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Dissertation thesis

NEUROACTIVE STEROIDS - PHYSIOLOGY AND PATHOPHYSIOLOGY

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CONTENTS

ABBREVIATIONS USED

3ß-HSD	– 3ß hydroxysteroid dehydrogenase
A2	–androstenedione
A3α5α	– androsterone (3α– hydroxy-5α–androstan-17-one)
A3α5β	– etiocholanone (3α–hydroxy-5β–androstan-17-one)
A3β5α	– epiandrosterone (3β–hydroxy-5α–androstan-17-one)
A3β5β	– epietiocholanone (3β–hydroxy-5β–androstane-17-one)
ACTH	– adrenocorticotropin hormone
AED	– antiepileptic drug(s)
Allo	– allopregnanolone, P3α5α, 3α–hydroxy-5α–pregnan-20-one
AS	- androstane steroids
CIL	- chemimmunoluminescence
CNS	– central nervous system
CRH	– corticotropin releasing hormone
CSF	– cerebrospinal fluid
DHEA	– dehydroepiandrosterone
DHEAS	– dehydroepiandrosterone sulfate
E_2	_ estradiol
ELISA	– enzyme-linked immuno sorbent assay
GABA	– gamma-aminobutyric asid
GS-MS	– gas chromatography-mass spectrometry
HPLC	– high pressure liquid chromatography
HSOR	– hydroxy steroid oxidoreductase
LH	– luteinizing hormone
MOX-TMS	– TMS, but oxo groups modified to methoxylamine

NAS – neuroactive steroid(s)

NMDA – N-methyl-D-aspartate

NS – neurosteroid(s)

P3α5β – pregnanolone, 3α–hydroxy-5β-pregnan-20-one

P3β5α – isopregnanolone, 3β–hydroxy-5α–pregnan-20-one

P3β5β – epipregnanolone. 3β–hydroxy-5β–pregnan-20-one

P – progesterone

PI – pregnanolone isomers

PICs – pregnanolone polar conjugates

PNAS –Proceeding of the National Academy of Sciences of the USA

PS – pregnan steroid(s)

PregS – pregnenolone sulfate

RIA – radioimmuno assay

S – steroid(s)

SHBG – sex hormone binding globulin

SSRIs – selective serotonin reuptake inhibitors

T – testosterone

THDOC3α5α – tetrahydrodeoxycorticosterone

TLC – thin layer chromatography

TMS – steroid hydroxyl groups modified to trimethylsilyl derivatives but intact oxo groups

1 Introduction

The brain, similarly to the gonads, adrenal glands and placenta, produces steroid hormones. Steroids which are synthesized in the central and peripheral nervous system are called neurosteroids (NS). Steroids which act on the nervous system, regardless of their site of synthesis, are known as neuroactive steroids (NAS). Some NS are able to easily pass through the blood-brain barrier (Bixo et al. 1997). Disorders in their biosynthesis or malfunctions in their interactions with target sites can be the cause of many pathologies, including psychiatric illnesses. In contrast with most common steroid hormones acting, NS largely affect non-genomic mechanisms and influence nerve excitability in both directions. Some NS, such as allopregnanolone (allo-) or dehydroepiandrosterone (DHEA) and its derivates, also show neuro-protective effects (Ciriza et al. 2004, Morfin and Starka 2001, Shi et al. 2000).

Already in the early 1970s, Backstrom demonstrated the relationship between premenstrual tension and changes in estradiol and progesterone levels (Backstrom and Mattsson 1975). In 1980, the term neurosteroid appeared for the first time in the Czech literature (Schreiber 1980), and a year later the term was first used in the international literature in connection with experiments based on the hypothesis concerning the direct biosynthesis of steroids in the brain independent of the peripheries (Corpechot et al. 1981). Three years later, Harrison and Simmonds published their work on the anesthetic effect mechanism of the synthetic pregnane steroid ganaxalone through modulation of the stimulation of the γ-aminobutyric acid receptor (GABA-r) (Harrison and Simmonds 1984). The following year, Majewska published the first study on the modulation effect of endogenous steroids on GABA-r, and thereby initiated an intense research effort focused on the mechanisms of neuroactive steroid activity which continues to this day (Majewska et al. 1985).

1.1. Mechanisms of the effects of pregnane steroids

Neuroactive steroids act mainly as modulators of ionotropic receptors in nerve cell membranes responsible for the permeability of relevant ions (Pisu and Serra 2004). Among the most well known receptors affected by NS are the γ-aminobutyric acid type A receptors

($GABA_A$-r). These receptors are responsible for the influx of chloride ions to nerve cells and subsequent inhibition of nerve function. $GABA_A$-r are positively modulated by pregnane and androstane steroids with hydroxylation at the 3α-position. Pregnane steroids (PS) with hydroxyl group at the 3β- position, however, reduce the increased capture of chloride ions caused by the positive modulation of $GABA_A$-r (Lundgren et al. 2003, Prince and Simmonds 1992, Wang et al. 2002). While 3α-PS are therefore extremely effective endogenous neuroinhibitors, 3β-PS and polar conjugates of all pregnane steroids act as their antagonists, and in some cases with comparable modulating activity (Park-Chung et al. 1999) if we take into account their higher concentration in body fluids (Havlikova et al. 2006, Hill et al. 2007). 3α-Pregnane steroids shorten the period of so-called paradox sleep, decrease the liberation of acetylcholine in the neocortex and hippocampus, suppress neurogenesis and worsen spatial memory. These effects are modulated through $GABA_A$-r (Mayo et al. 2003). 3α-androstane steroids, such as androsterone and 5α-androstan-3α, 17β-diol stimulate $GABA_A$-r, while 3β-androstane derivates, like isomers with hydroxylation in position 17α-, are non-effective (Bitran et al. 1996). In addition to pregnanolone isomers, $GABA_A$-r are also negatively modulated by polar conjugates of 3β-hydroxy-5-en steroids such as pregnenolone sulfate and DHEAS (Twede et al. 2007).

Another well-known type of receptor influenced primarily by polar conjugates of NS are N-methyl-D-aspartate receptors (NMDA-r) responsible for the influx of calcium ions to nerve cells and their activation. Conjugates of 5α-PS act as activators of NMDA-r, as do sulfate of 3β-hydroxy-5-en steroids, while conjugates of 5β-PS have the opposite effect (Park-Chung et al. 1994). This means that conjugates of 5α-pregnane steroids are neuroactive substances from this point-of-view, while 5β-pregnane steroid conjugates are neuroinhibitors. Aside from these effects on neuron membranes, some NS also bind to progesterone intracellular receptors, and thus also influence the gene expression of $GABA_A$-r subunits (Diano et al. 1997, Dubrovsky 2005, Gu et al. 1999). It must be emphasized that NMDA-r and $GABA_A$-r, the pair of receptors most often influenced by neuroactive steroids, are also present outside of the central nervous system (CNS) (Leung et al. 2002).

Pregnane steroids with a hydrogen in positions 5α and 5β- also block T-type calcium channels in peripheral neurons in rats, which play an important role in the sensing of pain, whereas 5β- isomers display increased modulation activity (Todorovic et al. 2004). These

mechanisms described above therefore also explain the antinociceptive activity of pregnane steroids at the peripheral level, independent of the modulation of $GABA_A$-r.

1.2. Enzymes participating in the biosynthesis of pregnane steroids

The biosynthesis of neuroactive steroids begins with the conversion of cholesterol sulfate to the neuroactive pregnenolone sulfate (PregS). Subsequently, the 5-en metabolic pathway continues through the action of C17-hydroxylases, C17,20-lyases, with the production of non-active 17-hydroxy-pregnenolone and its neuro-protective metabolite DHEA. An important metabolic step is the 3β-hydroxysteroid dehydrogenase (3β-HSD) conversion of pregnenolone to progesterone, which is a precursor of many neuroactive pregnane steroids (Fig.1). Through reducing metabolism, 5α- and 5β reductases contribute to the biosynthesis of progesterone, deoxycorticosterone, cortisol and testosterone with high activity in the liver, in nervous system cells, and in tissues related to pregnancy and birth (Kawahara et al. 1975, Lisboa and Holtermann 1976, Milewich et al. 1978, Stoffel-Wagner et al. 2000). Both enzymes irreversibly convert progesterone to the relevant dihydroprogesterones. The final metabolic step responsible for the biosynthesis of neuroactive PS is reversible and is performed by stereospecific 3α- and 3β- hydroxysteroid oxidoreductases (HSOR) (Campbell and Karavolas 1990, Krause and Karavolas 1980, Patte-Mensah et al. 2004, Rupprecht and Holsboer 1999, Stoffel-Wagner et al. 2000, Tsuruo 2005).

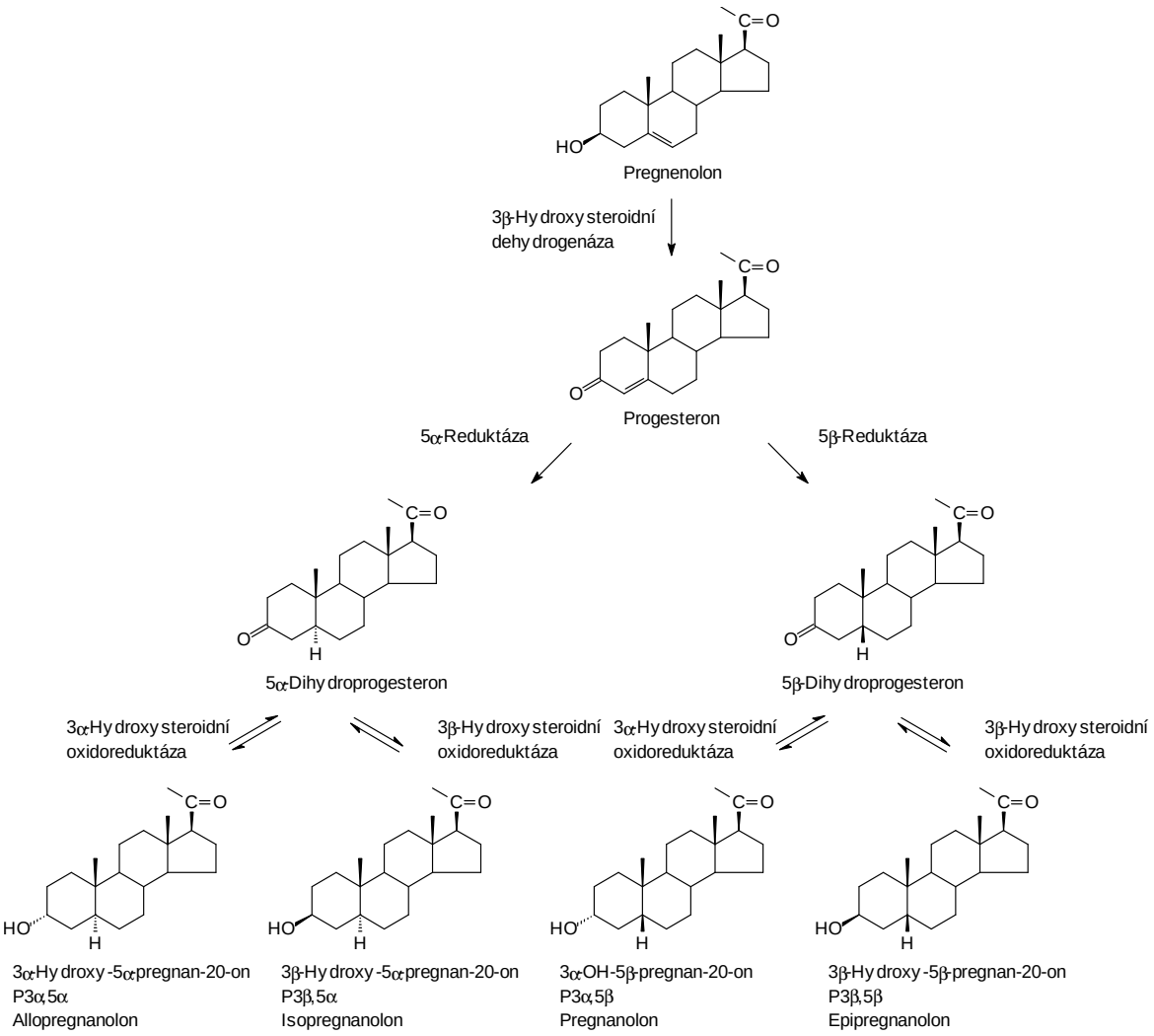

Figure 1. Biosynthesis of reduced Progesterone metabolites

1.3. Biosynthesis of neuroactive steroids in nervous system cells

An important landmark in the research of the biochemistry of neuroactive steroids was the discovery of their biosynthesis directly in the CNS (Corpechot et al. 1985, Le Goascogne et al. 1987, Schumacher et al. 2003). This gave rise to the consideration of neurosteroids, i.e. molecules produced in nerve or ancillary cells. In the CNS, neurosteroids are synthesized in the mitochondria of glial cells and neurons, both in the brain and peripheries (Schumacher et al. 2000). Enzymes that are essential for the synthesis of pregnane neurosteroids, such as 3β-HSD and 5α-reductase type 1 (5α-R1) and 2 (5α-R2), are present in the spinal cord as well as in the brain. The steroid sulfatase and sulfotransferase systems are important in mediating changes or even the reversal of the neuromodulating affect of PS. Relatively high sulfatase activity, though rather lower sulfotransferase activity, can be found in cells of the CNS (Compagnone et al. 1997).

1.4. Biosynthesis of pregnane steroids and their precursors in the gonads

The biosynthesis of neuroactive steroids varies depending on age and gender. In women, the largest proportion of pregnane type neuroactive steroids produced from progesterone metabolites are synthesized in the corpus luteum in the luteal phase of the menstrual cycle (Ottander et al. 2005). The dominant pregnane-type metabolite is allopregnanolone, i.e. 3α-hydroxy-5α-pregnane-20-one (Hill et al. 2005). Similarly to progesterone, its reduced metabolites show a decrease with age in women, with a qualitative change after menopause (Genazzani et al. 1998).

Changes in the concentration of progesterone that are also reflected in changes in the levels of its reduced metabolites are, together with the abstinence effect, an evident cause of premenstrual syndrome in women. With a sudden decrease in positive steroid modulators of $GABA_A$-r, the abstinence effect occurs fairly quickly, the same as when inducing dependence on these compounds. Changes in the concentrations of the relevant steroids are accompanied by changes in the expression of $GABA_A$-r subunits responsible for their affinity to these compounds.

These mechanisms clearly require good synchronization, as their malfunction could have significant neuropsychological or neuropsychiatric consequences. This is of course true not

only for the menstrual cycle, but also for other physiological or pathological situations, such as pregnancy, birth, lactation, the onset of menopause, trauma, endocrine disease, stress, or the use of medicines which affect some of the elements of this mechanism. Additionally, other influences may play a role, such as the subsequent metabolism of these compounds mainly through sulfatizing and oxido-reductive balance changes or reversals in receptor affinity to neuroactive steroids through the phosphorylation of specific receptor sites (Brussaard and Koksma 2003).

In men, it is clear that the main source of gonadal $GABA_A$-r modulation is testosterone, which in a mechanism similar to that for ovarian progesterone is converted in the first step to 5α- and 5β-dihydrotestosterone and further to androstane 17-oxo- and particularly 17β-hydroxy- metabolites, which act on $GABA_A$-r similarly to pregnane steroids (Reddy 2004).

1.5. The function of the adrenal cortex in the biosynthesis of pregnane steroids

The adrenal cortex produces most of the precursors of neuroactive steroids. In the zona glomerulosa and zona fasciculata, deoxycorticosterone is produced, with subsequent products being the neuroactive isomers of 3α,5α-tetrahydrodeoxycorticosterone (THDOC3α5α). In contrast to allopregnanolone, which reaches a level about 10% of the progesterone level, the basal levels of deoxycorticosterone and THDOC3α5α are similar

In humans (<0.5 nmol/l) and during acute stress can approach values sufficient for the initiation of a neuromodulatory effect (Reddy 2006). In the 5-en pathway, pregnenolone sulfate is produced in the adrenal zona fasciculata (40-800 nmol/l), correlating with the level of cortisol (de Peretti et al. 1986). Considering the quantity, pregnenolone sulfate is the most significant precursor of progesterone and deoxycorticosterone of adrenal origin. Progesterone is also a substrate for the production of 5α- and 5β-dihydroprogesterone, and further neuroactive pregnanolone isomers. These mechanisms can easily explain the higher levels of brain NS documented in patients having diagnoses connected to stress (Higashi et al. 2005). The above-given mechanisms, dependent on the peripheral production of neuroactive steroids and their precursors and transport across the blood-brain barrier, can be considered to be a physiological compensation for stress. Results from the literature show an evident connection between the production of some pregnane neuroactive steroids

and adrenal gland activity, particularly in women of fertile age in the follicular phase of the menstrual cycle (Hill et al. 2005), as well as children, men, and the elderly. However, the proportion of pregnane steroids dependent on adrenal gland activity is much lower when compared to the proportion dependent on production in the corpus luteum. In addition to pregnenolone sulfate, the adrenal gland zona fasciculata produces mainly cortisol, and similarly as for progesterone, its reduced metabolites can likewise demonstrate neuromodulatory activity through interaction with $GABA_A$-r (Stromberg et al. 2005). The final adrenal cortex zone, which is less dependent on stimulation from ACTH, the zona reticularis, produces rather androgenic precursors and, to a certain extent, testosterone. These substances, of course, are substrates for the production of 5α- and 5β-dihydrotestosterone and further neuroactive androstane metabolites.

1.6. Pregnane steroids and CNS disorders during birth and postpartum

Already in 1989, Majewska published a hypothesis on the role of steroid modulators of GABA-r in the pathogenesis of post-partum depression. This hypothesis was supported five years later in an Italian study (Nappi et al. 1994).

Pregnenolone sulfate is a very abundant neuroactive steroid in the maternal and especially fetal compartments. Its role is not straightforward, as it may act as an activator of nerve functions through the activation of NMDA-r or the negative modulation of $GABA_A$-r, but at the same time act as an activator of $GABA_A$-r, both through its role as a precursor of progesterone and in some cases even directly.

1.7. Neuroactive steroids and addictive substances

NS are important modulators of the affects of alcohol on $GABA_A$-r (Morrow et al. 2001). While both activators of $GABA_A$-r and their precursors following relevant conversions intensify these affects, negative modulators of $GABA_A$-r and activators of NMDA-r mitigate them (Vanover et al. 1999, Barbosa and Morato 2001, Czlonkowska et al. 2000, Mitchell et al. 2005,).

Another affect of alcohol, which can be influenced by neuroactive steroids, is appetence for alcohol. For instance, rats given allopregnanolone, showed increased

appetence for alcohol, while giving 5α-reductases i.e. one of the enzymes responsible for the synthesis of neuroactive 5α-pregnane steroids, had the opposite effect (Ford et al. 2005). Strong links can be found especially in the relationship of neuroactive steroids to the intensity of abstinence symptoms after withdrawal of alcohol. Withdrawal symptoms in alcohol-dependent rats taken off on alcohol were eliminated by giving allopregnanolone. By suddenly reducing allopregnanolone levels, an up to tripling in gene transcription coding the α_4 GABA receptor subunit was observed. Blocking the transcription of this gene led to the prevention of symptoms related to abstinence syndrome (Devaud et al. 1995, Smith et al. 1998).

For both abstinence symptoms and the protective effects of allopregnanolone there exist evident gender differences (Devaud et al. 1995), and in females there is also a difference in susceptibility to alcohol addiction with phases of the menstrual cycle. In the luteal phase, when levels of steroid activators of $GABA_A$-r are increased, even an increased sensitivity to alcohol has been observed in female primates. In females given higher alcohol doses, the sensitivity to allopregnanolone and alcohol was less affected by the menstrual cycle (Green et al. 1999). Some results indicate that chronic alcoholism influences anxiety through $GABA_A$-r in the hippocampus (Martin-Garcia and Pallares 2005).

In addition to the influences of nerve cell membrane receptor modulators on the effects of alcohol, influences in the opposite direction have also been documented. In rats, chronic alcohol use led to the up-regulation of NMDA-r in the brain (Kalluri et al. 1998). The activity of positive steroid modulators of NMDA-r is weakened by alcohol. This effect is specific for NMDA-r and was not related to changes in the expression of NMDA-r subunits. Negative modulation activity of pregnenolone sulfate (PregS) on $GABA_A$-r was not influenced by alcohol (Bowen and Grant 1998, Costa et al. 2000). In contrast, the binding of the non-steroid $GABA_A$-r activators flunitrazepam and muscimol was significantly higher in alcohol-dependent rats (Mehta and Ticku 1998).

Prenatal alcohol exposure led to lowered sensitivity of $GABA_A$-r to allopregnanolone, evidently as a result of addiction to this neuroactive steroid (Mehta and Ticku 1998).

In addition to the direct influence on the function of nerve cell membrane receptors, alcohol can also influence the concentration of neuroactive steroids in the brain. The acute use of alcohol leads to increased levels of allopregnanolone and progesterone in women

during both menstrual cycle phases (Torres and Ortega 2003) as well as in men (Torres and Ortega 2004). An anesthetic dose of alcohol also led to a marked increase in levels of progesterone and pregnenolone in the brain of rats. Furthermore, in hypophysectomized and adrenalectomized animals, these increased concentrations were not found. On the other hand, an increased level of these steroids after ACTH stimulation was seen in hypophysectomized animals, which in this case highlights the predominant adrenal origin of both these brain steroids. In rats of both genders, a distinct dose and time dependence on significantly increased concentrations of allopregnanolone in the cortex to pharmacologically relevant levels was observed after systematic exposure to alcohol (Morrow et al. 2001). The levels of allopregnanolone present correlated with the intensity of the hypnotic effect of alcohol. In contrast, inhibition of 5α-reductase with finasteride led to an inhibition of alcoholic effects.

Chronic alcoholism, however, has a completely opposite effect on the concentration of steroid activators of $GABA_A$-r. Considerably lower levels of $GABA_A$-r steroid activators have been described in both human genders (Hill et al. 2005, Morrow et al. 2001, Romeo et al. 1996). During anti-alcohol detoxification therapy, there was a significant tendency in women towards the restoration of pregnane steroid and progesterone levels to control values, even already after about 100 days of treatment (Hill et al. 2005). The most significant changes were seen in the ratio of the most abundant pregnane steroid and the most affective endogenous positive $GABA_A$-r modulator allopregnanolone and pregnenolone sulfate, which is a positive modulator of NMDA-r and can also negatively modulate $GABA_A$-r. Chronic alcohol use evidently induces a decrease in levels of pregnane neurosteroids in the hippocampus (Cagetti et al. 2004). While allopregnanolone is produced mainly in the corpus luteum, pregnenolone sulfate originates primarily in the adrenal zona fasciculata, the same as cortisol. As was already mentioned,

allopregnanolone lowers alcohol tolerance, increases the effect of alcohol on $GABA_A$-r, and increases alcohol appetence, while pregnenolone sulfate has the opposite effects.

1.8. Are neuroactive steroids addictive substances?

It is known from the literature that in addition to 3α-pregnane steroids, benzodiazepine and alcohol also have $GABA_A$-r modulating activity. All of these substances have anxiolytic, ataxic,

anticonvulsive, and hypnotic effects. Many studies on the ability of steroid $GABA_A$-r modulators to be addictive have been performed, showing quick induction mainly by neuroinhibitory 3α-pregnane steroids. Blocking the chronic function of 3α-pregnane steroids leads to symptoms similar to the abstinence effect of chronic alcoholism. The induction of the abstinence effect, however, leads to the gradual increase in originally lowered $GABA_A$-r concentrations brought about by the originally excessive levels of positive $GABA_A$-r modulators. This phenomenon thus reflects a likely compensation mechanism reacting to the sudden appearance of a deficiency in the influx of chloride to neurons in the hippocampus (Bitran and Smith 2005). In addition to increased $GABA_A$-r concentrations, there is also a specific increase in the expression of α4- subunits of $GABA_A$-r, the presence of which in relevant $GABA_A$-r subtypes is connected to an increased affinity to 3α-pregnane steroids. In contrast, alcohol use again decreases the expression of α4- subunits of $GABA_A$-r (Smith and Gong 2004).

1.9. Pregnane steroids and stress

Stress leads to the increased production of corticosteroids in plasma and in the brain. In addition to cortisol, the production of deoxycorticosterone is also increased. 3α-metabolites of deoxycorticosterone have anxiolytic and anticonvulsive effects through $GABA_A$-r. This could be important in the explanation of the mechanisms of some pathophysiologies related to stress situations, such as epilepsy, panic disorders, post-traumatic syndrome, and depression (Reddy 2006).

1.10. Pregnane steroids and CNS disorders

From the available information it is evident that epilepsy in women is related to reproductive endocrinology disorders, especially to hypogonadotropic hypogonadism, lowered or abnormal secretion of luteotropin hormone (LH), a higher incidence of anovulatory cycles, and disorders in the production of ovarian steroids (Morrell 1999, Stoffel-Wafner et al. 1998).

In the relationship of progesterone to its reduced metabolites, there is a well-know connection between increased ratios of serum estradiol to progesterone and the frequency of tonic-clonic epileptic seizures in preovulatory and premenstrual phases of the menstrual

cycle. In cases of anovulatory cycles, this frequency depends of the levels of estradiol regardless of menstrual cycle phase (Backstrom 1976). Women with catamenial epilepsy showed up to 50-60% reductions in estrogen levels (estradiol, estriol) and significantly lower levels of 5α- and 5β pregnanediols, which are lower-affectivity acting metabolites of neuroactive pregnanolone isomers (Buntner and Rosciszewska 1985).

In epileptics, lowered levels of progesterone in the luteal phase of the menstrual cycle have been documented (Herzog 1991). Insufficient progesterone likely leads to an insufficiency of neuroinhibitory 5α/β- reduced 3α- metabolites of progesterone, which could be the cause of an increased frequency of epileptic seizures. A lower ratio of neuroinhibitory 3α-pregnanolone isomers to neuroactive 3β-isomers has been found in patient serum (Stoffel-Wagner 2001). Most current studies show a relationship between catamenial epilepsy and disorders in the biosynthesis of progesterone and its reduced metabolites (Backstrom et al. 2003), particularly in the luteal phase of the menstrual cycle (Bonuccelli et al. 1989).

Testosterone modulates the propensity for epileptic seizures in both humans and animals. On the one hand, its aromatization to estradiol increases the likelihood of the onset of a seizure, while on the other hand its reduction produces neuroinhibitory anticonvulsive $GABA_A$-r positively modulating 3α-hydroxy-androstane metabolites such as androsterone or 3α,17β- androstanediol. Indometacin, as an inhibitor of the 3α-HSOR enzyme that is necessary for the synthesis of 3α-androstane metabolites, tends to increase the pro-convulsive effect of testosterone, as would be expected (Reddy 2004).

Levels of 3α-reduced neuroactive steroids in the plasma are lower in patients suffering from severe depression. Short-term therapy with selective serotonin reuptake inhibitors (SSRIs) leads to an increase in these steroid levels, while non-pharmacological treatments do not produce similar changes, which indicates a pharmacological effect of SSRIs on the production of levels of 3α-reduced neuroactive steroids regardless of the treatment outcome (Baghai et al. 2005). This increase in steroid levels is likely the result of the modulation activity of 3α-HSOR as well as through the activation of the hypothalamo-pituitary-adrenal system, leading to an influence on the increase of serotonin mediating neurotransmission (Van de Kar et al. 2001). Serotonin thus evidently stimulates the release

of CRH from the hypothalamus to the portal circulation, which is reflected in the increased intensity of peripheral steroid production (Pisu and Serra 2004).

1.11. Correlations between steroids in human blood and cerebrospinal fluid

Although the steroid transport between the peripheral blood circualation and the brain have been analyzed in humans and monkys (Marynick at al. 1976, Pardridge and Mietus 1979, Kullak-Ublick et.al. 1998, Murakami et al. 1999) the data available in the literature are still not complete, and usually concern only separate steroids. The expterriments tracing out to what extent the periphery steroids may contribute to the steroid metabolome in the CNS are rather scarce, and again two/three (George et al. 1994, Backstrom at al. 1976, Uzunova et al. 1998) or not more than five steroids put under investigations in one study (Schwarrz and Pohl 1992, Kim et al. 2003).

Methods for neuroactive steroids determination

The variety and range of application of neuroactive hormones assay methodology have expanded greatly during the last 10 years. Among them should be mentioned radioimmunoassay (RIA), enzymoimmunoassay (ELISA), chemimmunoluminescence (CIL), thin layer (TLC) or high-pressure liquid chromatography (HPLC) followed by RIA, gas chromatography alone or rather in combination with gas chromatography-mass spectrometry (GC-MS). These methods cover nearly, but not all of the known neuroactive steroids and their metabolites of endocrinological interest.

2. Scope and aims

As it was mentioned above there are a good variety of methods for determination of the neuroactive steroids. Most of the measurements and the data obtained, while working on the Dissertation thesis, were done by the method presented by Hill et al. /2000/, sometimes with minor changes. When they were applied they were pointed out elsewhere. To the best of our knowledge there was not a method for the quantification of serum 7-hydroxy-steroids

published before, with the exception of 7-hydroxy-DHEA epimers. Therefore a novel method for the measurement of the above mentioned neuroactive steroids had to be developed.

We believe that many NAS like progesterone, estrogens, their sulfate esters and several of their metabolites are involved in certain disorders, that there are not strictly male or female hormones, that a limited number of hormones have been traced some times in many investigations, hence a limited information might be obtained in this way, we hypothesis that a method suitable for the determination of as many as possible steroids in one assay would be helpful. As far as NAS have been found in different nervous system disorders, epilepsy, alcoholism in women and in reproduction, we speculate that the events are modulated by NAS, having mostly enhancing effects. It might be supposed that the altered relationship between steroid sulfate and their unconjugated analogues could influence formation of sex steroids. Similarly NAS, such as pregnanolone isomers, pregnenolone sulfate, DHEA, DHEAS, and other S biosynthesis might be influenced by chronic alcoholism as well as by subsequent alcohol detoxification therapy. It might be hypothesized also that many free and conjugated NAS, including some which have not been measured (like 7-hydroxy S) are age, sex, phase of the menstrual cycle and pregnancy stage dependent and correlated, and are able significantly to influence the circulating levels of free and active S in the same time.

Most frequently studied NS as allopregnanolone; DHEA and pregnanolone are modulating events as chronic alcoholism, alcohol detoxication therapy, epilepsy or pregnancy and delivery. Our hypothesis was that also minor metabolites of progesterone, DHEA or testosterone might be involved in those events.

Some authors have analyzed the steroids transport between the periphery and the brain, but the data are still insufficient. That is why we investigated and evaluated the correlations of the steroids between the periphery and the cerebrospinal fluid (CSF), considering also the effect of the steroid conjugation in the periphery. It is possible that for the diagnostics of CNS diseases, which in their turn probably are connected with an imbalance in peripheral steroidogenesis (like postpartum depressions, catamenial epilepsy, premenstrual syndrome, affective disorders) it would be much easier to collect peripheral blood, than CSF. In addition, it is of interest the correlations between the steroids within the

CSF to be followed, especially if peripheral steroids and particularly the NAS could be comparable with the predictivity of the CSF steroids.

AIMS:

1. Methods suitable for determination of some neuroactive steroids to be developed.

2. To investigate the possible linkage between epilepsy in women and the changes in steroidogenesis (especially in steroids sulfates and their unconjugated analogues).

3. To see if the deficiency in progesterone isomers (metabolites) reflects an impaired progesterone biosynthesis in women with severe alcohol addiction.

4. To evaluate the differences in the production of the individual (pregnanolone isomers) PI and their profiles during the reproduction.

5. To estimate to what extent the peripheral steroids may contribute to the steroid metabolome in the CNS and how important may be the contribution of the in situ brain synthesis.

3. Materials and Methods

3.1. Subjects (study population and data collection)

The studies were carried out in subjects from the stuff of the Institute of Endocrinology (IE), Prague, Department of Addiction Treatment General Faculty Hospital, Charles University (CUTH), Department of Gynecology and Obstetrics, First Medical Faculty, Charles University, St. Anne's University Hospital, Brno, Department of Neurosurgery, volunteers or outpatients (men – examined for fertility problems of the couple or participating in a study on iodine deficiency; women – regularly cycling, pregnant or during alcohol detoxification – according to the inclusion and exclusion criteria).The CSF was taken from patients operated for either tumorous or non-tumorous lesion.

A detailed list of the numbers of the subjects participating in the investigations are presented on Table 1. The numbers from 1 to 10 in the table corresponded to the experiments carried out and, to the attached author's publications list.

A total of 319 subjects were included in the investigations, as follows: 4 girls (age 6 - 10), 5 boys (age 6 – 10), 13 adolescent men (age 13 – 20), 29 men (age 16 – 66) and 253 women (age 18 – 48) in follicular phase, luteal phase and pregnant. From eight postmenopausal women (age 56-78) and 7 men (age 22-88) who underwent an endoscopic 3rd ventriculostomy CSF was collected.

The local ethical committees of the IE, CUTH and the St. Anne's University Hospital, Brno, approved the protocols for all the studies in concern. After signing written, informed consent, the subjects underwent blood sampling from the cubital vein and the respective group of women and men for CSF sampling from the third ventricle. All subjects were free of major medical problems or medication known to affect steroid metabolism.

N	GIRLS	BOYS	ADOLESCENT MEN	MEN	WOMEN				
					CONTRILS	FOLLICULAR PHASE	LUTEAL PHASE	PREGNANT	POSTMENOPAUSAL
1								138	
2	4	5	13			15	17		
3						20	20		
4					17	20	20		
5						15	16		
6				14					
7				15		15	16	30	
8				15					
9								30	
10				7					8

Tab.1. Number of subjects participating in the investigations

The patient group of women (experiments 3 and 4) during alcohol detoxification therapy at 5 stages (start, 3days, 14 days, 1 month, and 4 month after termination of therapy) were with known phase of menstrual cycle in order its influence to be considered. The presence of the ovulatory cycle was verified by P assay. Circulating NAS were examined in young man (experiment 6) with normal spermiogram before and 5-6 min after ejaculation provoked by masturbation. Pregnant volunteers were at age between 18 and 30 years with physiological pregnancy, cephalic presentation of the fetus and no disease history.

Cooled plastic tubes containing 100 µl 5 % EDTA and 50 aprotinin (Antilysin, Spofa, Prague) were used for blood and CSF sampling. The plasma was obtained after centrifugation for 5 min at 2 000 x g at 0^0 C. The plasma samples were stored at -20^0 C until analyzed.

3.2. Steroids, chemicals and equipment

The non-radioactive steroids and their conjugates were obtained from Steraloids (Wilton, NH, USA). The solvents for extraction, for HPLC and pyridine were of analytical grade and were purchased from Merck & Co (Darmstadt, Germany), while methoxylamine was from Sigma (St.Louis, MO, USA). The derivatization agent Sylon BFT and TMCS were purchased from Supelco (Bellefonte, PA, USA). When needed the internal standard 3β-hydroxyl(19-H^3)androst-5-en-17-one (trideuterated DHEA) and others were prepared using a method described elsewhere (I. Černý et al., 2004).

The gas chromatography-mass spectrometry (GC-MS) system was supplied by Shimadzu (Kyoto, Japan). The system consists of a GC17A gas chromatograph equipped with automatic flow control, ACC-20 autosampler, and, for the MS a OP 5050A quadrupole electron impact detector with a fixed electron voltage of 70 eV. A Zebron ZB-50 medium polarity capillary column (50% phenyl/50 % methylpolysiloxane) from Phenomenex (St.

Torrance, CA, USA) was used for analysis. The length of the column was 15m, the internal diameter was 0.25mm, and the film thickness was 15µm. The liquid scintillation spectrometer was supplied by Beckmann Coulter (Fullerton, CA)

3.3. Preparation of the plasma samples for the GC-MS free steroid analysis.

The samples (serum, CSF or plasma) were kept frozen at -20^0 C or -80^0 C until analysis. They were thawed and 1 mL of them was spiked with trideuterated DHEA or other steroid (17α-estradiol) as the internal standard to attain a concentration of 1 µg/mL. The spiked sample was extracted with 3mL of diethyl ether. The water phase was kept frozen in a mixture of solid carbon dioxide and ethanol, and the organic extracts were decanted into glass tubes and evaporated to dryness. The dry organic phase residue was used for the determination of free steroids. It was partitioned between 1 mL of 80% methanol with water and 1 mL of petroleum ether (or n-pentane) to eliminate the majority of lipids and sterols. The petroleum ether phase was discarded, while the methanol/water phase containing steroids for analysis was evaporated in a vacuum centrifuge. Sometimes the samples were prepared twice for further processing using two different derivatization techniques. The first was used for preparation of steroids with hydroxyl-groups modified to trimethylsilyl (TMS) derivatives and with hydroxy-groups modified as in the former case but, in addition, with oxo-groups modified by methoxylamine (MOX-TMS derivatives). The dry organic phase residue was used for the determination of free pregnenolone, DHEA, estradiol and PIs or derivatized components as described below.

Standard mixtures of the substances studied in three concentrations: 1 000, 100 and 10 µL/ were derivatized in the same way as the samples and used for calibration with an internal standard method. The blank was processed in the same way as the samples except for the use of water instead of serum. In all cases, the blank signal was zero or low. In the latter case, it was subtracted from the signal of the sample.

In case of neuroactive C21- and C19-steroids 1mL of plasma was extracted with 3mL of diethyl ether, the polar phase was frozen, the organic phase separated and evaporated, the dry residue was partitioned between 1 mL of 80% methanol and 1 mL of pentane. The upper pentane phase was discarded, while methanol-water phase containing free steroids was evaporated, and the dry residue derivatized.

3.4. Sample preparation for the GC-MS analysis of the steroid polar conjugates, derivatization

The frozen water phase in glass tubes was thawed and mixed with 1 mL methanol. The tubes were centrifuged, and the 1 mL aliquot of the supernatant was transferred into a glass tube and evaporated in a vacuum centrifuge. The steroid sulfates were hydrolyzed using a method described previously (Dehennin et al., 1996). The hydrolyzed sample was evaporated in a vacuum centrifuge; the dry residue was spiked with trideuterated DHEA (or estradiol) as an internal standard to attain a concentration of 1 μg/mL and further processed in the same way as in the free steroids.

Derivatization. The TMS derivatives of the steroids were prepared using the modified method of Hill et al. (2000), with some of the modifications reported recently (Havlíková et al. 2000). In summary, Sylon B/99% bis (trimethylsilyl)-trifluoroacetamide (BTSFA) + 1% trimethylchlorosilane (TMCS) (50 μL) was added to the dry residues from serum or plasma, mixed briefly and heated at 90^0 C for 45 min. The derivatization agent was evaporated under a stream of nitrogen. The dry residue was rinsed down with isooctane (50 μL) and the mixture was evaporated again. Finally, steroid derivatives were dissolved into 20 μL isooctane, and 4 μL portions were injected into the GC-MS system.

The MOX-TMS derivatives were prepared as follows: 50 μL of 2% solution of MOX-hydrochloride in pyridine were added to the dry residues from plasma, mixed briefly, and heated at 60^0 C for 2 hours. The mixture was then evaporated under a stream of nitrogen, before further TMS derivatization proceeded as described previously.

3.5. Temperature and pressure gradients for the GC-MS analysis, retention times and effective masses for the determination of steroids

The PI were measured by a method of Hill et al. (2000) originally described elsewhere with some modifications. The first one was the use of less steep temperature and pressure gradients; 1 min high pressure injection at 120^0 C and 100 kPa, followed by a pressure release to 30 kPa and a rapid linear gradient at 40^0 C and 8.5 kPa up to 220^0 C and 51 kPa, then a slow linear gradient at 2.9^0 C and 0.5 kPa up to 240^0 C and 54.5 kPa, and finally rapid linear gradient at 400^0 C and 9.0 kPa up to 310^0 C and 70 kPa with a 2 min delay. The other

modification consisted of the substitution of 17α-methyl-5androstane-3β,17β-diol as a internal standard for trideuterated dehydroepiandrosterone, added to the standard or to the sample in a 1 ng/mL concentration and recorded on an effective mass of 307. The overall time taken for the analysis was 14.2 min. The retention times differed depending of the PI analyzed. The last changes represented the substitution of micro-extraction in the vials by rapid drying of the derivatization agent under a stream of nitrogen.

TMS derivatives were analyzed by slightly modified procedure: 1 min high pressure injection at 120^0 C and 100 kPa, pressure release to 30 kPa, rapid linear gradient of 40^0 C/min and 8.5 kPa/min up to 200^0 C and 49.3 kPa, then slow linear gradient of 2.9^0 C/min and 0.5 kPa/min up to 220^0 C and 52.7 kPa/min, a medium gradient of 20^0 C/min and 8.0 kPa/min, up to 265^0 C and 70 kPa and a rapid linear gradient of 40^0 C/min and 10 kPa/min up to 310^0 C and 80.7 kPa followed by a 2 min delay. Respectively TMS-MOX derivatives were processed as follows: 1 min high pressure injection at 120^0 C and 100 kPa, pressure release to 30 kPa, rapid linear gradient of 40^0 C/min and 8.5 kPa/min up to 220^0 C and 51.0 kPa, then slow linear gradient of 2.9^0 C/min and 0.5 kPa/min up to 240^0 C and 54.5 kPa/min, a medium gradient of 40^0 C/min and 9.0 kPa/min, up to 310^0 C and 70 kPa and a 3 min delay. The overall time was – 15.2 min. To exploit the samples, the individual ones were applied 3 times in independent courses, in each case employing a part of the steroids under investigation. The types of gradients, effective masses for determination and quantification, order numbers of injections and retention times for the individual steroids are presented in Table 2.

The selectivity and sensitivity were sufficient for quantification of all steroids investigated with inter-assay coefficient of variance not exceeded 10 % for any steroid.

The twelve NAS and neuroprotective steroids (C21 and C19) were measured principally following the GS-MS procedure published by Klak et al (2003). Extraction was done by 3 mL of diethyl ether and the polar phase taken for further processing. To identify and simultaneously analyze 12 steroids at maximum sensitivity the protocol consisted of 4 sample or standard injections with identical temperature and pressure gradient were recorded but with different sets of the effective masses as listed in Tab. 3.

Table 2. Analytical criteria of the method for the multi-component quantification of neuroactive pregnanolone isomers and related steroids

No	Steroid	Form	Gradient/ derivatization	Injection	Retention time [min]	Effective mass [m/z]	Sensitivity [pg] (mean±SEM, n=5)
1	Pregnenolone	*F, C	**S	3	11.592	298, 388	3.67±0.35
2	Progesterone	F	MS	1	11.250, 11.392	100, 341, 372	2.09±0.27
3	5α-Dihydroprogesterone (P5α)	F	MS	1	10.975, 11.008	288, 343	1.26±0.11
4	5β-Dihydroprogesterone (P5β)	F	MS	1	10.396, 10.475	288, 343	3.43±0.61
5	Allopregnanolone (P3α5α)	F, C	S	1	10.758	285, 300, 375	1.79±0.26
6	Isopregnanolone (P3β5α)	F, C	S	1	11.563	285, 300, 375	1.93±0.24
7	Pregnanolone (P3α5β)	F, C	S	1	10.950	285, 300, 375	2.76±0.39
8	Epipregnanolone (P3β5β)	F, C	S	1	10.550	285, 300, 375	2.03±0.29
9	17α-Estradiol (internal standard)	----	S, (MS)	1-4	9.863	285, 416	1.42±0.17

** F…free steroid, C…conjugated steroid, ** S…trimethylsilyl derivatives, MS…methoxylamine - trimethylsilyl derivatives*

3.6. **Measurements of steroids by radioimmunoassay**

The concentrations of some steroids were evaluated by RIA. Blood progesterone levels were determined by a method originally described by Langer et al. (1978). The amount of pregnanolone sulfate was measured by a specific radioimmunoassay published by Hill et al. (2002). Pregnenolone sulfate, 17α-hydroxy-pregnenolone and progesterone were analyzed also by specific radioimmunoassay of Hill et al. (1999, 2002) and Langer et al. (1978). 17α–Hydroxy-progesterone and dehydroepiandrosterone were measured using RIA kits from Immunotech (Marseilles, France). Intra- and inter-assay coefficients of variation of 7.8 % and 5.7 % respectively, and a measurements range of 0.14 – 149 nmol/L characterized the data received by the applied kits.

3.7. Cerebrospinal fluid collection

The surgeries were performed by the staff of Dept. of Neurosurgery of the Faculty Hospital St. Anne's in Brno under a general endotracheal anesthesia. Neuroendoscopic system Wolf or Storz was used for the surgery. Neuroendoscopic access to the third ventricle was done following the methods of Longatti et al (2004) and Hellwig et al (2005).

Table 3. Characteristics of GC-MS analysis of the steroids

Substance	Injection No.	Retention time (min)	Effective mass (m/z)
Internal standard (trideuterated DHEA)	1-4	7.26	377
3α,17β-Dihydroxy-5α-androstane	1	5.16	241, 256
3α-Hydroxy-5α-androstan-17-one	1	6.29	270, 360
3α-Hydroxy-5β-androstan-17-one	1	6.39	270, 360
3β,17β-Androstendiol	2	5.87	215, 305, 344
17-Hydroxy-pregnenolone	2	7.10	270, 305, 360
DHEA	2	7.21, 7.27	260, 268, 305
Testosterone	2	7.97, 8.27	268, 389
Androstenedione	2	10.05, 10.45	313, 344
3α-Hydroxy-5α-pregnan-20-one	3	7.90	100, 388
Pregnenolone	3	9.09	100, 296, 386
7α-Hydroxy-DHEA	4	6.66	387
7β-Hydroxy-DHEA	4	7.82	387

3.8. Statistical analysis of the data

To evaluate changes in the steroid levels and eventually steroid ratios a one-way (Kruskal-Wallis test) and two-way ANOVA models were used depending on the number of factors under investigations. Multiple testing, when needed, was handle by nonparametric Kruskal-Wallis or Bonferroni multiple comparison to evaluate the differences between individual groups. Statistical computations were performed using NCSS 2002 statistical software (Number Cruncher Statistical Systems, Kaysville, UT, USA). Given the mostly non-Gaussian distribution and non-constant variance in most the steroids studied, the original data underwent a power transformation to attain symmetry and homoscedasticity in the data as well as in the residuals. The group mean values and their 95 % confidence intervals calculated in the transformed data were re-transformed to the original scale for graphical demonstration. For evaluating the difference, sometimes a robust Wilcoxon's paired test was

also applied. Statistical computations were performed using Statgraphics Plus v5.1 statistical software (Manugistics, Rockville, MA, USA). To chek the multivariate normal distribution in the transformed-data set, plots of the multivariate data were created using as well QC-Expert statistical software from Trilobyte (Pardubice, Czech Republic).

4. Results

Developed methods for steroid hormones determinations

The androstane steroids (AS) are important NAS, because like PS are modulators of membrane ionotropic receptors, mainly $GABA_{A\text{-}r}$, responsible for the chloride influx into the neuron. Studies based on AS determinations appeared so far in the scientific literature were using RIA or RIA combined with some type of chromatography to investigate one or at most several AS at once. A GC-MS method, here described, was developed for the simultaneously evaluation of 20 unconjugated S and 16 steroid polar conjugates as followes:

Steroid analysis

The levels of the 20 unconjugated steroids and 16 steroid polar conjugates were simultaneously measured in the serum from the 15 subjects using GC-MS. The unconjugated steroids measured were pregnenolone (Preg), 17-hydroxy-pregnenolone (Preg17), DHEA, 5-androstene-3β,17β-diol (Adiol), androstenedione, testosterone, 5α-androstane-3,17-dione (A5α), androsterone (A3α5α), epiandrosterone (3β-hydroxy-5α-androstan-17-one, A3β5α), etiocholanolone (3α-hydroxy-5β-androstan-17-one, A3α5β), epietiocholanolone (3β-hydroxy-5β-androstan-17-one, A3β5β), DHT5α, A3α5α17β, A3β5α17β, 5β-androstane-3α,17β-diol (A3α5β17β), 5β-androstane-3β,17β-diol (A3β5β17β), allopregnanolone (3α-hydroxy-5α-pregnan-20-one, P3α5α), pregnanolone (3α–hydroxy-5β–pregnan-20-one, P3α5β), epipregnanolone (3β-hydroxy-5β–pregnan-20-one, 3β5β) and isopregnanolone (3β-hydroxy-5α-pregnan-20-one, P3β5α). The polar conjugates of Preg (PregC), DHEA (DHEAC), Adiol (AdiolC), A3α5α (A3α5αC), A3β5α (A3β5αC), A3α5β (A3α5βC), A3β5β (A3β5βC), DHT5α (DHT5αC), A3α5α17β (A3α5α17βC), A3β5α17β (A3β5α17βC), A3α5β17β (A3α5β17βC), A3β5β17β (A3β5β17βC), P3α5α (P3α5αC), P3β5α (3β-hydroxy-5α-pregnan-20-one, P3β5αC),

pregnanolone (3α-hydroxy-5β-pregnan-20-one, P3α5βC) and epipregnanolone (3β-hydroxy-5β-pregnan-20-one, P3β5βC) were also monitored. In the GC-MS method some procedures, presented by Stárka et al. (2006) and for initial sample preparation by Havlíková et al. /2006/ were used. Derivatization was done first with methoxylamine-hydrochloride on oxo-groups and then by Sylon B/99% of bis-(trimethylsilyl)-trifluoroacetamide and 1 % of trimethylchlorosilane forming trimethylsilyl derivatives on hydroxyl-groups. A second aliquot of the sample was derivatized by Sylon B only.

Steroid conjugates remaining in the polar residue after diethyl-ether extraction were hydrolyzed as described by Dehennin et al. (1996) and further processed in the same way as the free steroids. The samples and polar phases after diethyl-ether extraction used for the quantification of the steroid conjugates were spiked with 17α-estradiol as an internal standard to attain a concentration of 1 ng/mL prior to further processing. The internal standard was recorded at effective masses m/z = 285 and 416. Adjusting the samples to the internal standard before processing ensured that the losses during the sample processing were not critical for steroid quantification. For the new validity of the new measurements to be checked, the sera were spiked with the steroid measured in amounts close to the mean value, found for each specific steroid and the recovery ranged from 79 % to 104 %.

The individual samples were processed usually 4 times each time in order best processing of biological materials to be obtained. The temperature and pressure gradients and the effective masses used for the measurement in selective ion monitoring mode was optimized to obtain maximum sensitivity and sufficient selectivity. In all cases mixtures of known standards, processed in the same way as the samples, were used to validate the applied method. The standards were injected in 3 different amounts for each steroid /40 pg, 400 pg and 4 000 pg/. In Tab. 4 are shown the temperatures and pressure gradients for the GC-MS analysis.

Table 4. Temperature and pressure gradients used for steroid analysis

Gradient[a]	Initial conditions (further increase [°C·min^{-1}], [kPa·min^{-1}])	Step				Overall time [min]
		1[b]	2	3	4[c]	
MS1, MS3	Temperature [°C]	120(40)	220(2.9)	240(40)	310(0)	14.19
	Pressure [kPa]	26.9(8.5)	47.9(0.5)	51.4(9)	66.9(0)	
MS2, S3	Temperature [°C]	120(40)	230(1.5)	240(40)	310(0)	14.19
	Pressure [kPa]	26.9(8.5)	47.9(0.5)	51.4(9)	66.9(0)	
MS4	Temperature [°C]	120(40)	240(2.9)	260(40)	310(0)	14.19
	Pressure [kPa]	26.9(8.5)	47.9(0.5)	51.6(9)	63.5(0)	
S1	Temperature [°C]	120(40)	220(3.5)	240(40)	310(0)	13.00
	Pressure [kPa]	23.0(9)	44.0(0.6)	47.5(9)	64.0(0)	
S2	Temperature [°C]	120(40)	200(1.0)	213(40)	310(0)	20.72
	Pressure [kPa]	20.0(9)	35.8(0.2)	38.5(9)	60.6(0)	

[a]MS...methoxylamine-trimethylsilyl derivatives, MS...trimethylsilyl derivatives, the numbers denote the order of injection (4 µL) from the 20 µL sample, i.e. S2 indicates the second injection from the sample of trimethylsilyl derivatives; [b]in all cases, the gradient started from a one-minute high pressure injection at 120°C and 100 kPa followed by pressure release; [c]the 4th step was followed by a two-minute delay at the final temperature and pressure for all gradients

The sensitivity of the method ranged mostly from hundreds of femtograms (fg) to tens of picograms, depending primarily on the steroid fragmentation pattern. As demonstrated in Tab. 5 and Fig. 2 the selectivity and sensitivity were sufficient for all the steroids measured, during the elaboration of the thesis.

Table 5. Analytical criteria of the method for quantification of free and conjugated androstane metabolites and related steroids

No	Steroid	Form	Gradient/ derivatization	Injection	Effective mass [m/z]	Peak No	Retention time [min]	Sensitivity [pg]	S/N, free steroids [%]	S/N, conjugates [%]	Inter-assay, free steroids C.V. [%]	Inter-assay, conjugates C.V. [%]
1	Pregnenolone	F, C[a]	S[b]	3	298[c], 388	1	10.64 [c]	2.85	264	6058	7.6	21.0
2	Preg17	F	MS	1	129, 231, 270, 304	1	10.02	18.8	37	---	26.8	---
3	DHEA	F, C	S	1	270, 304	1	9.64	1.65	2120	107286	10.2	11.9
4	Adiol	F, C	S	2	254, 344	1	14.48	2.99	177	45189	6.4	3.9
5	Androstenedione	F	MS	2	313, 344	1	11.78	5.24	283	---	28.1	---
						2	11.91	7.79	---	---	---	---
6	Testosterone	F	MS	2	268, 389	1	10.56	8.05	---	---	---	---
						2	10.84	10.1	623	---	15.3	---
7	A5α	F	S	4	244, 288	1	11.52	1.99	48	---	---	---
8	A3α5α	F, C	S	1	257, 272, 347	1	8.55	1.29	126	43311	10.8	14.3
9	A3β5α	F, C	S	1	257, 272, 347	1	9.65	0.54	79	10162	5.2	3.1
10	A3α5β	F, C	MS	1	270, 360	1	8.97	1.34	11	3164	15.9	13.7
11	A3β5β	F, C	S	1	257, 272, 347	1	8.36	1.02	4.0	1658	20.3	14.3
12	DHT5α	F, C	S	1	257, 272, 347	1	9.81	2.14	132	349	6.0	2.4
13	A3α5α17β	F, C	S	2	241, 256, 347, 421	1	11.57	2.02	54	43311	14.3	8.4
14	A3β5α17β	F, C	S	2	241, 256, 347, 421	1	14.34	4.75	8.7	4867	13.4	7.0
15	A3α5β17β	F, C	S	2	241, 256, 347, 421	1	11.77	2.13	8.4	1289	15.9	8.9
16	A3β5β17β	F, C	S	2	241, 256, 347, 421	1	11.15	2.4	7.3	227	10.3	7.7
17	P3α5α	F, C	MS	3	100, 388	1	10.93	0.707	115	226	13.2	5.0
18	P3β5α	F, C	MS	3	100, 388	1	11.54	0.686	85	368	12.5	7.2
19	P3α5β	C	MS	3	100, 388	1	11.01	1.52	---	890	---	6.5
20	P3β5β	C	MS	3	100, 388	1	10.77	1.23	---	109	---	6.7

Standard deviations for retention times and sensitivities were about 0.005 min and 10%, respectively; [a]F…free steroid, C…conjugated steroid; [b]S…derivatization of hydroxy-groups by trimethylsilylation, MS...derivatization of oxo-groups by methoxylamine and hydroxy-groups by trimethylsilylation; [c]the underlined text indicates the conditions used for quantification of steroids

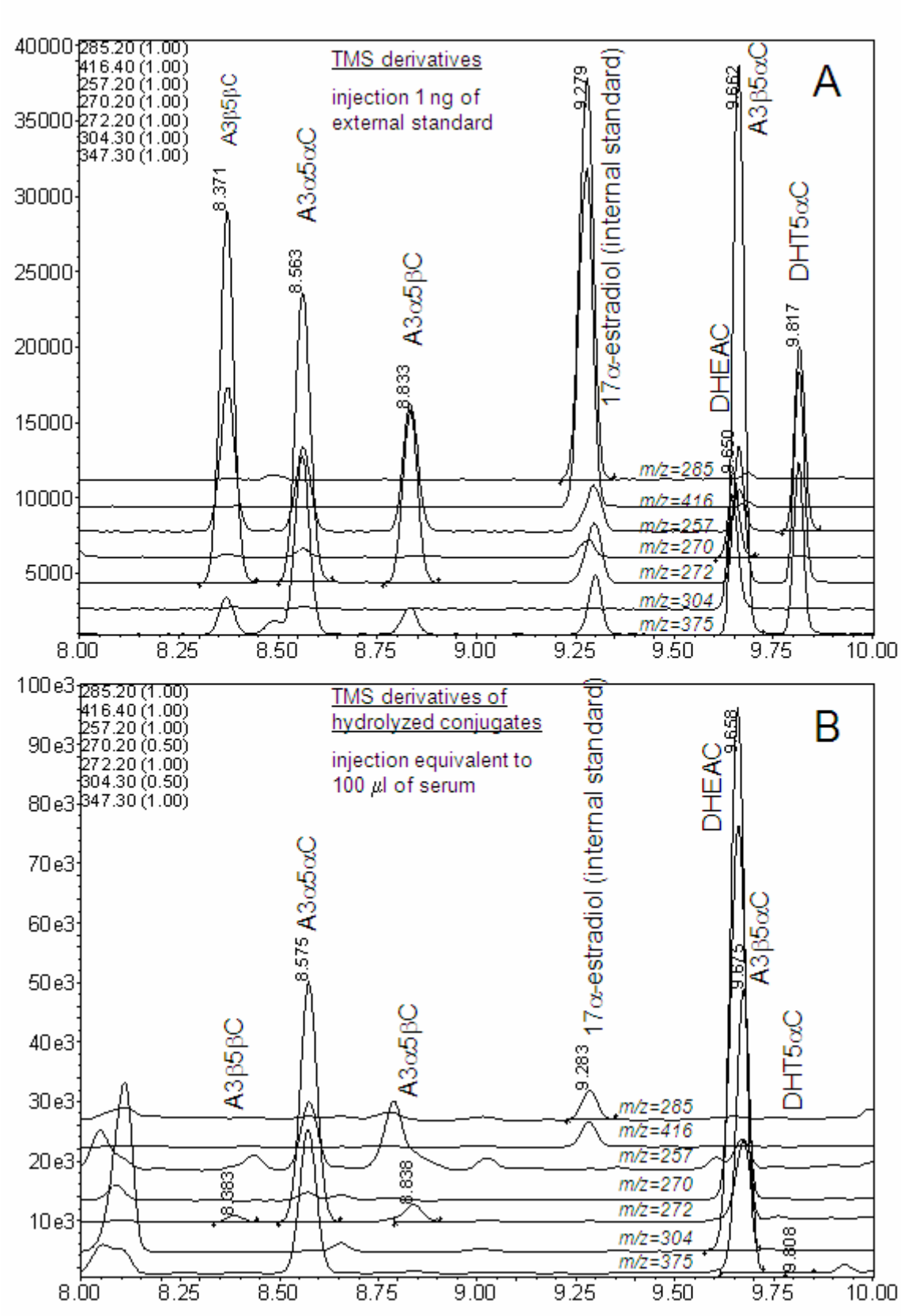

Figure 2. Chromatograms of 5α/β-reduced androstane steroids; section A…standard solution, section B…serum sample

4.2. Neuroactive steroids and epilepsy in women

This study was undertaking to give answers if the steroid metabolism of P and sex S is changed and whether there was an altered balance between steroid sulfates and their unconjugated analogues in women with epilepsy. Considerably lower levels of 17-hydroxy-pregnenolone ($p < 0.0001$, ANOVA; $p < 0.002$, Mann-Whitney test) and 17-hydroxy-progesterone ($p < 0.006$, ANOVA; $p < 0.002$, Mann-Whitney test) were found in patient with epilepsy compared to controls. In the all other S measured, including E_2 and P, not significantly differences were observed. Like in the follicular phase, during the luteal phase significant, although not so well pronounced, difference was noticed in 17-hydroxy pregnenolone ($p < 0.008$, ANOVA; $p < 0.05$, Mann-Whitney test). On the other hand, in comparison with follicular phase, even more profound lower levels of 17α-hydroxy-progesterone were detected in the patients in luteal phase ($p < 0.0001$, ANOVA; $p < 0.007$, Mann-Whitney test).

In both phases in menstrual cycle markedly elevated amounts of pregnenolone sulfate ($p < 0.0001$, ANOVA; were found in women with epilepsy along with almost not changed DHEAS levels. The NAS sulfate amounts in patients were independent in this study of menstrual cycle and no connection with catamenial seizures (linkage) was found by the ANOVA model with phase of the cycle, linkage, effect of treatment with anti epilepsy depressants and with phase X linkage interaction.

4.3. Profiles of serum neuroactive steroids in women with alcohol addiction

Alcohol abuse is connected with menstrual irregularities, related to the inhibition of progesterone (P) secretion, involved in regulation and organization of the menstrual cycle. During the study it became clear that P biosynthesis was impaired in premenopausal women with history of alcohol intake and subsequently treated for alcohol addiction. As it was expected this steroid rose in the luteal phase during treatment – Fig. 3.

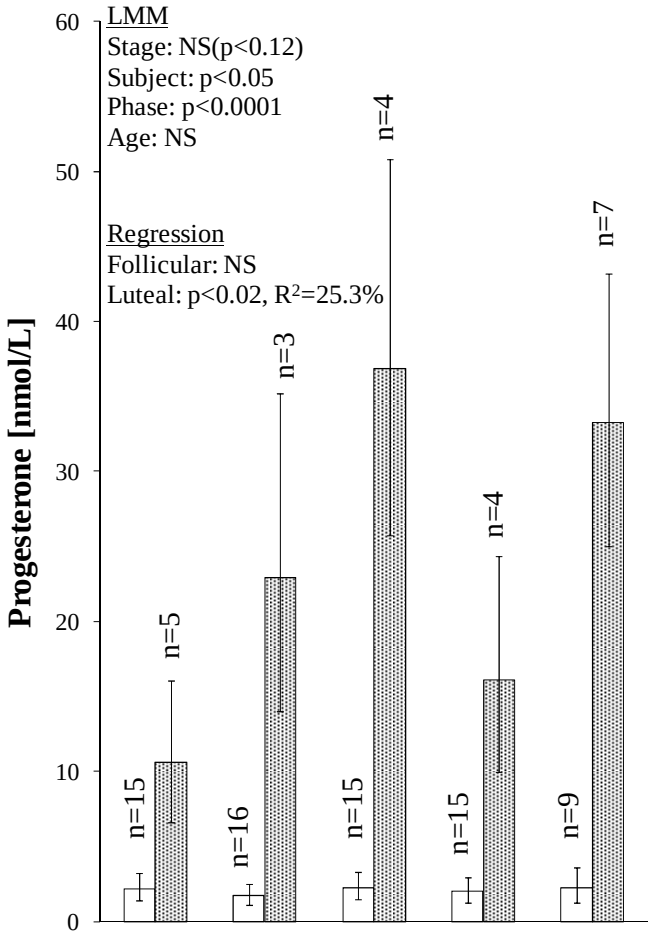

Figure 3. Changes in levels of progesterone in female serum during alcohol detoxication therapy .The empty and dotted bars, with error bars represent the retransformed mean values with their 95% confidence intervals in the follicular and luteal phases, resp.; R2 is the squared correlation coefficient of regression expressing the percentage of the total progesterone variability explained by the model. LMM is the linear mixed model, which was used for evaluation.

In the same time allopregnanolone, epipregnanolone and isopregnanolone increased in both phases of the menstrual cycle, but more well pronounced in luteal phase, whereas pregnanolone, E_2 and pregnanolone sulfate exhibited significant changes in either phase of the cycle.

The overall change in the ratio of 3α- to 3β-isomers, expressed as a square root of the ratio of the products of the 3α- and 3β-isomers, did not change during treatment. On the other hand, the overall change in ratio of the 3α- to 5β-isomers (expressed analogously as the square root of the ratio of products of the 5α- and 5β-isomers) showed a gradual increase in the luteal phase – Fig. 4.

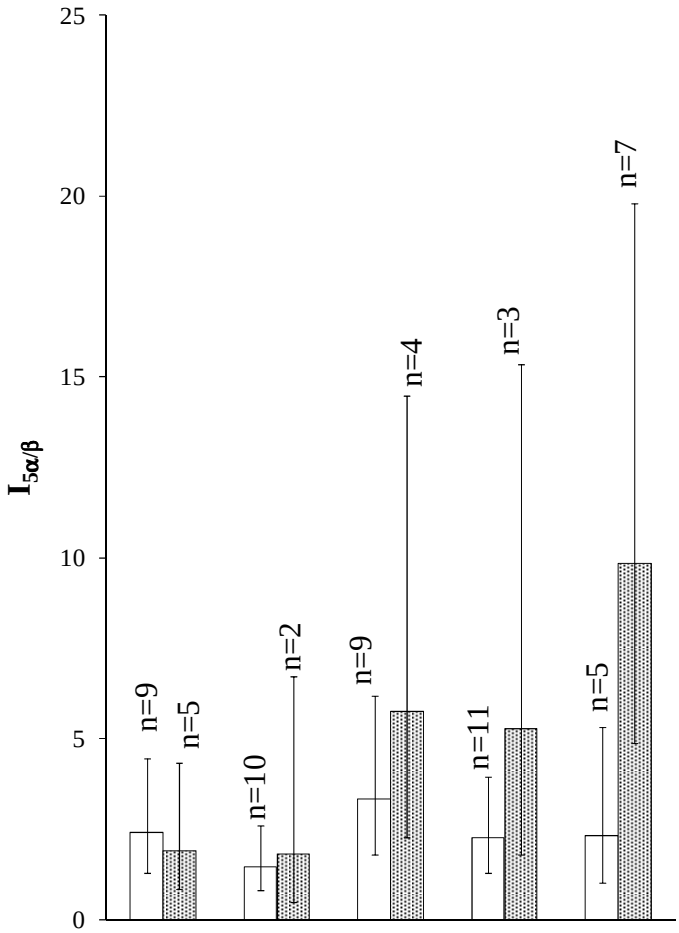

Figure 4. Changes in the I5α/β index characterizing the overall conjugated/free isomers ratio of serum pregnanolone isomers (defined as the square root of the ratio of product of the 5α to 5β-isomers) during alcohol detoxication therapy. The drawings and symbols are the same as for Figure 3.

Similarly, the ratio of neuroactivating pregnanolone sulfate to neuroinhibiting, the most abundant reduced progesterone metabolite allopregnanolone showed a regular decrease during therapy in both follicular and luteal phases of the menstrual cycle, as it is presented in Fig. 5.

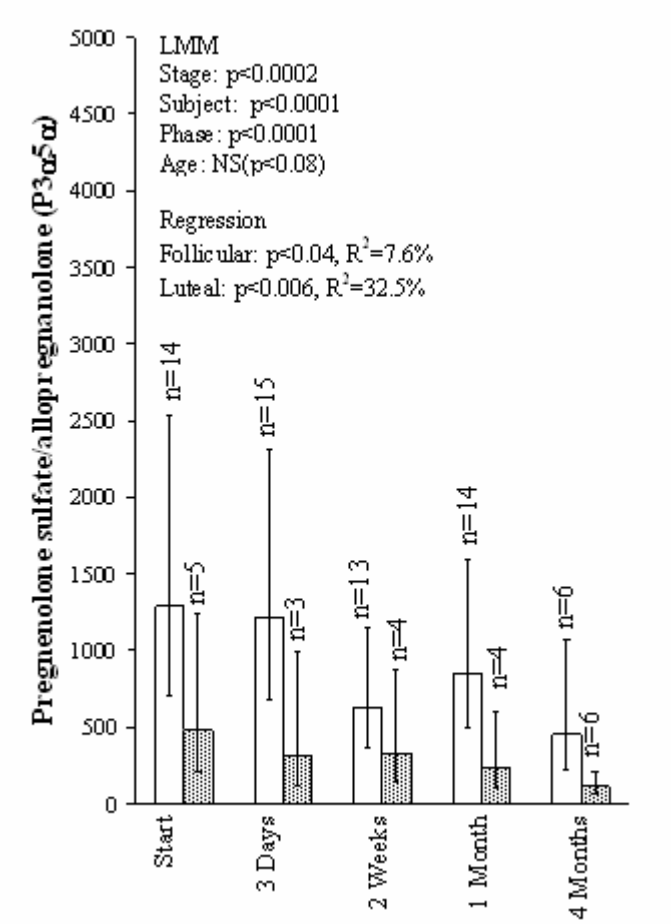

Fig.5. Changes in the ratio of neuro-activating pregnenolone sulfate to neuro-inhibiting allopregnanolone during alcohol detoxication therapy. The drawings and symbols are the same as for fig.3

The above cited results and especially the restoration of serum amounts of P, allopregnanolone and probably also epipregnanolone demonstrates the favorable effect of detoxification therapy on reproduction function and psychosomatic stability of premenopausal women treated for alcohol addiction.

The other aim of interest concerned altered profiles of serum neuroactive steroids in premenopausal women, also treated for alcohol addiction. The most elevated steroid in the patients in comparison to the controls, as it is seen on Fig. 6.A. is pregnenolone sulfate.

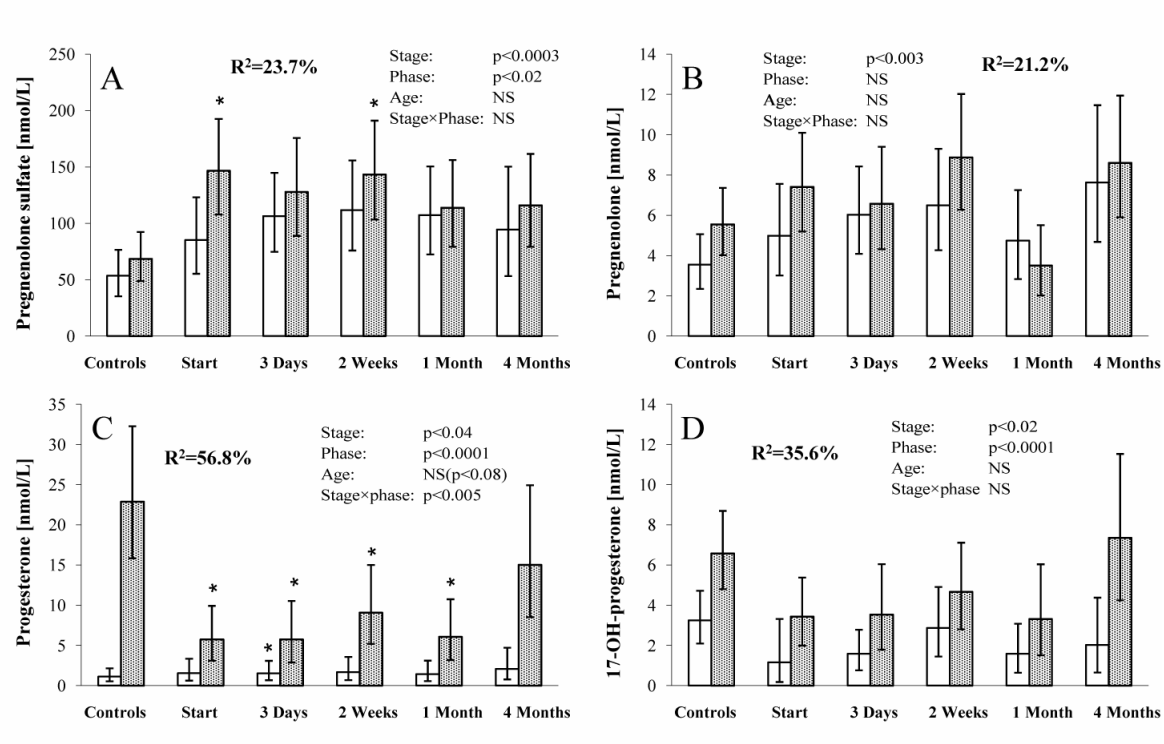

Figure 6. Serum levels of unsaturated C21-steroids in controls and in women during alcohol detoxication therapy. The empty and dotted bars, with error bars, represent the transformed mean values with their 95% confidence intervals in the follicular and luteal phases respectively; p is the level of statistical significance and R2 is the squared correlation coefficient expressing percentage of the total progesterone variability explained by an ANCOVA model consisting of subject status (controls or stage of treatment) and phase of menstrual cycle as factors, inter-factor interaction and age as a covariate. The ANCOVA was followed by Bonferroni (control vs. stage of the treatment) multiple comparisons and significant differences (p> 0.05) are labeled with an asterisk.

Quite similar situation appeared in unconjugated pregnenolone but was less pronounced – Fig. 6B. The changes in the above mentioned steroids during the treatment were not significant. No differences between the patients with alcohol abuse and controls and no changes during the treatment were detected in pregnenolone concentrations. By contrast, 3-oxo-4-ene steroids, P – Fig. 6. C and 17α-hydroxy progesterone – Fig. 6D in the luteal phase exhibited significant dependence on the subject status, this being more characteristic for P. Progesterone concentrations in the controls during the luteal phase were significantly higher than in the patient at the beginning of treatment, but no such difference was discovered when comparing the patients themselves after 4 months of treatments. The character of the differences in P levels significantly diverged in individual phase of menstrual cycle as indicated by the significant interaction between the factors subject status and phase of the cycle. In both luteal and follicular phases E_2 demonstrated a similar pattern of differences to the P in luteal phase but with less manifested differences in the follicular phase – Fig. 7.

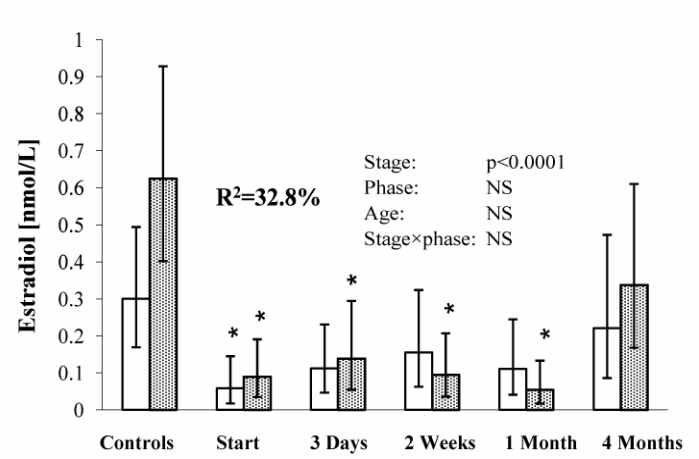

Figure 7. Serum levels of estradiol in controls and in the women during the alcohol detoxication therapy. The drawings and symbols are the same as for Figure 6.

The DHEA and DHEAS concentrations did not differ significantly between alcoholic subjects and controls and they did not present any changes during the treatment as well as for the levels of cortisol.

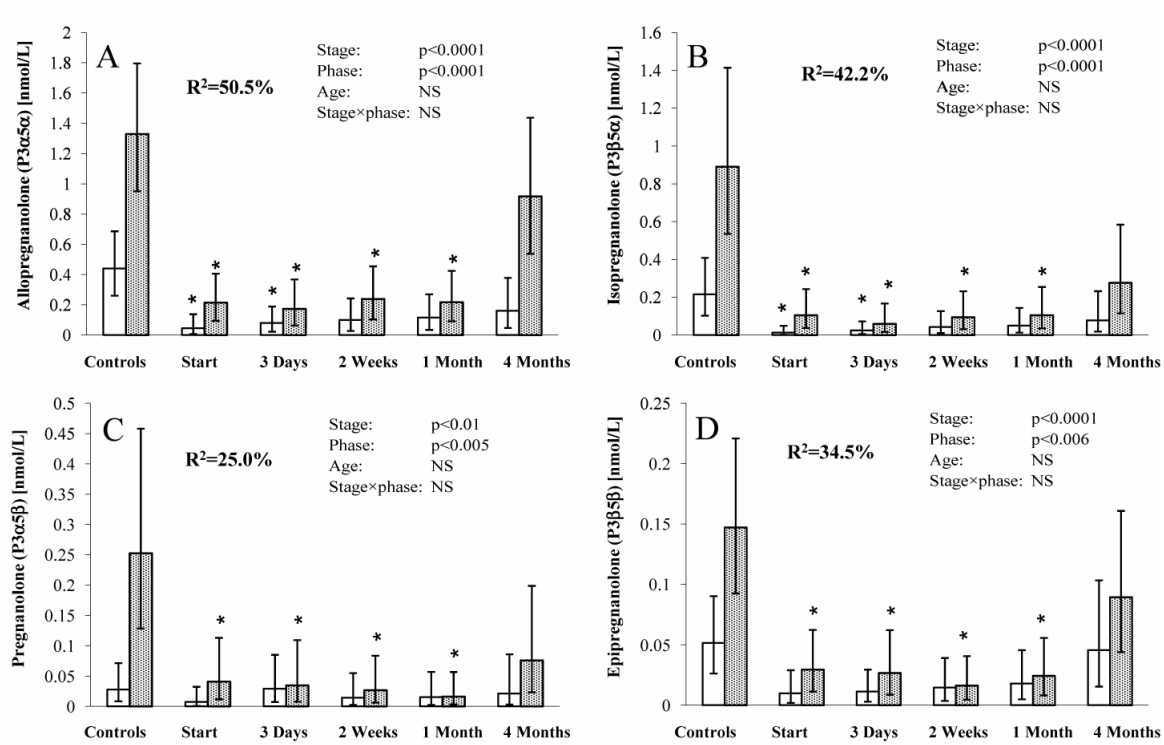

Figure 8. Serum levels of pregnanolone isomers in controls and in the women during the alcohol detoxication therapy. The drawings and symbols are the same as for Figure 3.

As it could be seen in Fig. 8 the differences in pregnanolone isomers (PI) did not simply reflect the variation in P levels, but were well prominent, particularly in the 5α-PI – Fig.8.A and B. This disparity between the controls and the untreated patients was relatively

less seen in the pregnanolone – Fig 8. C. Nevertheless, in contrast to P and similarly as in E_2, the character of the differences in PI did not diverge between follicular and luteal phase.

In order to identify in which steps of NS biosynthesis the alterations occur in women treated for alcohol addiction, the product/precursor ratios were traced in the pathway from pregnenolone and pregnenolone sulfate to PI. As it is apparent from Fig. 9, the ratio of pregnenolone sulfate to unconjugated pregnenolone displayed higher values in the patients, but with a tendency to compensation during treatment. The P/17α-hydroxy-progesterone and pregnenolone/17α-hydroxy-pregnenolone ratios present similar patterns in the luteal phase, with significantly decreased values at the beginning of treatment but with a trend to compensation during therapy.

The overall ratios of 3α- to 3β-PI and of 5α- to 5β-PI were also elevated, as were the proportions between neuroinhibiting (and most abundant/ PI allopregnanolone and neuroactivating 17α-hydroxy-progesterone. The ratio of the 3α- to 3β-PI presents no significant differences between the controls and patient during the study. By contrast, the ratio of the 5α– to 5β-PI showed a tendency to lower values in the patients and again a trend to compensation during their treatment – Fig. 10.A. As far as the ratio of the foremost neuroinhibiting PI allopregnanolone to neuroactivating 17α-hydroxy- progesterone is concerned a markedly depressed values in the patients, but with a trend to incomplete, but significant compensation is observed – Fig. 10.B.

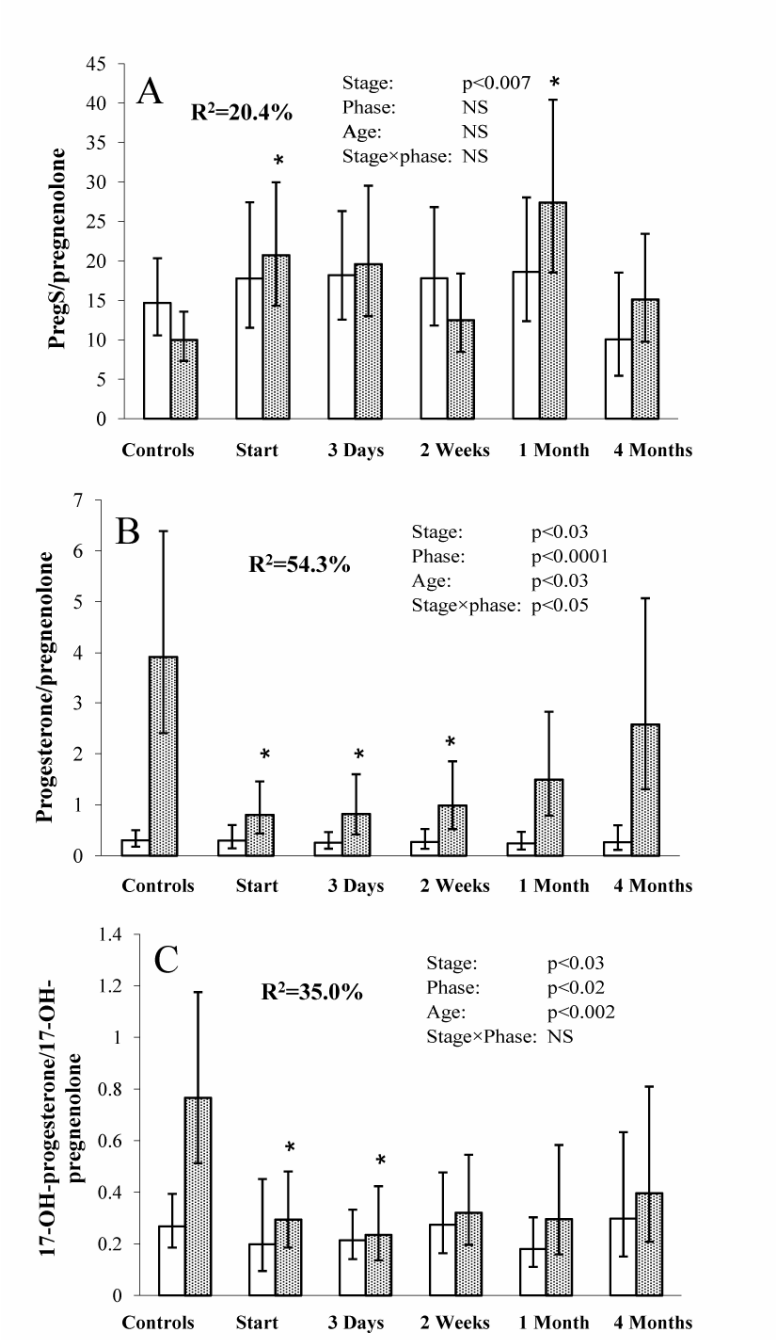

Figure 9. Serum steroid products/precursors ratios of unsaturated C21-steroids in controls and in women during alcohol detoxication therapy. The drawings and symbols are the same as for Figure.3

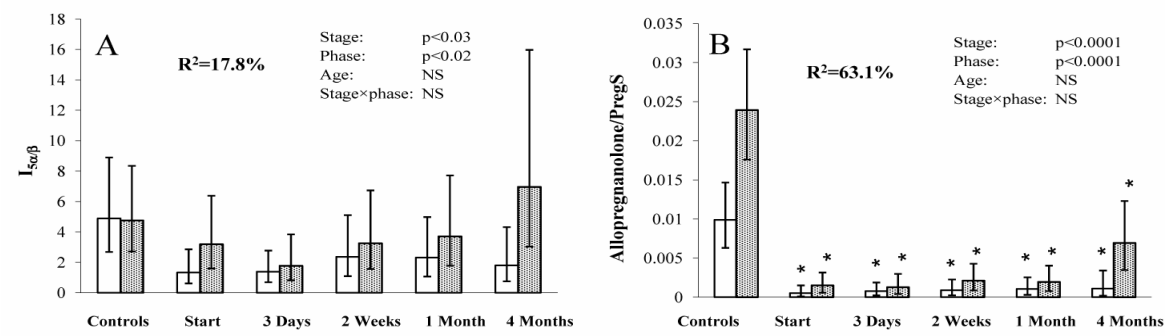

Figure 10. $I_{5\alpha/\beta}$ characterizing the overall conjugated/free isomer ratio of serum pregnanolone isomers (defined as the square root of the ratio of product of the 5α– to 5β-isomers) and the allopregnanolone/pregnanolone sulfate ratio in controls and in women during alcohol detoxication therapy. The drawings and symbols are the same as for Figure 3.

Neuroactive steroids in reproduction (status of sex, menstrual cycle end pregnancy)

It turned not true, during the recent years, that the 7-hydroxylated steroids were physiologically inactive metabolites. Moreover the best of our knowledge, no method for the simultaneous measurement of the serum 7-hydroxy-metabolites of pregnenolone, DHEA, 3β,17β-androstendiol and testosterone (T) including parent steroids has been reported at the time of the present study. The amounts of the steroids tested in the individual groups are shown in Figs.11, 12, 13 and 14

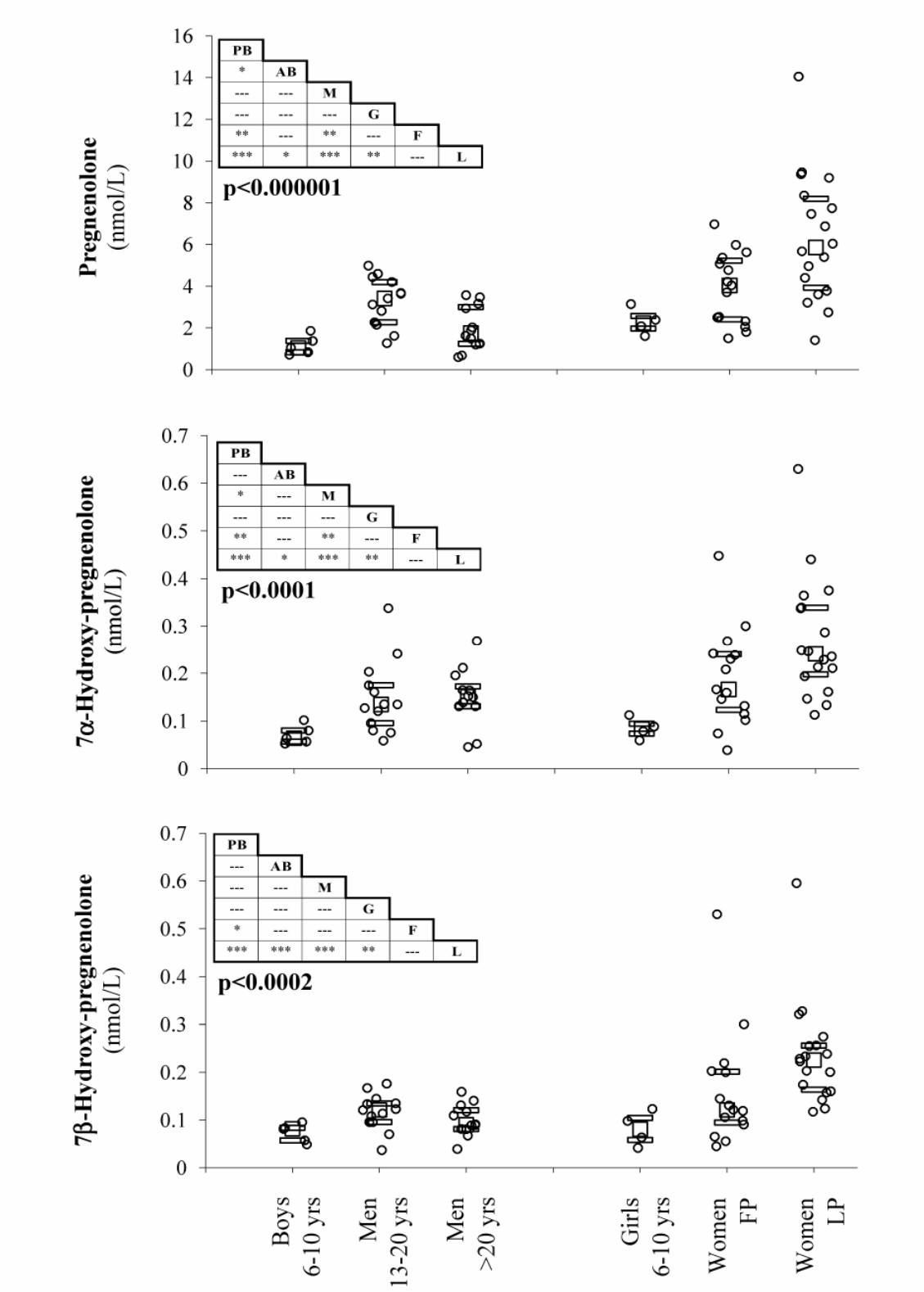

Figure 11. Serum levels of pregnanolone and its 17-hydroxy-metabolites as evaluated by one-way Kruskal- Wallis ANOVA followed by Kruskal- Wallis multiple comparisons; the p-value at the insert denotes significance of Kruskal- Wallis ANOVA; the table insets represents multiple comparisons: (*)p < 0.05, (**)p < 0.01, (***)p < 0.001, (-) not significant; the empty circles represent the individual subjects, while the empty squares with bold bars denote the group medians with quartiles; on the x-axis: FP-follicular phase of the menstrual cycle; LP, luteal phase of menstrual cycle; PB, prepubertal boys 6-10 years; AB, adolescent boys 13-20 years; M, men older than 20 years; G, pubertal girls 6-10 years.

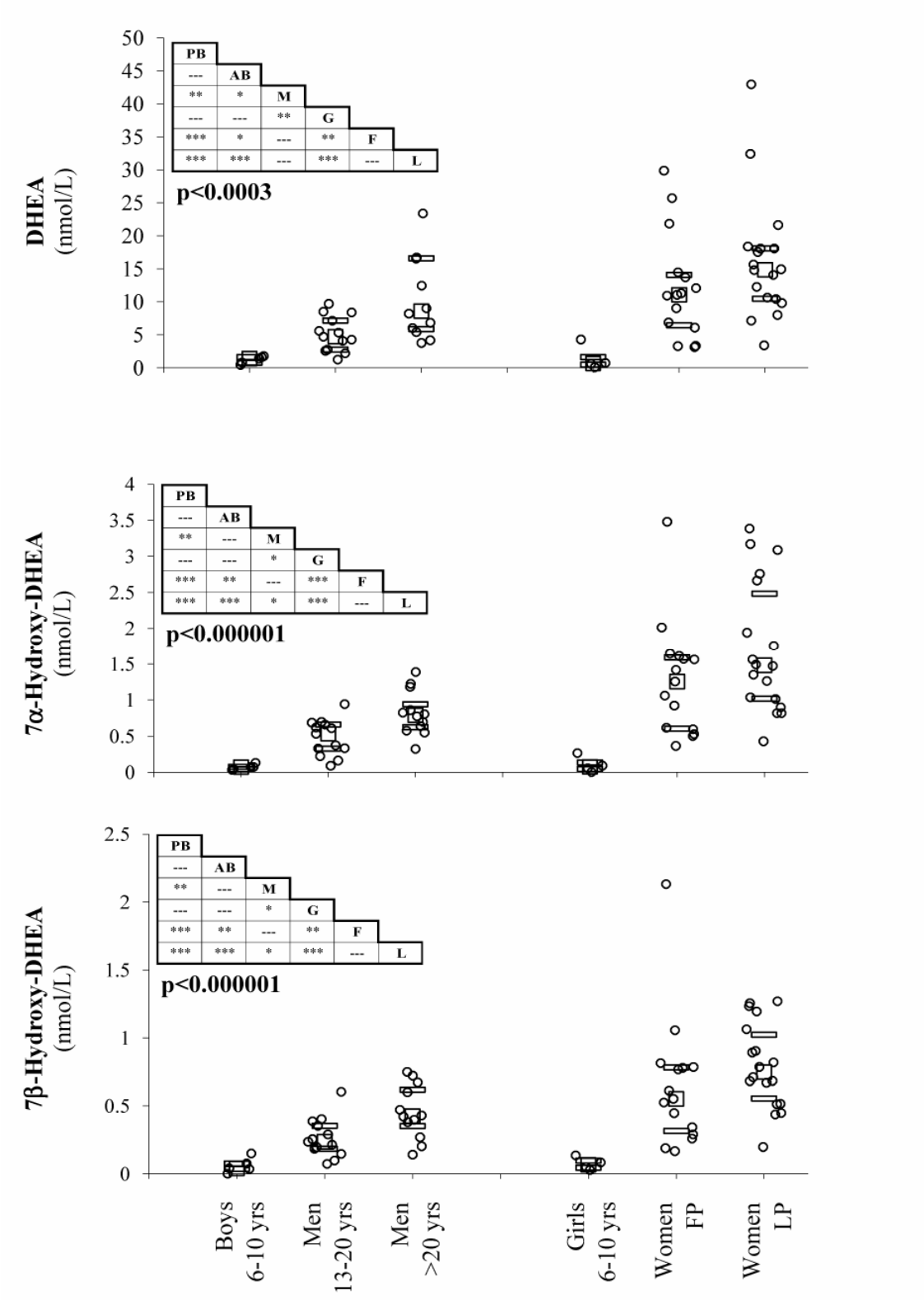

Figure 12. Serum levels of DHEA and its 17-hydroxy-metabolites; for legend see Figure 11.

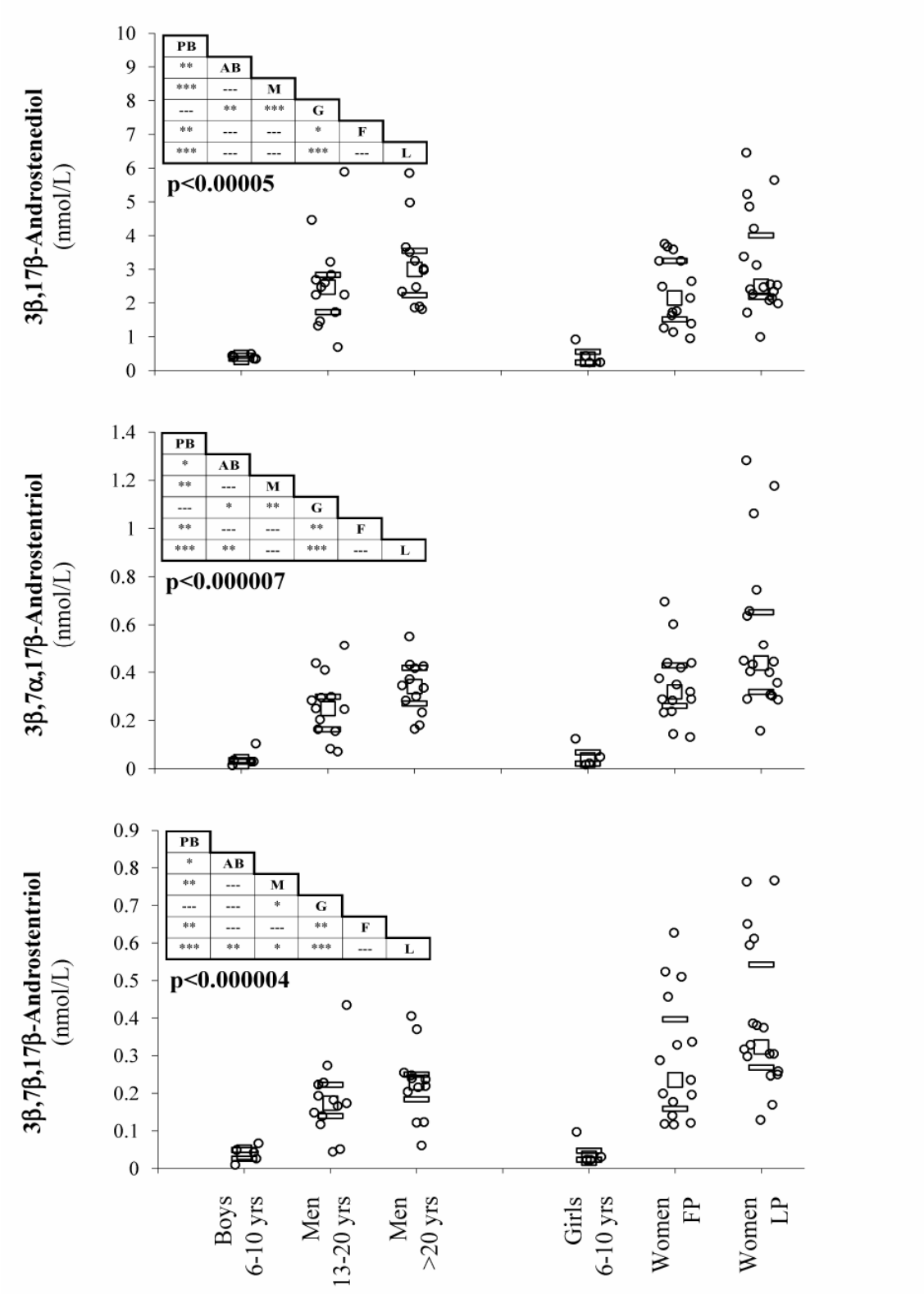

Figure 13. Serum levels of 3ϐ,17ϐ–androstendiol and its 7-hydroxy-metabolites; for legend see Figure 11.

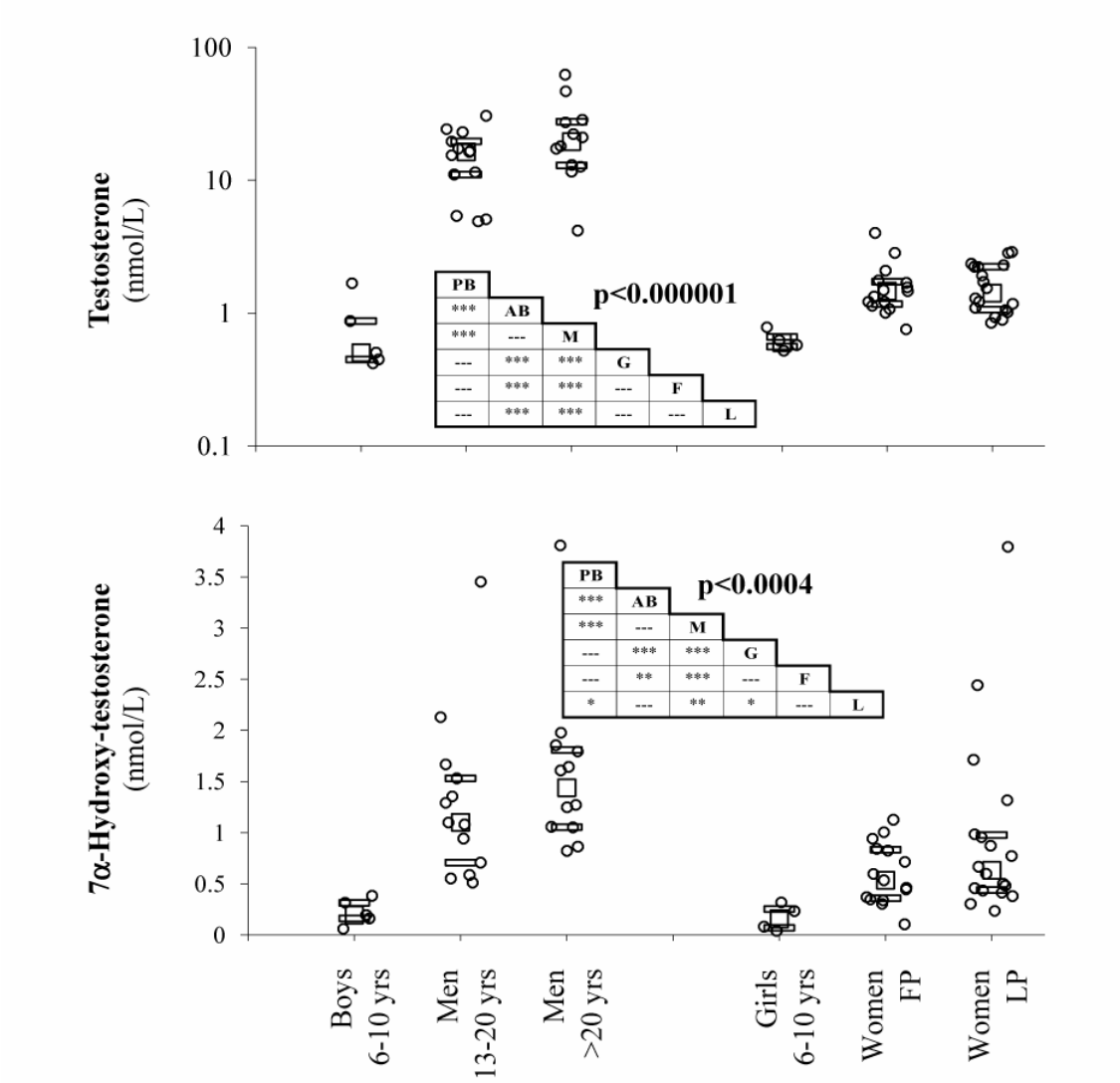

Figure 14. Serum levels of testosterone and its 7α–hydroxy-metabolite; for legend see Figure 11.

Given the number of subjects in the groups of prepubertal boys and girls, there was a low power of statistical analysis. In Fig. 11 are presented the concentrations of pregnenolone and its 7-hydroxylated metabolites. As expected, the minimum levels of the steroids were found in the group of prepubertal boys. Respectively the groups of adolescent boys and adult men did not differ in steroid levels. The women in the follicular phase of the menstrual cycle exhibited significantly higher levels of all the steroids in the prepubertal group, and higher levels of pregnenolone than in the group of men. In 7a-hydroxy-pregnenolone, a difference was also found between the female and girls groups. Women in the luteal phase had higher levels of all the steroids studied, than any other group, except the ones in follicular phase, significantly differing from the subjects of luteal phase group, but only in 7β-hydroxy-pregnenolone.

The serum concentrations of DHEA and its 7-hydroxylated metabolites are presented in Fig.12. As in the case of pregnenolone, the DHEA metabolites reflected the amount of DHEA. The women in follicular phase group showed no difference from the men group in any steroid, but women in luteal phase had higher levels of both DHEA metabolites than did the men group, but no difference were observed between the female in both follicular and luteal groups. Again, the levels of all steroids were very low in children, significantly differing from the groups of adults, but not from adolescent boys group for all of the steroids. The adolescent boys and men groups did not differ for any steroid.

Similarly, Fig.13 presents the amounts of 3β,17β-androstenediol and its 7-hydroxylated metabolites. It is well seen on this figure that the situation was similar to the case of DHEA but more pronounced. Again no significant difference was found between the women in the follicular and luteal phase of the menstrual cycle. The concentrations of all steroids were very low in children. On Fig. 14 the levels of T and 7α-hydroxy-testosterone were exhibited. As expected prominent inter-sexual differences were apparent in both steroids, although they were less pronounced for 7α-hydroxy-testosterone.

The results of given possible metabolic inter relationships, the 7α-epimers/parent steroids and 7α-epimer/7β-epimer ratio with respect to the differences between the steroid ratios for individual parent steroids as well of status of the subjects (age, sex, phase of the cycle) are shown in Fig. 15. For the 7α-epimer/parent steroid ratios, the overall trend was for the ratio to significant increase from pregnenolone to 3β,17β-androstenediol ($p < 0.0001$), and this trend was not the same within individual groups, as demonstrated by a significant inter-factor interaction ($p < 0.0001$). In prepubertal boys, 7α–epimer/parent steroid ratios were similar for all parent steroids. Striking differences among the subgroups of parent steroids were found within all of the female groups. Using ANOVA, a significantly increased trend was recorded in the 7α-hydroxylation of the parent steroids from childhood to adulthood in both males ($p < 0.0001$) and females ($p < 0.01$).

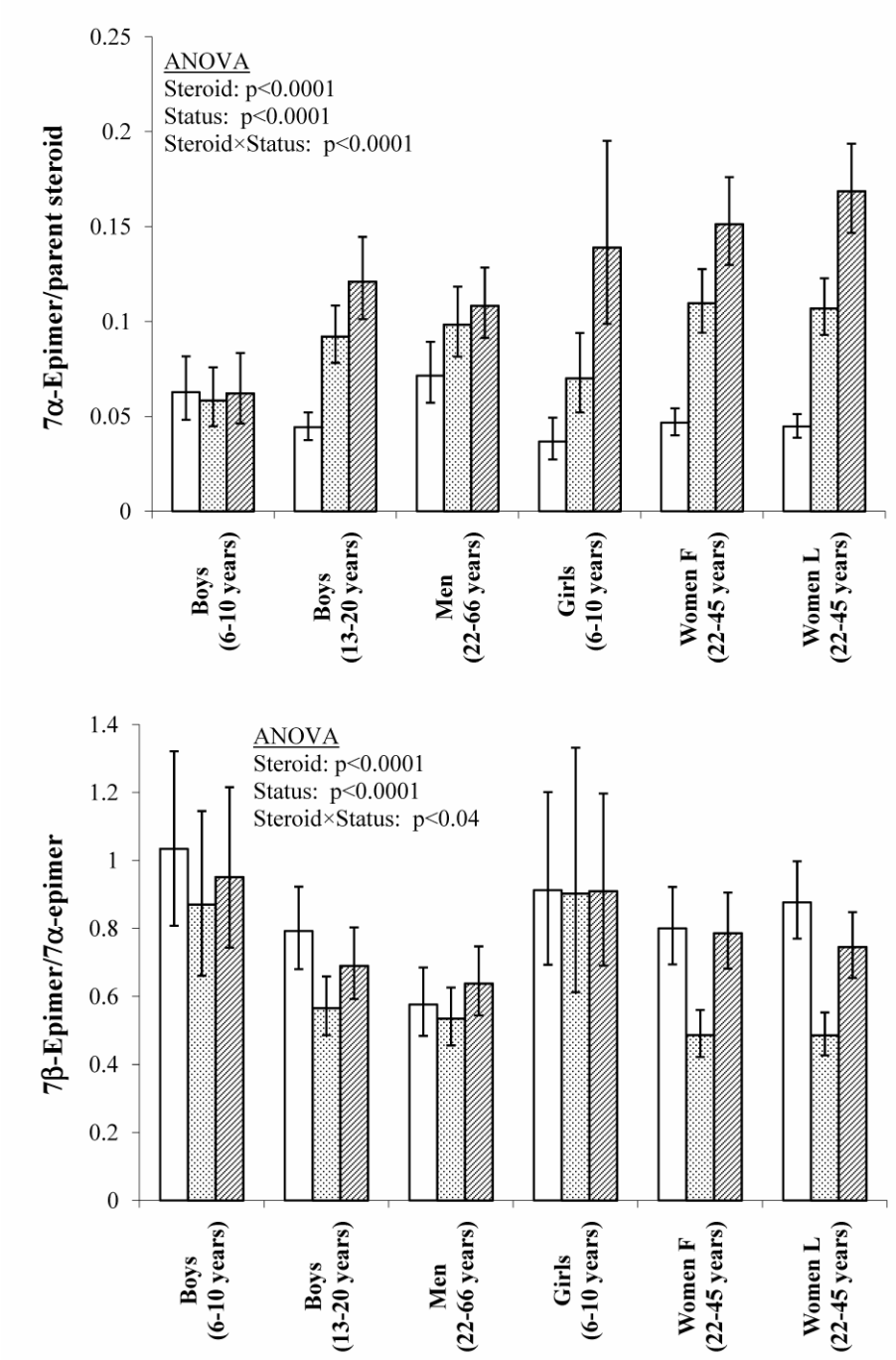

Figure 15. Product/precursor ratio for 7α–hydroxylation and conversion of 7α–hydroxylated steroids to 7β–epimers; on the x-axis: F, follicular phase of the menstrual cycle; L, luteal phase of the menstrual cycle. The bars with error bars represent the re-transformed mean values with their 95% confidence intervals; the empty dotted and hatched bars denote pregnenolone, DHEA and 3β,⍰–androstendiol as parent steroids, resp. The first factor „steroid" evaluates if the dependent variable differ according to the parent steroid (pregnenolone, DHEA and 3β,⍰–androstendiol) provided that the second factor is adjusted to a constant value and the between-factor interaction is absent. The second factor status evaluates if the dependent variable differs between the Gross according to the status (boys 6-10 years, boys 13-20 years, men, girl 6-10 years, women in follicular phase, women in luteal phase) provided that the first factor is adjusted to a constant value and the between-factor interaction is absent. The interaction steroid x status evaluates if the difference in the subgroups according to status chase from (parent) steroid to steroid or if the differences in the subgroups according to the parent steroid depend on the status.

In contrast to 7α-hydroxylation, the conversion of 7α-hydroxylated metabolites to the 7β-epimers was generally more balanced. As in the case of 7α-hydroxylation, no differences in the ratio related to phases of the menstrual cycle were observed.

The steroid levels in both sexes were highly correlated using Pearson's correlations, but as far as the C-21 steroids were concerned, in men weaker correlations were observed than in women.

Aiming to elucidate the physiological impact of endogenous neuroactive pregnanolone isomers and their polar conjugates in women, the levels of above mentioned steroids were measured in healthy premenopausal volunteers. As expected, the levels of unconjugated pregnenolone isomer in the follicular phase were low, with median values of 0.51, 0.27, 0.134 and 0.062 nmol/L for allopregnanolone, isopregnanolone, pregnanolone and epipregnanolone, while the level for the conjugates were markedly higher – 7.6, 10.0, 20.3 and 3.13 nmol/L respectively. When the amounts of free and conjugated steroids in luteal phase were compared with the ones in follicular phase, it was easily seen that they were quite elevated – 1.89, 1.12, 0.428 and 0.284 nmol/L and 28.8, 37.2, 51.2 and 6.5 nmol/L respectively for allopregnanolone, isopregnanolone, pregnanolone and epipregnanolone. The ratios of pregnanolone isomers in the luteal phase compared with those in the follicular phase, applying a linear model with the ratio as a dependent variable, containing the steroid status as the first factor and conjugation status as a second factor and also interaction between factors and age as a covariate, revealed that the factors and the interaction between factors were insignificant.

The differences in the ratio of conjugated to free steroids are shown in Fig. 16.

The model indicated highly significant differences between individual steroids ($p < 0.0005$), and conjugated to free steroids values rose to a greater or lesser degree in the follicular phase ($p < 0.007$). Of the interactions, phase of menstrual cycle X age reached significance ($p < 0.02$) indicating differences between younger and older subjects in respect of the factor phase of the cycle.

In the follicular phase, the correlations between pregnenolone isomers and their precursors were insignificant, with the exception of the borderline correlation between isopregnanolone and P. The opposite situation was found in the luteal phase, where

significant strong, medium or borderline correlations were recorded between P and pregnanolone isomers. In contrast to conjugated 3α-isomers significantly correlating with pregnenolone polar conjugates in the follicular phase, conjugated 3β-pregnenolone isomers did not.

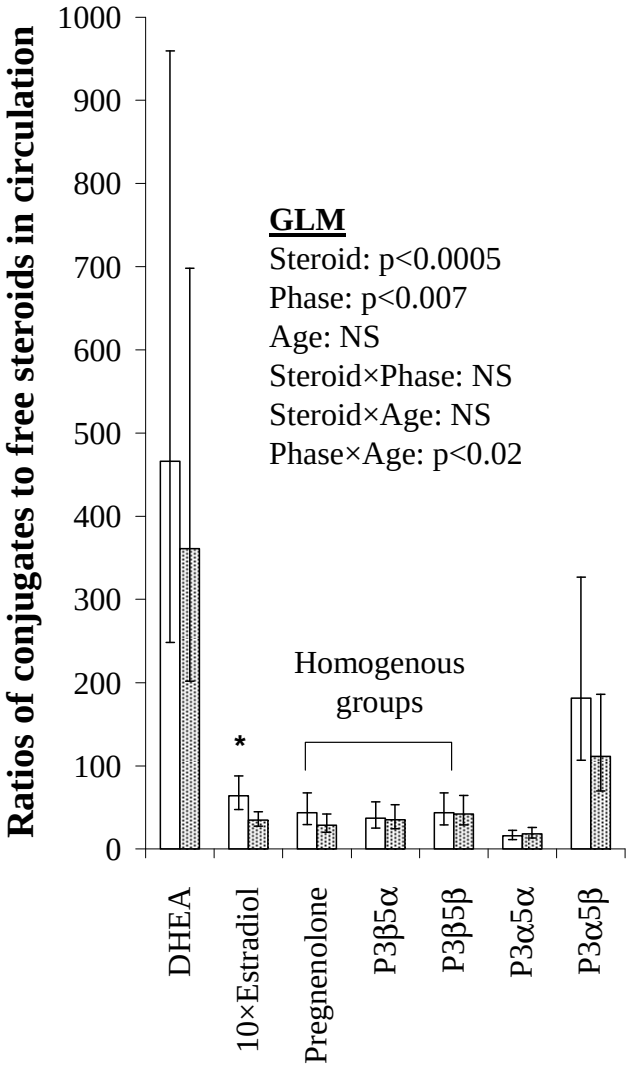

Figure 16. Ratios of polar conjugates to free steroids in DHEA, E2, pregnenolone, allopregnanolone, isopregnanolone, pregnanolone, epipregnanolone) in the circulation of non-pregnant women. A general linear model with the steroid and phase of the menstrual cycle as main factors, age as a covariate, and all combinations of second-order interactions was used to evaluate the effect of the factors and covariate. The differences between individual subgroups were evaluated using Bonferroni multiple comparisons. White and dotted bars with error bars represent retransformed mean values with 95% confidence intervals in the FP and LP, respectively.

Of the free and conjugated pregnenolone isomers, only the conjugated pregnanolone showed a significant age relationship, with decreasing values of the conjugate accompanying increasing age in the follicular phase. In terms of steroid ratios reflecting pregnenolone

isomers metabolism, the allopregnanolone conjugate/allopregnanolone ratio negatively correlated with age in the follicular phase. In the same phase 3α-pregnenolone isomers significantly increased with age, as did the ratio of 3α- to 3β-isomers.

The quantity of the above mentioned PI and their polar conjugates (PICs) and some respective precursors were measured by GC-MS in order their status of sex, phase of the menstrual cycle and pregnancy to be physiologically evaluated. Their levels are shown in Figs. – 17, 18 and 19.

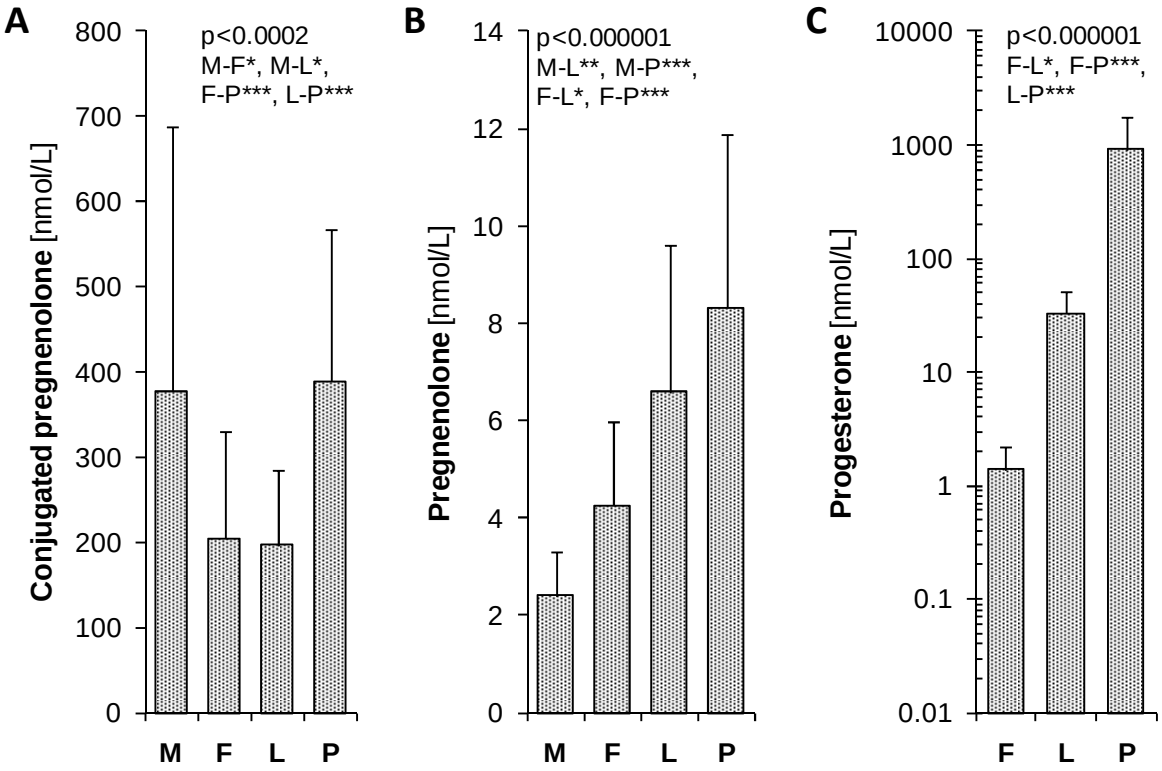

*Figure 17. Levels of polar conjugates of pregnanolone (A), unconjugated pregnanolone (B), and progesterone (C) in 15 adult men (M), 15 women in follicular phase of menstrual cycle (F), 16 women in the luteal phase of menstrual cycle (L), and 30 women in the 36th week of gestation (P). Bars with error bars represent group means with S.D.S. Owing to skewed data distribution and non-constant variance, but between-group were evaluated using robust Kruskal-Wallis ANOVA followed by Kruskal-Wallis multiple comparisons. The significance of ANOVA model is shown in the first line of embedded table, while significant inter-group differences as found by the multiple comparisons (P< 0.05) are shown in the further lines of this table (*P<0.05, **P<0.01, ***P<0.001).*

The values of isopregnanolone increased in the sequences: male subjects, female in follicular phase, female in luteal phase and pregnant. A similar situation was found in all PI and PICs steroids. Both 5α–PI exhibited higher conjugate to free steroid ratios for men and pregnant women, when compared with those of both phases of the cycle. In contrast to the situation in 5α-PI, the ratio of allopregnanolone conjugate to allopregnanolone isomer showed decreasing trend in the sequence: subjects in follicular phase, subjects in luteal

phase and in the pregnancy. The ratio of epipregnanolone conjugate to the same isomer exhibited no difference.

As far as the ratio of 3α- to 3β-isomers is concerned, the women in follicular and luteal phase showed more than two times lower values than the pregnant women. The respective differences in the conjugates were much less pronounced. The ratios of 5α-PI to 5β-PI showed higher values for follicular and luteal phases groups when compared with pregnancy group, although the situation was different in PICs, where the ratios were significantly higher in the subjects in luteal phase.

In the women in the follicular group the only significant correlation between conjugated and free PI was found for the epipregnanolone ($r = 0.536$, $P = 0.040$, $n = 15$), while in luteal phase group isopregnanolone significantly correlated with its conjugated form and the same was proved for epipregnanolone and epipregnanolone conjugates. The others tested correlations did not reach significance. Significant correlations between 3α- and 3β-PI were mostly found within all groups for 5α- and 5β-PI. Isopregnanolone and epipregnanolone significantly correlated in follicular group, luteal group and pregnant women group as well, while between isopregnanolone conjugate and epipregnanolone conjugate did not reach significance in the follicular group, but strongly correlated in luteal and pregnant groups. Roughly it was the same situation with the other correlations tested. As far as the correlation between isopregnanolone and P is concerned it did not reach significance in follicular group, but the S in most cases correlated in the other groups. Pregnanolone conjugate negatively correlated with the 5α-PI in men, attaining significance for isopregnanolone, but not for epipregnanolone.

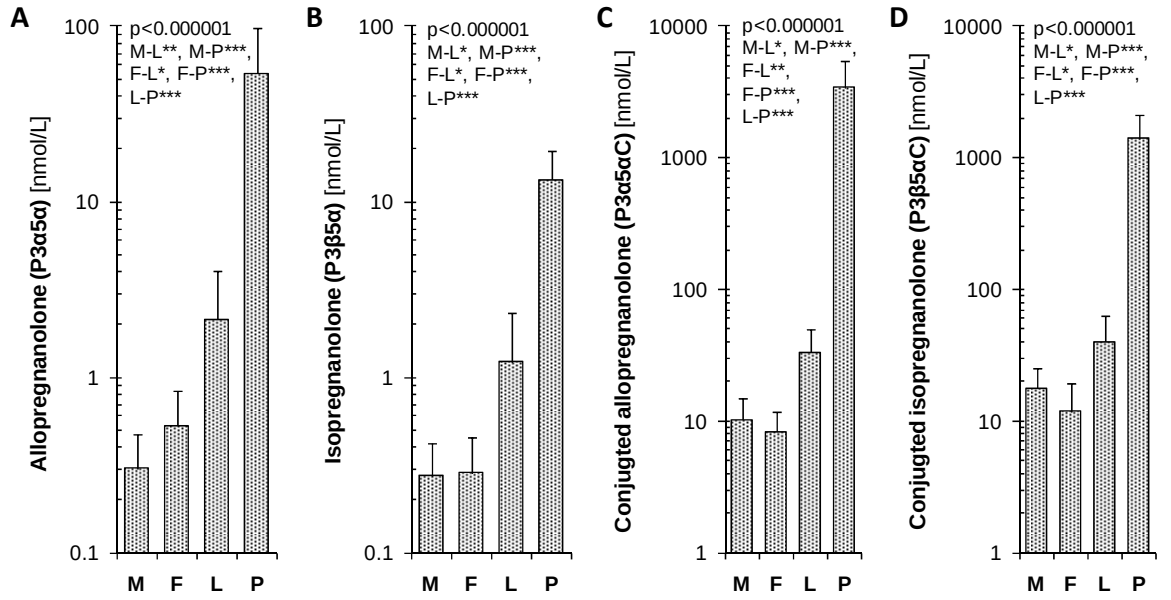

Figure 18. Levels of 5α-pregnanolone isomers (A and B) and their polar conjugates (C and D), in 15 adult men (M) 15 women in follicular phase of menstrual cycle (F), 16 women in the luteal phase of menstrual cycle (L), and 30 women in the 36th week of gestation (P). The drawings and symbols are the same as for Figure 17.

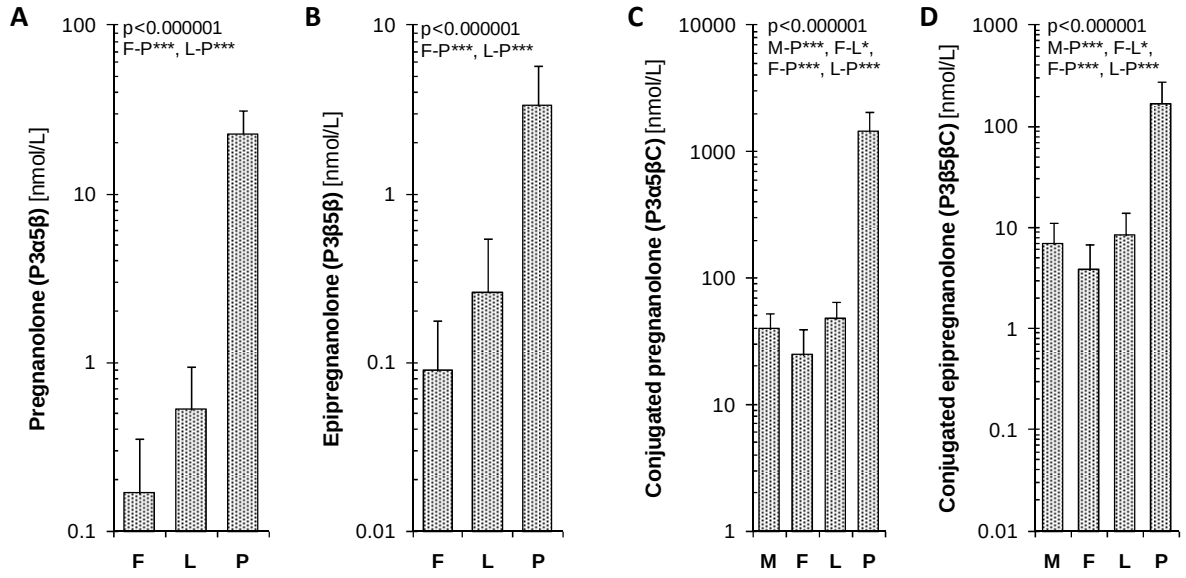

Figure 19. The levels of 5β-pregnanolone isomers (A and B) and their polar conjugates (C and D, in 15 adult men (M) 15 women in follicular phase of menstrual cycle (F), 16 women in the luteal phase of menstrual cycle (L), and 30 women in the 36th week of gestation(P). The drawings and symbols are the same as for Figure 17.

Some NA PI during pregnancy were also measured altogether with E_2, P and DHEA The time profiles for P, E_2 and E_2/P ratio are shown in Fig.20.

All PI, P and E_2 exhibited significantly increasing trends by Kruskal-Wallis test. In the 5α-PI a plateau occurred within the first and the fifth months of pregnancy. For pregnanolone and E_2 an accelerating increase was found within the first and the seventh

months, while for epipregnanolone a gradual increase was observed within the first and sixth month. Also a significant increase was observed from the fifth month in the 3α-isomes. Progesterone, as expected, steadily increased from the first to the tenth months of pregnancy. The ratio between 5α-PI and P amounts during pregnancy was constant, but the pregnanolone/P ratio showed an increasing trend within the first and fifth months. The changes of the ratios of the E_2 to PI are presented in Fig.21. With the exception of E_2/pregnanolone ratio being constant during pregnancy, all remaining ratios significantly increased up to the sixth or seventh month of pregnancy, with maximum for the E2/allopregnanolone ratio.

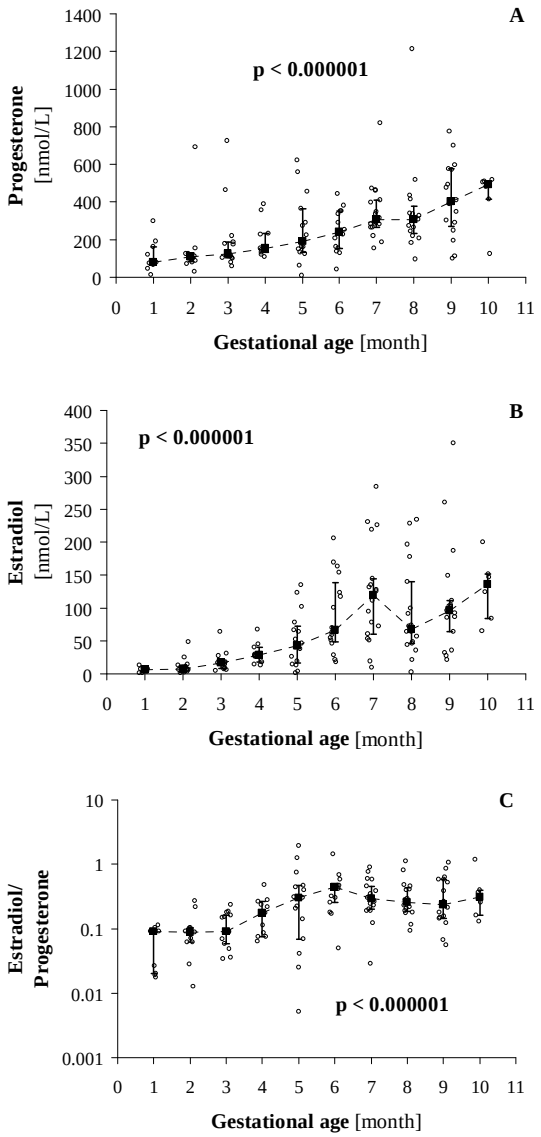

Figure 20. Changes in the plasma levels of progesterone, estradiol, and estradiol/progesterone ratio in the plasma during pregnancy. ○, Individual subjects; ■with error bars, group median with quartiles. P values represent the statistical significance of the overall trend, as found by Kruskal-Wallis robust ANOVA. The

numbers of subjects were 131, 138 and 130 for progesterone, estradiol and estradiol/progesterone ratio respectively.

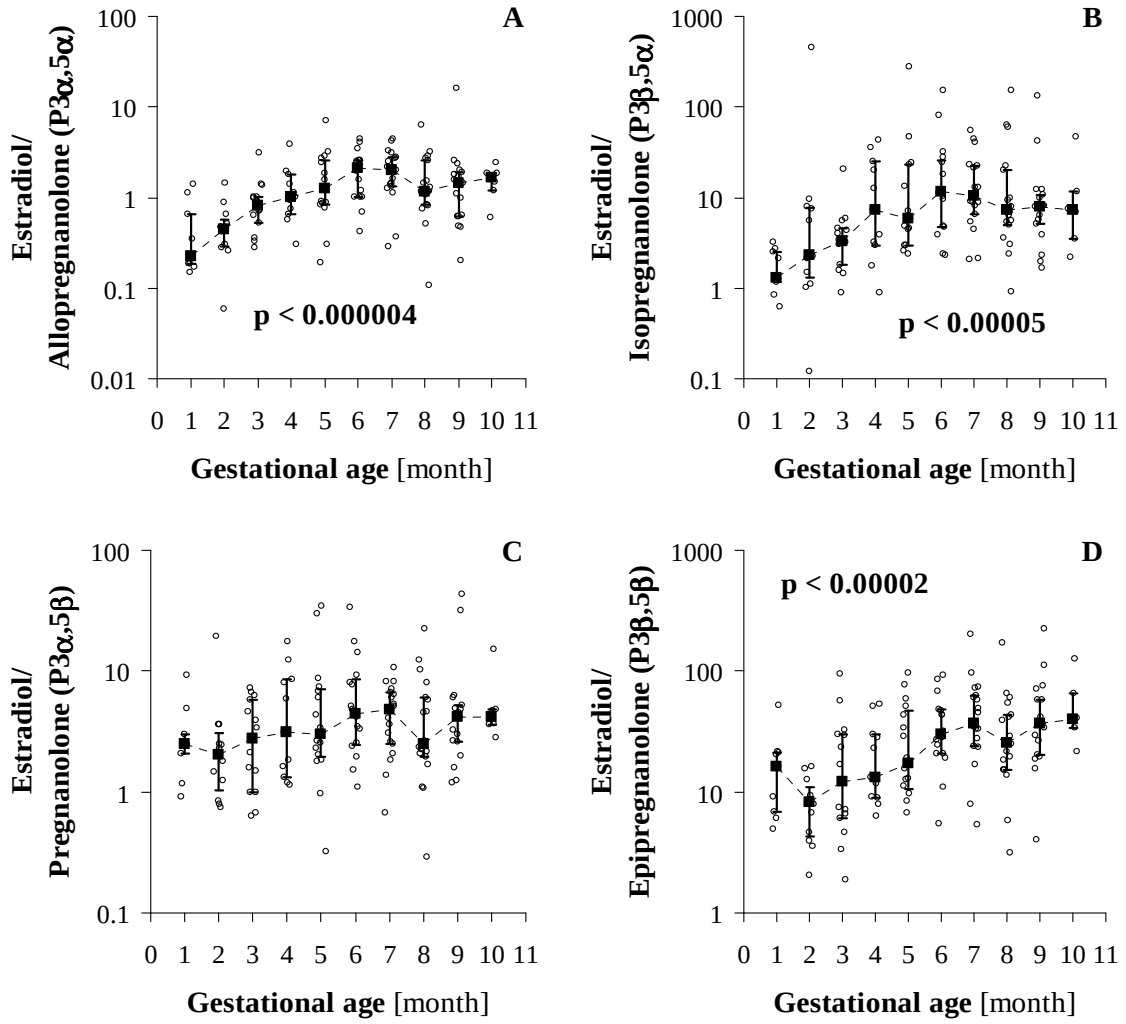

Figure 21. Changes in the ratios between estradiol and PI in maternal plasma during pregnancy. The symbols are explained in Fig. 20. The numbers of subjects were 138, 137, 138 and 138 for estradiol to allopregnanolone, isopregnanolone, pregnanolone and epipregnanolone ratios, respectively.

More attention was paid to circulating levels of PI during the third trimester of human pregnancy. Pronounced differences were observed between the 5α- and 5β-pregnane S. The 5α-dehydroprogesterone and 5α-PI showed a significant increase between the 30th and 36th weeks white the respective 5β-pregnane S displayed either no significant change (5β-dihydroprogesterone) or a decreasing trend in 5β-PI from the 36th week of gestation. The ratio of 3α- to 3β-PI did not also change significantly during the third trimester. A trend of borderline significance with a maximum in the 35th week showed the ratio of 5α-dehydroprogesterone to P, while the respective ratio for the 5β-dehydroprogesterone significantly decreased from the 31st week. Three of the conjugated PI

displayed significant increases from the 30th week to the 37th week, followed by a plateau. The only exception was pregnanolone.

Both ratios of conjugated 5a-PI to the respective free S gradually increased from 30th to the 37th weeks, and then showed a plateau up to the 40th week. The ratio for 5β-PI, i.e. isopregnanolone and epipregnanolone showed an increase from the 30th or 31st week

4.5. Correlations between steroids in human blood and cerebrospinal fluid from the 3rd ventricle

Eighteen unconjugated steroids in the CSF from the 3rd ventricle and 18 unconjugated steroids and 7 steroid polar conjugates were measured in 8 postmenopausal women and 7 men. Table 6 depicted the ratios of S levels to median values in the CSF and documented pronouncedly lower levels of the CSF steroids in comparison with the amounts in circulation.

Table 6. Ratios of steroid levels to median levels in the cerebrospinal fluid (CSF) and serum

		Serum, free steroids/CSF				Serum, conjugates/CSF				Serum, conjugates/free steroids			
				quartile				quartile				quartile	
No	Steroid	n	median	lower	upper	n	median	lower	upper	n	median	lower	upper
1	Preg	13	6.9	4.1	13.0	11	584	281	903	11	85	41	131
2	DHEA	13	29.3	14.2	57.5	11	19849	9332	26932	11	677	318	918
3	Prog	13	7.1	6.4	9.4								
4	Prog17	13	46.4	33.6	118.0								
5	A2	13	6.0	3.2	9.1								
6	T	13	29.7	18.1	55.7								
7	P3α5α	13	16.1	9.6	19.1	12	469	241	844	12	29.2	15.0	52.5
8	P3β5α	13	4.0	3.3	4.5	12	148	101	276	12	37.0	25.4	69.1
9	A3α5α	13	23.6	17.1	45.5	11	70129	45114	221196	11	2976	1915	9388
10	A3β5α	13	28.8	15.1	48.5	11	24597	17075	66532	11	853	592	2307
11	DHEA7α	13	2.6	1.7	4.3								
12	DHEA7β	13	8.5	4.6	12.0								
13	AT7α	13	14.0	5.9	24.7								
14	AT7β	13	4.2	2.8	6.9								
15	Preg16α	13	50.3	36.8	157.3								
16	DHEA16α	13	30.5	17.4	36.5	12	12149	5901	15934	12	399	194	523
17	Prog16α	13	34.8	23.4	42.9								
18	Cort	13	73.7	27.7	108.6								

The ratios of circulating S/CSF steroids showed values from 2.6 for DHEA7a to 77 for cortisol. Circulating S polar conjugates to unconjugated S in the CSF ratios demonstrated values from 175 for P3B5a to 70 000 for A3a5a. Similarly the correlations between the S in the CSF and serum are presented in Table 7. Generally, C19-3B-hydroxy-5-ene S showed significant correlations between circulation and CSF, particularly the 7α/β-hydroxy-metabolites of DHEA and androstenediol (Fig. 22). Borderline, but relatively strong correlations of pregnenolon and DHEA serum conjugates, being primarily of adrenal origin,

with the free S in the CSF may reflect the differences in the activity of the adrenal cortex between the subjects.

After adjustment to constant serum PregC, the partial correlation between CSF Preg and serum Preg was insignificantly negative (r = 0.390, p = 0.2) but the corresponding correlation between CSF Preg and serum PregC was significantly positive (r = 0.645, p=0.05, for constant serum Preg).

Table 7. Pearson's correlations[a)] between circulating steroids and steroids in the cerebrospinal fluid

	CSF vs. serum free steroids			**CSF vs. serum conjugates**			**Serum free steroids vs. serum conjgates**		
Steroid	r	p	n	r	p	n	r	p	n
Preg	0.225	0.459	13	0.588	0.057	11	**0.742**	**0.009**	11
DHEA	**0.820**	**0.001**	13	0.532	0.092	11	**0.620**	**0.042**	11
Prog	0.087	0.778	13	----	----	----	----	----	----
Prog17	0.586	0.097	9	----	----	----	----	----	----
A2	**0.558**	**0.047**	13	----	----	----	----	----	----
T	0.320	0.287	13	----	----	----	----	----	----
P3α5α	**0.740**	**0.004**	13	0.195	0.543	12	0.155	0.630	12
P3β5α	**0.656**	**0.015**	13	0.431	0.161	12	0.429	0.164	12
A3α5α	0.505	0.078	13	0.168	0.621	11	0.406	0.215	11
A3β5α	**0.820**	**0.001**	13	0.450	0.165	11	0.477	0.138	11
DHEA7α	**0.917**	**0.000**	13	----	----	----	----	----	----
DHEA7β	**0.941**	**0.000**	13	----	----	----	----	----	----
AT7α	**0.867**	**0.000**	13	----	----	----	----	----	----
AT7β	**0.890**	**0.000**	13	----	----	----	----	----	----
Preg16α	**0.843**	**0.000**	13	----	----	----	----	----	----
DHEA16α	**0.735**	**0.004**	13	-0.048	0.882	12	0.303	0.338	12
Prog16α	0.486	0.092	13	----	----	----	----	----	----
Cort	**0.889**	**0.000**	13	----	----	----	----	----	----

[a)] Pearson's correlation after data transformation providing Gaussian distribution and homoscedasticity; significant correlations (p<0.05) are in bold

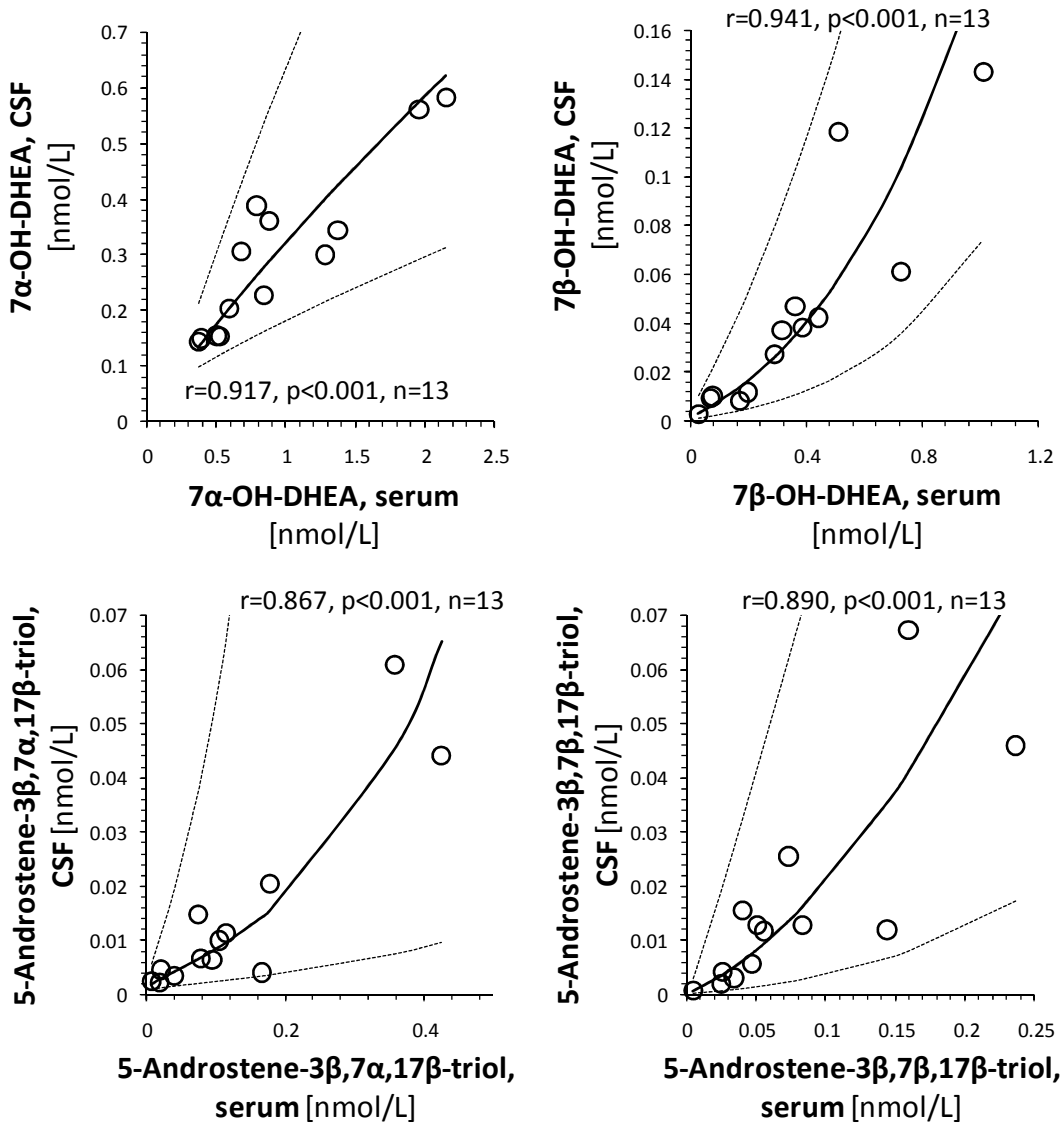

Figure 22. Relationships between the steroids in the cerebrospinal fluid from the 3rd brain ventricle (CSF) and serum for the levels of 7α-hydroxy-DHEA (section A), 7β-hydroxy-DHEA (section B), 5-androstene-3β,7α,17β-triol (section C), and 5-androstene-3β,7β,17β-triol (section D). The bold full curve represents the principal axis after retransformation to the original scale, while the thin dashed line is the retransformed 95% confidence ellipsoid. The correlation coefficient r is calculated from the data transformed by a power transformation to attain Gaussian data distribution and a constant variance.

In the CSF, we observed significant correlations between T and A2 (Fig. 23), and between serum and CSF A2 (Tab. 6) but insignificant correlation between serum and CSF testosterone (Tab. 6). These relationships might indicate that T levels in the CSF depend on the transport of A2 from the periphery to the CNS and on the conversion of A2 to T within the CNS and might be only partly dependent of serum T levels

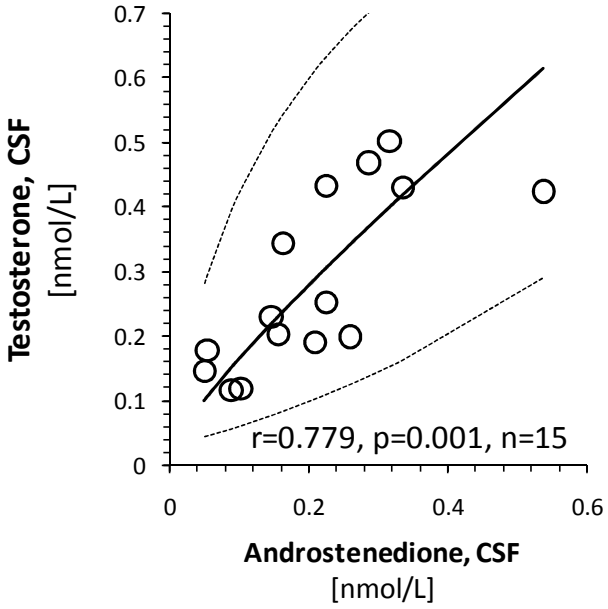

Fig. 23. Relationships between the testosterone and androstendione in the cerebrospinal fluid from the 3rd brain ventricle. The bold full curve represents the principal axis after retransformation to the original scale, while the dashed line is the retransformed 95% ellipsoid. The correlation coefficient r is calculated from the data transformed by a power transformation to attain Gaussian data distribution and a constant variance.

5. Discussion

To obtain a better knowledge of the role of androstane and pregnane metabolites in adult men and of the transformation of these predominantly NA substances to inactive or even reversely active conjugates, a method was developed suitable for the determination of 20 circulating S, including nearly all the C19 S hormones, their precursors and polar conjugates. It is important to notice that some of these hormones are known to act or may possibly operate in the modulation of signal transduction on ligand gated ion-channels in the central and peripheral nervous system.

The selectivity and sensitivity of the method were sufficient for the entire S measured. As far as sensitivity is concerned it ranged from hundreds of femtograms to tens of picograms, due to the S fragmentation pattern.

The retention times of DHEA and A3β5α17β are nearly identical and because their molecular masses differ very slightly (only by 2g/mol), there could be an overlap in some fragments. That is why in measuring comparatively high levels of DHEA, this fact could be

critical. In general, the unconjugated AS values measured using GC-MS were either comparable or lower, than the obtained by RIA (Meikle et al. 1979, Genazzani et al. 1998). Higher levels of most of the conjugated AS detected by the created GC-MS method point to the efficient hydrolysis of the S conjugates (Dehennin et al., 1996). At the same time the levels of conjugated AS were by two or three orders of magnitude higher than the free S.

Epilepsy in women is usually menstrual cycle-related seizure disorder. It is characterized by an increase in seizures during particular phases of the menstrual cycle. Many authors suggest that the cyclical changes of ovarian hormones estrogens, mainly E2, have proconvulsant role, while P and especially its conversion to the NAS allopregnanolone (Kokate et al. 1999; Veliskova 2007) have anticonvulsant, beneficial neuroprotective properties.

Considerably lower levels of P, 17α-hydroxy-pregnenolone and 17α-hydroxy-progesterone were found in our patients compared to controls. Regarding the significant differences in P concentrations, especially during the luteal phase, the results are in accordance with those of other authors (Rosciszewska et al. 1986 Bonucceli et al. 1990; Herzog 1991). No difference was observed in P levels between patients and controls in follicular phase. As far as changes in E2 amounts are concerned our results are also in agreement of those published by the above mentioned researchers. Galimberti et al. (2009) found that P and E2 were lower during the luteal phase in women with epilepsy, whereas SHBG levels were higher in the patients than in the controls.

The ratio E2/P was higher in the investigated patients during the both phases of the menstrual cycle. Recently Shen et al. (2005) claimed that estrogens enhance the effect of P, but the basis of this effect is still not well understood. Evidently the phenomenon suggests that in some physiological situation, as it might be in epilepsy, estrogens enhance the plasticity of GABAa receptors. At the same time it is well known that the NS can modulate most GABAa receptor isoforms (Lambert et al., 2003/)

In both phases of menstrual cycle in women with epilepsy elevated levels of 17-hydroxy-pregnenolone sulfate were detected when compared with controls at almost unchanged DHEAS. Most probably significant higher levels of circulating metabolite with proconvulsant activity might increases frequency of epileptic seizures, but eventually this influence is not connected with the stage of menstrual cycle.

Long-term alcohol abuse among women is a growing thread to their health and reproductive condition (Hugues et al. 1980; Torres and Ortega 2003). Highly sensitive and selective GC-MS method was used for the analysis of the progesterone isomers, pregnenolone and E2 in order to evaluate how the NAS reflect impaired P synthesis in women treated for alcohol addiction and whether during the therapy the female reproductive functions could be restored by reisnstatement of the steroid biosynthesis. Initially and during the first month of the treatment the levels of P and its reduced metabolites were significantly lower than in controls which was in accordance with the data of Pettersson et al. (1990)) and Sarcola et al. (1999). The beneficial influence of the therapy, as the obtained results point out, show its compensating effect in P, 17α-hydroxy-progesterone and progesterone isomers. Of them pregnanolone demonstrated the least pronounced differences, possibly due to its rapid conversation. (Parivar et al., 1996). The restoration of P and its isomers to the physiological levels during alcohol detoxification therapy could be explained by the regeneration of P synthesis impaired by alcohol abuse or by the recovery of enzyme activities.

A restoration of the P metabolism to P isomers is also likely. Third possibility could be the adaptation of the organism to a rising demand for $GABA_A$ receptor activating substances to the cessation of alcohol intake, having in mind the similar mechanism in the effect of alcohol and S activators on it (Morrow, 2001)

It is worth to notice that the ratios of the 5α- to 5β- isomers showed a gradual increase in the luteal phase, as well the ratio of neuroactivating pregnanolone sulfate to neuroinhibiting allopregnanolone was expressing a regular decreased during therapy in both phases of the menstrual cycle.

A similar pattern as in the P and its metabolites was found in E_2. This finding suggests that the restoration of ovarian activity during medication is not limited only to P and its metabolites. As mentioned before, estrogens are also neuroprotective substances. It is quite likely that the reduction of neuroinhibiting pregnanolone isomers in women treated for alcohol addiction is primarily connected with ovarian steroid biosynthesis impairment.

But it not be entirely ruled out that changes in the formation of neuroactive P metabolites and the decreased physiological requirements for neuroinhibiting S and by contrast the increased requirements for S neuroactivators might be also a reason. The

mechanism/s of the corresponding feedback loop/s remains in question. Nevertheless the restoration of S concentrations during detoxification therapy for alcohol addiction has a favorable effect on reproductive functions and the psychosomatic stability of premenopausal women.

The physiological impact of endogenous NA pregnanolone isomers, especially the polar conjugates has not be assessed at all, when their determination in serum of non-pregnant women of fertile age was undertook It is obvious that the levels of the pregnanolone isomers including the conjugates strongly depends of the menstrual cycle, following changes in P synthesis. There is close relation between free pregnanolone isomers, absolute levels of the S and the ratio of neuroactivating isomer conjugates to neuroinhibiting 3α-isomers, which should be taken into consideration. The levels of conjugates were markedly higher than the free S. The balance in proportion between neuroinhibiting 3α-pregnanolone isomers positively modulating $GABA_A$ receptors and the 3β-isomers reducing their uptake on the receptor needs evaluation. But in the same time it is clear that the amounts of circulating neuroactivating pregnanolone isomers are markedly prevalent over neuroinhibiting, keeping in mind that the proportions need not necessarily reflect S levels at the sites of their action. Moreover polar conjugates are more soluble than non-polar free analogs. The strong correlation between the 3α- and 3β-isomers in both phases of the menstrual cycle indicates their uncomplicated inter-conversation, all operating on $GABA_a$ receptors, but in opposite manners. The results also indicate adrenal origin of pregnanolone isomers in the follicular phases and gonadal source of the S in the luteal phase. The sulfatation of pregnanolone isomers and particularly of pregnanolone not only moderates free isomers levels bur also significantly restrains E_2 biosynthesis via degradation of P as a substrate. Substantial proportion of progesterone is metabolized in the sequence:

Progesterone → 5β-Dihydroprogesterone → Pregnanolone → Conjugated pregnanolone.

In men and women serum concentrations of pregnanolone, its conjugates, all pregnanolone isomers and their conjugates were measured. During pregnancy distinctive effects are to be expecting of pregnanolone isomers due to persistently elevated levels of 3α-pregnanolone isomers, resulting in decreased affinity of $GABA_A$ receptors for these NAS (Brussard et al., 2000; Koksma et al., 2003). In men and to large extend in women in the follicular phase role of adrenal activity and in situ brain synthesis of pregnanolone isomers can be expected. The

data received, suggest that the production of pregnanolone isomers in men depend on sulfatase activity, hence the major metabolic pathway in them to be expected is:

Conjugated pregnenolone → Pregnenolone → Progesterone → 5α-DHP → Allopregnanolone → Isopregnanolone

Women in the luteal phase and early pregnancy produce in bulk the isomers in corpus luteum (Ottander et al., 2005) After the luteo-placental shift, the primary precursor of the pregnanolone isomers and conjugated pregnanolone originates almost entirely from the fetal zone (see Fig 1)

Changes in neuroactive pregnanolone isomers during pregnancy were traced in order to elucidate if the balance between neuroinhibiting and neuroactivating isomers influence the timing of parturition. All of the isomers studied exhibited a significantly increasing trend during pregnancy, especially distinct in 3α-steroids, which are known to attenuate neuronal activity and reduce the excitotoxicity induced by N-methyl-D-aspartate (Lockhart et al., 2002) With reference to the timing of parturition the ratio of allopregnanolone to isopregnanolone in this study was not found to be changed. This fact weakens the hypothesis concerning the role of isopregnanolone in the onset in parturition, despite of its blocking effect on allopregnanolone function as a modulator of $GABA_A$ receptors (Lundgren et al., 2003). In contrast, the result shows that the ratio of E_2, uterine activity stimulator (Fang et al., 1996; Chaim and Mazor, 1998) to allopregnanolone, exhibiting the opposite activity (Leng and Russell, 1999; Brussard et al., 2000) gradually changed in favor of E_2 up to 6 – 7 month, reflecting the profile of the E_2/P ratio. Findings such as the rapid metabolism of pregnanolone (Hering et al., 1996) together with its high production rate, suggest the relatively dynamic balance between pregnanolone and E_2 might be one of the mechanisms that influences the timing of parturition.

The mean concentrations of free pregnanolone isomers ranged from 2 to 50 nmol/L, while the mean levels of their polar conjugates were 40 – 100 times higher. The ratio of 5α-isomers to P significantly culminated in the 35^{th} week. It is not to be entirely ruled out the involvement of pregnenolone in initiating of parturition in humans that might, like allopregnanolone, sustain the pregnancy via attenuation of hypothalamic $GABA_A$ receptors.

Androstane and pregnane metabolites in adult men modulate $GABA_A$ receptors (Darnaudery et al., 1999; Reddy, 2004). The 3α- and 5α-androstane steroids stimulate $GABA_A$ receptors, while 3β-isomers according to Bitran et al. (1996) are not operative. The S neuromodulators in men originate mainly from the 3-oxo-4-eneC_{19}-steroids, which are converted to their 3α- and 3β-hydroxy-5α/5β-reduced metabolites (Meike et al., 1979; Labrie et al., 1997; Genazzani et al., 1998). Some of the S have been measured in human for the first time during this study. A comparison of the others evaluated S, with the values reported elsewhere (Labrie et al., 1997; Person Murphy and Allison, 2000; Matsuzaki et al., 2004; Starka et al., 2006) generally agreed and especially when individual variation among subjects are taken in account (Matsuzaki et al., 2004). The levels of conjugated DHEA were in agreement with the studies of Labrie et al. (1997), T value with reported by Murphy (1988) but the androsterone quantities were three times higher compared to the data of Labrie et al. (1997).

The levels of neuroactive androstane metabolites in men were present in higher concentrations than the respective pregnane S. These results indicate that in male population, the physiological effect of androstane hormones may be of greater importance than the effect of reduced progesterone metabolites, the level of which are extremely low. The data obtained are compatible with the concept of an uncomplicated, reversible, oxidoreductive transformation between the 3α and 3β-metabolites via the 3-oxo-intermediate products, and the inter-conversion between free metabolites and their 3α/3β-hydroxy-groups for the oxidoreductive changes. The results also showed that conjugation may restrain the transport of neuroactive androstane metabolites across the blood-brain barrier into the central nervous system.

Our data from GC-MS and RIA analysis of 18 unconjugated S in CSF and 18 unconjugated S and 7 steroid polar conjugates are in fair agreement with the GC-MS results of other studies (Backstrom et al., 1976; Schwarz and Pohl, 1992; George et al., 1994;; Uzunova et al., 1998; Kim et al., 2003; Hennebert et al., 2007). They are most apparent in Preg, showing very close values to the ones of Usunova et al. (1998), but both are lower than the data of George et al. (1994) concerning the measured values by immunoassay. This is also valid for the DHEA CSF levels while compared to the published results of Kim at al. (2003) and Rasmusson et al. (2006). Progesterone CSF levels are also in good agreement with the data reported

elsewhere (Backstrom et al., 1976; George et al., 1994; Rasmusson et al., 2006), but our CSF androstenedione data, obtained by GC-MS are substantially lower than those of Schwarz et al. (1992). Testosterone, as expected, shows higher CSF levels in male subjects, as have been already proven by Backstrom et al. (1976), while allopregnanolone levels are lower (Uzunova et al., 1998), possibly due to the age distribution of our subjects.

Concerning the possibility of Preg conversion in the CNS, no significant correlation between Preg and DHEA in the CSF was found, but significant ones between circulating DHEA and DHEA in the CSF were observed ($r = 0.820$, $p = 0.001$). This data indicated the key role of peripheral C17-hydroxylase-C17-lyase activity for the ratios between DHEA and Preg in the CSF. The results also outlined that DHEA, in contrast to Preg, is exclusively of peripheral origin, which is in accordance with the concept of negligible or even missing activity of C17-hydroxylase, C17,20 lyase in the CNS.

In the CSF, we observed significant correlations between T and A2 (Fig. 23), and between serum and CSF A2 (Tab. 6) but insignificant correlation between serum and CSF testosterone (Tab. 6). These relationships might indicate that T levels in the CSF depend on the transport of A2 from the periphery to the CNS and on the conversion of A2 to T within the CNS and might be only partly dependent of serum T levels.

In the CSF, DHEA correlated with DHEA7α ($r=0.791$, $p<0.001$), DHEA7α correlated with DHEA7β ($r=0.957$, $p<0.001$) and AT7α correlated with AT7β ($r=0.946$, $p<0.001$) (Fig. 3). These relationships pointed to a brain in situ 7α-hydroxylation. 7α/β-Hydroxy-metabolites of DHEA and androstendiol showed tight correlations both between and within the body fluids. The correlations between DHEA7α and DHEA7β in the CSF, as well as between AT7α and AT7β in the CSF, indicates uncomplicated inter-conversion between 7α- and 7β-hydroxy metabolites (via 7-oxo- intermediate) in the CNS independently of their transport from the periphery.

The relatively close concentrations of some steroids in the CSF and serum, as well as the tight correlations between CSF and serum 7-hydroxy-steroids (Tab 9) demonstrated relatively uncomplicated transport of these substances between CSF and peripheral circulation.

In brief, the 7α-hydroxy-steroids may originate in the brain, may be further converted to 7β -hydroxy-metabolites but all of the already mentioned steroids may also penetrate from the periphery in the CNS.

While the proportion between in situ synthesized steroids and the steroids penetrating across the blood-brain-barrier from the periphery did not show pronounced differences for the 7-hydroxy-steroids, but this was not the case for 16α-hydroxy-metabolites. Certain amount of Preg16α and DHEA16α in the CSF appeared to be of a peripheral origin and a part of these steroids may be also synthesized in the CNS from DHEA. Like the Preg16α and DHEA16α, Prog16α significantly correlates with the parent steroid in the CSF, but Prog16α in the CSF shows only borderline correlation with the serum Prog16α. The data indicated that Prog16α synthesis in the CSF may be more important than the transport of the steroid from the periphery.

The 3α- and 3β-hydroxy-5α-reduced metabolites of C21 and C19 steroids significantly correlate between the CSF and the serum (Tab. 9) and show significant inter-correlations within the CSF. These relationships indicate mutual interconversion via 3-oxo intermediates in CNS and periphery as well as transport of the substances from periphery to the CNS. In conclusion, we found pronouncedly lower levels of the CSF steroids in comparison with the levels in circulation. However, major differences were found between the individual steroids. In humans, not the peripheral Preg but the PregC is of importance for Preg concentrations in the CNS is further supported by a great excess of PregC levels over Preg levels in the human circulation.

The situation appears to be different for DHEA. The results indicate the possibility of transport of DHEAS, however from the CNS, rather than into the CNS. This means that considering about 700 times higher serum DHEAC levels in comparison with those found for DHEA in our serum samples as well as almost 20000 fold higher DHEAC concentrations when compared to CSF we may also expect significant influx of DHEAS following this huge concentration gradient.

In general, our data are compatible with the concept that a part of the steroids may be synthesized de novo in the CNS. However, substantial part of the steroid metabolites may be synthesized in the CNS from the steroid precursors or directly transported from the periphery. The CNS synthesis and transport from periphery might be complementary in

some cases i.e. brain synthesis might provide minimum level of steroids, which are indispensable for the CNS functions. However, brain steroids of peripheral origin may reflect various physiological situations.

Conclusions

1. A novel and original GC-MS method with sensitivity and selectivity for simultaneous quantification of free and conjugated pregnane and androstane steroids has been developed and evaluated. The sensitivity ranged from hundreds of femtograms to tens of picograms, depending on the steroid fragmentation pattern.

2. Significantly ($p < 0006$) lower levels of 17α-hydroxy-pregnenolone, 17α –hydroxy-progesterone and higher values of pregnenolone sulfate ($p < 0.0001$) were found in both phase of the menstrual cycle of women with epilepsy when compared with controls. Progesterone concentrations did not differ in follicular phase, but they were depressed in luteal phases in women with epilepsy.

3. Premenopausal women with alcohol addiction showed depressed progesterone, 17α-hydroxy-progesterone, progesterone isomers and estradiol levels along with elevated pregnenolone sulfate values during the menstrual cycle in comparison with healthy controls. The reinstatement of serum progesterone, estradiol and progesterone isomers during the treatment for alcohol abusement and its favorable effect was demonstrated on both reproductive function and the psychosomatic stability of the patients.

4. The levels of 7-hydroxy-metabolites of dehydroepiandrosterone and 3β,17β-androstenediol are age and sex dependent, but independent of the phase of the menstrual cycle. Pregnanolone isomers are of adrenal origin during the follicular phase and of gonadal source during the follicular phase. These isomers and their basic levels are similar in men and women, but increasing in luteal phase (3 times) and escalating (over 38 times) during pregnancy, compared with follicular phase.

5. Simultaneous measurement of 20 conjugated steroids and 16 polar steroid conjugates in the serum of men revealed that the conjugated androstane steroids for the most part reached micromolar concentrations, one or two orders of magnitude higher than the values

for the corresponding pregnane steroids, which may restrain the transport of free androstane hormones from the periphery into central nervous system.

6. For the first time neuroactive steroids in cerebrospinal fluid from the 3rd ventricle were determined. The results allowed to derive how the steroids are transported through the hematoencephalic barrier. Part of the steroid metabolites issynthesized *de novo* in the central nervous system from the steroid precursors or directly transported from the periphery.

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Participation in International Congresses and Conferences

Kancheva L, Kančeva R, Hill M, Novak Z, Chrastina J, Starka L.

Steroid levels in the human cerebrospinal fluid and serum, poster

VII Croatian Congress on Gynaecological Endocrinology, Human Reproduction and Menopause with international participation

Brijuni, Croatia 10-13.09.09

Kancheva L

Účinek neurosteroidú na CNS a stabilitu těhotenství, přednaška

Pracovní setkaní krajských perinatologů České Republiky

Praha, 6.10.2008 4-6.10.2008

Kancheva L, Veliková M, Pařízek A, Hill M, Stárka L.

Cnojugated reduced progesterone metobolites in fetal umbilical, arterial and venouse blood, amniotic fluid and in the maternal blood at normal and premature labors., poster

18th International Symposium Of The Journal Of Steroid Biochemistry & Molecular Biology

Seefeld, Austria 18-21.9. 2008

Kancheva L, Hil Ml, Vrbíková J, Stárka L.

Neuroaktivní Androstanové Metabolity U Dospšlých Mužů, poster

XXX. Endokrinologické Dny s mezinárodní účastí

Špindlerův Mlyn, Česká Republika 4-6.10.2007

Kancheva L, Hill M, Pelíkanová T and L. Stárka

Neuroactive pregnane and androstane methabolites in men, poster

The 4th Croatian congress of endocrinology with international participation.

Rovinj, Croatia May 2-6, 2007

Kancheva L, Včelaková H, Hill M, Vrbíková J and Stárka L.

Neuroactive steroids in adult men, poster

4th International Meeting Steroids and Nervous System

Torino, Italy 17-21 February 2007

Kancheva L, Hilll M, Vrbikova J., Starka L.

Neuroactive Testosterone Methabolits in Adult Men, poster

12th International Congress on Hormonal Steroids and Hormones and Cancer

Athens, Greece 13-17.9.2006

Kancheva L, Cibula D, Hill D, Havlikova H, Fait T, Starka L.

Circulating Neuroactive Pregnanolone Isomers in the Third Trimester of Human Rregnancy, poster

12th World Congress of Gynecological Endocrinology

Florence, Italy March 2-5 2006

Kancheva L, Havliková H, Hill M, Vrbiková J.

Stanovení 7-hydroxilovaných metaboliů 3-hydroxy-5-en-steroidů a testosteronu v séru, poster

Olomolc, Česká Republika Endocrinologicke dny, 20-22.10.2005

Kancheva L, Cibula D, Hill M, Havlíková H, Fait T and Stárka.

Relationships between neuroactiv steroids , their precursors and metabolits in the third trimester of human pregnancy, poster

6th Athens Congress on Women's Health & Disease

Athens, Greece 22-25. 9. 2005

Havlikova H , Kancheva K, Šulcová J, Štulc T.

Léčba fenobarbitálem u mužů snižuje sérové hladiny dehydrepiandrosteronu, poster

VIII Kongres o Ateroskleróze

Špindlerův Mlýn, Česká Republika 2-4. 12. 2004

Hill M, Cibula D, Havlikova H, Kancheva L and Morfin R

Lamotrigine side effects in epilepsy treatment and alterations in 7-and 16alpha-hydroxylation of DHEA, poster

Proceeding of the 12th ISE

Lisabon,Portugal,August 31-September 4,2004

Havlikova H, Hill M, Cibula D,Kancheva L.Hampl R.

Pregnanolon isomers and progesterone in epileptic women, poster

Euromedlab

Barcelona, Spain 1-5.06.2003