Contents

1. Introduction

1.1. Hormones-endocrine system and endocrine regulations

As animals evolved from single cells to multicellular organisms, single cells took on specialized functions and became mutually dependent in order to satisfy the needs of the whole organism. Survival of an individual required the ability to adjust and adapt to hostile external environmental conditions and survival of a species required coordination of reproductive functions with those internal and external factors. Therefore, communication between the capacities of specialized cells became crucial to meeting these needs for survival of multicellular organisms.

Chemical signals that carry out such intercellular communication might be simple molecules such as modified amino acids or fatty acids, or they might be more complex peptides, proteins or steroids. In general, chemical substances that carry information between two or more cells could be ascribed as hormones. This communication can take place locally between cells within a tissue or organ, and at a longer distance in order to integrate the activities of cells or tissues in separate organs.

Since hormones are highly diluted in blood and extracellular fluid, achieving meaningful concentrations usually requires coordinated secretion by a mass of cells, commonly known as endocrine glands. The secretory products of endocrine glands are released into the extracellular space and diffuse across the capillary endothelium into the bloodstream.

Classical endocrine glands include the pituitary, thyroid, parathyroids, gonads, islets of Langerhans and adrenals. However, virtually even organ including brain, hearth and fat has its own endocrine function in addition to its commonly recognized role.

1.2. Steroid hormones

1.2.1. General aspects

The natural steroid hormones generally belong to a group of hydrophobic compounds that could be found within many eukaryotic organisms. In mammals steroid hormones (SHs)

are synthesized from cholesterol especially in placenta, gonads and adrenal glands. Furthermore, several SHs as well as their precursors could be synthesized also in non-endocrine tissues such as fat and brain from the same precursor.

1.2.2. Steroid hormone metabolism

Steroids in general are molecules derived from the cyclopentanoperhydrophenanthrene four-ring hydrocarbon nucleus. All SHs could be derived from cholesterol in a process that could be conceptualized as having five steps:

a) *The conversion of cholesterol to pregnenolone.* In humans only the adrenal cortex, testicular Leydig cells, ovarian theca cells, trophoblast cells of the placenta and specific glial and neuronal cells are able to cleave cholesterol (Chol) to pregnenolone (Pregn). Mobilization of Chol is a complex event that serves as a key point of regulation.
b) *Transformation of Pregn to active hormones, intermediates and exported steroid derivatives.* The enzymes involved in these steps qualitatively determine the steroids that are made. Since these steps are not kinetically limiting, it is step ***a*** that quantitatively regulates amount of SHs that are made at a given moment.
c) *Peripheral metabolism of hormones and precursors.* Several tissues that are not primarily steroidogenic possess ability to transform steroid precursors to active hormones.
d) *Target tissue metabolism.* Steroids can be activated as well as inactivated in appropriate target tissues.
e) *Catabolism.* These reactions lead to SHs with no or negligible biologic activity; most of them are products of hepatic transformations that are renally excreted.

Chol contains 27 carbons and acts as a proximate precursor to other SHs. These hormones can be further divided according to number of carbons into three groups:

a) **C21 steroids** (corticosteroids) are derived from pregnane with side chain on C_{17} and methyl groups on C_{10} and C_{13}. The physiologically important SHs of the C_{21}-series are in humans synthesized in adrenal cortex (cortisol, cortisone, corticosterone, 11-deoxycorticosterone and aldosterone) and in ovaries and placenta (progesterone). In humans these hormones are widely implicated in regulation of extracellular fluid homeostasis, stress response, immune response, inflammation and several reproduction processes.

b) **C19 steroids** (androgens) are derived from androstane containing methyl groups on C_{10} and C_{13}. These steroids are synthesized especially in testes (testosterone, 5α-dihydrotestosterone, androstenedione, and dehydroepiandrosterone), rarely in ovaries and adrenal cortex. C19 steroids are responsible for development and maintenance of accessory sexual structures responsible for nurturing gametes and development of secondary sexual characteristics of males including behavioral ones.
c) **C18 steroids** (estrogens) are derived from estrane with the only one methyl group on C_{13}. Estrogens (estrone, 17β-estradiol) are synthesized in ovaries and placenta where dehydroepiandrosterone (DHEA) acts as the main source (70 to 80%). Estrogen actions are intimately connected with ovulation and formation of corpus luteum. Furthermore, estrogens are responsible for development and maintenance of accessory sexual structures in females.

The groups of enzymes involved in SH biosynthesis are relatively limited. Almost all reactions are functionally if not absolutely unidirectional, so the accumulation of products does not drive flux back to precursors.

Steroidogenesis covers cytochrome P-450-mediated hydroxylations and C-C bond cleavage reactions that are physiologically irreversible as well as hydroxysteroid dehydrogenase reactions. However, pairs of several enzymes can drive the balance of a steroid pair in opposite directions such as 11β-hydroxysteroid dehydrogenase type 1 (reduction) and type 2 (oxidation). Furthermore, there are different enzymes that catalyze other reactions in opposite directions such as steroid sulfatase (STS) and steroid sulfotransferase (SULT).

1.2.3. Steroid hormone transport

As was previously emphasized, SHs are non-polar hydrophobic compounds and if unconjugated they can pass plasma membrane of eukaryotic cells by simple diffusion. On the other hand, SHs that are conjugated either with sulfuric acid (sulfate) or with glucuronic acid (glucuronide) can not quite easily pass the cell membrane and therefore remain in the bloodstream.

Conjugation of SHs is also closely related to their solubility. SH sulfates and glucuronides well dissolve in polar fluids and therefore can be transported independently in the bloodstream. Conversely, unconjugated SHs are commonly transported bound to specific

carrier proteins such as sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) or are unspecifically bound to human serum albumin.

1.2.4. Mechanisms of action of SHs

Hydrophobic hormones such as steroids have direct access to cellular interior where they can bind to soluble proteins. These receptors are actually ligand-regulated transcription factors that bind SHs and their derivatives. As a result, SHs directly influence expression of various structural and functional proteins including other transcription factors.

Since the genomic actions of SHs are well established, there is considerable evidence that in some cases steroids may act more directly on cellular metabolism without the need for gene induction. These effects are mediated by hormone binding to putative membrane receptors, enzymes and/or allosteric sites of ion channels and are described as nongenomic.

1.3. Neurosteroids

Since pregnenolone (Pregn) and dehydroepiandrosterone (DHEA) as well as their sulfated conjugates were found in the brain tissue of castrated and arenalectomized rats (Corpechot *et al.* 1981, Corpechot *et al.* 1983), these results established that the enzymes found in classic steroidogenic tissue are indeed found also in the nervous system. Consequently, neurosteroids were defined as steroids that are newly synthesized from Chol or another early precursor in the central and/or peripheral nervous system and are present there in substantial amounts regardless of removal of peripheral steroidogenic glands (Mensah-Nyagan *et al.* 1999).

Neurosteroids occur in nervous system as free unconjugated steroids, sulfated esters or fatty acid esters of steroids (Jo *et al.* 1989). These various forms of neurosteroids are involved in control of metabolic, behavioral and psychical processes including cognition, stress, anxiety and sleep (Majewska 1992).

As such, neurosteroids can be further classified into two groups: neuroactive and neuroinactive. Neuroactive steroids refer to a group of steroids that is active in neural tissue. They may therefore be synthesized endogenously in the brain or may be synthesized in classic endocrine tissues but act on neural tissues. Conversely, neuroinactive steroids are synthesized in the brain but are inactive on neural tissues (Mellon 1994).

As was previously emphasized, neurosteroids and therefore also neuroactive steroids rapidly exert their effects and thus can not involve steroid receptor-mediated activation of gene transcription. It was demonstrated that neurosteroids act as allosteric modulators of γ-amino butyric acid type A receptor ($GABA_A$ receptor), and act to modulate GABA-ergic effects by increasing (Mellon *et al.* 2002) or decreasing (Majewska *et al.* 1987) the frequency and duration of chloride channel openings. Additionally, the interactions of neurosteroid with other ligand-gated ion channels were found. N-methyl-D-aspartate (NMDA) receptors are positively modulated with pregnenolone sulfate (PregnS), which enhances the response of glutamate receptors (Shirakawa *et al.* 2002). As is true for the effects of neurosteroids on $GABA_A$ receptors, NMDA receptors are modulated by increasing the fractional open time of NMDA-activated channels, by increasing the frequency of opening and the duration of channel opening (Wu *et al.* 1991, Bowlby 1993). Furthermore, several other types of neurotransmitter receptors such as glycine (Wu *et al.* 1997), α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA) receptors (Shirakawa *et al.* 2005), kainate receptors (Wu *et al.* 1997), nicotinic (Valera *et al.* 1992), muscarinic (Klangkalya *et al.* 1988) and σ-receptors (Monnet *et al.* 1995) are modulated by neurosteroids.

Among other nongenomic action of neurosteroids it is possible to mention binding of pregnenolone to microtubule-associated protein 2, which results in enhancement of microtubules polymerization (Murakami *et al.* 2000) or the role of progesterone in axon remyelization (Schumacher *et al.* 1999).

1.3.1. DHEA and DHEAS as neuroactive steroids

Since this work is focused especially on DHEA and DHEAS interconversion in the central nervous system (CNS), further comments are restricted only to biosynthesis and activity of those two neuroactive steroids.

1.3.1.1. Proteins involved in DHEA/S biosynthesis in the brain

Most of the enzymes present in adrenal glands, gonads and placenta were also found in the brain. In the nervous system there is not the only region-specific expression of the steroidogenic enzymes but there is also a cell-type-specific and developmental specific expression of these enzymes. The brain contains additional steroid-metabolizing enzymes including sulfotransferases and sulfatases that can interconvert unconjugated neuroactive steroids and their sulfate conjugates. In many cases, both compounds exert different and distinct nongenomic actions especially on $GABA_A$ and NMDA receptors.

To determine the source of DHEA/S in the CNS two possibilities should be considered: free diffusion of steroids, synthesized in peripheral steroidogenic tissues, across the blood-brain barrier, or *de novo* steroid biosynthesis within the brain.

1.3.1.1.1. Cytochrome P450 side chain cleavage

Concerning *de novo* biosynthesis in the brain, in 1987 immunocytochemical localization of the cytochrome P450 side chain cleavage (EC 1.14.15.6, P450scc) was established in the white matter of the rat brain, suggesting that oligodendrocytes are the source of neurosteroids in brain (Le Goascogne *et al.* 1987). P450scc was first considered to be the rate-limiting and hormonally regulated step in steroidogenesis and it belongs to one of the slowest enzymes known. The reaction involves three successive steps: 20α-hydroxylation, 22-hydroxylation and C_{20}-C_{22} carbon bond scission. All reactions take place in a single active site of the enzyme that is in close contact with membrane bilayer. The human and rat genomes contain a single gene coding for P450scc, which is approximately 20 kbp long and contains nine exons (Morohashi *et al.* 1987, Oonk *et al.* 1990). P450scc mRNA encodes a 521 amino acid (AA) protein that is proteolytically cleaved, removing a 39 AA leader peptide that directs the protein to mitochondria. Mitochondrial inner membrane environment is strictly required for the enzyme activity (Black *et al.* 1994). Here P450scc functions along with electron donor adrenodoxin and adrenodoxin reductase.

1.3.1.1.2. Adrenodoxin/adrenodoxin reductase electron transport system

Despite P450scc functions as a terminal oxidase in a mitochondrial electron transport, electrons from nicotinamide adenine dinucleotide phosphate (NADPH) are first accepted by adrenodoxin reductase. This flavoprotein is located in the mitochondrial matrix and is closely associated with the inner membrane (Hanukoglu *et al.* 1986, Mitani *et al.* 1979). Adrenodoxin reductase provides electron transfer to an iron/sulfur (Fe_2S_2 type) protein-adrenodoxin-which is also located in the mitochondrial matrix.

Adrenodoxin forms complex with adrenodoxin reductase, dissociates after electron transfer, and then binds to P450scc or to the other mitochondrial terminal oxidases such as P450c11B1, P450c11AS or P450c11B3 (Bassett *et al.* 2004, Ogishima *et al.* 1992, Quinn *et al.* 1988). In humans, there is one gene coding for adrenodoxin reductase, found on chromosome 17, and multiple functional adrenodoxin genes on chromosome 11 (Sparkes *et al.* 1991). These genes code for identical mRNAs and proteins. Furthermore, there are two adrenodoxin pseudogenes on chromosome 20 that are not functional (Morel *et al.* 1988).

Adrenodoxin but not adrenodoxin reductase is regulated by trophic hormones on transcriptional level, especially with adrenocorticotrophic hormone (ACTH), acting via cyclic adenosine monophosphate (cAMP) (Sewer *et al.* 2003).

1.3.1.1.3. Steroidogenic acute regulatory protein (StAR)

StAR was recently identified as a protein important for cholesterol transport into the mitochondria in response to ACTH stimulation (Clark *et al.* 1994, Stocco *et al.* 1996). In the major steroidogenic tissues, StAR expression and function are critical for steroid hormones biosynthesis. As it does in peripheral steroidogenic glands, StAR plays a key role also in brain steroidogenesis.

Indeed, the delivery of Chol to the site of its conversion to Pregn, carried out by P450scc, was found to be the rate-limiting and hormonally acutely-regulated step in steroidogenesis (Jefcoate *et al.* 1987, Privalle *et al.* 1983). As a result, StAR was identified as a 30 kDa phosphoprotein associated with the mitochondrial intermembrane space. It is synthesized as a 37 kDa precursor protein containing N-terminal mitochondrial targeting sequence (King *et al.* 1995). In human, there is a single gene encoding StAR mRNA located to chromosomal region 8p11.2 and one pseudogene mapped to chromosome 13 (Sugawara *et al.* 1995). Individuals with mutations that inactivate StAR have severe impairments in steroidogenesis that lead to congenital lipoid adrenal hyperplasia (Saenger *et al.* 1995).

Recently, it was also established that altered StAR expression in several human brain tumors might be associated with intratumoral steroidogenesis and tumor progression especially in malignant astrocytomas (Kim *et al.* 2003).

1.3.1.1.4. Cytochrome P450 17α-hydroxylase/17, 20-lyase (P450c17)

As well as in classic steroidogenic glands, Pregn and progesterone (Prog) undergo 17α hydroxylation to produce corresponding derivatives also in brain. These steroids than undergo cleavage of the C_{17}-C_{20} bond to form DHEA (Figure 1, p. 13) and androstenedione. All these reactions are mediated by a single enzyme P450c17 (EC 1.14.99.9).

P450c17 is bound to the smooth endoplasmic reticulum and accepts electrons from cytochrome P450 oxidoreductase. The enzyme possesses both 17α-hydroxylase and C_{17}-C_{20}-lyase activities. The lyase activity of this enzymatic complex is posttranslationally modulated by serine phosphorylation and depends also on lipid environment (Nakajin *et al.* 1981,

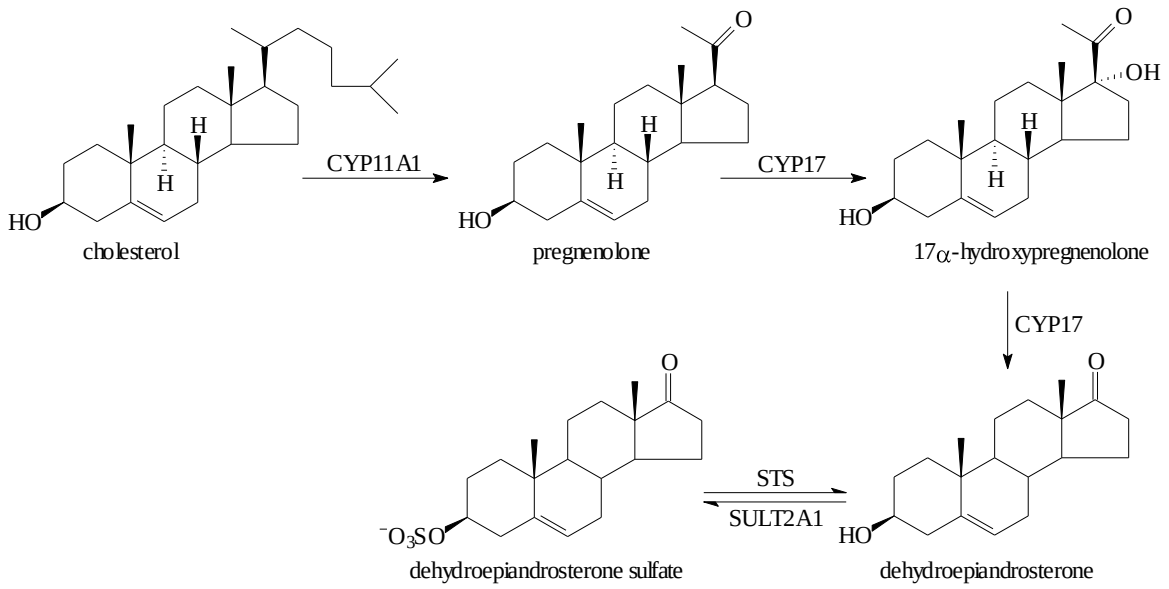

Figure 1. Metabolic pathway showing biosynthesis of DHEA from cholesterol. The involved enzymes are mentioned in detail in text.

Namiki *et al.* 1988). Several other factors are important for C_{17}-C_{20} bond cleavage such as presence of cofactors (cytochrome b_5) and possible competition for substrate (Miller 1988). Generally, 17α-hydroxylase reaction occurs more readily than C_{17}-C_{20}-lyase reaction while P450c17 prefers Δ^5 unsaturated substrates (17α-hydroxypregnenolone) rather than Δ^4 (17α-hydroxyprogesterone). This significantly contributes to large concentrations of DHEA in human adrenals.

In brain P450c17 was detected in various neuronal populations and brain regions (Stromstedt *et al.* 1995, Compagnone *et al.* 1995). However, recent research suggested possibility of P450c17-independent DHEA formation by an alternative pathway involving Fe^{2+} ions (Cascio *et al.* 1998).

Brain-derived DHEA may not be formed exactly according to the known peripheral pathways but also by an alternative process. Isolated astrocytes did not express P450c17 but responded to Fe^{2+} ions by producing DHEA. In contrast to glioma cells, P450c17 mRNA and protein were found in oligodendrocytes but Fe^{2+} ions enhanced DHEA production. These

findings suggest that in differentiating oligodendrocytes and astrocytes DHEA is formed via an oxidative stress-dependent alternative pathway (Cascio *et al.* 2000).

In human brain, P450c17 activity was restricted specifically to neurons while both, oligodendrocytes and astrocytes produced DHEA in response to Fe^{2+} (Brown *et al.* 2000). This suggests that alternative pathway of DHEA biosynthesis may be important in pathological conditions involving increased oxidative stress.

1.3.1.1.5. Cytochrome P450 oxidoreductase

Both enzymes P450c17 and P450c21 receive electrons from a membrane-associated protein P450 oxidoreductase, which is distinct from adrenodoxin reductase. P450 oxidoreductase, containing flavin dinucleotide (FAD), receives electrons from NADPH and transfers them one at a time via flavin mononucleitide (FMN) to the microsomal P450c17 or other P450 oxidoreductase. P450c17 therefore does not need another protein such as adrenodoxin used by P450scc (Miller 2005). The second electron can also be provided by cytochrome b_5.

Ratio of P450 oxidoreductase to P450c17 influences the activity of P450c17, while increased electron support favors both 17α hydroxylation and C_{17}-C_{20} cleavage resulting in DHEA formation (Lin *et al.* 1993, Yanagibashi *et al.* 1986).

1.3.1.1.6. Cytochrome b_5

Cytochrome b_5 is a small heme-containing protein, which donates electrons for many P450-catalyzed reactions. Cytochrome b_5 is found in both soluble form and membrane-associated form, which acts as microsomal electron donor playing the role of cofactor for P450c17 (Kominami *et al.* 1992). Cytochrome b_5 specifically influence P450c17 lyase activity not through electron donation but by enzyme allosteric modulation (Auchus *et al.* 1998). These results indicate that cytochrome b_5 expression may be one of the mechanisms by which P450c17 lyase activity, and consequently DHEA production, can be regulated in specific brain regions.

1.3.1.2. DHEA/DHEAS and membrane channels modulation

A fine interplay exists in the brain between the inhibitory and excitatory neuroactive steroids. They not only counteract each other's actions but are also metabolically linked, as excitatory steroids PregnS and DHEAS can be hydrolyzed and converted to inhibitory tetrahydroprogesterone and androsterone (Majewska 1992).

1.3.1.2.1. $GABA_A$ receptor modulation

The $GABA_A$ receptor ($GABA_A$-R) is the principal inhibitory neurotransmitter receptor in the brain. It is a pseudosymmetric pentamer (Schofield *et al.* 1987) spanning the membrane and forming the chloride ion channel. Important anxiolytic, anticonvulsant, and sedative/hypnotic drugs bind to specific sites on the $GABA_A$-R and allosterically potentiate its function (Macdonald *et al.* 1994). Activation by GABA opens the associated chloride ion channel, leading to increased chloride conductance and usually to hyperpolarization of neuronal membrane.

The $GABA_A$-R is also a target for various neurosteroids. Some of them enhance $GABA_A$-R function, and act as anxiolytic and anticonvulsant drugs (allopregnanolone and tetrahydroxydeoxycorticosterone). Others inhibit $GABA_A$-R function, and can improve cognitive function and reduce seizure threshold (sulfated neurosteroids pregnenolone sulfate and DHEAS) (Belelli *et al.* 2005). DHEAS but not DHEA acts as noncompetitive antagonist of the $GABA_A$-R and inhibits GABA-induced chloride transport (current) by a reduction of channel opening frequency. It indicates that steroid-binding sites are neither situated inside the ion channel nor compete with receptor agonists for their binding sites (Dingledine *et al.* 1999). These various sites, located on the outer receptor surface, may be occupied by various steroids resulting in enhancing or decreasing leakage of a particular ion channel.

The neurosteroid-binding site is therefore a potential target for new drugs to modulate $GABA_A$-R function. The structure-activity approach highlighted the significance of the functional group attached to C_3 and the stereochemistry of C_5 in the steroid A ring, and the functional group attached to C_{17} in the D ring (Akk *et al.* 2007, Morris *et al.* 2004, Hamilton 2002).

1.3.1.2.2. NMDA receptor modulation

DHEA, DHEAS and PregnS were also reported to act as positive allosteric modulators of the ionotropic glutamate, especially NMDA, receptors (NMDA-R) activity (Bergeron *et al.* 1996). As is true for the effects of neuroactive steroids on $GABA_A$-R, DHEAS modulates NMDA-R by increasing the fractional opening time of NMDA-activated channels, by increasing the frequency of openings and the duration of channel openings (Mellon 1994).

The NMDA-Rs are unique in their requirement for simultaneous binding of two different agonists for activation, glutamate and glycine (Stephenson 2006). The NMDA-R channels are highly permeable to calcium and sodium ions, thus after activation it leads to excitotoxic neuronal cell death, which may be influenced by NMDA-R antagonists (Gil-ad *et al.* 2001).

The activity of NMDA-Rs can be modulated by wide variety of physiological and pharmacological compounds, which can be further divided into three basic groups (Kemp *et al.* 2002):

a) competitive antagonists acting in the glutamate and/or glycine binding sites
b) uncompetitive antagonists/ion channel blockers with binding sites localized inside the ion channel (Mg^{2+}, memantine)
c) noncompetitive antagonists-binding sites are localized neither inside the ion channel nor compete with receptor agonists (Zn^{2+}, Cu^{2+}, H^{+}, cytoplasmic polyamines, and last but not least neuroactive steroids)

   As mentioned for $GABA_A$-R, we can observe similar structure-activity relationship in NMDA-R. It was found that 3β sulfate group in steroid A-ring is not essential for potentiation of the NMDA-R response, but a negatively charged group at C_3 is required for potentiating activity. In addition, ketone group at C_7 or C_{11} resulted in complete loss of NMDA-R-potentiating activity (Park-Chung *et al.* 1997). In addition, experiments with reduced PregnS metabolites revealed inhibitory effects on NMDA-R response (Park-Chung *et al.* 1994, Yaghoubi *et al.* 1998). Enzymatic activities that add or remove negatively charged group at C_3 are supposed to play important role in NMDA-R modulation.

Glutamate receptors may have also other outputs: soluble tyrosine kinases activation and/or mitogen-activated protein kinase pathways activation. Furthermore, NMDA-R in plasma membranes could be coupled to NO synthase (NOS) through the postsynaptic density protein 95 scaffold (Tochio *et al.* 2000). Treatment with DHEA causes NMDA stimulation which leads to a decrease in Ca^{2+}-sensitive NOS activity. Therefore, NO production is inhibited and as a consequence DHEA exerts a neuroprotective effect on cultured rat hippocampal neurons (Kurata *et al.* 2004).

However, unlike $GABA_A$-R interaction, the detailed studies of the interaction of neuroactive steroids with NMDA-R are not well documented and no specific interaction sites were described.

1.3.1.2.3. Other ligand-gated channels modulation

Subsequently, DHEA/S were found also to regulate other neurotransmitter receptors such as AMPA receptors, kainate receptors, nicotinic, muscarinic and σ-receptors (Shirakawa *et al.* 2005, Valera *et al.* 1992, Wu *et al.* 1997, Klangkalya *et al.* 1988, Monnet *et al.* 1995).

1.3.1.2.4. G-protein coupled receptors

It was proposed that neurosteroids could act on nerve cells via hypothesized surface receptors coupled with G-proteins or through specific membrane sites using calcium as an intracellular messenger (Orchinik *et al.* 1992, Ramirez *et al.* 1996). Recently, it was showed that σ 1 receptor-like protein activity was modulated by PregnS. This protein is coupled with $G_{i/o}$ protein, and as a result this interaction leads to enhancement in short-term presynaptic facilitation onto adult hippocampal CA1 neurons (Schiess *et al.* 2005).

More recently, evidence for high affinity surface receptor for DHEA was found in endothelial cells (Liu *et al.* 2002). This receptor is G_i-protein-coupled and activates endothelial NOS (Liu *et al.* 2004). However, the exact mechanism of endothelial NOS activation remains unknown.

1.4. Interconversion of DHEA and DHEAS

As was emphasized on previous pages, unconjugated and sulfated steroid molecules act in many cases in opposite ways on the same receptor or have distinct actions in different targets. Therefore, the role of steroid sulfates and sulfotransferase is crucial in regulation of the equilibrium between sulfated and free neuroactive steroids in brain.

1.4.1. Steroid sulfatase (STS)

STS is an almost ubiquitous enzyme of steroid metabolism. It is an important factor influencing many physiological and pathophysiological processes regulated by steroid hormones. A number of them have been revealed recently (Stanway *et al.* 2007, Hazan *et al.* 2005, Weidler *et al.* 2005, Nakamura *et al.* 2006).

1.4.1.1. STS gene and protein

STS belongs to a superfamily of sixteen different mammalian sulfatases (Obaya 2006). The gene encoding STS is pseudoautosomal in mice, but not in humans, and escapes X

inactivation in both species. The functional nucleotide sequence maps to Xp22.3-Xpter (Yen *et al.* 1987, Meroni *et al.* 1996).

The DNA sequence coding for STS on the X-chromosome was cloned, sequenced and well characterized. The 2.4 kbp cDNA encodes a protein of 583 amino acid residues with a short signal peptide of 21-23 amino acids and four potential N-glycosylation motifs N-X-S/T. Probably two of these sites (N_{47} and N_{259}) are used for N-linked oligosaccharide connection.

STS is synthesized as a membrane-bound 63.5 kDa polypeptide. Newly synthesized polypeptide is processed to a mature 61 kDa form. The decrease in size is due to the processing of the oligosaccharide chains, which are cleavable by endoglucosaminidase as well as mannosidase(s) (Conary *et al.* 1986). Recently, STS was purified and crystallized from the membrane bound part of the human placenta (Hernandez-Guzman *et al.* 2001) and shortly

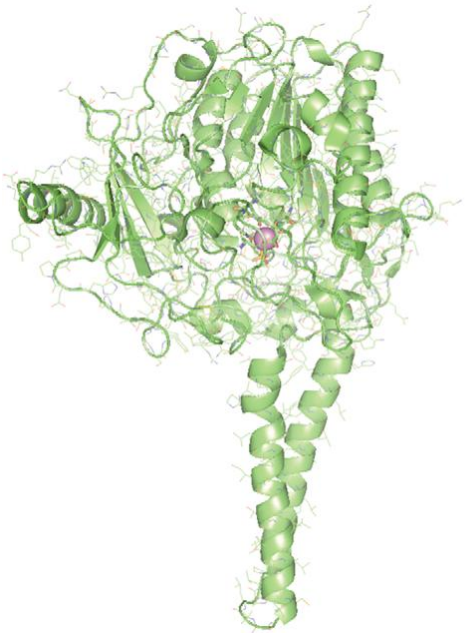

Figure 2. The crystal structure of STS showing the course of polypeptide backbone through α-helices, β-sheets and loop regions. Ca^{2+} cation at the active site is shown as a sphere. Adapted from Ghosh D. *Methods Enzymol.* (2005);400:273-93.

after, three-dimensional structures of three human sulfatases were released (Ghosh 2005). The representative structure of STS is shown in Figure 2.

Concerning membrane topology, the enzyme has two membrane-spanning antiparallel hydrophobic α-helices with proline 212 serving as a turn point at the cytosolic side of the endoplasmic reticulum membrane. The polar catalytic domain of the enzyme is situated to the lumen side of the lipid bilayer (Hernandez-Guzman *et al.* 2003). Nevertheless, participation of the lipid bilayer in maintenance of the active site integrity, passage of the substrate and/or product release was still not exactly determined.

1.4.1.2. STS enzyme activity

Sulfatases are a unique group of enzymes that carry at their catalytic site a post-translational modification, an alpha-formylglycine residue that is essential for enzyme activity. Formylglycine is generated by oxidation of a highly conserved cysteine or, in some prokaryotic sulfatases, serine residue (Dierks *et al.* 1998, Miech *et al.* 1998).

In eukaryotes, this oxidation occurs in the endoplasmic reticulum during or shortly after import of the nascent sulfatase polypeptide. The mechanisms that are responsible for the oxidation are probably associated with the protein translocation apparatus. *In vitro* studies for arylsulfatase A revealed short linear motif (CTPSR), starting with the cysteine residue, which acts as a target site for the post-translational modification. Proline and arginine play crucial roles for appropriate modification as well as seven additional residues (AALLTGR) directly following the CTPSR sequence (Dierks *et al.* 1999, Knaust *et al.* 1998).

The proposed catalytic mechanism also includes the presence of bivalent Ca^{2+} rather than Mg^{2+} cation in the active site of the enzyme. Therefore, the reaction mechanism, which STS uses for steroid sulfate hydrolysis, closely resembles the mechanism that was proposed for arylsulfatases (Boltes *et al.* 2001). Recent results show that also N-terminal and C-terminal regions have important contribution to the STS enzyme activity (Sugawara *et al.* 2006).

1.4.1.3. Subcellular localization of STS

In the above mentioned studies STS was found mainly in the rough endoplasmic reticulum. In cultured human fibroblasts, immunohistochemistry proved that the enzyme exists in Golgi cisternae and in the trans-Golgi, where it is co-distributed with lysosomal enzymes and the mannose 6-phosphate receptor. STS was also found at the plasma membrane (Kawano *et al.* 1989) and in the coated pits, endosomes and multivesicular endosomes. These structures may be the sites where sulfated estrogen and/or androgen precursors are hydrolyzed.

It also co-localizes with lysosomal enzymes and the mannose 6-phosphate receptor here. STS activity was also found in lysosomes (Stein *et al.* 1989). Sulfatase activity in lysosomes and the appropriate protein was isolated and characterized as N-acetylgalactosamine-4-sulfatase (Bond *et al.* 1997). The stability of sulfatases in subcellular compartments with low pH is possibly due to their membrane topology. It could be related to the high homology of two the luminal domains of STS with the lysosomal sulfatases

arylsulfatase A and B. However, the transport between trans-Golgi and lysosomes possibly uses a mannose receptor-independent mechanism, that was proposed as a result of the absence of mannose-6-phosphate in STS carbohydrate moiety (Stein *et al.* 1989).

1.4.1.4. STS occurrence in tissues

Enzymatic activity of STS was demonstrated for the first time in rat liver microsomes (Dodgson *et al.* 1954). Later, this enzyme was found in testes (Payne *et al.* 1969), ovaries (Clemens *et al.* 2000), adrenal glands, placenta, prostate, skin, kidney, fetal lung (Hobkirk *et al.* 1982), viscera, endomerium, aorta, bone, peripheral blood leukocytes (Han *et al.* 1987) and brain tissue (Iwamori *et al.* 1976). Now STS is believed to be an almost ubiquitous enzyme.

Tissue and organ distribution varies considerably between different mammalian species. Placenta was found to be the richest source of STS. However, the findings differ in dependence on methodological approaches.

Biochemically, sulfatase activity was detected in microsome fraction and/or tissue homogenates. These approaches were used for identification and characterization of STS activity in rat testis (Payne *et al.* 1969), ovarian granulosa cells (Clemens *et al.* 2000), leukocytes (Han *et al.* 1987) and brain tissue (Iwamori *et al.* 1976). More recently, a biochemical approach was used for determination and characterization of STS activity in *Macaca* brain regions (Kriz *et al.* 2005)

1.4.2. Steroid sulfotransferase (SULT)

Conjugation of drugs, xenobiotics and endogenous compounds with a sulfonate moiety belongs to an important reaction in their biotransformation. Generally, sulfonation leads to decrease in biological activity of these compounds but in certain cases it can result in new, previously unexpected properties. Concerning neuroactive steroids, sulfated esters are hydrophilic compounds that hardly cross the blood-brain-barrier (BBB) and therefore they are considered to be synthesized locally in the brain.

1.4.2.1. Steroid sulfotransferases: genes and protein

In humans, ten SULT genes have been characterized. These can be divided into four different families and contain thirteen distinct members (Blanchard *et al.* 2004, Hildebrandt *et al.* 2004). The nucleotide sequences encoding the functional protein map to chromosomes 2q (SULT1C2, SULT1C4), 4q (SULT1B1 and SULT1E1) and a short arm of chromosome 16,

where are thought to arise from gene duplication or gene duplication following recombination events (SULT1A1-4), which represent SULT1 family (Gamage *et al.* 2006). SULT2 family is encoded in chromosome locus 19q13.3 and contains nucleotide sequences for SULT2A1 and SULT2B1 (Her *et al.* 1997, Otterness *et al.* 1992). The third human sulfotransferase family accounts for SULT4A1 mapping to chromosome region 22q13.1-13.2 (Falany *et al.* 2000). Finally, SULT6B1 was identified. However, neither protein nor its enzymatic activity was characterized (Freimuth *et al.* 2004). Although the family members share considerable sequence and structural similarity, they appear to have different biological functions.

The regulation of SULT expression includes endogenous ligands and also xenobiotics that are effective transcription inducers and exert their effect via nuclear receptors. B-naphtoflavone, 3-methylchloranthrene and others were shown to reduce phenol (SULT1A) and hydroxysteroid (SULT2A) sulfotransferase activities and mRNA (Runge-Morris 1998). On the other hand, polycyclic aromatic hydrocarbons had no effect on SULT1A, 2A and 1E expression (Lampen *et al.* 2004). However, physiological consequences of transcriptional regulations by these compounds are not clear, as is not apparent if sulfonation contributes to detoxification or bioactivation process.

Concerning endogenous ligands, evidence suggests that several SULT genes are under hormonal control. Progesterone was found to control the cyclical expression of human SULT1E1 (Falany *et al.* 2006) and estrogens as well as tamoxifen (commonly used antiestrogen) activate hydroxysteroid sulfotransferase (SULT2) expression (Hellriegel *et al.* 1996). It was demonstrated that both pregnane X receptor and glucocorticoid receptor act directly on human SULT2A1 promoter and induce mRNA transcription (Duanmu *et al.* 2002). Furthermore, retinoic acid positively affects expression of human SULT1A1, 1E1 and 2A1 genes (Kruger *et al.* 2005).

Recently, human SULT2A1 was found to be regulated by bile acids via Farnesoid X receptor (Francis *et al.* 2003) and peroxisome proliferator-activated receptor (Fang *et al.* 2005).

Overall SULT enzyme structure was resolved. SULTs are generally globular proteins with a single α/β domain that forms five-stranded parallel β-structure surrounded on either side with α-helices. The catalytic residues are conserved across membrane-bound and cytosolic SULTs (Negishi *et al.* 2001). Most of the cytosolic SULTs exist mainly as homodimers but heterodimers are also possible (Petrotchenko *et al.* 2001). Physiological significance of dimerization was, however, not identified.

1.4.2.2. SULT enzyme activity

Enzymes carrying out the transfer of a sulfonate group must interact with two substrates: sulfonate donor and acceptor molecules. Sequence analysis showed that the sequence motif GxxGxxK, present in almost all SULTs, might constitute 3-phosphoadenosine 5-phosphosulfate (PAPS) binding site (Hashimoto *et al.* 1992). The motif is positioned within a highly conserved region located near the C-terminus. In mammals, PAPS represents the universal sulfonate donor for all SULT reactions (Farooqui 1980). Thus the rate of sulfoconjugation is determined not only by the activity of individual SULT but also by the local availability of PAPS.

The biosynthesis of PAPS involves two-step reaction and is widespread regardless the species or tissue of origin (Schiff *et al.* 1987). The enzymes that catalyze PAPS formation as well as steroid SULTs are present in the cytosolic fraction of tissue preparation.

In contrast to the PAPS binding site, which is characterized by conserved residues among all SULTs, the substrate binding site of SULTs displays a great deal of variability. It is presumably due to broad, in many cases overlapping, substrate specifity, though a given enzyme can be characterized by preferring a specific substrate where it often demonstrates strict regiospecifity. In the SULT2A subfamily, crystal structures with PAP and DHEA were resolved (Figure 3, p. 23) and two substrate-binding orientations for DHEA were identified (Rehse *et al.* 2002). Several hydrophobic amino acid residues were proposed to form the DHEA binding site in which crucial catalytic role of His-99 was established. Sulfonate transfer occurs without formation of intermediates and employs random or ordered bi-bi mechanism (Whittemore *et al.* 1986, Varin *et al.* 1992).

At high concentrations of preferred substrates, substrate inhibition was observed as a characteristic feature of SULTs (Raftogianis *et al.* 1999).

1.4.2.3. SULT subcellular and tissue localization

As was previously indicated, two broad classes of SULTs were identified according to subcellular localization of these enzymes:

a) membrane-bound SULTs that are located in the Golgi apparatus and are responsible for sulfonation of peptides, proteins and aminoglycans (Negishi *et al.* 2001)
b) cytosolic SULTs that are responsible for the biotransformation of xenobiotics, and sulfonation of small endogenous ligands such as steroids, bile acids and neurotransmitters (Nowell *et al.* 2006).

The tissue distribution and expression in humans can not be easily generalized. There are remarkable differences in tissue expression depending on species, age, sex, and developmental and physical status (Strott 1996). Concerning SULT2A subfamily, mRNA was

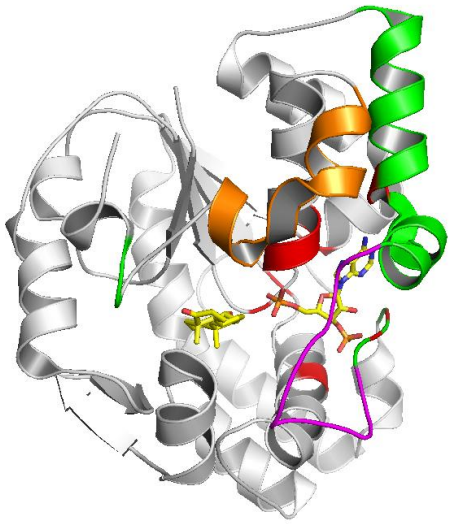

Figure 3. Representative structure of human SULT2A1 showing the course of polypeptide backbone through α-helices, β-sheets and loop regions. DHEA and PAP as cosubstrates are shown as stick models colored per element. Adapted from Protein Data Bank.

found in high amounts in human steroidogenic organs, androgen-depending tissue, kidney, liver and gastrointestinal tract (Kennerson *et al.* 1983, Sasano *et al.* 1995, Tashiro *et al.* 2000). SULT2A1 was also determined in brain and bone marrow but is absent in skin, where the mRNA for SULT2B is expressed (Javitt *et al.* 2001).

Recently, an exclusive brain localization of SULT4A1 was detected in mice, rats and humans (Sakakibara *et al.* 2002, Liyou *et al.* 2003). However, now it is suggested its wider tissue distribution.

1.5. Homocysteine

Homocysteine (Hcy) as well as cysteine (Cys) are sulfur-containing amino acids involved in the metabolism of the essential amino acid L-methionine. Hcy is particularly considered to be a risk factor for cerebrovascular and cardiovascular diseases. In some neurological disorders Hcy accumulates in the brain and acts as a potent neurotoxin. Furthermore, Hcy can induce an oxidative injury to nerve cell terminals, which involves NMDA-R stimulation and NOS activation associated with free radicals formation.

1.5.1. Homocysteine metabolism

Hcy is metabolised via two major pathways. In the excessive methionine uptake, Hcy undergoes trans-sulfuration pathway that results in the production of cystathionine and subsequently Cys. This process requires pyridoxine as a cofactor for cystathionase and cystathionine-β-synthase. Excessive Cys is oxidized to taurine or sulfate and eliminated.

However, in low levels of methionine, Hcy is mainly metabolised via a methionine-conserving pathway. In most tissues, this involves remethylation of Hcy to form methionine in a process requiring methyltetrahydrofolate and vitamin B_{12} as cofactors (Langman *et al.* 1999).

These two pathways are mediated by S-adenosylmethionine (SAM), which acts as the main source of activated methyl groups for all methylation reactions within the cell. S-adenosylhomocysteine (SAH), the by-product of methylation reactions, is hydrolysed regenerating Hcy, which is then available for other methyl group transfer.

High levels of Hcy are associated with reduced methylation potential, whereas folate and vitamin B_{12} increase this potential. Changes in the concentration of methionine in the body, particularly as a result of dietary intake of methionine, affect the rate of SAM synthesis, as well as the metabolism of Hcy.

1.5.2. Hcy action in brain tissue

High levels of Hcy, which is released from the extrahepatic tissues that lack complete trans-sulfuration pathway, cause hyperhomocysteinemia with possible subsequent accumulation of Hcy in the brain tissue (Miller 2003). The nervous system is particularly sensitive to extracellular Hcy, because it contributes to excitotoxicity via NMDA-R stimulation, which can lead to neuronal DNA damage and further apoptotic events (Ho *et al.* 2002, Kruman *et al.* 2000).

1.5.2.1. Homocysteine and NMDA-R

Early studies on animals revealed that in brain, in addition to simple diffusion, Hcy can be also transported via specific saturable receptor (Grieve *et al.* 1992, Zeise *et al.* 1988). Current knowledge indicates that Hcy can be transported from the plasma into the brain and vice versa via specific cellular transporters (Grieve *et al.* 1992). Furthermore, under certain conditions such as folate deprivation, Hcy can be produced in enhanced amounts within the brain itself (Ho *et al.* 2003).

As previously emphasized, Hcy acts as endogenous glutamate receptor agonist, which is prone to function especially on NMDA-R subtype. Hcy oxidative product (homocysteic acid) also functions as an excitatory neurotransmitter by activating NMDA-R. As well as neuroactive steroids, Hcy indirectly enhances Ca^{2+} influx by binding NMDA-R. The neurotoxicity can be blocked by selective NMDA antagonists (Kim *et al.* 1987).

Interestingly, the neurotoxicity of Hcy was observed only in high Hcy concentrations. In presence of normal (10 μM) glycine concentrations, Hcy acts as a weak antagonist of the glycine-binding site and inhibits receptor-mediated Ca^{2+} influx (Lipton *et al.* 1997). As a result, Hcy may act either as the glutamate site antagonist or glycine site blocker depending on glycine concentration. Recently, it was suggested that Hcy can activate also group I metabotrophic glutamate receptors (Zieminska *et al.* 2003). By activating these groups of glutamate receptors, Hcy indirectly increases intracellular Ca^{2+} levels, thereby activating several Ca^{2+} dependent protein kinases (Robert *et al.* 2005). Taking together, a clear link exists between Hcy as a risk factor and steroid actions on brain receptors.

1.5.2.2. Oxidative stress-role of Hcy

Development of oxidative stress is associated with impairments in antioxidants levels and free radicals formation. Defects in trans-sulfuration pathway influence redox homeostasis and contribute to neuronal damage. As such, Hcy undergoes autooxidation process and therefore disrupts redox homeostasis. This can lead to impairments in signalling pathways of vascular and neural cells (Perna *et al.* 2003). The effects of Hcy are explained not only by modulation of glycine brain receptors but also by enhanced production of reactive oxygen species (ROS) and oxidative deactivation of NO.

Additionally, Hcy in submilimolar concentrations caused brain lipid peroxidation as a result of NMDA-R stimulation, NOS activation and associated ROS formation (Jara-Prado *et al.* 2003). It is also of interest to mention the role of Hcy and subsequently oxidative stress in neurodegenerative disorders such as Alzheimer disease (Tchantchou 2006). With respect to antioxidative properties of DHEA (Araghiniknam *et al.* 1996, Aragno *et al.* 2000) and its involvement in NO action, it points to further connection between DHEA and Hcy.

1.5.2.3. Hypomethylation and Hcy

Hypomethylation of DNA that consequently results in altered gene expression also belongs to important mechanisms that lead to neuronal damage caused by elevated Hcy levels. Low DNA methylation was observed in gene coding for presenilin 1. This protein is a

member of γ-secretase group of proteases involved in β-amyloid peptide formation from β-amyloid precursor protein (Fuso *et al.* 2005). Acceleration in β-amyloid precursor protein processing and β-amyloid peptide formation occurs via the up regulation of the presenilin 1 gene. The gene can be silenced with SAM administration. This silencing was considered as a possible mechanism of Alzheimer's disease treatment (Scarpa *et al.* 2003).

1.6. Hcy and neuroactive steroids in brain tissue

Neuroactive steroid formation and metabolism of steroid intermediates belong to critical points in normal brain functions. Any alterations in brain steroidogenesis may lead to impairments in concentrations of neuroactive steroids resulting in imbalance between excitatory and inhibitory synaptic transmission. Modulation of either activation or inhibition mediated primarily by $GABA_A$-R could be expected to result in altered synaptic pathways leading to altered nervous system function.

Excessive activation of excitatory glutamate receptors can result in neuronal damage via excitotoxic mechanisms where also alterations in sulphur-containing amino acid metabolism play significant role. Since the neuroactive steroids exert rapid direct effects on the function of excitatory as well as inhibitory neurotransmitter receptors, it is reasonable that modulation of balance between excitatory and inhibitory neurotransmission could be provided also by modulation of neuroactive steroid-producing enzymes.

As previously emphasized, 3β-sulfated and nonconjugated steroids exert distinct or in several cases opposite (Pregn and PregnS) effects especially on synaptic transmission. Therefore, the enzymes, which are able to interconvert free neuroactive steroids and their sulfate conjugates, may play a crucial regulatory role in synaptic transmission by modulating the balance between excitatory and inhibitory neurotransmission. Consequently, STS and SULT activities not only in healthy brain tissue but also in its pathologically changed states should be assessed as a possible target of novel therapeutic neuromodulators.

Concerning the crucial roles of STS and SULT in modulating the neurotransmission in brain tissue we determined both enzymes activities in monkey brain tissue and selected human brain tumors. In addition to the enzyme activity, we determined Hcy concentration in brain tissue to find out further connections between steroid sulfonation and alterations in various methylation reactions.

2. Aims of the study

From the above mentioned brief outline it is obvious that to date no comprehensive data on STS and SULT activities in mammalian brain and its regions were published. It is a consequence of both lack of appropriate methodical approach and ethical reasons connected with the use of intact primate brain tissue. In order to contribute to the present knowledge about connection of cancerous processes with steroid metabolism, we focused on equilibrium unconjugated steroid-steroid sulfate. Particularly, the following points were risen:

1. Development of efficient methodology for the determination of free and sulfate-conjugated steroid metabolites. The methodology was developed for the purpose of determination of specific activities (if any) of STS and SULT in intact primate brain tissue.
2. Finding the optimal conditions for the determination of STS and SULT enzyme activities and characteristics.
3. Application of the methods to assessment of STS and SULT enzyme activities in primate brain tissue and selected human brain tumors in order to find possible differences in enzyme activities and to find if obtained data on enzyme activities correlate with tumor aggressivity and/or malignity.
4. Development of methodology for Hcy determination in cancer tissue in order to evaluate the levels of Hcy in human brain tumors.
5. Search for possible correlation between STS and SULT activities and Hcy levels with severity of brain tumors.

3. Methods

For the determination of enzyme activities of STS and SULT in brain tissue at least partial enzyme purification appeared to be necessary. For the STS assay we obtained microsomal preparation by differential centrifugation of homogenized tissues. Here several pitfalls rose as a consequence of use of the intact tissue.

To avoid partial or full proteolysis of studied enzymes we used protease inhibitors that affected a broad spectrum of proteases likely present in obtained microsomal and mainly cytosolic fractions. Peptide derivatives Leupeptin and Pepstatin A appeared to be the best solution. However, addition of chelating agents such as ethylenediaminetetraacetic acid (commonly used as a metalloprotease inhibitor) led to a significant decrease especially in STS activity. It was a result of removal of Ca^{2+} from the STS substrate binding site. Further dialysis against the reaction buffer containing 10 mM or 100 mM $CaCl_2$ did not lead to a reconstitution of the enzyme activity. The use of other protease inhibitors such as PMSF led to almost complete loss of STS activity as well as use of phosphate-containing buffers.

Additional attempts to purify STS and SULT failed because any further treatment of microsomal or cytosolic fractions led to a decrease in specific enzyme activities. Here solubilisation of membrane-bound STS appeared to be the crucial step in maintaining enzyme activity. Treatment of microsomal fraction with Triton-X 100, Tween 20 and Tween 80 resulted in complete loss of STS activity. Since the intact brain tissue especially from human brain tumors was not commonly available and also the amounts were limited we focused on determination of the enzyme activities in subcellular fractions.

Prior to particular determination of enzyme activities we determined optimal reaction conditions. We focused on pH of reaction buffer, temperature, incubation time, subcellular preparation quantity and substrate concentrations. For these assessments we employed appropriately diluted ^{3}H radioactive substrates to simplify the determinations.

When separating the reaction product from the complex matrix we used liquid-liquid extraction rather than solid phase micro extraction (SPME). It was a result of both economical and practical reasons. Optimizing SPME seemed to be more complex than finding optimal solvents for extraction of nonpolar steroids such as DHEA. Two aspects were taken into account: the highest extraction of nonpolar DHEA from the reaction mixture and the lowest co-extraction of polar DHEAS. The best results were obtained with TMP/ethylacetate (1:1, v/v) and diethylether. The former was therefore chosen for experiments. However,

diethylether was also highly efficient in extraction of nonpolar compounds, while the co-extraction of sulfates was slightly higher but still satisfactory (4.61 ± 0.22% for STS assay and 3.98 ± 0.31% for SULT assay) in comparison with the former extraction mixture.

Determination of STS activity, and therefore DHEA unequivocal quantification, was carried out using GC-MS system. As such, direct DHEA quantification was impossible due to rather low molecular weight of the compound, so the treatment with derivatization agents was necessary. We used both C_3 hydroxy- and C_{17} oxo- groups for conjugation, while further data revealed that only silylation on C_3 was necessary. However, prior to derivatization step it is advisable to use liquid-liquid extraction to avoid sample contamination with membrane lipids, fatty acids and cholesterol including its derivatives co-extracted with DHEA from the reaction mixture. These compounds in several cases caused serious problems in detection of appropriate fragments. For correction to losses we employed steroids that are regularly not formed in unchanged tissues. For this purpose it is also possible to use deuterated steroids resemble DHEA such as deuterated forms of androstenedione or androstenediol with retention times similar to DHEA.

In determination of SULT activity we focused on direct determination of DHEA rather than the reaction product DHEAS. Direct detection of DHEAS by radioimmunoassay failed as a consequence of low amounts of DHEAS formed and a low sensitivity of the immunoassay. Possibility for substitution of immunoassay for LC-MS analysis was also considered. However, regarding the financial aspect of such analyses and a lack of appropriate methodology we used a biochemical approach that included removal of unconverted substrate by liquid-liquid extraction followed by specific enzymatic cleavage of the reaction product (DHEAS) remaining in the aqueous phase. The particular determination of DHEA released in the sulfatase/glucuronidase-catalyzed reaction was carried out with high sensitive radioimmunoassay. In this case we avoided GC-MS quantification to minimize the losses of DHEA during the sample processing. Furthermore, the sensitivity and specificity of the immunoassay was satisfactory for monitoring the changes in DHEA concentrations.

For the determination of Hcy and other aminothiols we also used gas chromatography with flame ionization detection rendering similar quantitation limits like commonly used MS detection. However, in this case, SPME with subsequent derivatization provided reliable data. We used recently published method with fast and simple sample preparation, which employed substituted chloroformate in derivatization step. Unfortunately, this method could not have been used for derivatization of steroids prior GC-MS analysis. It was caused by poor fragmentation of obtained derivatives. To make the procedure suitable also for GC-MS

analysis of steroids, different compounds based on chloroformate and fluoroformate derivatives are currently tested with promising results.

Since we focused on biochemical approach with quantitative analysis of metabolites, it is also necessary to mention molecular techniques such as real time PCR that could be used for quantitation of enzyme expression in particular tissues. This approach, however, reflects only changes in gene expression but possible alterations in protein folding and/or changes in tertiary structure that may affect enzyme activity are not covered.

4. Publication 1

5. Publication 2

6. Publication 3

7. Discussion

Enzymatic activities of steroid sulfatase and sulfuryl transferase, responsible for maintaining actual concentrations of two neuroactive steroids acting in an opposite way on $GABA_A$ receptors, were measured in tissues from three different regions of primate brain with DHEA and its sulfate as substrates. Steroid sulfates can activate NMDA receptor expression and thus, the influx of calcium into cells resulting in arachidonate release and finally, formation of leukotrienes (Compagnone *et al.* 2000). These compounds can further take place in an immune response and can participate in reactive oxygen radical and lipid peroxide formation, which may be one of the mediators of neurodegeneration.

Careful attention was paid to the development of methodology in view of its further use for human tissues from brain tumors. Slightly modified assay conditions described for bovine brain tissues (Park *et al.* 1997) were employed; the protease inhibitors were added to the microsome and cytosolic preparations to avoid partial proteolysis caused by endogenous, non-specific proteases that are commonly present in these fractions. 2, 2, 4-Trimethylpentane-ethyl acetate (1:1, v/v) appeared to be the optimal system for extraction of the unconjugated steroids from the incubation mixtures with respect to their maximal yields and with minimal co-extraction of steroid sulfates. Almost the same results in extraction efficiency were achieved with diethylether. However, the amount of extracted unconjugated steroids was slightly higher than in the former extraction mixture (data not shown). Nevertheless, with respect to observed results on STS and SULT activities, it is reasonable to use diethylether for extraction of unconjugated steroids due to considerably simplified sample processing. Previous sample deproteination was necessary to reduce or avoid possible emulsion formation during solvent partition. As an alternative to measurement of ^3H radioactivity for determination of sulfatase specific activity, 19-tri-deuterated substrate (d_3-DHEAS) was employed, using the fragments m/z 426.4 and 429.4 for quantification. No isotopic effect was found as demonstrated by a DHEA/d_3-DHEA ratio close to 1. These results showed that d_3-DHEAS could be used as a sole substrate for determination of STS activity in biological samples. Exchange of three hydrogens for deuterium on C_{19} had no effect on STS activity towards slightly changed substrate. This is especially useful in systems where it is difficult to avoid endogenous DHEAS contamination that could affect obtained results.

Concerning estimation of sulfotransferase activity, determination of DHEAS as the reaction product directly in the incubation medium by radioimmunoassay failed, because the

concentration of DHEAS was below the detection limit of the assay. Therefore, the product was converted to the unconjugated form, which was analyzed by radioimmunoassay. The sensitivity of the DHEA RIA was much higher than that for DHEAS. Use of arylsulfatase/glucuronidase preparation from *Helix pomatia* (crude solution) for DHEAS cleavage was preferred to solvolysis, because the latter appeared to partially destroy the steroid molecule. Treatment of unextracted steroid sulfates with concentrated sulfuric acid led to irreversible changes in steroid ring that were responsible for decreased binding affinity to monoclonal antibody directed against DHEA. Consequently, we used alternative non-specific enzyme hydrolysis instead of commonly used solvolysis. The crude solution contained β-glucuronidase as well as sulfatase activity. However, contribution of DHEA-glucuronide cleavage to final DHEA concentration is negligible. This is the result of low activity of UDP-glucuronosyltransferase in the brain tissue (De Leon 2003) and absence of UDP-glucuronide in the reaction buffer. UDP-glucuronosyltransferase activity occurs in brain capillaries but passing of DHEA glucuronide, if present, across the blood-brain-barrier is very limited due to its polarity. Therefore, the amount of other DHEA conjugates than DHEAS formed *in situ* should be very low. Another disadvantage of the latter method was the necessity of more steps leading to higher losses during processing. Recently, acid solvolysis became also a matter of controversy. Since liquid-liquid extraction followed by acid solvolysis of the aqueous phase is used to separate unconjugated and conjugated steroids into organic and aqueous phases, subsequent analysis of aqueous phase does not exactly reflect the concentration of steroid sulfates (Liu *et al.* 2003). It is a contribution of both, steroid sulfates and other steroid conjugates, which are distinct from these compounds. It was suggested that lipoidal steroid derivatives, presumably in form of micelles, significantly contribute to overall concentration of putative sulfated steroids remaining in the aqueous phase (Liere *et al.* 2004). Therefore, these should not be considered as sulfates but more clearly as steroid conjugates. The use of glucuronidase/arylsulfatase reaction leads to more specific cleavage of DHEAS rather than solvolysis. The use of nonradioactive substrate (DHEA) in the sulfuryl transferase assay and measurement of enzymatically released DHEA appeared to be even more sensitive than the method based on incubation of tritiated substrate and measurement of formed ^{3}H-DHEAS.

Since only scarce data is available on methodology of STS and SULT activities determination in human brain tissues, attention was devoted to find out optimal assay conditions. Tritiated substrates were used in these experiments, while for determination of

enzymatic activities in brain regions and tumors more sensitive detection of reaction products by GC-MS (STS activity) and highly sensitive radioimmunoassay (SULT) were used.

We found that STS was not as pH sensitive as SULT. This observation was similar for both, monkeys and humans. The course of enzyme activity depending on pH significantly differs for STS and SULT. pH Optimum for STS markedly differed from optimal pH value for SULT. While STS preferred neutral or slightly basic conditions (pH around 8.0), SULT activity was strictly limited to mild acidic conditions around pH 6.0. Even small changes in pH away from the optimum value significantly decreased SULT activity, which is especially obvious in human cytosolic fraction. This is not true for STS, where pH range from 7.5 to 9.0 in monkeys and from 7.0 to 8.5 in human tumors did not largely affect enzyme activity. This difference in pH optimum for both enzymes is probably due to the different subcellular localization of both enzymes. Furthermore, studied enzymes contain distinct amino acids in their reaction centre and therefore favor completely different reaction conditions. Low activity of STS in acidic conditions could be explained by the presence of three aspartate residues in the reaction centre that in their negatively charged forms participate on binding of steroid sulfate moiety via calcium ion (Hernandez-Guzman *et al.* 2003). Since pK_a for aspartate γ-COO^- group averages around 3.9, we observed only moderate decrease of enzyme activity with decreasing pH. On the other hand, SULT contains strongly conserved histidine residue in its reaction centre that forms a hydrogen bond to the steroid O-3 through N_ϵ-2 (Rehse *et al.* 2002). This is only possible in case of protonation of histidine N_ϵ-2 on imidazole side chain with pK 6.04. Enhancing pH resulted in deprotonation of histidine N_ϵ-2 and thereby decreased DHEA binding. On the other hand, additional decrease in pH led to enzyme denaturation and loss of its activity. Mildly acidic conditions therefore increase probability of steroid binding and positioning in the SULT active site and consequently were determined as optimal.

In temperature optimization, we found maximum sulfatase activity at temperatures as high as 50°C. For SULT the optimum averaged around physiological temperature. Both enzymes showed similar temperature characteristics for monkeys and humans. Here STS with high temperature optimum indicated considerably higher thermal stability in comparison with SULT (temperature optimum 33-37°C). A likely explanation of quite low SULT temperature optimum is probably not thermal instability of the enzyme as itself. It could be a combination of both (thermal) instability of SULT cofactor PAPS powered by decreased pH and lower stability of SULT at higher temperatures (Ozawa *et al.* 1999, Leyh 1993).

Despite the addition of protease inhibitors, enzyme activity decreased with time, probably as a result of non-specific proteolytic digestion. This was observed especially in the sulfatase assay at 50°C where prolonged incubation led to a decreased DHEA/DHEAS ratio. One reason for this may be due to thermal instability of the steroid substrate at higher temperatures. However, this effect was not observed in human subcellular preparation so we can speculate that the time-dependent decrease in STS activity in monkey resulted from proteolytic cleavage rather than enzyme denaturation or substrate decomposition. Surprisingly, prolonged incubation time in the SULT assay did not lead to significant increase of proteolytic activity in monkeys and humans. In SULT assay the highest yields were obtained after a 60 min incubation period and then decreased, probably due to chemical instability of PAPS (Leyh 1993) rather than the enzyme degradation. It is surprising that this effect was not observed in cytosolic preparation from healthy monkey brains.

Concerning the optimal concentration of substrate, we received surprising results especially on STS assay. The product formation continuously enhanced with enhancing substrate concentration up to DHEAS concentration of 50 μM. However, further increase in substrate concentration led to high-substrate inhibition of STS activity. This was apparent in STS from monkey brains but especially from human brain tumors. Here DHEAS concentration of 250 μM led to almost complete inhibition of the enzyme activity.

These results suggest that two sites are located on the STS enzyme, one productive site (with high affinity for steroid substrate) and one inhibitory site (with low affinity for DHEAS). The substrate that inhibits STS activity should bind the site distinct from that used for substrate hydrolysis although these sites might be situated closely. In fact, substrate inhibition appeared only at the DHEAS concentrations that were appropriate for binding the low-affinity inhibitory site. As we used purified substrate, high-substrate inhibition seemed to be the most likely explanation of the observed effect rather than possible sample contamination. However, physiological relevance (if any) of this phenomenon can not be directly drawn from these results. Recently, structure-function relationship emerged for estrone sulfatase (Hernandez-Guzman *et al.* 2003). However, to date no inhibitory binding site was revealed.

Finally, we tested the possible range for protein concentration in both, monkey and human assays. As demonstrated, STS enzyme activity increased linearly from 33 μg to 660 μg in monkey microsomal fractions and from 50 μg to 370 μg of total protein. Narrow range in human brain tumors might be caused by inevitably changed expression of proteases in brain tumors that, despite added inhibitors, could cause partial enzyme cleavage. Since altered

expression of proteases is often associated with tumor grade (Newton 2004), the use of mixed subcellular fractions from different tumor types seemed helpful in optimization studies. The effect of partial STS cleavage was however observed neither in monkey brain cytosolic fractions nor in cytosolic fractions from human brain tumors. Here total protein content averaged from 42 μg to 630 μg and from 180 to 720 μg for SULT assays carried out with monkey and human cytosolic fractions, respectively.

Using this methodology, the specific activity of steroid sulfatase was determined in three primate brain regions – the cerebral cortex, subcortex (basal ganglia and striatum), and cerebellum. In female monkey brain tissue, the specific activity declined in the sequence cerebellum, cortex, and subcortex, while in male cerebral regions, only small differences between each region were found. Surprisingly, there was about a two to three-fold higher sulfatase activity found in female brain regions than in male ones. These observations do not correspond with results previously reported (Lakshmi *et al.* 1981). In this report a little higher STS activity was found in male cerebral cortex than in the same female region. These results (including two animals of each sex) also showed high inter-animal variability that could cause the discrepancy. Furthermore, the enzyme assay was carried out under slightly different conditions without finding any optimal conditions.

We are aware of low number of animals used for the experiments because of both, economical and ethical reasons. As a result we were not able to avoid possible inter-animal variability.

Furthermore, the steroid sulfatase kinetic characteristics, K_M and V_{max} were determined. Apparent average K_M value for the monkey brain steroid sulfatase with DHEAS as a substrate found in our experiment was about three times lower than that measured in bovine brain tissue (K_M 113 ± 21 μM) (Park *et al.* 1997), but it was higher than the value reported by Noel *et al*. for human placental enzyme (K_M = 14 μM) (Noel *et al.* 1983). These differences may be explained by the fact that in both experiments we used 50°C incubation temperature instead of commonly used 37°C, as a result of optimization tests.

Concerning enzyme characteristics of sulfatase and sulfotransferase, we were able to determine only the K_M of the former enzyme, since the activity of sulfotransferase was too low. The physiological relevance of two enzymes with different activities responsible for formation and hydrolysis of DHEAS may reflect the fact that a certain portion of DHEAS (its concentration in human blood is more than 200 times higher compared to DHEA) may cross the blood-brain barrier, in spite of its hydrophilic nature (Asaba *et al.* 2000). The results of

cooperation of both enzymes (with much higher sulfatase activity) are the physiological concentrations of both steroids. As published previously, in brains of control humans unconjugated DHEA prevails (Weill-Engerer *et al.* 2002).

Similar to sulfatase activity, the specific activity of steroid sulfotransferase was determined in the above-mentioned cerebral regions. In the female brain, the highest sulfonation activity was observed in cortex, followed by subcortex and cerebellum, in contrast to male brain tissue, where the highest specific activity was found especially in cerebellum, and much lower activities were found in cerebral cortex and subcortex. Due to too low sulfotransferase activity, we were not able to determine SULT kinetic characteristics in brain cytosolic fraction.

After developing a novel method for determination of STS and SULT activities in brain tissue, we focused on assessment of these activities in pathologically changed human brain. In addition, we measured total homocysteine (t-Hcy) levels to reveal possible associations between STS and/or SULT activities and t-Hcy levels in human brain tumors.

Selected aminothiols were therefore chosen for explication of possible impairments in pathologically changed brain tissue. The attention was especially focused on Hcy as the main neuronal destroyer. We also studied the relationship between concentrations of methionine, cysteine and homocysteine.

For quantification of these compounds we used gas chromatography analysis with flame ionization detection. The levels of t-Cys and t-Hcy were examined using prior reduction of disulfide bonds. Therefore, we did not distinguish between reduced and oxidized forms, which are present in tissues under physiological conditions and may interconvert also during the tissue processing. Therefore we did not point to the contribution of the compounds with free –SH group and their oxidized forms.

Hcy plays one of the key roles in methionine metabolism. Possible misbalance in t-Hcy concentrations may reflect alterations in both pathways in tumor metabolism. Moreover, high Hcy levels may up-regulate tissue factors leading to venous thrombosis and/or pulmonary embolism, mainly in brain tumors, and therefore cause further serious complications for patients, as recently demonstrated (Sciacca *et al.* 2004).

The latter authors found higher plasma Hcy levels in patients with high-grade gliomas then in healthy subjects. This finding is consistent with our results in brain tissues. Our results showed significantly higher t-Hcy concentration in glioblastomas compared with low-grade gliomas ($p = 0.0046$) and other tumors except for pituitary adenomas. Fast GBM growing and higher methionine uptake could explain enhanced t-Hcy levels in this type of tumors. As tumor cells divide, DNA-methylases require increased supplementation with S-adenosyl methionine, where Met serves as proximate precursor. This was apparent also from enhanced Met levels found in GBMs when compared with low grade gliomas. Recently, it was proposed that Met deprivation could lead to selective elimination of cancer cells that are unable to re-use Hcy as a source of Met (Kokkinakis *et al.* 2001, Kokkinakis *et al.* 2002).

Frequently, glioblastomas are seriously complicated with venous thromboembolism. This risk may rise also as a consequence of high Met turnover leading to Hcy formation, which is not efficiently degraded especially in tumors. Modulation of Hcy transforming pathways was therefore proposed to be one of the possible strategies that could be used to decrease venous complications in glioblastoma treatment. Unfortunately, current results showed that lowering of the Hcy levels had no significant impact on the risk of recurrence of venous or arterial diseases (Ray *et al.* 2007, Cattaneo 2006). Nevertheless, hyperhomocysteinemia still remains serious risk factor for venous thrombosis not only in patients with brain tumors but also in other states connected with advanced cancer (Gatt *et al.* 2007).

Interestingly in non-secreting pituitary adenoma as typically benign neoplasm of epithelial origin, growing comparatively slowly, tissue Met and t-Hcy levels were also high. We can conclude that each tumor type may have its specific Met turnover probably independent on the rate of cell division.

In other neoplastic tissues, we found low levels of aminothiols without significant differences among the all studied types of tumors. All results were not related to sex because the investigated tumors did not differ in males and women. It could be also caused by relatively low number of samples.

Unfortunately, we could not obtain results of the tissue concentrations of studied compounds in healthy human brains. Possibilities to substitute human tissues for other primate brains were not adequate as well as tissues obtained from cadaverous subjects.

Altered properties of ion channels caused by misbalance in steroid concentrations may influence tumor metabolism and/or its growth. In addition, high activity of STS along with

low activity of SULT in brain results in local excess of unconjugated DHEA, which may serve as a substrate for estradiol. Estradiol is known to enhance glutamate receptor-mediated excitatory neurotransmission and (at the same time) decreases GABAergic inhibition (Reddy 2003).

Although several reports appeared concerning roles of STS and/or SULT in various human tissues including brain, no analysis of both enzyme's activities in a given brain tumor type is available. This is due to the complex and variable nature of tumors; tumors with similar pathological characteristics often display different clinical outcomes.

Glioblastoma multiforme belongs to the most common primary malignant type of brain tumor in adults and, undoubtedly, one of the most lethal of all cancers. GBMs tend to grow rapidly, spreading diffusely into the surrounding brain tissue. Microvascular proliferation, extensive necrosis and high resistance to common cancer therapies due to enhanced apoptosis resistance lead to a rapid clinical progression, often to death (Zhu *et al.* 2002). This type of tumor either arise as *de novo* disease (primary GBM) or progresses from low-grade tumor to an indistinguishable high-grade phenotype (secondary GBM) (Louis *et al.* 2007). Generally, epidermal growth factor receptor amplification and phosphatase and tensin homologue (PTEN) mutation are typical for this type of tumor (Andersson *et al.* 2004, Homma *et al.* 2006).

In GBMs we have found low activities of both enzymes when compared with other investigated tumors. Our results indicate that unconjugated steroids produced in this type of tumor slightly prevail. When activated by DHEA, NMDA receptor subtype of glutamate-gated ion channels possesses high Ca^{2+} permeability (Monyer *et al.* 1992). Wang et al. have shown in cultured cells that upregulation of NMDA receptors in brain tissue led to increased NMDA-induced apoptotic events (Wang *et al.* 2000). However, no measurable NMDA-activated current was detected in human GBM cells (Markert *et al.* 2001). Therefore one may speculate that there are no DHEA-sensitive NMDA isoforms that could activate apoptotic pathways in GBMs. In addition, DHEA could activate the phosphatidyl inositol 3-kinase (PI3K)/Akt kinase pathway in cultured embryonic forebrain cells (Zhang *et al.* 2002). Downstream effects of phosphorylated (active) Akt are primarily mediated by S6 signalling and thus further enhancing cell proliferation as shown in human GBM cells (Riemenschneider *et al.* 2006). Furthermore, most GBMs express elevated levels of Akt as a result of loss of PTEN activity and action of PI3K as found in human GBM cell lines (Haas-Kogan *et al.* 1998). Lack of functional NMDA receptors combined with activation of PI3K/Akt pathway

represent a selective advantage for tumor cell survival and progression that could contribute to the explanation of such rapid and uncontrolled growth of GBMs.

Conversely, the highest STS activity was found in astrocytomas compared with other tumor types. Brain tissue is a complex of various cell types; the cells in the tissues that were used for this experiment were primarily derived from astrocytes. Considering the relatively low SULT activity found, our results suggest that steroids and especially DHEA, which is produced in large amounts in astrocytes, occur mainly in their unconjugated forms. Despite the fact that astrocytes in cooperation with oligodendrocytes are capable of producing DHEA in remarkable amounts, we can speculate that it is directly converted to androgens and/or estrogens (von Schoultz *et al.* 1990). These pathways probably do not involve sulfated intermediates. The decreased ability in conversion of DHEA to DHEAS and other especially hydroxylated metabolites also contributes to its accumulation in brain and decreases possible neuroprotective effects of steroid sulfates and hydroxylated steroids such as 7-OH-DHEA derivatives. It may be of interest that increased DHEA levels along with decreased DHEAS levels were also determined in other pathological states such as Alzheimer's disease and vascular dementia (Kim *et al.* 2003). Taken together, ASTs can significantly contribute to local androgen and/or estrogen production in the brain with various consequences that are still not exactly understood.

Interestingly, in pituitary adenomas, typically benign neoplasms of epithelial origin and comparatively slow growth, we have found the highest level of SULT activity among all investigated tumors. In addition, PIAs exhibited the lowest STS activity resulting in the highest SULT/STS ratio. Therefore, we suggest that the equilibrium of unconjugated to sulfated steroids considerably favors sulfate formation. Decreased ability of growth in these brain tumors seems to be consistent with pro-apoptotic and anti-proliferative effects of steroid sulfates, especially DHEAS. Evidence was brought that DHEAS in rat neuronal precursor cells decreased activated serine-threonine proteinkinase Akt levels and therefore increased apoptosis (Zhang *et al.* 2002).

Moreover in human PIAs, unlike in other tissues, vascularization is lower compared to the normal gland (de la Torre *et al.* 2004). If we consider the role of DHEAS on vascular endothelial cell proliferation in humans (Hinson *et al.* 2004), we suggest that enhanced SULT levels may contribute to decreased vascularization in PIAs via DHEAS action. Furthermore, in PIAs we found higher levels of all investigated aminothiols, including Hcy, in comparison with other types of brain tumors. Elevated Hcy levels in PIAs may also contribute to increased differentiation of endothelial cells rather than their enhanced proliferation

(Rodriguez-Nieto *et al.* 2002). Our results indicate that enhanced SULT activity, and consequently DHEAS action, together with Hcy action may influence PIA growth and progress. However, we are aware that the process of angiogenesis is the result of equilibrium between many other stimulating and inhibiting factors.

Meningiomas (WHO grade I) are slow-growing benign brain tumors. Typically about 70% of MENs possess progesterone receptors while less than 30% contain estrogen receptors. In women these tumors are frequently associated with breast cancer (Wahab *et al.* 2003). In our experiments, both enzymatic activities were relatively high when compared with the other tumor types. As mentioned above, DHEA could trigger increase in phosphorylated Akt levels and consequently decrease apoptosis. This is consistent with a rare occurrence of apoptotic cells especially in high-grade MENs (Mawrin *et al.* 2005).

Taking into account STS and SULT activities in the investigated tumor samples we revealed that the data could split up into four different clusters. As apparent from Table 1 and Figure 3 (Publication 3, p. 33), all investigated GBM samples are unequally distributed in clusters 1 and 2. These clusters could be characterized with low levels of STS as well as SULT activities. In cluster 3 all samples exhibited higher STS activities but lower SULT levels. This cluster contained 72.7% of investigated PIA samples. Cluster 4, with higher SULT activity and average STS activity, included all MEN samples, all AST samples and 27.3% of PIA samples. We may speculate that the tumor types in cluster 4 are very similar in their steroid metabolizing pathways as concerns STS and SULT activities. These tumor types probably share some similarities in their steroid metabolism. From the cluster analysis highly individual metabolic characteristics of GBMs and a majority of PIAs also emerged.

None of the test results showed any relationship to gender. The investigated tumors did not exhibit significant differences on STS and SULT activities in males and females. Other types of tumors, especially metastases, were not considered because of their different sites of origin.

Our results may serve in the development of novel therapeutic agents, especially in connection with their stability and efficiency inside tumor cells. However, further studies including mRNA expression and immunohistochemistry of studied enzymes in cancer tissue are needed to confirm these results. Now STS antibody that should be useful in such experiments is available (Selcer *et al.* 2007).

8. Conclusion

A novel methodology for the determination of steroid sulfatase and sulfotransferase activities in primate brain tissue has been developed and evaluated. The final task was its use for assessment of the above enzymatic activities in human brain tumors, as new markers of severity of cancer process and prognosis of its treatment.

Determination of the former enzyme activity was based on the enzyme assay followed by separation of the reaction product by liquid-liquid extraction, derivatization and GC-MS analysis. Determination of sulfotransferase activity included measurement of converted substrate after liquid-liquid extraction of unconverted substrate and a specific hydrolytic release of the reaction product, followed by its determination by highly sensitive radioimmunoassay. This methodology has not yet been applied for determination of STS and SULT activities.

For the sulfatase assay the use of deuterated substrate was proposed to avoid possible contamination of the reaction product with endogenous DHEA from subcellular preparations.

Optimal conditions for both STS and SULT assays were found. The obtained data corresponded well to previously reported results concerning both enzymes structures. Strong structure-activity relationships in both enzyme activities have been observed. Furthermore, so far unidentified high-substrate inhibition of STS was revealed. Regarding physiological consequences a hypothesis was suggested that high-substrate inhibition of STS activity could have protective effect on tissue level to avoid further conversion of DHEA to androgens and/or estrogens known to act as multiple gene activators.

When testing the method on monkey brain regions, differences were observed between male and female monkeys in both enzyme activities. The differences were also found among various brain regions. Unfortunately, statistic approaches did not enable to evaluate the significance of the differences. In addition, STS kinetic characteristics under optimal conditions were determined.

With respect to the known role in pathogenesis of brain diseases, Hcy concentrations were measured in human brain tumor tissue, in addition to STS and SULT activities. Significantly enhanced Hcy levels were found in pituitary adenoma and glioblastoma multiforme. Concentrations of other aminothiols did not vary significantly according to diagnosis. No significant differences were found with respect to sex and age.

Determinations of STS and SULT activities in brain tumors revealed significant differences among studied brain tumors. Further analysis of the data revealed spontaneous tendency to form clusters according to diagnoses. Cluster formation indicated individual metabolic behavior especially for glioblastomas, which are considered the most dangerous type of brain tumor.

This was the first attempt in assessing the enzymatic activities of two enzymes with opposite effect directly inside the tumor tissues. In contrast to experiments carried out on tissue cultures, clinically interesting data that well reflect the situation inside the tumor cells were obtained. With respect to changes in DHEA and DHEAS plasma levels in males and females during the life span, it is also clinically important that STS and SULT activities as well as Hcy levels in brain tumors depend neither on age nor on sex of the studied subjects. These findings may serve as a starting point for further clinical studies. The enzyme activities that catalyze conversion of transcriptionally inactive precursors to multiple gene activators may be used as therapeutically interesting targets in anticancer research.

Concerning future perspectives, further studies including mRNA expression and immunohistochemistry in individual tumor cells are required to confirm these results.

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10. List of abbreviations

AA	amino acid
ACTH	adrenocorticotrphic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AST	astrocytoma
BBB	blood-brain barrier
bp	base pair
cAMP	cyclic adenosine monophosphate
CBG	corticosteroid-binding globulin
cDNA	complementary deoxyribonucleic acid
Chol	cholesterol
CNS	central nervous system
Cys	cysteine
d_3-DHEA	[19,19,19 ^{2}H]-dehydroepiandrosterone
d_3-DHEAS	[19,19,19 ^{2}H]-dehydroepiandrosterone sulfate
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DNA	deoxyribonucleic acid
FAD	flavin adenine dinucleotide
FMN	flavin adenine mononucleotide
GABA	γ-amino butyric acid
$GABA_A$-R	γ-amino butyric acid type A receptor
GBM	glioblastoma multiforme
GC-MS	gas chromatography-mass spectrometry
GLI	glioma
Hcy	homocysteine
kDa	kilodalton (1000 daltons)
K_M	Michaelis constant
LC-MS	liquid chromatography-mass spectrometry
MEN	meningioma
Met	methionine
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartate

NMDA-R	N-methyl-D-aspartate receptor
NO	nitric oxide
NOS	nitric oxide synthase
P450scc	cytochrome P450 side chain cleavage
PAP	3-phosphoadenosine-5-phosphate
PAPS	3-phosphoadenosine-5-phosphosulfate
PCR	polymerase chain reaction
PI3K	phosphatidyl inositol 3-kinase
PIA	pituitary adenoma
PMSF	phenylmethylsulfonyl fluoride
Pregn	pregnenolone
PregnS	pregnenolone sulfate
Prog	progesterone
PTEN	phosphatase and tensin homologue
ROS	reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SH	steroid hormone
SHBG	sex hormone-binding globulin
SPME	solid phase microextraction
StAR	steroidogenic acute regulatory protein
STS	steroid sulfatase
SULT	steroid sulfotransferase
t-Cys	total cysteine
t-Hcy	total homocysteine
TMP	2, 2, 4-trimethylpentane
UDP	uridyl diphosphate
V_{max}	maximum velocity
WHO	World Health Organization

11. Presentation of the results

11.1. Publications

Kriz L, Bicikova M, Mohapl M, Hill M, Cerny I, Hampl R. **Steroid sulfatase and sulfuryl transferase activities in human brain tumors.** J Steroid Biochem Mol Biol. 2007; E-pub ahead of print.

Kriz L, Bicikova M, Hampl R. **Roles of steroid sulfatase in brain and other tissues.** Physiol Res. 2007; E-pub ahead of print.

Bicikova M, **Kriz L**, Mohapl M, Burkonova D, Tallova J, Husek P. **Aminothiols in human brain tumors.** Clin Chem Lab Med. 2006;44(8):978-82.

Kriz L, Bicikova M, Hill M, Hampl R. **Steroid sulfatase and sulfuryl transferase activity in monkey brain tissue.** Steroids. 2005 Dec 15;70(14):960-9.

11.2. Peer reviewed

Heracek J, Hampl R, Kuncova J, Urban M, Lukes M, Zachoval R, Zalesky M, **Kriz L**, Hill M, Starka L, Eis V, Mandys V. **Tissue and serum levels of principal androgens in patients with localized and locally advanced prostate cancer.**

Drbalova K, Matucha P, Matejkova-Behanova M, Bilek R, **Kriz L**, Kazihnitkova H, Hampl R. **Immunoprotective steroids and SHBG in non-treated hypothyroidism and their relationship to autoimmune thyroid disorders.**

Simunkova K, Starka L, Hill M, **Kriz L**, Hampl R, Vondra K. **Comparison of total and salivary cortisol in a low-dose ACTH (Synacthen) test: Influence of three-month oral contraceptives administration to healthy women**.

11.3. Conferences

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2005 13th International Thyroid Congress, Buenos Aires, Argentina

2003 Advances in Organic, Bioorganic and Pharmaceutical Chemistry, Liblice, Czech Republic

Faculty of Science, Charles University, Prague
Institute of Endocrinology, Prague

Sulfation and desulfation of neuroactive steroids in normal and neoplastic primate brain tissues.

Ph.D. Thesis

Prague 2008 Lubomír Kříž

Declaration

I affirm that I worked out my thesis alone and I presented all the references used. No part of this thesis has been submitted elsewhere for any degree or qualification.

Prague, January 2008

Lubomír Kříž

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