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Steroids and steroid-metabolizing enzymes in the nervous system

Special focus on cell survival and sex hormone synthesis

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Abstract

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Some steroids in the brain and peripheral nervous system have been shown to have neuroprotective effects but the knowledge is limited. The present study examines the effects of steroids including oxysterols, vitamin D and vitamin D analogs on cell viability/growth and steroidogenesis in the nervous system.

Both 24- and 27-hydroxycholesterol reduced staurosporine-induced toxicity in human neuroblastoma SH-SY5Y cells. In addition, 27-hydroxycholesterol decreased the staurosporine-mediated induction of caspases, known to be important in apoptotic events. From the findings it may be concluded that effects of oxysterols on cellular viability are dependent on the concentration and on the type of oxysterol. 24-Hydroxycholesterol was also found to attenuate oxidative stress both in SH-SY5Y cells and astrocytes. The results indicate that during some conditions, oxysterols may have neuroprotective effects.

The vitamin D analogs tacalcitol and calcipotriol strongly reduced proliferation, cell viability and migration of human glioblastoma T98G cells, similarly as $1,25(OH)_2D_3$, the physiological form of vitamin D. Glioblastoma is the most lethal type of primary tumors in the CNS. These findings suggest that vitamin D analogs are potential candidates in treatment of brain tumors, most likely in combination with other therapies.

Astrocytes were found to be a major site for expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) whereas expression of CYP17A1 was found in both astrocytes and neurons. 3β-HSD and CYP17A1 are important steroidogenic enzymes. Vitamin D inhibited both CYP17A1- and 3β-HSD -mediated activity and mRNA levels, with a stronger effect on mRNA expression than on enzyme activity. This indicates that $1,25(OH)_2D_3$ could affect the production of sex hormones in the brain.

In summary, results from this thesis contribute to the knowledge on the effects of oxysterols on cell viability and oxidative stress in cells from the CNS. Also the results provide data on the effects of vitamin D in the brain and suggest that vitamin D analogs may be promising candidates for treatment of certain brain tumors.

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For Ivar and Maj

List of Papers

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

I **Emanuelsson, I.,** Norlin, M. (2012) Protective effects of 27- and 24-hydroxycholesterol against staurosporine-induced cell death in undifferentiated neuroblastoma SH-SY5Y cells. *Neuroscience Letters,* 525 (2012): 44– 48

II **Emanuelsson, I.,** Wikvall, K., Friman, T., Norlin, M. Vitamin D analogs tacalcitol and calcipotriol inhibit proliferation and migration of T98G human glioblastoma cells. *Submitted*

III **Emanuelsson, I.*,** Almokhtar, M.*, Wikvall, K., Grönbladh, A., Nylander, E., Svensson, A., Fex Svenningsen, Å., Norlin, M. Expression and regulation of CYP17A1 and 3β-hydroxysteroid dehydrogenase in cells of the nervous system: potential effects of vitamin D on brain steroidogenesis. *Submitted*

*Authors contributed equally to the work

IV **Emanuelsson, I.,** Wikvall, K., Rönnqvist, K., Norlin, M. Effects of 24-hydroxycholesterol on oxidative stress in human neuroblastoma SH-SY5Y cells and primary rat astrocytes. *Manuscript*

Contents

Abbreviations

AD	Alzheimer's disease
ANOVA	Analysis of variance
Androstenediol	5-Androstene-3β,17β-diol
Androstenedione	4-Androstene-3,17-dione
AR	Androgen receptor
BBB	Blood brain barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
$1,25(OH)_2D_3$	1α,25-Dihydroxyvitamin D_3
DMSO	Dimethyl sulfoxide
Estradiol	17β-Estradiol
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERE	Estrogen response element
FBS	Fetal bovine serum
GABA	Gamma-amino butyric acid
GBM	Glioblastoma multiforme
GC–MS	Gas chromatography–mass spectrometry
HEK	Human embryonic kidney
3β-HSD	3β-Hydroxysteroid dehydrogenase
17β-HSD	17β-Hydroxysteroid dehydrogenase
27-Hydroxycholesterol	5-Cholestene-3β,27-diol
24-Hydroxycholesterol	5-Cholestene-3β,24-diol
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LXR	Liver X receptor
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
7α-OH-DHEA	7α-Hydroxydehydroepiandrosterone
17-OH-PREG	17α-Hydroxypregnenolone

17-OH-PROG	17α-Hydroxyprogesterone
PD	Parkinson's disease
SERM	Selective estrogen receptor modulator
TLC	Thin layer chromatography
VDR	Vitamin D receptor

Introduction

Steroids in the nervous system

Neurosteroids are steroids synthesized by the brain and peripheral nervous system. They are synthesized from cholesterol into many different types of neurosteroids such as dehydroepiandrosterone (DHEA), pregnenolone, androstenedione, testosterone, estradiol and vitamin D [1, 2]. Steroids with neural activities but from a different origin than the brain are referred to as neuroactive steroids [3]. Neurosteroids regulate gene expression by binding to nuclear receptors or affecting neurotransmission by binding to $GABA_A$ receptors, NMDA receptors, sigma receptors or voltage-dependent calcium channels [4]. As a result they are involved in a range of different functions in the brain including neuronal growth, cell survival and differentiation [5]. Some steroids such as DHEA and pregnenolone have also been shown to have neuroprotective properties and could therefore potentially be used in the treatment of neurodegenerative diseases [6-8]. However, the knowledge of the effects of neurosteroids and enzymes on the central nervous system (CNS) is limited.

Enzymes in steroid metabolism

Enzymes involved in steroid hormone biosynthesis are oxidoreductases including both cytochrome P450 and non-P450 enzymes [2]. Most of the steroidogenic enzymes present in the adrenals, gonads, and placenta have also been found in the brain. The expression of steroidogenic enzymes is region- and cell type-specific in the nervous system. Steroidogenic enzymes may be regulated e.g. by development and signals from the pituitary [2, 9]. The cytochrome P450 (CYP) superfamily consists of oxygenases, which catalyze monooxygenase reactions, mostly hydroxylations, and are involved in the oxidative conversion of steroids, lipids, drugs and environmental toxins [10]. The non-*P*450 enzymes in the steroid metabolism consist of dehydrogenases such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and17β-hydroxysteroid dehydrogenase (17β-HSD). Dehydrogenases in steroid metabolism catalyze reactions that involve oxidation and/or isomerization [11, 12].

Cholesterol and oxysterols

Cholesterol in the brain

Cholesterol is an essential structural component in all cell membranes. The brain contains large amount of cholesterol and is in fact the most cholesterol-rich organ in the body with the majority of brain cholesterol being present in the myelin membranes. Myelin isolates the axons in neuronal cells facilitating rapid transmitting and receiving of electrical signals between neurons [13]. The rest of the cholesterol is present in plasma membranes of astrocytes and neurons to maintain normal neuronal function and morphology. Cholesterol is synthesized in both astrocytes and neurons but in the adult brain primarily synthesized in astrocytes [14-17]. It is important to maintain a constant level of cholesterol in the brain for normal brain function and defects in brain cholesterol metabolism have been associated with neurological syndromes, such as Alzheimer's disease and Parkinson's disease [18, 19].

Cholesterol transport

Cholesterol is essential for the body as a whole. It is not only involved in membrane structure and function, it is also a precursor for the formation of bile acids, steroid hormones and vitamin D in various tissues [20]. The cholesterol in the brain is separated from the cholesterol in the body by the blood-brain barrier (BBB). In the body cholesterol is transported with lipoproteins, where low-density lipoprotein (LDL) is the major transporter. High concentration of LDL can lead to atherosclerotic plaque formation in blood vessels. High-density lipoprotein (HDL) particles however transport cholesterol back to the liver where it is either used as a precursor for synthesis of bile acid and hormones or eliminated from the body [17]. In the brain cholesterol is transported mainly by the apolipoprotein E (ApoE) [21].

Oxysterols- hydroxylated derivatives of cholesterol

Oxysterols are hydroxylated derivatives of cholesterol. They can be formed in the body through different oxidation processes both enzymatically and non-enzymatically. They could also be absorbed from the diet due to lipid oxidation reactions in food [22]. Oxysterols are intermediates in bile acid synthesis and important for cholesterol transport and elimination [23, 24]. In addition they have been shown to be involved in regulation of atherosclerosis, neurodegeneration and inflammation [25-28]. Some well-known oxysterols are 7α- ,7β-, 24-, 25- and 27- hydroxycholesterol and 7-ketocholesterol. The brain is the major source of circulating 24-hydroxycholesterol. Cholesterol is too hydrophobic to pass the blood-brain barrier and is therefore metabolized to 24- and 27-hydroxycholesterol which

are less hydrophobic [29]. The oxysterols studied in this thesis are mainly 24- and 27-hydroxycholesterol, which may be considered the most physiologically relevant for the brain.

Biosynthesis and transport of oxysterols

24-Hydroxycholesterol is only synthesized in the brain and is transported from the brain into the circulation. 27-Hydroxycholesterol is made in many tissues and enters CNS from the circulation [30-32]. Inside the brain 27-hydroxycholesterol is further metabolized to 7α-hydroxy-3-oxo-4-cholestenoic acid, which can efficiently pass the BBB to the circulation [33]. Cholesterol is metabolized to 24-hydroxycholesterol by the enzyme CYP46A1 and to 27-hydroxycholesterol by the enzyme CYP27A1 (Fig. 1). The expression of CYP46A1 is restricted to the brain in humans primarily in neurons and astrocytes, while CYP27A1 is expressed in most cells and tissues in the body [34, 35]. In vitro studies indicate that both 24- and 27-hydroxycholesterol bind to the liver X receptor (LXR), a receptor that control transcription of several genes involved in the cholesterol, fatty acid, and glucose homeostasis [36, 37]. 24-Hydroxycholesterol is transported by low-density lipoprotein (LDL) to the liver where it is converted into bile acid intermediates and primary bile acids [38].

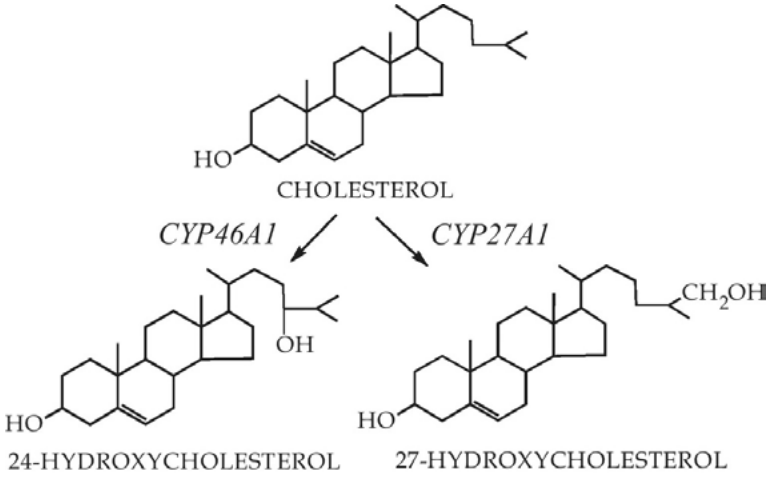


Figure 1. Formation of 24- and 27-hydroxycholesterol from cholesterol.

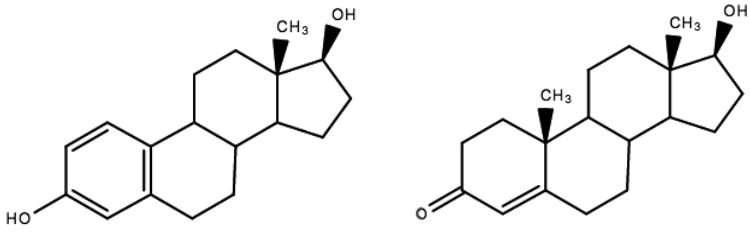
Oxysterols and neurodegenerative disease

There is a relation between cholesterol turnover and hypercholesterolemia as well as neurodegenerative diseases such as Alzheimer's disease and Parkin-

son's disease [28, 39, 40]. A cholesterol-rich diet increases plasma cholesterol levels but does not affect brain cholesterol levels [14, 41, 42]. Instead disturbed levels of oxysterols have been discovered in several neurodegenerative conditions. The levels of 27-hydroxycholesterol are dramatically increased in AD brains and PD brains [43, 44]. 24-Hydroxycholesterol has been shown to reduce the formation of amyloid β production while 27-hydroxycholesterol has been shown to increase the Aβ production and tau phosphorylation, two primary biochemical events in the development of AD [45, 46]. 27-Hydroxycholesterol has also been shown to increase α-synuclein levels through activation of LXRs while 24-hydroxycholesterol has been shown to reduce α-synuclein levels [47]. Accumulation of the protein α-synuclein forms Lewy bodies which causes the death of dopaminergic neurons in PD [48]. Studies have reported that 24-hydroxycholesterol may protect cells against 7-ketocholesterol-induced toxicity via activation of the LXR pathway [49]. However concentrations above 10 μM of 24-hydroxycholesterol have been reported to have cytotoxic effects and induce apoptosis [50, 51]. Thus, reported effects of oxysterols on viability are conflicting.

Estrogens and androgens

The female sex hormones, estrogens, and the male sex hormones, androgens, are involved in the formation of female and male characteristics in reproduction and development. Estrogens and androgens are present in both sexes but in different concentrations and both have important functions in both female and male physiology and pathology [52]. Estrogens exert their biological effects mainly by binding to the estrogen receptors ERα and ERβ and affecting gene expression [1]. Apart from the effects on the female reproductive functions, estrogens also play a significant role in the regulation of bone development, cell proliferation, immune response and functions in the CNS [53-56]. High estrogen blood levels are correlated with increased risk of developing breast cancer [57, 58]. The two most important androgens are testosterone and the more potent form 5α-dihydrotestosterone (DHT). Both regulate male development, behavior and testicular functions by binding to the androgen receptor (AR) [59-61]. High androgen blood levels are correlated with increased risk of developing prostate cancer [62]. Estrogens and androgens have also been shown to have neuroprotective properties and stimulate neurogenesis [63, 64]. Altered levels of estrogens and androgens in the brain have been detected in neurodegenerative diseases such as AD [65, 66].



Estradiol Testosterone

Figure 2. The chemical structure of the sex hormones estradiol and testosterone.

Sex hormone production

An overview of sex hormone production is shown in Fig. 3. The first and rate-limiting step in sex hormone biosynthesis is performed by CYP11A1 that catalyzes the conversion of cholesterol to pregnenolone. Pregnenolone can then either be converted to progesterone by the enzyme 3β-HSD or to 17-hydroxypregnenolone by the enzyme CYP17A1. CYP17A1 also catalyzes the conversion of 17-hydroxypregnenolone to DHEA. Several steroids are synthesized from DHEA. One of these is androstenedione which is formed by 3β-HSD and another is 5-androstene-3β,17β-diol (androstenediol) which is formed by 17β-HSD. 17β-HSD can also convert androstenedione to testosterone [67]. A third metabolite of DHEA is 7α-hydroxy-DHEA, which is formed after a 7α-hydroxylation by CYP7B1. CYP7B1 is an enzyme that also 7α-hydroxylates pregnenolone to 7α-hydroxy-pregnenolone and androstenediol to 5-androstene-3β,7α,17β-triol (androstenetriol) [68]. Androstenediol is further converted to testosterone by 3β-HSD. Aromatase (CYP19A1) converts androstenedione to estrone as well as testosterone to estradiol [67].

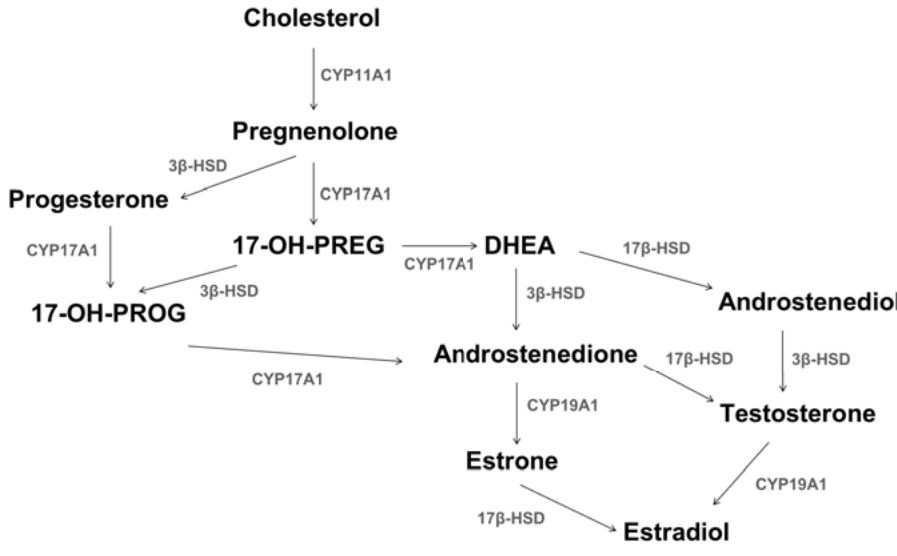


Figure 3. Pathways for biosynthesis of sex hormones.
DHEA, dehydroepiandrosterone; 17-OH-PREG, 17α-hydroxypregnenolone; 17-OH-PROG, 17α-hydroxyprogesterone.

Vitamin D

History

Rickets, a disease of vitamin D deficiency, was epidemic across Europe amongst children living in industrialized and polluted cities in late 1800s. In the 1920s it was observed that foods rich in fat-soluble vitamin A (cod-liver oil, butter, and whole milk) as well as sunlight or food irradiated with ultraviolet light were able to prevent rickets [69]. These discoveries were intriguing since both food and sunlight had the same effect on treatment of rickets. In the 1930s vitamin D was isolated and found to be structurally different from vitamin A. It was also discovered that vitamin D could be formed by UV irradiation of 7-dehydrocholesterol. The form of vitamin D produced in skin and present in animal food is called vitamin D_3. In the search to understand how this steroid could prevent rickets it was discovered that vitamin D increases the absorption of calcium from the intestine and causes the mobilization of calcium from the bone into the plasma [70]. In the late 60s and beginning of the 70s it was discovered that the natural form of vitamin D was not metabolically active. To become active vitamin D has to be activated by two metabolic steps in the body [71, 72].

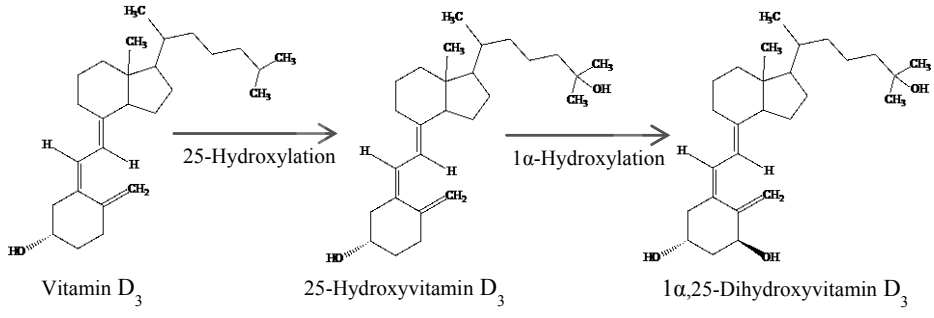


Figure 4. Bioactivation of vitamin D_3 to 1α,25-dihydroxyvitamin D_3.

Bioactivation and physiological function

The classical function of vitamin D is to regulate calcium and phosphate homeostasis in serum, necessary for bone mineralization but also for neuromuscular function [73]. Vitamin D becomes active by two enzymatic steps in the body. The first step is a 25-hydroxylation performed mainly in the liver by 25-hydroxylases (CYP2R1, CYP27A1, CYP3A4, CYP2J2) and the second step is a 1α-hydroxylation performed by CYP27B1 mainly in the kidney (Fig 4.) [74, 75]. The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is excreted into the circulation and transported by vitamin D-binding protein (DBP) [76]. The serum level of $1,25(OH)_2D_3$ is about 50-125 pM and strictly regulated by a feed-back mechanism where $1,25(OH)_2D_3$ decreases the formation of CYP27B1 and increases the formation of the enzyme CYP24A1 [74, 77]. CYP24A1 inactivates $1,25(OH)_2D_3$ by converting it into a series of 24- and 23-hydroxylated products leading to calcitroic acid targeted for excretion [78].

Effects on gene expression via VDR

The bioactivated form of vitamin D (1α,25-dihydroxyvitamin D_3) exerts its biological effects by binding to the vitamin D receptor (VDR) which affects gene expression of a large number of genes [79, 80]. VDR is a member of the steroid hormone nuclear receptor family. The VDR receptor is expressed in most cells and tissues including the central nervous system [81-84]. Upon activation VDR forms a heterodimer with the retinoid X receptor. The complex together with coactivators binds to a vitamin D responsive element (VDRE) in the gene promoter and acts as a transcription factor that activates or in some cases suppress transcription [85]. It has been suggested that $1,25(OH)_2D_3$ also may have VDR-independent effects via binding receptors located on the cell surface but the physiological relevance of this potential mechanism remains unclear [86].

Recently discovered functions of vitamin D

The VDR receptor is expressed in most cells and tissues and it has been revealed that vitamin D affects a wide range of physiological processes in the body apart from the effect on calcium homeostasis. Recent discoveries have shown that vitamin D has several other functions in the body including effects on cell proliferation and differentiation, the immune system, hormone regulation, cancer and the cardiovascular system [77, 81, 87, 88]. Vitamin D insufficiency is associated with an increased risk of developing cancer and other chronic illnesses such as dementia and Parkinson's disease [89, 90]. Compounds influencing the vitamin D system could potentially be used in treatment of many diseases and understanding vitamin Ds impact on human physiology and pathology is therefore important.

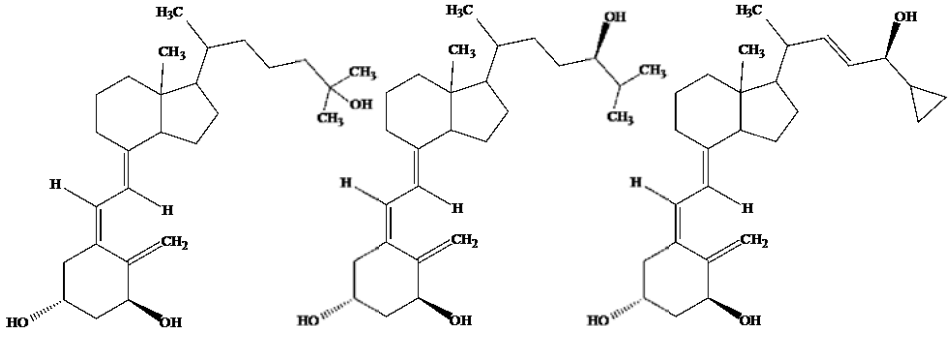
Vitamin D and cancer

Several clinical studies indicate that vitamin D deficiency increases the risk of developing cancer. There is an inverse relationship between solar exposure and many types of cancer with the exception of skin cancer. Epidemiological and early clinical trials are however inconsistent, they do not definitively support a beneficial role for vitamin D [91]. The most consistent protective effect has been shown on the incidence of colon rectal cancer in clinical trials [92]. There is an association between VDR expression and patient prognosis with a reduced risk of death in various tumor types [93, 94]. Several cancer cells also have an abnormal expression of the $1,25OH_2D_3$-inactivating enzyme CYP24A1 which disturbs the action of $1,25(OH)_2D_3$ [95, 96]. Studies both in vitro and in vivo show beneficial effects for either vitamin D supplementation or administration of $1,25OH_2D_3$ and its analogs on cancer progression [97-99]. In vitro, $1,25OH_2D_3$ inhibits cell proliferation and reduce the progression of several tumor types such as breast-, prostate-, colon- and ovarian cancer cells. $1,25(OH)_2D_3$ also promotes the apoptosis of a number of cell types [100-102].

Vitamin D analogs

Vitamin D analogs are chemically modified synthetic VDR-binding molecules but with reduced effect on calcium metabolism. Synthesis of analogs has been initiated since hyper-physiological doses of $1,25(OH)_2D_3$ can cause the side effect of hypercalcemia. Several analogs have been shown to inhibit cell proliferation and apoptosis [103, 104]. Tacalcitol and calcipotriol are two synthetic $1,25(OH)_2D_3$ analogs with less hypercalcemic activities but with similar affinity for VDR than that of $1,25(OH)_2D_3$ (Fig.5). In vivo, calcipotriol is 100–200 times less active in raising serum calcium. Calcipotriol and tacalcitol are currently used in treatment for psoriasis [105-107]. Exam-

ples of other analogs are alfacalcidol, doxercalciferol and oxacalcitriol, which are clinically used for osteoporosis and renal failure patients [108].



1,25(OH)$_2$D$_3$ Tacalcitol Calcipotriol

Figure 5. The structural difference between 1,25(OH)$_2$D$_3$ and the vitamin D analogs tacalcitol and calcipotriol.

Cell growth and cell survival

The growth and division of cells are referred to as the cell cycle. Somatic cells reproduce by dividing into two identical daughter cells. The first part of the cell cycle starts with the G_1 phase where cells grow larger. The G_1 is followed by the S phase during which DNA replication takes place. After DNA replication cells enter into the G_2 phase where cell growth continues and proteins are synthesized in preparation for mitosis. During mitosis daughter chromosomes are separated and the cytoplasm divided, forming two new cells. After mitosis, the cell must decide whether it will once again start a new round of active growth and division or enter a non-growing state called Go [109]. The progression of the cell cycle is tightly controlled by a series of cyclin-dependent kinases (Cdks). The cyclin levels rise and fall during the cell cycle and are influenced by external and internal signals. The most important of these Cdk regulators are cyclin proteins. Each phase has checkpoints controls to permit the cell to proceed to the next step in the cycle only if the previous step has been completed successfully [110, 111].

Cells divide a limited number of times before they stop and enter a non-dividing state. Cancer cells have an abnormal proliferation which is commonly caused by defects in the regulation of the cell cycle [112]. Many growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) activate the Ras pathway which stimulates cell division by allowing S-phase to begin. Activation of the Ras pathway leads to the

activation of mitogen-activated protein kinase (MAP kinase) cascade [113]. Map kinases such as c-jun terminal kinase (JNK), regulate cell proliferation, survival, differentiation, inflammation and cell death. Growth factors also activate PI3-kinase-Akt pathways, which promotes cell survival and proliferation by phosphorylating target proteins that inhibits apoptosis and cell cycle arrest [114, 115]. Cell proliferation means an increase of cell number and cell division. Cell viability is defined as the amount of healthy cells in a sample. The cell growth can be detected by a variety of methods e.g. by manual cell counting, MTT assay, and LDH assay [116-118].

Cell death and cytotoxicity

Cells that die as a result of acute injury, swell and burst and undergo necrosis, a passive cell death. Membranes are disrupted and cellular content leaks out leading to tissue damage and inflammation. Cells that are a threat to the organism or no longer needed are destroyed in a programmed cell death called apoptosis. In apoptosis, the cell shrinks, condenses and undergoes phagocytosis. It is then recycled by the cell that ingests it [119]. Apoptosis is triggered by the activation of proteolytic enzymes called caspases. They exist in all cells as inactive precursors and are activated by cleavage by other caspases starting a proteolytic caspase cascade. Caspase activation is regulated by either extracellular or intracellular death signals by members of the Bcl-2 and IAP (inhibitor of apoptosis) protein families that are activated when the cell is damaged. Some members of the Bcl-2 family inhibit apoptosis and some promote pro-caspase activation and cell death. IAP binds to some procaspases and inhibits apoptosis. Apoptosis is important in the prevention of abnormal proliferation and oncogenic transformation of cells [120-122].

Aims of the present investigation

The overall aim of this research was to study the effects of steroids including oxysterols and vitamin D on cell viability/growth in the nervous system and to study the effects of vitamin D on enzymes in biosynthesis of sex steroids in the brain.

The specific aims were:

I. To examine the effect of oxysterols on viability of human neuroblastoma SH-SY5Y cells treated with staurosporine, a toxic substance that induces apoptosis.

II. To analyze the effects of vitamin D analogs on cell viability, proliferation and migration in the human glioblastoma cell line T98G.

III. To study the cellular localization of the enzymes CYP17A1 and 3β-HSD in different cells of the nervous system and to investigate potential effects on these enzymes by vitamin D.

IV. To study the effects of 24-hydroxycholesterol on oxidative stress in human neuroblastoma SH-SY5Y cells and primary rat astrocytes and to investigate if 24-hydroxycholesterol can affect estrogen receptor signaling.

Experimental procedures

Materials

27-Hydroxycholesterol, prepared from kryptogenin was a kind gift from Dr. L. Tökes, Syntex, Palo Alto, CA, USA. 24-Hydroxycholesterol was generously provided by Prof. I Björkhem and was synthesized as described [123]. The purity of the oxysterols was >95% as determined by GC–MS. The human ERα and ERβ expression vectors and the ERE (estrogen response element) luciferase reporter vector were generous gifts from Dr. P. Chambon, Institute de génétique et de biologie moléculaire et cellulaire, Strasbourg, France and Dr. K. Arcaro, University of Massachusetts, MA, USA, respectively. All other chemicals were of analytical grade and purchased from various commercial sources.

Cell culture and treatment

Human neuroblastoma SH-SY5Y cells (CRL-2266), human glioblastoma T98G cells (CRL-1690) and human embryonic kidney HEK 293 cells (CRL-1573) were purchased from American Type Culture Collection. Primary rat astrocytes were prepared from whole brains of newborn rat pups (day 1-3) as described by Fex Svenningsen et al. [68] and were cultured in poly-L-lysine coated flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (10%), L-glutamine (0.3%) and antibiotics/antimycotics (1%). Primary cortical cell cultures from rat brain were prepared from embryos of pregnant Sprague-Dawley rats, removed at embryonic day 17 as described by Nylander et al. [124] and cells were cultured on poly-L-lysine coated plates in Neurobasal media supplemented with B-27 (2%), glutamine (600 μM) and antibiotics/antimycotics. Animal studies were approved by the regional ethics committee for research on animals in Uppsala (Sweden) and carried out in accordance with the policy of the Society for Neuroscience. SH-SY5Y and T98G cells were cultured in DMEM with the addition of 10% fetal bovine serum and 1% antibiotic–antimycotic. Cells were cultured at 37°C with 5% CO_2 in a humidified environment and subcultured twice a week. Prior to experiments cells were seeded in 96-well plates or 6-well plates and treated the following day with steroid hormones or other substances dissolved in ethanol or dimethyl sulfoxide (DMSO). The control

group was treated with vehicle (ethanol/DMSO). Protein concentrations were determined using BCA assay kit according to the manufacturer's instructions.

Measurement of cell viability and proliferation

In order to analyze the effect of 24- and 27-hydroxycholesterol, tacalcitol and calcipotriol on cell viability and proliferation the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was used. MTT is reduced in the mitochondria in living cells to water-insoluble formazan crystals. When these crystals are dissolved with isopropanol it is possible to quantitatively determine the cell viability/proliferation by measuring absorbance. SH-SY5Y cells were pretreated with 24- and 27-hydroxycholesterol and then exposed to the protein kinase inhibitor, staurosporine. T98G cells were treated with $1,25(OH)_2D_3$, tacalcitol or calcipotriol. MTT was added to cells and reduced in living cells to purple formazan product which were dissolved with isopropanol containing 0.7% SDS. The absorbance was measured at 570 nm using a microplate reader (POLARstar Optima BMG Labtech). Proliferation was also assessed by manual cell counting and by ^{3}H-thymidine incorporation assay. ^{3}H-Thymidine incorporation assay specifically measure proliferation and was used to study the effect of tacalcitol in T98G cells. Radioactive thymidine is incorporated into new strands of chromosomal DNA during mitotic cell division. ^{3}H-thymidine incorporation assay was carried out as described in paper II [125].

Measurement of cytotoxicity and apoptosis

To measure the effects of 24- and 27-hydroxycholesterol, tacalcitol and calcipotriol on cytotoxicity the LDH activity was determined by an enzymatic test (Cytotoxicity Detection Kit PLUS, Roche Diagnostics GmbH). The enzyme LDH exists in the cytosol in all living cells and is released in the media when the cell membrane is disrupted due to injury or exposure of toxic substances. SH-SY5Y cells were pretreated with 24- and 27-hydroxycholesterol and then exposed to staurosporine. T98G cells were treated with $1,25(OH)_2D_3$, tacalcitol or calcipotriol. Analysis was then carried out according to the manufacturer's recommendations and the absorbance was measured at 450 nm using a microplate reader. In order to investigate the effects of 24- and 27-hydroxycholesterol, tacalcitol and calcipotriol on apoptosis an assay measuring the caspase-3 and -7 activities (Caspase-Glo® 3/7 Assay kit, Promega) was used. This is a luminescence assay where a proluminescent caspase-3/7 substrate is cleaved, generating a luminescent signal proportional to the caspase activity. SH-SY5Y cells were pretreated with 24-

and 27-hydroxycholesterol and then exposed to staurosporine. T98G cells were treated with $1,25(OH)_2D_3$, tacalcitol or calcipotriol. The caspase activity was then measured according to the manufacturers recommendations. The luminescence was measured using a TD-20/20 luminometer (Turner Designs).

Assay of enzyme activities

The enzyme activities of 3β-HSD, CYP17A1, CYP19A1, 17β-HSD and CYP7B1 were measured in SH-SY5Y cells, primary rat astrocytes and primary neuron-enriched cerebral cortex cells. 3β-HSD activity and CYP7B1 activity was measured by addition of ^{3}H-labeled DHEA which was converted to androstenedione and 7α-hydroxy-DHEA by the respective enzymes. To assay CYP17A1-mediated enzyme activity, cells were incubated with ^{3}H-progesterone and the metabolites 17α-hydroxyprogesterone and androstenedione were analyzed. Assay of CYP19A1 activity and 17β-HSD activity was carried out by incubation of cells with ^{3}H-androstenedione which was converted to estrone and testosterone. After incubation for 24 h the cell media were collected and extracted with ethyl acetate. The substrates and metabolites were separated using thin layer chromatography (TLC). The extracts were applied to silica gel plates (Merck) and analyzed with chloroform/ethyl acetate 80:20 (v/v) as the mobile phase. The silica gel plates were scanned using an AR2000 TLC Radio Scanner (Eckert-Ziegler).

Measurement of oxidative stress

To analyze the effect of 24-hydroxycholesterol on oxidative stress in SH-SY5Y cells and rat astrocytes, a chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was used as molecular probe. CM-H2DCFDA passively diffuses into cells and becomes fluorescent after acetate groups are removed by oxidation. The oxidized probe is much better retained by cells than the parent compound. Oxidation of this probe can be detected by monitoring the increase in fluorescence. SH-SY5Y cells and astrocytes were pretreated with 24-hydroxycholesterol. The cells were probed with CM-H2DCFDA and then exposed to tert-butyl hydroperoxide (t-BHP) alone and in combination with 24-hydroxycholesterol. The fluorescence at 492/520nm was measured using a microplate reader (POLARstar Optima BMG Labtech).

Analysis of cell migration

Wound healing assay is a method used to characterize cell migration which is an important biological process in cancer metastasis. In this assay, confluent monolayers of cells are scraped with a micropipette to create a cell-free area which induces the cells to migrate into the gap. The uncovered wound area is imaged after several hours and quantified. Glioblastoma cells are highly invasive and grows rapidly. In this study human glioblastoma cells T98G were pretreated with $1,25(OH)_2D_3$, tacalcitol or calcipotriol and then scratched with a pipette tip. The uncovered wound area was measured and quantified at different intervals with ImageJ 1.37v.

Reverse Transcription-PCR (RT-PCR) and real time quantitative PCR (qPCR)

The VDR expression in T98G cells was evaluated with semi quantitative RT-PCR with human kidney as positive control. Total RNA was isolated by RNeasy Mini kit (Qiagen,) and reverse transcribed to cDNA by Reverse Transcription System (Promega). The PCR amplification was performed with AmpliTaq Gold DNA Polymerase (Applied Biosystems). In order to investigate potential effects on CYP17A1 and 3β-HSD mRNA levels by vitamin D real-time RT-PCR was used. Cells were pretreated with $1,25(OH)_2D_3$ and untreated cells received ethanol (control group). TATA box binding protein (TBP) was used as control (housekeeping) gene for normalization. All real-time RT-PCR data on CYP17A1 and 3β-HSD expressions were normalized to the control (housekeeping) gene. The RT-PCR analysis was performed with iQ SYBR Green Supermix (Bio-Rad) using an iQ Real-Time PCR Detection System (Bio-Rad) in accordance to the manufacturer's recommendations. The relative mRNA level was calculated with the ΔΔ–Ct method with a stepwise diluted standard curve and expressed as – fold change compared to vehicle-treated cells.

Western blotting

The effect of 24-hydroxycholesterol on the phosphorylation level of JNK, a protein that regulates apoptosis, was examined using Western blotting. Total protein extract was denatured and separated by SDS- polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membrane and then hybridized with antibodies against JNK and phosphorylated-JNK (Thr 183/Tyr 185) (Cell Signaling) at 4°C overnight. Horseradish peroxidase-conjugated secondary antibodies were added and antibody binding was de-

tected using the SuperSignal West Pico Chemiluminescent Substrate kit. Membranes were placed on FUJI medical X-ray film and protein bands were quantified by densitometry using ImageJ.

Reporter assay

Estrogen receptor activation was determined using an ER-dependent ERE (estrogen response element) -luciferase reporter assay as described as in paper IV [126, 127].

Statistical analysis

Analysis of statistical significance was performed using ANOVA, Kruskal-Wallis test or Student's t-test in Minitab 17 or Excel. P-values <0.05 were considered statistically significant.

Results and discussion

Effects of oxysterols on cell viability (paper I)

In paper I, effects of oxysterols, in particular 27- and 24-hydroxycholesterol, on the viability of human neuroblastoma SH-SY5Y cells were examined. Cells were treated with either oxysterols alone or in combination with staurosporine, which is a toxic substance that induces apoptosis. Cholesterol cannot pass the blood-brain barrier and must be converted into 24- and 27-hydroxycholesterol which are less hydrophobic. These oxysterols can pass the blood brain barrier and are therefore very important for the cholesterol balance in the brain [24]. Abnormal levels of oxysterols have also been reported in various neurodegenerative conditions [26, 28, 128]. Staurosporine has been used in many studies to induce apoptosis. There are, however, no previous reports on oxysterol-mediated effects on viability of neuronal cells using staurosporine. Other neurosteroids such as DHEA and pregnenolone have been shown to be neuroprotective against staurosporine-induced toxicity [129]. The aim with paper I was to examine the effect of oxysterols on viability of human neuroblastoma SH-SY5Y cells in the presence and absence of the protein kinase inhibitor staurosporine. The SH-SY5Y cell line is derived from a human tumor (neuroblastoma) and is a commonly used cell line for studies of neuronal viability.

The methods to study cell viability and cytotoxicity were MTT and LDH assay. Apoptosis was analyzed by Caspase 3/7-assay since caspases are essential in apoptotic events. Results showed that 27-hydroxycholesterol significantly reduced the staurosporine- induced toxic effect at concentrations between 100 nM-20 μM when measured with MTT assay (Fig. 6). 27-Hydroxycholesterol displayed the highest protective effect in concentrations between 10-20 μM. Treatment with 27-hydroxycholesterol alone had no effect on cell viability. 27-Hydroxycholesterol also showed a protective effect when measured with LDH assay. The concentrations of 10 μM 27-hydroxycholesterol reduced the staurosporine-induced LDH activity nearly to the same levels as untreated controls. Assay of caspase activity showed that 27-hydroxycholesterol can reverse the apoptotic event of staurosporine. Caspases are a family of protease enzymes that are activated by apoptosis. Treatment with staurosporine for 4 h increased caspase 3/-7 activity by about 4-fold compared to controls. Pretreatment with 10-20 μM of 27-

hydroxycholesterol antagonized this effect significantly. 24-Hydroxycholesterol also displayed a protective effect on staurosporine-induced toxicity but with highest protective effect in lower concentrations (10 nM-100 nM) (Fig. 7). At higher concentration (10 μM) of 24-hydroxycholesterol enhanced the toxic effect of staurosporine and decreased the cell viability. Treatment with 24-hydroxycholesterol alone had no effect on cell viability.

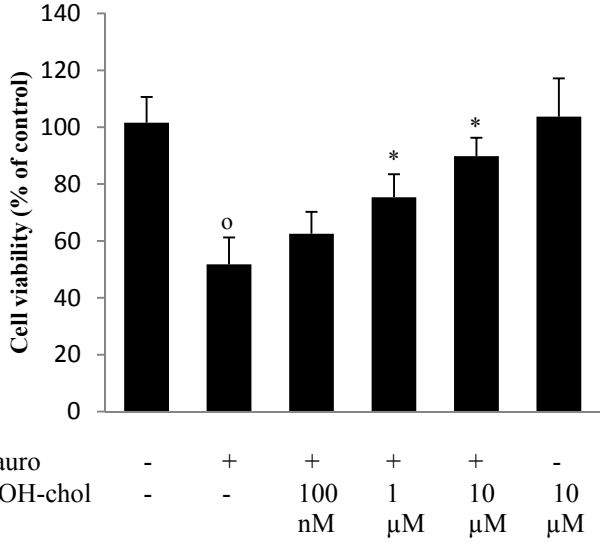


Figure 6. Effect of 27-hydroxycholesterol (27OH-chol) on SH-SY5Y cells treated with staurosporine (Stauro), measured by MTT assay.
◦Statistically significant change compared to treatment with vehicle ($p < 0.05$). *Statistically significant change compared to treatment with staurosporine without steroid ($p < 0.05$).

The oxysterols 7β-hydroxycholesterol, 7-ketocholesterol and 25-hydroxycholesterol had similar protective effects on staurosporine- induced toxicity as 27-hydroxycholesterol. These oxysterols are however less important in the CNS. The results indicate that oxysterols have protective effects on staurosporine-induced cytotoxicity in SH-SY5Y cells. These findings are contradictory to some other studies on effects of oxysterols. In previous studies, oxysterols have been reported to influence apoptosis, necrosis and oxidative stress resulting in decreased viability [28, 39, 130]. A study on co-cultured SH-SY5Y cells and C6 cells showed 27-hydroxycholesterol to inhibit cell viability at concentration between 25-200 μM, where a concentration of 200 μM reduced viability with 50% [131]. 27-Hydroxycholesterol, but not 24-hydroxycholesterol, is also involved in the formation of Aβ peptides in human neuroblastoma cells [39]. In this study the effects of 24- and 27-hydroxycholesterol were dose dependent. A higher concentration of 27-

hydroxycholesterol was required to protect the cells against staurosporine compared to 24-hydroxycholesterol. Previous studies have reported that the concentration of 24-hydroxycholesterol reaches 4-15 ng/mg wet weight in brain tissue while the concentration of 27-hydroxycholesterol is 10-20 % of the concentration of 24-hydroxycholesterol [30, 32]. In view of these measurements, the concentrations showing effects in the present study may be considered to be within or near physiological levels and the concentration seems important for oxysterol-mediated effects. Previous studies have shown that concentrations above 10 μM of 24-hydroxycholesterol can

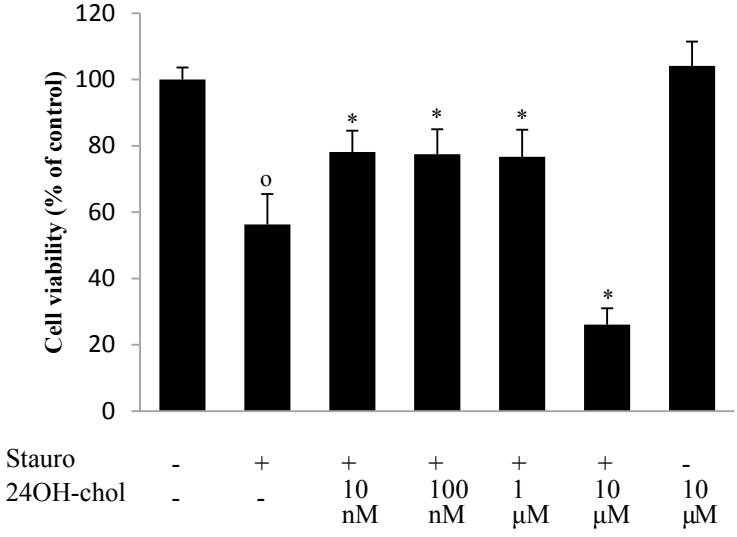


Figure 7. Effect of 24-hydroxycholesterol (24OH-chol) on SH-SY5Y cells treated with staurosporine (Stauro), measured by MTT assay.
◦Statistically significant change compared to treatment with vehicle ($p < 0.05$). *Statistically significant change compared to treatment with staurosporine without steroid ($p < 0.05$).

have cytotoxic effects and induce apoptosis [49]. This was however, not confirmed in this study, neither 27-hydroxycholesterol nor 24-hydroxycholesterol had any effects on cell viability on their own. The data indicate that, during some conditions, oxysterols may have neuroprotective effects.

Effects of vitamin D analogs on cell proliferation and migration (paper II)

In paper II, the effects of the vitamin D analogs tacalcitol and calcipotriol were studied in human T98G glioblastoma cells. Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor of the CNS and vitamin D has shown potential for various applications in cancer prevention and treatment [132]. In vitro studies have shown that $1,25(OH)_2D_3$ inhibits cell proliferation and induces apoptosis in several tumor cell lines, however much of the work on vitamin D as an anticancer agent has been focused on colorectal-, breast- and prostate cancer [100, 102]. A small number of studies have previously indicated a response to vitamin D in human and rat glioma cells [133]. Both tacalcitol and calcipotriol are synthetic $1,25(OH)_2D_3$ analogs with less systemic side effects than $1,25(OH)_2D_3$ [134]. Both are currently used for treatment of psoriasis but their effect on glioblastoma cells has not previously been investigated.

Since $1,25(OH)_2D_3$ exerts its effect by binding to VDR, the first step in this study was to confirm that T98G cells expressed VDR using RT-PCR. The results showed that T98G cells expressed VDR. Cells were treated with $1,25(OH)_2D_3$, tacalcitol or calcipotriol for six days. The results showed that all three compounds had a significant, dose-dependent, inhibitory effect on cell viability/growth when measured using MTT assay. A significant effect was detected in concentrations as low as 1 nM with the highest effect detected at 10 μM for all three compounds. Tacalcitol showed the strongest inhibitory effect on cell viability/growth. In a concentration of 10 μM the cell viability/growth was reduced to 34% compared to the control (Fig. 8A). Calcipotriol was less active but inhibited viability/growth to 64% in the same concentration. The MTT assay does not distinguish between effects on cell viability and cell growth. Therefore, to be able to detect the effect on cell growth only, manual cell counting and ^{3}H-thymidine incorporation assay were used. The results of experiments using manual cell counting indicate that tacalcitol and calcipotriol strongly inhibits T98G cell growth, to about 10% in a concentration of 10 μM (Fig. 8B). Treating cells for a shorter time period than six days had no inhibiting effect on cell viability or growth. The effect of tacalcitol on cell proliferation was further analyzed using ^{3}H-thymidine incorporation. ^{3}H-Thymidine incorporation is a method which measures cell proliferation by directly measuring DNA synthesis and cell division [135]. T98G cells were treated for 48 h with concentrations of tacalcitol ranging from 100 nM to 10 μM. The highest concentration of 10 μM significantly reduced cell proliferation by about 50 % (Fig. 8C). A concentration of 1 μM showed a tendency to inhibit proliferation but results were not significant

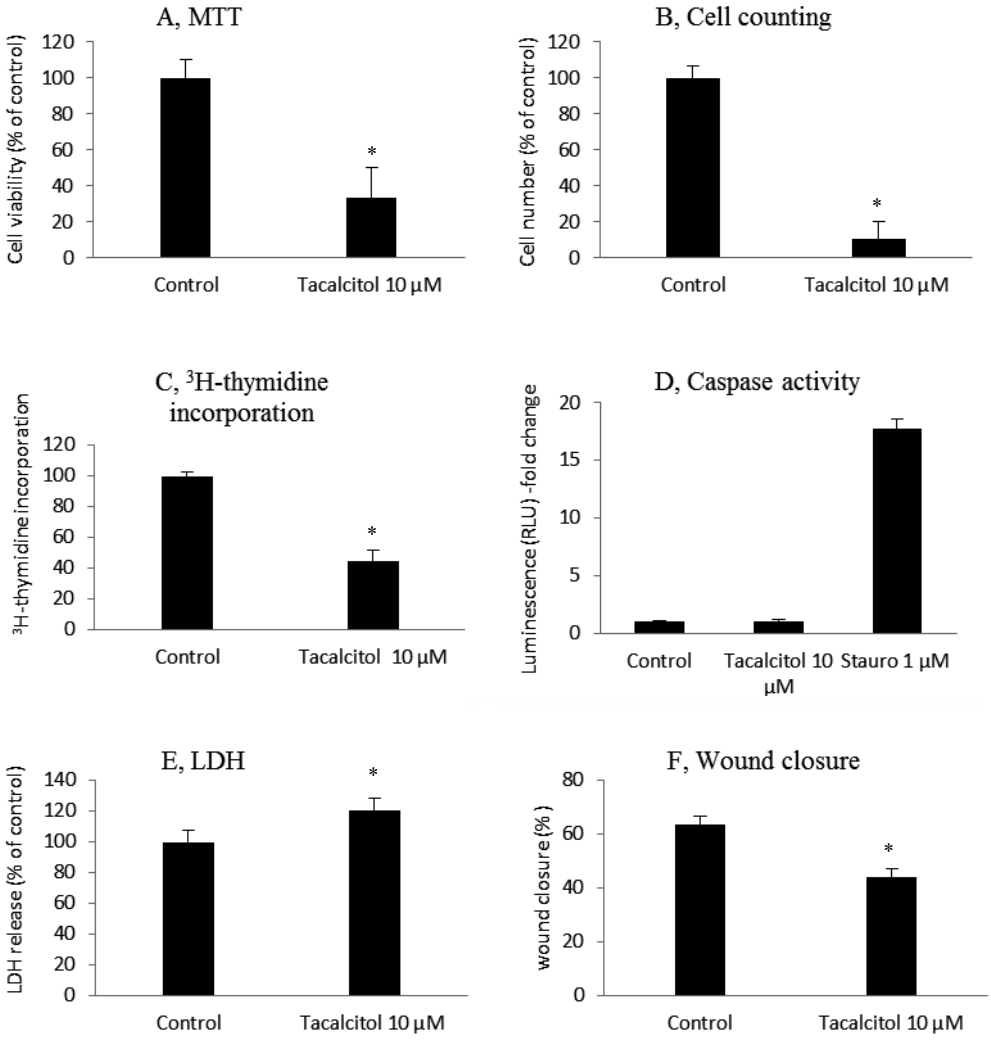


Figure 8. The effects of tacalcitol on human glioblastoma T98G cells analyzed with different methods. *Statistically significant change compared to control.

Previous studies have indicated that vitamin D may be involved in apoptotic events [136]. Therefore, in order to establish if this is also true for the analogs used in this study, caspase activity was measured using caspase 3/7-assay. T98G cells were treated with 1α,25(OH)$_2$D$_3$, tacalcitol or calcipotriol for 24 h. Staurosporine was used as positive control. Neither 1,25(OH)$_2$D$_3$ nor any of the analogs had any measurable effect on caspase activity while staurosporine increased the caspase activity 17-fold (Fig. 8D). Cells were also treated for a longer time period than 24 h but these conditions only resulted in a reduced luminescence signal compared to control. The reason for

this is probably that these cultures had a reduced cell number and therefore generated a weaker signal. Treating cells for less than 24 h did not affect caspase activity. This implies that the growth inhibitory effect does not involve apoptosis. Effects of $1,25(OH)_2D_3$, tacalcitol and calcipotriol on cytotoxicity was also analyzed using LDH assay. Increased LDH-activity is an indicator for cytotoxicity. A concentration of 10 µM of all three compounds increased LDH release, but only by about 25-30% compared to vehicle-treated controls (Fig 8E). Concentrations below 10 µM did not affect LDH-release. Cells that were treated for 72 h, 96 h and 120 h all generated similar results on LDH release and there was no significant difference between the three tested substances. Treating cells for less than 72 h did not affect LDH activity. From the results of these experiments it seems that the effect of these compounds is stronger on growth than on cell survival.

Glioblastoma cells are highly invasive cells and it was therefore interesting to study if $1\alpha,25(OH)_2D_3$, tacalcitol and calcipotriol had any effect on cellular migration. This was done using wound healing assay, where cells are grown to confluency and then scraped with a micropipette. The uncovered wound area is measured and quantified at different time intervals. T98G cells were first treated with 10 µM of $1\alpha,25(OH)_2D_3$, tacalcitol or calcipotriol for 24 h and then scraped. After 17 h all three compounds had reduced the migration rate of T98G cells compared to untreated control. Control cells had a wound closure of about 60% while treated cells had a wound closure of about 35-45% (Fig 8F).

This study indicates that the two vitamin D analogs tacalcitol and calcipotriol have growth inhibitory effects in human glioblastoma T98G cells. They seem foremost to have a regulatory effect on proliferation and cell cycle by inhibiting cell division; however an effect on cell viability cannot be excluded. They did also have an inhibiting effect on cellular migration. They do not appear to be cytotoxic or to activate apoptosis. All three substances tested showed the strongest effect at the highest concentration (10 µM) but significant inhibitory effect on cell growth/cell viability was detected at concentrations as low as 1 nM when measured with MTT assay. The normal serum level of $1,25(OH)_2D_3$ is 50-125 pM [77] and in comparison, 10 µM can be considered as a hyperphysiologic dose. In the case of $1,25(OH)_2D_3$ this dose would lead to dangerous side effects such as hypercalcemia. Tacalcitol has been shown to have similar affinity for VDR as $1,25(OH)_2D_3$ but with less calcemic activity and a shorter half-life [105]. However, a short half-life can be both positive and negative. A short half-life may result in a reduced risk of toxicity but also decreased therapeutic effect. Several newer vitamin D analogs with less calcemic effects have been synthesized, some of which suppress CYP24A1 activity in order to increase the half-life [137]. In vivo studies have indicated that calcipotriol enhances the anti-cancer effect of the

cancer therapeutic agent gemcitabine in an animal model of pancreatic cancer [138]. Similar synergistic effects were observed with treatment of a C6 glioblastoma cell line with vitamin D and temolozomid. Temolozomid is used as initial treatment for high-grade glioblastoma. The antitumor effect was significantly increased with a combined treatment with temolozomid and vitamin D compared with either vitamin D or temolozomid alone [139]. The results from this and other studies indicate that vitamin D analogs are potential candidates in treatment of glioblastomas, most likely in combination with other therapies.

Expression and regulation of CYP17A1 and 3β-HSD (Paper III)

In paper III, the expression and activity of the enzymes CYP17A1 and 3β-HSD in different cells from the CNS were analyzed. Potential regulatory effects on CYP17A1 and 3β-HSD expression and activity by vitamin D were also investigated.

Enzyme activity of CYP17A1 and 3β-HSD in different cells of the nervous system

The enzyme CYP17A1 exerts two types of enzyme activities, a 17α-hydroxylase activity and a 17,20 lyase activity. CYP17A1 is involved in several reactions in steroid hormone synthesis, especially the formation of sex hormones (Fig. 2) [140]. 3β-HSD is involved in all basic steroidogenic pathways and is responsible for the production of the 3-oxo-group which is present in most of the mature hormones (Fig. 2) [11]. It has previously been reported that steroid hormones can be enzymatically produced in neurons, astrocytes and oligodendrocytes [9]. However, there have been conflicting results about which cell type is most important for the expression of CYP17A1 and 3β-HSD. Therefore, in this study the expression and enzyme activity of CYP17A1 and 3β-HSD were investigated in rat astrocytes, in neuron-enriched mixed cells from rat cerebral cortex and in human neuroblastoma SH-SY5Y cells. Cells were treated with ^{3}H-labeled progesterone and ^{3}H-labeled DHEA and the CYP17A1-mediated conversion of progesterone to 17OH-progesterone and androstenedione (Fig. 9), and the 3β-HSD-mediated conversion of DHEA to androstenedione (Fig. 10) were analyzed by TLC.

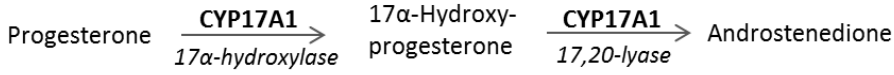


Figure 9. The formation of 17α-hydroxyprogesterone and androstenedione by CYP17A1.

The results showed that CYP17A1 was expressed in all three types of cell cultures and displayed both a 17α-hydroxylase activity and a 17,20-lyase activity. All three cell types produced 17OH-progesterone at similar rates, between 30-40 pmol/mg protein/h. They also displayed a similar 17,20-lyase activity and androstenedione was produced at about 30-60 pmol/mg protein/h. The 3β-HSD activity was measured by analyzing the conversion of DHEA to androstenedione and the results showed that only astrocytes had a 3β-HSD activity. The formation of androstenedione was about 82-120 pmol/mg protein/h. In SH-SY5Y cells and neuron-enriched cerebral cortex cells the 3β-HSD activity was below the limit of detection (≤ 5 pmol/mg protein/h). SH-SY5Y cells, which are a commonly used model for neuronal function and differentiation, were also differentiated using retinoic acid to be more similar to mature neurons. This did however not have impacts on 3β-HSD activity.

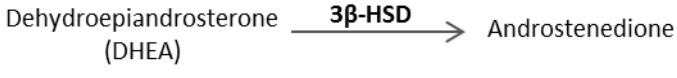


Figure 10. The formation of androstenedione by 3β-HSD.

The results indicate that both neurons and astrocytes express the enzyme CYP17A1 and show CYP17A1 activity. In previous studies, CYP17A1 expression has been reported in neurons from rat and frog brain [141, 142]. A study with cells from the cerebral cortex of neonatal rat brain showed a higher mRNA-level of CYP17A1 in astrocytes than neurons which had a low transcript of CYP17A1 mRNA. Astrocytes had also a significant higher enzyme activity than that of neurons [143]. Other studies, however, have reported no CYP17A1 mRNA levels in astrocytes and mixed glial cells [144]. Rat is a common model to study neurosteroid biosynthesis. A study by Brown *et al.* [145] on a human glioma cell line as well as on primary normal human astrocytes and purified human neurons showed expression of CYP17A1 mRNA but no CYP17A1 enzyme activity. Potential species difference cannot be excluded.

In the present study, 3β-HSD activity was detected only in astrocytes. The neuron-enriched mixed cortex cells contain about 60% neurons in addition to glial cells. If neurons would have been involved in 3β-HSD activity, this activity would have increased compared to astrocytes alone but instead the activity decreased. This indicates that neurons are not involved in 3β-HSD activity. Neurons are dependent on glial cells and will only survive for a limited time without the support of these cells and therefore a mixed cell culture may be considered to be more similar to a physiological culture. SH-SY5Y cells expressed 3β-HSD mRNA but had no 3β-HSD enzyme activity. The same result was obtained in differentiated SH-SY5Y cells. Conflicting results concerning 3β-HSD expression have been published. The results from the present investigation are supported by several studies reporting 3β-HSD expression in human, rat and dog brain [146-149]. However, other studies have reported that 3β-HSD is expressed only in neurons in rat brain [150]. More studies are needed to evaluate the contribution of these cell types to 3β-HSD mediated activity in the nervous system.

Effects of vitamin D on CYP17A1 and 3β-HSD expression and activity in CNS

The current knowledge of CYP17A1 and 3β-HSD regulation in CNS is limited and studies have mostly been focused on regulatory effects by estrogens. Vitamin D has previously been shown to have regulatory effects on steroid-producing enzymes in adrenocortical and breast cancer MCF-7 cells but the effects of vitamin D have not been studied in cells from CNS [151, 152]. The aim with this investigation was therefore to analyze the effects of vitamin D on CYP17A1 and 3β-HSD activities and expression. Astrocytes, neuron-enriched cortex cells and SH-SY5Y cells were treated with 10 nM $1,25(OH)_2D_3$ and the enzyme activity was measured using TLC.

The results showed that 10 nM of $1,25(OH)_2D_3$ suppressed the CYP17A1-mediated conversion of progesterone into 17α-hydroxyprogesterone and androstenedione with about 20% in SH-SY5Y cells and astrocytes. In neuron-enriched cerebral cortex cells, $1,25(OH)_2D_3$ had no significant effect. Since $1,25(OH)_2D_3$ slightly suppressed CYP17A1 activity in SH-SY5Y cells and astrocytes, the effect on mRNA levels were investigated. The results showed that suppression of CYP17A1 mRNA levels was considerably stronger, about 50% in SH-SY5Y cells and 75% in astrocytes compared to untreated controls. The effect of $1,25(OH)_2D_3$ on the rate of 3β-HSD-mediated conversion of DHEA into androstenedione was also investigated in the three different cell lines. The results showed that $1,25(OH)_2D_3$ significantly suppressed 3β-HSD activity in astrocytes by 20%. In SH-SY5Y cells and neuron-enriched cerebral cortex cells the enzyme activity was below the

limit of detection and was not affected by the treatment of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ decreased the 3β-HSD mRNA levels by about 60% in astrocytes but had no effect on the 3β-HSD mRNA levels in SH-SY5Y cells.

Table 1.

	CYP17A1 mediated activity (% of control)	3β-HSD mediated activity (% of control)	CYP17A1 mRNA expression (% of control)	3β-HSD mRNA expression (% of control)
SH-SY5Y				
Control (vehicle)	100	ND	100	100
$1,25OH_2D_3$	80±10*	ND	56±11*	92±11
Astrocytes				
Control (vehicle)	100	100	100	100
$1,25OH_2D_3$	80±30	80±10*	29±9*	36±7*
Neuron-enriched cerebral cortex cells				
Control (vehicle)	100	ND	nm	nm
$1,25OH_2D_3$	105 (100-110)	ND	nm	nm

Effects of $1,25(OH)_2D_3$ on enzyme activities and mRNA expression of CYP17A1 and 3β-HSD. *Statistically significant compared to control.
ND, not detectable; nm, not measured

Other metabolic pathways for DHEA and androstenedione

The results showed that $1,25(OH)_2D_3$ had a stronger effect on the mRNA expression than on the enzyme activities of CYP17A1 and 3β-HSD. This might be due to further metabolism of the analyzed products. In order to investigate if androstenedione and DHEA are metabolized by other enzymes, experiments analyzing CYP19A1, 17β-HSD and CYP7B1 activities were also performed. CYP19A1 converts androstenedione to estrone. The formation of estrone was about 132 pmol/mg/h in human SH-SY5Y cells and 254 pmol/mg/h in neuron-enriched rat cerebral cortex cells but was very low in rat astrocytes (12 pmol/mg/h). 17β-HSD converts androstenedione to testosterone. The formation of testosterone varied between 21 and 72 pmol/mg/h in the three cell cultures. CYP7B1 catalyzes the formation of 7OH-DHEA from DHEA. It has previously been reported that 7OH-DHEA is the major metabolite in astrocytes [153] and the current results showed that the formation of 7OH-DHEA was 343±33 pmol/mg/h in astrocytes.

$1,25(OH)_2D_3$ did not significantly affect any of the CYP19A1, 17β-HSD or CYP7B1 activities.

In the present study, $1,25(OH)_2D_3$ was shown to have an inhibitory effect on 3β-HSD and CYP17A1 in cells of the nervous system with a stronger effect on mRNA expression than on enzyme activity. This indicates that $1,25(OH)_2D_3$ may be able to regulate the functions of these enzymes in the CNS and potentially could affect the production of sex hormones in the brain. Two previous studies, one in human cumulus granulosa cells and one in human ovarian cells, showed that $1\alpha,25(OH)_2D_3$ can increase 3β-HSD mRNA levels and 3β-HSD enzyme activity [152, 154]. This suggests that the effect of $1,25(OH)_2D_3$ on steroidogenesis is highly cell specific. As mentioned above, the currently observed effects were stronger on mRNA than on enzyme activity (for both 3β-HSD and CYP17A1). The reason(s) for this remain at present unclear. Experiments on the effect of $1,25(OH)_2D_3$ on other enzymatic activities involving DHEA and androstenedione did not provide results that can explain these findings. The possible presence of additional unknown metabolic processes cannot be excluded. The effect of $1,25(OH)_2D_3$ on the expression of these enzymes may be complex and should be subject to further studies.

Effect of 24-hydroxycholesterol on oxidative stress (paper IV)

In paper IV, the effect of 24-hydroxycholesterol on oxidative stress was analyzed. The brain is especially sensitive to oxidative damage due to high lipid content and high oxygen consumption. Reactive oxygen species (ROS) are involved in many neurodegenerative processes including aging, ischemic stroke and Alzheimer's disease [155]. 24-Hydroxycholesterol is important for the cholesterol level in the brain but altered levels of this oxysterol has been associated with disease. For example decreased levels of 24-hydroxycholesterol in plasma and cerebrospinal fluid has been detected in patients with Alzheimer's disease and Huntington's disease [156, 157]. Several different effects of 24-hydroxycholesterol in the CNS have been reported. 24-Hydroxycholesterol has been shown to have neuroprotective effects and ability to reduce the formation of β-amyloid plaques but also to induce neuronal cell death [45, 50]. The aim of this study was therefore to investigate the effects of 24-hydroxycholesterol on oxidative stress in human neuroblastoma SH-SY5Y cells and primary rat astrocytes. Tert-butyl hydroperoxide (t-BHP) was used to induce oxidative stress and raloxifene was used as a positive control. Raloxifene is a selective estrogen receptor modulator (SERM) and well known to protect against oxidative stress [158]. Cells were

pretreated with 10 μM 24-hydroxycholesterol for 24h and then exposed to t-BHP for 45 min. The results showed that t-BHP induced oxidative stress about 2 to 3-fold in both SH-SY5Y cells and astrocytes compared to untreated controls. In SH-SY5Y cells, 24-hydroxycholesterol significantly attenuated this effect by 70%. The strongest effect was observed in concentrations between 10-20 μM (Fig. 11). A concentration of 1 μM had a tendency to inhibit oxidative stress but the effect was not significant. Raloxifene, used as a positive control, attenuated t-BHP induced oxidative stress by about 40%. Treatment with 24-hydroxycholesterol alone did not generate oxidative stress. Astrocytes are neuronal helper cells and the main glial cells in CNS. In astrocytes a concentration of 10 μM 24-hydroxycholesterol significantly attenuated t-BHP induced oxidative stress by about 60% (Fig. 11).

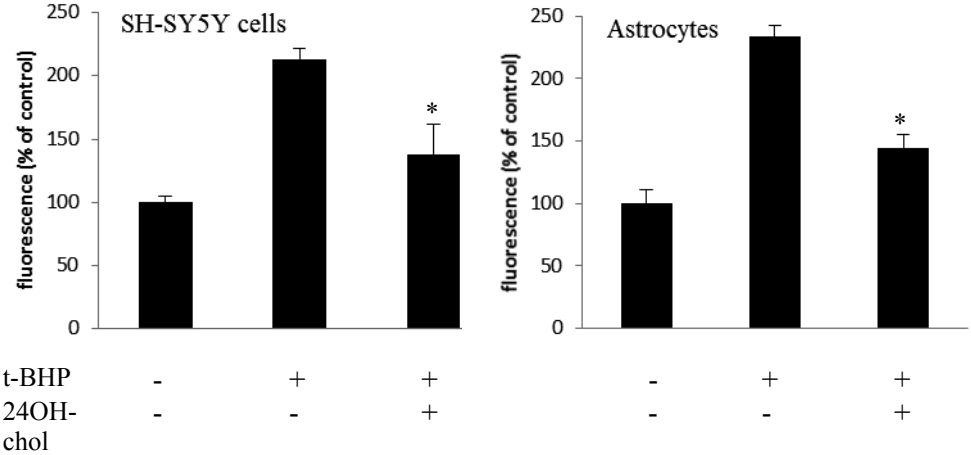


Figure 11. Effect of 24-hydroxycholesterol (24OH-chol) on t-Butyl hydroperoxide (t-BHP) induced oxidative stress in SH-SY5Y cells and astrocytes.
*Statistically significant change compared to treatment with t-BHP alone, ($p<0.05$).

Estrogens have shown to be involved in both prevention and protection against oxidative stress [158]. Recent studies have also revealed 27-hydroxycholesterol to be able to bind and modulate estrogen receptors ERα and ERβ signaling and to be the first endogenous selective estrogen receptor modulator (SERM) [159]. To investigate if 24-hydroxycholesterol can affect estrogen receptor signaling, human embryonic kidney cells (HEK 293) were co-transfected with an ERE-luciferase reporter plasmid and vectors containing cDNA for either ERα or ERβ. Cells were then treated with estradiol as positive control and/or 24-hydroxycholesterol. HEK 293 cells have previously been reported not to contain any endogenous ER [160]. The result showed a significant stimulation of both ERα and ERβ by estradiol while 24-hydroxycholesterol did not stimulate any of the estrogen receptors. However, an interesting finding was that a combined treatment with estradiol and 24-

hydroxycholesterol resulted in a reduced response to estradiol, implicating that 24-hydroxycholesterol could have an antagonistic effect on estrogen receptors. Similar results were obtained in a study by Umetani *et al.* [161], focusing on cardiovascular effects by oxysterols, where 24-hydroxycholesterol in combination with estradiol had an antagonistic effect on ERα and ERβ after 24 h of treatment. However, this study did not examine effects of 24-hydroxycholesterol alone. Considering that 24-hydroxycholesterol is synthesized only in the brain, any potential effects of this oxysterol on estrogen signaling should be more relevant in the nervous system than in other tissues.

To investigate if the mechanism for the effect of 24-hydroxycholesterol on oxidative stress involves induction of ER, SH-SY5Y cells were treated with 24-hydroxycholesterol, with and without the estrogen receptor antagonist ICI 182, 780. The cells were then exposed to t-BHP for 45 min. It has previously been shown that SH-SY5Y cells express both ERα and ERβ [162]. The estrogen receptor antagonist had no impact on the effect of 24-hydroxycholesterol on oxidative stress; however the antagonist showed a protective effect on oxidative stress on its own. This finding was surprising since estrogens are reported to protect against oxidative stress. The anti-oxidative effects of estrogens are reported to be mediated via ER and alter genes that play a role in apoptosis [163]. It has also been reported that ER in the membrane or cytoplasm activates intracellular signaling cascade such as mitogen-activated protein kinase (MAPK), B-ras and G-proteins [163]. It cannot be excluded that ICI 182, 780 may have additional effects on the cell than the inhibition of estrogen receptors. In summary the results suggest that 24-hydroxycholesterol may function as an estrogen receptor antagonist or that it may be able to modulate estrogen receptors. The response to oxysterols may vary in different cell types. 27-Hydroxycholesterol has previously been shown to be an antagonist in bovine aortic endothelial cells (BAEC) and MCF-7 human breast cancer cells and to be an agonist in HepG2, Caco-2, and HeLa cells [130, 159].

Oxidative stress is involved in neuronal apoptosis [164]. C-Jun N-terminal kinase (JNK) belongs to the MAP kinase family and is a regulator of cell proliferation and apoptosis [114]. It was therefore studied whether the anti-oxidative effect of 24-hydroxycholesterol involved activation of JNK signaling. This was analyzed using Western blotting with antibodies against JNK and phosphorylated JNK. SH-SY5Y cells were pretreated with and without 24-hydroxycholesterol for 24 h and cells were then treated with t-BHP for 60 min. The results suggest that t-BHP may increase the phosphorylation level of JNK compared to untreated control and 24-hydroxycholesterol might be able to attenuate this phosphorylation (activation).

The results from this study showed that 24-hydroxycholesterol can counteract the t-BHP-induced oxidative stress in the human neuroblastoma cell line SH-SY5Y and in primary rat astrocytes. 24-Hydroxycholesterol even exerted a stronger anti-oxidative effect than the well-studied raloxifene. Previous studies have reported that 24-hydroxycholesterol may protect cells against 7-ketocholesterol-induced toxicity via activation of the LXR pathway [49]. However concentrations above 10 μM of 24-hydroxycholesterol have been reported to have cytotoxic effects and induce apoptosis in SH-SY5Y cells [50]. Thus, reported effects of oxysterols on viability are conflicting. In this study the anti-oxidative effect was only significant in concentrations of 10 μM. The concentration of 24-hydroxycholesterol in plasma is about 0.25 μM, but the concentration in the brain has been reported to be much higher. In fact, the ratio between cholesterol and 24-hydroxycholesterol has been reported to be up to 40 times higher in brain [24, 30]. 24-Hydroxycholesterol is synthesized by CYP46A1 in the brain and previous studies have shown that inhibition of CYP46A1 expression in vivo leads to a decrease of the 24-hydroxycholesterol content and accumulation of neuronal cholesterol. This causes an increase in production of Aß peptides, abnormal tau protein and neuronal death. CYP46A1 has therefore been suggested to be a potential therapeutic target [165]. A study with *Cyp46a1*$^{-/-}$ knockout mice showed an increased production of reactive oxygen species in retina [166]. Results from this thesis indicate protective effects of 24-hydroxycholesterol on both cell viability and on oxidative stress on cells from the nervous system (Paper I and Paper IV). More studies are needed to fully clarify the effect(s) of 24-hydroxycholesterol in the brain. However, from this and previous studies the concentration seems very important for the observed effects of oxysterols. This indicates that it is important to maintain a balance of oxysterols in the brain.

Conclusions

In this thesis, effects of steroids including oxysterols and vitamin D on cell viability/growth in CNS and regulatory effects of vitamin D on brain steroidogenesis were investigated. Based on the results presented in this thesis, the main conclusions can be summarized as follows:

I. 24- and 27-Hydroxycholesterol reduced the staurosporine-induced toxic effect in SH-SY5Y cells. The data indicate that, during some conditions, oxysterols may have neuroprotective effects and can reverse apoptotic events.

II. The vitamin D analogs tacalcitol and calcipotriol strongly reduced proliferation and migration of human glioblastoma T98G cells. These findings suggest a potential therapeutic role for this type of compounds in treatment of brain tumors.

III. Astrocytes were found to be a major site for expression of 3β-HSD whereas expression of CYP17A1 was found in both astrocytes and neurons. Vitamin D suppressed both CYP17A1 and 3β-HSD -mediated activity and mRNA levels. The results indicate that vitamin D may have a suppressive effect on the expression of some of the enzymes in the brain steroidogenesis affecting the formation of sex hormones.

IV. 24-Hydroxycholesterol attenuated oxidative stress in SH-SY5Y cells and astrocytes. The effects of 24-hydroxycholesterol on oxidative stress may be mediated by JNK signaling. Also, the results suggest that 24-hydroxycholesterol may function as an estrogen receptor antagonist. This implies that 24-hydroxycholesterol may prevent and reduce oxidative stress in connection with brain disease.

Svensk sammanfattning/Summary in Swedish

Denna avhandling handlar om effekter av steroider, vitamin D och vitamin D analoger (syntetiska ämnen som har liknande struktur som vitamin D) på cellöverlevnad och celltillväxt i nervsystemet. Studier har också gjorts på vitamin D:s effekter på enzymer involverade i steroidsyntesen dvs. bildning av steroider. Steroider som bildas i hjärnan kallas för neurosteroider. Exempel på neurosteroider är könshormonerna östradiol och testosteron. Enzymer är proteiner som fungerar som biologiska katalysatorer i kroppen. Många av de enzymer som bildar steroider tillhör cytokrom P450 (CYP450) familjen. Neurosteroider har flera olika funktioner i hjärnan. De stimulerar bl.a. signalering, tillväxt och överlevnad av nervceller. Många steroider har också visat sig ha nervskyddande effekter och har föreslagits kunna vara potentiella läkemedel i behandling av sjukdomar som bryter ner nervceller som Alzheimers- och Parkinsons sjukdom. Kunskapen om neurosteroider och steroidproducerande enzymer i hjärnan är begränsad och det behövs mer forskning om neurosteroiders funktion i hjärnan. Startmaterialet för bildning av alla steroider och vitamin D är kolesterol. Hjärnan är det organ som innehåller mest kolesterol i kroppen. I hjärnan är det mesta kolesterolet lokaliserat i myelinmembranen som isolerar nervcellens utskott (axon), vilket medför att nervsignalering kan ske mycket snabbt. Kolesterol är alltför fettlöslig för att kunna passera blod-hjärnbarriären men genom att en OH-grupp adderas så omvandlas kolesterol till 24-hydroxykolesterol eller 27-hydroxykolesterol som inte är lika fettlösliga och därför kan passera blod-hjärnbarriären. Dessa två föreningar är viktiga för kolesterolbalansen i hjärnan. De har också visat sig vara betydelsefulla i neurodegenerativa sjukdomar. Syftet med Arbete I var att studera 24- och 27-hydroxykolesterols effekter på cellöverlevnad i SH-SY5Y celler, som blivit behandlade med det toxiska ämnet staurosporin. SH-SY5Y är en human cellinje, tagen från en tumör i nervsystemet och är en vanlig cellmodell för studier av nervceller. Både 24- och 27-hydroxykolesterol har i detta arbete visat sig ha en skyddande effekt på inducerad toxicitet i SH-SY5Y celler. 27-Hydroxykolesterols skyddande effekt medförde hämning av caspas aktivitet. Caspaser aktiveras vid apoptos som är cellens programmerade celldöd. Detta kan tyda på att 24- och 27-hydroxykolesterol under vissa förhållanden har nervskyddande effekter i hjärnan.

Vitamin D är också en förening som bildas i samband med kolesterolsyntesen. Vi får i oss vitamin D antingen genom födan eller genom produktion i

huden via UV-ljus. Den klassiska funktionen av vitamin D är att upprätthålla kalciumbalansen i kroppen som är viktig för skelettet och musklernas funktion. Vitamin D fungerar som ett steroidhormon vilket innebär att den binder till en receptor (protein som kan binda molekyl och vidarebefordra signaler) i cellkärnan och påverkar genuttrycket för många olika proteiner. Senare tids forskning har visat att vitamin D också påverkar cellöverlevnad, immunsystemet, cancer samt hjärt- och kärlsystemet. Syftet med Arbete II var att studera effekterna av vitamin D-analogerna tacalcitol och calcipotriol på cellöverlevnad och migrering i glioblastom-celler. Tacalcitol och calcipotriol är syntetiska vitamin D föreningar som används för behandling av psoriasis. Glioblastom är en mycket aggressiv och dödlig hjärntumör. Cancerceller med förmågan att migrera bildar metastaser som sprider cancertillväxten i kroppen. Både tacalcitol och calcipotriol hämmade celltillväxten och migrationen av glioblastom-celler men påverkade inte apoptos (programmerad celldöd). Detta kan innebära att vitamin D-analoger i kombination med andra behandlingsformer är möjliga läkemedel för behandling av hjärntumörer.

I Arbete III undersöktes bildning och enzymaktivitet hos enzymerna 3β-HSD och CYP17A1 i olika celler från nervsystemet. 3β-HSD och CYP17A1 är viktiga för bildning av steroidhormoner; framförallt för bildning av könshormoner. Vitamin D:s effekt på genuttryck och aktivitet av 3β-HSD och CYP17A1 undersöktes också. Resultaten visade att CYP17A1 är aktiv i både nervceller och astrocytceller. Astrocyter är celler som hjälper nervceller att fungera, de förser nervceller med syre, näring och för bort restprodukter. 3β-HSD var bara aktiv i astrocyter men inte i nervceller. Vitamin D sänkte genuttrycket av både 3β-HSD och CYP17A1 i astrocyter. I nervceller var det bara CYP17A1 som påverkades av vitamin D. Vitamin D hämmade också enzymaktiviteten i nervceller och astrocyter vilket skulle kunna påverka bildningen av bl.a. könshormoner. Få studier har utförts av vitamin D:s effekter i hjärnan. Resultaten i Arbete III tyder på att vitamin D skulle kunna reglera bildningen av steroider i hjärnan.

I Arbete IV studerades effekter av 24-hydroxykolesterol på oxidativ stress i SH-SY5Y celler och astrocyter. Hjärnan är särskilt utsatt för oxidativ stress pga. dess höga syreomsättning och höga lipid innehåll. Oxidativ stress är involverad i många tillstånd som bryter ned nervceller som stroke och Alzheimers sjukdom. Resultaten i Arbete IV visade att 24-hydroxykolesterol sänkte t-butyl hydroperoxid-inducerad oxidativ stress med så mycket som 70% i SH-SY5Y celler och med 60% i astrocyter. Studien tyder på att mekanismen för 24-hydroxykolesterols antioxidativa effekt skulle kunna involvera proteinet JNK (c-Jun N-terminal kinas), ett protein som reglerar cellöverlevnad. 24-Hydroxykolesterol visade sig också ha en hämmande effekt på receptorer för östrogener, som är viktiga för nervsystemets funktion.

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