

Novel route for elimination of brain oxysterols across the blood-brain barrier: conversion into 7 α -hydroxy-3-oxo-4-cholestenoic acid

Steve Meaney,^{1,*} Maura Heverin,^{1,*} Ute Panzenboeck,[†] Lena Ekström,[§] Magnus Axelsson,^{**} Ulla Andersson,^{*} Ulf Diczfalusy,^{*} Irina Pikuleva,^{††} John Wahren,^{§§} Wolfgang Sattler,^{***} and Ingemar Björkhem^{2,*}

Division of Clinical Chemistry* and Division of Clinical Pharmacology,[§] Karolinska University Hospital, Huddinge, Sweden; Division of Clinical Chemistry** and Division of Surgical Sciences,^{§§} Karolinska University Hospital, Solna, Sweden; Department of Pharmacology and Toxicology,^{††} University of Texas Medical Branch, Galveston, TX; and Institute of Pathophysiology[†] and Institute of Molecular Biology and Biochemistry, Center of Molecular Medicine,^{***} Medical University Graz, Graz, Austria

Abstract Recently, we demonstrated a net blood-to-brain passage of the oxysterol 27-hydroxycholesterol corresponding to 4–5 mg/day. As the steady-state levels of this sterol are only 1–2 μ g/g brain tissue, we hypothesized that it is metabolized and subsequently eliminated from the brain. To explore this concept, we first measured the capacity of *in vitro* systems representing the major cell populations found in the brain to metabolize 27-hydroxycholesterol. We show here that 27-hydroxycholesterol is metabolized into the known C₂₇ steroidal acid 7 α -hydroxy-3-oxo-4-cholestenoic acid by neuronal cell models only. Using an *in vitro* model of the blood-brain barrier, we demonstrate that 7 α -hydroxy-3-oxo-4-cholestenoic acid is efficiently transferred across monolayers of primary brain microvascular endothelial cells. Finally, we measured the concentration of 7 α -hydroxy-3-oxo-4-cholestenoic acid in plasma from the internal jugular vein and brachial artery of healthy volunteers. Calculation of the arteriovenous concentration difference revealed a significant *in vivo* flux of this steroid from the brain into the circulation in human. Together, these studies identify a novel metabolic route for the elimination of 27-hydroxylated sterols from the brain. Given the emerging connections between cholesterol and neurodegeneration, this pathway may be of importance for the development of these conditions.—Meaney, S., M. Heverin, U. Panzenboeck, L. Ekström, M. Axelsson, U. Andersson, U. Diczfalusy, I. Pikuleva, J. Wahren, W. Sattler, and I. Björkhem. Novel route for elimination of brain oxysterols across the blood-brain barrier: conversion into 7 α -hydroxy-3-oxo-4-cholestenoic acid. *J. Lipid Res.* 2007. 48: 944–951.

Supplementary key words brain cholesterol homeostasis • 27-hydroxycholesterol • CYP7B1 • CYP27

Cholesterol is essential for the correct function of the brain. The necessity to maintain tight control of brain cholesterol levels has led to the evolution of specialized mechanisms for the control of cholesterol levels within the central nervous system. First, the blood-brain barrier prevents the exchange of cholesterol between the brain and plasma. Conversion of cholesterol to side chain oxidized oxysterols, however, facilitates its passage across the blood-brain barrier, and it is well documented that the most important mechanism by which the brain eliminates excess cholesterol is via the formation and secretion of 24S-hydroxycholesterol (for reviews, see Refs. 1, 2).

Using established physiological methods to investigate blood-to-brain transport, we recently made the surprising discovery that 27-hydroxycholesterol passes from the circulation into the central nervous system (3). This finding is in agreement with our previous observations that labeled 27-hydroxycholesterol passed from the circulation into cerebrospinal fluid in a healthy volunteer and that the levels of 27-hydroxycholesterol in the circulation and the cerebrospinal fluid were correlated (4). However, despite the fact that 4–5 mg of 27-hydroxycholesterol passes into the brain each day, its levels are only 1–2 μ g/g (5). Absence of a functional sterol 27-hydroxylase, which occurs in cerebrotendinous xanthomatosis, results in the accumulation of cholesterol and cholestanol in the form of brain xanthomas (6).

The discrepancy between the expected and observed levels of intracerebral 27-hydroxycholesterol suggested to us that this oxysterol must be further metabolized. It is well known that as part of bile acid synthesis 27-hydroxycho-

Manuscript received 13 December 2006 and in revised form 23 January 2007.
Published, JLR Papers in Press, January 24, 2007.
DOI 10.1194/jlr.M600529-JLR200

¹S. Meaney and M. Heverin contributed equally to this work.

²To whom correspondence should be addressed.

e-mail: ingemar.bjorkhem@karolinska.se

Copyright © 2007 by the American Society for Biochemistry and Molecular Biology, Inc.

lesterol may be metabolized into a number of C₂₇ steroidal acids, which are also present at micromolar levels in the plasma (7). Using primary rat astrocytes, Zhang et al. (8) demonstrated the conversion of radiolabeled 27-hydroxycholesterol to several of these acidic intermediates. However, only trace amounts of 7 α -hydroxy-3-oxo-4-cholestenoic acid, the terminal metabolite in this pathway, were found. To date, only one other biological compartment has been shown to contain appreciable amounts of this steroidal acid: Nagata et al. (9) showed that subdural hematomas contain more than five times as much 7 α -hydroxy-3-oxo-4-cholestenoic acid as the general circulation. In addition, there was an absence of this steroid in normal cerebrospinal fluid (10).

We hypothesized, based on the available data, that 27-hydroxycholesterol within the CNS is metabolized to a steroidal acid before being eliminated from the brain. Using different *in vitro* approaches, we show that 27-hydroxycholesterol is metabolized to 7 α -hydroxy-3-oxo-4-cholestenoic acid and that this acid can rapidly traverse a model of the blood-brain barrier. Finally, by measuring the concentrations of all of the cholestenic acids in the internal jugular vein and in the brachial artery of healthy volunteers, we show a net flux of 7 α -hydroxy-3-oxo-4-cholestenoic acid from the brain to the circulation. Together, these results are consistent with 7 α -hydroxy-3-oxo-4-cholestenoic acid being an important terminal metabolite of 27-hydroxycholesterol present in the brain.

MATERIALS AND METHODS

Materials

All organic solvents used were of gas chromatography or high performance liquid chromatography grade.

Synthesis of labeled steroids

Deuterium-labeled internal standards and unlabeled reference compounds were synthesized as described previously (11). [7 β -³H]7 α -hydroxy-3-oxo-4-cholestenoic acid was prepared from [7 β -³H]7 α -hydroxycholesterol (with a specific activity of 100 μ Ci/mg) via sequential treatment with bacterial cholesterol oxidase and recombinant human sterol 27-hydroxylase, with a 40% yield of the desired product. The radiolabeled product was purified by HPLC using a YMC-Pack ODS-A 250 \times 4.6 mm inner diameter S-5 μ m, 120A column and a mobile phase of 15 mM potassium phosphate, pH 5.4, buffer-methanol (1:3, v/v). The final product was pure as judged by radio-HPLC and GC-MS, and it had a mass spectrum identical to that of the authentic compound (7).

Cell culture

SH-SY5Y neuroblastoma cells were routinely cultured in DMEM with 10% (v/v) fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. CHME-3 microglia [SV-40 large T-antigen immortalized human fetal microglia (12)] and D-384 astrocytoma cells [a clonal cell line established from a human astrocytoma (13)] were cultured in high-glucose DMEM (4,500 mg/l glucose), 10% fetal calf serum, 2 μ M Glutamax™, 100 U/ml penicillin, and 100 μ g/ml streptomycin. All cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

Expression profiling of different cell preparations

Total RNA was extracted from dishes of the cells described above treated with vehicle or 27-hydroxycholesterol (27-OHC) using Trizol® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcriptase-PCR was performed with Superscript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen) with gene-specific primers and 250 ng of total RNA, with the exception of sterol 27-hydroxylase (CYP27A1) (1 μ g of total RNA). Primers, product sizes, and annealing temperatures (T_a) were as follows. For CYP27A1 (223 bp), sense (5'-AAGCGATACCTGGATGGTTG-3') and antisense (5'-TGTTGGATGTCGTGTC-CACT-3'); T_a = 57.7°C. For oxysterol 7 α -hydroxylase (CYP7B1) (152 bp), sense (5'-GTCTACATGGTGACCCTGA-3') and antisense (5'-CATTTGCTGGTTCCAGTTCC-3'); T_a = 52°C. For 3 β -hydroxy-C27-steroid dehydrogenase/isomerase (HSD3B7) (209 bp), sense (5'-TCCAGGAGCCTTGAGTCTGT-3') and antisense (5'-GTAAGCCTGTGGAGCCTCTG-3'); T_a = 57.7°C. Human adult brain total RNA (Clontech, Mountain View, CA) was used as a positive control. The temperature program was per the manufacturer's instructions, and samples were amplified for either 36 or 38 cycles. Products were run on a 2% agarose gel and visualized with ethidium bromide and an ultraviolet transilluminator.

Incubations with 27-hydroxycholesterol

For metabolic transformation experiments, complete medium was removed from the cells and replaced with serum-free medium containing 20 μ g of 27-hydroxycholesterol dissolved in 45% (w/v) 2-hydroxypropyl- β -cyclodextrin and ethanol. In no case did the vehicle exceed 0.5% of the total medium. Triplicate 100 mm dishes were incubated for 24, 48, and 72 h. After the appropriate incubation period, cell medium was aspirated from the cells and immediately frozen at -20°C until required.

Analysis of cell medium

Determination of steroid content in the cell medium was performed as described previously (7). Briefly, 300 ng of norcholestenoic acid was added as internal standard to 7.5 ml of cell medium before extraction with 30 ml of chloroform-methanol (2:1, v/v) in a separatory funnel. The organic phase was removed, dried under vacuum, and redissolved in 0.5 ml of chloroform. This material was then applied to a Bond-Elut™ column (Varian) previously conditioned with 4 ml of *n*-hexane and allowed to enter the column matrix under gravity. Neutral steroids were eluted with 4 ml of chloroform-isopropanol (2:1, v/v), and acidic steroids were eluted with 4 ml of acetic acid-diethyl ether (1:50, v/v). Each of the fractions was stored under argon at -20°C until required. Derivatization and analysis of these fractions were performed as described previously (7).

Isolation of porcine brain microvascular endothelial cells

Porcine brains were obtained from freshly slaughtered pigs. After removal of the meninges and secretory areas, the gray and white matter of the cerebral cortex were minced and porcine brain microvascular endothelial cells were isolated by sequential enzymatic digestion and centrifugation steps as described (14). Clusters from one brain were seeded in medium A (M199 containing 10% ox serum, 1% penicillin/streptomycin, and 1% gentamycin) on six 75 cm² calf skin collagen-coated flasks (60 μ g/ml). After 1 day *in vitro*, cells were washed with PBS and cultivated in medium B (identical to medium A except lacking gentamycin).

In vitro blood-brain barrier efflux experiments

Cells were seeded on calf skin collagen-coated Transwell inserts on 12-well cell cluster plates at a density of 40,000 cells/cm²

in medium B. After 3 days, induction of tight junctions was initiated via overnight incubation in DMEM/Ham's F12 supplemented with 150 nM hydrocortisone, 1% penicillin/streptomycin, and 0.25% glutamine. The integrity of the monolayer was ascertained by measuring the transendothelial electrical resistance using an Endohm electrode. For efflux experiments, [^3H]7 α -hydroxy-3-oxo-4-cholestenoic, [^3H]24S-hydroxycholesterol, or [^3H]cholesterol was added to the basolateral compartment, together with a defined amount of unlabeled material to maintain a constant sterol concentration (0.5 $\mu\text{g}/1.5\text{ ml}$) in the basolateral compartment. Fatty acid-free BSA (0.5 and 1 mg/ml) or human serum (1%, v/v) was added as sterol acceptor to the apical compartment. At the indicated time points, 100 μl of the apical medium was removed for the determination of transferred radioactivity and replaced with fresh medium. At the end of the incubations, transendothelial electrical resistances were measured, cells were washed with PBS, transferred to a new 12-well chamber, and lysed in 0.3 N NaOH by overnight incubation on an orbital shaker at 4°C, and the cell-associated radioactivity was measured.

Catheterization experiments

Ten healthy males, mean age 29 years (range, 21–38 years), were recruited for this study. Because of analytical problems and lack of material, only 9 of the 10 patients could be included in the study. After an overnight fast, blood samples were taken simultaneously from two catheters inserted percutaneously. One Cournand catheter was inserted at the level of the inguinal ligament, and the tip was advanced under fluoroscopic control to the internal jugular vein at the base of the skull. A second thin Teflon catheter was introduced into the brachial artery in the antecubital fossa.

Measurement of plasma sterols

Plasma levels of the steroidal acids known to be formed from 27-hydroxycholesterol were measured essentially as described by Axelson, Mörk, and Sjövall (7). Norcholestenoic acid was used as an internal standard, and the samples were analyzed as methyl esters-trimethylsilyl ethers by combined gas chromatography-mass spectrometry using the selected ion-monitoring mode (7). The following ions were monitored: m/z 488 (norcholestenoic acid), m/z 500 (3 β ,7 α -dihydroxy-5-cholestenoic acid), m/z 412 (3 β -hydroxy-5-cholestenoic acid), and m/z 426 (7 α -hydroxy-3-oxo-4-cholestenoic acid). To quantify the other acids using this standard curve, compensation factors of 0.33 and 0.78 were applied to correct for the intensity of the m/z 500 ion of 3 β ,7 α -dihydroxy-5-cholestenoic acid relative to the m/z 412 ion of 3 β -hydroxy-5-cholestenoic acid and for the intensity of the m/z 426 ion of 7 α -hydroxy-3-oxo-4-cholestenoic acid relative to the m/z 412 ion of 3 β -hydroxy-5-cholestenoic acid, respectively. In addition, the neutral steroid 7 α -hydroxy-4-cholesten-3-one, another possible precursor, was measured by HPLC using an ultraviolet detector. Finally, cholesterol and albumin levels were measured using an enzymatic colorimetric assay and a colorimetric assay, respectively, on the Roche/Hitachi modular routine analyzer.

Statistical evaluations

Results are presented as means \pm SEM. In our evaluation of the metabolite profiling, we used the one-tailed Student's *t*-test to evaluate the significance of differences, in accordance with our hypothesis of a net efflux of some metabolite of 27-hydroxycholesterol from the brain. $P < 0.05$ and $P < 0.01$ were considered significant.

Ethical aspects

All subjects were informed of the nature, purpose, and possible risks of the study before giving their voluntary consent to participate. The study protocol was reviewed and approved by the institutional ethics committee.

RESULTS

Cell-specific metabolism of 27-hydroxycholesterol

As a first step in the investigation of the metabolism of 27-hydroxycholesterol within the human brain, we screened several commonly used cell systems for suitability as models of neurons, astrocytes, and microglia. RT-PCR-based profiling of enzymes known to be involved in the metabolism of 27-hydroxycholesterol (i.e., CYP27A1, CYP7B1, and HSD3B7) in different cell types revealed that SH-SH5Y, D-384, and CHME-3 cells had expression profiles consistent with those described in vivo (15–17) (Fig. 1A). Notably, only SH-SY5Y neuroblastoma cells expressed CYP7B1. Thus, we considered these cell systems suitable models for the exploration of the metabolism of 27-hydroxycholesterol.

Incubation of each of the cell types with 27-hydroxycholesterol led to a time-dependent formation of both 3 β -hydroxy-5-cholestenoic and 3 β ,7 α -dihydroxy-5-cholestenoic acids (Fig. 1B). However, only SH-SH5Y cells were capable of metabolizing 27-hydroxycholesterol to 7 α -hydroxy-3-oxo-4-cholestenoic acid, albeit at amounts close to the limits of detection of our gas chromatography-mass spectrometry technique (results not shown). In parallel experiments, trace amounts (50 ng) of radiolabeled 27-hydroxycholesterol were incubated with SH-SY5Y cells. Under these conditions, which may be regarded as considerably more physiological than the saturating conditions described previously, almost 6% of the 27-hydroxycholesterol was converted to 7 α -hydroxy-3-oxo-4-cholestenoic acid (Fig. 1C). The conversion into the immediate precursor, 7 α -hydroxy-cholestenoic acid, was \sim 50%. In a separate experiment, D-384 and CHME-3 cells were incubated with a mixture of 7 α - and 7 β -hydroxycholestenoic acid. Under these conditions, D-384 cells were able to metabolize \sim 50% of the added material to 7 α -hydroxy-3-oxo-4-cholestenoic acid (results not shown).

Efflux of 7 α -hydroxy-3-oxo-4-cholestenoic acid across porcine brain microvascular endothelial cell monolayers

To gain some insight into whether 7 α -hydroxy-3-oxo-4-cholestenoic acid is a viable transport form of 27-hydroxycholesterol, we used an established in vitro model of the blood-brain barrier consisting of high-resistance monolayers of porcine cerebral microvascular endothelial cells (14). We demonstrated that the basolateral-to-apical transfer of radiolabeled 7 α -hydroxy-3-oxo-4-cholestenoic acid was apparently nonsaturable and time-dependent and occurred in both the presence and absence of serum proteins (Fig. 2A). Importantly, this transfer was significantly faster than that of 24S-hydroxycholesterol (Fig. 2B), an oxysterol that is well known to efficiently traverse the

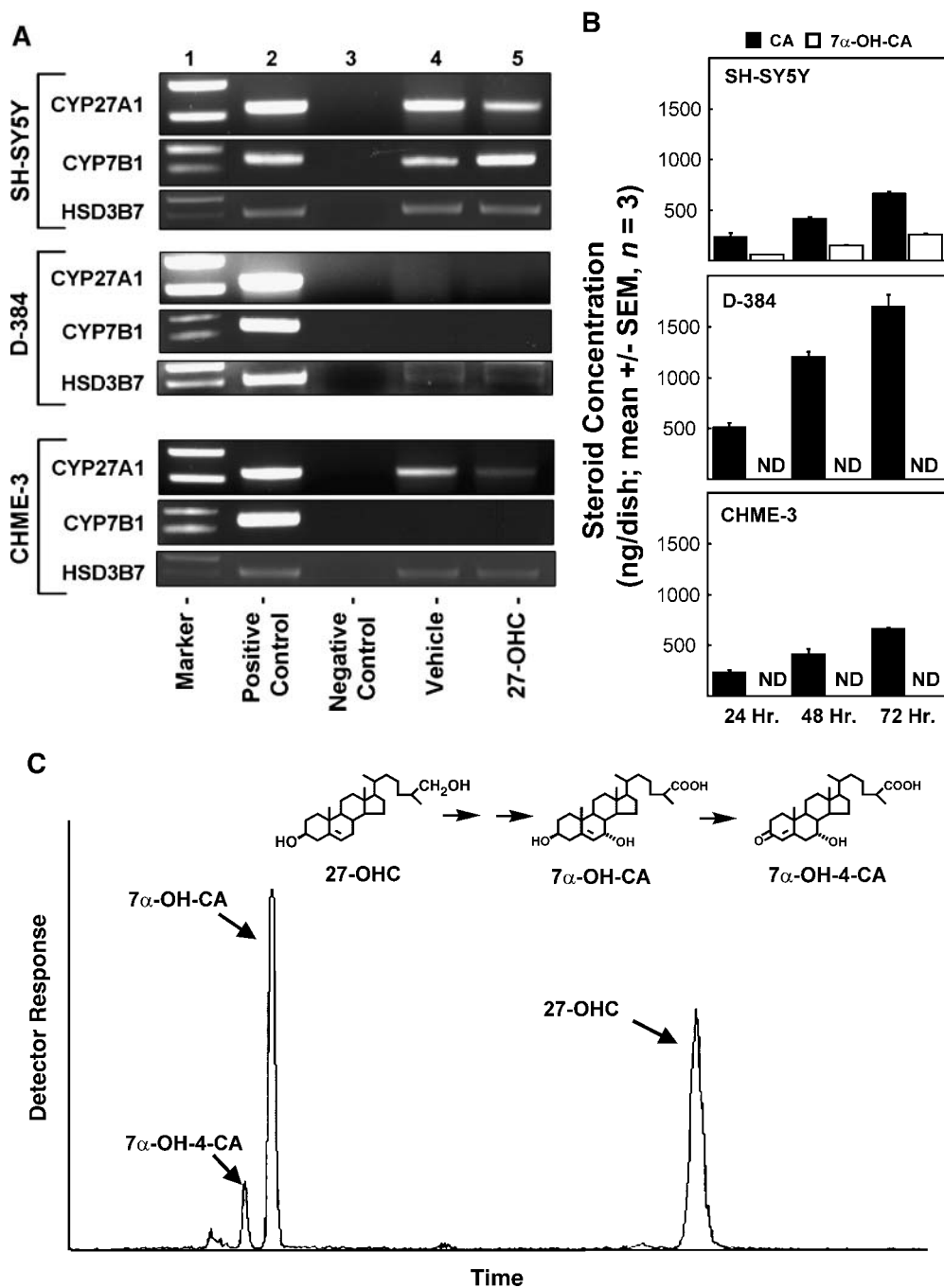


Fig. 1. Intracerebral routes for the formation of C_{27} steroidal acids. **A:** Expression of genes involved in the formation of 7α-hydroxy-3-oxo-4-cholestenoic acid (7α-OH-4-CA) in cell culture systems. Although human brain contained substantial amounts of each message, only SH-SY5Y cells expressed the complete metabolic pathway of the genes required for the formation of 7α-OH-4-CA. These expression profiles are consistent with those described previously. **B:** Incubation of SH-SY5Y cells with saturating concentrations of 27-hydroxycholesterol (27-OHC) resulted in a significant time-dependent production of cholestenic acid (CA) and 7α-hydroxy-cholestenoic acid (7α-OH-CA). In addition, there was also a very low formation of 7α-OH-4-CA. In contrast, incubations of D-384 and CHME-3 cells under identical conditions resulted in the formation of cholestenic acid only, with little or no formation of other C_{27} steroidal acids. Values shown are means \pm SEM. **C:** Metabolism of [^3H]27-OHC by SH-SY5Y cells. The radio-HPLC chromatogram shows the conversion of [^3H]27-OHC to 7α-OH-CA ($\sim 45\%$) and 7α-OH-4-CA ($\sim 6\%$).

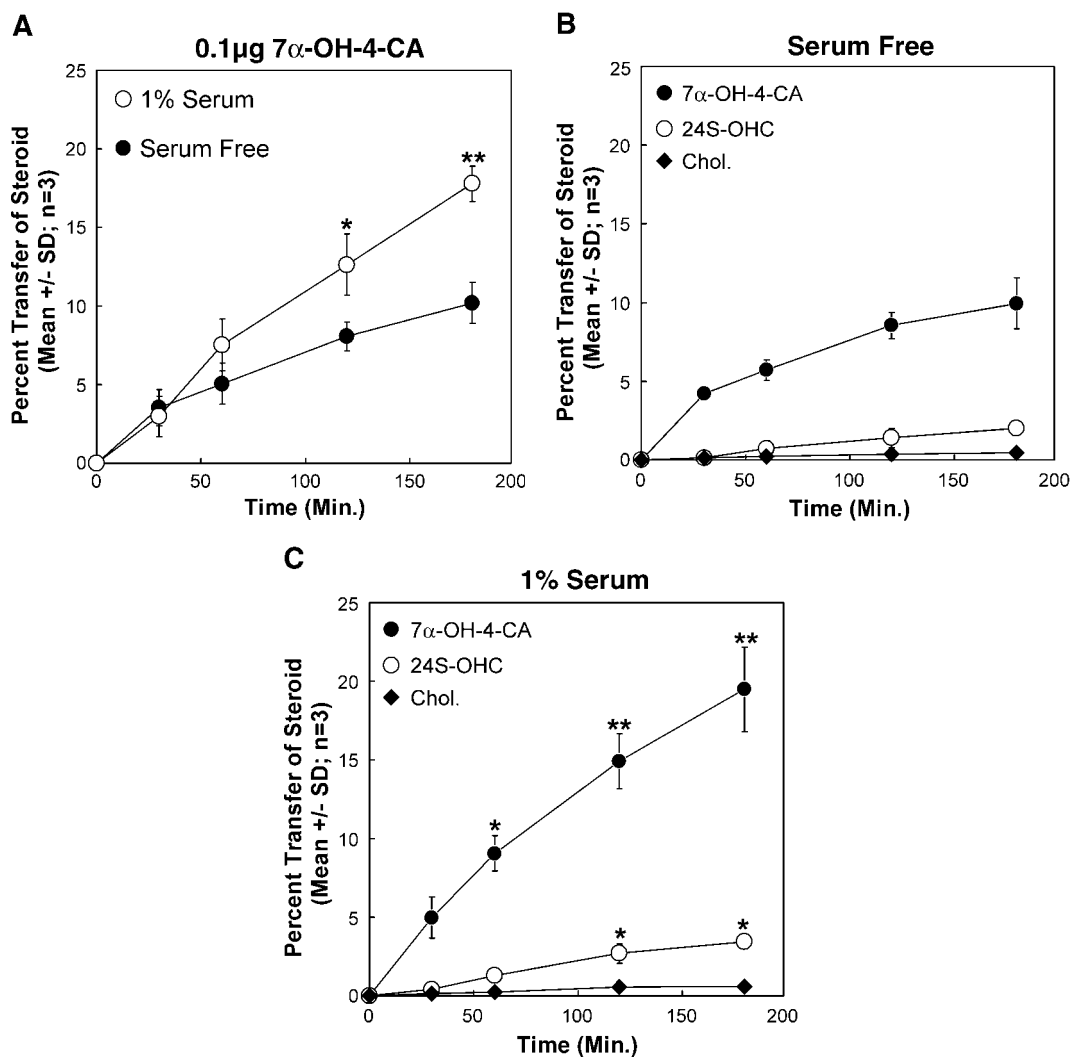


Fig. 2. Transfer of 7 α -hydroxy-3-oxo-4-cholestenoic acid (7 α -OH-4-CA) across an in vitro model of the blood-brain barrier. A: Basolateral-to-apical transfer of [3 H]7 α -OH-4-CA across porcine brain microvascular endothelial cells under serum-free conditions and in the presence of 1% serum. Addition of either serum or fatty acid-free albumin significantly stimulated the rate of transfer. Retention of the label by the cells was negligible. B, C: Comparative transfer of [3 H]cholesterol, [3 H]24S-OHC, and [3 H]7 α -OH-4-CA across porcine brain microvascular endothelial cell monolayers. Addition of serum to the apical compartment led to a 2-fold increase in 7 α -OH-4-CA transfer, whereas 24S-hydroxycholesterol (24S-OHC) and cholesterol were only marginally stimulated (C). Transfer of radiolabeled steroids at each time point was estimated as a percentage of the total (medium + cells) radioactivity present. Values represent means \pm SD of three experiments. * $P < 0.05$, ** $P < 0.01$.

blood-brain barrier in vivo (1). As expected, there was almost no measurable transport of cholesterol across the cell system. Addition of either serum or albumin to the acceptor compartment potentially stimulated the transfer of 7 α -hydroxy-3-oxo-4-cholestenoic acid but had minor effects on 24S-hydroxycholesterol (Fig. 2C and results not shown), consistent with the transport of the acidic sterol in the circulation in a complex with albumin (18). In agreement with this, addition of a trace amount of labeled 7 α -hydroxy-3-oxo-4-cholestenoic acid to human plasma, followed by the selective removal of albumin by immunoadsorption, resulted in loss of >95% of the radioactivity (results not shown). Thus, most 7 α -hydroxy-3-oxo-4-cholestenoic acid is likely to be bound to albumin in the human circulation.

In the experiments shown in Fig. 2A–C, we also measured the percentage of cell-associated radioactivity. In all of these experiments, except those with 24S-hydroxycholesterol, the percentage of cell-associated radioactivity varied between 0.4% and 1.2% of the radioactivity added to the basolateral compartment. In the experiments with 24S-hydroxycholesterol, however, the corresponding figures varied between 3% and 4% of the added radioactivity.

Arteriovenous differences of plasma sterols

To investigate the possibility that any of the steroidal acids shown in Fig. 3 and identified in the cell experiments are excreted from the human brain, we used an established technique based on sampling plasma from the jugular vein and the brachial artery of healthy volunteers.

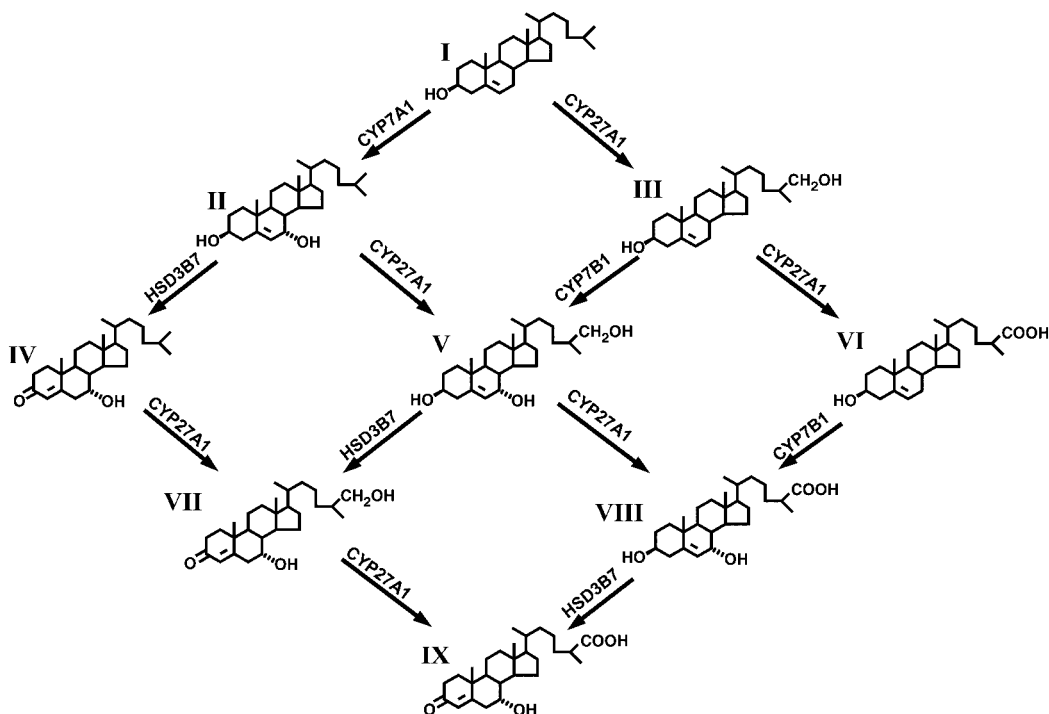


Fig. 3. Metabolism of cholesterol (I) and 27-hydroxycholesterol (III) into 7 α -hydroxy-3-oxo-4-cholestenic acid (7 α -OH-4-CA) (IX). Based on previously described pathways in other tissues, the formation of 7 α -OH-4-CA (IX) may proceed via an initial formation of 7 α -hydroxycholesterol (II), 7 α -hydroxy-4-cholesten-3-one (IV), and 7 α ,27-dihydroxy-4-cholesten-3-one (VII) or via the formation of 27-hydroxycholesterol (27-OHC) (III), cholestenic acid (VI), and 7 α -hydroxy-cholestenic acid (VIII). There is considerable potential for crossover between the different pathways. CYP7A1, cholesterol 7 α -hydroxylase; CYP7B1, oxysterol 7 α -hydroxylase; CYP27A1, sterol 27-hydroxylase; HSD3B7, 3 β -hydroxy-C27-steroid dehydrogenase/isomerase; 27-OHC, 27-hydroxycholesterol.

Analysis by GC-MS revealed that all of the major metabolites of 27-hydroxycholesterol known to be present in the general circulation were also present in the arterial and jugular plasma. Calculation of the net transfer of each sterol revealed that only one compound was found at significantly higher levels in the venous circulation ($P = 0.03$), which is equivalent to an efflux from the brain (**Table 1**). No significant concentration difference was found for any other C₂₇ steroidal acid or for the terminal metabolites of 27-hydroxycholesterol (i.e., bile acids). Moreover, despite intensive efforts, only trace amounts of possible hydroxyl-

ated metabolites of 27-hydroxycholesterol (i.e., 24,27-dihydroxycholesterol and 7 α ,27-dihydroxycholesterol) were found, indicating that these are unlikely to be participants in the elimination of 27-hydroxycholesterol from the brain (results not shown).

As these steroidal acids are likely to be transported bound to albumin, we also measured albumin concentrations in the arterial and venous plasma. To our surprise, we observed a small but significant difference in albumin concentrations between the internal jugular vein and the artery. To the best of our knowledge, a decrease in albu-

TABLE 1. Absolute and albumin-related concentrations of 27-oxygenated steroids in brachial artery and jugular vein

Steroid	Brachial Artery	Jugular Vein	Arteriovenous Difference	<i>P</i>
Analyte concentration				
Cholesterol (mg/ml)	1.56 \pm 0.04	1.53 \pm 0.04	0.02 \pm 0.02	NS
Albumin (mg/ml)	42.7 \pm 1.15	41.6 \pm 0.95	1.1 \pm 0.42	<0.05
7 α -Hydroxy-4-cholesten-3-one (ng/ml)	24.4 \pm 5.4	24.5 \pm 5.4	-0.01 \pm 0.83	NS
3 β -Hydroxy-5-cholestenic acid (ng/ml)	75.5 \pm 5.9	74.4 \pm 5.5	1.1 \pm 1.4	NS
3 β ,7 α -Dihydroxy-5-cholestenic acid (ng/ml)	29.5 \pm 4.9	28.9 \pm 4.9	0.6 \pm 0.7	NS
7 α -Hydroxy-3-oxo-4-cholestenic acid (ng/ml)	27.4 \pm 3.5	30.3 \pm 3.1	-2.8 \pm 1.3	<0.05
Analyte-albumin ratio				
3 β -Hydroxy-5-cholestenic acid (ng/mg)	1.79 \pm 0.17	1.81 \pm 0.16	-0.02 \pm 0.03	NS
3 β ,7 α -Dihydroxy-5-cholestenic acid (ng/mg)	0.69 \pm 0.12	0.69 \pm 0.12	-0.004 \pm 0.02	NS
7 α -Hydroxy-3-oxo-4-cholestenic acid (ng/mg)	0.65 \pm 0.09	0.73 \pm 0.08	-0.08 \pm 0.03	<0.05

Values shown are means \pm SEM ($n = 9$), except for 7 α -hydroxy-4-cholesten-3-one, for which $n = 6$.

min concentration in the jugular vein has not been reported previously. Correction of the levels described above for the C₂₇ steroidal acids for changes in albumin levels did not alter the pattern revealed by the absolute concentrations: 7 α -hydroxy-3-oxo-4-cholestenoic acid remained the only metabolite present at significantly greater concentrations ($P = 0.015$) in the venous circulation. Based on these results, the flux of 7 α -hydroxy-3-oxo-4-cholestenoic acid from the brain was calculated to be ~ 2 mg/24 h.

DISCUSSION

It is well established that conversion of cholesterol into 24S-hydroxycholesterol is of importance for cholesterol homeostasis in the brain (for review, see Ref. 1). However, this mechanism appears to be responsible for the removal of only two-thirds of newly synthesized cholesterol in rodents (19, 20). To compensate for continuing cholesterol synthesis in the brain, additional mechanisms are likely to exist. The occurrence of brain xanthoma in patients with cerebrotendinous xanthomatosis is consistent with the possibility that sterol 27-hydroxylase is involved in such an additional removal mechanism.

It is well known that sterol 27-hydroxylase is involved in cholesterol elimination in cells such as macrophages and endothelial cells. We have shown previously that there is a centripetal flux of 27-oxygenated steroids to the liver, where they are taken up and integrated into bile acid synthesis. However, uptake of 7 α -hydroxy-3-oxo-4-cholestenoic acid is extremely efficient, with an apparent extraction of $>40\%$ in a single pass (21). The present results show that secretion of 7 α -hydroxy-3-oxo-4-cholestenoic acid by the brain is also highly efficient, implying that this steroid is well adapted to fulfill a role as a transport form for sterols.

This contention is supported by the present results using porcine brain microvascular endothelial cells. Transfer of 7 α -hydroxy-3-oxo-4-cholestenoic acid proceeds by an apparently nonsaturable mechanism. Interestingly, the rate of transfer was considerably greater than that of 24S-

hydroxycholesterol, an oxysterol known to flux continuously from the brain to the circulation. The addition of albumin (or serum) to the system increased the flux of the acid across the brain endothelial cell monolayer.

It is evident that the magnitude of the flux of 7 α -hydroxy-3-oxo-4-cholestenoic acid from the brain is lower than could be expected if this were the only metabolite of 27-hydroxycholesterol leaving the brain. We were unable to demonstrate a net flux of the precursors of 7 α -hydroxy-3-oxo-4-cholestenoic acid shown in Fig. 3 from the brain into the circulation. In view of the relatively great variations and small sample size, our results do not completely exclude the presence of such a flux. It should be mentioned that there may be some cerebral production of chenodeoxycholic acid, as there was also an apparent enrichment of this acid in subdural hematomas (9). We have failed to demonstrate such a production in our *in vitro* studies, and preliminary *in vivo* experiments in our laboratory have also failed to show a net excretion of chenodeoxycholic acid from the human brain into the circulation (our unpublished observation).

In view of the effects of side chain oxidized oxysterols on the generation of amyloid (22), the flux of 27-hydroxycholesterol over the blood-brain barrier has been suggested to be the missing link between hypercholesterolemia and Alzheimer's disease (23). Because there is a good correlation between the levels of cholesterol and 27-hydroxycholesterol in the circulation, it is likely that hypercholesterolemia is associated with an increased flux of 27-hydroxycholesterol into the brain. In this connection, it is interesting that we have demonstrated an accumulation of this specific oxysterol in the brain of patients with Alzheimer's disease as well as in the brain of mice with the Swedish mutation in the amyloid precursor gene (5). The accumulation may be attributable to an increased potential for flux of this steroid from the circulation into the brain as a consequence of a defect in the blood-brain barrier. Another possibility is a reduced intracerebral metabolism by CYP7B1, and according to recent reports, the levels of this cytochrome in the brain of patients with Alzheimer's disease are reduced (24, 25). The possibility may also be considered that a normal regulatory response

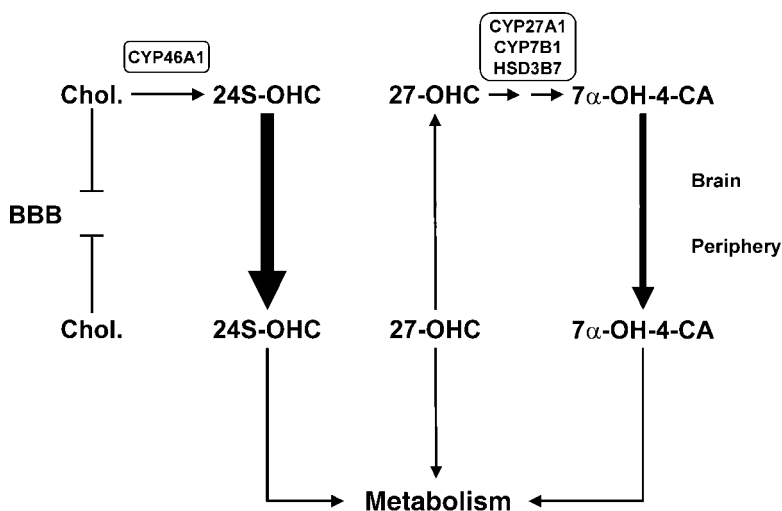


Fig. 4. Crosstalk of oxysterols over the blood-brain barrier (BBB) in humans. The efficient blood-brain barrier restricts the movement of cholesterol (Chol.) into and out of the brain. However, side chain oxidized oxysterols are capable of crossing this barrier. 24S-Hydroxycholesterol can exit the brain and serve as an excretory form of brain cholesterol, whereas 27-hydroxycholesterol may pass from the general circulation into the brain. Highly efficient systems exist to rapidly metabolize 27-hydroxylated sterols present in the brain to the major metabolite 7 α -hydroxy-3-oxo-4-cholestenoic acid. CYP46A1, cholesterol 24S-hydroxylase; CYP7B1, oxysterol 7 α -hydroxylase; CYP27A1, sterol 27-hydroxylase; HSD3B7, 3 β -hydroxy-C27-steroid dehydrogenase/isomerase.

to an increased flux of 27-hydroxycholesterol is lost in the brain of Alzheimer's patients. In any case, it is evident that the present pathway for the elimination of 27-hydroxylated sterols must be important in connection with the increased amounts of 27-hydroxycholesterol in the Alzheimer's brain.

To summarize, we have identified a new mechanism for the elimination of 27-hydroxylated sterols, and potentially also cholesterol, from the brain. The essential features of this mechanism are depicted in **Fig. 4**. According to the present data, this new pathway corresponds to a steroid flux that is about one-third of that of 24S-hydroxycholesterol. However, once 7 α -hydroxy-3-oxo-4-cholestenoic acid has been formed, it is eliminated very efficiently, and the importance of the conversion may be greater under pathological conditions. It is tempting to suggest that the lack of this mechanism in patients with cerebrotendinous xanthomatosis may be part of the explanation for the accumulation of brain xanthomas in this disease. **■**

This work was supported by the Swedish Science Council, the Heart-Lung Foundation, the Brain Foundation, Brain Power, the Foundations "Gamla Tjänarinnor," Thuring, Lars Hiertas, and Stohnes, the Austrian Science Fund (P17474-B0), National Institutes of Health Grants GM-62882 and AG-024336, and in part by Pfizer. The gift of the CHME-3 microglial cell line from Prof. M. Tardieu (Université Paris Sud, France) is gratefully acknowledged.

REFERENCES

- Dietschy, J. M., and S. D. Turley. 2004. Thematic review series: brain lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. *J. Lipid Res.* **45**: 1375–1397.
- Björkhem, I., and S. Meaney. 2004. Brain cholesterol: long secret life behind a barrier. *Arterioscler. Thromb. Vasc. Biol.* **24**: 806–815.
- Heverin, M., S. Meaney, D. Lütjohann, U. Diczfalusy, J. Wahren, and I. Björkhem. 2005. Crossing the barrier: net flux of 27-hydroxycholesterol into the human brain. *J. Lipid Res.* **46**: 1047–1052.
- Leoni, V., T. Masterman, P. Patel, S. Meaney, U. Diczfalusy, and I. Björkhem. 2003. Side chain oxidized oxysterols in cerebrospinal fluid and the integrity of blood-brain and blood-cerebrospinal fluid barriers. *J. Lipid Res.* **44**: 793–799.
- Heverin, M., N. Bogdanovic, D. Lütjohann, T. Bayer, I. Pikuleva, L. Bretillon, U. Diczfalusy, B. Winblad, and I. Björkhem. 2004. Changes in the levels of cerebral and extracerebral sterols in the brain of patients with Alzheimer's disease. *J. Lipid Res.* **45**: 186–193.
- Björkhem, I., K. Muri Boberg, and E. Leitersdorf. 2001. The Metabolic and Molecular Bases of Inherited Disease. 8th edition. McGraw Hill, New York.
- Axelsson, M., B. Mörk, and J. Sjövall. 1988. Occurrence of 3 β -hydroxy-5-cholestenoic acid, 3 β ,7 α -dihydroxy-5-cholestenoic acid, and 7 α -hydroxy-3-oxo-4-cholestenoic acid as normal constituents in human blood. *J. Lipid Res.* **29**: 629–641.
- Zhang, J., Y. Akwa, M. el-Etr, E. E. Baulieu, and J. Sjövall. 1997. Metabolism of 27-, 25- and 24-hydroxycholesterol in rat glial cells and neurons. *Biochem. J.* **322**: 175–184.
- Nagata, K., K. Takakura, T. Asano, Y. Seyama, H. Hirota, N. Shigematsu, I. Shima, T. Kasama, and T. Shimizu. 1992. Identification of 7 α -hydroxy-3-oxo-4-cholestenoic acid in chronic subdural hematoma. *Biochim. Biophys. Acta.* **1126**: 229–236.
- Nagata, K., Y. Seyama, and T. Shimizu. 1995. Changes in the level of 7 α -hydroxy-3-oxo-4-cholestenoic acid in cerebrospinal fluid after subarachnoid hemorrhage. *Neurol. Med. Chir. (Tokyo).* **35**: 294–297.
- Björkhem, I. 1969. On the mechanism of the enzymatic conversion of cholest-5-ene-3 β ,7 α -diol into 7 α -hydroxycholest-4-en-3-one. *Eur. J. Biochem.* **8**: 337–344.
- Janabi, N., S. Peudenier, B. Heron, K. H. Ng, and M. Tardieu. 1995. Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. *Neurosci. Lett.* **195**: 105–108.
- Balmforth, A. J., S. G. Ball, R. I. Freshney, D. I. Graham, H. B. McNamee, and P. F. Vaughan. 1986. D-1 dopaminergic and beta-adrenergic stimulation of adenylate cyclase in a clone derived from the human astrocytoma cell line G-CCM. *J. Neurochem.* **47**: 715–719.
- Balazs, Z., U. Panzenboeck, A. Hammer, A. Sovic, O. Quehenberger, E. Malle, and W. Sattler. 2004. Uptake and transport of high-density lipoprotein (HDL) and HDL-associated alpha-tocopherol by an in vitro blood-brain barrier model. *J. Neurochem.* **89**: 939–950.
- Jellinck, P. H., M. Kaufmann, A. Gottfried-Blackmore, G. Croft, V. Byford, B. S. McEwen, G. Jones, and K. Bulloch. 2006. Dehydroepiandrosterone (DHEA) metabolism in the brain: identification by liquid chromatography/mass spectrometry of the delta-4-isomer of DHEA and related steroids formed from androstenedione by mouse BV2 microglia. *J. Steroid Biochem. Mol. Biol.* **98**: 41–47.
- Steckelbroeck, S., M. Watzka, D. Lütjohann, P. Makiola, A. Nassen, V. H. Hans, H. Clusmann, A. Reissinger, M. Ludwig, L. Siekmann, et al. 2002. Characterization of the dehydroepiandrosterone (DHEA) metabolism via oxysterol 7 α -hydroxylase and 17-ketosteroid reductase activity in the human brain. *J. Neurochem.* **83**: 713–726.
- Trap, C., F. Nato, S. Chalbot, S. B. Kim, P. Lafaye, and R. Morfin. 2005. Immunohistochemical detection of the human cytochrome P4507B1: production of a monoclonal antibody after cDNA immunization. *J. Neuroimmunol.* **159**: 41–47.
- Babiker, A., and U. Diczfalusy. 1998. Transport of side-chain oxidized oxysterols in the human circulation. *Biochim. Biophys. Acta.* **1392**: 333–339.
- Björkhem, I., D. Lütjohann, O. Breuer, A. Sakinis, and A. Wennmalm. 1997. Importance of a novel oxidative mechanism for elimination of brain cholesterol. Turnover of cholesterol and 24(S)-hydroxycholesterol in rat brain as measured with ¹⁸O₂ techniques in vivo and in vitro. *J. Biol. Chem.* **272**: 30178–30184.
- Lund, E. G., C. Xie, T. Kotti, S. D. Turley, J. M. Dietschy, and D. W. Russell. 2003. Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specific mechanism of cholesterol turnover. *J. Biol. Chem.* **278**: 22980–22988.
- Lund, E., O. Andersson, J. Zhang, A. Babiker, G. Ahlberg, U. Diczfalusy, K. Einarsson, J. Sjövall, and I. Björkhem. 1996. Importance of a novel oxidative mechanism for elimination of intracellular cholesterol in humans. *Arterioscler. Thromb. Vasc. Biol.* **16**: 208–212.
- Brown, J., 3rd, C. Theisler, S. Silberman, D. Magnuson, N. Gottardi-Littell, J. M. Lee, D. Yager, J. Crowley, K. Sambamurti, M. M. Rahman, et al. 2004. Differential expression of cholesterol hydroxylases in Alzheimer's disease. *J. Biol. Chem.* **279**: 34674–34681.
- Björkhem, I., M. Heverin, V. Leoni, S. Meaney, and U. Diczfalusy. 2006. Oxysterols and Alzheimer's disease. *Acta Neurol. Scand. Suppl.* **185**: 43–49.
- Yau, J. L., S. Rasmuson, R. Andrew, M. Graham, J. Noble, T. Olsson, E. Fuchs, R. Lathe, and J. R. Seckl. 2003. Dehydroepiandrosterone 7-hydroxylase CYP7B: predominant expression in primate hippocampus and reduced expression in Alzheimer's disease. *Neuroscience.* **121**: 307–314.
- Schumacher, M., S. Weill-Engerer, P. Liere, F. Robert, R. J. Franklin, L. M. Garcia-Segura, J. J. Lambert, W. Mayo, R. C. Melcangi, A. Parducz, et al. 2003. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. *Prog. Neurobiol.* **71**: 3–29.