Expression and regulation of CYP17A1 and 3β-hydroxysteroid dehydrogenase in cells of the nervous system
Potential effects of vitamin D on brain steroidogenesis.

Emanuelsson, I; Almokhtar, M; Wikvall, K; Grönbladh, A; Nylander, E; Svensson, AL; Fex Svenningsen, Åsa; Norlin, Maria

Published in:
Neurochemistry International

DOI:
10.1016/j.neuint.2017.11.007

Publication date:
2018

Document version:
Accepted manuscript

Document license:
CC BY-NC-ND

Citation for published version (APA):

Go to publication entry in University of Southern Denmark's Research Portal

Terms of use
This work is brought to you by the University of Southern Denmark.
Unless otherwise specified it has been shared according to the terms for self-archiving.
If no other license is stated, these terms apply:

- You may download this work for personal use only.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying this open access version

If you believe that this document breaches copyright please contact us providing details and we will investigate your claim.
Please direct all enquiries to puresupport@bib.sdu.dk

Download date: 01. Dec. 2021
Expression and regulation of CYP17A1 and 3β-hydroxysteroid dehydrogenase in cells of the nervous system: Potential effects of vitamin D on brain steroidogenesis

Ida Emanuelsson, Mokhtar Almokhtar, Kjell Wikvall, Alfhild Grönbladh, Erik Nylander, Anne-Lie Svensson, Åsa Fex Svenningsen, Maria Norlin

PII: S0197-0186(17)30334-0
DOI: 10.1016/j.neuint.2017.11.007
Reference: NCI 4166

To appear in: Neurochemistry International

Received Date: 2 June 2017
Revised Date: 16 October 2017
Accepted Date: 16 November 2017


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Expression and regulation of CYP17A1 and 3β-hydroxysteroid dehydrogenase in cells of the nervous system: potential effects of vitamin D on brain steroidogenesis

Ida Emanuelsson¹*, Mokhtar Almokhtar¹*, Kjell Wikvall¹, Alfhild Grönbladh¹, Erik Nylander¹, Anne-Lie Svensson¹, Åsa Fex Svenningsen² and Maria Norlin¹§

*contributed equally

¹Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden
²Department of Molecular Medicine, Neurobiology Research, University of Southern Denmark, Odense, Denmark

§To whom correspondence should be addressed:
Maria Norlin
Department of Pharmaceutical Biosciences, Uppsala University
Box 591, S-751 24 Uppsala, Sweden
Telephone: +46-18-471 4331
Email: Maria.Norlin@farmbio.uu.se

Keywords
vitamin D, brain, metabolism, neurons, astrocytes, neurosteroids

Abbreviations:
CNS, central nervous system; CYP, cytochrome P450; DHEA, dehydroepiandrosterone; 3β-HSD, 3β-hydroxysteroid dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA box binding protein; TLC, thin layer chromatography
Abstract

Steroids are reported to have diverse functions in the nervous system. Enzymatic production of steroid hormones has been reported in different cell types, including astrocytes and neurons. However, the information on some of the steroidogenic enzymes involved is insufficient in many respects. Contradictory results have been reported concerning the relative importance of different cell types in the nervous system for expression of CYP17A1 and 3β-hydroxysteroid dehydrogenase (3β-HSD). 3β-HSD is important in all basic steroidogenic pathways and CYP17A1 is required to form sex hormones. In the current investigation we studied the expression of these enzymes in cultured primary rat astrocytes, in neuron-enriched cells from rat cerebral cortex and in human neuroblastoma SH-SY5Y cells, a cell line often used as an in vitro model of neuronal function and differentiation. As part of this study we also examined potential effects on CYP17A1 and 3β-HSD by vitamin D, a compound previously shown to have regulatory effects in steroid hormone-producing cells outside the brain. The results of our study indicate that astrocytes are a major site for expression of 3β-HSD whereas expression of CYP17A1 is found in both astrocytes and neurons. The current data suggest that neurons, contrary to some previous reports, are not involved in 3β-HSD reactions. Previous studies have shown that vitamin D can influence gene expression and hormone production by steroidogenic enzymes in some cells. We found that vitamin D suppressed CYP17A1-mediated activity by 20% in SH-SY5Y cells and astrocytes. Suppression of CYP17A1 mRNA levels was considerably stronger, about 50% in SH-SY5Y cells and 75% in astrocytes. In astrocytes 3β-HSD was also suppressed by vitamin D, about 20% at the enzyme activity level and 60% at the mRNA level. These data suggest that vitamin D-mediated regulation of CYP17A1 and 3β-HSD, particularly on the transcriptional level, may play a role in the nervous system.
1. Introduction

Endogenous steroids, such as estrogens and androgens, are reported to have diverse functions in the nervous system, including effects on neuronal survival, inflammation, proliferation, brain plasticity and modulation of synaptic transmission (Arnold and Beyer, 2009; Brann et al., 2007; Porcu et al., 2016). Several metabolic pathways for biosynthesis of different neurosteroids exist in cells of the central nervous system (CNS). However, data indicate that, in line with tissue-specific differences observed in other parts of the body, metabolism varies in different brain cell types (Cascio et al., 2000; Hojo et al., 2004; Manca et al., 2012; Zwain and Yen, 1999).

Cells of the CNS consist of neurons and glial cells. The most abundant cell types in the CNS are the astrocytes and the oligodendrocytes (Brann et al., 2007; Jessen, 2004). Oligodendrocytes are important in the formation of myelin. The enzymatic production of steroid hormones have previously been reported in neurons, astrocytes and oligodendrocytes (Zwain and Yen, 1999). Synthesis of steroid hormones, using cholesterol as starting material, requires several enzymes (Fig. 1) (Benkert et al., 2015; Lundqvist et al., 2010). The first step of this synthesis forms pregnenolone, which is then metabolized into various types of hormones via different pathways. A critical enzyme in these metabolic pathways is 3β-hydroxysteroid dehydrogenase (3β-HSD), necessary for production of the 3-oxo-group present in most of the mature hormones or in their immediate precursors. Deficiency of 3β-HSD leads to disturbed levels of steroid hormones, in particular a decreased or absent production of glucocorticoids and mineralocorticoids which, if untreated, can lead to death in infancy (Labrie et al., 1992).

Certain of the steroidogenic enzymes, such as CYP19A1 (aromatase) which catalyzes aromatization of the steroid A-ring of androstenedione and testosterone to form estrogens, have been well characterized (Garcia-Ovejero et al., 2005). However, for some of the other steroidogenic enzymes information is insufficient in many respects. For instance, conflicting results are reported regarding which cell type in the nervous system is most important for the expression of CYP17A1 and 3β-HSD. CYP17A1 is required to form dehydroepiandrosterone (DHEA) as well as androstenedione, the immediate precursor to testosterone and estrone. Several studies have reported expression of CYP17A1 almost exclusively in neurons (Do Rego et al., 2007; Hojo et al., 2004; Manca et al., 2012). In contrast, another study reported expression of CYP17A1 mRNA and enzyme activity mainly in astrocytes (Zwain and Yen,
1999), whereas yet other authors report undetectable levels of CYP17A1 mRNA in astrocytes (Cascio et al., 2000), or cultures of glial cells (Mellon and Deschepper, 1993).

As described above 3β-HSD has an essential role in all pathways (Fig. 1). Astrocytes appear to be a major site of 3β-HSD expression (Sinchak et al., 2003). However, it has also been reported that this enzyme is expressed mainly in neurons in the rat brain (Schumacher et al., 2004).

In addition to the confusion on which cell type in the nervous system is most important for CYP17A1 and 3β-HSD expression, the knowledge of their regulation is limited, with studies focusing mainly on effects by estrogens (Micevych et al., 2007; Sorwell et al., 2012).

In the current investigation, we studied the expression of CYP17A1 and 3β-HSD in different cells of the nervous system. In addition, we examined potential effects on these enzymes by vitamin D, a compound previously shown to have regulatory effects on genes and enzymes in steroid hormone-producing cells outside the brain (Lundqvist et al., 2010; Merhi et al., 2014). To our knowledge there are very few studies on potential influence of vitamin D in regulation of the metabolism of neurosteroids (Yagishita et al., 2012). We find this of particular interest since levels of vitamin D have been linked to several diseases that affect the brain (Eyles et al., 2013).

2. Materials and methods

2.1. Materials

Human neuroblastoma SH-SY5Y cells (ATCC CRL-2266) were purchased from the American Type Culture Collection (Manassas, VA, USA). Materials for cell culturing were obtained from Thermo Fisher Scientific (Life Technologies). 1α,25-Dihydroxyvitamin D₃ (sc-202877) was purchased from Santa Cruz Biotechnology Inc. Radiolabeled [1,2,6,7-³H(N)]-dehydroepiandrosterone (NET814), [1β-³H(N)]-androst-4-ene-3,17-dione (NET469) and [1,2,6,7-³H(N)]-progesterone (NET381) were obtained from Perkin Elmer. Retinoic acid and unlabeled steroid hormones were purchased from Sigma.

2.2. Animals

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. Rats (Sprague-Dawley) were obtained from Charles River, Germany. Primary rat astrocytes and neuron-enriched cells from rat cerebral cortex were prepared from brain tissue of these animals as described below.

2.3. Preparation of neuron-enriched cortical cell cultures from rat embryos

Primary cortical cell cultures from rat brain, containing neurons and glia at a ratio of approximately 60/40, were prepared from embryos of pregnant Sprague-Dawley rats, removed at embryonic day 17, as described by Nylander et al. (Nylander et al., 2016), Diwakarla et al. (Diwakarla et al., 2016) and Kindlundh-Högberg et al. (Kindlundh-Högberg et al., 2010). The obtained cortical cells were cultured on poly-L-lysine coated plates for 2 weeks in Neurobasal media (which favors neuronal cell growth), supplemented with B-27 (2%), glutamine (600 µM) and antibiotics/antimycotics (1%), prior to experimentation. Media changes were performed twice a week. The amount of neurons in these cultures were determined by assay of microtubule-associated protein 2-positive cells (Nylander et al., 2016).

2.4. Preparation of astrocyte cultures from neonatal rat brain

Primary cultures of astrocytes were prepared from whole brains of newborn rat pups (day 1-3) as described by Fex-Svenningsen et al. (Fex Svenningsen et al., 2011) and McCarthy and de Vellis (McCarthy and de Vellis, 1980). The obtained cells were cultured in poly-L-lysine coated flasks for 7-10 days in DMEM supplemented with fetal bovine serum (10%), L-glutamine (0.3%) and antibiotics/antimycotics (1%). After this time period, the cell culture flasks were shaken at 200 rpm for 18 h in a 37°C shaker incubator to detach and remove microglia and oligodendrocytes from the cultures, leaving the strongly attached astrocytes.

2.5. Cultures of human neuroblastoma SH-SY5Y cells

The study includes experiments on the human neuroblastoma cell line SH-SY5Y. This cell line is often used as an in vitro model of neuronal function and differentiation. SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics/antimycotics (1%) and, in most experiments, with fetal bovine serum (10%). In a separate set of experiments, we attempted to increase the activity by differentiating the SH-SY5Y cells, using retinoic acid, to obtain a more mature neuronal cell type. For these experiments SH-SY5Y cells were differentiated by treatment with retinoic acid (10 µM) for 7 days.
2.6. Incubations with cell cultures

Incubations for assay of enzymatic conversions and mRNA levels in the different cell cultures (see sections 2.3 - 2.5) were carried out in 6-well plates with 1.5 ml of appropriate medium per well, in the presence or absence of 1α,25-dihydroxyvitamin D₃, in concentrations of 10-100 nM. Most of our experiments were carried out with 10 nM of 1α,25-dihydroxyvitamin D₃. Cells were treated for 24 h with 10 nM of 1α,25-dihydroxyvitamin D₃ dissolved in ethanol. Vehicle-treated cells received ethanol (control group).

In some experiments we replaced the normal serum with charcoal-stripped serum to study if this would lead to changes in the observed results, considering that serum contains lipids, including small amounts of steroids, which might potentially influence the assays of steroid metabolism. Results obtained with cells cultured with normal serum and charcoal-stripped serum were however essentially the same.

2.7. Assay of conversion of DHEA to androstenedione (3β-hydroxysteroid dehydrogenase activity) and 7α-hydroxy-DHEA (7α-hydroxylase activity)

Assay of enzymatic conversions mediated by 3β-HSD and 7α-hydroxylase (CYP7B1) was carried out by incubation of cells for 24 h with ³H-DHEA (7 µM, 3 µCi) dissolved in ethanol, followed by extraction of the culture media with ethyl acetate and analysis of formed androstenedione and 7α-DHEA by silica gel thin layer chromatography as described below.

2.8. Assay of conversion of progesterone to 17α-hydroxyprogesterone and androstenedione (CYP17A1 activity)

Assay of CYP17A1 activity was carried out by incubation of cells for 24 h with ³H-progesterone (6 µM, 3 µCi) dissolved in ethanol, followed by extraction of the culture media with ethyl acetate and analysis of formed 17α-hydroxyprogesterone and androstenedione by silica gel thin layer chromatography as described below.

2.9. Assay of conversion of androstenedione to estrone (CYP19A1 activity) and testosterone (17β-hydroxysteroid dehydrogenase activity)

Assay of CYP19A1 activity and 17β-hydroxysteroid dehydrogenase activity was carried out by incubation of cells for 24 h with ³H-androstenedione (7 µM, 3 µCi) dissolved in ethanol,
followed by extraction of the culture media with ethyl acetate and analysis of formed estrone and testosterone by silica gel thin layer chromatography as described below.

2.10. Thin-layer chromatography (TLC)

Thin layer chromatography was carried out essentially as described by Lundqvist and Norlin (Lundqvist and Norlin, 2012), using a mobile phase consisting of chloroform/ethyl acetate 80:20 (v/v). The Rf values for DHEA, 7α-DHEA, androstenedione, progesterone, 17α-hydroxyprogesterone, estrone and testosterone in this system were 0.52, 0.17, 0.64, 0.74, 0.45, 0.74, and 0.41, respectively. In all experiments authentic, unlabeled steroids were used as reference compounds and developed together with the radiolabeled samples. The TLC-plates were scanned for localization and quantitation of the radioactive products, using a AR2000 TLC Imaging Radio scanner (Eckert-Ziegler), followed by exposure to iodine vapors (o/n) to visualize the unlabeled steroids and comparison of Rf values for reference compounds with the samples. Typical TLC-images for analyses of the CYP17A1-mediated conversions of progesterone to 17α-hydroxyprogesterone and androstenedione and the 3β-HSD-mediated conversion of DHEA to androstenedione and 7α-DHEA are shown in Fig. 2 and 3.

2.11. Preparation of mRNA and RT-PCR

Total RNA was isolated from the cells using RNeasy Mini kit (Qiagen) following the manufacturer's protocol and cDNA was prepared from 1 µg of RNA as previously described (Almokhtar et al., 2016). Real-time RT-PCR was used to quantitate the expression levels of mRNA for CYP17A1 and 3β-HSD in SH-SY5Y cells, neuron-enriched rat cortex cells and rat astrocytes. In some experiments we also used semi-quantitative RT-PCR for qualitative analysis of mRNA expression. The primers used for human and rat CYP17A1 and 3β-HSD are shown in Table 1. All real-time RT-PCR data on CYP17A1 and 3β-HSD expressions were normalized to a control (housekeeping) gene. In most of the real-time RT-PCR experiments TATA box binding protein (TBP) was used as control (housekeeping) gene for normalization. In some experiments we also used GAPDH, in order to obtain data for an additional housekeeping gene and examine if changing the control gene might influence the observed results. The reason for choosing TBP in most of our experiments is that from previous studies we have found this a robust control gene that is not easily affected by altered culture conditions or treatment with hormones. When comparing TBP and GAPDH we obtained similar results for all experiments except assay of CYP17A1 mRNA in SH-SY5Y cells where we observed significant suppression by 1α,25-dihydroxyvitamin D₃ when using TBP but not
when using GAPDH. The real-time 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 ΔΔCt method with a stepwise diluted standard curve and expressed as -fold change compared to vehicle-treated cells.

2.12. Immunoblotting

For extraction of proteins, cells were lysed in RIPA buffer containing a mixture of protease inhibitors (Sigma). Total protein extract (15 µg) was denatured in Bolt LDS sample Buffer at 70°C and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Human recombinant 3β-HSD (Novus Biologicals, H00003283-P01), 0.1 µg, was used as positive control. Proteins were transferred onto PVDF membrane and were then hybridized with antibodies against 3β-HSD (Novus Biologicals, NB-78644) overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were added and the membrane was incubated for 1 h at room temperature. Antibody binding was then detected using a SuperSignal West Pico Chemiluminescent Substrate kit.

2.13. Other methods

Analysis of statistical significance was performed using two-tailed Student’s t-test. P values < 0.05 were considered statistically significant. Assay of protein concentrations in cell homogenates, to prepare samples for immunoblotting and to determine specific enzyme activity expressed per mg protein and hour, was performed using a BCA assay kit (Thermo Fisher) according to the manufacturer’s instructions.

3. Results

3.1. CYP17A1-mediated enzyme activity in human SH-SY5Y cells, primary rat astrocytes and neuron-enriched rat cerebral cortex cells

CYP17A1-dependent enzyme activity was measured by incubation of the different cell cultures with ³H-labeled progesterone. CYP17A1 possesses two types of enzyme activities, a hydroxylase activity and a 17,20-lyase activity converting 17α-hydroxypregnenolone and 17α-hydroxyprogesterone into DHEA and androstenedione, respectively, by splitting the side-chain off the steroid nucleus (Fig. 1). All the three types of cell cultures produced the 17α-hydroxylated product in similar rates, about 30-40 pmol/mg protein/h. In addition, all
three cell cultures showed 17,20-lyase activity by producing androstenedione in comparable rates, 30-60 pmol/mg protein/h. The results demonstrate that the two enzyme activities of CYP17A1 are expressed in all of the cell cultures.

3.2. 3β-Hydroxysteroid dehydrogenase-mediated enzyme activity and protein levels in human SH-SY5Y cells, primary rat astrocytes and neuron-enriched rat cerebral cortex cells

The 3β-HSD activity was measured by incubation of the different cell cultures with 3H-labeled DHEA. The astrocytes showed a marked conversion of DHEA into androstenedione, 82-120 pmol/mg protein/h, whereas the activity in SH-SY5Y cells and neuron-enriched cerebral cortex cells was below or at the limit of detection (≤5 pmol/mg protein/h). Treatment of SH-SY5Y cells with retinoic acid, in order to differentiate cells, did not influence 3β-HSD activity. Expression of 3β-HSD was also analyzed by immunoblotting (Fig. 4). Results of immunoblotting support the data from assay of enzyme activity showing expression of 3β-HSD protein in astrocytes but not in SH-SY5Y cells. A weak band for this protein was also observed in neuron-enriched rat cerebral cortex, indicating low but detectable expression.

3.3. Effects of 1α,25-dihydroxyvitamin D3 on CYP17A1 enzyme activity and mRNA levels

The three different cell cultures (SH-SY5Y, astrocytes and neuron-enriched cerebral cortex cells) were treated with 10 nM of 1α,25-dihydroxyvitamin D3, to study its effect on the CYP17A1-mediated hydroxylase activity (rate of conversion of progesterone into 17α-hydroxyprogesterone). The results are summarized in Table 2. The relative CYP17A1 hydroxylase activities between 1α,25-dihydroxyvitamin D3-treated and vehicle-treated cells are given (as –fold change) in Table 2. The results show suppression of CYP17A1 activity of about 20% in SH-SY5Y cells and astrocytes. However, the CYP17A1 activity in neuron-enriched cerebral cortex cells was not significantly influenced by treatment with 1α,25-dihydroxyvitamin D3. The CYP17A1-mediated conversion of progesterone into androstenedione was similarly suppressed by 1α,25-dihydroxyvitamin D3 (data not shown).

In most of the experiments we used a concentration of 10 nM of this compound but we also performed experiments with 100 nM 1α,25-dihydroxyvitamin D3. Increasing the concentration of 1α,25-dihydroxyvitamin D3 to 100 nM did not result in more suppression, instead the results were inconsistent, in some cases showing no effect, in some cases suppression and in some cases even a slight increase of CYP17A1 activity. Because CYP17A1-mediated enzyme activity was slightly suppressed by 1α,25-dihydroxyvitamin D3
in the experiments with SH-SY5Y cells and astrocytes (cf. Table 2) real time RT-PCR was used to investigate the effects of 1α,25-dihydroxyvitamin D₃ on the expression of mRNA for CYP17A1 in SH-SY5Y cells and astrocytes. For human SH-SY5Y cells, treatment with 10 nM 1α,25-dihydroxyvitamin D₃ decreased the mRNA levels by about 50% (Fig. 5A). Upon treatment of rat astrocytes with 1α,25-dihydroxyvitamin D₃, the mRNA levels of CYP17A1 decreased by 75% compared with the levels in vehicle-treated control astrocytes (Fig. 5B). No effect of 1α,25-dihydroxyvitamin D₃ on CYP17A1 mRNA was found in samples prepared from cultures of neuron-enriched cerebral cortex cells.

3.4. Effects of 1α,25-dihydroxyvitamin D₃ on 3β-hydroxysteroid dehydrogenase enzyme activity and mRNA levels

The SH-SY5Y cells, astrocytes and neuron-enriched cerebral cortex cells were also treated with 10 nM-100 nM 1α,25-dihydroxyvitamin D₃ to study its effect on the 3β-HSD activity (rate of conversion of DHEA into androstenedione). The results are summarized in Table 3. The data on the enzyme activities in the different sets of experiments were compared in a similar manner as in Table 2. The 3β-HSD activity in astrocytes was significantly suppressed by 20% in cells treated with 10 nM 1α,25-dihydroxyvitamin D₃. Similarly as in the assay of CYP17A1 activity, increasing the concentration of 1α,25-dihydroxyvitamin D₃ to 100 nM did not further increase the suppressive effect. The enzyme activity in SH-SY5Y cells and neuron-enriched cerebral cortex cells was below or at the limit of detection and was not significantly influenced by treatment with 1α,25-dihydroxyvitamin D₃.

Because the 3β-HSD activity in astrocytes was significantly suppressed by treatment with 1α,25-dihydroxyvitamin D₃ (cf. Table 3), the effect of this treatment on the mRNA levels were also examined. For human SH-SY5Y cells, treatment with 10 nM 1α,25-dihydroxyvitamin D₃ showed no effect on the 3β-HSD expression (Fig. 6A). In neuron-enriched rat cerebral cortex cells the 3β-HSD mRNA levels were very low and we could not detect any effect by treatment with 1α,25-dihydroxyvitamin D₃. However, upon treatment of the rat astrocytes with 1α,25-dihydroxyvitamin D₃, the mRNA levels of 3β-HSD decreased by about 60% compared with the levels in vehicle-treated control astrocytes (Fig. 6B).
3.5. Other metabolic pathways for DHEA and androstenedione

The experiments with 1α,25-dihydroxyvitamin D₃ treatment revealed that the CYP17A1 mRNA expression and 3β-HSD expression were markedly suppressed in astrocytes, whereas the enzyme activities were only slightly suppressed. To further investigate whether this discrepancy could be due to metabolism of androstenedione and DHEA by other enzymes, experiments were conducted on CYP19A1, 17β-hydroxysteroid dehydrogenase and CYP7B1. CYP19A1 (aromatase) catalyzes aromatization of the steroid A-ring of androstenedione to estrone. The CYP19A1 activity, measured as formation of estrone from androstenedione, was 132 ± 18 pmol/mg/h in human SH-SY5Y cells and 254 ± 19 pmol/mg/h in neuron-enriched rat cerebral cortex cells. The CYP19A1 activity was low in rat astrocytes (12 ± 2 pmol/mg/h). The 17β-hydroxysteroid dehydrogenase activity, measured as formation of testosterone from androstenedione, varied between 21 and 72 pmol/mg/h in the three cell cultures (72, 21 and 46 pmol/mg/h, respectively, in SH-SY5Y cells, astrocytes and neuron-enriched cerebral cortex cells). Treatment with 10 nM 1α,25-dihydroxyvitamin D₃ did not significantly influence the CYP19A1 or 17β-hydroxysteroid dehydrogenase activities (data not shown).

It has previously been reported that 7α-hydroxy-DHEA, formed by action of CYP7B1, is the major DHEA metabolite in rat astrocytes (Wicher and Norlin, 2015). In the current study, we observed a formation of 7α-hydroxy-DHEA in astrocytes at a rate of 343 ± 33 pmol/mg/h. Treatment with 10 nM 1α,25-dihydroxyvitamin D₃ did not significantly influence the CYP7B1-dependent hydroxylation of DHEA (data not shown).

4. Discussion

This study addresses important questions regarding the expression of 3β-HSD and CYP17A1, two enzymes essential for production of sex steroids in the CNS, and the potential role for vitamin D in their regulation. Contradictory results have previously been reported concerning the relative importance of different cell types in the nervous system for expression of these two enzymes. The current results indicate that astrocytes are a major site for expression of 3β-HSD whereas expression of CYP17A1 was found in both astrocytes and neurons. For 3β-HSD, the primary rat astrocytes expressed mRNA and protein (as measured by immunoblotting) and catalyzed conversion of DHEA into androstenedione. The neuron-enriched cerebral cortex cells, on the other hand, lacked detectable 3β-HSD-mediated activity, suggesting that neurons do not express 3β-HSD. The neuron-enriched cortex cells contained
approximately 60% neurons in addition to glial cells, the latter including astrocytes. These findings suggest that neurons may not be an important site for 3β-HSD activity. If neurons were active in 3β-HSD-catalyzed reactions one would expect to observe this activity in a culture where the majority of cells are neurons. Instead the neuron-enriched culture showed no detectable activity and very little 3β-HSD mRNA. Furthermore, in other experiments we found that neuroblastoma SH-SY5Y cells, originating from human neural tissue, showed expression of mRNA for 3β-HSD but no detectable 3β-HSD protein or 3β-HSD-mediated activity.

Primary neurons may survive for a limited time without the presence of supporting glial cells, but are dependent on astrocytes for long time survival (Gottschling et al., 2016; Sobieski et al., 2015). In the current study we chose to conduct studies with neuron-enriched cultures, which are co-cultures containing both glia and neurons but with a majority of the latter cells. Using a culture such as this may be considered to have advantages and disadvantages. Neurons in vivo are dependent on interaction with astrocytes and other glia, thus a cell culture containing all cells together would - at least in some respects - resemble a physiological situation. This is in particular because co-cultures allow communication between different cell types, such as is the case for cells in vivo in the brain. A disadvantage with our experimental setup is that it makes it more difficult, even though not impossible, to distinguish between contributions between individual cell types in the cultures containing both glia and neurons. Also, the different cell types within a co-culture may affect each other, and thus we cannot conclude that the findings on expression would necessarily be the same if we cultured the neurons with little or no glial cells present.

Taken together, our current data suggest that neurons are not involved in 3β-HSD reactions. The results do not support two previous studies reporting that 3β-HSD is expressed in rat brain mainly in neurons (Schumacher et al., 2004) and in tadpole brain by both neurons and glial cells (Bruzzone et al., 2010). The results are, however, in agreement with other reports showing expression of 3β-HSD in astrocytes in human, rat and dog brain (Lucetti et al., 2014; Micevych et al., 2007; Sinchak et al., 2003; Yarim and Kabakci, 2002).

We were surprised by the findings of the present study showing that astrocyte cultures clearly express 3β-HSD mRNA, 3β-HSD protein and 3β-HSD-mediated enzyme activity, whereas no activity and very low mRNA and protein levels for this enzyme was found in the neuron-enriched cultures, which contained about 30-40% glial cells. Although it at present must remain a speculation, the neurons present in the co-cultures might produce some agent that
suppresses 3β-HSD expression in the astrocytes. Another possibility might be that other glial cells present in lower amounts in the neuron-enriched cultures but not in the pure astrocyte cultures, could in some way affect the expression of 3β-HSD in astrocytes, at least under the culturing conditions used here. A third possibility, considering that astrocytes are prepared from newborns but neuron-enriched cultures from embryos, might be that before birth this enzyme function is perhaps not fully developed or in some way suppressed. Considering the complex function and interplay of cells in the brain it may be suggested that one of the possible reasons for the contrasting results in the literature of this field is that results could be highly dependent on methodology and culturing conditions.

The current results strongly suggest that both astrocytes and neurons are responsible for CYP17A1 expression. Thus, expression of CYP17A1 and conversion of progesterone into the CYP17A1-mediated products 17α-hydroxyprogesterone and androstenedione were found in cell cultures of rat astrocytes as well as in neuron-enriched cerebral cortex cells. The rates of formation of the CYP17A1-mediated products were comparable in these two different cell cultures. Additionally, undifferentiated and differentiated human SH-SY5Y cells expressed mRNA for CYP17A1 and efficient CYP17A1-mediated enzyme activity. To our knowledge this is the first report showing the expression of CYP17A1 in the human neuroblastoma cell line SH-SY5Y. The current data do not support results from several other studies, reporting no detectable amount of CYP17A1 mRNA in astrocytes (Cascio et al., 2000) and glial cells (Mellon and Deschepper, 1993) or expression of CYP17A1 almost exclusively in the neurons of the adult rat hippocampus (Hojo et al., 2004), frog brain (Do Rego et al., 2007), and cerebellar nuclei of adult male rats (Manca et al., 2012). Our results are similar but not identical to another previous study, reporting expression of CYP17A1 mRNA and formation of the CYP17A1-mediated products DHEA and androstenedione in cultured primary rat astrocytes as well as in neurons (Zwain and Yen, 1999). In that study however, the activity was reported to be higher in astrocytes than in neurons. Potential species differences in expression of these enzymes cannot be excluded.

As outlined above, different culture systems have advantages and disadvantages. Therefore results from different studies may be difficult to compare. Even though primary cultures are in principal normal cells, the cells might not behave normally while in cultures in vitro. Our study provides results that contribute to our understanding of cell specificity in brain steroidogenesis, but more information is clearly needed to fully clarify these processes.

Steroids may impact the brain and peripheral nervous system in several ways. Neurosteroids
and neuroactive steroids are involved in a range of different functions, including neuronal growth, cell survival and neurotransmission (Baulieu, 1998; Brann et al., 2007). Receptors that are affected or modulated by steroids include GABA$_A$, NMDA receptors and sigma receptors. In order to better understand these processes and perhaps target them in therapy to treat disease, e.g. to treat neurodegenerative conditions, much more basic information is needed on the basic functions of cells in the nervous system.

The active vitamin D hormone 1α,25-dihydroxyvitamin D$_3$ regulates the expression of a great number of genes via the vitamin D receptor (VDR) that is expressed in cells of virtually all tissues (Eyles et al., 2013; Lundqvist et al., 2010). Previous studies have shown that vitamin D can influence gene expression and hormone production of steroidogenic enzymes in human adrenocortical NCI-H295R cells as well as in ovarian, breast and prostate cells (Lundqvist et al., 2011; Merhi et al., 2014). There is, however, little information about possible vitamin D-mediated regulatory effects on genes and enzymes of the steroidogenesis in the brain. The current data concerning effects by 1α,25-dihydroxyvitamin D$_3$ on brain steroidogenesis showed a downregulation of the expression of mRNA and enzyme activities for both CYP17A1 and 3β-HSD in primary rat astrocytes. For astrocytes treated with 1α,25-dihydroxyvitamin D$_3$ the mRNA levels of CYP17A1 and 3β-HSD were markedly suppressed (60-70%) compared with vehicle-treated control cells. The downregulation of the corresponding enzyme activities was considerably less (about 20%). The reason for the discrepancy between the stronger vitamin D-mediated effects on mRNA levels compared with the enzyme activity remains to be established. It is possible that these steroids may undergo several additional metabolic reactions in brain cells, perhaps more efficiently than those catalyzed by CYP17A1 or 3β-HSD. If so, other potential metabolizing enzymes may or may not be regulated by 1α,25-dihydroxyvitamin D$_3$ and this might confuse the results. In an effort to examine alternative pathways for metabolism of androstenedione and DHEA, we carried out experiments to study the effect of 1α,25-dihydroxyvitamin D$_3$ on CYP19A1 (aromatase), CYP7B1 and 17β-hydroxysteroid dehydrogenase. However, no vitamin D-regulating effects on these enzyme activities could be observed. It is possible, however, that unknown metabolic events could play a role.

In this study we observed reproducible results in experiments when cells were treated with a concentration of 10 nM 1α,25-dihydroxyvitamin D$_3$. Somewhat surprisingly, increasing the concentration to 100 nM did not result in enhanced suppression. It may be speculated that a concentration as high as 100 nM, which must be considered far from physiological levels,
might lead to multiple effects on the cells. The concentration of 1α,25-dihydroxyvitamin D₃ in individual tissues is unclear although the serum concentration of this compound is about 0.1 nM. The main precursor for this active form of vitamin D₃, 25-hydroxyvitamin D₃, is present in serum in a concentration of about 50-100 nM. This precursor can be taken up by different cells and converted to 1α,25-dihydroxyvitamin D₃. The concentration of 10 nM, that was used this study, was chosen because it is common in studies of effects of active vitamin D₃ and has been used in many published reports on effects of vitamin D in different cells.

Formation and regulation of steroids in the nervous system is not fully understood, despite the many reported functions of these compounds, and therefore more information is needed in this field of research. From the current results, the role of vitamin D-mediated effects in steroidogenesis remain somewhat unclear and would require further investigation to obtain a complete picture of potential roles in vivo. Interestingly, the vitamin D hormone has been proposed to act as a neuroactive hormone and to have multiple functions in the nervous system including effects on neurotransmission and neuroprotection (DeLuca et al., 2013). Low serum levels of vitamin D have been linked to Parkinson’s disease, Alzheimer’s disease, depression and multiple sclerosis (DeLuca et al., 2013; Eyles et al., 2013). Furthermore low prenatal vitamin D levels tend to increase the risk of developing neuronal disorders such as schizophrenia (Eyles et al., 2013). Mechanisms for effects of vitamin D in the brain that might explain these findings are largely unknown. The current data showing a vitamin D-mediated regulation of CYP17A1 and 3β-HSD, particularly on the transcriptional level, contribute to the knowledge on effect of vitamin D in cells of the nervous system and suggest that actions of vitamin D may involve effects on brain steroidogenesis. If this is true for brain cells in vivo, active vitamin D may be a physiological regulator controlling the production of for instance estradiol, a steroid known for its multiple effects in the brain (Arnold & Beyer 2009; Brann et al 2007). The ability for careful regulation of the amount of this and other brain steroids may be essential for the many complex and sensitive functions of the nervous system and if dysfunctional might be expected to lead to disease. Understanding these processes better may hopefully provide better tool for future treatment of conditions where brain function is disturbed.
References


Nylander, E., Grönbladh, A., Zelleroth, S., Diwakarla, S., Nyberg, F., Hallberg, M., 2016. Growth hormone is protective against acute methadone-induced toxicity by


### Tables

**Table 1. Primers used for real-time RT-PCR experiments**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer-F (5’-3’)</th>
<th>Primer-R (5’-3’)</th>
<th>Annealing temp (°C)</th>
<th>No. of cycles</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3β-HSD (human)</td>
<td>5’- GGAATCTGAAAAACGGGC GGC-3’</td>
<td>5’- CTGAGATAAGTAGAAGCTTGT CTCGGAATG-3’</td>
<td>60</td>
<td>28</td>
<td>(Hammer et al., 2005)</td>
</tr>
<tr>
<td>CYP17A1 (human)</td>
<td>5’- GCC ATC AGA AGC TGG AGA AGT-3’</td>
<td>5’- CCA CAG AGG TGG TGG TCT C-3’</td>
<td>56</td>
<td>39</td>
<td>(Lundqvist et al., 2010)</td>
</tr>
<tr>
<td>3β-HSD (rat)</td>
<td>5’- GCAAAAAAGATGCGCGA GAA-3’</td>
<td>5’- GGC ACA AGT ATG CAA TGT GCC-3’</td>
<td>60</td>
<td>40</td>
<td>(Chauvigné et al., 2011)</td>
</tr>
<tr>
<td>CYP17A1 (rat)</td>
<td>5’- TGG CTT TCC TGG TGC ACA ATC-3’</td>
<td>5’- TGA AAG TTG GTG TTC GCC TGA AG-3’</td>
<td>60</td>
<td>40</td>
<td>(Chauvigné et al., 2011)</td>
</tr>
</tbody>
</table>
Table 2

Effect of treatment with $1\alpha,25$-dihydroxyvitamin D$_3$ (10 nM) on the CYP17A1-mediated enzyme activity in human SH-SY5Y cells, primary rat astrocytes and neuron-enriched rat cerebral cortex cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>17$\alpha$-hydroxyprogesterone (-fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SH-SY5Y</strong></td>
<td></td>
</tr>
<tr>
<td>Without $1\alpha,25$-dihydroxyvitamin D$_3$</td>
<td>1.0</td>
</tr>
<tr>
<td>+ $1\alpha,25$-dihydroxyvitamin D$_3$</td>
<td>0.8 ± 0.1*</td>
</tr>
<tr>
<td><strong>Astrocytes</strong></td>
<td></td>
</tr>
<tr>
<td>Without $1\alpha,25$-dihydroxyvitamin D$_3$</td>
<td>1.0</td>
</tr>
<tr>
<td>+ $1\alpha,25$-dihydroxyvitamin D$_3$</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td><strong>Neuron-enriched cerebral cortex cells</strong></td>
<td></td>
</tr>
<tr>
<td>Without $1\alpha,25$-dihydroxyvitamin D$_3$</td>
<td>1.0</td>
</tr>
<tr>
<td>+ $1\alpha,25$-dihydroxyvitamin D$_3$</td>
<td>1.1 (1.0-1.1)</td>
</tr>
</tbody>
</table>

$^3$H-Labeled progesterone (6 µM) was incubated with respective cell culture for 24 h with or without $1\alpha,25$-dihydroxyvitamin D$_3$ (10 nM). The labeled CYP17A1 product, 17$\alpha$-hydroxyprogesterone, was analyzed as described in Materials and methods. The relative CYP17A1 activities in treated cells compared with control cells (without $1\alpha,25$-dihydroxyvitamin D$_3$) are given as -fold change. The mean value of activity in controls for respective cell culture is set to 1. Values for SH-5YSY cells and astrocytes are means ± standard deviation (n=3-5). Values for neuron-enriched cortex cells are means with range (n=2). *, Statistically significant difference between treated cells and cells without $1\alpha,25$-dihydroxyvitamin D$_3$ treatment, p<0.05.
Table 3

Effect of treatment with 1α,25-dihydroxyvitamin D₃ (10 nM) on the 3β-hydroxysteroid dehydrogenase-mediated enzyme activity in human SH-SY5Y cells, primary rat astrocytes and neuron-enriched rat cerebral cortex cells.

<table>
<thead>
<tr>
<th>Androstenedione ( -fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SH-SY5Y</strong></td>
</tr>
<tr>
<td>Without 1α,25-dihydroxyvitamin D₃</td>
</tr>
<tr>
<td>+ 1α,25-dihydroxyvitamin D₃</td>
</tr>
<tr>
<td><strong>Astrocytes</strong></td>
</tr>
<tr>
<td>Without 1α,25-dihydroxyvitamin D₃</td>
</tr>
<tr>
<td>+ 1α,25-dihydroxyvitamin D₃</td>
</tr>
<tr>
<td><strong>Neuron-enriched cerebral cortex cells</strong></td>
</tr>
<tr>
<td>Without 1α,25-dihydroxyvitamin D₃</td>
</tr>
<tr>
<td>+ 1α,25-dihydroxyvitamin D₃</td>
</tr>
</tbody>
</table>

³H-Labeled DHEA (7 μM) was incubated with respective cell culture for 24 h with or without 1α,25-dihydroxyvitamin D₃ (10 nM). The labeled 3β-hydroxysteroid dehydrogenase product, androstenedione, was analyzed as described in Materials and methods. The relative 3β-hydroxysteroid dehydrogenase activities in treated cells compared with control cells (without 1α,25-dihydroxyvitamin D₃) are given as –fold change. The mean value of activity in controls for respective cell culture is set to 1. Values for astrocytes are means ± standard deviation (n=10). *, Statistically significant difference between treated cells and cells without 1α,25-dihydroxyvitamin D₃ treatment, p<0.05. a, activity below or at the limit of detection, no influence by 1α,25-dihydroxyvitamin D₃ treatment.
Figure captions

Fig. 1. Overview of enzymes in steroidogenesis

Fig. 2. TLC analysis of conversion of progesterone into 17α-hydroxyprogesterone and androstenedione in incubations with astrocytes for (A) 0 and (B) 48 h.

Fig. 3. TLC analysis of conversion of DHEA into androstenedione and 7α-hydroxy-DHEA in incubations with astrocytes for (A) 0 and (A) 48 h.

Fig. 4. Analysis of 3β-HSD protein levels in extracts of SH-SY5Y cells, astrocytes and neuron-enriched cortex cells. Cell lysates (15 µg) were analyzed by immunoblotting as described in Materials and methods, using an antibody against human and rat 3β-HSD. Human recombinant 3β-HSD (0.1 µg) was used as positive control. 1, SH-SY5Y cells; 2, astrocytes; 3, neuron-enriched cells; 4, recombinant 3β-HSD (positive control)

Fig. 5. Treatment with 1α,25-dihydroxyvitamin D₃ (1.25D₃) decreased CYP17A1 mRNA levels in SH-SY5Y cells (A) and astrocytes (B). Cells were treated for 24 h with 10 nM of 1α,25-dihydroxyvitamin D₃. After 24 h of treatment, RNA was extracted and the mRNA levels were determined using real-time RT-PCR as described in Materials and methods. TBP was used as housekeeping gene for normalization. The mRNA levels are shown as fold change compared to vehicle-treated cells (control). Data are given as mean ± standard deviation (n = 3). *Statistically significant difference (p < 0.05).

Fig. 6. Treatment with 1α,25-dihydroxyvitamin D₃ (1.25D₃) had no effect on 3β-HSD mRNA level in SH-SY5Y cells (A) but decreased 3β-HSD mRNA level in astrocytes (B). Cells were treated for 24 h with 10 nM of 1α,25-dihydroxyvitamin D₃. After 24 h of treatment, mRNA was extracted and the RNA levels were determined using real-time RT-PCR as described in Materials and methods. TBP was used as housekeeping gene for normalization. The mRNA levels are shown as -fold change compared to vehicle-treated cells (control). Data are given as mean ± standard deviation (n = 3). *Statistically significant difference (p < 0.05).
unspecific bands

3β-HSD

1 2 3 4
Highlights

* Expression of steroidogenic enzymes were studied in different CNS cell cultures
* CYP17A1 activity was observed in both neuronal cell cultures and astrocytes
* 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was found only in astrocytes
* Vitamin D suppressed mRNA levels and activities of 3β-HSD and CYP17A1
* The biological actions of vitamin D may involve effects on brain steroidogenesis