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Non-stereo-selective cytosolic human brain tissue 3-ketosteroid reductase is refractory to inhibition by AKR1C inhibitors

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Abstract

Cerebral 3 α -hydroxysteroid dehydrogenase (3 α -HSD) activity was suggested to be responsible for the local directed formation of neuroactive 5 α ,3 α -tetrahydrosteroids (5 α ,3 α -THSs) from 5 α -dihydrosteroids. We show for the first time that within human brain tissue 5 α -dihydroprogesterone and 5 α -dihydrotestosterone are converted via non-stereo-selective 3-ketosteroid reductase activity to produce the respective 5 α ,3 α -THSs and 5 α ,3 β -THSs. Apart from this, we prove that within the human temporal lobe and limbic system cytochrome P450c17 and 3 β -HSD/ Δ^5 - Δ^4 ketosteroid isomerase are not expressed. Thus, it appears that these brain regions are unable to conduct *de novo* biosynthesis of Δ^4 -3-ketosteroids from Δ^5 -3 β -hydroxysteroids. Consequently, the local formation of THSs will depend on the uptake of circulating Δ^4 -3-ketosteroids such as progesterone and testosterone. 3 α - and 3 β -HSD activity were (i) equally enriched in the cytosol, (ii) showed equal distribution between cerebral neocortex and subcortical white matter without sex- or age-dependency, (iii) demonstrated a strong and significant positive correlation when comparing 46 different specimens and (iv) exhibited similar sensitivities to different inhibitors of enzyme activity. These findings led to the assumption that cerebral 3-ketosteroid reductase activity might be catalyzed by a single enzyme and is possibly attributed to the expression of a soluble AKR1C aldo-keto reductase. AKR1Cs are known to act as non-stereo-selective 3-ketosteroid reductases; low AKR1C mRNA expression was detected. However, the cerebral 3-ketosteroid reductase was clearly refractory to inhibition by AKR1C inhibitors indicating the expression of a currently unidentified enzyme. Its lack of stereo-selectivity is of physiological significance, since only 5 α ,3 α -THSs enhance the effect of GABA on the GABA_A receptor, whereas 5 α ,3 β -THSs are antagonists.

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Keywords

Human brain; 3 α /3 β -hydroxysteroid dehydrogenase; HSD; aldo-keto reductase; stereo-selective; GABA-A receptor

1. Introduction

The reduction of Δ^4 -3-ketosteroids into 5 α (β)-dihydrosteroids (5 α (β)-DHSs) via ubiquitous 5 α -reductase (EC 1.3.1.30) or hepatic 5 β -reductase (EC 1.3.99.6) activity is a prerequisite for the subsequent reduction of their 3-keto-moiety via 3 α /3 β -HSD activity [1]. Besides their important catabolic function in the inactivation of Δ^4 -3-ketosteroid hormones, combined 5 α -reductase and 3 α -HSD (EC 1.1.1.50) activities were also suggested to be responsible for the formation of 5 α ,3 α -THSs as neuroactive compounds [2]. 5 α ,3 α -THSs act as potent positive allosteric mediators of the GABA_A receptor resembling the effects of benzodiazepines and barbiturates [3,4] and bind to specific transmembrane sites of the GABA_A receptor [5] leading to a potentiation of GABA-gated chloride conductance. As a consequence, they exhibit anesthetic, analgesic, anticonvulsant, anxiolytic and antidepressant properties [3,6–9].

Previous studies with murid rodents and birds suggested a possible intracerebral *de novo* biosynthesis of 5 α ,3 α -THSs from cholesterol via consecutive cytochrome P-450_{scc} (EC 1.14.15.6), 3 β -HSD/ Δ^5 - Δ^4 ketosteroid isomerase (3 β -HSD/KSI; EC 1.1.1.145), cytochrome P450_{c17} (EC 1.14.99.9), 5 α -reductase and 3 α -HSD activities [10]. Apart from this, the following observations suggested an intracerebral formation of neuroactive 5 α ,3 α -THSs from Δ^4 -3-ketosteroids: (i) the inhibitory neurotransmission caused by Δ^4 -3-ketosteroids does not require nuclear receptors [11], (ii) the sedative-anesthetic effects of Δ^4 -3-ketosteroids are mediated by their 5 α -DHS derivatives as well as by the subsequently formed 5 α ,3 α -THS derivatives [12,13], (iii) the behavioral and electrophysiological responses to Δ^4 -3-ketosteroids were attenuated by inhibitors of 5 α -reductase or 3 α -HSD activities, whereas the responses to the respective 5 α ,3 α -THS derivatives were not affected [2,13–15], (iv) GABA_A receptor mediating effects of Δ^4 -3-ketosteroids were not observed in the 5 α -reductase type 1 knockout mouse [16], and (v) previous animal studies demonstrated the cerebral co-expression of 5 α -reductase and 3 α -HSD activity [17]. In accordance with this, we previously revealed relatively high expression of 5 α -reductase type 1 in the human temporal lobe [18,19], whereas human brain tissue 3-ketosteroid reductase has not yet been specifically investigated to date.

Four highly homologous human enzymes of the AKR1C subfamily in the aldo-keto reductase (AKR) superfamily are known to act *in vivo* as NADPH-dependent non-positional-specific ketosteroid reductases in an isoform-specific manner [20–22]. Contrary to their stereo-selective 17 β -HSD (EC 1.1.1.51; unpublished data) and 20 α -HSD (EC 1.1.1.149) activities [23], soluble AKR1Cs act as non-stereo-selective 3 α /3 β -HSDs catalyzing the reduction of 3-ketosteroids into 3 α - and 3 β -hydroxy-derivatives [22]. *In vitro*, AKR1C1 was shown to function as a 3 β -HSD with subsidiary 3 α -HSD activity, AKR1C2 as an efficient 3 α -HSD with negligible 3 β -HSD activity, AKR1C3 as a weak 3 α /3 β -HSD, and AKR1C4 as a highly efficient 3 α -HSD with subsidiary 3 β -HSD activity using 5 α -dihydrotestosterone (5 α -DHT) as substrate [22].

The major purpose of the present study was to elucidate the characteristics of human brain tissue 3-ketosteroid reductase with the aim to possibly clarify if AKR1C expression is responsible. Investigating surgical biopsies of the human temporal lobe and limbic system we show for the first time that within brain tissue 5 α -DHSs are reduced via non-stereo-selective cytosolic 3-ketosteroid reductase activity. Moreover, we could clearly demonstrate that this activity is most likely not attributed to the expression of AKR1Cs but possibly to a currently unidentified AKR-related protein. A secondary aim of the study was to prove if human brain

tissue is able to conduct *de novo* biosynthesis of neuroactive THSs from cholesterol. Here, we show that 3 β -HSD/KSI and cytochrome P450c17 are absent indicating that the local formation of THSs requires a remote source of Δ^4 -ketosteroids.

2. Human tissues and cell lines

As described previously [24], brain tissue was obtained from patients suffering from temporal lobe epilepsy undergoing therapeutic partial temporal lobectomy or amygdalo-hippocampectomy. In general, tissue situated around the presumed epileptic focus was not used and we only included specimens that appeared macroscopically and microscopically inconspicuous. Exceptions were made in the 3 β -HSD/KSI studies, where we also investigated inevitably non-normal parahippocampal gyrus and hippocampus specimens. Histological signs of tumor growth or inflammation always led to exclusion from the study [24]. Adrenal tissue was obtained from a 45-yr-old female patient with kidney cancer undergoing nephrectomy. Heart muscle tissue of a 68-yr-old female patient and liver tissue of a 59-yr-old female patient were obtained from biopsies carried out to rule out disease. Human term placenta was obtained immediately following cesarean section. The U-87 astrocytoma and the JEG3 choriocarcinoma cell lines were purchased from ATCC (Manassas, VA, USA). Surgical removal of all tissues utilized in this study was clinically indicated. The study was approved by the local ethics committee. Written informed consent was received from all tissue donors or their guardians.

3. Chemicals

Radioactively labeled steroids were obtained from PerkinElmer Life Sciences (Zaventem, Belgium), non-radioactive steroids from SigmaTM Chemical Company (Deisenhofen, Germany) and Steraloids Inc. (Newport, Rhode Island, USA), respectively. Pyridine nucleotides, PCR buffer, the expand long template PCR system, deoxyribonucleotides and the DNA size markers were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Trizol LS reagent and SuperscriptTM II First-Strand Synthesis System for RT-PCR were purchased from Invitrogen GmbH (Karlsruhe, Germany). The QuantiTect SYBR Green PCR Kit and the QIAquick gel extraction kit were obtained from Qiagen (Hilden, Germany). All other chemicals and solvents were purchased from SigmaTM Chemical Company or Merck AG (Darmstadt, Germany) and were of American Chemical Society grade or better.

4. Laboratory methods

4.1. Tissue preparation and incubation procedures

Tissue preparation and incubation procedures were identical to previously described protocols [18,24]. Besides buffered brain tissue homogenate, the reaction mixtures contained a saturating source of cofactor and one of the following substrates: (i) 1 μ mol/L [4-¹⁴C]progesterone, (ii) 0.1 μ mol/L [1 β ,2 β -³H]5 α -DHT plus unlabeled 5 α -DHT to achieve final substrate concentrations, (iii) 0.1 μ mol/L [1 β -³H] Δ^4 -androstenedione, (iv) 2 μ mol/L [4-¹⁴C]DHEA, or (v) 0.1 μ mol/L [1,2-³H] Δ^5 -androstenediol. All incubations were performed at pH 7.5 and 37 °C. The progesterone metabolism assays were terminated by the addition of 0.3 mL ice-cold water and 2.5 mL ice-cold ethyl acetate. All other reactions were terminated by the addition of 1 mL ice-cold chloroform:methanol (2:1, vol/vol). Steroids were extracted as described [24].

4.2. Thin layer chromatography (TLC)

The organic extracts of the progesterone metabolism assays were entirely evaporated under a stream of nitrogen and then dissolved in 110 μ L acetonitrile/ethanol (9:2; v/v) containing 25 μ g each of non-radioactive reference steroids. To achieve two-dimensional separation of metabolites and reference standards on F₂₅₄-TLC plates (Merck AG, Darmstadt, Germany),

chromatograms were developed twice in chloroform/ethyl acetate (88:11; v/v) in the Y-direction, and then once in dichloromethane/diethyl ether (77:23, v/v) in the X-direction. In all other assays, 200 μ L aliquots of the respective extracts were evaporated and dissolved in 50 μ L chloroform/ethanol (35:15; v/v) containing the respective reference standards. To achieve one-dimensional separation on LK6D TLC plates (Whatman International Ltd, Kent, UK), chromatograms were developed as follows: (i) thrice in dichloromethane/diethyl ether (11:1, v/v) in the 3-ketosteroid reductase assays; (ii) once in dichloromethane/acetone (9:1, v/v) in the 3 β -HSD/KSI assays; (iii) once in dichloromethane/acetone (92.5:7.5, v/v) in the 5 α -reductase assays [18]; and twice in chloroform/ethyl acetate (88:11; v/v) in the 20 α -HSD assays.

4.3. Metabolite identification and quantification

Following two-dimensional TLC, chromatograms were exposed to X-ray film at -80°C for two weeks. Subsequently, the Molecular Imager GS-800 densitometer (Bio-Rad, München, Germany) was used to estimate metabolite formation by calculating the staining intensities of the radiosignals on the X-ray film after background correction. The positions of radioactive steroid signals were verified by staining of co-chromatographed authentic non-radioactive reference standards as described previously [18].

To detect radioactive metabolites following one-dimensional TLC, the radio-distribution in the Y-direction was scanned with the automatic LB 285 TLC-linear analyzer (EG&G Berthold, Wildbad, Germany) as described [24]. Enzyme activities were assessed by quantifying the formation of: (i) 3 α - and 3 β -androstenediol from 5 α -DHT in the 3-ketosteroid reductase assays; (ii) Δ^4 -androstenedione from DHEA or testosterone from Δ^5 -androstenediol in the 3 β -HSD/KSI assays; (iii) 5 α -androstenedione plus androsterone from Δ^4 -androstenedione (or 5 α -DHP plus 5 α ,20 α -THP, 5 α ,3 α -THP and 5 α ,3 β -THP from progesterone) in the 5 α -reductase assays; and (iv) 20 α -hydroxyprogesterone plus 5 α ,20 α -THP from progesterone in the 20 α -HSD assays.

4.4. Gas chromatography mass spectrometry (GC-MS)

To investigate 5 α -DHT metabolites by GC-MS analysis, a cytosolic subcellular fraction was prepared from pooled subcortical white matter (SC) specimens of an 11-yr-old and a 54-yr-old male patient and incubated using an 8 ml incubation system with 5 α -DHT (20 μ mol/L final concentration) and saturating NADPH. Negative control incubations were conducted without the addition of 5 α -DHT. Radioactive metabolites co-migrating in TLC with authentic 3 α - and 3 β -androstenediol, respectively, were eluted from silica gel and GC-MS analysis was performed after derivatization of hydroxy-groups to trimethylsilyl ethers as described previously [24]. The identity of the reaction products were qualified by comparison of their retention times and spectra with those of authentic 3 α - and 3 β -androstenediol, respectively.

4.5. mRNA detection

Total RNA was extracted, RT was performed, and PCR products were resolved and visualized as described previously [25]. Specific amplification of 3 β -HSD/KSI cDNA was achieved utilizing a 35-cycle RT-PCR protocol for the simultaneous amplification of both isoforms [26]. Isoform-specific quantification of AKR1C mRNA expression was carried out according to a previously described real-time PCR protocol [27] using the ABI 7700 Instrument (Applied Biosystems, Weiterstadt, Germany) and the non-specific DNA binding dye SYBR green I for the detection of PCR products. Initial differences in the amount of RNA subjected to RT were corrected by calculating the mRNA expression ratio of the investigated gene to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [25].

5. Data analysis

Statistical differences were calculated using either the Mann-Whitney U test (sex differences) or the Wilcoxon rank test (differences between enzyme activity in cerebral neocortex [CX] and SC). *P* values < 0.05 were considered to reflect statistical significance. Curve-fitting and kinetic analyses were performed with computer-assisted, non-linear curve-fitting methods using the Fig. P 2.7 program (Biosoft, Cambridge, UK).

6. Results

6.1. Cerebral 3-ketosteroid reductase activity catalyzes non-stereo-selectively the conversion of 5 α -DHSs into 3 α /3 β -THSs

First of all, we aimed at further supporting the hypothesis that combined 5 α -reductase and 3 α -HSD activity are responsible for the local formation of neuroactive THSs from 3 Δ^4 -3-ketosteroids also in the human brain by using progesterone as source substrate. In previous studies with brain tissue of different mammalian species, reduction of progesterone via 5 α -reductase, 3 α -HSD and 20 α -HSD activities was observed [28,29]. If cell-free tissue preparations are incubated with a source of NADPH conversion of progesterone via cytochrome P450c17 (17 α -hydroxylase/17,20-lyase) and 21-hydroxylase (EC 1.14.99.10) activities might also be observed [30]. Consequently, we developed a two-dimensional TLC method that enabled separation of the respective reference standards.

Cell-free CX as well as SC homogenates of temporal lobe tissue from a 39-yr-old woman and a 22-yr-old man were incubated with progesterone and saturating NADPH (re-generating system). Differences between the two genders were not observed. The formation of 5 α -DHP and 20 α -hydroxyprogesterone as well as their subsequent reduction to yield 5 α ,20 α -THP verifies the co-expression of 5 α -reductase and 20 α -HSD activities (Figs. 1B & 1C). Besides, 5 α -DHP was further reduced via non-stereo-selective 3-ketosteroid reductase activity to yield both 5 α ,3 α - and 5 α ,3 β -THP (for specific activities see Table 1). 3 β -HSD activity clearly dominated over 3 α -HSD activity in CX as well as in SC with a mean ratio of 3.1. In addition, the formation of a minor unidentified metabolite migrating in close proximity to 5 α -DHP was observed. Neither cytochrome P450c17 nor 21-hydroxylase activity was detected.

To verify the expression of non-stereo-selective 3-ketosteroid reductase activity in human brain tissue, we substituted [1 β ,2 β -³H]5 α -DHT for 5 α -DHP, since the latter was unavailable in a radioactively labeled form. To begin with, we compared the NADPH-dependent reduction of 5 α -DHT in temporal lobe tissue (pooled from an 18-yr-old woman and a 26-yr-old man) with its conversion in tissues known to express 3 α -HSD and/or 3 β -HSD activity (Fig. 1D). 3 β -Androstenediol was the major metabolite in adrenal cortex and placenta, most likely reflecting *in vitro* 3-ketosteroid reductase activity of highly expressed 3 β -HSD/KSI in these tissues [31]. Although 3 β -HSD/KSI prefers NAD(H) over NAD(P)(H) [32,33], detection of its activity with the incorrect cofactor can be explained by the incubation of relatively large amounts of un-dialyzed cell-free homogenates containing high whole-cell protein concentration. As a consequence, mitochondrial nicotinamide nucleotide transhydrogenase will interconvert added NADPH and tissue NAD⁺ to gain NADH and NADP⁺ [34]. Besides, oxidative 17 β -HSD activity with enzyme-generated NADP⁺ was also observed in the experiment with adrenal cortex converting 5 α -DHT into 5 α -androstenedione and 3 β -androstenediol into epiandrosterone. In contrast to the assays with adrenal cortex and placenta, 5 α -DHT was reduced into both 3 α - and 3 β -androstenediol using liver and brain tissue homogenates (for specific activities see Table 1). Since no differences in cerebral 3-ketosteroid reductase activity were observed when saturating NADPH (3 mmol/L) was substituted for the NADPH re-generating system (data not shown), subsequent studies were performed with only NADPH.

6.2. Characteristics of cerebral 3-ketosteroid reductase activity

To decipher the subcellular distribution of cerebral 3-ketosteroid reductase activity, whole cell homogenates as well as nuclear, membrane and cytosolic fractions from temporal lobe tissue were incubated with 5 α -DHT and saturating NADPH. As a control for subcellular fractionation, we show that microsomal 5 α -reductase activity to reduce Δ^4 -androstenedione was enriched in the membrane fraction (Fig. 2C). In contrast, 3 α - and 3 β -HSD activities were equally enriched in the cytosolic fraction (Figs. 2A & 2B) with a ratio of 3 α - versus 3 β -HSD activity of 3.6.

To determine if differences exist in the specific tissue distribution patterns of cerebral 3 α - and 3 β -HSD activity, we investigated the NADPH-dependent reduction of 5 α -DHT in cell-free preparations of CX and SC specimens from thirteen women and ten men. Data analyses found no sex differences or age-dependency. Therefore, we combined all data from female and male patients to investigate if differences exist between CX and SC. Both, 3 α - and 3 β -HSD activity were found to be significantly higher in SC than in CX (Fig. 3A & 3B; for specific activities see Table 1). A scatter-plot of 3 α -HSD activity in all the specimens relative to the respective 3 β -HSD activity revealed a strong and significant correlation between the two activities (Fig. 3C) with a constant ratio of 3 α -HSD activity versus 3 β -HSD activity of 3.6 ± 0.6 (mean $\pm$ SD).

6.3. GC-MS analysis verifies the non-stereo-selective conversion of 5 α -DHT

To unequivocally verify non-stereo-selective cerebral 3-ketosteroid reductase activity by GC-MS analysis, we conducted long term large, scale incubation of brain tissue cytosol with 5 α -DHT and saturating NADPH. Digital autoradiography detected the formation of two major metabolites with R_f -values in TLC identical to authentic 3 α - and 3 β -androstenediol (Fig. 4A). Due to the long incubation period, the formation of additional minor metabolites was observed with R_f -values in TLC identical to authentic (epi)androsterone and 5 α -androstenedione reflecting the presence of oxidative cerebral 17 β -HSD activity using enzyme-generated NADP⁺.

GC-MS analyses (Fig. 4C – 4J) of the trimethylsilyl ethers of the two major radioactive 5 α -DHT metabolites detected compounds with retention times and characteristic ion fragment patterns identical to those of the trimethylsilyl ethers of authentic 3 α - and 3 β -androstenediol. Metabolites with retention times and characteristic ion fragment patterns identical to 3 α - and 3 β -androstenediol were not detected when the homogenate was incubated in the absence of 5 α -DHT (negative control; data not shown).

6.4. 3 β -HSD/KSI is not expressed in the human temporal lobe

To exclude that membrane-associated 3 β -HSD/KSI is responsible for the observed cerebral 3 β -HSD activity towards 5 α -DHTs, we specifically investigated its expression in human brain tissue. High mRNA expression was observed in the positive controls. In contrast, legitimate mRNA expression levels were not detectable in the human temporal lobe (Fig. 5A), parahippocampal gyrus and hippocampus (data not shown). Sequence analyses verified the expression of the type 1 isoform in the JEG3 choriocarcinoma cell line and the type 2 isoform in the adrenal cortex and heart muscle.

To verify the absence of functional 3 β -HSD/KSI, we also investigated if DHEA was converted into Δ^4 -androstenedione and if Δ^5 -androstenediol was converted into testosterone, respectively. Adrenal cortex was used as positive control, temporal lobe specimens as the unknowns. As expected, high NAD⁺-dependent 3 β -HSD/KSI activity converting DHEA and Δ^5 -androstenediol was observed in the positive control (Figs. 5B & 5C). The detected specific activity (see Table 1) confirms the efficacy of our assay: it (i) exceeds the value previously reported for purified human placental microsomes by one order of magnitude [33], and (ii)

falls below the value previously reported for submitochondrial rat adrenal cortex particles by only one order of magnitude [35]. In contrast to adrenal cortex, 3β -HSD/KSI activity was not detectable in human brain tissue. Instead, in the presence of NADP^+ DHEA was reduced via 17β -HSD activity to produce Δ^5 -androstenediol (Fig. 5B), most likely reflecting enzyme-generated formation of NADPH from added NADP^+ . Moreover, cerebral 17β -HSD activity oxidizing Δ^5 -androstenediol into DHEA was observed in the presence of either cofactor (Fig. 5C).

Additional experiments verified the absence of 3β -HSD/KSI activity in subcellular fractions from CX, SC, parahippocampal gyrus and hippocampus specimens of four female (aged 1, 24, 25, 38 yrs) and four male patients (aged 1, 25, 30, 38 yrs). These were incubated with DHEA as well as pregnenolone as a further substrate of 3β -HSD/KSI. Although increasing incubation periods from 1 h to 4 h were applied, 3β -HSD/KSI activity was again not detectable (data not shown). The limit of detection corresponded to a specific activity five orders of magnitude less than in the respective adrenal cortex positive control.

6.5. Soluble AKR1C isoforms are expressed in the human temporal lobe

We hypothesized that expression of soluble AKR1Cs might be responsible for the observed cerebral $3\alpha/3\beta$ -HSD activity. Previously, we quantified AKR1C3 mRNA expression in the human temporal lobe [25,36]. To address the whole human AKR1C subfamily, we now investigated the expression of the remaining three isoforms by real-time RT-PCR analysis. Overall, AKR1C1 and AKR1C2 demonstrated low expression (Table 2), whereas AKR1C4 was not detectable.

6.6. Cerebral 3-ketosteroid reductase activity is refractory to inhibition by AKR1C inhibitors

To further phenotype cerebral 3-ketosteroid reductase, we used a panel of known AKR1C inhibitors, i.e. phenolphthalein (AKR1C4-selective), indomethacin (AKR1C3-specific), flufenamic acid (inhibitor of AKR1C1–1C3 activity), and 5β -cholan-3 α ,7 α -diol (AKR1C2-specific) [22,37–39]. We investigated the inhibitory effects of excess amounts of these substances on the NADPH-dependent reduction of 5α -DHT using a cytosolic brain tissue preparation. Surprisingly, none of the compounds acted as a significant inhibitor of cerebral 3-ketosteroid reductase activity (Fig. 6A). By contrast, the reduction of 5α -DHT within liver cytosol was potently inhibited by phenolphthalein and to a lesser extent by flufenamic (Fig. 6B) reflecting that the predominant activity was AKR1C4 with some minor contribution from AKR1C1–1C3. In the co-incubation experiment of brain and liver cytosol, the reduction of 5α -DHT was obviously catalyzed by both, cytosolic brain and liver enzymes, but it appeared that solely hepatic AKR1C activity was inhibited by phenolphthalein and flufenamic acid (compare Figs. 6A, 6B & 6C).

Finally, we compared the inhibitory effects of flufenamic acid on human brain tissue 3- and 20-ketosteroid reductase activities with those of quercetine and hydroxymercuribenzoate (two previously described inhibitors of human brain tissue aldehyde reductase activity [40]). Using a cytosolic subcellular SC fraction as the enzyme source, we detected NADPH-dependent 20α -HSD activity, which reduced progesterone into 20α -hydroxyprogesterone (> 95% of the detectable metabolites). In contrast to cerebral $3\alpha/3\beta$ -HSD activity (Figs. 6D & 6E), cerebral 20α -HSD activity was potently inhibited by flufenamic acid (Fig. 6F). Moreover, quercetine acted as a moderate and hydroxymercuribenzoate as a strong inhibitor of 3α -, 3β -, and 20α -HSD activities.

7. Discussion

Neuroactive 3 α -THSs offer new therapies for GABA_A receptor related disorders [9,41]. Consequently, knowledge on the enzymes responsible for their cerebral formation and/or metabolism is desirable. Within mature human brain tissue, the Δ^4 -ene of Δ^4 -3-ketosteroids is reduced via 5 α -reductase type 1 activity [18,19], whereas 5 β -reductase is not expressed [18,29,42]. The final step in the cerebral formation of neuroactive THSs - i.e. the reduction of the 3-keto-group of 5 α -dihydrosteroids via 3 α /3 β -HSD activity - has not yet been specifically investigated to date.

7. 1. 5 α -DHSs are non-stereo-selectively reduced via cerebral 3-ketosteroid reductase activity

The detected NADPH-dependent reduction of progesterone via co-expressed 5 α -reductase, 20 α -HSD and 3-ketosteroid reductase activities (Figs. 1B & 1C) is consistent with the results of previous studies investigating human fetal brain tissue homogenates [29] or rat brain tissue slices [28]. However, contrary to these studies, which reported conversion of 5 α -DHP via only 3 α -HSD activity, we detected the formation of both 5 α ,3 α -THP and 5 α ,3 β -THP. Moreover, 3 β -HSD activity prevailed over 3 α -HSD activity leading to a predominant formation of 5 α ,3 β -THP. Non-stereo-selective conversion of 5 α -DHSs was verified substituting 5 α -DHT for 5 α -DHP (Fig. 1D). Here, the cerebral 3 α -HSD activity appeared to predominate over 3 β -HSD activity indicating a substrate-dependent inversion of the stereo-chemical course in comparison to the experiment with progesterone. Subsequent GC-MS analyses unequivocally verified the cerebral conversion of 5 α -DHT via non-stereo-selective 3-ketosteroid reductase activity (Fig. 4).

7. 2. Cerebral 3 α -HSD and 3 β -HSD activity appear to be catalyzed by a single enzyme

3 α -HSD and 3 β -HSD activity were equally enriched in the cytosolic subcellular fraction (Fig. 2) indicating that the same enzyme(s) might be responsible for the two reactions. This assumption is corroborated by the finding that the two reactions showed equal distribution in CX and SC without sex- or age-dependency (Figs. 3A & 3B). This manifests in a strong and significant correlation between 3 α - and 3 β -HSD activities in the investigated 46 different human temporal lobe specimens (Fig. 3C). Moreover, both reactions were equally affected by different inhibitors of enzyme activity (Fig. 6).

7. 3. Co-expression of 5 α -reductase and 3-ketosteroid reductase activity in the human brain

Previously, we revealed sex- and age-independent expression of microsomal 5 α -reductase type 1 in the human temporal lobe with a significantly higher activity in CX compared to SC, which suggests a neuronal expression of the enzyme [18]. In contrast, cytosolic 3-ketosteroid reductase activity was found to be significantly higher in SC than in CX suggesting glial expression (Figs. 3A & 3B). Hence, 5 α -reductase and 3-ketosteroid reductase activity are co-expressed within the human temporal lobe, but are localized in different compartments and possibly also different cell types.

7. 4. Human brain tissue is unable to catalyze *de novo* the biosynthesis of 3 α /3 β -THSs

Functional expression of NADPH-dependent cytochrome P450c17 and NAD⁺-dependent 3 β -HSD/KSI is a prerequisite for the formation of steroid hormones from pregnenolone - the immediate Δ^5 -3 β -hydroxysteroid derivative of cholesterol [30]. Contrary to the hypothesis of an intracerebral *de novo* biosynthesis of THSs, we neither detected conversion of progesterone (Fig. 1A & 1B) or pregnenolone (unpublished data) via cytochrome P450c17 activity nor did we detect cytochrome P450c17 mRNA expression in human mature temporal lobe [43] or parahippocampal gyrus and hippocampus specimens (unpublished data). These results are

consistent with the previous observation that cytochrome P450c17 expression can be detected only in the embryonic, but not in the postnatal rat brain [44].

Moreover, 3β -HSD/KSI is also not expressed in the mature human temporal lobe (Fig. 5) and limbic system (data not shown) contradicting previous studies with frog, bird and rat brain. In these studies low expression of the enzyme was observed within the animal CNS [31,45,46]. Moreover, our result seems to contradict a previous 50-cycles real-time RT-PCR study that detected mRNA expression levels in human brain tissue at about five orders of magnitude lower than in the adrenals [47]. However, these extremely low levels possibly reflect the amplification of illegitimate transcripts. Notably, the observed lack of functional 3β -HSD/KSI expression in the human brain is consistent with a previous adult rhesus monkey brain study, which did not observe functional 3β -HSD/KSI expression in the cerebral cortex, cerebrum and cerebellum [48].

The absence of cytochrome P450c17 and 3β -HSD/KSI indicates that the investigated human brain regions are unable to catalyze *de novo* the biosynthesis of $3\alpha/3\beta$ -THSs from pregnenolone. Hence, their local formation will most likely depend upon the uptake of circulating Δ^4 -3-ketosteroids across the blood-brain barrier either by diffusion or carrier-mediated transport [49]. This assumption is corroborated by the results of a previous post-mortem study investigating human female brain tissue, which suggested that the cerebral levels of progesterone, 5α -DHP and $5\alpha,3\alpha$ -THP depend upon the level of glandular steroidogenesis [50].

7. 5. The detection of AKR1C mRNA suggested that aldo-keto reductases may be responsible for cerebral $3\alpha/3\beta$ -HSD activity

The soluble character of cerebral 3-ketosteroid reductase, its lack of stereo-selectivity and the observed substrate-dependent inversion of the stereo-chemical course of reaction suggested that AKR1C expression might be responsible [22,23,39]. Previously, we found that AKR1C3 mRNA is expressed in the human temporal lobe at a level of about 10–20 % of the expression in liver [25,36]. Its sex-independent expression with significantly higher levels in SC compared to CX indicated that cerebral $3\alpha/3\beta$ -HSD activity may be attributed to AKR1C3 expression. However, recombinant AKR1C3 acts *in vitro* only as a weak $3\alpha/3\beta$ -HSD [22]. We now verified that AKR1C1 and AKR1C2 are also expressed at a low mRNA level (Table 2), which initially suggested that they might contribute to the observed 3-ketosteroid reductase activity. However, contrary to AKR1C3, their expression profiles did not match the observed tissue distribution patterns of cerebral 3-ketosteroid reductase activity.

7. 6. Cerebral 3-ketosteroid reductase activity is refractory to inhibition by AKR1C inhibitors

To clearly elucidate if human brain tissue 3-ketosteroid reductase activity was attributed to the cerebral expression of AKR1Cs, we exploited the use of AKR1C inhibitors. In contrast to purified cytosolic rat brain tissue 3α -HSD [51] and cytosolic human liver $3\alpha/3\beta$ -HSD (Fig. 6B), human brain tissue 3-ketosteroid reductase was not significantly affected by any of the AKR1C inhibitors (Fig. 6A). Co-incubation experiments with brain and liver cytosol excluded the possibility that the compounds were bound non-specifically to cerebral proteins thus preventing their inhibitory effects (compare Figs. 6A – 6C). This assumption is corroborated by the finding that flufenamic acid very well acted as a potent inhibitor of cytosolic cerebral 20α -HSD activity, which suggests that AKR1C expression is responsible for this reaction (Fig. 6F). Together, these results prove that AKR1C expression does not contribute to human brain tissue $3\alpha/3\beta$ -HSD activity.

7. 7. Conclusions

The insensitivity of cerebral 3-ketosteroid reductase to inhibitors of AKR1C activity indicates that the expression of AKR1C1-1C3 is not sufficient to explain the observed 3 α /3 β -HSD activity. Nevertheless, cerebral 3-ketosteroid reductase shares a number of features with AKR1Cs. This suggests that the cerebral 3 α /3 β -HSD might be an AKR-related enzyme, which is corroborated by the finding that cerebral 3 α /3 β -HSD and 20 α -HSD activities were equally inhibited by known inhibitors of aldehyde reductase activity (Figs. 6D–F).

The cerebral expression of a non-stereo-selective 3-ketosteroid reductase is of physiological significance, since only 5 α ,3 α -THSs enhance the effect of GABA on the GABA_A receptor, whereas 5 α ,3 β -THSs are antagonists [4,8,52]. The fact that RoDH 5 and RoDH-like 3 α -HSD - two membrane-associated short-chain dehydrogenases/reductases that *in vivo* act as 3 α -hydroxysteroid oxidases [20] - are expressed within the human CNS [53,54] suggests that a gradual epimerization of 5 α ,3 α -THSs into 5 α ,3 β -THSs might occur. Accordingly, 3-ketosteroids might undergo enzymatic cycling in the human brain via oxidation into the respective 5 α -DHS and subsequent reduction via non-stereo-selective 3-ketosteroid reductase activity. Therefore, protecting the 3 α -hydroxy-moiety of neuroactive steroidal drugs from oxidation by 3 β -substitution should substantially improve their pharmacokinetic profile - just as it was observed for the anticonvulsant drug ganaxolone, which is the 3 β -methyl substituted synthetic analog of endogenous allopregnanolone [55].

Research Highlights

In the human brain 5 α -dihydrosteroids are converted via 3 α /3 β -HSD activity.
 The human brain is unable to conduct *de novo* biosynthesis of Δ^4 -3-ketosteroids.
 The local formation of tetrahydrosteroids depends on the uptake of Δ^4 -3-ketosteroids.
 Cytosolic cerebral 3 α /3 β -HSD activity might be catalyzed by a single enzyme.
 AKR1Cs are expressed.
 The cerebral 3 α /3 β -HSD activity is not affected by AKR1C inhibitors.
 The lack of stereo-selectivity is of physiological significance.
 Only 5 α ,3 α -tetrahydrosteroids enhance the effect of GABA on the GABA_A receptor.
 5 α ,3 β -tetrahydrosteroids are antagonists.

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Abbreviations

(Δ^4 -androstenedione)	Androst-4-ene-3,17-dione
(5 α -androstenedione)	5 α -androstane-3,17-dione
(epi/androsterone)	3 β /3 α -hydroxy-5 α -androstane-17-one
(testosterone)	17 β -hydroxyandrost-4-ene-3-one
(5 α -dihydrotestosterone [5 α -DHT])	17 β -hydroxy-5 α -androstane-3-one

(3 β /3 α -androstenediol)	5 α -androstane-3 β /3 α ,17 β -diol
(dehydroepiandrosterone [DHEA])	3 β -hydroxyandrost-5-ene-17-one
(Δ^5 -androstenediol)	androst-5-ene-3 β ,17 β -diol
(progesterone)	preg-4-ene-3,20-dione
(5 α -dihydroprogesterone [5 α -DHP]also known as allopregnanedione)	5 α -pregnane-3,20-dione
(5 α 3 β /3 α -tetrahydroprogesterone [5 α ,3 β /3 α -THP], also known as epi/allopregnanolone)	3 β /3 α -hydroxy-5 α -pregnane-20-one
(5 α 20 α -tetrahydroprogesterone, [5 α ,20 α -THP])	20 α -hydroxy-5 α -pregnane-3-one
(pregnenolone)	3 β -hydroxypreg-5-ene-20-one
(17 α -hydroxyprogesterone [17 α -OHP])	17 α -hydroxypreg-4-ene-3,20-dione
(20 α -hydroxyprogesterone)	20 α -hydroxypreg-4-ene-3-one
(11-deoxycorticosterone [DOC])	21-hydroxypreg-4-ene-3,20-dione
(pregnenolone)	3 β -hydroxypregn-5-ene-20-one
(AKR)	aldo-keto reductase
(CX)	cerebral neocortex
(DHS)	dihydrosteroid
(GAPDH)	glyceraldehyde-3-phosphate dehydrogenase
(HSD)	hydroxysteroid dehydrogenase
(KSI)	ketosteroid isomerase
(SC)	subcortical white matter
(THS)	tetrahydrosteroid

9. References

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Fig. 1. Non-stereo-selective formation of 3 α /3 β -THSs from 5 α -DHSs in human brain tissue
Panels A–C, autoradiograms of [4-¹⁴C]progesterone and metabolites following two-dimensional TLC separation. The radiosignals result from 3 hour incubations of 1 μ mol/L progesterone in the presence of saturating NADPH (re-generating system). Assays were conducted with (Panel A) a no-tissue control, (Panel B) a cell-free cerebral neocortex preparation or (Panel C) a cell-free subcortical white matter preparation. The plotted rims are assigned to co-chromatographed and stained reference standards added to the incubation extracts: (Δ^4 -A) Δ^4 -androstenedione, (P) progesterone, (5α) 5 α -dihydroprogesterone, ($5\alpha,3\beta/3\alpha$) 5 $\alpha,3\beta/3\alpha$ -tetrahydroprogesterone, (20α) 20 α -hydroxyprogesterone, ($5\alpha,20\alpha$) 5 α -pregnane-20 α -ol-3-one, ($5\alpha,3\beta/3\alpha,20\alpha$) 5 α -pregnane-3 $\beta/3\alpha,20\alpha$ -diol, (17α) 17 α -

hydroxyprogesterone and (DOC) 11-deoxycorticosterone; (?) unidentified metabolite; (OR) origins of TLC. *Panel D*, digital autoradiogram of [$1\beta,2\beta$ - ^3H]5 α -DHT and metabolites following one-dimensional TLC separation. The radiosignals result from incubations of 0.1 $\mu\text{mol/L}$ 5 α -DHT in the presence of saturating NADPH (re-generating system). Assays were conducted with (C) a no-tissue control, or with cell-free preparations of (AC) adrenal cortex (72 μg protein per assay, incubation period of 30 min), (Pl) placenta (28 μg , 30 min), (Li) liver (7 μg , 30 min), and (Br) brain tissue (300 μg , 60 min). The displayed photography shows co-chromatographed and stained reference standards: (5 α -A) 5 α -androstanedione, (Δ^4 -A) Δ^4 -androstanedione, (A) androsterone and (3 α /3 β -Diol) 3 α /3 β -androstanediol; (Epi-A) position of authentic epiandrosterone; (OR) origin of TLC.

Fig. 2. Cerebral 3 α - and 3 β -HSD activities are both enriched in the cytosolic subcellular fraction (Panel A) 3 α -HSD, (Panel B) 3 β -HSD and (Panel C) 5 α -reductase activities in (WH) whole cell homogenates as well as (Nu) nuclear, (Me) membrane and (Cy) cytosolic subcellular fractions of temporal lobe tissue from a (open bars) 33-yr-old woman or a (solid bars) 35-yr-old man. Enzyme activities were determined using 3 mmol/L NADPH and 0.1 μ mol/L substrate (either [1 β ,2 β - 3 H]5 α -DHT for 3 α /3 β -HSD activity or [1 β - 3 H] Δ^4 -androstenedione for 5 α -reductase activity). The results represent mean values of assays performed in duplicate.

Fig. 3. The tissue distribution patterns of cerebral 3α- and 3β-HSD activity suggest that a single enzyme is responsible

The cerebral reduction of 2 μmol/L 5α-DHT via (Panel A) 3α- and (Panel B) 3β-HSD activity was investigated in the presence of 3 mmol/L NADPH using cell-free temporal lobe preparations from (solid symbols) cerebral neocortex and (open symbols) subcortical white matter specimens of (circles) thirteen women (aged 22–47 yrs) and (squares) ten men (aged 18–52 yrs), respectively. A scattered plot of 3α-HSD relative to 3β-HSD activity is given in Panel C. The results represent mean values of assays performed in duplicate.

Fig. 4. Verification of non-stereo-selective cerebral 3-ketosteroid reductase activity by GC-MS analysis

Products of cerebral 3-ketosteroid reductase were generated by bulk incubation of brain tissue cytosol with 20 $\mu\text{mol/L}$ 5 α -DHT and 3 mmol/L NADPH. *Panel A*, linear radio-distribution of [$1\beta,2\beta$ - ^3H]5 α -DHT and metabolites following one-dimensional TLC; positions of authentic reference standards are marked (5 α -A) 5 α -androstanedione, (Epi-A/A) epi/androsterone, (3 α /3 β -Diol) 3 α /3 β -androstanediol; (OR) origin of TLC; *Panel B*, no-tissue control incubation. Silica gel elutes of the zones with radiosignals corresponding to the positions of authentic 3 α - or 3 β -androstanediol were subjected to GC-MS analysis. *Panel C*, GC retention time (10.118 min) of the trimethylsilyl ether of authentic 3 α -androstanediol. *Panel D*, mass spectrum of the trimethylsilyl ether of authentic 3 α -androstanediol. *Panel E*, GC retention time of trimethylsilyl ethers of organic brain tissue metabolites with the same R_F -value in TLC as authentic 3 α -androstanediol. *Panel F*, mass spectrum of the trimethylsilyl ether of the 5 α -DHT metabolite with the same R_F -value in TLC and the same retention time in GC as authentic 3 α -androstanediol. *Panel G*, GC retention time (10.712 min) of the trimethylsilyl ether of authentic 3 β -androstanediol. *Panel H*, mass spectrum of the trimethylsilyl ether of authentic 3 β -androstanediol. *Panel I*, GC retention time of trimethylsilyl ethers of organic brain tissue metabolites with the same R_F -value in TLC as authentic 3 β -androstanediol. *Panel J*, mass spectrum of the trimethylsilyl ether of the 5 α -DHT metabolite with the same R_F -value in TLC and the same retention time in GC as authentic 3 β -androstanediol.

Fig. 5. Lack of 3β-HSD/KSI expression in the human temporal lobe

Panel A, RT-PCR based detection of 3β-HSD/KSI and GAPDH cDNA, respectively. Total brain tissue RNA extracted from either eight female (aged 11–60 yrs) or eight male patients (aged 5–58 yrs) was pooled prior to RT; (CX) cerebral neocortex, (SC) subcortical white matter, (w) women, (m) men, (U-87) U-87 human astrocytoma cell line, (AdCx) human adrenal cortex, (JEG3) JEG3 human choriocarcinoma cell line, (NTC) H₂O no-template negative control. The positions of the 250-bp fragment (50-bp ladder) and the 1000-bp DNA-fragment (100-bp ladder) of DNA size markers (M) are indicated. *Panels B & C*, digital autoradiogram of radioactively labelled Δ⁵-3β-hydroxysteroid and metabolites following one-dimensional TLC separation. The radiosignals result from incubations of either (Panel B) 2 μmol/L [4-¹⁴C] DHEA or (Panel C) 0.1 μmol/L [1,2-³H]Δ⁵-androstenediol in the presence of 3 mmol/L NAD⁺ and NADP⁺, respectively. Assays were conducted with cell-free preparations of (Br, w

& m) temporal lobe from a 38-yr-old woman and a 38-yr-old man (incubation period of 60 min; 398 μ g and 382 μ g protein per assay, respectively) and (AdCx 1 & 2) adrenal cortex (30 min; 6 μ g & 10.5 μ g per assay, respectively); (NTC) no-tissue control. The displayed photography shows co-chromatographed and stained non-radioactive reference standards added to the incubation extracts: (Δ^4 -A) Δ^4 -androstenedione, (T) testosterone, and (Δ^5 -A) Δ^5 -androstenediol; (Ch) stained cerebral cholesterol extracted from brain tissue incubations; (OR) origin of TLC.

Fig. 6. Cerebral 3-ketosteroid reductase is refractory to inhibition by AKR1C inhibitors

Panels A–C, inhibitory effects of excess amounts (100 $\mu\text{mol/L}$) of (Ind) indomethacin, (5 β C) 5 β -cholanic acid 3 α ,7 α -diol, (Phe) phenolphthalein, and (Flu) flufenamic acid on the NADPH-dependent (3 mmol/L) reduction of 5 α -DHT (11.7 $\mu\text{mol/L}$) were monitored by measuring the formation of (open bars) 3 α - and (solid bars) 3 β -androstenediol; (NoI) no inhibitor controls. Assays were conducted using (Panel A) brain tissue cytosol (pooled subcortical white matter of a 51-yr-old woman and a 57-yr-old man; 0.1 mg protein per assay), (Panel B) liver cytosol (0.01 mg protein per assay), and (Panel C) brain and liver cytosol in combination (0.1 mg cytosolic brain tissue proteins plus 0.01 mg cytosolic liver proteins). *Panels D–F*, inhibitory effects of excess amounts (100 $\mu\text{mol/L}$) of (Flu) flufenamic acid, (Quer) quercetin, and (HMB) hydroxymercuribenzoate on NADPH-dependent (3 mmol/L) cytosolic cerebral ketosteroid reductases: (Panel D & E) reduction of 5 α -DHT (1 $\mu\text{mol/L}$) via 3 α - and 3 β -HSD activity, respectively; (Panel F) reduction of progesterone (1 $\mu\text{mol/L}$) via 20 α -HSD activity. The results represent mean values of assays performed in duplicate.

Table 1Summary of specific enzyme activities measured in the present study (in nmol/h/mg_{protein})

ad section 6.1 and Figs. 1B & 1C:

NADPH-dependent (re-generating system) conversion of progesterone (1 µmol/L) in cell-free preparations of cerebral neocortex (CX) and subcortical white matter (SC) from a 22-yr-old man.

	20 α -HSD activity	5 α -reductase activity	3 α -HSD activity	3 β -HSD activity
CX	0.007	0.020	0.001	0.005
SC	0.019	0.015	0.002	0.005

ad section 6.1 and Fig. 1D:

NADPH-dependent (re-generating system) conversion of 5 α -DHT (0.1 µmol/L) in cell-free human tissue homogenates.

	Reduction into 3 α -androstanediol	Reduction into 3 β -androstanediol	Formation of 5 α -androstanedione	Formation of epiandrosterone
Adrenal Cortex	–	0.550	0.020	0.130
Placenta	–	1.370	–	–
Liver	10.440	0.450	–	–
Brain	0.019	0.006	–	–

ad section 6.2 and Fig. 3:

NADPH-dependent (3 mmol/L) conversion of 5 α -DHT (2.0 µmol/L) in linked CX and SC homogenates of 23 human temporal lobe biopsies (mean $\pm$ SD).

	Reduction into 3 α -androstanediol	Reduction into 3 β -androstanediol
CX	0.160 $\pm$ 0.048	0.045 $\pm$ 0.013
SC	0.397 $\pm$ 0.090	0.109 $\pm$ 0.027

ad section 6.4 and Figs. 5B & 5C:

NAD⁺/NADP⁺-dependent (3 mmol/L) conversion of DHEA (2.0 µmol/L) or Δ^5 -androstenediol (0.1 µmol/L) in cell-free human tissue homogenates; (Δ^4 -A) Δ^4 -androstenedione; (Δ^5 -A) Δ^5 -androstenediol.

	NAD ⁺ -dependent conversion of DHEA into Δ^4 -A	Reduction of DHEA into Δ^5 -A in presence of NADP ⁺	NAD ⁺ -dependent conversion of Δ^5 -A into testosterone	NAD ⁺ - (NADP ⁺ -) dependent oxidation of Δ^5 -A into DHEA
Brain (man)	–	0.019	–	0.002 (< 0.001)
Brain (woman)	–	0.018	–	0.001 (< 0.001)
Adrenal Cortex	49.0	–	2.1	–(–)

Table 2

Expression of AKR1C1, AKR1C2 and AKR1C4 in cerebral neocortex (CX) and subcortical white matter (SC) of six female (f) and six male patients (m) was investigated by real-time RT-PCR analyses relative to the expression of GAPDH.

	AKR1C1	AKR1C2	AKR1C4	GAPDH
CX (f)	2.8 ± 0.6 (<i>29.0 ± 0.6</i>)	$4.1 \pm 0.7^{\#}$ (<i>29.8 ± 0.3</i>)	ND	(<i>20.1 ± 0.1</i>)
SC (f)	$2.9 \pm 1.0^*$ (<i>30.2 ± 0.8</i>)	1.5 ± 0.4 (<i>32.5 ± 0.7</i>)	ND	(<i>21.1 ± 0.3</i>)
CX	0.9 ± 0.2 (<i>29.9 ± 0.3</i>)	$2.2 \pm 0.3^{\#}$ (<i>30.0 ± 0.4</i>)	ND	(<i>19.3 ± 0.4</i>)
SC (m)	0.8 ± 0.3 (<i>31.8 ± 0.7</i>)	0.9 ± 0.3 (<i>32.4 ± 0.4</i>)	ND	(<i>20.4 ± 0.4</i>)
Liver	100.0 (<i>23.1</i>)	100.0 (<i>24.7</i>)	(<i>22.4</i>)	(<i>20.0</i>)

Data are given as arbitrary units (mean ± SEM) with the expression in liver set as 100 per cent; threshold cycles are given in (*italics*)

* significantly higher ($P < 0.05$) in SC (f) than in SC (m)

[#] significantly higher ($P < 0.05$) in CX than in SC; ND, not detectable.