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Obstetrics and Gynecology
Department of Clinical Sciences
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GABA-steroid effects in healthy subjects and women with polycystic ovary syndrome

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To my family with love

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ABSTRACT

Background: The progesterone metabolite allopregnanolone is involved in several clinical conditions in women, e.g. premenstrual dysphoric disorder. It is a very potent GABA-steroid with GABA-A receptor effects similar to other GABA-agonists, e.g. benzodiazepines, and it causes sedation. An objective way to examine effects on the GABA-A receptor in humans is to measure saccadic eye velocity (SEV), which is reduced by GABA-agonists, e.g. allopregnanolone. Animal studies suggest that allopregnanolone is involved in the regulation of gonadotropin secretion via the GABA-A receptor, but this has not been studied in humans. Polycystic ovary syndrome (PCOS) is the most common endocrine disturbance among women of fertile age (5–10%), characterized by polycystic ovaries, menstrual dysfunction, hyperandrogenity, and 50% have obesity. Studies have shown higher allopregnanolone levels in overweight people. PCOS women have increased levels of androstenediol, an androgen metabolite which is a GABA-A receptor agonist. Tolerance often occurs when persons are exposed to high levels of GABAergic modulators. It has not been studied whether GABA-A receptor sensitivity in PCOS women is changed. Another progesterone metabolite, isoallopregnanolone, is the stereoisomere of allopregnanolone but has not been shown to have any GABA-A receptor effect of its own. Instead it has often been used to control steroid specificity to allopregnanolone.

Aims: To compare the effects of allopregnanolone and isoallopregnanolone on gonadotropin secretion. To compare allopregnanolone levels, GABA-A receptor sensitivity to allopregnanolone and effects on gonadotropin secretion in both cycle phases and PCOS conditions. To examine pharmacokinetics and pharmacodynamic properties for isoallopregnanolone.

Method: In the follicular phase healthy women were examined for the effect of allopregnanolone or isoallopregnanolone on gonadotropin secretion. PCOS women and healthy women in both cycle phases were given allopregnanolone and the differences in effects on SEV were examined, as well as changes in serum levels of gonadotropins and allopregnanolone at baseline and during the test day. Pharmacokinetics and GABA-A receptor sensitivity using SEV were explored for isoallopregnanolone in healthy women.

Results: Allopregnanolone decreases gonadotropin serum levels in healthy controls in both cycle phases, but has no effect on gonadotropin secretion in

women with PCOS. PCOS women have higher baseline serum levels of allopregnanolone than follicular phase controls, but lower levels than luteal phase controls. PCOS women show greater reduction in SEV to allopregnanolone than controls. Isoallopregnanolone has no effect on gonadotropin secretion. There is an effect of isoallopregnanolone on SEV, explained by a metabolism of isoallopregnanolone into allopregnanolone.

Conclusion: There are significant differences in the GABA-A receptor response to a GABA-steroid in different endocrine conditions in women of fertile age examined with saccadic eye velocity. The GABA-steroid allopregnanolone decreases gonadotropin serum levels in healthy women but not in PCOS women. The lack of effect on gonadotropins by isoallopregnanolone suggests an involvement of the GABA-A receptor.

SAMMANFATTNING PÅ SVENSKA

Bakgrund

Det kvinnliga könshormonet progesteron (gulkroppshormon) har flera nedbrytningsprodukter i kroppen varav en, allopregnanolon, är speciellt potent och involverad i flera kliniska tillstånd hos kvinnor, till exempel vid premenstruellt syndrom. Allopregnanolon är en stark aktiverare av GABA-A-receptorn i hjärnan, där GABA-systemet är ett hämmande receptorsystem. Allopregnanolon är en så kallad agonist, vilket innebär att den har samma stimulerande effekt av receptorn, som de substanser som normalt verkar på den. Andra agonister på GABA-A-receptorn är bensodiazepiner (lugnande, sömngivande och ångestlindrande), barbiturater (narkosmedel) och alkohol och de ger sedation (trötthet). Allopregnanolons koncentrationer varierar parallellt med progesteron under menscykeln med högst nivåer efter ägglossningen i menscykelns lutealfas. Ett objektiva sätt att mäta allopregnanolonets effekt på GABA-A-receptorn hos människa är att mäta ögonsackadastigheter (SEV), vilken minskas av GABA-agonister t.ex. allopregnanolon. Djurstudier har visat att allopregnanolon är involverat i regleringen av reproduktionscykeln genom påverkan på luteiniserande hormon (LH) och follikelstimulerande hormon (FSH) via GABA-A-receptorn. Detta är inte studerat tidigare hos människan.

Polycystiskt ovarial syndrom (PCOS) är den vanligaste endokrina rubbningen bland kvinnor i fertil ålder (5–10 %), och karakteriseras av polycystiska ovarier, amenorré eller oligomenorré (utebliven eller gles menstruation) och ökad androgenaktivitet. 50 % av kvinnor med PCOS är obesa. Studier har påvisat högre allopregnanolonnivåer hos överviktiga flickor jämfört med smala flickor och hos överviktiga vuxna jämfört med normalviktiga vuxna. PCOS har förhöjda nivåer av androstanediol, en androgenmetabolit och GABA-A-receptoragonist. Tolerans kan uppkomma när en person är utsatt för höga nivåer av GABA-A receptorstimulerare, till exempel bensodiazepiner. Det är inte studerat huruvida GABA-A-receptor-känsligheten är förändrad hos kvinnor med PCOS.

En annan progesteronnedbrytningsprodukt är isoallopregnanolon som är en stereoisomer (spegelbild) av allopregnanolon i sin struktur. Den saknar egen effekt på GABA-A-receptorn i *laboratorie- och djur*-studier. Isoallopregnanolon har ofta använts som negativ kontroll för allopregnanolon när allopregnanolons GABA-A-receptorspecificitet studerats.

Mål

Att jämföra effekten av allopregnanolon och isoallopregnanolon på sekretionen av gonadotropinerna LH och FSH hos friska fertila kvinnor i menscykelns follikelfas (före ägglossningen). Att jämföra skillnader i basalnivåer av allopregnanolon, GABA-A-receptorkänslighet för allopregnanolon och effekten av allopregnanolon på gonadotropinsekretion mellan friska fertila kontroller och kvinnor med PCOS. Att undersöka farmakokinetikparametrar och GABA-A-receptorkänslighet för isoallopregnanolon.

Metod

Friska fertila kvinnor undersöktes i follikelfas avseende effekten av intravenöst givet allopregnanolon och isoallopregnanolon på gonadotropinsekretionen. Farmakokinetikparametrar och GABA-A-receptorkänslighet med SEV undersöktes hos friska kvinnor i follikelfas efter intravenöst givet isoallopregnanolon. PCOS kvinnor jämfördes med friska kontroller i båda menscykelfaserna avseende skillnader i effekter på SEV liksom förändringar i gonadotropinnivåer basalt samt under testdagen efter intravenöst givet allopregnanolon.

Resultat

Allopregnanolon sänker LH och FSH serumnivåer hos friska fertila kvinnor i båda menscykelfaserna men har ingen gonadotropinsänkande effekt hos kvinnor med PCOS. PCOS-kvinnor har högre basalnivåer av allopregnanolon jämfört med follikelfaskontroller men lägre än nivåerna i lutealfas. PCOS-kvinnor har mer uttalad minskning i SEV av allopregnanolon jämfört med kontrollerna. Isoallopregnanolon har ingen effekt på gonadotropinsekretionen. Det ses en effekt på SEV av isoallopregnanolon, men den kan förklaras av en metabolisering av isoallopregnanolon till allopregnanolon.

Slutsats

Det finns signifikanta skillnader i GABA-A-receptorrespons av GABA-steroider vid olika endokrina tillstånd hos kvinnor i fertil ålder undersökt med mätning av ögonsackadhastigheter. GABA-steroiden allopregnanolon sänker gonadotropinserumnivåer hos friska kontroller men inte hos kvinnor med PCOS. Den uteblivna effekten av isoallopregnanolon på gonadotropinerna antyder att GABA-A-receptorn är involverad i gonadotropinregleringen.

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AES	the Androgen Excess Society
ANOVA	analysis of variance
ASI	Anxiety Sensitivity Inventory
BMI	body mass index
CL	clearance
C _{max}	maximum serum concentration
DHEAS	dehydroepiandrosterone sulfate
DMPA	depot medroxyprogesterone acetat
DSM-IV	Diagnostic and Statistic Manual of Mental Disorders, 4th ed.
EOG	electrooculography
FAI	free androgen index
fMRI	functional magnetic resonance imaging
FSH	follicle-stimulating hormone
GABA-A	gamma amino butyric acid A
GnRH	gonadotropin-releasing hormone
HPG-axis	hypothalamic-pituitary-gonadal axis
HPLC	high performance liquid chromatography
HRT	hormone replacement therapy
LH	luteinizing hormone
LSDT	least significant difference test
MRS	mood rating scales
NIH	National Institutes of Health
OC	oral contraceptives
PCOS	polycystic ovary syndrome
PMDD	premenstrual dysphoric disorder
PMS	premenstrual syndrome
PRIME-MD	Primary Care Evaluation of Mental Disorders
PSS	Panic Symptom Scale
PTSD	posttraumatic stress disorder
RIA	radioimmunoassay
SADS	State Anxiety and Discomfort Scale
SD	standard deviation
SEM	standard error of mean
SEV	saccadic eye velocity
SHBG	sex-hormone-binding globulin
STAI	State-Trait Anxiety Inventory
t _{1/2}	elimination half-life time
VAS	visual analog scale
V _d	volumes of distribution

ORIGINAL PAPERS

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

- I. Hedström H, Bixo M, Nyberg S, Spigset O, Zingmark E, Bäckström T. **Studies of pharmacokinetics and pharmacodynamic properties of isoallopregnanolone in healthy women.** *Psychopharmacology (Berl)* 2009 Mar;203(1):85–98
- II. 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*, 2010 Dec 29. (E-pub ahead of print)
**These authors have contributed equally in this work*
- III. Hedström H, Bäckström T, Bixo M, Nyberg S, Turkmen S, Wang M. **Does chronic endogenous exposure to neuroactive steroids change GABA_A receptor sensitivity to allopregnanolone in humans?** Manuscript
- IV. Hedström H, Bäckström T, Wang M, Nyberg S, Bixo M. **Allopregnanolone, a GABA-A receptor agonist, decreases gonadotropin levels in healthy fertile women but not in women with polycystic ovary syndrome.** Manuscript

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INTRODUCTION

It is fascinating, when you think about it, every month during the fertile part of a woman's life an egg is ready to be fertilized and eventually give rise to a new human being. The events regulating this complex procedure are partly the topic of this thesis, and also what could be the reason when it goes wrong.

Hypothalamic-pituitary-gonadal axis

The menstrual cycle is regulated by the hypothalamic-pituitary-gonadal (HPG) axis via specific hormones. The hypothalamus serves as the primary site for the integration and regulation of many important physiological processes, including reproduction. The hypothalamus stimulates and controls the release of gonadotropins from the anterior pituitary gland by regulating the secretion of gonadotropin-releasing hormone (GnRH) into the portal system. GnRH is normally released in discrete pulses that lead to optimal stimulation of gonadotropins [1, 2]. Low frequencies favor synthesis and secretion of follicle-stimulating hormone (FSH), whereas higher frequencies tend to favor those of luteinizing hormone (LH) [3]. LH and FSH stimulate the production of estradiol and progesterone from the ovaries. The hypothalamus contains receptors for estrogen, progesterone and androgens, but GnRH neurons also express a wide variety of other receptors, i.e. norepinephrine, glutamate and gamma amino butyric acid (GABA) [4]. Kisspeptin neurons interact with GnRH neurons and influence GnRH secretion by several different mechanisms, and it is possible that episodic kisspeptin activity drives GnRH pulses [5]. The loss of GnRH or kisspeptin leads to hypogonadotropic hypogonadism, a condition in which the reproductive system is completely shut down [5]. The regulation of the GnRH secretion is very complex and the exact mechanism is not clearly understood.

Feedback mechanisms from the ovaries

In humans, hormonal events during the menstrual cycle are controlled by the ovaries. Transmission of information from the gonads to the brain is referred to as feedback regulation (Figure 1). Depending on the sex of the individual and the stage of the reproductive cycle, the feedback signal can either inhibit (negative feedback) or stimulate (positive feedback) GnRH secretion, and subsequently the secretion of gonadotropins. Gonadal steroids also have feedback influence on the pituitary to modulate its response to GnRH. In females, estradiol levels reflect the status of developing follicles

and progesterone levels indicate the occurrence of ovulation and provide information about the status of the corpus luteum. Estradiol is the main steroid that exerts feedback. Progesterone by itself does not exert inhibition of gonadotropin secretion in most species but does appear to have suppressing effects on gonadotropins in the luteal phase in the presence of estradiol [2, 6, 7]. Estradiol acts via nucleus estrogen receptors that regulate gene transcription within the target cell, though a nongenomic receptor has recently been identified, GPR30 [8]. Progesterone acts on the gonadotropin secretion through nuclear receptors [9].

The mechanisms by which steroids control GnRH release in humans are for the most part unclear. There are fundamental aspects of the neuroendocrine regulation of reproduction that are dramatically different among species and several are unique to higher species and humans. Caution must be exercised when making generalizations and drawing inferences based on work performed in certain laboratory animals because the data may or may not apply to humans.

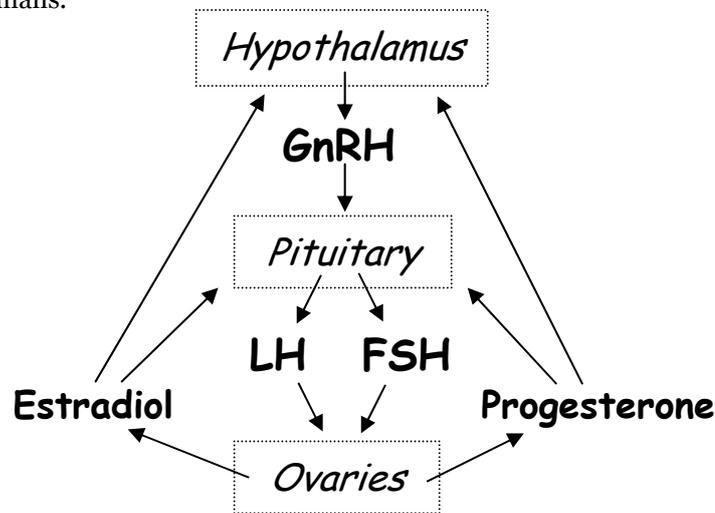


Figure 1. Schematic illustration of the HPG axis and feedback regulation by estradiol and progesterone on the hypothalamus and anterior pituitary.

Hormones during the menstrual cycle

The menstrual cycle is approximately 28 days long (Figure 2). The first part is the follicular phase, and after the ovulation (usually around day 14) the luteal phase starts. At the beginning of the menstrual cycle, there are very low levels of estradiol and no negative feedback, and so FSH is released. FSH stimulates growth of follicles that produce estradiol. Eventually, high enough levels of estradiol are produced from the dominant follicle to turn the

feedback regulation into positive feedback, resulting in the LH surge. The LH surge occurs 10–12 hours prior to the ovulation. After the ovulation, the dominant follicle forms the corpus luteum which produces both progesterone and estradiol. The highest level of progesterone is reached about eight days post ovulation and progesterone levels above 35 nmol/l (day 21) indicates a normal corpus luteum function. Low/medium low estradiol levels give negative feedback and decreases FSH release. In late luteal phase, progesterone and estradiol levels decline due to luteolysis. When estradiol levels are almost zero, FSH levels start to increase again. [2].

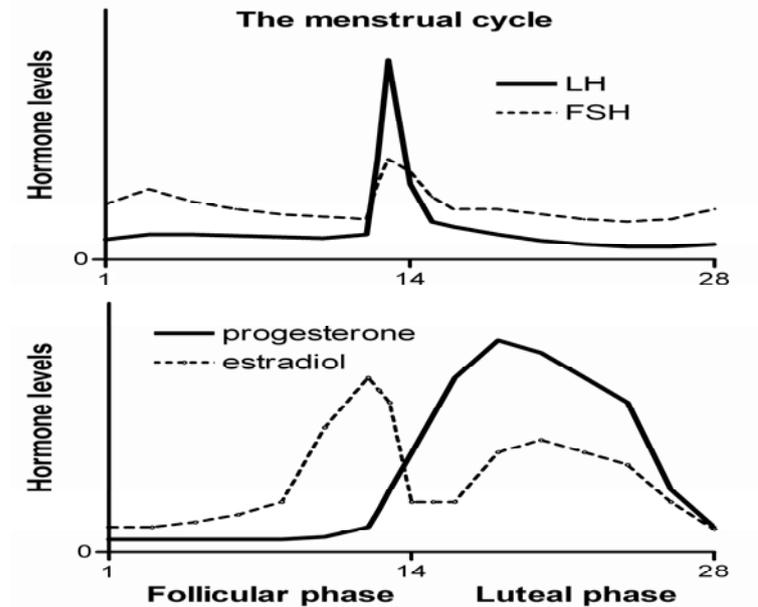


Figure 2 shows hormone changes during the follicular and luteal phases of the menstrual cycle.

The metabolism of progesterone

The gonadal steroids, estradiol and progesterone, are metabolized in different steps, and in this thesis I will mainly focus on progesterone and its metabolites. Progesterone is produced from cholesterol in high levels from the corpus luteum in the ovaries during the luteal phase, during pregnancy from the placenta, from the adrenals during stress and also in the brain [10, 11]. There are several target organs for its actions: the endometrium of the uterus, the breasts and, also, actions on different levels in the brain are known. Progesterone has several interesting metabolites that also have actions by themselves for instance on brain function, though not as hormones via the classic nuclear progesterone receptors [9, 10].

Progesterone is metabolized in two steps (Figure 3). First there is a reduction at the 5-position using the enzymes 5 α - or 5 β -Reductase. In the next step there is a hydroxylation in the 3-position using the enzyme 3 α - or 3 β -hydroxy steroid dehydrogenase (HSD). The metabolic reduction along the 5 α or 5 β pathway is similar for all natural steroids having a keto group at the 3-position and a double bond between carbon atoms 4 and 5 in the steroid molecule [12]. In the 5 α pathway progesterone is metabolized to 5 α -Dihydroprogesterone (5 α -DHP) and in the 5 β pathway to 5 β -Dihydroprogesterone (5 β -DHP). During the hydroxylation it can either form a 3 α or a 3 β structure. From the 5 α pathway allopregnanolone (3 α -hydroxy-5 α -pregnan-20-one) or isoallopregnanolone (3 β -hydroxy-5 α -pregnan-20-one) are formed, and from the 5 β pathway pregnanolone (3 α -hydroxy-5 β -pregnan-20-one) or epipregnanolone (3 β -hydroxy-5 β -pregnan-20-one).

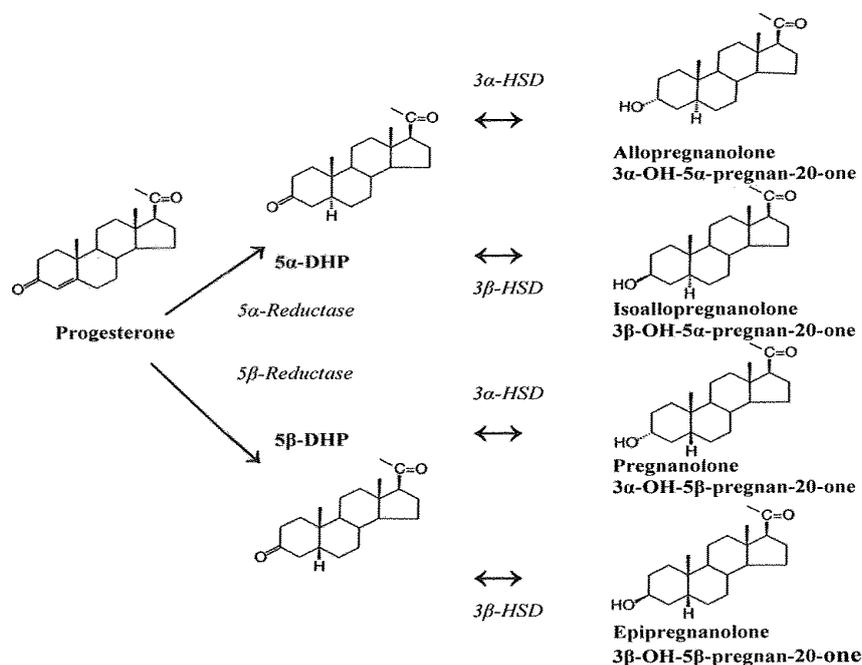


Figure 3 shows the metabolism of progesterone forming different isomers via either 5 α - or 5 β -Reductase in the first step and either 3 α - or 3 β -HSD (hydroxy steroid dehydrogenase) in the second step. The 3 α - and 3 β -HSD are active in both directions.

The main interest in this thesis will be in allopregnanolone and its stereoisomere isoallopregnanolone, where the only structural difference between these stereoisomers is the hydroxylgroup in 3- α or 3- β position [13].

Levels of allopregnanolone and isoallopregnanolone

Allopregnanolone and isoallopregnanolone serum concentrations vary during the menstrual cycle in parallel to progesterone with an elevation in the luteal phase [14-16] as a reflection of steroid synthesis in the ovaries [17, 18]. During the luteal phase, allopregnanolone released from the corpus luteum [18] shows a temporal coupling with LH and cortisol, but in the follicular phase and in amenorrheic women, allopregnanolone shows a temporal coupling only with cortisol [19]. Besides in the luteal phase, the highest physiological levels of allopregnanolone and isoallopregnanolone are reached during late pregnancy, produced from the placenta and fetus [20-23].

Mean physiological levels of allopregnanolone in healthy women are 0.3–1.9 nmol/l in the follicular phase and 1.1–3.7 nmol/l in the luteal phase [14-16, 23-25], showing a cyclical pattern. During late pregnancy, maternal mean levels in plasma are 45–75 nmol/l with an inter-individual variation between 20 and more than 150 nmol/l [21, 22]. In addition, both allopregnanolone and isoallopregnanolone are secreted from the adrenal cortex and can be synthesized *de novo* in the central nervous system from cholesterol [10, 17]. Allopregnanolone secretion from the adrenal cortex increases during stress, as shown in both animals and humans [26, 27]. In humans suffering from acute stress, endogenous allopregnanolone levels have been shown to rise from 1.0 to 1.3 nmol/l [27]. Basal levels in men are 0.2–0.3 nmol/l, which was comparable to women in the follicular phase of the menstrual cycle in the same report [14, 23, 28]. In postmenopausal women, serum allopregnanolone levels were in the same range as those in age-matched men and fertile women in the follicular phase [14]. The allopregnanolone levels do not change in women with age, while in men an age-related decrease was seen, reaching the lowest levels after 60 years of age [14]. The brain concentrations of allopregnanolone are significantly higher than the plasma levels in both cycling and pregnant female rats, and both brain and plasma levels of allopregnanolone temporally follow those of progesterone [17]. Post-mortem studies in fertile and postmenopausal women indicate that allopregnanolone is accumulated in the brain, with the highest levels in the substantia nigra and basal hypothalamus with concentrations ranging from 35–40 ng/g [29]. Isoallopregnanolone levels vary in parallel to progesterone and allopregnanolone during the menstrual cycle (follicular phase 0.1–0.3 nmol/l and luteal phase 0.14–1.2 nmol/l) and in men 0.2–0.3 nmol/l [15, 23]. Isoallopregnanolone likewise shows the highest physiological levels during late pregnancy, 5–25 nmol/l [21, 23, 28] and rises during stress in rats [30].

Neurosteroids / Neuroactive steroids / GABA-steroids

Neurosteroids are steroids synthesized from cholesterol in the CNS in neuronal and glial cells [31] or from steroid hormone precursors imported from peripheral sources [10, 18, 32]. Neuroactive steroids, especially metabolites of progesterone, are potent modulators of the gamma-aminobutyric acid A (GABA-A) receptor and exercise rapid effects on brain function [33], exerting anxiolytic, anticonvulsant, sedative-hypnotic and anesthetic effects [34]. These steroids are positive modulators/enhancers of GABA's actions on the GABA-A receptor in the brain and are also called GABA-steroids [33, 35, 36]. The $5\alpha/5\beta$ reduced and 3α hydroxylated pregnane steroids are endogenous ligands of the GABA-A receptor [33] and do not interact with classical intracellular steroid receptors in the brain but act as GABA agonists [34, 35]. The alpha metabolites are flat and the beta metabolites are angulated. The structure of the isomere is of great importance for their actions on the GABA-A receptor [37]. Allopregnanolone, which is a flat molecule, is one of the most potent agonists on the GABA-A receptor, in contrast to isoallopregnanolone, which is an angulated molecule and lacks effects of its own on the GABA-A receptor [13, 38]. Allopregnanolone and pregnanolone are the most potent and selective endogenous modulators of the action of GABA on brain GABA-A receptors [39].

The GABA-A receptor

The GABA-transmitter system is the major inhibitory system in the mammalian CNS, and GABA has been estimated to be released at as many as 30% of the synapses in the brain [34]. GABA activates three different receptors in the brain, the GABA-A, GABA-B, and GABA-C receptor. In this thesis I will concentrate only on the GABA-A receptor since it can be modulated by GABA-steroids, e.g. allopregnanolone. The GABA-A receptor is also the target receptor for benzodiazepines and barbiturates and mediates some of the effects of alcohol. Activation of the GABA-A receptor causes sedative and anticonvulsant effects. The receptor is a chloride ion channel consisting of five subunits forming a ligand gated chloride channel [40] (Figure 4). A total of 19 mammalian genes code for different GABA receptor subunits, of which 16 genes are coding for subunits in the GABA-A receptor. Eight subunit classes have been cloned: α_1 - α_6 , β_1 - β_3 , γ_1 - γ_3 , δ , ϵ , θ and π . The additional three subunits found, ρ_1 - ρ_3 , contribute to the GABA-C receptor. Other species have additional subunits [41]. The five subunits can vary in many different combinations, the most prevalent subtype being the $\alpha_1 \beta_2 \gamma_2$ isoform [42]. The receptor can be located in the synaptic cleft or extrasynaptically, that is, outside of the synaptic cleft [43].

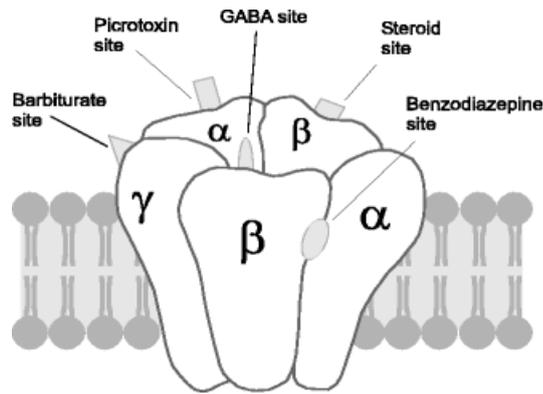


Figure 4 shows the GABA-A receptor with five subunits. Reprinted with kind permission from Springer Science+Business Media, Psychopharmacology 2006 [44].

Besides GABA there are other substances that have actions on the GABA-A receptor. Benzodiazepines, alcohol, barbiturates, neurosteroids, anticonvulsants and anesthetic agents [45] bind to the receptor at specific binding sites, which depend on the subunit composition [45]. The subunit composition varies between different parts of the CNS and subsequently gives different responses and functions in different brain areas [42, 46, 47]. Sedative, amnesic, probably ataxic and partly anticonvulsant actions of benzodiazepines are mediated by the receptor containing the α_1 subunit [48], whereas the benzodiazepine anxiolytic action takes place via receptors containing the α_2 subunit [49]. The α_5 subunit in the hippocampus can be involved in memory impairment [50]. Body motor control involves the α_6 subunit [51], and the β_2 or β_3 subunit is required for anesthesia [42, 47].

Changed GABA-A sensitivity in different endocrine conditions

It is important to notice that the GABA-A receptor subunit composition and sensitivity seem to be changed during different endocrine conditions such as over the estrus cycle [52] and in puberty in mice [53, 54]. During oral contraceptive usage a reduction in progesterone, pregnanolone and allopregnanolone levels was seen in plasma in both women and rats. In the rats reduced levels of progesterone, pregnanolone and allopregnanolone were also found in the cerebral cortex, as well as changed expression of subunits of the GABA-A receptor [55]. Other studies on rats showed a plasticity of the GABA-A receptors during pregnancy and after delivery related to physiological changes in plasma and brain concentrations of neurosteroids [56, 57]. In a rat model of progesterone withdrawal to mimic premenstrual syndrome and the postpartum period, alterations in the expression of GABA-A receptor subunits were found, as the α_4 subunit gene transcription was enhanced [58]. The rat model also showed signs of

increased anxiety after progesterone withdrawal, decreased response to benzodiazepines, and exacerbation of seizure activity [59-61]. This is in accordance with decreased saccadic eye velocity sensitivity in response to benzodiazepines, pregnanolone and alcohol among women with PMS/PMDD in the late luteal phase of the menstrual cycle [62-64]. Chronic alcohol consumption also changes the GABA-A receptor subunit expression [65] as well as during treatment with allopregnanolone [66]. Estrogen treatment increases the α_4 subunit in the GABA-A receptor in the hippocampus [67]. In female mice exposed to anabolic androgenic steroids the expression of α_5 subunits was up-regulated [68]. Anabolic androgen steroids are also allosteric modulators of the GABA-A receptor [69].

Effects of allopregnanolone and isoallopregnanolone

Allopregnanolone exerts sedative [25, 70], anxiolytic [71, 72] and antiepileptic effects [73]. The stereoisomere isoallopregnanolone, however, is as far as we know today, without hormonal or GABA-A receptor effects [38, 74]. Instead, isoallopregnanolone has often been used as a control when testing the specificity of allopregnanolone and its GABA-A receptor enhancing effect, to show the major importance of the sterical configuration for the effect of the steroid [37]. No isoallopregnanolone influence has been noted on anesthesia [75], anxiolysis [71, 72, 76, 77], antiepileptic effect [78], hyperphagic effect [79] or stimulation of gastric acid secretion [80]. Also, in *in vitro* experiments no effect of isoallopregnanolone of its own has been detected [13, 74, 81-85]. The effects of allopregnanolone on the GABA-A receptor and its pharmacokinetic properties are known in humans [25], but for isoallopregnanolone this has not been studied in humans before.

Tolerance

Tolerance is defined as a decrease over time in the ability of a drug to produce the same degree of pharmacological effect. Tolerance often occurs when persons are exposed to high levels or prolonged stimulation of GABAergic modulators, e.g. benzodiazepines, alcohol and neurosteroids [86], and they often show a cross-tolerance in both animals and humans [61, 62, 64]. Short-term exposure can change the subunit composition of the GABA-A receptor and lead to changes in the sensitivity of neuroactive steroids [66]. Long-term stimulation of the GABA-A receptor by these substances decreases the mRNA expression of GABA-A receptor subunits [87]. Allopregnanolone may produce a down-regulation of the GABA-A receptor and reduce the binding sites for benzodiazepines and allopregnanolone or change the subunit composition and thus the sensitivity to stimulating compounds [66, 88].

Clinical aspects of allopregnanolone and cyclic variations

Allopregnanolone seems to be involved in several clinical conditions in both women and men. Numerous studies have reported relationships between the plasma levels of progesterone/allopregnanolone and the occurrence of certain conditions.

Epilepsy in women can have variations during the menstrual cycle related to steroid variations [89]. Women with persisting petit mal absence epilepsy have more seizures during the luteal phase [90]. Partial epilepsy and **migraine** are often catamenial, which means that the women have more seizures during the menstruation [36, 91, 92]. Progesterone, allopregnanolone and pregnanolone have antiepileptic effects [73, 93].

Allopregnanolone presence or concentrations have been studied in women in psychiatric conditions such as **premenstrual dysphoric disorder** (PMDD), which is present in about 3–8% of fertile women [94]. The symptoms are present only in ovulatory cycles [95]. The etiology is not clearly known, but the symptoms are strongly related to allopregnanolone concentrations in a biphasic pattern [24, 36, 64, 96-100]. The most likely explanation is a paradoxical effect of the GABA-A active compounds acting on the GABA-A receptor [101]. PMDD patients have less sensitivity on the GABA-A receptor to neurosteroids, benzodiazepines and alcohol during the luteal phase of the menstrual cycle when allopregnanolone levels are high compared to during the follicular phase when GABA steroids are absent [62, 64, 102]. In addition neural circuits involved in emotional processing and stress sensitivity are changed with progesterone/allopregnanolone and during the menstrual cycle [99, 103-105].

Oral contraceptive usage (OC) has well known side effects in negative mood symptoms [106]. There is an association between PMDD and mood side effects during OC [36]. Long-term daily administration of OC induced changes in the GABA-A receptor subunit composition in rats [55]. During **hormone replacement therapy** (HRT) of climacteric symptoms, negative mood changes are clinically well-known side effects when progesterone is added to the treatment [107, 108]. There is a relation between earlier PMDD and the experience of negative mood symptoms during HRT [108, 109]. Oral progesterone is metabolized in a high degree to allopregnanolone [110]. The impaired sensitivity of neurosteroids on the GABA-A receptor in PMDD patients might be an explanation for the negative mood changes during OC and HRT [64].

Allopregnanolone increases substantially during pregnancy [22] and during the first part of a pregnancy marked **sleepiness** is observed. This sleepiness is decreased later in pregnancy even though progesterone and allopregnanolone levels are increasing to serum levels where sedation would have been seen in a non-pregnant woman [25]. These findings indicate a tolerance development during pregnancy [111]. In animals, GABA-A receptor subunit composition changes in parallel to increasing allopregnanolone concentrations during pregnancy [56].

Around labor progesterone/allopregnanolone levels decrease abruptly [22]. Women experiencing **postpartum “blues”** showed significantly decreased allopregnanolone levels three days postpartum compared to women who experienced no postpartum “blues” [112]. A history of PMDD and earlier depressive symptoms were found to be associated with the occurrence of postpartum mood disorders [113].

Depressive disorders are more common in females than in males, indicating that sex steroids may contribute to the sex differences in depression [114]. **Major depression** has been shown to be associated with low serum levels of allopregnanolone and 60% lower levels in the cerebrospinal liquid compared to healthy subjects [115, 116]. The allopregnanolone levels were normalized after fluoxetine treatment and also after treatment with selective serotonin reuptake inhibitors, and this was correlated to improvement of depressive symptoms [115-117]. Patients with **bipolar dysphoric disorders** in a state of well-being have higher levels of allopregnanolone compared to healthy controls and those with major depression [118]. In patients with **mixed anxiety-depressive disorders** [119] or **generalized anxiety disorders** [120], though no differences in allopregnanolone levels were seen compared to healthy controls. Women with **panic disorders**, however, had elevated levels in both menstrual phases [121]. Panic disorders are twice as common in females as in males, which suggests a sex-specific vulnerability involved in the etiology of this disorder [122]. Women are at greater risk than men for most anxiety disorders, panic disorder, agoraphobia, generalized anxiety disorder and posttraumatic stress disorder [122].

Elevated levels of allopregnanolone have also been noted in humans during **stress** [27]. Women with a history of former depression, both with or without PMDD, are associated with alterations in allopregnanolone response to stress, thus they fail to produce the normal increase in allopregnanolone levels as a response to stress [123]. In premenopausal women with **posttraumatic stress disorder** (PTSD) there is a decrease by 39% in levels of allopregnanolone and pregnanolone in cerebrospinal fluid

compared to healthy women [124], and the lowest levels were seen in patients suffering from both PTSD and a comorbid depression [124].

Women with **eating disorders** such as anorexia, binge eating and bulimia have elevated levels of allopregnanolone [125, 126]. **Food intake and cravings** fluctuate across the menstrual cycle phases in women. Women eat less food during the follicular phase of the menstrual cycle compared to the luteal phase, the only phase of the menstrual cycle in which progesterone is elevated [127].

Antagonistic effects of isoallopregnanolone

Why would a progesterone metabolite, with no effects of its own on the GABA-A receptor, be of interest to study? Isoallopregnanolone can be used as a control to study allopregnanolone specificity. Studying the specificity of allopregnanolone is important to understand whether the effects apply to all sex and stress steroids or not. In addition, isoallopregnanolone has in recent years been shown to have antagonistic effects to allopregnanolone on the GABA-A receptor shown *in vitro* [74, 128], *in vitro* on slices of brain tissue [129] and *in vivo*, as it inhibits allopregnanolone-mediated induction of anesthesia in rats [130]. The antagonizing effect of isoallopregnanolone on allopregnanolone seems specific as GABA, benzodiazepine or barbiturate effects are not inhibited by isoallopregnanolone [38, 74]. To be able to compare isoallopregnanolone effects with allopregnanolone in humans the pharmacokinetics and GABA-A receptor effects of isoallopregnanolone must first be studied alone in humans.

Allopregnanolone GABA and gonadotropin release

The pulsatile secretion of LH and FSH from the pituitary reflects the pulsatile secretion of GnRH from the hypothalamus. The regulation of GnRH production is a remarkably complex system and includes several hormones, receptors and neurotransmitters. Glutamate and GABA are two opposing neurotransmitters that play a key role in the control of the GnRH neurons and are mainly expressed in GnRH neurons which are exposed to negative feedback [131]. Glutamate and GABA are regulators of developmental-, adulthood-, and age-related control of GnRH function [131].

GABA acts through the GABA-A receptor, which is in turn made up of subunits whose expression varies by developmental age, sex and region [132-135]. In addition, the GABA-A receptor subunit composition and sensitivity seem to be changed during different endocrine conditions, pharmacological

influences and pregnancy [52-54, 68, 111] [55, 56]. At the time of puberty in female mice, the effect of GABA on GnRH neurons switches from depolarizing to hyperpolarizing actions [53]. Allopregnanolone levels increase in humans during puberty [136]. During the luteal phase, allopregnanolone release shows a temporal coupling with LH and cortisol. However, in the follicular phase and in amenorrheic women, e.g. women with hypothalamic amenorrhea and amenorrheic PCOS women, allopregnanolone shows a temporal coupling with cortisol [19, 137].

The question of the role of GABA in the pulsatile release of LH from the pituitary has been extensively studied and the results are variable. *In vitro* studies suggest that the release is affected by neuroactive steroids, such as allopregnanolone, via the GABA-A receptor [84, 138-140]. In female rats, ovulation was suppressed by allopregnanolone injections into the cerebral ventricles (icv), evidenced by a decreased number of oocytes collected at estrus. When endogenous allopregnanolone was blocked, the number of oocytes was subsequently increased [141]. In ovariectomized rats, primed with estrogen and progesterone, LH serum levels decreased following icv allopregnanolone, and the authors suggest an involvement of the GABA-A receptor and the dopamine system [142]. Allopregnanolone suppresses the release of hypothalamic GnRH *in vitro* and it is suggested to be mediated via the GABA-A receptor [84]. Other well-known GABA-A agonists, e.g. barbiturates have been shown to inhibit ovulation in rats through the GABA-A receptor [143], and muscimol, a specific GABA-A receptor agonist, decreases LH levels in ewes [144].

A failure in GnRH secretion results in hypogonadotropic hypogonadism [145]. Allopregnanolone levels are changed in endocrine conditions where there is a failure in the reproductive cycle. High serum levels of allopregnanolone have been shown in girls with central precocious puberty [146], and lower levels of allopregnanolone have been shown in precocious pubarche compared to healthy controls and girls with central precocious puberty [147]. Both lower and higher allopregnanolone levels are reported in patients with hypothalamic amenorrhea compared to healthy controls in the follicular phase [19, 148]. In hypothalamic amenorrhea the adrenals are the major source of allopregnanolone, temporal coupled to cortisol [19]. Moreover, higher serum levels of allopregnanolone were found in patients with premature ovarian failure compared to both postmenopausal and fertile women [149]. Women with polycystic ovary syndrome (PCOS) have been suggested to have elevated serum levels of allopregnanolone compared to allopregnanolone levels in healthy women in other studies [137].

Hypothalamic amenorrhea is a functional and reversible disorder, with blocked ovaries and hyperactive adrenal glands, in which the impairment of pulsatile GnRH secretion plays a key role. It is the most common cause of secondary amenorrhea in adolescent girls [150]. There are three main types, stress-related, weight-loss-related and exercise-related amenorrhea [150, 151]. The spectrum of disturbances in the regulatory action of GnRH on LH secretion is broad and includes lower mean frequencies of LH pulses, complete absence of LH pulsatility, normal-appearing secretion pattern and higher mean frequency of LH pulses [19, 152, 153]. Clinical diagnostic criteria include amenorrhea for more than six months and very low levels of LH and FSH, and it is diagnosed by exclusion of other causes of anovulation. The precise mechanisms of the underlying pathology are very complex and still unclear. Whether allopregnanolone is involved in the regulation of gonadotropin secretion and whether it differs during the menstrual cycle or in anovulatory women is not known. Studies on humans are rare, so further studies are warranted.

Measuring neurosteroid sensitivity in humans

Most studies on neurosteroid influence on the GABA-A receptor have been conducted on animals. In humans, measurements of peripheral levels of neurosteroids in plasma, serum or cerebrospinal fluid are as far as we can get, besides autopsy materials, when it comes to invasive methods in humans. Indirectly, though, the GABA-A receptor sensitivity is possible to measure in humans as described below.

A way to estimate the functional GABA-A receptor sensitivity indirectly is by measuring saccadic eye velocity (SEV) in response to sedative drugs operating via the GABA-A receptor [154]. The velocity of the saccade is controlled by the frontal eye field, substantia nigra, superior colliculus, pontine reticular formation and cerebellum [155]. A saccade is a fast movement made by the eye in order to change focus of the fovea. Maximal SEV has a large variation between subjects [156], but is stable within subjects, both within a testing period and between tests [102, 157, 158]. SEV is not under voluntary control [157]. Thus SEV has been proposed as an objective and sensitive measure of CNS depression, benzodiazepine effect, and consequently GABA-A receptor sensitivity in humans [154, 159]. Because the method is stable within subjects, both within and between tests, but has large variations between subjects, the most appropriate way to study the effect is to calculate the change (delta values) from baseline to each time point during the test for each individual. This is the way the method has been used in our laboratory for several years [25, 62, 64, 102, 160-163].

SEV is reduced in a dose-dependent manner by benzodiazepines [154], barbiturates [164], inhaled anesthetics [165], propofol [166] and alcohol [167]. The benzodiazepine effect is reversed by the benzodiazepine antagonist Flumazenil [159]. Pregnanolone [63], allopregnanolone [25], and oral progesterone [168] also reduces SEV in a dose-dependent fashion. There is no drug that is considered to be able to increase the SEV above the baseline [169].

Self-rated sedation (intoxication, in the alcohol study) is also a way to estimate sedation highly correlated to SEV. Benzodiazepines, oral progesterone, pregnanolone, allopregnanolone and alcohol increase self-rated sedation [25, 63, 64, 154, 168]. Thyrotropin-releasing hormone, though, has been shown not to reverse benzodiazepine-induced slowing of SEV, whereas the self-ratings of sedation were almost completely reversed [170].

Functional magnetic resonance imaging (fMRI) is another way to study the effects of gonadal hormones in the human brain. Using this technique it is possible to compare responses in different areas in the brain, in different phases of the menstrual cycle and after administration of gonadal hormones, e.g. progesterone [104].

POLYCYSTIC OVARY SYNDROME

The condition was first described as the Stein-Leventhal syndrome, named after its discoverers in the 1930's. Polycystic ovary syndrome (PCOS) is nowadays a condition that can include the metabolic syndrome with hyperinsulinemia, hyperlipidemia, diabetes mellitus, and possibly cardiac disease, as well as the more conventionally recognized increase in androgen levels, cosmetic problems, anovulation, infertility, endometrial cancer and obesity [171]. PCOS is the most common endocrine disturbance, affecting 6–8% of women in fertile age [172], and higher prevalences can be seen in certain populations e.g. Mexican American women [173].

The PCOS diagnosis

In 1990 diagnostic criteria for PCOS were first established by the National Institutes of Health [174] and included:

1. Menstrual abnormalities and anovulation
2. Clinical and/or biochemical hyperandrogenemia

Differential diagnoses such as, hyperprolactinemia, thyroid disease, late-onset congenital adrenal hyperplasia and Cushing's syndrome were to be excluded.

The diagnostic criteria for PCOS were controversial, and at an expert conference in Rotterdam in 2003 [175] new criteria were established, including at least two out of three of the following:

1. Polycystic ovaries on ultrasound examination
2. Clinical or biochemical hyperandrogenism
3. Menstrual dysfunction with anovulation

The differential diagnoses described above were excluded.

The diagnostic criteria are still controversial because, according to this new definition of PCOS, hyperandrogenism is no longer necessary for the PCOS diagnosis. Because of this the Androgen Excess Society (AES) 2006 [176] suggested new criteria for the diagnosis of PCOS, including these three:

1. Hyperandrogenism: Hirsutism and/or hyperandrogenemia
2. Ovarian dysfunction: Oligo-anovulation and/or polycystic ovaries
3. Exclusion of other androgen excess or related disorders

Menstrual abnormalities include oligomenorrhea, defined as less than eight periods per year, or cycles that are longer than 35 days, and amenorrhea, defined as absence of menstruation for more than three months without pregnancy [173].

Polycystic ovaries are defined as at least one ovary with ≥ 12 follicles ranging from 2–9 mm in diameter, or increased ovarian volume (>10 ml) [173] (Figure 5). Polycystic ovaries are seen in 90-100% of women with PCOS [173, 177] but also observed in 20-30% of women of fertile age in general [172].

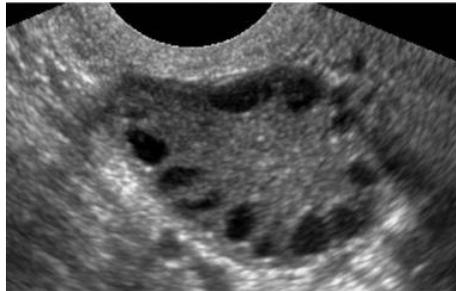


Figure 5 shows a typical polycystic ovary on ultrasound. Picture reprinted with kind permission from the copyright holder.

Clinical hyperandrogenism includes hirsutism, which is presented in 60–75% of PCOS women but varies greatly in different ethnic populations and is rarely present in Asian women [178, 179]. The degree of hirsutism can be assessed using the Ferriman-Gallwey score [180] and the chin and abdomen seems to be the most important areas [181]. In one study, more than 70% of women with hirsutism were estimated to suffer from PCOS [179]. Among women with androgen excess two large studies have shown 70-80% prevalence of PCOS [182, 183]. Other signs of hyperandrogenism, but more unspecific, are acne and male-pattern alopecia [173].

Biochemical hyperandrogenemia is most commonly assessed by measurement of free androgen index (FAI) in serum, which is elevated in 60–80% of PCOS women [173]. FAI is a ratio between total serum testosterone and sex-hormone-binding protein (SHBG). Levels of SHBG are usually low in PCOS, the more obese the lower levels [184]. Measurements of other androgens alone are often of little value. Dehydroepiandrosterone sulfate (DHEAS) is often high in women with PCOS. In the biochemical investigation a raised LH/FSH ratio is often found mainly in the non-obese PCOS women [184-186].

Pathogenesis and clinical symptoms

The pathogenesis of PCOS is poorly understood, but the primary defect may be insulin resistance leading to hyperinsulinemia. Circulating levels of insulin and LH are generally raised. The theca cells in the ovary respond to

this stimulation and produce androgens for conversion to estrogen in the ovary. The rise in LH levels is thought to be caused by relatively high and unchanged concentrations of androgens and estrogen that may alter the control of LH by the HPG axis [171].

PCOS is a condition with effects throughout the life of a woman. Girls born small for gestational age have an increased risk of developing insulin resistance and hyperandrogenism in adolescence [187]. In childhood there is a connection between premature pubarche and PCOS [188]. In the teens, PCOS women often have oligo-amenorrhea, hirsutism, acne and weight disorders. There is an increased frequency of PCOS in women with bulimia, 17% compared to 2% in controls [189, 190]. In reproductive age, infertility, overweight and hirsutism are more common symptoms, and later on the metabolic syndrome including diabetes, hypertension and dyslipidemia can occur [171]. High free testosterone is a risk factor for higher insulin levels [184, 191]. PCOS women also have an increased risk of depressive disorders [192].

PCOS is the second most frequent cause of menstrual disorders among adolescent girls [150], but is generally under-diagnosed, and many young women with bleeding disorders and oligomenorrhea will be prescribed oral contraceptive pills, which mask the condition until they try to achieve pregnancy. Symptoms of PCOS may however improve with age, if the patient's BMI (body mass index) does not increase, and this phenomenon is explained by a decrease in the size of the follicle cohort due to ovarian aging [193].

Obesity in PCOS

Obesity is a growing health problem. According to the SBU report for 2002 on obesity, 8% of adults in Sweden were obese and 4% of children and adolescents [194]. In the USA in 2007–2008 the prevalence of obesity among adults was 33.8% [195].

Underweight	BMI <18.5
Normal weight	BMI 18.5–24.9
Overweight	BMI 25–29.9
Obesity	BMI 30–34.9
Severe obesity	BMI 35–39.9
Very severe obesity	BMI >40

The definition of overweight and obesity from the SBU report 2002 is in accordance with the WHO definition from 1997 [194]. BMI is easy to count and useful since it puts weight in relation to height. The limits, though, have been widely discussed and different definitions have been used in several studies. BMI increases with age, is higher in males than in females, and not applicable in children. BMI underestimates overweight in short people and the opposite in tall people and it does not say anything about the distribution of the fat in the body or the relation between body fat and muscles [194].

Obesity is common and observed in about 50% of PCOS women [171, 172, 184], and the fat seem to be abdominal in its distribution [171]. In a study from Spain, the prevalence of PCOS in overweight and obese women was 28.3% compared to 5.5% in lean women [196]. There are several theories trying to explain the increased prevalence of overweight in PCOS women. One theory is that women with PCOS have a genetic predisposition for accumulating energy in the body [197]. Another theory is that PCOS women have an impaired adipocyte lipolysis [198] and the third; a changed appetite regulation [199]. Losing weight is often difficult for PCOS patients. Nevertheless, as little as a five-percent decrease in weight often improves ovarian function [200, 201].

High chronic GABA-steroid levels in PCOS

Women with PCOS have elevated levels of androgens. One of these androgens, androstenediol ($3\alpha,17\beta$ -dihydroxy- 5α -androstan), is increased in PCOS women in particular those with hirsutism, but also in non hirsute PCOS women [202, 203]. Androstenediol is particularly interesting because it is a neuroactive steroid modulating the GABA-A receptor, a GABA-steroid with actions similar to benzodiazepines, barbiturates, alcohol and other neurosteroids on the GABA-A receptor [39, 204]. Androstenediol is a 5α -reduced and 3α -hydroxylated metabolite of testosterone, or from androstenedione via testosterone. Androstenediol levels are not affected by BMI [203]. In addition to the elevated levels of the GABA-steroid androstenediol there are indications of elevated levels of another GABA-steroid, namely allopregnanolone. Elevated allopregnanolone levels have been reported in non-obese PCOS women compared to allopregnanolone levels in healthy women in other studies [137]. Thus, PCOS women with hyperandrogenemia probably have a situation with high chronic GABA-steroid levels, which may affect the sensitivity of the GABA-A receptor. Tolerance often occurs when persons are exposed to high levels/or prolonged stimulation of GABAergic modulators [86], and one can expect, and our hypothesis is, that the sensitivity to GABAergic modulators may be changed in conditions with high GABA-steroid production.

Allopregnanolone and GABA-A receptor sensitivity in PCOS

Overweight and obesity might be a reason for elevated allopregnanolone levels since obese girls have a higher serum level of allopregnanolone than normal weight controls [205, 206]. Also obese men and women showed higher allopregnanolone levels than normal weight control subjects [207]. Metformin treatment (an insulin-sensitizing compound) in non-obese women with PCOS decreased allopregnanolone levels [137] and positively modulated the pulsatile release of LH, cortisol and allopregnanolone. This supports the hypothesis that hyperinsulinism affects the adrenal function and neurosteroid production in PCOS, and that metformin administration modulates cortisol episodic secretion and reduces plasma allopregnanolone levels. In addition, metformin modulates both the ovarian production of allopregnanolone and the temporal coupling with LH pulsatile release [137]. In contrast, another study on overweight PCOS women, showed increased allopregnanolone levels after metformin treatment even though progesterone was unchanged and BMI was reduced [208]. The author explains these conflicting results by the fact that in the study on non-obese PCOS women the women were amenorrheic and had elevated plasma allopregnanolone levels in the same range as women with stress-induced functional amenorrhea [137]. However, in the latter study on overweight PCOS women the women were oligomenorrheic or eumenorrheic and had plasma levels in baseline conditions in the same range as fertile women in the literature [208]. Thus, in these two studies the allopregnanolone levels does not seem to be explained by, or associated with, overweight.

As mentioned earlier, the GABA-A receptor can change its sensitivity influenced by different endocrine conditions. Androstanediol might influence changes in subunit expression [52]. PCOS conditions differ from the normal hormonal milieu during the menstrual cycle, and the chronic GABA-A steroid stimulation might give PCOS women a different GABA-A receptor sensitivity from healthy controls. GABA-A receptor sensitivity is known for several GABAergic substances in healthy women, but GABA-A receptor sensitivity has not been studied in hyperandrogenic PCOS women.

The GABA-A receptor seems also to be involved in the regulation of gonadotropins. PCOS women often have anovulation, often raised LH levels and a situation with high chronic levels of GABAergic substances. The situation in PCOS women differs from healthy controls, so it is interesting to study the influence on the gonadotropins via the GABA-A receptor.

Obesity and appetite regulation in PCOS

The regulation of appetite is complicated and not fully understood, and several hormones, peptides and neurosteroids are involved. In my thesis I will mainly focus on what is most relevant in PCOS and on the relation to GABA-steroids.

A huge number of PCOS women show a condition of insulin resistance and hyperinsulinemia. Insulin stimulates ovarian steroidogenesis and inhibits SHBG synthesis in the liver, which results in high levels of estrogen and androgens. Insulin stimulates growth factors and obesity seems to amplify the degree of insulin resistance and hyperinsulinemia in PCOS women. Also, obese PCOS women tend to be more hyperandrogenic than their normal-weight counterparts [184]. Androgens stimulate appetite and PCOS women also have a disturbed appetite regulation [190, 199, 209]. Food cravings are more intense in PCOS women with a high androgen profile [202].

Cholecystokinin is a satiety hormone secreted as a response to ingested food, and PCOS women have reduced postprandial cholecystokinin secretion and deranged appetite regulation associated with increased levels of testosterone [199]. Cholecystokinin levels are decreased in women with bulimia [210]. Impaired cholecystokinin secretion may play a role in the greater frequency of binge eating and overweight in women with PCOS, and it has a relation to increased testosterone levels [190, 199]. Androgens impair impulse control [190] and there is a relation to bulimia [189, 190]. Treatment with antiandrogenic oral contraceptives in women with bulimia resulted in decreased testosterone levels and hampered bulimic behavior [211]. The anorexigenic, satiety-promoting hormone leptin is linked to both subclinical eating disorders and PCOS [189].

Ghrelin is orexigenic, hunger-promoting and stimulates food intake [212-214]. Women with PCOS may have a dysregulation of ghrelin with lower fasting ghrelin concentrations and smaller postprandial reduction in ghrelin, especially in overweight women with PCOS compared to control subjects [209, 215]. The low ghrelin levels in PCOS women have a connection to insulin resistance and high testosterone levels typical for the PCOS condition [209, 216, 217]. Decreased ghrelin levels were also seen in obese patients compared with normal subjects [217]. A strong negative correlation between ghrelin and androstenedion suggests involvement of the gonads in appetite regulation [216].

During pregnancy, for instance, there are great adaptations to provide growing of the fetus, placenta and fat storage. These adaptations are thought

to be mediated by neurosteroids, especially allopregnanolone, which is highly increased during pregnancy [218]. Energy intake and food cravings are also higher in the luteal phase of eumenorrheic women [127] and this is abolished in anovulatory cycles [219], an involvement of sex steroids and probably allopregnanolone is suggested.

Increased appetite and lack of satiety are two major reasons for obesity development [220]. It was recently shown that GABAergic transmission plays an important role in the regulation of food intake [221]. The hypothalamus is an important regulatory centre of feeding behaviour and integrates signals from different hormones, such as ghrelin and leptin and other signals. The effect of ghrelin is mediated by the GABA system [221] and GABA also inhibits the sensation of satiety [222]. The importance of the GABAergic transmission in feeding has been shown by investigations where a deletion of GABAergic transmission led to an attenuation of hyperphagic response to ghrelin and resistance to diet-induced obesity [221]. Especially two populations of neurons are likely targets for allopregnanolone, namely POMC neurons of the arcuate nucleus and neurons in the paraventricular nucleus. The hunger-promoting neurons, stimulated by ghrelin, project to the paraventricular nucleus and promote feeding via a GABAergic inhibition of satiety promoting neurons. Activation of GABA neurons in the paraventricular nucleus increases feeding, evidenced by the finding that local applications of muscimol (a GABA-A receptor agonist) in the paraventricular nucleus induce overeating [223]. The other group of neurons in the arcuate nucleus contains the hunger-inhibiting POMC neurons, which are activated by satiety signals such as the hormone leptin, and project to the paraventricular nucleus, resulting in a satiety signal. In the arcuate nucleus, hunger-promoting neurons inhibit POMC neurons via GABA release and GABA thus inhibits the sensation of satiety [222].

Taken together, it is known that activation of the GABA-A receptor stimulates feeding [79] and allopregnanolone has hyperphagic effects in rats [79]. Hypersecretion of GABA-steroids in obese patients may represent one of the mechanisms underlying obesity [207]. Therefore, especially women with PCOS and hyperandrogenism are suitable to investigate in terms of changed GABA-A receptor sensitivity which may contribute to the obesity.

Weight gain in relation to pharmacological drugs

Pharmacological drugs also have relations with weight gain. Female contraceptive hormones in relation to weight gain has been widely discussed and examined in several studies and the results are variable. A two year follow up comparing depot medroxyprogesterone acetate (DMPA) and non

hormonal methods showed weight gain in both groups but significantly more in the DMPA group [224]. A large study with more than 2000 women treated with DMPA showed a modest weight gain [225]. In a study from Brazil users of DMPA compared to copper intrauterine device showed an increased weight after five years [226], but a study from Thailand showed no significant difference in weight gain after 10 years [227]. Adolescent women using DMPA gained more weight than those using combined oral contraceptives (OC) and those who were already overweight were at greater risk [228]. Weight gain during OC has shown divergent results [228, 229]. Antipsychotic drugs, such as haloperidol, clozapine and olanzapine, could cause weight gain [230-232], and an involvement of the GABA-A receptor is suggested [230]. Diazepam can also induce food intake [230] in rats.

A growing body of literature indicates an association between valproic acid, weight gain and PCOS. Valproat is a highly effective antiepileptic drug used widely to treat epilepsy, bipolar disorders and migraines. Women seem to be more susceptible than men to weight gain, and the mechanism through which valproic acid induces weight gain is still unclear [233]. Prolonged use of valproic acid seems to be associated with an increased risk of developing PCOS in predisposed women [234]. On the other hand, the incidence of PCOS appears to be higher among women with epilepsy than other women [235]. PCOS occurs in 10–25% of women with epilepsy, even if they are not treated with antiepileptic drugs, as compared to 5–6% in the general population [233, 236]. The risk of developing PCOS during valproic acid treatment seems to be higher in women with epilepsy than in women with bipolar disorders, maybe due to an underlying neuroendocrine dysfunction related to the seizure disorder [234, 237]. More women using valproic acid compared to lamotrigine developed ovarian dysfunction (54% versus 38%), and 9% in the valproic acid group developed PCOS compared to 2% in the lamotrigine group after one year of treatment [238]. Development of hyperandrogenism occurred more frequently with valproic acid than lamotrigine, especially if medication was started before the age of 26 [238].

These are substances that can act via the GABA-A receptor, and activation of the GABA-A receptor stimulates feeding [79]. Whether obese women have a different sensitivity of the GABA-A receptor is interesting to study since it could give us clues to further research leading to the prevention of overweight and obesity.

AIMS

- To examine the pharmacokinetics and GABA-A receptor sensitivity to isoallopregnanolone and to explore effects of isoallopregnanolone when given to women.
- To investigate the effect of allopregnanolone and isoallopregnanolone on gonadotropin secretion in the follicular phase in healthy controls.
- To measure the effect of allopregnanolone on gonadotropin secretion in anovulatory, hyperandrogenic PCOS women compared to healthy controls in both menstrual cycle phases.
- To measure baseline serum levels of allopregnanolone in anovulatory, hyperandrogenic PCOS women compared to healthy controls in both menstrual cycle phases.
- To compare the GABA-A receptor sensitivity to allopregnanolone in hyperandrogenic PCOS women and healthy controls in the follicular and luteal phase of the menstrual cycle.

MATERIALS AND METHODS

All studies were conducted at the Department of Clinical Sciences, Obstetrics and Gynecology, Umeå University, Umeå, Sweden. The subjects were recruited through advertisement or personal communication. The women were informed in detail, both orally and in writing, about the purpose and performance of the study and gave their oral and written informed consent before inclusion. The study procedures were in accordance with ethical standards for human experimentation, established by the Declaration of Helsinki 1975, revised in 1983. The Regional Ethical Review Board, Umeå University, and the Medical Products Agency of Sweden approved the studies (Dnr 02-315 2002-09-19, LVFS 2003:6 204-11-25, Dnr 05-041M 2005-05-30, LVFS 2003:6 2005-05-02, Dnr 04-125M 2004-11-02).

Subjects

In these studies, a total of 39 subjects have been examined. All four studies (I–IV) were conducted on healthy controls, and in studies III and IV ten women with PCOS were compared to ten healthy controls. The women in study III and IV were the same. The women in study I also participated in the isoallopregnanolone arm in study II. The women in the allopregnanolone arm in study II had participated in an allopregnanolone challenge to explore the steroids' pharmacokinetic and pharmacodynamic properties [25]. The subjects were enrolled in the studies during 2003–2008.

Healthy controls (papers I–IV)

Inclusion criteria were healthy women, aged 18 to 40, with regular menstrual cycles. Exclusion criteria were treatment with any steroid compound (including oral contraceptives and hormonal intrauterine devices) during the last six months prior to enrollment in the study, treatment with benzodiazepines or other psychotropic drugs during the last three months preceding inclusion, and treatment with any drug (including over-the-counter drugs) during the last four weeks before inclusion. Women trying to become pregnant, women with night work or women who experienced jet-lag during the last week before the study day were also excluded. Further exclusion criteria were any current or previous somatic disease, any mental disorder (including PMDD), during the last six months, or a history of drug abuse or alcohol use more than 72 grams (4–6 glasses of wine/3–4 of beer) during one day the last four weeks before the study day. The presence of psychiatric disorders was evaluated using a structured psychiatric interview

PRIME-MD [239], which has been validated for use in primary care settings and conforms to the DSM-IV criteria [240]. Physical and gynecological examinations were performed, as well as routine blood chemistry screens. Women with systolic blood pressure below 90 or above 170 mmHg or diastolic blood pressure below 50 or over 100 mmHg were excluded. All subjects had negative pregnancy tests and normal blood chemistry screens.

Hyperandrogenic PCOS women (papers III–IV)

Inclusion criteria in the PCOS group were women, aged 18–40, with PCOS. The PCOS diagnosis was based on the appearance of biochemical and/or clinical hyperandrogenism, oligo- or amenorrhea and at least one polycystic ovary on gynecological ultrasound examination, thus adhering to both the NIH and Rotterdam criteria [174, 175]. All PCOS women in our studies met all three criteria, thus they were all hyperandrogenic. In paper III, the PCOS women will be referred to as the hyperandrogenic group since the interesting hypothesis was to study the effect of the elevated GABAergic androgens. In paper IV they will be referred to as the PCOS group. All PCOS women had BMI >25. Exclusion criteria in the PCOS group were, besides the same exclusion criteria as in the controls, other causes of anovulation, e.g. hyperprolactinemia, hypo- or hyperthyroidism and premature ovarian failure.

Experimental design

Paper I

Study I was a phase I+II trial on isoallopregnanolone to explore its pharmacokinetic properties and the pharmacodynamic effects on saccadic eye velocity movements and self-rated sedation. Seven women were included and the experiment was conducted in the follicular phase (days 6–13 of the menstrual cycle). To avoid interference of diurnal variations, all study patients were tested at the same time of day. Three intravenous injections of isoallopregnanolone were given at 30-minute intervals (0.04, 0.06 and 0.10 mg/kg), thus giving a cumulative dose of 0.20 mg/kg. Blood samples were drawn from the contralateral arm for analyses of isoallopregnanolone and allopregnanolone in serum at baseline and with short intervals until 780 minutes and again the next day. SEV, self-rated sedation and mood rating scales (MRS) were measured at baseline and repeatedly during the test. Vital functions were checked and adverse events reported throughout the experiment until the next day.

Paper II

Study II was an investigation of the effects of allopregnanolone and isoallopregnanolone on concentrations of gonadotropins, estradiol and progesterone. Twelve women were included in the allopregnanolone challenge and another seven in the isoallopregnanolone challenge. Steroid administration was performed in the follicular phase with the first injection at 08.30 a.m. In both groups, serum levels of allopregnanolone and/or isoallopregnanolone, FSH, LH, estradiol and progesterone were drawn at baseline and repeatedly throughout the experiment, until 780 minutes. In the ALLO group, three intravenous injections of allopregnanolone were given at 30-minute intervals (0.015, 0.03 and 0.045 mg/kg), producing a cumulative dose of 0.09 mg/kg. In the ISOALLO group, three intravenous injections of isoallopregnanolone were given at 30-minute intervals (0.04, 0.06 and 0.10 mg/kg), producing a cumulative dose of 0.20 mg/kg.

Papers III and IV

In studies III and IV, ten women with PCOS, all anovulatory, performed the test once, and ten controls with regular menstrual cycles performed the test in both the follicular and the luteal phase. To avoid diurnal variations, all study patients were tested at the same time of the day starting between 7.50 and 8.40 a.m. One single intravenous injection of allopregnanolone, 0.050 mg/kg, was given. In study III, allopregnanolone serum levels, SEV, self-rated sedation and MRS were performed at baseline, and repeatedly throughout the test until 180 minutes after the allopregnanolone injection. In study IV, serum levels of allopregnanolone, FSH, LH, progesterone and estradiol were drawn at baseline and repeatedly throughout the test until 180 minutes after the allopregnanolone injection.

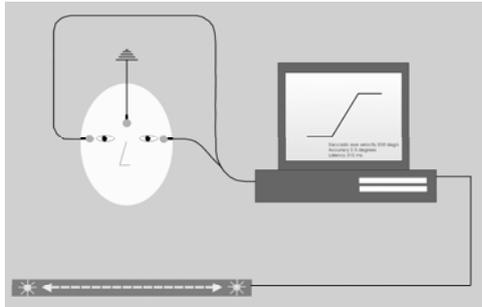
Medications

The experimental medications for intravenous administration were prepared by the Umeå University Hospital Pharmacy. The isoallopregnanolone solution was formulated with isoallopregnanolone (3 β -hydroxy-5 α -pregnan-20-one, Umecrine AB, Umeå, Sweden). Eight milligrams were dissolved in 100 ml albumin solution (Albumin, 200 mg/ml) using an ultrasound bath and the solution was then filtered through two sterile filters. The final solution contained 0.0736 ± 0.00807 mg/ml (mean \pm SD) isoallopregnanolone (n = 6). The allopregnanolone solution was formulated with allopregnanolone (3 α -hydroxy-5 α -pregnan-20-one, Umecrine AB, Umeå, Sweden). Thirteen milligrams were dissolved in 100 ml albumin

solution (Albumin, 200 mg/ml) using an ultrasound bath. The final solution contained 0.126 ± 0.003 mg/ml (mean \pm SEM) allopregnanolone ($n = 9$). Concentrations of allopregnanolone and isoallopregnanolone, in each batch, were determined using high-performance liquid chromatography (HPLC) and UV absorbance [241]. Details of the solutions are given in the papers.

Saccadic eye movement measurements (Papers I and III)

Measurement of saccadic eye velocity is a validated and objective way to estimate sedation with small intra-individual variation [154]. SEV is measured using electro-oculography (EOG) with the CSGAAS5 system, fully documented elsewhere [242-244]. The basic concept is to measure the difference in electric potential between the cornea and the retina. The computer program that generates and calculates the saccades was developed by Cardiff Clinical Trials Ltd and is well documented [245]. The technique has been used in our laboratory for several years [25, 62, 64, 102, 160-163]. Several parameters are registered with the technique and in this thesis only SEV parameters are used. SEV was measured at baseline and at intervals during the test; for details see papers I and III. The test was performed with the patient sitting in a comfortable chair in a quiet, semi-lit room. A pillow was used to support the head of the patient to prevent movements. EEG cup electrodes (Synetics AB, Stockholm, Sweden) with a small amount of electrode gel (Elefix, Nihon Kohden Europe, Rosbach, Germany) were used and placed 1 cm lateral of the outer canthus of both eyes, with one common electrode in the center of the forehead. Before application of the electrodes, the skin was exfoliated with Skinpure cream (Nihon Kohden). Electrode impedance was measured and confirmed to be less than 5 k Ω . The subject was instructed to watch an array of light-emitting diodes placed at eye level, 67 cm from the glabella. The target for the eye movements was an illuminated LED, and 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. The subjects were instructed not to anticipate targets. A fixed sequence of 4 \times 24 targets, producing target steps of 10, 20, 30, and 40 degrees, was displayed with a brief rest in between. The target movements took place at 1.5-second intervals. 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 test procedure. The EOG was DC amplified and low-pass filtered (-3 dB at 50 Hz) before being digitized to 12-bit resolution at a sampling frequency of 250 Hz. A personal computer controlled the target movements and digitized the waveform using an analogue-digital converter.



Figures 6 and 7 show a subject in action and schematic illustration of the SEV arrangements.

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 [243]. 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 milliseconds after target movements were included, and to be considered a saccade, the recorded eye movement had to display a velocity of more than 100 degrees/second. 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 the 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, known as the main sequence [246]. 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 is fitted by a quadratic equation to the peak velocity data using the calculated saccade angle as the independent variable. Carrying out the fitting procedure twice and weighting the second fit with the inverse of the square of the residuals from the first fit minimized the influence of outliers in the data. The values of peak velocity for 10-, 20-, 30- and 40-degree saccades were calculated by interpolation. Saccades with amplitudes of 20 and 30 degrees were chosen for further analyses as SEV [246].

Subjective rating scales

Visual analogue ratings of sedation (papers I and III)

Subjective ratings of sedation were done by the test persons using a visual analogue scale (VAS) [247]. The scale measured from 0 to 10 cm, where 0 represented complete absence of sleepiness and 10 represented falling asleep. The ratings were made at baseline and at the same time points as the saccadic eye measurements.

Mood rating scales (papers I and III)

At baseline, three scales were used to measure the subject's tendency to develop anxiety and the actual anxiety level, State-Trait Anxiety Inventory (STAI, state subscale), Anxiety Sensitivity Inventory (ASI) and Panic Symptom Scale (PSS). The difference in anxiety level during the test was measured using the State Anxiety and Discomfort Scale (SADS). STAI and PSS were also used after the test to register anxiety symptoms or relief of anxiety during the first 24 hours after the test.

State-Trait Anxiety Inventory

STAI comprises 20 items tapping anxiety proneness and anxiety in relation to the experimental situation, with a total range of scores from 20 to 80 [248].

Anxiety Sensitivity Index

ASI comprise 16 items that express concerns about the possible adverse consequences of anxiety symptoms [249]. Respondents indicate their degree of endorsement on 5-point scales that range from 0 (very little) to 4 (very much), and total scores range from 0–64. The ASI manual reports a mean of 19.01 (SD=9.11) for healthy subjects [250]. The mean score for panic disorder patients was reported to be 36.2 [251].

Panic Symptom Scale

PSS is a DSM-IV-derived panic symptom scale [252], where subjects retrospectively rate the maximum intensity of panic symptoms on 18 items (0=not present, 1=mild, 2=moderate, 3=severe, 4=extremely severe). The sum of intensity ratings was measured by summarizing each item score on the PSS (range 0–72).

State Anxiety and Discomfort Scale

SADS is a global measure of subjective discomfort. This scale has previously been validated in pharmacological tests on humans, where quick changes in

anxiety levels could be detected [253]. The SADS is a self-rating scale, ranging from 0 to 5 (0=no discomfort, 1=slight, 2=moderate, 3=severe, 4=very severe to 5=worst imaginable discomfort), and a global measure of the three aspects of anxiety, namely effect, somatic symptoms and cognition [254]. SADS was used bedside repeatedly during the test.

Likert scale for prospective symptom ratings (Paper I)

The subjects performed daily mood ratings each evening from the first day of menstruation in the actual menstrual cycle until the next menstruation started. This scale (graded 0–8) was used to measure day-to-day fluctuations in mood and has been used in several prior studies [108, 109, 161, 163, 255–259]. The scale comprises menstrual bleeding and 15 physical and mood symptoms relevant for the diagnosis Premenstrual Dysphoric Disorder (PMDD) according to DSM-IV [240, 260, 261]. In the statistical analyses the symptoms are grouped together as summarized symptom scores. Negative mood symptoms included tension, irritability, depression and fatigue. Positive mood symptoms included cheerfulness, happiness, energy and interest in daily activities. Physical symptoms included breast tenderness, change in appetite and bloating.

Steroid assays

Isoallopregnanolone and allopregnanolone assays (Papers I–II)

The method is described in detail in paper I. Briefly, the samples are measured by radio-immunoassay (RIA) after pre-assay diethylether extraction and separation with high-performance liquid chromatography (HPLC). In the isoallopregnanolone assays a separation using HPLC is necessary and allopregnanolone is also eluted. In the allopregnanolone assay, an easier separation using celite chromatography is an alternative, and was used in the studies where allopregnanolone only was analyzed (II, III and IV).

HPLC separation

Fractions of 1.5 ml were symmetrically collected around the peak retention time for isoallopregnanolone and allopregnanolone for further analysis with RIA. No cross-reacting steroids had retention times close to the collected fractions. Retention time for isoallopregnanolone is 18.9 minutes and for allopregnanolone 24.2 min. The recovery of the extraction and HPLC procedure was 95% for isoallopregnanolone, and 98% for allopregnanolone and the results were compensated for recovery.

Retention time for cross-reacting steroids	min
Isoallopregnanolone (5 α -pregnan-3 β -ol-20-one)	18.9
Allopregnanolone (5 α -pregnan-3 α -ol-20-one)	24.2
5 α -pregnan-20 β -ol-3-one	26.7
5 β -pregnan-3 α -ol-20-one	22.5
5 β -pregnan-3,20-dione	19.4
4-pregnen-3 α -ol-20-one	18.5
5 β -pregnan-3 β -ol-20-one	17.9
5 α -pregnan-3,20-dione	17.0
5-pregnen-3 β -ol-20-one (pregnenolone)	14.7
5 α -pregnan-3 α ,21-diol-20-one	10.1
preg-4-ene-3,20-dione (progesterone)	9.4
4-pregnen-20 α -ol-3-one	9.2

Isoallopregnanolone RIA

An antibody against pregnenolone (pregnenolone-3-monoheemisuccinate-HSA; ICN Pharmaceuticals, Inc Orangeburg, NY, USA) was used as it also binds to isoallopregnanolone. Cross-reactivity with isoallopregnanolone was 26.6% and allopregnanolone 13%. The sensitivity of the assay (2 SD above the blank mean) was 0.11 pmol or 35.8 pg, with an intra-assay coefficient of variation of 8.5% and an inter-assay coefficient of variation of 10.7%.

Cross-reactivity to antibody	%
Pregnenolone	100
Isoallopregnanolone	26.6
Allopregnanolone	13
5 α -pregnan-3,20-dione	7
5 β -pregnan-3 β -ol-20-one	<1
5 β -pregnan-3 α -ol-20-one	<1
5 α -pregnan-3 α ,20 α -diol	<1

Allopregnanolone RIA

Allopregnanolone was measured using a polyclonal rabbit antiserum raised against 3 α -hydroxy-20-oxo-5 α -pregnan-11-yl carboxymethyl ether coupled to bovine serum albumin, provided by R. H. Purdy, (The Scripps Research Institute, La Jolla, CA, USA)[262]. The method in detail and cross-reactivity of the antibody are shown in earlier publications [25, 262]. The sensitivity of the assay was 25 pg with an intra-assay coefficient of variation for allopregnanolone of 6.5% and an inter-assay coefficient of variation of 8.5%. The recovery of allopregnanolone was 98% and the results are compensated for recovery.

Allopregnanolone assay (Papers II–IV)

Briefly, the samples were extracted with diethylether, separated from cross-reacting steroids with celite chromatography, and measured by RIA as described in the section above, in paper II and previously in detail [25]. This method was used in the ALLO group in study II and in study III and IV.

Hormone analyses (Papers II and IV)

Serum concentrations of LH and FSH were analyzed with a solid phase, two-site chemiluminescent immunometric assay (Immulite®). Serum estradiol was measured using a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite®) and serum progesterone with a sequential competitive immunoassay (Immulite®). All analysis kits were purchased from Diagnostic Products Corporation, Corporate Offices, Los Angeles, CA, USA.

Pharmacokinetic analysis (Paper I)

For details see paper I. Briefly, pharmacokinetic parameters for isoallopregnanolone and the allopregnanolone produced were examined. Baseline serum concentrations (C_0), concentrations 5 minutes after each intravenous injection (C_5 , C_{35} and C_{65}), maximum serum concentrations (C_{max}), and the time to achieve maximum serum concentrations (t_{max}), were obtained directly from the measured values. The baseline isoallopregnanolone and allopregnanolone concentrations were subtracted from the measured values obtained 5–1860 minutes later before the pharmacokinetic calculations were carried out, so that only the net concentrations were used for further pharmacokinetic analyses. Baseline isoallopregnanolone concentrations were below 1.9% of the maximum concentrations and baseline allopregnanolone concentrations were below 1.3%. Pharmacokinetic parameters were calculated by means of the Kinetica program package, version 4.3 (InnaPhase Corporation, Philadelphia, PA, USA).

Statistics

Statistics are described in detail in each paper. In papers I and III saccadic eye velocity parameters and self-rating scores of sedation were calculated as a difference from baseline at each time-point, e.g. delta degrees/second and delta sedation scores. SEV parameters and sedation were analyzed by one-way ANOVA (analysis of variance) with repeated measures. Least significant

difference test (LSDT) was used as post hoc test when the initial ANOVA was significant. Isoallopregnanolone and allopregnanolone concentrations, as well as concentrations of FSH, LH, progesterone and estradiol, were described as mean±SEM. Changes in each group from baseline at different time-points were analyzed by one-way ANOVA with repeated measures and LSDT as post hoc test. Differences between groups were tested with two-way ANOVA with repeated measures. In the ANOVA, time-point was used as an independent factor and steroid/hormone concentration as a dependent factor. To explore differences between single measurements in the study groups, the Mann-Whitney U-test was used. To explore differences between follicular and luteal phase, Wilcoxon Signed Rank Test was used. Spearman Rank correlation test was used to explore relationships between concentrations of allopregnanolone, isoallopregnanolone and gonadotropins. In paper I daily symptom rating scores were centered around the day of menstrual bleeding onset and the test day. The SPSS version 11 (paper I), 13 (paper II) and 17 (papers III and IV) statistical package was used for the analyses in the different papers. P values less than 0.05 were considered to be statistically significant.

RESULTS

Isoallopregnanolone challenge (paper I)

Out of seven included women, six completed the study. The seventh subject was excluded on the test day because of a vasovagal reaction when the intravenous cannula was inserted; she was never given any test injection.

Isoallopregnanolone pharmacokinetics

The pharmacokinetic parameters of isoallopregnanolone are described in table 1. Table 1 and figure 8 bottom panel show the isoallopregnanolone concentrations at baseline and after each of the three intravenous injections of isoallopregnanolone (0.04 mg/kg, 0.06 mg/kg and 0.10 mg/kg). The concentration values indicate a dose-related increase in the serum concentration of isoallopregnanolone. In parallel to the increasing concentrations of isoallopregnanolone there was a rise in allopregnanolone concentrations which is significantly correlated ($r = 0.484$; $p < 0.001$).

Table 1 shows pharmacokinetic parameters for isoallopregnanolone and allopregnanolone.

	Isoallopregnanolone Mean \pm SD	Allopregnanolone Mean \pm SD
C₀ , nmol/L	1.01 \pm 0.30	0.80 \pm 0.13
C₅ , nmol/L	27.5 \pm 6.7	3.7 \pm 2.1
C₃₅ , nmol/L	60.8 \pm 17.7	6.6 \pm 4.7
C₆₅ , nmol/L	138 \pm 71.7	19.0 \pm 18.4
C_{max} , nmol/L	143 \pm 73.0	22.8 \pm 22.5
t_{max} , min	67.7 \pm 4.1	74.0 \pm 21.9
t_{1/2} , min	847 \pm 599	1079 \pm 527

Maximum serum concentration of isoallopregnanolone was 143 nmol/L, achieved at 67.7 minutes. Maximum serum concentration of allopregnanolone was 22.8 nmol/L, which is 16% of the maximum concentration of isoallopregnanolone, and occurred 6 minutes later. The rise in allopregnanolone serum levels indicates that a metabolism of isoallopregnanolone into allopregnanolone took place.

Effects on saccadic eye velocity and sedation

There was an overall significant decrease in SEV ($F(14,70) = 3.27$; $p < 0.001$). The post hoc test showed significant decreases in SEV 5 and 13 minutes after the first injection ($p = 0.014$ – 0.027) and 5 and 18 minutes after the second injection ($p = 0.007$ – 0.024). After the third injection, there was a significant decrease during the first 35 minutes ($p = 0.019$ – 0.049), not at 45 minutes, but then again at 55 minutes ($p = 0.008$). From 150 minutes after the first injection SEV had returned to baseline (figure 8, middle panel).

No significant changes occurred in sedation following the three injections of isoallopregnanolone (figure 8, top panel).

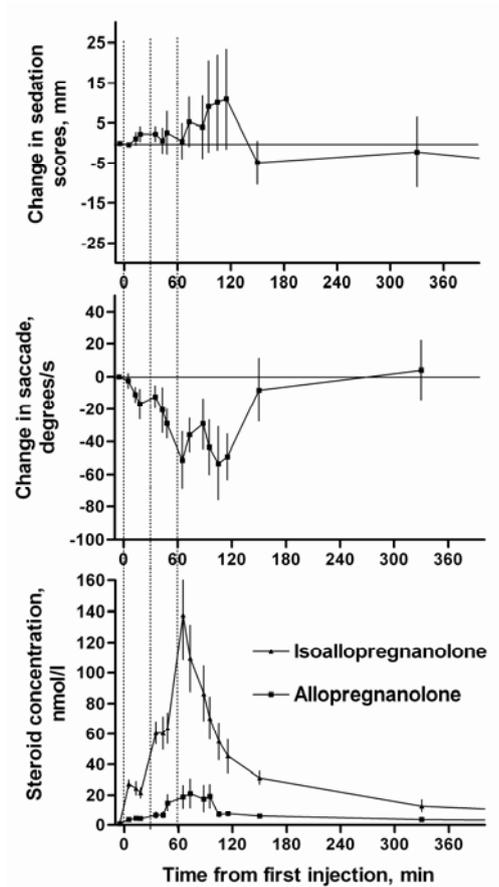


Figure 8 shows changes from baseline in sedation scores (top panel) and SEV (middle panel) and the corresponding steroid concentrations during the test (bottom panel). Injections of isoallopregnanolone were given at 0, 30 and 60 minutes as indicated in the figure.

Safety parameters of isoallopregnanolone from different aspects

During the study day there was a significant decrease in heart rate ($F(5,25)=6.12;p<0.001$), but there were no significant changes in systolic and diastolic blood pressure or respiration frequency. The subjects reported no symptoms of discomfort.

The menstrual cycle length of the test cycle was not significantly different from the reported cycle length before the test (27.7 ± 1.1 days versus 28.8 ± 0.6 days).

The ASI test showed that all women were within a low anxiety range (score 0–10), except one subject with a score of 12 (total range of the ASI test is 0–64, [263]). PSS and STAI revealed no significant changes from baseline until the day after the test. No fluctuations in anxiety level during the test were revealed by SADS.

To investigate whether isoallopregnanolone had any effect on mood symptoms the data were centered around the day of the test injection. Summarized (Σ) positive, negative and physical symptoms as well as the individual symptoms were investigated from five days before the test until ten days after the test. There were no significant changes in any symptoms after the injections (figure 9).

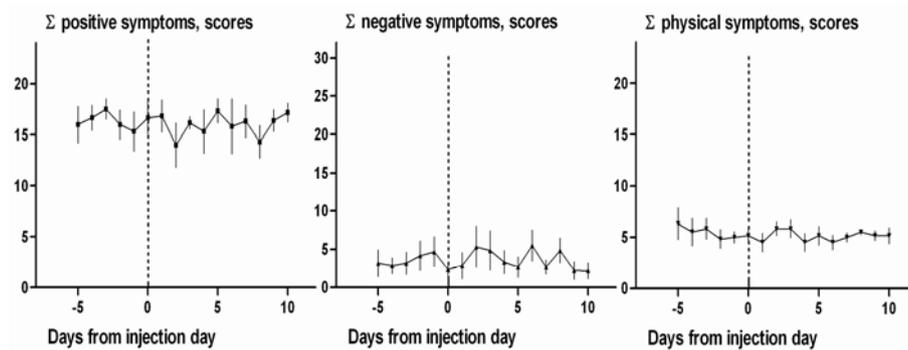


Figure 9 shows the summarized (Σ) positive (cheerfulness, happiness, energy and interest in daily activities), negative (tension, irritability, depression and fatigue) and physical symptoms (breast tenderness, change in appetite and bloating) from five days before the test until ten days after the isoallopregnanolone test.

Steroid effects on gonadotropins in the follicular phase (Paper II)

Out of twelve included women, ten completed the allopregnanolone (ALLO) study; two never performed the experiment because of changes of residency. Out of seven included women, five completed the isoallopregnanolone (ISOALLO) study. One was excluded because of a vasovagal reaction on the test day at insertion of the cannulas and was never given any test injection, and the other was excluded to avoid interference with the preovulatory LH peak which was ongoing on the planned test day.

Table 2. Demographic data and baseline hormone and allopregnanolone levels in both groups.

	ALLO group	ISOALLO group	p-value
Age , years, median (range)	26 (19–30)	30 (23–39)	NS
Body mass index , median (range)	22 (20–25)	24 (22–32)	p=0.01
Menstrual cycle length , days, median (range)	30 (28–36)	29 (27–31)	NS
Cycle day at test , day, range	5–10	6–12	NS
Tobacco users , %	40	0	
Estradiol , pmol/L, mean \pm SEM	100.3 \pm 36.6	133.2 \pm 31.0	p=0.043
Progesterone , nmol/L, mean \pm SEM	2.20 \pm 0.26	1.47 \pm 0.14	NS
FSH , IU/L, mean \pm SEM	6.20 \pm 0.25	4.34 \pm 0.70	p=0.05
LH , IU/L, mean \pm SEM	6.60 \pm 0.68	4.64 \pm 1.02	NS
Allopregnanolone , nmol/L, mean \pm SEM	0.46 \pm 0.05	0.77 \pm 0.06	p=0.01

Effects on gonadotropin levels

Following the allopregnanolone injections in the ALLO group, FSH was significantly reduced between 5 and 105 minutes after the first injection ($F(16,144) = 2.18$, $p = 0.008$, figure 10, left top panel), and again at 330 minutes ($p < 0.05$). Likewise, LH was significantly reduced between 5 and 81 minutes after the first injection, except for 43 and 51 minutes ($F(16,144) = 2.63$, $p = 0.001$, figure 10, left middle panel), and again at 330 minutes ($p < 0.01$), 600 and 780 minutes ($p < 0.05$).

Following the isoallopregnanolone injection in the ISOALLO group, however, no significant changes were seen in FSH and LH during the first 150 minutes or at 780 minutes (figure 10, right top and middle panels).

Corresponding serum concentrations of steroids after each injection in both groups are shown in figure 10, bottom panels.

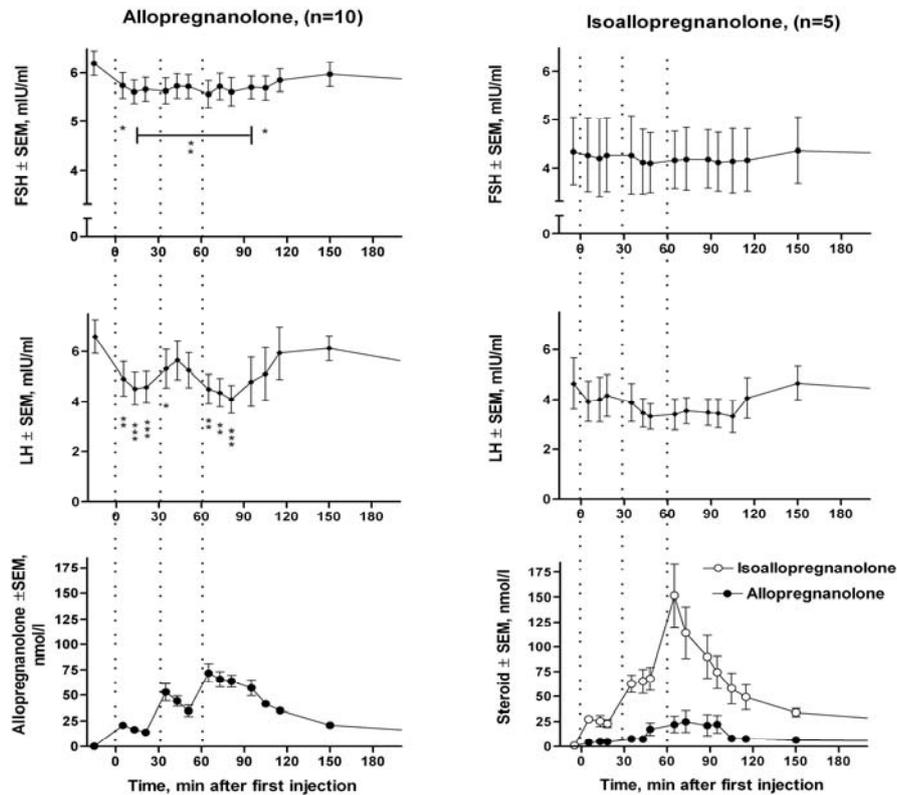


Figure 10 shows the changes in both groups in FSH (top panels) and LH (middle panels) and the corresponding steroid concentration (bottom panels). Dotted lines indicate the injections.

Effects on estradiol and progesterone

In the ALLO group, progesterone concentrations were decreased ($F(16,144) = 6.15, p < 0.001$) and post hoc analyses revealed a significant change at 5 minutes ($p < 0.05$, figure 11, left top panel) and at 780 min ($p < 0.05$). In the ISOALLO group, concentrations of progesterone were however increased ($F(16,64) = 4.54, p < 0.001$) between 35 and 73 minutes, at 95 and 115 minutes (figure 11, right top panel), and again at 780 min ($p < 0.05$). Estradiol concentrations were not affected by injections of allopregnanolone or isoallopregnanolone (figure 11, bottom panels).

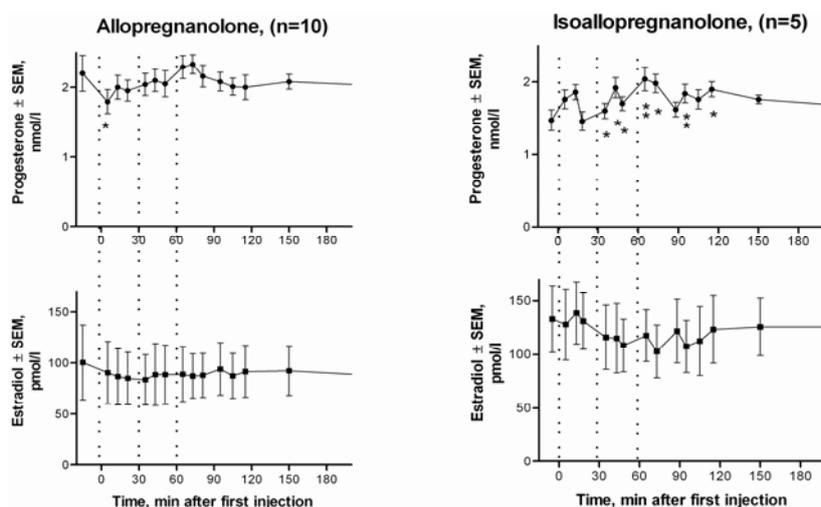


Figure 11 shows changes in both groups of concentrations of progesterone (top panels) and estradiol (bottom panels) after the steroid injections. Dotted lines indicate the injections.

Allopregnanolone effects on gonadotropins in PCOS and controls (Paper IV)

Ten women were included in each group (table 3). One of the PCOS women was taking metformin and was therefore excluded, thus nine PCOS women completed the test. In the controls, two were excluded due to no signs of ovulation in the luteal phase, thus eight were included.

Table 3. Demographic data in both groups.

	PCOS group	Control group	p-value
Age , years, median (range)	27 (20–38)	26 (21–31)	NS
Body mass index , median (range)	31 (28–37)	21 (19–35)	p=0.016
Weight , kg, median (range)	84 (63–102)	61 (52–95)	p=0.027
Menstrual cycle length , days, median (range)	---a/	30 (25–32)	
Cycle day at test , day median (range)	---a/	Follicular 8 (6–11) Luteal 24 (22–28)	
Anovulation , %	100	0	

a/ The women were oligo- or amenorrheic.

The PCOS women (table 4) had anovulation, polycystic ovaries, clinical and/or biochemical signs of hyperandrogenism, in paper IV referred to as hyperandrogenic. Eight out of nine women had hirsutism and 3/9 acne.

Table 4. Hormonal characteristics of the PCOS women.

Id	Oligo- / amenorrhea	PCO	Clin HA	LH/FSH ratio >2	FAI	SHBG nmol/L (26-110)	Testo nmol/L (0.2-3.0)	DHEAS μ mol/L (2.7-9.2)
1	+	+	+	3.2	0.05	28	1.3	5.3
2	+	+	+	2.5	0.04	39	1.6	6.5
3	+	+	+	0.7	0.07	19	1.4	3.0
4	+	+	+	2.5	0.05	28	1.4	2.7
5	+	+	+	2.9	0.05	30	1.4	4.3
6	+	+	+	3.0	0.07	15	1.0	7.0
7	+	+	+	1.6	0.15	13	1.9	--
8	+	+	+	2.9	0.11	18	2.0	6.6
9	+	+	+	3.0	0.09	29	2.5	17.5

PCO – polycystic ovary on ultrasound, Clin HA – clinical signs of hyperandrogenism, FAI – free androgen index, Testo – testosterone

Baseline hormone levels

Baseline allopregnanolone levels in the PCOS group (0.80 ± 0.1 nmol/l) were significantly higher than in the follicular phase (0.44 ± 0.02 nmol/l), but lower than in the luteal phase (1.60 ± 0.2 nmol/l) of controls ($p=0.034$ and $p=0.004$; see table 2 in paper III). Progesterone baseline levels were significantly higher in the PCOS group compared to the follicular phase controls (4.7 ± 0.9 nmol/l vs 2.2 ± 0.5 nmol/l, $p=0.012$). As expected, progesterone concentrations were higher in the luteal phase than in both the follicular phase and the PCOS group (27 ± 4.6 nmol/l, $p=0.012$ and $p=0.001$). Follicular phase estradiol levels were lower than in the luteal phase ($p=0.012$), and there was a trend towards lower levels compared to the PCOS group ($p=0.054$). S-FSH was higher in the follicular phase than in the luteal phase ($p=0.018$), and tended to be higher than in the PCOS group ($p=0.054$). S-LH showed no significant differences between the groups at baseline. For further information see table 2 in paper IV.

Effects on gonadotropin levels

In the **follicular phase of controls**, there was a significant decrease in LH concentration ($F_{7,49}=2.80$; $p=0.016$) compared to baseline, at 5–30 minutes, and again at 60 and 180 minutes ($p<0.05$). S-FSH was not significantly changed. In the **luteal phase of controls**, there was a significant decrease

from baseline in both LH and FSH levels during the whole test period ($F_{7,49}=8.50;p<0.001$ and $F_{7,49}=5.67;p<0.001$). In the PCOS group, LH and FSH levels were not significantly changed following the allopregnanolone injection. See figure 1 in paper IV.

Effects on estradiol and progesterone

In the follicular phase, S-progesterone showed a significant increase, but the changes were small ($F_{7,49}=3.38; p=0.005$). Estradiol levels were not significantly changed. In the luteal phase, S-estradiol was significantly decreased from baseline to 5 minutes ($F_{7,49}=2.39;p=0.035$), but S-progesterone was not changed. In the PCOS group, S-estradiol was significantly reduced ($F_{7,49}=2.52;p=0.025$) from baseline to 5 minutes and again between 30 and 60 minutes, but S-progesterone was not significantly changed. For illustration see figure 2 in paper IV. No correlations between serum levels of allopregnanolone and progesterone, estradiol, FSH or LH were shown in either of the phases in the controls or in the PCOS group.

Saccade changes in hyperandrogenic PCOS women and controls (Paper III)

Mean allopregnanolone levels at baseline were, as described in the previous section, significantly higher in the hyperandrogenic PCOS women than in the follicular phase of controls, but significantly lower than in the luteal phase of controls (see table 2 in paper III). BMI and weight were, as expected and described in the previous section, significantly higher in the hyperandrogenic PCOS group than in the controls (table 3). The ratios between baseline allopregnanolone/BMI and baseline allopregnanolone/weight were significantly different between the PCOS group and the controls in the luteal phase ($p=0.002$ and $p=0.004$), but not in the follicular phase. Absolute values of SEV and sedation did not differ between the hyperandrogenic PCOS group and the controls at baseline (see table 2 in paper III). Description of the hyperandrogenic PCOS women is given in table 4.

After the single allopregnanolone injection of 0.050 mg/kg, the allopregnanolone concentrations ([Allo]) in the hyperandrogenic PCOS group were significantly higher than in the controls in both phases ($F(1,15)=10.2;p=0.006$ and $F(1,15)=23.8;p<0.001$, respectively; see bottom panel figure 1, paper III).

Saccadic eye velocity sensitivity and sedation

There was a significantly higher response in delta (Δ) SEV at 20° in the hyperandrogenic PCOS group compared to the controls in the luteal phase, $F(1,15)=5.1;p=0.039$. The difference was especially notable later in the test period. The SEV in the hyperandrogenic group was significantly decreased between 5 and 60 minutes. However, in follicular phase controls there was a decrease only at 13 and 18 minutes and in the luteal phase only between 18 and 30 minutes. The SEV was thus decreased during a shorter period after the allopregnanolone injection in the controls compared to the hyperandrogenic group (see figure 1 top and middle panel in paper III). The Δ SEV at 30° likewise gave a greater response in the hyperandrogenic group than in the luteal phase controls, $F(1,15)=4.8;p=0.046$ (data not shown). Δ sedation VAS however showed no significant difference between the groups (see figure 2, paper III).

SEV related to the allopregnanolone concentration

The produced levels of allopregnanolone [Allo] after the injection were higher in the hyperandrogenic PCOS group than in controls (see figure 1, bottom panel in paper III). This occurred even though, or because, the dosage given was related to the body weight of the individual. To further investigate differences between the hyperandrogenic group and the controls in effect per unit allopregnanolone, a ratio (degrees/s divided by nmol/l) between the SEV and the corresponding [Allo] on each test occasion was calculated for each individual woman. With this procedure SEV became related to [Allo].

The ratios (SEV20°/[Allo]) over time were significantly lower in the hyperandrogenic PCOS group compared to controls in the follicular phase, $F(1,15)=10.2;p=0.006$, and luteal phase, $F(1,15)=19.2;p=0.001$, (see figure 3 in paper III). These differences were especially notable later in the test period. The interpretation is that there was a larger response per serum allopregnanolone unit nmol/l in the hyperandrogenic group compared to the controls. Similarly, the SEV30°/[Allo] ratio over time was different in the hyperandrogenic group compared to follicular phase controls, $F(1,15)=8.9;p=0.009$, and luteal phase, $F(1,15)=16.8;p=0.001$ (data not shown). The sedation/[Allo] ratio did not differ between the groups.

DISCUSSION

Isoallopregnanolone GABA-A receptor sensitivity and pharmacokinetics

Allopregnanolone have effects on the GABA-A receptor in humans, and the pharmacokinetics for allopregnanolone in humans is known [25]. Its stereoisomere isoallopregnanolone has been studied in several *in vitro* and *in vivo* studies in animals and has not been shown to have any effects on the GABA-A receptor [13, 71, 72, 74-85]. Instead, isoallopregnanolone has been used as a control to establish the specificity for allopregnanolone on the GABA-A receptor [37]. The pharmacokinetics and GABA-A receptor sensitivity to isoallopregnanolone has not been studied previously in humans, but needs to be investigated to evaluate the specificity of allopregnanolone on the GABA-A receptor in order to better interpret future studies and results.

After the intravenous isoallopregnanolone injections there was a dose-proportional increase in serum concentration of isoallopregnanolone and in parallel, significantly correlated, but with a slight delay, also a rise in serum concentration of allopregnanolone (figure 8, bottom panel). The obvious explanation is that some isoallopregnanolone has been metabolized into allopregnanolone. This theory is supported by earlier findings showing that allopregnanolone and isoallopregnanolone serum concentrations are highly correlated in both phases of the menstrual cycle [15]. Furthermore, the 3 α hydroxy steroid dehydrogenase is effective in both directions (figure 3), and when allopregnanolone is given to animals there is an increase in isoallopregnanolone concentration so one can expect that when isoallopregnanolone is given allopregnanolone should increase [264].

The study was carried out in the follicular phase of the menstrual cycle when endogenous concentrations of isoallopregnanolone and allopregnanolone are low. However, the baseline endogenous isoallopregnanolone concentration obtained in the present study was in the upper range of concentrations reported in the literature when using HPLC/RIA and gas chromatography-mass spectrometry as analytical techniques [15, 115, 265-267]. In addition, the allopregnanolone concentrations at baseline are in the upper normal range and higher than levels during mid-follicular phase in women but in the same range as samples taken in early follicular phase [16]. Two factors may explain the baseline levels. Firstly, the baseline samples are all taken early in the morning when ACTH-stimulated steroid production from the adrenals is high. During the follicular phase, the ACTH-stimulated adrenals is the major

source of serum allopregnanolone and most likely also of isoallopregnanolone [19, 268]. Secondly, the situation was somewhat stressful because the subjects knew that they were about to receive a compound never given to humans before, electrodes for oculography had been mounted and intravenous cannulas inserted. It is known that stress increases levels of allopregnanolone and isoallopregnanolone [26, 27, 30]. These factors may explain the somewhat higher baseline concentrations of isoallopregnanolone and allopregnanolone in the follicular phase in the present study.

The current study showed a significant decrease in saccadic eye velocity during the test. This was not anticipated, as earlier *in vitro* studies by our group have not shown any effect of isoallopregnanolone whatsoever directly on the GABA-A receptor [74]. A possible explanation for this finding is that a metabolism of isoallopregnanolone to allopregnanolone occurred and that the effect on the saccadic velocity actually was an effect of allopregnanolone. This theory is supported by the course of events during the study. The effect on SEV lasted from the first injection of isoallopregnanolone until 150 minutes after the last injection (figure 8, middle panel), which is longer than the effect produced by allopregnanolone directly where the effect declined after just 45 minutes [25]. Also, in the controls in paper III the effect of allopregnanolone had declined at 30 minutes. The concentration curve showed the same pattern but the dosage of allopregnanolone was a bit lower. However, in the present study, the concentration of isoallopregnanolone decreased rapidly, but for allopregnanolone the concentration reached a plateau and was almost constant until 35 minutes after the last isoallopregnanolone injection (figure 8, bottom panel). In addition, the cumulative dose of isoallopregnanolone was quite high, which might facilitate a substantial metabolism to allopregnanolone. Finally, an additional metabolism of isoallopregnanolone to allopregnanolone might also take place within the central nervous system without being detected in the periphery. The 3 α hydroxy steroid dehydrogenase acts in both directions, and the conversion of isoallopregnanolone to allopregnanolone is probably higher in the brain than in plasma [264]. Due to a high concentration of isoallopregnanolone the metabolism is most probably in the direction from isoallopregnanolone to allopregnanolone, and a higher allopregnanolone concentration can be assumed in the brain than in plasma [264]. However, we cannot exclude the possibility that isoallopregnanolone has an effect of its own, even though earlier *in vivo* and *in vitro* studies have not been able to show it [38, 74, 75].

Interestingly, no effect on self-rated sedation was noted in the present study (figure 8, top panel), although a significant decrease in SEV was observed.

One possible explanation for the lack of effect on sedation is that the concentration of allopregnanolone was less than one third of the concentrations produced in earlier studies where a distinct effect on sedation was found [25]. Higher concentrations than in the present study are probably necessary to produce an effect on self-rated sedation. SEV might also be a more sensitive measurement of sedation than subjective VAS scores. It has previously been demonstrated that sedation and saccadic eye velocity can show different responses to GABA-A stimulation [170]. An explanation for the differential effects on sedation and SEV might be differences in allopregnanolone accumulation, metabolism, receptor interaction and subunit composition in different areas of the brain involved in sedation compared to areas controlling the saccadic eye velocity.

Steroid effects on gonadotropins in the follicular phase

The main finding was that allopregnanolone, a GABA-A-active steroid, decreased the secretion of FSH and LH when given as intravenous injections to healthy women in the follicular phase, while isoallopregnanolone injections did not (figure 10). Two groups were compared, one receiving allopregnanolone (ALLO) and the other receiving isoallopregnanolone (ISOALLO). The divergent results between the two groups corroborate that the allopregnanolone decreasing effect on gonadotropin secretion is mediated through a specific GABA-A receptor activation and not an unspecific steroidal effect [38].

There was a slight difference in baseline levels of estradiol and FSH between the groups (table 2). A plausible explanation for the higher baseline levels of estradiol in the ISOALLO group is that they had higher BMI, which increases the estrogen levels [269] and the fact that 40% of the subjects in the ALLO group were tobacco smokers compared to none in the ISOALLO group and smoking decreases estrogen levels [270].

The effect of steroid injections on gonadotropin concentrations are in accordance with earlier *in vitro* and animal studies where changed GnRH secretion, decreased gonadotropin levels and suppression of ovulation have been noted following allopregnanolone administration [84, 138, 139, 142, 153]. Also, other GABA-A agonists have been shown to inhibit ovulation in rats [143] and decrease LH levels in ewes [144]. Several of the above-mentioned studies have reported no effect of isoallopregnanolone on neuroendocrine function, and it was thus concluded that the allopregnanolone effect is an exclusive GABA-A receptor mediated mechanism [84, 138, 139]. A limited number of studies have shown absence

of, or opposite effects, of allopregnanolone on neuroendocrine function *in vitro* and *in vivo* [271-274]. One explanation for these discrepancies might be that the subcomposition of the GABA-A receptor is different during different endocrine conditions [52-54].

In the ALLO group, there was no increase in progesterone or estradiol levels, thus the decrease in FSH and LH was not caused by negative feedback on the HPG-axis. In the ISOALLO group, however, there was an increase in progesterone levels, but this increase did not affect the gonadotropin levels by negative feedback (figure 11). This is in accordance with an earlier study where pregnanolone injections were given to women in the follicular phase and an increase in progesterone levels was found but no effect on gonadotropin secretion or estradiol levels [275].

An important question is whether the effect on the gonadotropins seen here with supraphysiological concentrations of allopregnanolone could be applied to the physiological situation. The decrease in gonadotropins in the ALLO group was present already at 5 minutes after the first injection of allopregnanolone, when serum levels of allopregnanolone were approximately 20 nmol/l. This is a physiological level during pregnancy [21, 22], but considerably higher than normal follicular phase or luteal phase levels [14-16, 24]. Still, as the changes in gonadotropin levels were detectable at the first time point after injection, and effects of lower concentrations between baseline and the 5 min value remain unstudied, an effect on gonadotropin levels in the physiological interval of allopregnanolone cannot be excluded. Further studies with lower doses are warranted, as well as studies during the luteal phase and in anovulatory conditions.

Allopregnanolone effects on gonadotropins in both menstrual cycle phases

The main finding was that in the luteal phase of healthy eumenorrheic women, allopregnanolone significantly decreased LH and FSH levels, and in the follicular phase allopregnanolone significantly decreased LH, but not FSH levels (see figure 1 in paper IV). The impact on gonadotropins was thus more pronounced in the luteal phase, when endogenous levels of progesterone and allopregnanolone are higher than in the follicular phase [14, 15].

The influence of allopregnanolone on gonadotropins is partly in accordance with our previous study in the follicular phase (paper II), where allopregnanolone significantly decreased the gonadotropins, both LH and

FSH. However, in the present study, the dose of allopregnanolone was lower than in the previous report. When looking at the graph, it seems as if there was a decrease in FSH in both phases of controls but the decrease was not significant in the follicular phase, probably due to a wide spread (see figure 1 in paper IV). The mean maximum concentration of allopregnanolone was 65 nmol/l, but the impact on the gonadotropins was still present three hours after the allopregnanolone injection, when the allopregnanolone serum concentration had declined to below 5 nmol/l in the follicular phase and below 7 nmol/l in the luteal phase, which is quite close to physiological concentrations of allopregnanolone during the menstrual cycle [14-16]. During the menstrual cycle, the main source of allopregnanolone differs between the phases. In the follicular phase, the adrenals are the main source and allopregnanolone is temporally coupled to serum cortisol. In the luteal phase, allopregnanolone is produced by the corpus luteum in the ovaries and temporally coupled to both serum LH and cortisol [19].

Interestingly, in this healthy eumenorrheic group, 20% (two out of ten), turned out to have no signs of ovulation, as detected in the luteal phase, and were thus excluded. In both cases the allopregnanolone injection had been administered in the follicular phase of the actual cycle and one could speculate as to whether the allopregnanolone injection in the follicular phase decreased the LH surge and subsequently prevented the ovulation.

The effect of allopregnanolone on the GnRH neurons is considered to be mediated via the GABA-A receptor [84, 138-140]. The difference in allopregnanolone effect in different cycle phases could be explained by different sensitivity of the GABA-A receptor in different endocrine conditions due to changed subunit composition. The subunit composition of the GABA-A receptor, and probably also the sensitivity, has been shown to change over the estrus cycle in mice [52].

Allopregnanolone effects on gonadotropins in anovulatory PCOS conditions

This is, to our knowledge, the first time allopregnanolone effects on gonadotropin secretion have been tested in PCOS, as a model for hyperandrogenic and anovulatory conditions in women. In both cycle phases of eumenorrheic women, allopregnanolone decreased LH, but in PCOS women allopregnanolone did not affect the levels of either LH or FSH (see figure 1 right panels in paper IV). Thus, it seems as if women with PCOS are resistant to the influence of allopregnanolone on gonadotropin secretion.

At baseline, elevated serum concentrations of allopregnanolone were observed in the PCOS women compared to follicular phase controls (see table 2 in paper III). One explanation could be the difference in BMI, as the PCOS women had higher BMI than the controls. Also, higher levels of allopregnanolone have been reported in obese, compared to lean, girls [205, 206], and in overweight men and women compared to controls [207]. However, the ratio between baseline allopregnanolone level and BMI was not significantly different between the PCOS women and the follicular phase controls, suggesting that the weight factors (fat tissue) might be one reason for the higher baseline concentrations of allopregnanolone. This is also in accordance with a significant difference in baseline allopregnanolone and BMI between the healthy groups in paper II.

Another finding was that women with PCOS had higher baseline levels of progesterone compared to follicular phase controls (see table 2 in paper IV). Since allopregnanolone is a progesterone metabolite, this is in accordance with higher allopregnanolone serum levels in PCOS women compared to controls in the follicular phase. Previous studies have shown that while allopregnanolone levels were increased in obese adults and girls compared to non-obese subjects [205, 207], progesterone levels did not differ between obese and non-obese girls [205]. Therefore, we conclude that the elevation of baseline progesterone levels in the PCOS women in this study could probably not be explained by their overweight.

Another explanation for elevated allopregnanolone levels could be that the PCOS women are anovulatory. In women with amenorrhea, the main source of allopregnanolone is presumed to be the adrenals [19]. There is a limited body of evidence indicating a relationship between levels of endogenous allopregnanolone and anovulation in humans. Higher baseline serum levels of allopregnanolone have been reported in subjects suffering from hypothalamic amenorrhea compared to the follicular phase in healthy controls [19]. Moreover, elevated serum levels of allopregnanolone were found in patients with premature ovarian failure compared to both postmenopausal and fertile women [149]. Besides the physiological increase of allopregnanolone levels during the luteal phase of the menstrual cycle and pregnancy, serum levels of allopregnanolone increase during stress, anxiety and panic disorders, binge eating, anorexia and bulimia nervosa [27, 121, 125, 126]. In humans suffering from acute stress, endogenous allopregnanolone levels have been shown to rise from 1.0 to 1.3 nmol/l [27]. Although the increase is discrete, the time point in relation to menstrual cycle phase, as well as whether the rise is constant or not, might be significant for effects on the ovulatory mechanism.

However, the concentrations of gonadotropins in PCOS women showed no decrease during the allopregnanolone challenge, even though the produced serum levels of allopregnanolone after the injection were higher in the PCOS women than in the controls (see figure 1 bottom panels in paper IV). One explanation for this difference might be that the PCOS women seem to have a different endocrine milieu, dominated by a chronic hyperandrogenism [202, 203], but also, as shown in the present thesis, a situation with high allopregnanolone levels and anovulation, compared to eumenorrheic controls. Androstenediol and allopregnanolone have agonistic effects on the GABA-A receptor [39, 204]. It is important to notice that the GABA-A receptor subunit composition and sensitivity seems to be changed during exposure to neurosteroids and other GABA-A receptor active compounds [111], but also during different endocrine conditions such as over the estrus cycle [52], during androstenediol influence [52] and in puberty in mice [53, 54]. In female mice exposed to anabolic androgenic steroids, the GABA-A receptor was changed [68] and anabolic androgen steroids are also allosteric modulators of the GABA-A receptor [69].

Tolerance development could be one explanation for the lack of effect in the PCOS group. However, tolerance was not noted in the study measuring saccadic eye velocity in the same women (paper IV). On the other hand, we do not know whether there are different concentrations of allopregnanolone in the areas responsible for gonadotropin or SEV control, or whether the GABA-A receptor subtype responsible for the effect on the gonadotropin secretion and the saccade velocity is different. Different subtypes may express different tolerance behavior.

Saccade changes in hyperandrogenic PCOS women and controls

In the present study, we measured saccadic eye movement during an allopregnanolone challenge in eumenorrheic controls in both cycle phases and PCOS women, as a model for a hyperandrogenic condition. We found that the sensitivity to allopregnanolone, expressed as a greater reduction of SEV per concentration unit of serum allopregnanolone, was higher in hyperandrogenic women than in both follicular and luteal phase controls (see figure 3 paper III). There were no differences between the PCOS and control groups in self-rated sedation.

The hyperandrogenic women turned out to have higher baseline levels of allopregnanolone compared to the follicular phase controls, but the PCOS women also had a higher BMI, which could explain the difference in baseline allopregnanolone concentrations [205, 206]. It is known that

hyperandrogenic PCOS women have elevated levels of a GABAergic androgen metabolite [202, 203], and obese PCOS women tend to be more hyperandrogenic than their normal-weight counterparts [184]. Taken together, the hyperandrogenic women seem to have higher levels of GABAergic modulators in their circulation, which could influence the GABA-A receptor sensitivity.

After the allopregnanolone injection, a larger increase in serum allopregnanolone concentration ([Allo]) was observed in the hyperandrogenic group compared to the controls in both cycle phases (see figure 1 bottom panel in paper III). In animal studies of allopregnanolone-mediated anesthesia there was a very high accumulation of allopregnanolone in fat tissue after 90 minutes of anesthesia, but the initial concentration in fat after 30 minutes of anesthesia was low [276]. Thus, there seems to be a delay in the accumulation of allopregnanolone in fat tissue, at least in rats. Nevertheless, this might also be the case in humans, and could be the reason for higher levels in women with higher BMI compared to slimmer women. However, in this study we only explored the concentrations of allopregnanolone in the peripheral serum compartment; the difference in allopregnanolone levels in the brain and fat tissue remains unknown.

In order to examine whether the increase in SEV response was solely related to the elevated allopregnanolone serum concentrations ([Allo]) and not dependent on a changed sensitivity to allopregnanolone, a ratio between SEV and the [Allo] was calculated to ascertain degrees/second per nmol/l of allopregnanolone (see figure 3 in paper III). The ratio was lower in the hyperandrogenic group, indicating that the increased SEV response was not linked to the higher [Allo] after the injection, but instead indicating a true difference in the allopregnanolone sensitivity. Thus, we can conclude that the sensitivity to allopregnanolone is higher in hyperandrogenic women than in controls. Moreover, we have investigated the difference between the groups related to the obtained serum concentration of allopregnanolone, and adjusted for the increased concentration obtained due to the higher BMI, and not related the effect only to the dosage given. Therefore, we are confident that it is a pharmacodynamic effect on receptor sensitivity to allopregnanolone that we observe, and not only an effect related to higher serum concentrations. In addition, the self-rated sedation scores revealed no difference between the hyperandrogenic and the control group, and if the CNS effect was only due to increased serum concentration we would have expected a similar difference between the groups in the self-rated sedation as in SEV. Thus, this lack of difference in effect on self-rated sedation supports the interpretation that a pharmacodynamic mechanism explains the difference between the hyperandrogenic and the control groups.

It is known that activation of the GABA-A receptor stimulates feeding [79] and allopregnanolone has hyperphagic effects in rats [79]. In women, energy intake is higher in the luteal phase, when allopregnanolone levels are increased [127]. In these hyperandrogenic, overweight PCOS women, we found elevated baseline allopregnanolone levels compared to follicular phase controls. Earlier studies on PCOS women have shown elevated levels of the androgen metabolite androstenediol [202, 203], which stimulates the GABA-A receptor [39, 204]. Furthermore, the tendency is that in PCOS women, the more obese, the more hyperandrogenic they are [184]. In the present study, we have also found an increased GABA-A receptor sensitivity to allopregnanolone, as shown with saccadic eye velocity measurements. Taken together, it is interesting to speculate on whether these are factors that contribute to the higher degree of overweight found in PCOS women.

It has been reported earlier that high doses or chronic exposure to allopregnanolone induced tolerance of the GABA-A receptor in animals [86]. Therefore, our hypothesis was that the GABA-A receptor sensitivity would decrease in parallel to a tolerance development due to the chronic exposure to GABAergic substances in hyperandrogenic women. Instead the reverse, an increased sensitivity in hyperandrogenic women, was found. However, the increase of baseline allopregnanolone in the hyperandrogenic group was modest, and androstenediol is a weak GABA-A receptor agonist compared to allopregnanolone [39] and tolerance development is dependent on high and constant exposure [111]. It is possible that the concentrations of androstenediol were not high enough to induce tolerance development. Another explanation is that hyperandrogenic women have similar GABA-A receptor sensitivity compared to healthy postmenopausal women who show increased GABA-A receptor sensitivity to benzodiazepines and pregnanolone following oral progesterone or progestagen treatment [161, 277]. Oral progesterone is metabolized, to a high degree, into allopregnanolone [110].

The sensitivity of the GABA-A receptor could also depend on its subunit composition. Animal studies have shown that hormonal state can alter the subunit composition of the GABA-A receptor shown after androstenediol influence and over the estrus cycle, during oral contraceptive usage, during pregnancy and after delivery and under chronic exposure to anabolic androgenic steroids [52, 55-57, 68].

Methodological considerations

In papers III and IV the PCOS women in both studies are the same; all of them are anovulatory and have hyperandrogenism. In paper III, they are also referred to as the hyperandrogenic group, which in this thesis can be a bit confusing. The reason is that in study III we wanted to examine the effects of GABAergic androgens on the GABA-A receptor, and clearly indicate this difference between the groups. In paper IV, the difference between the groups is basically seen from the reproductive perspective. In studies III and IV it would have been more appropriate if the PCOS group and a control group had been weight-matched. Our aim, though, was to examine PCOS women with high levels of circulating GABA-agonists, i.e. the hyperandrogenic PCOS women. The higher BMI a PCOS woman has, the more hyperandrogenic she is [184]. All of the PCOS women had received their diagnoses before the inclusion visit. They all met the PCOS criteria very strictly which is a benefit in these studies. A grading of the hirsutism using the Ferriman Goldway scale would have been elegant in both PCOS women and controls, as well as if the controls also had been biochemically controlled for hyperandrogenity and not only with clinical signs.

In study II, daily ratings of prospective symptoms were done in the ISOALLO group, and this includes data on when the next menstrual period occurred after the steroid injection. It would have been more complete, and probably also interesting, if the women receiving allopregnanolone had filled in such a diary and the date of the next period had been established. The results with decreasing levels of LH and FSH after the allopregnanolone injection were not anticipated, and unfortunately we do not have the data in the ALLO group on whether the next period was on time or delayed.

One obvious limitation in these studies is the number of study subjects. A higher number would of course give better power, but still, even though the number of subjects, for example, in the gonadotropin studies is low it shows significant changes. In study II compared to study IV the decrease in LH levels is confirmed in both studies but the decrease in FSH is only present in the first one (Paper II). Perhaps the explanation could be that the number of subjects was smaller in study IV, eight compared to ten, and the allopregnanolone dose was slightly lower. In addition, if study IV also had explored only the situation in the follicular phase, the two subjects who were excluded due to lack of signs of ovulation in the following luteal phase would not have been excluded. Maybe those two subjects had the most pronounced effect of allopregnanolone.

Future investigations

The results with decreased levels of gonadotropins after allopregnanolone injections are very interesting. Therefore, further studies are needed to explore whether lower doses of allopregnanolone, closer to non-pregnant physiological ranges, can affect the gonadotropins and thereby perhaps establish the link between stress and hypothalamic amenorrhea.

The research field of obesity, appetite and food intake regulation, including the GABA-A receptor, would be very interesting to investigate further to establish the role of allopregnanolone. Does allopregnanolone also increase food intake in humans? Does allopregnanolone have a role in obesity development? Perhaps in this way one could find clues on how to prevent obesity development and useful ways for treatment.

Studies of the distribution of different GABA-A receptor subunits in the brain and concentrations of allopregnanolone in different brain areas are possible to conduct in research animals but more difficult in humans. Functional MRI and perhaps also positron emission tomography – computed tomography (PET-CT) of the brain can be tools in future brain research.

Also, it would be interesting to investigate possibilities in humans to manipulate allopregnanolone levels, for instance with antagonists, and whether these substances can be used to develop treatments for conditions where allopregnanolone is involved.

GENERAL CONCLUSIONS

- Isoallopregnanolone has no effect on self-rated sedation, mood ratings and daily symptom ratings. The subjects reported no symptoms of discomfort. There was a slight pulse frequency decrease during the test, which we interpret as due to rest. There is a metabolism of isoallopregnanolone into allopregnanolone, which we believe explains the effect on saccadic eye velocity that we detected during the test.
- Allopregnanolone decreases gonadotropins in the follicular and luteal phase in healthy women, and the effect is more pronounced in the luteal phase. Isoallopregnanolone has no effect on the gonadotropins in the follicular phase. This finding suggests that the allopregnanolone effect most likely is mediated by the GABA-A receptor, since allopregnanolone has such specific effects on the GABA-A receptor, and since no effect is seen with isoallopregnanolone.
- In anovulatory, hyperandrogenic PCOS women, the gonadotropin regulation is not influenced by allopregnanolone. This finding can be explained by a tolerance development due to a chronic situation with high levels of GABAergic substances, e.g. the androgen metabolite androstandiol (where elevated levels in PCOS women are shown in previous studies made by others), and as we found in the PCOS women, elevated levels of allopregnanolone. The changed sensitivity can also be explained by the fact that the GABA-A receptor can change its subunit composition in different endocrine conditions.
- PCOS women, compared to healthy women, have an increased sensitivity to allopregnanolone on the GABA-A receptor, explored with the indirect method saccadic eye velocity. In this situation, though, there are no signs of tolerance to allopregnanolone, and a possible explanation for this difference is that those two functions, the regulation of gonadotropins and saccades, involve two rather different areas in the brain. Gonadotropin secretion is regulated from the hypothalamus and saccades are controlled from areas including the mesencephalon, pons and cerebellum. GABA-A receptors in different parts of the brain probably have different sensitivity to allopregnanolone, and this can be explained by different receptor subunit composition in the regulating regions, or differences in concentrations and metabolism in different areas.

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