placebo-controlled cross-over study (Wang *et al.*, 1995). Furthermore, there has been a variety of treatments for PMS over the years where the claimed effects on symptoms are supported by little or no evidence. Progesterone treatment used to be widely prescribed, at least in the UK, but the meta-analysis of a systematic review found no support for progesterone treatment in PMDD (Wyatt *et al.*, 2001). Additionally, a systematic review of 62 herbs, vitamins, and minerals claiming amelioration of PMS symptoms only found evidence from randomized control trials for 10, and there was only enough evidence quality for the use of calcium in PMS (Whelan *et al.*, 2009).

Trying to understand PMDD by *in vivo* and *in vitro* research

Etiological considerations in PMDD

The variety of treatment options for PMDD over time is a reflection of the past and present pathophysiological hypotheses of PMDD. Initially, much attention was focused on possible alterations in sex steroid blood levels in PMDD patients, but results yielded no consistency. Progesterone was not found to be the eliciting factor as the progesterone receptor antagonist, mifepristone, did not remove the cyclical symptoms (Schmidt *et al.*, 1991; Chan *et al.*, 1994). Nevertheless, PMDD symptoms are dependent on the formation of a corpus luteum after ovulation. Cyclical mood changes are not displayed during spontaneously anovulatory cycles, neither during GnRH analogue induced anovulatory cycles (Hammarback and Backstrom, 1988; Hammarback *et al.*, 1991; Schmidt *et al.*, 1998), nor after bilateral oophorectomy (Casson *et al.*, 1990). In the absence of evidence for progesterone effects by its classical intracellular receptor, an interest in central effects of progesterone metabolites emerged. Progesterone can be metabolized into allopregnanolone (3α-hydroxy-5α-pregnane-20-one or 3α5α-tetrahydroxyprogesterone, THP) (Biggio and Purdy, 2001), which is an allosteric modulator of the membrane-bound $GABA_A$ receptor and facilitates the effect of γ-aminobutyric acid (GABA) (Majewska *et al.*, 1986).

Other neurotransmitter systems proposed to underlie the characteristics of PMDD are mainly the serotonergic system, but also the glutamate and beta-endorphin systems (Cunningham *et al.*, 2009). The efficacy of SSRI treatment is a major argument for a disturbed serotenergic function in PMDD patients. Additional arguments are the findings that serotonin depletion through tryptophan-free diet, or by treatment with a serotonin-receptor antagonist aggravate premenstrual symptoms (Yonkers *et al.*, 2008). Indeed, various studies report aberrant serotonergic function in

women with PMDD (Bixo *et al.*, 2001; Melke *et al.*, 2003; Wihlback *et al.*, 2004; Eriksson *et al.*, 2006; Jovanovic *et al.*, 2006; Gingnell *et al.*, 2010). Unfortunately, causes and effects are not easily distinguished in PMDD findings. In fact, there are links between sex steroids and the serotonergic transmitter system that may include both the serotonergic and GABAergic hypotheses. For example, allopregnanolone has been shown to inhibit serotonergic neurons in the dorsal raphe nuclei in female rats (Kaura *et al.*, 2007). In addition, the difference in onset of the effect of SSRI treatment between major depression and PMDD is striking. This divergence in treatment response may be attributed to preclinical findings of direct effects by SSRIs on the enzymes involved in the metabolism of progesterone to allopregnanolone (Griffin and Mellon, 1999; Pinna *et al.*, 2006), and not the serotonergic effect per se. Moreover, Sundstrom and Backstrom reported that citalopram treatment of PMDD patients during the luteal phase increased the functional $GABA_A$ receptor sensitivity to pregnanolone, the 5β epimer to allopregnanolone, measured by saccadic eye velocity (SEV) (1998a). The focus in this thesis is on allopregnanolone and the $GABA_A$ receptor, and other neurotransmitter systems will not be further discussed in the context of PMDD.

Brain imaging in PMDD patients across the menstrual cycle

There are growing knowledge from functional brain imaging studies on how sex steroids regulate the emotion neurocircuitry women, for a review see van Wingen *et al.* (2011). Expanding the findings of menstrual cycle phase associated brain plasticity in healthy women (Fernandez *et al.*, 2003; Protopopescu *et al.*, 2005; Ossewaarde *et al.*, 2010a; Ossewaarde *et al.*, 2010b) into subjects with PMDD has illustrated parts of the neurobiology of the disorder (Protopopescu *et al.*, 2008; Rapkin *et al.*, 2011). However, the complexity of the human brain is evident and interpretations are difficult. In contrast to the enhanced fMRI response to an emotional linguistic task in the orbitofrontal cortex during the premenstrual phase in asymptomatic women, PMDD patients showed the opposite pattern across the menstrual cycle (Protopopescu *et al.*, 2008). Positron emission tomography (PET) measuring glucose metabolism showed an increased cerebellar activity from the follicular to the luteal phase among women with PMDD but not in control women (Rapkin *et al.*, 2011). The authors discuss this finding in relation to earlier findings of increased cerebellar activity in unipolar and bipolar depressed patients as well as in relation to the large presentation of $GABA_A$ receptors in the cerebellum (Rapkin *et al.*, 2011).

Connections between the GABA system and PMDD

The basis for the GABA system to be involved in PMDD pathophysiology is the presence of potent progesterone metabolites active on the $GABA_A$ receptor during the symptomatic luteal phase of the menstrual cycle together with the indications of a GABAergic dysfunction in PMDD patients (Sundstrom *et al.*, 1997a; Sundstrom *et al.*, 1997b; Sundstrom *et al.*, 1998; Epperson *et al.*, 2002; Smith *et al.*, 2003; Nyberg *et al.*, 2004; Epperson *et al.*, 2007; Kask *et al.*, 2008b). However, direct measures of GABAergic activity in humans are for evident reasons not easily performed, and much work has been done in animal models (Smith *et al.*, 1998b; Gulinello *et al.*, 2001; Lovick *et al.*, 2005; Maguire *et al.*, 2005; Mostallino *et al.*, 2006; Smith *et al.*, 2006; Maguire and Mody, 2007). Nevertheless, there are some correlates of $GABA_A$ receptor activation measurable in humans, and studies suggest dysregulation of the GABAergic function in PMDD patients. The motor evoked electropotential following transcranial magnetic stimulation reflects the integration of synaptic inputs by cortical and spinal motorneurons, and the relative degree of inhibition and facilitation represents a model for GABAergic activity. Healthy women had more facilitation and less inhibition in the follicular phase, possibly related to the presence of unopposed estrogen (Smith *et al.*, 1999; Smith *et al.*, 2002). It is well established that estradiol increases excitability through its facilitating influence on glutamate, the main excitatory neurotransmitter (Finocchi and Ferrari, 2011). Following transcranial magnetic stimulation during the luteal phase, the balance has been shown to shift towards inhibition, interpreted as a reflection of the present progesterone metabolites in this phase of the menstrual cycle (Smith *et al.*, 1999; Smith *et al.*, 2002). By contrast, PMDD patients displayed an opposite effect, with less inhibition and more facilitation in the luteal phase, suggesting an abnormal brain response to endogenous progesterone metabolites (Smith *et al.*, 2003). Quantitative measurements of GABA levels in the occipital cortex by proton magnetic resonance spectroscopy (^{1}H-MRS) showed a decrease in GABA levels from the follicular to the luteal phase among healthy women, while the change in PMDD patients was in the reverse direction (Epperson *et al.*, 2002). The acoustic startle response is a measurement of arousal and is recorded as the eyeblink following a noxious (auditory) stimulus. The startle response is increased in patients with anxiety disorders as well as in animal models of these disorders (Morgan *et al.*, 1996; Schwegler *et al.*, 1997). Progesterone, presumably by its $GABA_A$ active metabolite allopregnanolone, has been demonstrated to decrease the CRF-enhanced startle response (Toufexis *et al.*, 2004). Hence, the startle response is thought to be regulated by sex steroids. In PMDD patients, the acoustic startle response was increased compared to control women during the luteal phase of the menstrual cycle

(Epperson *et al.*, 2007). In a study with twice as many PMDD patients this finding was replicated and present in both phases of the menstrual cycle (Kask *et al.*, 2008b). In a cross-over intervention study with intermittent low dosages of GnRH agonists inhibiting the production of allopregnanolone by about 30%, a significant decrease in symptom severity related to the allopregnanolone decrease was obtained, irrespective of whether the allopregnanolone decrease occurred in the placebo treatment or GnRH treatment (Nyberg *et al.*, 2007). Results from brain imaging studies show that progesterone, or more likely allopregnanolone, selectively increased amygdala reactivity. Furthermore, functional connectivity analyses indicate that progesterone modulated functional coupling of the amygdala with distant brain regions. These results reveal a neural mechanism by which progesterone may mediate adverse effects on anxiety and mood (van Wingen *et al.*, 2008).

Our group has previously investigated the functional $GABA_A$ receptor sensitivity to several $GABA_A$ receptor agonists by the method of saccadic eye velocity (SEV) in relation to the menstrual cycle, PMDD, and postmenopausal replacement therapy. In a pilot study, healthy women exposed to diazepam showed an increased SEV response (increased $GABA_A$ receptor sensitivity) during the luteal phase of the menstrual cycle, in contrast to PMDD patients who did not change the SEV response from the follicular to the luteal phase (Sundstrom *et al.*, 1997a). However, comparing each menstrual cycle phase separately, the SEV response to diazepam was reduced among PMDD patients compared to controls in the luteal phase but not in the follicular phase (Sundstrom *et al.*, 1997a). A subsequent study including a larger number of participants, and testing the effect of the more water-soluble benzodiazepine midazolam, showed a reduced SEV sensitivity among PMDD patients in the follicular phase compared to control subjects, but no significant within-group differences in the SEV response across the menstrual cycle (Sundstrom *et al.*, 1997b). In another study, PMDD patients had a decreased SEV response to a low dose infusion of alcohol during the late luteal phase, compared to the follicular phase, while the responsiveness among control subjects was unaltered across the menstrual cycle phases (Nyberg *et al.*, 2004). The SEV response to pregnanolone, the 5β epimer to allopregnanolone with similar actions on the $GABA_A$ receptor, was increased during the luteal phase among controls, but not in PMDD patients (Sundstrom *et al.*, 1998). In line with this, the SEV response to pregnanolone was increased when a progestogen was added to a postmenopausal hormone replacement therapy (HRT) (Wihlback *et al.*, 2001). However, in a subsequent cross-over designed study using natural progesterone instead of synthetic progestogens for postmenopausal HRT, the SEV response was increased by estradiol-only as well as by the combination of estradiol and

progesterone, as compared to pre-treatment measurements (Wihlback *et al.*, 2005). Additionally, women with prospectively confirmed cyclical negative mood induced by progesterone during HRT were more sensitive to pregnanolone than women without progesterone-induced negative mood (Wihlback *et al.*, 2005). In summary, the change of $GABA_A$ receptor sensitivity across the menstrual cycle measured by SEV has consistently been reported to differ in PMDD patients compared to women without PMDD. However, in some studies the functional $GABA_A$ receptor sensitivity (to various $GABA_A$ agonists) has been demonstrated to depend on the menstrual cycle phase among healthy women, and in some studies among PMDD patients.

Neurosteroids

The report of rapid non-genomic steroid actions by progesterone metabolites on the GABA transmitter system, i.e. the main inhibitory transmitter system within the brain, was the start of a new research area (Majewska *et al.*, 1986). Later, the term neurosteroids was introduced, referring to steroidal molecules with synthesis and actions within the central nervous system (Baulieu, 1997). Another term, "neuroactive steroids", is used for defining steroids that are active in the brain no matter where they are produced. From the perspective of this thesis, where the steroids are produced by the ovary but acting in the brain, this term is perhaps the most adequate (Paul and Purdy, 1992). One of the most potent progesterone metabolites is allopregnanolone, which is an allosteric positive modulator of the $GABA_A$ receptor (Majewska *et al.*, 1986; Rahman *et al.*, 2008). Allopregnanolone is synthesized from progesterone in two enzymatic steps, by 5α-reductase and then by 3α-hydroxysteroid dehydrogenase (3α-HSD) (Biggio and Purdy, 2001).

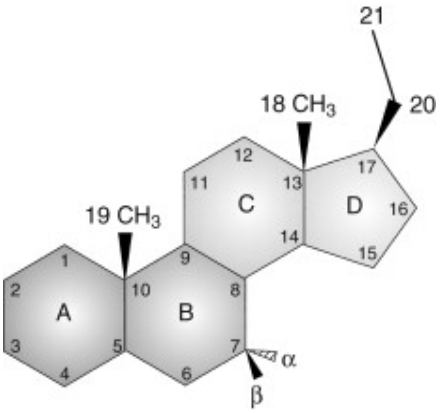

Figure 3. The principle of the steroid structure with the numerical positions of the carbon atoms, α position below the steroid plane and β position above the steroid plane. From Strauss and Barbieri (2009c). Reprinted with permission from Elsevier.

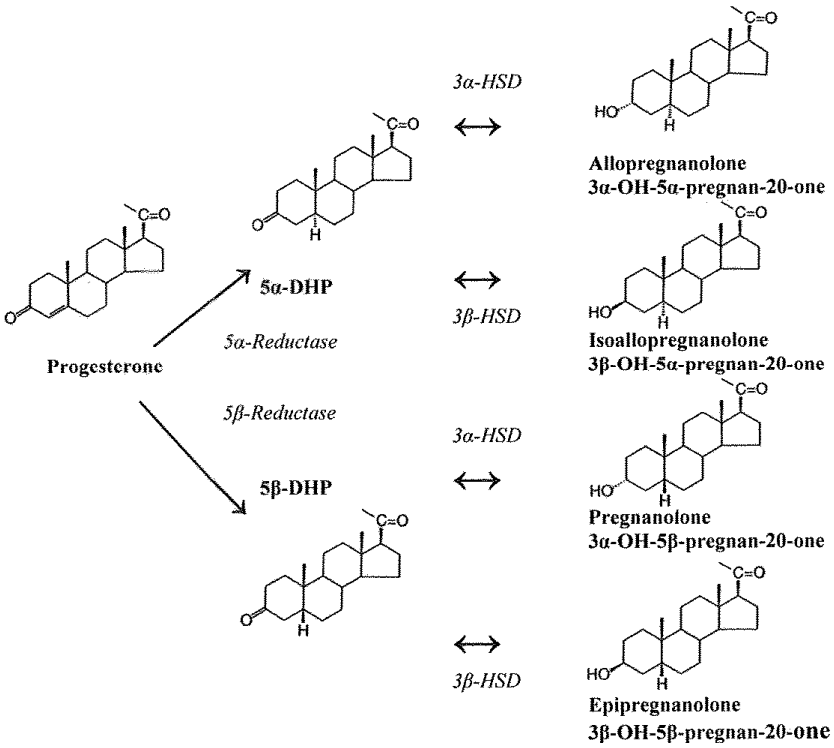

Figure 4. The metabolic pathways from progesterone into its 3α/β-OH-5α/β derivatives. DHP: dihydroprogesterone, HSD: hydroxysteroid dehydrogenase.

In focus: allopregnanolone

Allopregnanolone synthesis

In non-pregnant women, allopregnanolone is mainly secreted by the corpus luteum (Ottander *et al.*, 2005) and the circulating levels correspond to the serum levels of progesterone (Wang *et al.*, 1996). Allopregnanolone concentrations in serum are approximately 0.2–0.6 nmol/l during the follicular phase, rising to about 1–4 nmol/l during the luteal phase of the menstrual cycle (Nyberg *et al.*, 2007). As a consequence of the placental steroid production, allopregnanolone levels rise in parallel to progesterone throughout pregnancy and maternal levels of 100 nmol/l have been measured (Parizek *et al.*, 2005; Hill *et al.*, 2007; Kancheva *et al.*, 2007). Allopregnanolone levels in the umbilical cord are similar to maternal concentrations (Hill *et al.*, 2000) and are probably of importance for the developing fetal brain and neonate (Hirst *et al.*, 2008).

Allopregnanolone levels during the follicular phase of the menstrual cycle are similar to the levels in postmenopausal women and men (Genazzani *et*

al., 1998; Nyberg *et al.*, 2007). Thus, allopregnanolone is also produced by the adrenal glands and levels increase in rat brain after stress exposure (Purdy *et al.*, 1991). The adrenal origin of peripheral allopregnanolone and its correlation to cortisol secretion have been verified in humans by experiments using CRF, ACTH and GnRH tests (Genazzani *et al.*, 1998; Genazzani *et al.*, 2002). In addition, peripheral allopregnanolone levels rose significantly during stress in both men and women (Droogleever Fortuyn *et al.*, 2004). Allopregnanolone is a neurosteroid, which in addition to synthesis in peripheral glands, can be synthesized within the central nervous system *de novo* from cholesterol or from peripheral progesterone substrates (Stoffel-Wagner, 2001). A high correlation between allopregnanolone levels in human cerebrospinal fluid and plasma has been reported (Kim *et al.*, 2000). A post-mortem study clearly showed that peripheral allopregnanolone levels are reflected in the brain (Bixo *et al.*, 1997). Moreover, the central amounts of allopregnanolone were not merely a function of peripheral levels, but accumulation of allopregnanolone in specific brain regions was observed, thus enabling a possibility of specific uptake mechanisms or site-specific production (Bixo *et al.*, 1997). Indeed, rapid cerebral increases of allopregnanolone following stress exposure have been detectable in rats irrespective of plasma levels (Purdy *et al.*, 1991).

Allopregnanolone effects

Endogenous allopregnanolone effects

The effects of endogenous allopregnanolone production are poorly understood in humans. As previously mentioned, the massive placental secretion is likely of great relevance to the fetal central nervous system. Experimental research on sheep and guinea pigs has reported that allopregnanolone govern the normal fetal sleep pattern recorded by intrauterine electroencephalography (Crossley *et al.*, 1997; Nicol *et al.*, 2001). In addition, allopregnanolone in the fetomaternal circulation has been reported to protect the sheep fetal brain from cell death related to asphyxia (Yawno *et al.*, 2011). The finding that allopregnanolone is secreted during stress (Purdy *et al.*, 1991; Genazzani *et al.*, 1998; Droogleever Fortuyn *et al.*, 2004) has led some researchers to suggest that the physiological role of allopregnanolone is to counterbalance the effect of adrenalin, cortisol, and norepinephrine (Biggio *et al.*, 2007). Several studies have reported altered peripheral levels of allopregnanolone in PMDD patients (Rapkin *et al.*, 1997; Bicikova *et al.*, 1998; Monteleone *et al.*, 2000; Girdler *et al.*, 2001), whereas others reported no differences compared to control subjects (Schmidt *et al.*, 1994; Wang *et al.*, 1996; Sundstrom and Backstrom, 1998b). The current view of PMDD suggests an abnormal response to normal ovarian cyclicity

and not different circulating levels of steroids in PMDD patients (Halbreich, 2003). Allopregnanolone seems to have implications in other affective disorders. It has been reported that humans with prior depression fail to mount a proper allopregnanolone increase in response to stress, for a review see Girdler and Klatzkin (2007), and moreover, the findings of altered allopregnanolone levels in major depression and panic disorder are reviewed by van Broekhoven and Verkes (2003) and by Eser *et al.* (2006).

Exogenous allopregnanolone effects

Allopregnanolone has sparsely been administered to humans. Prior to the implementation of study I in this thesis, it had only been given to postmenopausal women as a vaginal gel in a study on hormonal replacement therapy, and there was no measurement of allopregnanolone absorption into serum (Navarro *et al.*, 2003). The outcome was endometrial histology, which was not different from estradiol-only treatment, and there were no adverse effects that easily could be distinguished from estradiol-related side effects (Navarro *et al.*, 2003). After the publication of study I, the sedative effect of allopregnanolone has also been reported in women on OCs and in men (van Broekhoven *et al.*, 2007). Allopregnanolone injections have been shown to deteriorate episodic memory in healthy women (Kask *et al.*, 2008a), but did not affect the startle response in PMDD patients or healthy women (Kask *et al.*, 2009). In studies evaluating the serum levels of allopregnanolone following progesterone administration, a significant sedative effect (Freeman *et al.*, 1993; de Wit *et al.*, 2001; Soderpalm *et al.*, 2004) and anti-epileptic properties (Backstrom *et al.*, 1984) have been reported. In laboratory animals, allopregnanolone has profound pharmacological actions including anesthetic (Korneyev and Costa, 1996; Norberg *et al.*, 1999), anxiolytic (Akwa *et al.*, 1999) and anti-epileptic (Landgren *et al.*, 1978; Landgren *et al.*, 1987; Frye, 1995) properties. Moreover, allopregnanolone has shown neuroprotective characteristics in animal models of traumatic brain injury, and there are plans for a large RCT on progesterone treatment to patients with severe traumatic brain injury in the US (Sayeed and Stein, 2009).

Isoallopregnanolone

The serum levels of isoallopregnanolone, the 3β epimer of allopregnanolone, rise in parallel to progesterone and allopregnanolone (Corpechot *et al.*, 1993; Parizek *et al.*, 2005; Havlikova *et al.*, 2006; Hill *et al.*, 2007). Isoallopregnanolone has not been shown to exert hormonal or $GABA_A$ receptor effects by its own (Lundgren *et al.*, 2003; Stromberg *et al.*, 2006). Isoallopregnanolone has previously been used to confirm the specificity of allopregnanolone on the $GABA_A$ receptor, i.e. the relation of $GABA_A$ receptor activation and the sterical α-configuration of the 3-hydroxy group (Gee *et al.*,

1987). No isoallopregnanolone influence has been noted on anxiolysis or seizures (Bitran *et al.*, 1991; Kokate *et al.*, 1994), and furthermore, no effect of its own has been demonstrated *in vitro* (Lambert *et al.*, 1995; Calogero *et al.*, 1998; Concas *et al.*, 1998b). However, when administered concurrently with allopregnanolone, it has been reported that isoallopregnanolone antagonized the $GABA_A$ receptor enhancing effect of allopregnanolone, *in vitro* (Wang *et al.*, 2000; Wang *et al.*, 2002; Lundgren *et al.*, 2003), and *in vivo* during the allopregnanolone-induced anesthesia in rats (Backstrom *et al.*, 2005). This effect seems specific, as the effects of other $GABA_A$ agonists, e.g. benzodiazepines and barbiturates, on the $GABA_A$ receptor are not antagonized by isoallopregnanolone (Lundgren *et al.*, 2003; Stromberg *et al.*, 2006).

The GABA transmitter system

The γ-amino butyric acid (GABA) system is the main inhibitory transmitter system in the brain, and it has been estimated that GABA serves as a transmitter at about 30% of all the synapses in the central nervous system (Rang *et al.*, 1995b).

The $GABA_A$ receptor

GABA acts predominantly on the $GABA_A$ receptor which augments the intracellular chloride concentrations when activated. The influx of chloride ions hyperpolarizes the neuron and inhibits neurotransmission. There are a number of endogenous (i.e. neurosteroids) and exogenous (e.g. benzodiazepines, barbiturates, alcohol) $GABA_A$ receptor ligands enhancing the effect of GABA. The $GABA_A$ receptor is a membrane-bound receptor composed of five subunits, forming a chloride channel. Most native $GABA_A$ receptors comprise two α, two β and one γ subunit, though to date 19 subunits have been identified (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3) making numerous combinations possible (Olsen and Sieghart, 2008). $GABA_A$ receptors are ubiquitously distributed within the brain and could be located both synaptically and extra-synaptically (Farrant and Nusser, 2005). The composition of $GABA_A$ receptor subunits varies in a site-specific way throughout the different areas of the brain (Pirker *et al.*, 2000; Backstrom *et al.*, 2003). Different $GABA_A$ receptor subtypes can exert different pharmacological responses (Belelli and Lambert, 2005; Olsen and Sieghart, 2009; Uusi-Oukari and Korpi, 2010). The $GABA_A$ receptor δ subunit expression in rodents is associated with the physiological variations in sex steroid hormones over the ovarian cycles (Lovick *et al.*, 2005; Maguire *et al.*, 2005; Maguire and Mody, 2007), and during pregnancy, as well as the post-partum period (Maguire and Mody, 2008; Sanna *et al.*, 2009). The $GABA_A$ receptor plasticity over the ovarian cycle has been shown to be mediated by

allopregnanolone (Maguire and Mody, 2007). In addition, exogenous steroid exposure, tolerance development, and steroid withdrawal have been reported to change the α4, γ2, or δ subunit expression of the $GABA_A$ receptor in rats (Smith *et al.*, 1998b; Follesa *et al.*, 2001; Follesa *et al.*, 2002; Birzniece *et al.*, 2006; Mostallino *et al.*, 2006). The $GABA_A$ receptors located synaptically exert phasic (transient) inhibition in contrast to the $GABA_A$ receptors located extra-synaptically which exert tonic (constant) inhibition on neurotransmission (Farrant and Nusser, 2005). The δ subunit is present in the extra-synaptic $GABA_A$ receptors. $GABA_A$ receptors with one δ subunit have been reported to be very sensitive to low, nanomolar, concentrations of GABA (Zheleznova *et al.*, 2009) and to be most sensitive to allopregnanolone (Belelli *et al.*, 2002). In knock-out (δ^-/δ^-) mice, a reduced sensitivity to neurosteroids together with a concomitant reduction of hippocampal α4 subunit levels was shown (Spigelman *et al.*, 2003). During pregnancy, homozygote and heterozygote mice for this genotype showed increased anxiety and failed to govern optimal breeding behavior after delivery, which was ameliorated in heterozygotes treated with a selective δ subunit agonist (Maguire and Mody, 2008).

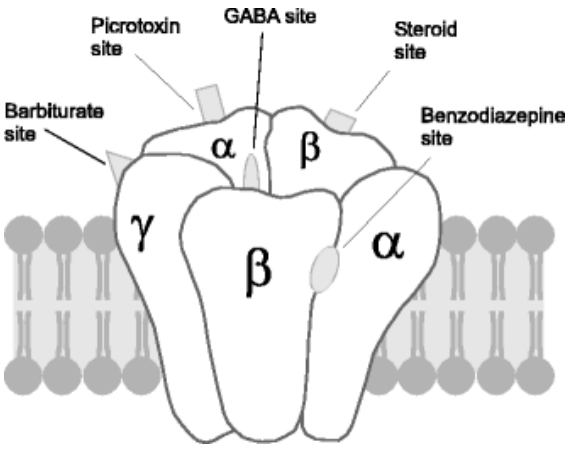

Figure 5. The $GABA_A$ receptor located in the cellular membrane comprised five subunits forming a chloride channel. From AC *et al.* (2006), reprinted with kind permission from Springer Science+Business Media. NB, the allopregnanolone binding sites have recently, in contrast to the figure, been identified on the α subunit.

$GABA_A$ receptor agonists and antagonists

$GABA_A$ receptor agonists are benzodiazepines, barbiturates, alcohol, and endogenous neurosteroids. They have different binding sites, and the binding sites for allopregnanolone have recently been identified on the α

subunit (Hosie *et al.*, 2006; Hosie *et al.*, 2009). The 5β epimer of allopregnanolone, pregnanolone, whose serum concentrations are related to progesterone (Wang *et al.*, 1996), is a $GABA_A$ receptor agonist with similar actions to allopregnanolone, but suggested to have lower potency (Zhu *et al.*, 2001; Rahman *et al.*, 2008). In pharmacological research, the principal $GABA_A$ receptor agonist is muscimol, whereas bicucilline is a competitive antagonist, and picrotoxin is a non-competitive antagonist (Rang *et al.*, 1995b).

Tolerance development at the $GABA_A$ receptor – relevance for PMDD?

In the clinic, tolerance development to $GABA_A$ receptor active agents, such as benzodiazepines and alcohol is a well-known problem. Tolerance means that an increasing dose is needed to maintain the initial pharmacological response, and moreover that disruption of exposure could be associated with withdrawal symptoms (Petursson, 1994; Kan *et al.*, 2004). The mechanisms of tolerance development could briefly be attributed to pharmacokinetic changes (increased elimination of the drug), or pharmacodynamic changes (decreased sensitivity to the drug at the receptor level). There is substantial preclinical evidence for benzodiazepine tolerance development being associated with altered GABAergic transmission (Miller *et al.*, 1988; Allison and Pratt, 2003). Induced changes in the $GABA_A$ receptor subunit composition might provide the simplest explanation for tolerance development, and the regulation of $GABA_A$ receptor subunit expression by various pharmacological agents, including benzodiazepines, barbiturates, alcohol, and neurosteroids is thoroughly reviewed by Uusi-Oukari and Korpi (2010).

Tolerance is classified into chronic tolerance (after repeated exposures) and acute tolerance (during/after single exposure) (Kalant *et al.*, 1971). Chronic tolerance development to allopregnanolone exposure has been found in rats tested in the Morris water maze (Turkmen *et al.*, 2006), and in selected mouse lines regarding allopregnanolone-induced hypothermia (Palmer *et al.*, 2002). Acute tolerance to allopregnanolone has been reported in rats during anesthesia (Zhu *et al.*, 2004; Turkmen *et al.*, 2008). Tolerance development was associated with a decreased mRNA expression of the α4 subunit in the ventral posteriomedial nucleus of the thalamus (Birzniece *et al.*, 2006; Turkmen *et al.*, 2008). During progesterone and allopregnanolone withdrawal, increased anxiety has been reported in rodents, in parallel to increased levels of hippocampal α4 subunits and benzodiazepine insensitivity (Smith *et al.*, 1998b; Smith *et al.*, 2006). In addition, blocking of the α4 gene transcript suppressed the withdrawal properties of allopregnanolone including the induced benzodiazepine insensitivity (Smith

et al., 1998a). The authors suggested allopregnanolone withdrawal as a model for PMDD (Smith *et al.*, 1998a; Smith *et al.*, 2006). Additional support is the reduced sensitivity to diazepam in PMDD patients reported by Sundstrom and colleagues Sundstrom (1997a). However, this withdrawal hypothesis does not explain why PMDD symptoms can begin shortly after ovulation when progesterone and allopregnanolone levels are still increasing.

Studies of tolerance development to neurosteroids in humans have not been performed. The question whether physiological fluctuations in sex steroid levels across female reproductive life could be associated with mechanisms of tolerance development in women remains to be answered. However, the commonly observed marked first trimester fatigue, which declines in spite of increasing allopregnanolone serum concentrations throughout pregnancy, supports the possibility of a physiological tolerance development across reproductive events. Hypothetically, the $GABA_A$ receptor subunit composition may alter the physiological adaptation to sex steroid exposure in vulnerable women.

The GABA paradox – relevance for PMDD?

In two studies of postmenopausal women, the allopregnanolone serum levels following progesterone administration were associated with negative mood symptoms induced by the progesterone content in hormonal replacement therapy (Andreen et al., 2005; Andreen et al., 2006). An inverted U-shaped association between negative mood and serum levels of allopregnanolone was displayed, and the most prominent negative mood symptoms corresponded to allopregnanolone levels within the physiological range during the luteal phase of the menstrual cycle (Andreen et al., 2006). This is paradoxical in relation to the sedative pharmacological effects of progesterone in humans (Freeman et al., 1993; de Wit et al., 2001; Soderpalm et al., 2004), and the anesthetic, antiepileptic, and anxiolytic effects of allopregnanolone in animals (Frye, 1995; Akwa et al., 1999; Norberg et al., 1999). However, paradoxical reactions are not a novelty concerning exposure to other $GABA_A$ receptor agonists in humans, e.g. benzodiazepines (Ben-Porath and Taylor, 2002), and alcohol (Cherek et al., 1992; Dougherty et al., 1996). Paradoxical reactions to $GABA_A$ receptor agonists, including allopregnanolone, are further reported in laboratory animals (Fish et al., 2002; Miczek et al., 2003). Biphasic responses, i.e. aggressive response to lower doses of benzodiazepines or alcohol, and calming effects of higher doses, seem consistent across species (Miczek et al., 2002). These findings have been called the GABA paradox (Andreen et al., 2009; Backstrom et al., 2011). In female mice, allopregnanolone was found to increase anxiety during puberty, in contrast to the anxiolytic effect in pre-

pubertal and adult animals (Shen et al., 2007). The reverse allopregnanolone effect was attributed to the increased expression of α4β2δ $GABA_A$ receptor subunits that generated an outward current in a chloride-dependent manner (Shen et al., 2007). The paradoxical effect of allopregnanolone thus depended on the $GABA_A$ receptor subunit composition and the intracellular chloride concentration. Another controversy has been the divergence in the electrophysiological response to GABA and $GABA_A$ receptor active substances including allopregnanolone in GnRH neurons (Han et al., 2004; Moenter and DeFazio, 2005). This debate throws light upon the complexity of in vitro experimentation and has expanded into new insights (Herbison and Moenter, 2011). Here, the authors stress that the importance of the intracellular concentration of chloride ions is fundamental for the resulting hyperpolarizing or depolarizing effect of $GABA_A$ receptor gating. The intracellular chloride concentration in turn differs in immature and adult neurons depending on the relationship between the sodium-potassium-chloride co-transporter 1 (NKCC1, chloride brought into the cell), and the potassium-chloride co-transporter 2 (KCC2, chloride removed from the cell) (Rivera et al., 1999). To speculate, in vulnerable individuals, the NKCC1/KCC2 ratio may be altered, potentially as a consequence of a different set of $GABA_A$ receptor subunits, and could subsequently produce paradoxical effects to $GABA_A$ steroids.

Measuring $GABA_A$ receptor sensitivity in humans

It is difficult to measure central actions by neurosteroids in humans. Much work on neurosteroids and the $GABA_A$ receptor has been performed in laboratory animals. The promising new technologies of brain imaging presented in the brain plasticity sections earlier in this thesis provide new opportunities for insights into central GABA levels and correlates of GABAergic function in humans (Epperson et al., 2002; Fernandez et al., 2003; van Wingen et al., 2008; Ossewaarde et al., 2010a; Ossewaarde et al., 2010b; Rapkin et al., 2011). One method that is used to measure the functional activation of the $GABA_A$ receptor is saccadic eye velocity (SEV). The method was introduced in the evaluation of benzodiazepine sensitivity about 25 years ago (Griffiths et al., 1984; Hommer et al., 1986), and has been used by our group in several studies over the last 15 years (Sundstrom et al., 1997a; Sundstrom et al., 1997b; Sundstrom et al., 1998; Sundstrom and Backstrom, 1998a, b; Wihlback et al., 2001; Nyberg et al., 2004; Wihlback et al., 2005).

Saccadic eye velocity

A saccade is a quick, jerky movement of the eye in order to change the focus of the fovea. This movement can be measured by electrooculography with

three electrodes applied on the subject's head (figure 7, methods section). There is an electric potential over the eyeball, with the cornea being positive and the retina negative. When the eye moves, the vector is changed in relation to the reference electrode on the forehead and the electric amplitude is registered. The signal is amplified, filtered and converted into velocity terms according to the CSGAAS5 system (Marshall *et al.*, 1985; Marshall and Richens, 1989). The frontal eye fields, substantia nigra, superior colliculus, pontine reticular formation and cerebellum have been shown to control saccades (Becker, 1989). Hikosaka and Wurtz have shown that injected GABA agonists in the superior colliculus reduce SEV, while in the substantia nigra they give irrepressible saccades (1985b, a). The explanation for this was that neurons from the substantia nigra have inhibitory actions on the superior colliculus neurons.

Once the saccade has started it is presumed to be out of voluntary control (Gentles and Thomas, 1971). Maximal SEV ranges from 200–600 deg/s for different individuals (Baloh *et al.*, 1975) but is stable within subjects, also between testing periods (Gentles and Thomas, 1971; Roy-Byrne *et al.*, 1990; Sundstrom and Backstrom, 1998b). A tiring effect after 30–60 min of repetitive testing has been detected, which was removed by amphetamine, a stimulant of the central nervous system (Tedeschi *et al.*, 1986). However, no drugs have been able to increase the baseline SEV. This suggests that the saccade system is set at its maximum function (Glue *et al.*, 1991). $GABA_A$ agonists, such as benzodiazepines, reduced SEV dose-dependently (Hommer *et al.*, 1986). Flumazenil, a benzodiazepine antidote, reversed the decreased SEV response to benzodiazepines (Ball *et al.*, 1991). SEV is considered to be an objective method to measure sedation, benzodiazepine and neurosteroid effect, and thus functional $GABA_A$ receptor sensitivity (Hommer *et al.*, 1986; Sundstrom *et al.*, 1998). There is a correlation between the SEV reduction by benzodiazepines or pregnanolone, and subjective increased feeling of sedation (Hommer *et al.*, 1986; Sundstrom *et al.*, 1997b; Sundstrom *et al.*, 1998). Studies have shown that the relation between sedation and SEV reduction is complex however. Thyrotropin-releasing hormone (TRH) was able to almost completely reverse the subjective increased sedation scores following benzodiazepine administration, but not to reverse the decreased SEV (Glue *et al.*, 1992).

Combined oral contraceptives

Combined oral contraceptives (OCs) constitute a daily intake, days 1–21, of ethinylestradiol and a progestogen in order to inhibit ovulation by the mechanism of negative feedback on the HPG axis. Usually, no pills are taken days 22–28 which in turn leads to endometrial shedding as sex steroid concentrations are declining, i.e. the withdrawal bleeding. Combined oral

contraceptives are widely used by women, in Sweden 56% within the ages of 18–24 and 29% within the ages of 25–34 among women without wishes for pregnancy (Lewin et al., 2000). More than 100 million women worldwide use OCs (Petitti, 2003), predominantly for contraception but also for dysmenorrhea, menorrhagia, endometriosis, polycystic ovary syndrome, and premenstrual dysphoria.

OCs and mood

Negative mood side effects by OCs were reported soon after their introduction and were shown to be dependent on the progestogen component (Cullberg, 1972). However, most women report improved mood stability in prospective studies of OC users (Oinonen and Mazmanian, 2002; Rapkin *et al.*, 2006). Despite the widespread OC use following the commencement half a century ago, negative mood side effects are still one of the major reasons for disruption of OC use (Sanders *et al.*, 2001; Lindh *et al.*, 2009). The discontinuation proportion after six months was one-third among new OCs starters in two prospective studies (Rosenberg and Waugh, 1998; Sanders *et al.*, 2001). Emotional side effects were spontaneously reported by 33% among women who discontinued OC treatment (Sanders *et al.*, 2001). Women with a history of negative mood premenstrually have been reported to be more vulnerable to negative mood side effects during OC treatment (Cullberg, 1972), during hormonal replacement therapy after induced anovulation (Schmidt *et al.*, 1998), as well as during postmenopausal hormonal replacement therapy (Bjorn *et al.*, 1999; Bjorn *et al.*, 2006). Other studies have not found any association between PMDD and OC-induced negative mood, but on the other hand have reported significant associations between mood/anxiety disorders in general and OC-induced adverse mood effects (Joffe *et al.*, 2003; Segebladh *et al.*, 2009b). OC preparations can differ mostly regarding the progestogen component, but also in the dose ratio between ethinyl estradiol and the progestogen, as well as the progestogen amount across the cycle. Hence, the progestogen component in OCs can be constant throughout the cycle (monophasic), or vary in order to mimic the natural fluctuations of progesterone during the menstrual cycle (biphasic or triphasic) (Petitti, 2003). Nowadays, most OCs include 19-nortestosterone derivatives, or the spironolactone derivative drospirenone. One study with a double-blind cross-over design that prospectively rated mood symptoms before and during OC treatment reported more beneficial mood effects in the monophasic desogestrel preparation, compared to the monophasic and triphasic levonorgestrel preparations in women with premenstrual symptoms prior to OC use (Backstrom *et al.*, 1992). Drospirenone was reported superior to levonorgestrel in constant combinations with ethinyl estradiol regarding

mood symptoms in a randomized comparative study (Sangthawan and Taneepanichskul, 2005). In fact, studies of negative mood side effects during OC use mostly report improved mood, although there are weaknesses in assessing PMS/PMDD before inclusion. For a review of studies in healthy subjects and in relation to premenstrual symptoms see Kurshan and Epperson (2006).

So far, the neurobiology for OC-induced negative mood has not been much investigated. It is conceivable that the similarities between premenstrual mood symptoms and OC-induced negative mood might share underlying mechanisms. Indeed, it has been shown that PMDD patients have lower levels of prepulse inhibition (briefly, the ability to screen a sensory stimulus, in this case as a part of the acoustic startle response), during the symptomatic luteal phase as compared to controls (Kask *et al.*, 2008b). This finding was replicated in OC users with adverse mood effects compared to OC users without adverse mood effects (Borgstrom *et al.*, 2008). However, there was no difference in prepulse inhibitions levels between prior OC users who had disrupted OC use because of adverse mood, and prior OC users who had discontinued the OC use for other reasons than adverse mood (Borgstrom *et al.*, 2008).

OCs and neurosteroids

The use of OCs represents a more stable endocrine state compared to the natural menstrual cycle, and the levels of endogenous gonadal steroids and metabolites including peripheral allopregnanolone are low (Follesa *et al.*, 2002; Paoletti *et al.*, 2004; Rapkin *et al.*, 2006). Two studies have investigated neurosteroid levels during OC use in women in relation to mood parameters (Paoletti *et al.*, 2004; Rapkin *et al.*, 2006). Rapkin and colleagues enrolled healthy women without underlying affective disorder to a treatment with 0.02 mg ethinyl estradiol and 0.1 mg levonorgestrel OC formulation, and reported lowered serum levels of allopregnanolone and progesterone following treatment. On closer look, there was no decrease in allopregnanolone concentrations compared to the follicular phase levels before OC exposure, and additionally no effect on mood was detected. Hence, the authors conclude that reduced levels of allopregnanolone were not associated with adverse mood in this sample of 31 healthy women with a mean age of 27 years (Rapkin *et al.*, 2006). Paoletti and co-workers compared the effect of a 0.03 mg ethinyl estradiol and 3 mg drospirenone OC combination on allopregnanolone, progesterone, allotetrahydrodeoxycorticosterone (THDOC), and dihydroepiandrostendione sulfate (DHEAS) serum levels and psychological effects on the SCL-90 global score in 10 healthy women, compared to 12 untreated control women (Paoletti *et al.*, 2004). In the OC group, anxiety scores were reduced, in

parallel to reduced neurosteroid levels. The authors found decreased levels of DHEAS, which in contrast to allopregnanolone and THDOC is a negative allosteric modulator of the $GABA_A$ receptor (i.e. antagonist), and they claim that the decreased DHEAS may explain the improved mood parameters in the OC group (Paoletti *et al.*, 2004). Given the reported frequency of OC-induced mood deterioration of 16.3% in a case-control study (Joffe *et al.*, 2003) and the association with prior mood disorders (Oinonen and Mazmanian, 2002; Joffe *et al.*, 2003; Segebladh *et al.*, 2009b), the studies by Rapkin and Paoletti could have been limited by firstly, the small sample sizes and secondly by including only young and healthy women. The question whether OC users who experience adverse mood differ in neurosteroid levels during OC treatment compared to OC users without adverse mood effects remains to be investigated.

In female rats, OC treatment (0.03 mg ethinyl estradiol + 0.125 mg levonorgestrel daily for 6 weeks) increased anxiety behavior in the elevated plus-maze and lowered allopregnanolone concentrations in plasma as well as the cerebral cortex (Follesa *et al.*, 2002). Moreover, the γ2L and γ2S $GABA_A$ receptor subunit expression was increased in the cerebral cortex, in contrast to the α1, α3, α4, β1, β2, β3 subunit expression which remained unchanged by OC treatment (Follesa *et al.*, 2002). In line with this, the increase of allopregnanolone brain concentrations during pregnancy in rats was associated with a decrease of γ2 subunit expression (Concas *et al.*, 1998a). In summary, given the similarities between adverse mood effects induced by synthetic progestogens and PMDD it is reasonable to explore the functional $GABA_A$ receptor sensitivity during OC treatment.

Allopregnanolone and the HPG axis

HPG axis

The female menstrual cycle is regulated by feedback mechanisms affecting the hypothalamus-pituitary-gonad (HPG) axis. GnRH from the hypothalamus regulates the secretion of FSH and LH from the anterior pituitary. The gonadotropins, FSH and LH, in turn regulate the production of sex steroid hormones, estradiol and progesterone, in the ovaries. FSH stimulates follicular development, and promotes estradiol production, which in turn (by positive feedbeack) elicits the LH peak that precedes ovulation. Estradiol proliferates the endometrium during the follicular (or proliferative) phase. After ovulation, a corpus luteum producing progesterone and estradiol is formed. The endometrium develops secretory characteristics under the influence of progesterone during the luteal (or secretory) phase of the menstrual cycle. Estradiol and progesterone in combination exert negative feedback on gonadotropin secretion.

GnRH and gonadotropin secretion

GnRH, LH and FSH secretion is pulsatile and the pulse frequency changes with the different phases of the menstrual cycle (Backstrom *et al.*, 1982). This secretion pattern is crucial for maintaining reproductive function. If GnRH pulsatility is disturbed, as is the case in hypothalamic amenorrhea (Perkins *et al.*, 1999), or during long-term treatment with GnRH analogues (Lemay *et al.*, 1988), the HPG axis is down-regulated and the woman is put into a state of hypogonadotropic hypogonadism. There is a circadian rhythm in gonadotropin secretion. Gonadotropin levels decline during night in the follicular phase, in contrast to the circadian pattern of the other pituitary hormones, adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), growth hormone (GH) and prolactin (PRL), with nocturnal rises. The gonadotropin pattern is probably mediated by endogenous opiates. While the secretion of FSH maintains this diurnal pattern over the menstrual cycle, LH secretion only displays this pattern in the early follicular phase (Mortola *et al.*, 1992). There is also a pattern over the menstrual cycle in the pulsatility of GnRH secretion and thus in LH and FSH pulsatility (Backstrom *et al.*, 1982). In the early follicular phase, the mean intervals between GnRH pulses are approximately 90–100 min, which shorten to about 60 min during the mid-follicular phase, and then are markedly prolonged during the luteal phase. LH pulsatile secretion is highly correlated to GnRH pulses in women. FSH secretion is favored by longer GnRH pulse intervals, and the early follicular phase is characterized by markedly decreased GnRH pulse intervals during sleep. FSH secretion is also dependent on estradiol and inhibin negative feedback mechanisms, while estradiol exerts positive feedback on LH promoting the LH surge (Strauss and Barbieri, 2009a).

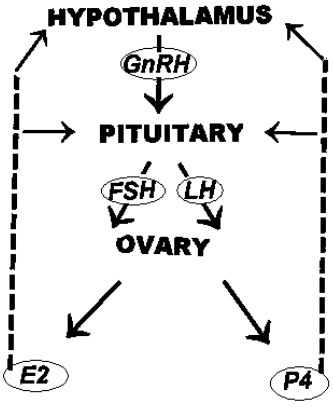

Figure 6. Levels and feedback mechanisms at the HPG-axis. E2: estradiol, P4: progesterone, FSH: follicle stimulating hormone, LH: luteinizing hormone, GnRH: gonadotropin releasing hormone.

The GABA transmitter system and gonadotropin secretion

The GABA transmitter system has been suggested to have regulatory effects on the reproductive axis in animals. Local infusion of the $GABA_A$ receptor agonist muscimol into the medial preoptic area in ewes decreased the GnRH release and lowered the plasma concentration of LH as well as the LH pulse amplitude during the estrous cycle (Tomaszewska-Zaremba *et al.*, 2003). Allopregnanolone injected into the intracerebral ventricles (icv) of female rats decreased the number of oocytes collected at estrous, a finding which was reversed when endogenous allopregnanolone was blocked (Genazzani *et al.*, 1995). In ovariectomized rats primed with estrogen and progesterone, icv injections of allopregnanolone reduced serum levels of LH while the $GABA_A$ receptor antagonist bicuculline was ineffective alone, but blocked the allopregnanolone-induced reduction of LH when injected concomitantly with allopregnanolone (Laconi and Cabrera, 2002). The GnRH release from individually incubated rat hemi-hypothalami was suppressed by allopregnanolone and muscimol but unchanged by its inactive 3β epimer, isoallopregnanolone (Calogero *et al.*, 1998). However, the GnRH release was stimulated by allopregnanolone in immortalized GT1-1 GnRH neurons *in vitro* (el-Etr *et al.*, 1995). Moreover, the electrophysiological response in GnRH neurons to GABA and $GABA_A$ receptor active substances including allopregnanolone has been both excitatory and inhibitory (Han *et al.*, 2004; Moenter and DeFazio, 2005). Acute administration of the 5β epimer to allopregnanolone, pregnanolone, did not influence gonadotropin levels in women (Sundstrom and Backstrom, 1999).

Hypothalamic amenorrhea

The condition of hypothalamic amenorrhea (i.e. hypogonadotropic hypogonadism) should only be stated after exclusion of other causes of amenorrhea. Hypothalamic amenorrhea is complicated by osteoporosis and infertility. The underlying mechanism resulting in hypothalamic amenorrhea is not fully known, but there are associations with physical, nutritional, and psychobiological stress, although in some cases no reason can be identified. However, the hypothalamus-pituitary-adrenal (HPA) axis, activated by stress, has been shown to interfere with the HPG axis (Fritz and Speroff, 2011a). For instance, women with hypothalamic amenorrhea display increased cortisol secretion (Biller et al., 1990; Berga et al., 1997). In rhesus monkeys, corticotropin-releasing hormone (CRH) was shown to inhibit GnRH secretion (Olster and Ferin, 1987). Negative energy balance, the common characteristic of restricted food intake and intense exercise, is proposed to arouse CRH secretion, leading to disturbed GnRH secretion (Loucks, 2003). In addition, endogenous endorphins from strenuous exercise can contribute to disturbances in GnRH secretion (Laatikainen et

al., 1986). Women with hypothalamic amenorrhea displayed not only higher levels of cortisol but also higher concentrations of allopregnanolone, with both episodic and concurrent release (Genazzani et al., 2002). Moreover, stress is associated with increased levels of allopregnanolone, as previously described in this thesis (Purdy et al., 1991; Droogleever Fortuyn et al., 2004). Taken together, these findings promote the hypothesis that allopregnanolone may be involved in the regulation of the HPG axis in women.

Aims of the thesis

- To investigate the pharmacokinetic and pharmacodynamic effects of intravenous allopregnanolone injections in healthy women (study I).
- To study whether the sensitivity to allopregnanolone in terms of saccadic eye velocity and subjectively rated sedation differs in the follicular compared to the luteal phase of the menstrual cycle in healthy regularly menstruating women (study II).
- To study whether the sensitivity to allopregnanolone in terms of saccadic eye velocity and subjectively rated sedation differs in women using combined oral contraceptives (OCs) compared to healthy regularly menstruating women (study II).
- To study whether the sensitivity to allopregnanolone in terms of saccadic eye velocity and subjectively rated sedation across the menstrual cycle differs in women with premenstrual dysphoric disorder (PMDD) compared to healthy regularly menstruating women (study III).
- To study the effect of intravenous allopregnanolone on the HPG axis in healthy women (study IV).

Material and methods

Subjects

All participants were volunteer women recruited through advertisements at Umeå University Hospital and in the local newspaper. To be included, they had to be physically and mentally healthy and aged 18–40. Physical and gynecological examinations as well as routine blood chemistry screens (total blood cell count, serum glucose, liver enzymes, creatinine, sodium, and potassium) were performed and found normal before inclusion. All participants had negative pregnancy tests in urine, and planning for pregnancy was an exclusion criterion. Further exclusion criteria were treatment with any steroid compound (the only exception was treatment with oral contraceptives in the OC group in study II) within the last six months and treatment with benzodiazepines or other psychotropic drugs within the last three months before enrolment in the study. Any somatic disease including liver, kidney, heart, lung, neurological or gynecological disease, or any ophthalmological disease that would render measurement of eye movements impossible, were exclusion criteria as well as language difficulties and a history of alcohol or drug abuse. The presence of psychiatric disorders was evaluated by the use of Primary Care Evaluation of Mental Disorders (PRIME-MD), a structured interview which has been validated for use in primary care settings, and conforms to the criteria in the Diagnostic and Statistical Manual of Mental Disorders 4th edition (Spitzer et al., 1994). All subjects gave written informed consent prior to inclusion. Studies were in accordance with the Declaration of Helsinki. The Regional Ethical Review Board, University of Umeå, and the Medical Products Agency of Sweden approved the studies.

Studies I and IV

The ten healthy women in studies I and IV reported regular menstrual cycles, and PRIME-MD did not reveal any psychiatric disease.

Study II

This study included 12 healthy naturally cycling women with regular menstrual cycles and 10 women on combined oral contraceptives (OCs). Ovulatory cycles were confirmed in the naturally cycling subjects by a baseline progesterone value exceeding 10 nmol/L in serum on the luteal phase test day. Two women were excluded due to not confirmed ovulation, and thus the study group of healthy naturally cycling women comprised 10

subjects. PRIME-MD interviews were negative for psychiatric disorders in both groups.

Study III

Study III included 10 women with PMDD. Any other psychiatric disorder was ruled out by negative PRIME-MD interviews. The women were included after two cycles of prospective scoring on the Cyclicity Diagnoser (CD) scale meeting the criteria for PMDD (APA, 1994). The CD form includes daily ratings of mainly mood-related symptoms on a Likert scale in 9 steps (0-8) (Segebladh *et al.*, 2009b). Absence of a particular symptom is scored as 0 and maximal severity of a particular symptom is scored as 8. There were eight negative mood parameters: depression, irritability, mood lability, sleep disturbances, lack of impulse control, tension/anxiety, difficulties in concentration, fatigue: there were three positive mood parameters: cheerfulness, energy and interest in daily activities: and four somatic symptoms: bloating, breast tenderness, appetite/cravings and menstrual bleeding. An additional parameter is a severity parameter, i.e. how actual symptoms impair everyday family life and work. The impairment scale also ranges from 0 to 8, i.e., no impairment (0), impairs her self (1), family notices and makes arrangements (2), disturbs family life (3), impairs social life and relations to friends (4–5) and work activities; if symptoms impair work performance but work was done OK (6), if work was done properly but the patient had to endeavor (7), if the work performance was not done properly or the patient stayed at home (8). Ovulatory serum-progesterone values in the luteal phase were obtained during scoring on the CD scale before inclusion. In addition, the test cycle was confirmed as ovulatory with a baseline progesterone value of at least 10 nmol/L in serum on the luteal phase test day. The control group in study III is the healthy naturally cycling, regularly menstruating women included in study II.

Study Protocols

All studies were small explorative studies of effects of intravenous injections of allopregnanolone. The experimental setting was the gynecological outpatient department of Umeå University Hospital. Subjects arrived at 8.00 a.m. Two intravenous cannulae were inserted in each forearm, one for allopregnanolone injections and the other for repeated blood samples. Electrodes for SEV measurements were applied. Baseline levels of serum allopregnanolone, progesterone, estradiol, FSH and LH were taken. Three sets of SEV measurements were performed before the allopregnanolone injection. The mean value of these three recordings was set as the baseline value for each individual. Subjects also rated feelings of sedation on a visual analogue scale (VAS) before injection as the baseline value. No night work or

jet lag journeys were allowed the week before the experimental session, and caffeine was restricted during the test day.

Studies I and IV

Study I and IV were based on the same study population and the same experimental setting. To the best of our knowledge this was the first time allopregnanolone was given as intravenous injections to human beings. The experimental session was performed during the follicular phase when endogenous levels of allopregnanolone are low. For pharmacokinetic and safety reasons allopregnanolone injections were administered in three increasing doses of 0.015, 0.03, 0.045 mg/kg at 30 min intervals, thus giving a cumulative dose of 0.09 mg/kg. SEV parameters were recorded at 5, 13, and 21 min after each allopregnanolone injection, and subjects reported their subjective feeling of sedation on a visual analogue scale after each SEV recording. Blood samples were drawn at the same intervals. Additional SEV recordings, subjectively rated sedation, and blood sampling were performed at 95, 105, 115, 150, and 330 min after the first allopregnanolone injection. Following the measurements at 150 min, subjects were allowed to walk around freely at the department. After the 330 min measurements, they returned home and came back to the hospital for completion with blood samples at 600 and 780 minutes, i.e. the last blood sample was taken 13 h after the first injection. For the analysis of gonadotropin secretion after allopregnanolone injections, a group of five women who had been challenged with intravenous injections of isoallopregnanolone were included in paper IV. The women in the isoallopregnanolone group (ISOALLO group) originated from a previous pharmacokinetic study of isoallopregnanolone by our group, described by Hedstrom *et al.* (2009). The isoallopregnanolone injections were given in three increasing doses of 0.04, 0.06, and 0.10 mg/kg, producing a cumulative dose of 0.20 mg/kg. Blood samples were drawn at 5, 13, 18, 35, 43, 48, 65, 73, 88, 95, 105, 115, 150, 330, 600, and 780 min after the first injection of isoallopregnanolone.

Studies II and III

Studies II and III have the same study protocol. The experimental session was performed once in the follicular phase and once in the luteal phase among regularly menstruating women with and without PMDD. In the OC group, the experiments were performed only once, i.e. during the latter half of the OC blister strip. Intravenous allopregnanolone was given as a single dose of 0.05 mg/kg, calculated to correspond to the cumulative dose given in study I. Before the allopregnanolone injection, subjects scored baseline values on the Anxiety Sensitivity Index (ASI), Panic Symptom Scale (PSS), State-Trait Anxiety Inventory (STAI) and State Anxiety and Discomfort Scale

(SADS). The infusion rate was 20 mg/ml. After the allopregnanolone injection, SEV recordings and subjectively rated sedation were performed at 5, 13, 18, 25, 30, 45, 60, 120, and 180 min. SADS was scored at 13, 25, 30, 45, 60, 120, and 180 min after injection. Blood samples were drawn at 5, 18, 30, 45, 60, 120, and 180 min. Between the measurements at 120 and 180 min the subjects were allowed to walk around at the department. When subjects returned home, PSS, STAI, and SADS scales were enclosed for ratings after 24 hours.

Preparation of study solutions

The Umeå University Hospital Pharmacy prepared the experimental solutions. Concentrations of allopregnanolone and isoallopregnanolone were determined using high-performance liquid chromatography (HPLC) and UV absorbance according to the description in Turkmen *et al* (2004).

Allopregnanolone solution

The allopregnanolone solution was formulated with GMP-made allopregnanolone (Umecrine AB, Umeå Sweden). Thirteen milligrams of allopregnanolone was dissolved in 100 ml albumin (200 mg/ml) solution using an ultrasound bath. The solution was filtered through two sterile filters. The final solution contained 0.126 ± 0.003 mg/ml (mean ± SEM) allopregnanolone (n=9).

Isoallopregnanolone solution

The isoallopregnanolone solution was formulated with GMP-made isoallopregnanolone (Umecrine AB, Umeå, Sweden). Eight milligrams of isoallopregnanolone was dissolved in 100 ml albumin (200 mg/ml) solution using an ultrasound bath. The solution was filtered through two sterile filters. The final solution contained 0.0736 ± 0.00807 mg/ml (mean ± SD) isoallopregnanolone (n=6).

Dosage of allopregnanolone

The overall aim was to choose an allopregnanolone dose which would produce a measurable effect in terms of decreased saccadic eye velocity, without causing pronounced sedation.

Studies I and IV

The dose of allopregnanolone in study I was based on earlier studies on its 5β epimer, pregnanolone, which had been preliminary evaluated as an anesthetic agent (Carl *et al.*, 1990; Gray *et al.*, 1992; Carl *et al.*, 1994; Parivar *et al.*, 1996). Based on that, Sundstrom *et al.* gave three consecutive

intravenous doses of 0.03, 0.06, and 0.09 mg/kg pregnanolone at 25 min intervals to women and recorded a dose-dependent decrease in SEV, and no serious adverse events (1998). This cumulative dose of 0.18 mg/kg pregnanolone corresponded to one fourth of the dose for anesthetic induction in humans (Carl *et al.*, 1990). Given the indications from laboratory animals that allopregnanolone might be about 35% more potent than pregnanolone (Norberg *et al.*, 1987; Paul and Purdy, 1992; Zhu *et al.*, 2001), half of the previously given pregnanolone dose was chosen, i.e. three consecutive doses of 0.015, 0.030, and 0.045 mg/kg allopregnanolone at 30 min intervals, producing a cumulative dose of 0.09 mg/kg allopregnanolone.

Studies II and III

The single dose of allopregnanolone (0.05 mg/kg) used in studies II and III was chosen on the basis of the cumulative dose (0.09 mg/kg) in study I, taking into account continuous metabolism during cumulative dosages. Additionally, the 5β epimer, pregnanolone, had been administered as a single dose of 0.10 mg/kg evaluating the SEV response in postmenopausal women with different hormone replacement therapies, and corresponded to the previous cumulative dose of 0.18 mg/kg pregnanolone (Wihlback *et al.*, 2001; Wihlback *et al.*, 2005).

Saccadic Eye Velocity

The pharmacodynamic effect of intravenous allopregnanolone injections was measured by recording saccadic eye velocity (SEV) and subjectively rated sedation on a visual analogue scale. The regulation of saccadic eye movements has previously been described in the introduction part. From each saccadic eye movement, it is possible to calculate several parameters in addition to SEV. Saccade latency is the time from movement of target to saccade initiation, i.e. the reaction time. It usually lasts between 150 and 300 ms. Saccade accuracy is the difference between the target position and the actual position of the eyeball at the end of the saccade, i.e. the precision. Most often there is a slight undershoot corrected by a new saccade to locate the final target. Saccade acceleration is the increase of SEV after the saccade start and saccade deceleration is the slowing of SEV before stopping at the target. In the present studies, saccadic latency, saccadic accuracy, peak saccadic eye velocity, and saccadic acceleration/deceleration were measured.

Saccadic eye velocity (SEV) was measured using electrooculography (EOG) with the CSGAAS5 system, fully documented elsewhere (Marshall et al., 1985; Marshall and Richens, 1989). The experiment was performed in a quiet, semi-lit room with the patient sitting in a comfortable chair supporting the head. EEG cup electrodes (Synetics AB, Stockholm, Sweden)

with a small amount of electrode gel (Elefix, Nihon Kohden) were used. The topical skin area was exfoliated with Skinpure cream (Nihon Kohden) before the electrodes were placed 1 cm lateral to the outer canthus of both eyes, and one electrode centrally on 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. The target movements took place at 1.5 second intervals. A fixed, non-random sequence of 4×24 targets producing target steps of 10°, 20°, 30°, and 40° with a brief rest in between, was displayed. 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 (−3 dB at 50 Hz) before being digitized into a 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 4×20 target steps, were stored and analyzed off-line according to the method of Marshall and Richens (1989). Firstly, the digitized data from each target displacement were processed to locate saccades. To avoid preemptive saccades and blinking artifacts, only saccades initiated 50 to 400 ms after target movements were included. Also, to be considered as a saccade, the recorded eye movement had to display a velocity of more than 100°/s. Secondly, each saccade was analyzed 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. SEV was further processed by plotting a velocity–saccade size curve, called the main sequence (Baloh *et al.*, 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 weighting 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° saccades were then calculated by interpolation. Saccades with amplitudes of 30° were chosen for further analyses as SEV reaches a maximum at approximately 30–35° of angular movement (Baloh *et al.*, 1975).

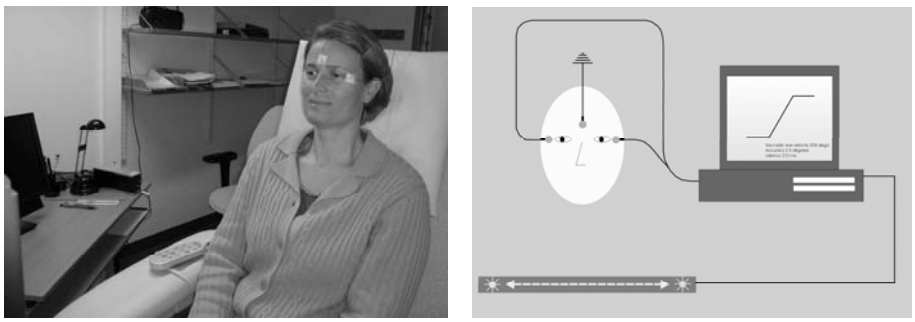

Figure 7. SEV recordings, in real life and in schematic view, that show the electrode equipment in the subject in the experimental chair, looking at the array of light-emitting diodes. The schematic view illustrates the saccade movement, y-axis: degree, x-axis: time.

Visual analogue scale of sedation

Subjects rated their feeling of sedation on a visual analogue scale from 0 to 100 mm. 0 representing no feeling of sedation at all and 100 representing falling asleep. Subjectively rated sedation was performed after each SEV recording.

Assays of allopregnanolone and isoallopregnanolone

Serum concentrations of allopregnanolone were measured with radioimmunoassay (RIA) after diethylether extraction and celite chromatographic or high-performance liqiud chromatographic purification of samples.

Extraction and separation of cross reacting steroids

Serum was pipetted into a cylindrical flat-bottom glass vial; then water (0.5 ml) and diethyl ether (3.0 ml) was added. The samples were left on an orbital shaker for ten minutes. Following the liquid/liquid extraction, the vials were transferred into an ethanol/dry ice bath. The water phase was frozen and the ether phase was decanted and evaporated under a stream of nitrogen gas.

Celite chromatography

In studies I, II, III, and IV (ALLO group), allopregnanolone was separated from cross-reacting steroids with celite chromatography. The evaporated sample was dissolved in 1.0 ml isooctane (Merck) saturated with ethylene glycol (J.T. Baker), before application to the column. Celite column chromatography was performed as described in papers I and II. The allopregnanolone-containing fraction was evaporated under nitrogen. Recovery was determined for each assay using 300–500 cpm of tritium-

labeled allopregnanolone, [9,11,12-^{3}H(N)]-5α-pregnan-3α-ol-20-one (Perkin-Elmer Life Sciences, Boston, USA), added to a serum sample before extraction and by measuring the amount recovered after chromatography. The recovery of allopregnanolone averaged 78% (studies I and IV) and 70% (studies II and III). The results were compensated for recovery.

High performance liquid chromatography

In study IV, isoallopregnanolone and allopregnanolone in the ISOALLO group was separated from cross reacting steroids with high performance liquid chromatography (HPLC). The fractions were symmetrically collected around the peak retention time for isoallopregnanolone or allopregnanolone for further analysis with radioimmunoassay (RIA). No cross-reacting steroids had retention times close to the collected fractions. The recovery of the extraction and HPLC procedure was for isoallopregnanolone 95%. The recovery of allopregnanolone was 98% and the results were compensated for recovery. The method has been described in detail earlier (Hedstrom *et al.*, 2009).

Radioimmunoassays of allopregnanolone and isoallopregnanolone

Allopregnanolone

Allopregnanolone was measured by RIA using a polyclonal rabbit antisera against 3α-hydroxy-20-oxo-5α-pregnan-11-yl carboxymethyl ether coupled to bovine serum albumin, made by Dr R.H. Purdy at The Scripps Research Institute, La Jolla, CA, USA (Purdy *et al.*, 1990). The standard curve was established by preparing duplicate tubes containing eight concentrations of unlabelled allopregnanolone to give a range from 0 to 5,000 pg. Rabbit antiserum was used in a dilution of 1/5,000. The antibody solutions were prepared using [9,11,12-^{3}H(N)]-5α-pregnan-3α-ol-20-one, 3×10^{6} cpm/32 ml solution containing 65 mM boric acid (Merck) buffer, pH=8.0, bovine serum albumin 100 mg/ml (Sigma, St Louis, USA), human gamma globulin solution 20 mg/ml (Octapharma, Sweden) and antibody in milliliter ratio, rabbit antisera solution: 30:1:1:0.006. The solution was allowed to equilibrate overnight at 8°C. Antibody solution (0.2 ml) was added to all standard and sample tubes, and the mixture allowed to stand overnight at 8°C. After the addition of 0.2 ml saturated ammonium sulfate, each tube was again mixed and centrifuged at 20,000 RPM for 20 min. Thereafter, the supernatant was aliquoted into a counting vial and diluted with 3.0 ml Optiphase scintillation medium (Wallac, Finland). The samples were counted in a RackBeta (Wallac) scintillation counter. The sensitivity of the assay was 25 pg, with an intraassay coefficient of variation for allopregnanolone of 6.5% and an interassay coefficient of variation of 8.5%.

In addition to the method described, a new egg-yolk antiserum (Agrisera AB, Vännäs, Sweden) was evaluated in study I, described in detail in paper I.

Isoallopregnanolone

Isoallopregnanolone was measured by RIA using an antibody raised against pregnenolone (pregnenolone-3-monohemisuccinate-HAS; ICN Pharmaceuticals, Inc Orangeburg, NY, USA) as it also binds to isoallopregnanolone. Cross-reactivity with isoallopregnanolone was 26.6%, allopregnanolone 13%, 5α-pregnane-3,20-dione 7%, 5β-pregnane-3β-ol-20-one <1%, 5β-pregnane-3α-ol-20-one <1%, 5α-pregnane-3α,20α-diol <1%, pregnenolone 100%. The sensitivity of the assay 35.8 pg, with an intra-assay coefficient of variation of 8.5% and an inter-assay coefficient of variation of 10.7%. The method has been described in detail earlier (Hedstrom *et al.*, 2009).

Analyses of progesterone, estradiol, FSH and LH

Concentrations of FSH and LH were analyzed with a solid-phase, two-site chemiluminescent immunometric assay (Immulite®). According to the manufacturer the intra- and inter-assay variation coefficients are 2.6 and 5.8% respectively for the FSH analysis whereas the intra-assay variation is 4.8-6.5% and the inter-assay variation is 7.2-11.6% for the LH analysis. Progesterone levels were measured with a sequential competitive immunoassay and estradiol with a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite®). All these analysis kits were manufactured by Diagnostic Products Corporation, Corporate Offices, Los Angeles, CA, USA.

Anxiety scales

Several scales were used by the subjects to rate anxiety and personality before and during the test to be able to find any paradoxical reactions. Anxiety Sensitivity Index (ASI) was used prior to injection to find fear of panic or anxiety (Reiss et al., 1986). Total score ranges from 0 to 64 and an ASI mean score of 19.01 has been reported for a normal sample (Peterson and Reiss, 1992), while the mean score for panic disorder patients has been reported to be 36.2 (Taylor et al., 1992). The DSM-IV derived Panic Symptoms Scale (PSS) includes 18 symptoms related to panic, each being scored from 0 to 4, and thus has a total range of 0–72. PSS mean score in the follicular phase in 12 healthy controls receiving placebo injections has been reported as 1.02 and in the luteal phase 2.27 (Bell et al., 2004). State-Trait Anxiety Inventory (STAI) with a total range of scores from 20 to 80 (Spielberger et al., 1970) was also used before injection and 24 hours after injection. In the current study, we used the state anxiety part, i.e. how the

subject feels in the immediate present situation. A total score below 40 indicates low, between 40 and 59 moderate, and 60 or more severe state anxiety. Actual feelings of anxiety or panic were also rated by subjects throughout the experimental hours (see the study protocol part), using the State Anxiety and Discomfort Scale (SADS). SADS is a global measure of subjective discomfort and has previously been tested in pharmacological tests on humans for detection of quick changes in anxiety level (Radu et al., 2002).

Pharmacokinetics

Before the pharmacokinetic calculations were undertaken in study I, the baseline allopregnanolone concentration (C_0) was subtracted from the measured values obtained 5–780 min later. Baseline allopregnanolone levels were less than 1% of the maximum concentration at the same sampling occasion. Pharmacokinetic parameters were calculated by means of the Kinetica version 4.3 program (InnaPhase Corporation, Philadelphia, PA, USA), using a two-compartment model. For details see paper I.

Calculations of BMI and menstrual cycle day

BMI was calculated as body weight (kg) divided by the squared body height (m^2). The onset of menstrual bleeding was defined as day 1 of the menstrual cycle.

Statistics

The number of subjects in study II and III were based on a statistical power analysis with α=0.05 and β=0.90. In earlier studies, it had been shown that the standard deviation of SEV within individuals is ± 7°/s and the smallest SEV change detectable from baseline was 15°/s. Based on this, the statistical power analysis calculated a study size of seven subjects in each group. We included ten subjects in each group.

Saccadic eye movement parameters and subjectively rated sedation were calculated as delta values, i.e. the baseline value was defined as 0. Delta values were compared within the group in study I. In studies II and III, delta values were compared between the two menstrual cycle phases as well as between the groups. In studies I and IV, repeated measures with analyses of variance (ANOVA) were performed using time-point as within-subject factor. In studies II and III, a mixed model was used to analyze the SEV and subjectively rated response to the allopregnanolone injection. In study II separate mixed models were used to explore the sedative response between the menstrual cycle phases within the naturally cycling women and between the OC group and each menstrual cycle phase. In study III separate mixed

models were used to compare the SEV and subjectively rated sedation response between the menstrual cycle phases within the PMDD group and within the control group, and additionally between-group comparisons were made for the follicular and the luteal phase separately. The mixed model handles variables nested within an individual (time after injection, menstrual cycle phase) as well as non-equivalent time intervals. In the mixed model, subjects' intercept was used as random coefficient, and time, menstrual cycle phase, and group as fixed coefficients. Interaction variables were calculated and analyzed in the mixed model when plausible. Because of the small sample sizes, non-parametric tests were preferred when other parameters than SEV, subjectively rated sedation, or serum concentrations were analyzed and repeated measures over time were not required. The non-parametric tests used were the Mann-Whitney U-test, or when plausible (same individual measured twice over time) Wilcoxon's signed-rank sum test, and Spearman's correlation test: for details see the individual papers. The SPSS software (version 17 in studies I and IV, and IBM SPSS statistics 19 in studies II and III) was used for all statistical analyses. P-values less than 0.05 were considered significant.

Results

Demographics of the study groups

Demographic parameters, including age, BMI, and parity of all study groups included in this thesis are shown in table 2. Women on oral contraceptives were significantly younger than the naturally cycling women in the same study. PMDD patients were significantly older than healthy controls with regular menstrual cycles. BMI and parity did not differ significantly in women on OCs or women with PMDD compared to healthy naturally cycling women.

Table 2. Demographic characteristics of the study groups.

	HW_1, studies I and IV n=10	OC, study II n=10	HW_2, study II and III n=10	PMDD, study III n=10
Age, years	26 (19–30)	23 (20–38)*	26.5 (21–32)	35.5 (24–38)**
BMI	22 (20–25)	23 (21–31), ns	23 (19–35)	25 (20–37), ns
Parity, children	0 (0–2)	0 (0–2), ns	0 (0–2)	1.5 (0–4), ns

HW_{1-2}: naturally cycling healthy women, OC: healthy women using combined oral contraceptives, PMDD: otherwise healthy women with premenstrual dysphoric disorder, BMI: body mass index. All values expressed as median (range). The Mann-Whitney U-test tested differences between the OC and HW_2 groups in study II, and between PMDD and HW_2 groups in study III. * $p<0.05$, ** $p<0.01$, ns=non significant.

PMDD diagnosis

Before inclusion in the PMDD group, the women performed daily ratings on the Cyclicity Diagnoser scale (Segebladh *et al.*, 2009b) for two ovulatory cycles meeting the DSM IV diagnostic criteria for PMDD. Confirmation of diagnosis and inclusion in the study were done by an experienced gynecologist. Visual inspection of the CD scale and patient's history were the basis for diagnosis. Also, the impact on daily life had to be at least at the level that the family is disturbed for at least three days during the late luteal phase (Nyberg *et al.*, 2004). All PMDD women displayed at least one week with sparse symptoms during the follicular phase. Cyclicity in major mood symptoms is presented in figure 8.

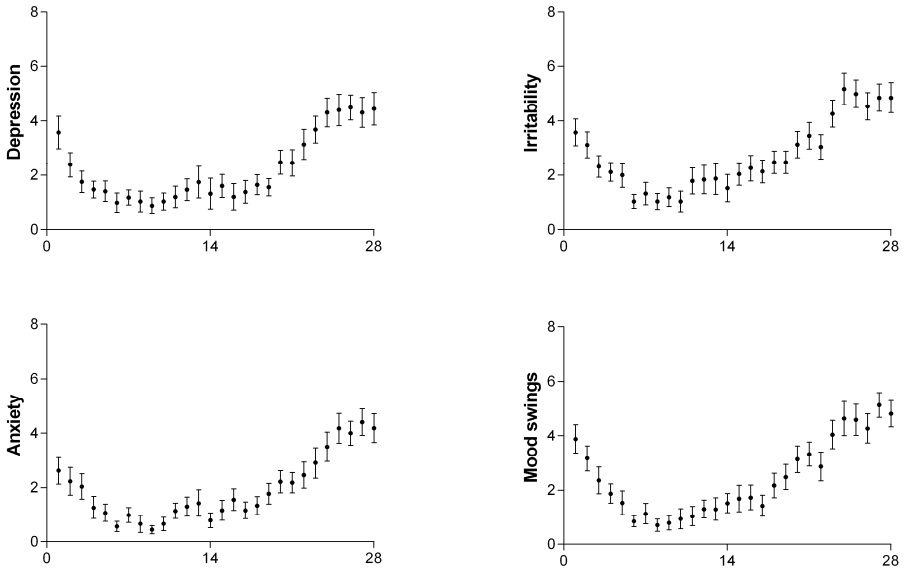

Figure 8. Daily assessed ratings of the items depression, anxiety, irritability, and mood swings on the Cyclicity Diagnoser (CD) scale performed by the PMDD patients included in study III, transformed into an idealized 28-day menstrual cycle. All values expressed as mean ± SEM.

Progestogen components in the oral contraceptives

All OCs were combinations of ethinyl estradiol and a progestogen. The ethinyl estradiol content was 30–40 μg. The progestogen components were of different types: five women used oral contraceptives with levonorgestrel, four women OCs with norethisterone acetate, and one with desogestrel. In addition, there were differences in the progestogen doses across the blister strip. Eight women had triphasic combinations of OCs and two women used monophasic OCs.

Timing of allopregnanolone challenge

Table 3 shows the actual cycle day for the test sessions in all study groups. There were no significant differences in cycle day testing in the follicular or luteal phase between the naturally cycling women with and without PMDD. All women on OCs were tested during the latter part of the blister strip.

Table 3. Timing of experiments in relation to menstrual cycle phase and OC blister strip.

	HW_1, studies I and IV n=10	HW_2, studies II and III n=10		PMDD, study III n=10		OC, study II n=10
	foll	foll	lut	foll	lut	blister day
Cycle day	6 (5–10)	8.5 (6–11)	25 (22–28)	8.5 (6–12)	22 (17–28)	19 (12–22)

HW_{1-2}: healthy naturally cycling women, PMDD: otherwise healthy women with premenstrual dysphoric disorder, OC: women using combined oral contraceptives, foll: follicular phase, lut: luteal phase of the menstrual cycle. One OC subject did not stop OC intake for withdrawal bleeding at day 21 and was tested on day 22. Values expressed as median (range).

Allopregnanolone concentrations at baseline

Blood samples for baseline serum concentrations of allopregnanolone were drawn in all subjects on the test day, before administration of intravenous allopregnanolone, and the results are summarized in table 4. Every menstruating subject had a higher allopregnanolone concentration at baseline in the luteal phase compared to the follicular phase. In addition, women on OCs had lower allopregnanolone concentrations than naturally cycling women in the follicular phase in study II. There was no difference in baseline serum concentrations of allopregnanolone between healthy women and women with PMDD, neither in the follicular phase nor in the luteal phase.

Table 4. Baseline serum concentrations of allopregnanolone.

	HW_1, studies I and IV n=10	HW_2, studies II and III n=10	PMDD, study III n=10	OC, study II n=10
Follicular phase	0.40 (0.30–0.79)	0.44 (0.32–0.62)	0.46 (0.20–2.74)	X
Luteal phase[a]	X	1.70 (0.82–6.20)**	2.15 (0.74–5.18)**	X
OC use[b]	X	X	X	0.19 (0.12–0.61)**

HW_{1-2}: healthy naturally cycling women, PMDD: otherwise healthy women with premenstrual dysphoric disorder, OC: women using combined oral contraceptives, X: not applicable. [a]The Wilcoxon signed-rank sum test tested differences between the follicular and luteal phases within the HW_2 group and within the PMDD group, [b]The Mann-Whitney U-test tested differences between the OC and the HW_2 groups. **p<0.01. All values in nmol/l, expressed as median (range).

Pharmacokinetics of intravenous allopregnanolone

To the best of our knowledge, allopregnanolone was administered intravenously to humans for the first time in study I. Pharmacokinetic parameters of intravenous allopregnanolone is shown in table 5. The distribution volume is large, indicating that allopregnanolone is distributed into fat tissue. Serum concentrations of allopregnanolone after the

cumulative injections are within the physiological range during third-trimester pregnancy.

Table 5. Pharmacokinetic parameters after three intravenous injections, representing a cumulative dose of 0.09 mg/kg allopregnanolone, to ten healthy women in the follicular phase of the menstrual cycle.

	Healthy women (n=10), mean ± SD
C_0	0.46 ± 0.2 nmol/l
C_5	20.9 ± 9.7 nmol/l
C_{35}	52.9 ± 26.2 nmol/l
C_{65}	71.8 ± 29.0 nmol/l
$t_{1/2,1}$	43.9 ± 7.3 min
$t_{1/2,z}$	261 ± 100 min
AUC	8897 ± 1467 nmol/(l x min)
CL	32.6 ± 5.8 ml/(min x kg)
V_z	12.5 ± 6.3 l/kg
V_{ss}	7.3 ± 2.5 l/kg

C_0: endogenous serum concentration of allopregnanolone; C_5, C_{35}, C_{65}: serum concentration of allopregnanolone 5 minutes after each allopregnanolone injection; $t_{1/2,1}$: distribution phase half-life; $t_{1/2,z}$: elimination phase half-life; CL: clearance; V_z: volume of distribution in the elimination phase; V_{ss}: volume of distribution at steady state; AUC: area under the serum concentration/time curve.

Serum concentrations of allopregnanolone after injections

Both the cumulative dose of 0.09 mg/kg allopregnanolone in studies I and IV, and the single dose of 0.05 mg/kg allopregnanolone in studies II and III, produced markedly increased serum concentrations of allopregnanolone in all subjects, comparable to endogenous levels during late pregnancy (Hill *et al.*, 2007). Maximum serum concentrations of allopregnanolone were displayed five minutes after the single injection in all study groups in studies II and III (table 6). No significant differences between or within the study groups were observed. However, a wide range was measured, and the implication of that will be discussed further on in this thesis.

Table 6. Peak serum concentrations of allopregnanolone.

	HW_2, n=10		PMDD, n=10		OC, n=10
	foll	lut	foll	lut	
C_5, median (range), nmol/l	63.6	57.5	90.3	81.3	73.5
	(37.4–98.3)	(31.1–153.6)	(39.4-148.3)	(62.1-158.9)	(47.5-128.0)
C_5, mean±SEM, nmol/l	67.5±6.4	73.5±13.2	87.7±9.6	93.1±10.5	79.8±7.3

Serum concentrations of allopregnanolone 5 min (C_5) after intravenous injection of 0.05 mg/kg allopregnanolone in the participants in studies II and III. For completeness, both median (range) and mean±SEM values are presented. OC: women using combined oral contraceptives in study II, HW_2: healthy naturally cycling women in studies II and III, PMDD: naturally cycling otherwise healthy PMDD patients in study III.

Sedative effects of allopregnanolone in women

The main finding in study I is that intravenous injections of allopregnanolone have sedative effects in women during the follicular phase of the menstrual cycle. The sedative effect was dose-dependent and measured by a significant decrease in SEV and an increase in subjectively rated sedation.

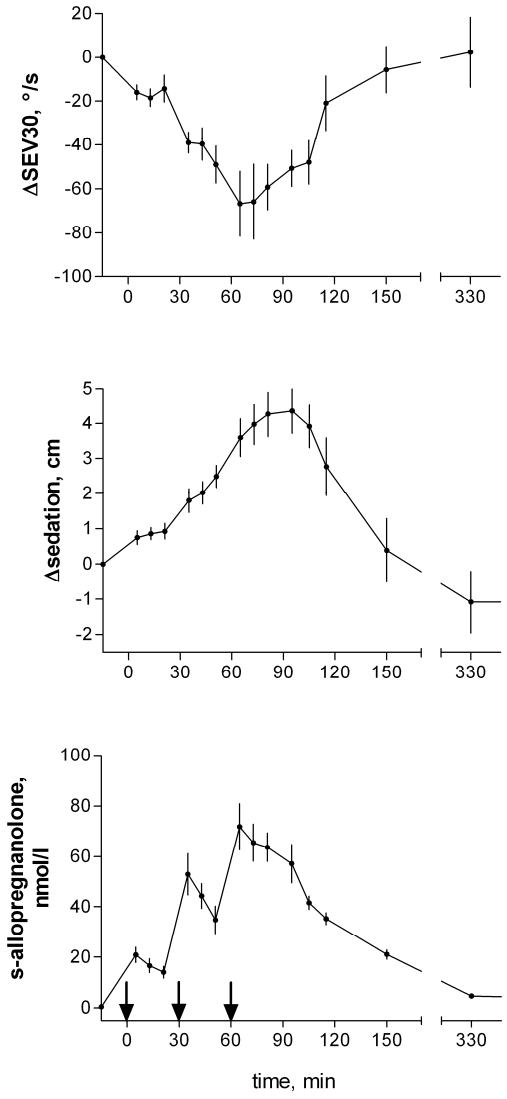

Figure 9. Sedative responses measured by decreased saccadic eye velocity (ΔSEV) and increased subjectively rated sedation (Δsedation) to three increasing doses of intravenous allopregnanolone (0.015, 0.03, 0.045 mg/kg), administered at 0, 30 and 60 minutes (indicated by arrows). Data based on measurements from 10 naturally cycling women in the follicular phase (study I). All values expressed as mean ± SEM.

The single dose 0.05 mg/kg of allopregnanolone in studies II and III was confirmed as sedative in terms of decreased SEV and increased subjectively rated sedation in both phases of the menstrual cycle as well as during use of oral contraceptives.

Allopregnanolone sensitivity over the menstrual cycle in healthy women

In healthy naturally cycling women, there was a significant association between menstrual cycle phase and the SEV response to the allopregnanolone injection, detected by a mixed model analysis of data, $F(1, 158.45)=5.70$, $p=0.018$. The SEV response estimation (β) by the mixed model was 10.2°/s less in the luteal phase than in the follicular phase, $t(158.45)=-2.39$, $p=0.018$. There was no significant phase x time interaction in the SEV response among women with regular menses, suggesting that the SEV responses were parallel during the follicular and luteal phase test sessions, but less pronounced in the luteal phase. Menstrual cycle phase was not associated with the subjectively rated sedation response to the allopregnanolone injection, and there was no phase x time interaction for subjectively rated sedation scores. There was no difference in baseline SEV values before the allopregnanolone injection between the follicular and luteal phases of the menstrual cycle in healthy women.

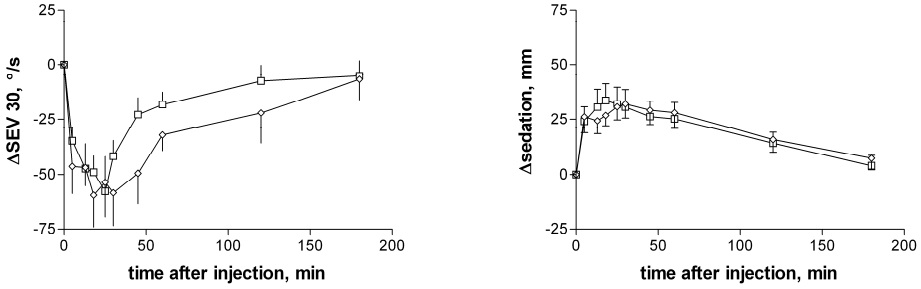

Figure 10. Sedative response to allopregnanolone, 0.05 mg/kg i.v. at 0 minutes, in 10 healthy menstruating women. ◇: delta values after injection in the follicular phase (error bars below) and □: delta values after exposure in the luteal phase (error bars above) of the menstrual cycle. All values expressed as mean ± SEM. The statistic results are presented in table 2 in paper II.

According to the separate lines of the SEV delta values for the follicular and luteal phases (figure 10), it seemed as if the difference between phases appeared after the maximum effect had occurred, e.g. about 30 minutes after the allopregnanolone injection. Mixed model analysis includes no post hoc options and therefore we chose to further analyze the data before and after 30 minutes after injection by two separate mixed model analyses. Looking at

the data in this way, we saw no significant association between menstrual cycle phase and the SEV response for the first 30 minutes. However, from 30 minutes onwards there was a significant difference in the SEV response depending on menstrual cycle phase, $F(1,81.85)=7.61$, $p=0.007$, with an estimation (β) of 15.5°/s lesser response in the luteal phase, $t(81.85)=-2.76$, $p=0.007$.

Allopregnanolone sensitivity in women on oral contraceptives

The responses on SEV and subjectively rated sedation together with the serum concentrations following allopregnanolone injection in women on OCs are shown in paper II, figure 1. The mixed model showed no significant differences in SEV response or subjectively rated sedation response to the allopregnanolone injection between the OC group and the individual menstrual cycle phases of healthy naturally cycling women. There was no group x time interaction regarding SEV response between the OC group and any of the menstrual cycle phases. This result indicates that the SEV response in women on OCs was parallel to the SEV response in both the follicular and the luteal phase among the naturally menstruating women. However, there was a significant group x time interaction in the subjectively rated sedation score between the OC group and the follicular phase, $F(1,158)=7.37$, $p=0.007$. Also, between the OC group and the luteal phase, the group x time interaction variable for subjectively rated sedation approached significance, $F(1,158)=3.03$, $p=0.084$. The group x time interactions could indicate that the response in subjectively rated sedation scores was not parallel between women on OCs and naturally menstruating women, at least not compared to the follicular phase. In addition, women on OCs showed a non-significant tendency to reach higher allopregnanolone serum concentrations after injection, see figure 1 in paper II. Still, their sedative responses were not augmented compared to menstruating women.

Allopregnanolone sensitivity in PMDD patients

Women with PMDD were significantly less sensitive to the allopregnanolone injection in the follicular phase, as compared to the luteal phase, in terms of SEV response, $F(1,168)=7.776$, $p=0.006$, and in terms of subjectively rated sedation, $F(1, 168)=13.735$, $p<0.001$. The mixed model estimation (β) of the lesser SEV response in the follicular phase was 7.8°/s, $t(168)=2.789$, $p=0.006$ and for the lesser subjectively rated sedation response β was –11.1 mm, $t(168)=-3.706$, $p<0.001$.

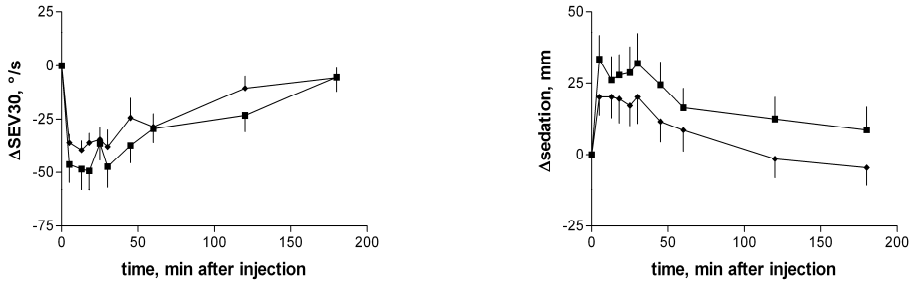

Figure 11. SEV responses and subjectively rated sedation responses over the menstrual cycle to a single injection of 0.05 mg/kg allopregnanolone i.v. at 0 min in 10 PMDD patients (◆follicular phase, ■luteal phase). All values presented as mean delta value ± SEM.

Allopregnanolone sensitivity in PMDD patients compared to healthy subjects

When PMDD patients were compared with controls, there was a highly significant group x phase interaction regarding the SEV response to allopregnanolone, $F(1, 327.489)=12.747$, $p<0.001$. The group x phase interaction indicates that the SEV responses were not parallel across the menstrual cycle phases in PMDD patients compared to women without premenstrual complaints. As presented above, PMDD women were less sensitive to allopregnanolone exposure in terms of SEV change and subjectively rated sedation in the follicular phase compared to the luteal phase. By contrast, healthy women were less sensitive to allopregnanolone exposure in terms of reduced SEV in the luteal phase compared to the follicular phase. There were no significant phase x time or group x time interactions. Considering each menstrual cycle phase separately, there were no significant between-group differences in the SEV response or the subjectively rated sedation response.

Allopregnanolone and the HPG axis

Allopregnanolone injections in study IV were associated with a significant decrease in FSH, $F(16, 144)=2.18$, $p=0.008$, and with a decrease in LH, $F(16, 144)=2.63$, $p=0.001$, based on analysis of variance (ANOVA). Moreover, there was a small but significant negative correlation between allopregnanolone concentrations and FSH levels, $r_s=-0.17$, $p=0.031$ (Spearman's correlation coefficient). By contrast, in subjects exposed to isoallopregnanolone injections, the gonadotropin concentrations (FSH and LH) did not change. Concerning the sex steroids, progesterone concentrations decreased after allopregnanolone injections, $F(16,144)=6.15$, $p<0.001$, but increased after isoallopregnanolone injections, $F(16, 64)=4.54$,

p<0.001. Estradiol concentrations did not change significantly after allopregnanolone or after isoallopregnanolone injections.

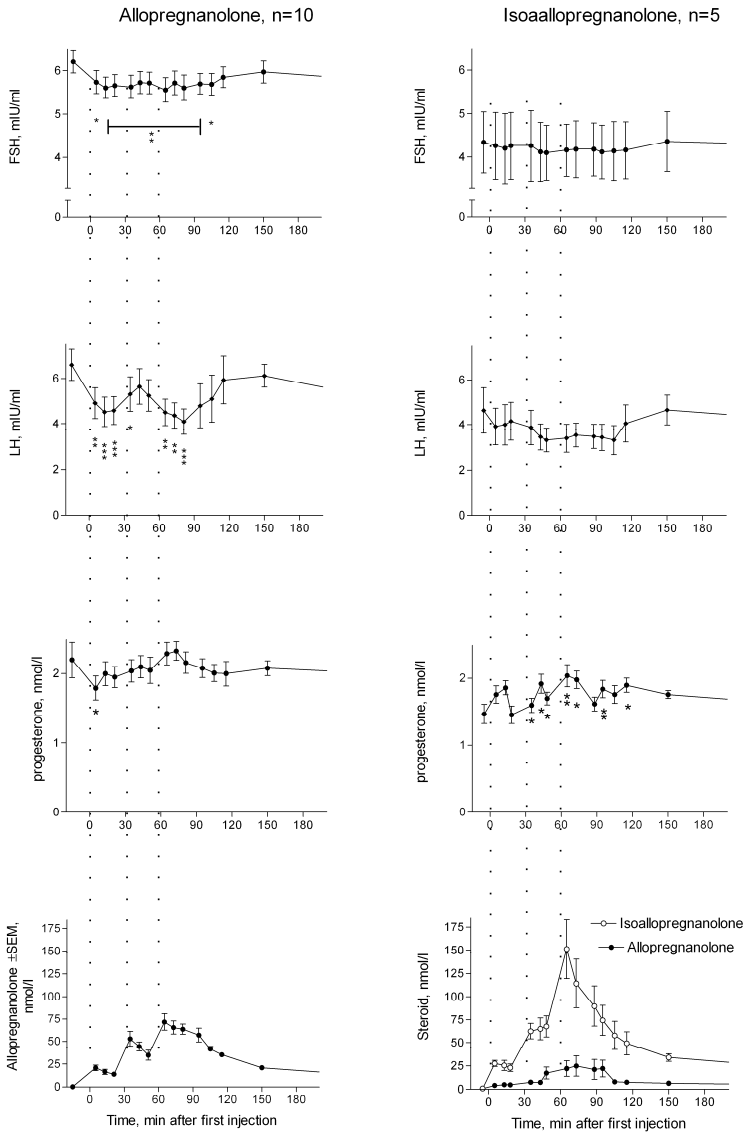

Figure 12. Serum concentrations of FSH, LH and progesterone after 0.015, 0.030 and 0.045 mg/kg doses of allopregnanolone (n=10), and after 0.04, 0.06, and 0.10 mg/kg doses of isoallopregnanolone (n=5). Injections indicated as dotted lines at 0, 30 and 60 min. Bottom panel shows subsequent increasing serum concentrations of allopregnanolone and isoallopregnanolone. Post hoc (LSD, least significant difference) analyses of changes compared to baseline indicated as *p<0.05 and **p<0.01. All values presented as mean ± SEM. Reprinted with kind permission from Informa Healthcare.

Anxiety Scales

One woman reported an anxiety attack within the 24 hours following allopregnanolone exposure in study I. At the time, this was considered a potential withdrawal effect comparable with those described after exposure to short-acting benzodiazepines, e.g. triazolam (Rang *et al.*, 1995a). To be able to detect paradoxical reactions to allopregnanolone injections, several anxiety scales were included in the subsequent studies (II and III). These scales are described in the introduction and the results are presented in each paper. Although there were some small changes within some individuals, during and after the experiment, these changes were not significant and not considered clinically relevant. In summary, no paradoxical reactions were detected during the test sessions or during the 24 hours following allopregnanolone exposure, neither in the follicular or luteal phase of the menstrual cycle, nor in the other study groups.

Side effects

Besides the expected feeling of sedation, non-severe side effects of allopregnanolone injections were reported by some subjects. In the first study, three women reported mild feelings of alcohol-like intoxication during the test session, three women experienced mild nausea and one woman reported flushing following the allopregnanolone injections. One woman reported an anxiety attack within 24 hours after allopregnanolone exposure. In the following single-dose studies, four women with PMDD reported mild feelings of alcohol-like intoxication. Brief vertigo was experienced by three women in the control group, by three women on oral contraceptives and by seven women in the PMDD group. Vertigo lasted generally for about five minutes and in no case longer than 15 minutes after allopregnanolone injection. Mild nausea was reported by two women, in one case prolonged for 14 days after the test session in the follicular phase. Other sensations reported by subjects were a short duration of strange taste (two control women, experienced during test sessions in both menstrual cycle phases), brief experience of double vision (two PMDD women, recurrent during both test sessions) and one woman on OCs who perceived a transient distortion of hearing. Two women with PMDD reported worsened acne following the test sessions in the follicular phase. A couple of hours after the test session in the follicular phase, one woman in the PMDD group had a sudden close-to-fainting feeling and had to sit down for a while. Documented side effects were based on spontaneous reports by subjects during the test sessions and follow-up, and as these reactions were sporadic no differences between study groups or across menstrual cycle phases were detected. No serious adverse events were reported or observed.

Discussion

Allopregnanolone sensitivity over the menstrual cycle

Allopregnanolone sensitivity in healthy women

We have demonstrated that allopregnanolone sensitivity in terms of SEV response is associated with menstrual cycle phase in healthy naturally cycling women. Allopregnanolone has rarely been administered to humans, and we believe that allopregnanolone was given intravenously to humans for the first time in study I, demonstrating unambiguous sedative effects. There are a few previous studies investigating the SEV response as a measurement of $GABA_A$ receptor sensitivity to some other $GABA_A$ steroids, besides allopregnanolone, over the menstrual cycle in healthy women without PMDD published by our group (Sundstrom *et al.*, 1997a; Sundstrom *et al.*, 1997b; Sundstrom *et al.*, 1998; Nyberg *et al.*, 2004). Our current results will primarily be discussed in relation to these previous studies. In contrast to earlier findings of an increased SEV response to allopregnanolone's 5β epimer, pregnanolone, given to healthy women in the luteal phase (Sundstrom *et al.*, 1998), we hereby report a decreased SEV response to allopregnanolone during the luteal phase among the healthy women given allopregnanolone. The increased SEV response to pregnanolone during the luteal phase among naturally cycling women was consistent with the finding that the SEV response to pregnanolone was increased when a synthetic progestogen was added to postmenopausal hormone replacement therapy (Wihlback *et al.*, 2001). However, in a similar study when vaginal progesterone was used instead of synthetic progestogens, the pregnanolone sensitivity in terms of SEV was increased by estradiol alone as well as in combination with natural progesterone compared to pre-treatment values (Wihlback *et al.*, 2005). The earlier finding of pregnanolone sensitivity over the menstrual cycle in healthy fertile women was in line with a report of increased SEV response in the luteal phase compared to the follicular phase in healthy women exposed to diazepam (Sundstrom *et al.*, 1997a). However, healthy women did not alter their SEV response to midazolam (Sundstrom *et al.*, 1997b) or to a low dose infusion of alcohol (Nyberg *et al.*, 2004) over the menstrual cycle.

These findings may seem inconsistent with the current finding of a decreased SEV response to allopregnanolone in the luteal phase compared to the follicular phase in healthy naturally cycling women. However, our results should not be compromised by these, in part contradictory, findings in earlier studies. Firstly, none of the above-mentioned studies actually

administered allopregnanolone, instead using other $GABA_A$ receptor agonists. Pregnanolone differs merely in the position of the hydrogen atom at the fifth carbon which is in β-position in pregnanolone and in α-position in allopregnanolone. The difference is important as the 5β-position gives a chair-like molecular structure while the α-position gives a planar molecular structure. They have similar actions at the $GABA_A$ receptor, although allopregnanolone is more potent in some respects, as shown in animals (Zhu *et al.*, 2001). Moreover, allopregnanolone and pregnanolone have been shown to inhibit each other when given simultaneously in an anesthetic model, suggesting that the binding properties to the receptor are not exactly the same (Norberg *et al.*, 1999). Furthermore, the pregnanolone solution, but not the allopregnanolone solution, contained a small amount of alcohol. The alcohol content could have acted in synergy with pregnanolone on the $GABA_A$ receptor (Sundstrom *et al.*, 1998). In addition to this, study protocols were not the same regarding intervals between injections during the experiments, and not regarding intervals between SEV recordings and, of major importance, the complete observation time after exposure was shorter in the previously mentioned studies.

Theories of tolerance

Theoretically, a continuous exposure to a ligand, such as allopregnanolone, during the luteal phase, would very likely decrease the sensitivity of the receptor to maintain a homeostatic balance. This is recognized during many different pharmaceutical treatments and the phenomenon is called tolerance. Tolerance development is a common phenomenon during chronic exposure to a number of different $GABA_A$ receptor agonists. In humans, repeated treatment with benzodiazepines and abuse of alcohol (both active at the $GABA_A$ receptor), resulting in chronic tolerance, is a well-known problem. Acute tolerance means that the sensitivity to a substance is reduced after/during single exposures (Kalant *et al.*, 1971). Acute tolerance to allopregnanolone has been reported in rats during anesthesia (Zhu *et al.*, 2004). Indications of acute tolerance to midazolam have been found in studies measuring SEV in humans (Ball *et al.*, 1991; Sundstrom *et al.*, 1997b). Interestingly, this was observed only in healthy women after the second injection (30 min after the initial injection), and only during the luteal phase (Sundstrom *et al.*, 1997b). In animal models testing anesthetic properties, acute tolerance to allopregnanolone did not occur earlier than 30 minutes after the start of exposure (Zhu *et al.*, 2004; Turkmen *et al.*, 2008). From visual inspection of the SEV graphs in healthy naturally cycling women in the present study, the impression is that the phase difference appears for the first time at 30 minutes, and that is why we have further explored the data by splitting the time at 30 minutes. Another reason for making this

extended exploration of our data is that the mixed model analysis does not offer any post hoc assumptions. When the time intervals from 5 to 30 minutes and from 30 to 180 min after the allopregnanolone injection were analyzed separately, a significant difference only from 30 minutes onwards was revealed. It is plausible that this finding is a demonstration of acute tolerance occurring in the luteal phase of the menstrual cycle as a consequence of circulating endogenous allopregnanolone, altering the central responsiveness to allopregnanolone. The phenomenon was recorded after 30 minutes of exogenous exposure, but not earlier, consistent with the animal findings and the findings in healthy women during the luteal phase in the midazolam study (Sundstrom *et al.*, 1997b; Zhu *et al.*, 2004).

Allopregnanolone sensitivity in PMDD patients

We have also demonstrated that allopregnanolone sensitivity in terms of SEV is associated with menstrual cycle phase in PMDD patients, with a lesser SEV response in the follicular than the luteal phase. The change in SEV response to allopregnanolone across the menstrual cycle is in the reverse direction in PMDD patients compared to healthy women, demonstrated by significant within-group differences and moreover, by a highly significant group x phase interaction.

There have consistently been findings of an altered SEV response among PMDD patients over the menstrual cycle compared to healthy women in the previous studies of sensitivity to other $GABA_A$ receptor agonists than allopregnanolone (Sundstrom *et al.*, 1997a; Sundstrom *et al.*, 1997b; Sundstrom *et al.*, 1998; Nyberg *et al.*, 2004). However, on closer look, the results are somewhat divergent (Sundstrom *et al.*, 1997a; Sundstrom *et al.*, 1997b; Sundstrom *et al.*, 1998; Nyberg *et al.*, 2004) and deserve to be scrutinized in the context of the current studies. For instance, PMDD patients did not alter their SEV response to pregnanolone or diazepam over the menstrual cycle as did healthy subjects; see above (Sundstrom *et al.*, 1997a; Sundstrom *et al.*, 1998). However, PMDD patients displayed a blunted SEV response to a low dose infusion of alcohol in the late luteal phase compared to the follicular phase (Nyberg *et al.*, 2004). In a study of midazolam, the SEV response was decreased in the follicular phase among PMDD patients compared to healthy women (Sundstrom *et al.*, 1997b). On the other hand, healthy women did not increase their SEV response to midazolam in the luteal phase compared to the follicular phase (Sundstrom *et al.*, 1997b), as they did to diazepam (Sundstrom *et al.*, 1997a). Moreover, the fact that the reduced SEV response to diazepam during the luteal phase in PMDD patients not was replicated for midazolam is interesting in the light of the different SEV responses to pregnanolone and allopregnanolone across the menstrual cycle. At the $GABA_A$ receptor, midazolam is a more potent

benzodiazepine than diazepam (Buhrer *et al.*, 1990), and allopregnanolone a more potent steroid than pregnanolone (Zhu *et al.*, 2001). Given the indications that tolerance is more easily developed to more potent substances, this could be an explanation for the divergent SEV responses within the benzodiazepine group of substances, as well as within the 5α- and 5β epimers (Turkmen *et al.*, 2011). In that way, failure to replicate initial findings within each substance group could in fact be dependent on basal pharmacological properties. The possibility exists that in the healthy women the endogenous allopregnanolone production during the luteal phase will change the $GABA_A$ receptor expression and thus change the response to more potent agonists but not to less potent compounds. PMDD patients on the other hand, might differ in $GABA_A$ receptor plasticity compared to healthy women.

The finding of a lesser SEV response to allopregnanolone within the PMDD patients during the follicular phase is in line with a lesser SEV response to midazolam in the follicular phase among PMDD patients. Thus, the $GABA_A$ receptor dysfunction among PMDD women might not be restricted to the luteal phase. In contrast, the earlier findings of an increased $GABA_A$ receptor sensitivity in healthy women during the luteal phase might be misleading in the interpretation of the physiological response to $GABA_A$ steroid cyclicity. Presume that the brain physiologically adapts to ovarian cyclicity by mechanisms of tolerance development to endogenous $GABA_A$ receptor agonists, and that this phenomenon is more or less pronounced across the spectrum of $GABA_A$ steroids. It then seems reasonable that a lesser SEV response in the luteal phase among healthy women could be due to tolerance to endogenous circulating $GABA_A$ receptor agonists, and represents a physiological response to $GABA_A$ steroids produced by the corpus luteum during the luteal phase. The marked first-trimester fatigue which declines in spite of increasing allopregnanolone serum concentrations throughout pregnancy is a common phenomenon supporting the theory of a physiologically tolerance development. Moreover, allopregnanolone activates the $GABA_A$ receptor at physiological low nM concentrations (Lambert *et al.*, 2001), and peripheral levels are reflected in the brain (Bixo *et al.*, 1997). Looking at PMDD as a deficient adaptation to normal ovarian cyclicity, the implication of our present results is that women with PMDD fail to develop physiological tolerance to endogenous circulating allopregnanolone during the luteal phase. Though there are divergences in previous and current results exploring $GABA_A$ receptor sensitivity in relation to the menstrual cycle and PMDD, the general conclusion from all of them is completely uniform, namely that $GABA_A$ receptor sensitivity in PMDD patients differs from that in healthy women. Our results add further evidence

to the indication of a link between the vulnerability to normal ovarian cyclicity in PMDD women and aberrant GABAergic transmission.

Allopregnanolone sensitivity in women on combined oral contraceptives

Our interest in allopregnanolone sensitivity in women on OCs originated from numerous studies showing inevitable links between the progestogen component and side effects, such as negative mood (Cullberg, 1972; Sanders *et al.*, 2001). The mechanism by which OCs exert negative effects on mood is not known (Kurshan and Neill Epperson, 2006). The temporal association between negative mood and the progesterone production after ovulation in PMDD is obvious, and we hypothesized that the GABAergic transmitter system, which is the focus of this thesis, might be involved in OC-induced negative mood. In addition, OCs have been reported to change $GABA_A$ receptor subunit composition in rats (Follesa *et al.*, 2002). Thus, we wanted to explore if the sensitivity to allopregnanolone was altered by the potent synthetic steroids in OCs.

Women on OCs did not differ significantly in their SEV response, or the subjectively rated sedation response to allopregnanolone compared to any of the menstrual cycle phases of healthy naturally cycling women in study II. However, there were group x time interactions in the subjectively rated sedation response between women on OCs and naturally cycling women. The group x time interaction was significant in the follicular phase and close to significant in the luteal phase. This indicates that allopregnanolone sensitivity potentially could be altered by synthetic progestogens. To speculate, tolerance to $GABA_A$ receptor substances and thereby a decrease in the $GABA_A$ receptor sensitivity induced by synthetic steroids may occur. The use of OCs means a daily intake of potent synthetic hormones (ethinyl estradiol and a progestogen) for 3 out of 4 weeks, and represents a more stable endocrine state compared to the variations in ovarian hormones during the natural menstrual cycle. During OC treatment, the levels of ovarian hormones and metabolites including peripheral allopregnanolone are low (Follesa *et al.*, 2002; Rapkin *et al.*, 2006). Our results regarding allopregnanolone sensitivity in women on OCs are rather vague, and also reveal the possibility of a difference between SEV reduction and increased subjectively rated sedation in the same individuals. SEV is thus not solely a measurement of sedation, evidenced by the finding that thyrotropin-releasing hormone (TRH) reversed the subjectively rated sedation but not the SEV decrease induced by benzodiazepines (Glue *et al.*, 1992).

Adverse mood susceptibility

Although a potential role for estrogens in PMDD cannot be ruled out, the role of progesterone and synthetic progestogens in deteriorating mood is solid (Cullberg, 1972; Bjorn *et al.*, 2000; Sanders *et al.*, 2001; Andreen *et al.*, 2003; Wihlback *et al.*, 2005). Some studies found that women with PMS/PMDD report more negative mood side effects from synthetic progestogens (Cullberg, 1972; Bjorn *et al.*, 1999), while others did not find associations between prior OC-induced negative mood and prospectively rated PMS symptoms in ovulatory cycles (Segebladh *et al.*, 2009b). In most women, OCs have a stabilizing effect on mood (Oinonen and Mazmanian, 2002; Joffe *et al.*, 2003). Also, a majority of women recognize mild changes in some symptoms premenstrually but are not at all on the verge of PMDD (Sveindottir and Backstrom, 2000; Wittchen *et al.*, 2002; Duenas *et al.*, 2011). As stated in the introduction, PMDD pathophysiology is not fully known, and other transmitter systems in addition to the GABA system are proposed to be aberrant in PMDD patients. In the clinic, SSRI treatment is currently prescribed to PMDD patients, but in contrast to treatment of major depression, efficacy is rapid and evident during intermittent treatment restricted to the luteal phase (Brown *et al.*, 2009). In another SEV study, the SEV response to pregnanolone was increased in PMDD patients by the selective serotonin reuptake inhibitor (SSRI) citalopram (Sundstrom and Backstrom, 1998a), suggesting links between the serotonergic and GABAergic transmitter systems. In fact, there is evidence from preclinical studies that SSRIs not only have an impact on the serotonin transmitter system, but directly affect the enzymatic efficiency of 3α-hydroxysteroid dehydrogenase, which is the last of two enzymatic steps in allopregnanolone formation from progesterone (Griffin and Mellon, 1999; Pinna *et al.*, 2006). This supports the hypothesis that the benefits of SSRIs in PMDD do not merely depend on effects on serotonergic transmission.

Women with PMDD seem to have an adverse response to normal ovarian cyclicity and disturbances in GABAergic transmission is proposed to be an underlying mechanism that could explain their vulnerability to effects of ovarian steroids (Sundstrom *et al.*, 1998; Gulinello *et al.*, 2001; Smith *et al.*, 2006; Kaura *et al.*, 2007). Paradoxical reactions to several $GABA_A$ receptor agonists, including allopregnanolone, have been described, both in laboratory animals (Fish *et al.*, 2002; Miczek *et al.*, 2002; Miczek *et al.*, 2003) and in humans (Cherek *et al.*, 1992; Dougherty *et al.*, 1996; Ben-Porath and Taylor, 2002; Andreen *et al.*, 2006). Notably, similar frequencies of paradoxical reactions to midazolam (10.2% in a sample of 58 patients undergoing surgery) and PMDD (3–8%) are reported (Weinbroum *et al.*, 2001; Halbreich *et al.*, 2003; Backstrom *et al.*, 2011). An explanation could be a disturbed $GABA_A$ receptor function in these subpopulations of

individuals. The findings of induced changes of the $GABA_A$ receptor subunit composition by the estrus cycle (Lovick *et al.*, 2005; Maguire *et al.*, 2005), by exogenous steroids (Follesa *et al.*, 2002; Smith *et al.*, 2006), and by $GABA_A$ receptor active pharmaceutics (Uusi-Oukari and Korpi, 2010) point to changes in $GABA_A$ receptor plasticity as a possible explanation. $GABA_A$ receptor plasticity may vary across individuals making some individuals liable to paradoxical reactions. In study I, one subject reported an anxiety attack within the 24 hours following allopregnanolone exposure. We then included several anxiety scales in the subsequent studies II and III, but were unable to detect any paradoxical reactions to allopregnanolone in the study groups. However, as the allopregnanolone dose resulted in allopregnanolone serum concentrations well above physiological levels across the menstrual cycle, we are not able to conclude, from the present studies, how reactions would be to lower allopregnanolone doses.

Allopregnanolone decreases gonadotropin levels

The novel finding of decreased levels of gonadotropins after allopregnanolone injections was somewhat unexpected, especially as the 5β epimer to allopregnanolone, pregnanolone, had no impact on gonadotropin levels in a previous study (Sundstrom and Backstrom, 1999). On the other hand, several preclinical investigations have shown that allopregnanolone affects the female reproductive axis (Genazzani *et al.*, 1995; Calogero *et al.*, 1998; Sim *et al.*, 2001; Laconi and Cabrera, 2002; Sullivan and Moenter, 2003). In addition to this, the reports of increased allopregnanolone levels during hypothalamic amenorrhea (Genazzani *et al.*, 2002) and during premature ovarian failure (Bernardi *et al.*, 1998) made us realize the importance of avoiding a statistical type 1 error in study IV. Therefore, we included women that had been exposed to the 3β epimer of allopregnanolone, i.e. isoallopregnanolone, known to have no agonistic effect but in some studies an antagonistic effect on the allopregnanolone enhancing $GABA_A$ receptor effect (Gee *et al.*, 1987; Backstrom *et al.*, 2005; Stromberg *et al.*, 2006). There was no difference in gonadotropin levels after isoallopregnanolone injections in these women who were exposed during the follicular phase of the menstrual cycle. Furthermore, the levels of progesterone and estradiol did not increase after allopregnanolone injections. Thus, the lower gonadotropin levels could not possibly be a result of negative feedback on the HPG axis. Notably, the measurements of lower FSH and LH are present already after the first allopregnanolone injection when the produced serum concentrations of allopregnanolone correspond to a physiological range. In addition, serum concentrations of allopregnanolone rise to a similar level after the third injection of isoallopregnanolone in the isoallopregnanolone group. However, among the women who received

isoallopregnanolone injections, FSH and LH were unaffected by the rise in allopregnanolone. This finding indicates that the effect on FSH and LH levels in the allopregnanolone group of women is mediated via the $GABA_A$ receptor.

The finding of lowered levels of FSH and LH after allopregnanolone exposure is indeed interesting. The mechanism behind hypothalamic amenorrhea is not fully known, but there are connections between the HPA axis and the HPG axis (Fritz and Speroff, 2011a). Women with this condition have increased allopregnanolone levels in serum (Genazzani *et al.*, 2002). In addition, allopregnanolone is released during stress in both sexes (Droogleever Fortuyn *et al.*, 2004). Given the preclinical results on impaired reproduction by allopregnanolone, our finding points to a potential mechanism behind stress-induced hypothalamic amenorrhea, worth further investigations. In summary, we argue for an impact on circulating gonadotropin levels by allopregnanolone exposure via the $GABA_A$ receptor in paper IV. Whether this impact consists of reduced synthesis, decreased secretion, altered pulsatility, or increased metabolism remains unclear. With respect to the limitations of study IV, the result should be interpreted with caution until replicated.

Methodological considerations

Size of study population

Based on earlier finding of a standard deviation of SEV within individuals of ± 7°/s, statistical power analysis calculated a study size of seven subjects in each group in studies II and III. However, in the present study group of ten healthy naturally cycling women, the mean intra-individual standard deviation was larger, namely 16.4°/s and 13.3°/s in the follicular and luteal phase respectively, and in the ten women on OCs 16.6°/s. Obviously, this discrepancy from calculations based on earlier studies might explain why we were unable to detect any between-group differences considering each menstrual cycle phase separately in studies II and III. On the other hand, the statistical power calculation was performed to detect differences within groups over the menstrual cycle, and not between groups.

Absence of placebo control

We chose not to include placebo control in the present studies and this was based on the results from earlier studies of $GABA_A$ receptor sensitivity measured by SEV response to benzodiazepines and alcohol which did not record any effects by placebo (Sundstrom et al., 1997a; Sundstrom et al., 1997b; Nyberg et al., 2004).

Number and order of test sessions

In studies II and III, the number of test sessions, in the naturally cycling women was two, and among women on OCs one. As group comparisons of the sedative response measured by SEV and subjectively rated sedation were made between OCs and each of the menstrual cycle phases separately, it does not seem very likely that the different number of test sessions in study II might have influenced the group comparisons. The order of the test sessions was random among naturally cycling women. Six healthy women had their first test in the follicular phase and four in the luteal phase, while seven women with PMDD had their first test in the follicular phase and three in the luteal phase. The fact that the SEV after allopregnanolone exposure returned to baseline levels already during the test session in studies I, II and III indicates that the primary allopregnanolone exposure did not influence the SEV response on the second test occasion, which was at least two weeks removed.

Influence of body weight and other possible confounders

Doses of allopregnanolone were consistently based on body weight. A cumulative dose of 0.09 mg/kg was used in studies I and IV, and a single dose of 0.05 mg/kg in studies II and III. The serum concentrations of allopregnanolone following allopregnanolone injections varied between individuals, and also between menstrual cycle phases within some individuals in a non-consistent way. Women on OCs and women with PMDD tended to reach higher peak serum concentrations of allopregnanolone, although the change was not significant compared to the control subjects. No significant differences in BMI could be detected between the groups that would have explained the wide range in allopregnanolone serum concentrations post-injection. However, the BMI range within the groups was broad, and an influence on the results from body weight dosing of allopregnanolone cannot be totally ruled out. Nevertheless, dosing per kg body weight is the most common way to determine the dosages of a pharmaceutical to an individual, although there are alternatives, e.g. dosages determined by lean body mass.

Other explanations for the wide range in post-injection allopregnanolone serum concentrations could be individual capacities for protein binding or poor quality of the allopregnanolone solution. The former explanation is not investigated, but it is well known that steroid hormones bind to plasma proteins (Strauss and Barbieri, 2009b). For pregnanolone, protein binding is about 1.1% in women (Dale *et al.*, 1999), but similar data for allopregnanolone protein binding are lacking. Poor quality of experimental substance should not be an issue to consider, as the study solutions were

prepared rigorously by the University Hospital Pharmacy. This production has earlier been proven reliable and the measured concentrations in the solution showed a very small variance, see methods section. Another fact to be considered is the rate of metabolism which might vary between the OC group and naturally cycling women. Allopregnanolone is metabolised by glucuronidation and excreted to urine (Sear, 1998). During the use of OCs, the liver receives high amounts of synthetic steroids by first-passage metabolism. This might lower the metabolic capacity of allopregnanolone in women on OCs and explain the tendency towards higher serum concentrations of allopregnanolone compared to controls five minutes after injection.

Impact of different progestogens

The progestogen content in the OCs in study III was heterogeneous, a circumstance that might blur our result. Progestogens and their metabolites may differ from progesterone and allopregnanolone concerning their effects in the central nervous system. The relation between synthetic progestogens and their impact on neurosteroid synthesis and metabolism is complex, and studies in humans are scarce. However, animal studies have shown that the progestogen medroxyprogesterone acetate (MPA) can induce anesthesia and also increase central allopregnanolone levels (Meyerson, 1967; Bernardi *et al.*, 2006). This effect was hypothesized to be due to the formation of progesterone resembling metabolites (i.e. allopregnanolone). The molecular structure of the progestogen is obviously important for the central effects and the influence on neurosteroids. For instance, the testosterone 19-nor derivatives norethisterone acetate (NETA) and levonorgestrel (LNG) induced a lower rise in serum allopregnanolone compared to progesterone derivatives such as MPA during postmenopausal hormonal replacement therapy (Bernardi *et al.*, 2003), and this effect was not different from the impact of estrogen-only replacement therapy. Likewise, the spironolacton derived progestogen drospirenone did not interfere with allopregnanolone production in female rats (Genazzani *et al.*, 2007). Skin 5α-reductase was blocked by oral contraceptive progestogens (e.g. LNG) *in vitro*, providing another mechanism for these compounds to affect neurosteroid metabolism as 5α-reductase is crucial in the formation of allopregnanolone (Rabe *et al.*, 2000). In addition, MPA has been shown to block 3α-hydroxysteroid dehydrogenase, which is the other enzyme involved in allopregnanolone formation (Bernardi *et al.*, 2003). In retrospect, a more homogenous composition of progestogens in the OC group would have been preferable in study II.

Healthy women as control subjects

Premenstrual symptoms are common, especially physical complaints, but also mildly deteriorated mood (Sveindottir and Backstrom, 2000; Lete *et al.*, 2011). However, no premenstrual psychological symptoms whatsoever were allowed in the healthy women in studies II and III. Daily assessed scorings on the CD scale during at least one ovulatory cycle prior to inclusion would preferably have been an inclusion criterion in the controls, but this was not done for practical reasons. However, they were carefully asked about premenstrual symptoms during the screening visit. Further, the PRIME-MD questionnaire was used to exclude psychiatric disorders and this diagnostic tool includes a question about premenstrual symptoms.

Concluding remarks and future perspectives

In this thesis, we demonstrate that intravenous administration of allopregnanolone, rapidly increasing the allopregnanolone serum concentrations to levels found during pregnancy, is sedative in women. We also demonstrate that the sensitivity to allopregnanolone is associated with menstrual cycle phase in naturally cycling women with and without PMDD. However, the allopregnanolone sensitivity changed in reverse directions across the menstrual cycle within the group of healthy women who had a decreased sensitivity during the luteal phase and within the PMDD patient group who, by contrast, had a decreased sensitivity during the follicular phase. This divergence in allopregnanolone sensitivity across the menstrual cycle within the two groups indicates that PMDD patients have a dysregulation in $GABA_A$ receptor function. The implication of our results is that women with PMDD lack the ability to adapt to the endogenous circulation of $GABA_A$ receptor steroids during ovulatory cycles. We suggest that a kind of "physiological tolerance" to endogenous allopregnanolone could be a part of the normal menstrual cycle. Studies of SEV through pregnancy and postpartum would be interesting to perform in healthy women. Moreover, it would be of interest to further elucidate correlates of $GABA_A$ receptor function, e.g. by brain imaging techniques, in women with PMDD. Extended knowledge about the $GABA_A$ receptor function in PMDD patients compared to controls is needed and possibly, in the future, such knowledge would enable a new target for treatment. In women on OCs, allopregnanolone sensitivity might be influenced by the exposure to synthetic steroids during OC treatment, but no definitive conclusion can be drawn from the present studies. Nevertheless, adverse mood induced by synthetic progestogens has similarities to PMDD, and future studies of OC treatment and a possible connection to the $GABA_A$ receptor system are warranted. Such studies should be designed to compare women with adverse mood effects by OCs and women without adverse mood effects by OCs.

Moreover, it is still unclear whether women who experience adverse mood effects by synthetic progestogens are the same women that are afflicted by PMDD, and it would be interesting to find out possible genetic factors. The current results on allopregnanolone exposure and decreased gonadotropin levels are preliminary and worth investigating further. New knowledge of the mechanisms behind hypothalamic amenorrhea might offer new targets for therapies. For women who do not want, or are unable to use, hormone replacement therapy, other therapeutic options are needed, especially as this condition is complicated by osteoporosis and infertility when untreated. However, when facing the patient, stress elimination must be the first step.

General Conclusions

- Allopregnanolone administered intravenously had dose dependent sedative effects in women measured by decreased saccadic eye velocity (SEV) and increased subjectively rated sedation.
- The sensitivity to exogenous allopregnanolone in terms of SEV response was less pronounced during the luteal phase than in the follicular phase of the menstrual cycle in healthy women without PMDD.
- The sensitivity to exogenous allopregnanolone in terms of SEV response did not differ in women on OCs compared to naturally cycling women, but there were indications of an altered subjectively rated sedation response in women on OCs.
- Allopregnanolone sensitivity in terms of SEV and subjectively rated sedation was less pronounced in the follicular phase than in the luteal phase of the menstrual cycle in women with PMDD, as opposed to the result within the group of healthy women without PMDD. Thus, a dysregulation of $GABA_A$ receptor function in PMDD patients is suggested.
- Allopregnanolone injections decreased gonadotropin levels in healthy women in the follicular phase of the menstrual cycle.

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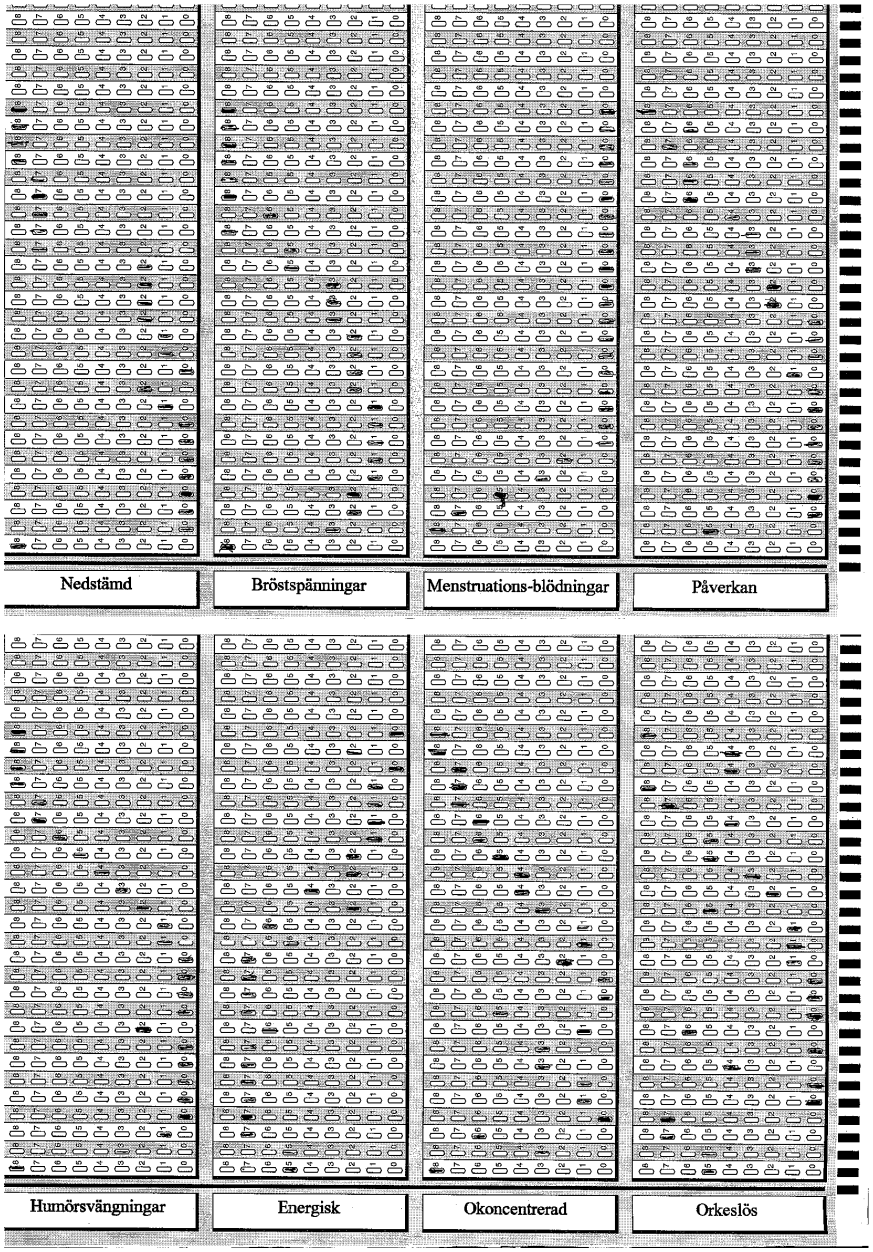
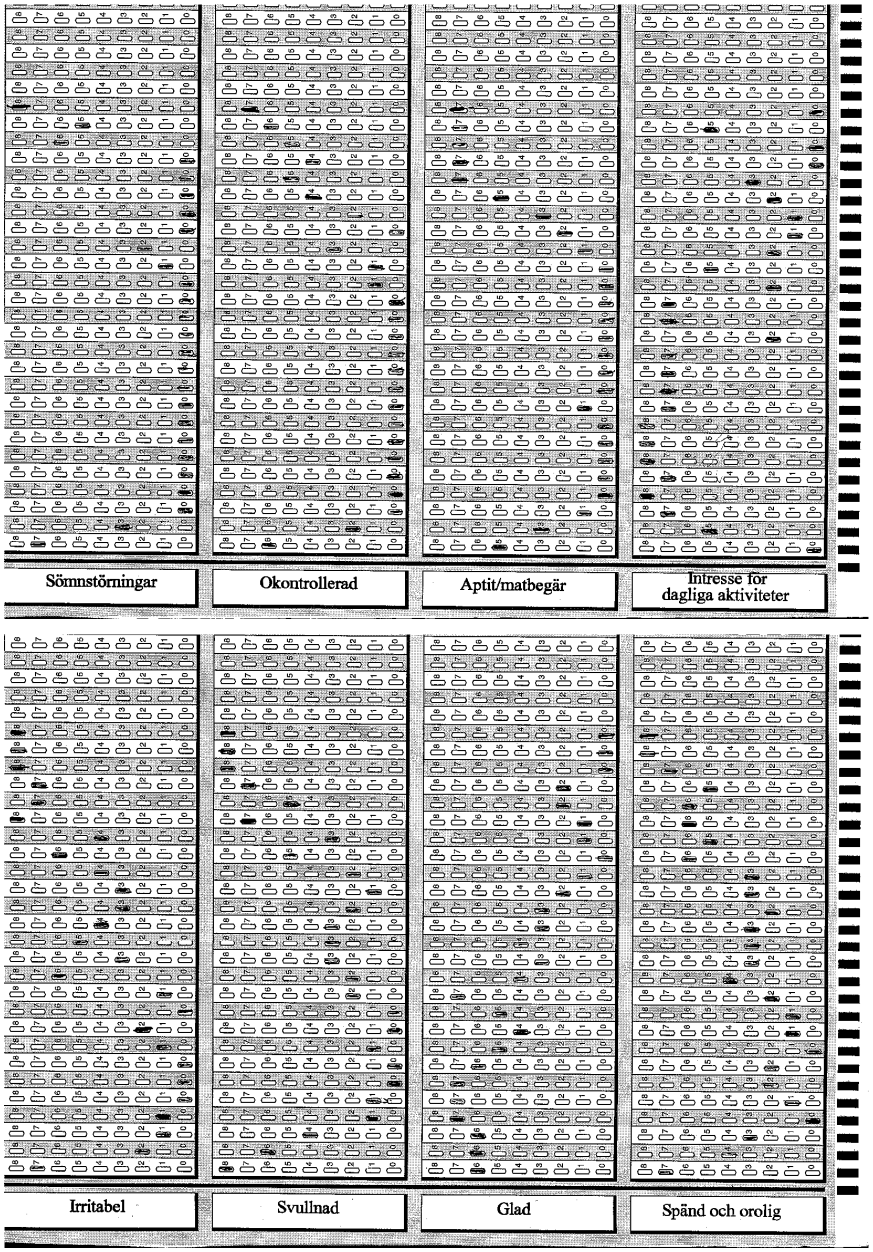
Appendix

One PMDD patient's scoring on the Cyclicity Diagnoser scale during one 26-day ovulatory menstrual cycle prior to inclusion in the PMDD group in study III.




Sömnstörningar
Okontrollerad
Aptit/matbegär
Intresse för dagliga aktiviteter
Irritabel
Svullnad
Glad
Spänd och orolig