Cholesterol metabolism changes under long-term dietary restrictions while the cholesterol homeostasis remains unaffected in the cortex and hippocampus of aging rats

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Abstract Maintaining cholesterol homeostasis in the brain is vital for its proper functioning. While it is well documented that dietary restriction modulates the metabolism of cholesterol peripherally, little is known as to how it can affect cholesterol metabolism in the brain. The present study was designed to elucidate the impact of long-term dietary restriction on brain cholesterol metabolism. Three-month-old male Wistar rats were exposed to long-term dietary restriction until 12 and 24 months of age. The concentrations of cholesterol, its precursors and metabolites, and food-derived phytosterols were measured in the serum, cortex, and hippocampus by gas

chromatography/mass spectrometry. Relative changes in the levels of proteins involved in cholesterol synthesis, transport, and degradation were determined by Western blot analysis. Reduced food intake influenced the expression patterns of proteins implicated in cholesterol metabolism in the brain in a region-specific manner. Dietary restriction decreased the concentrations of cholesterol precursors, lanosterol in the cortex, and lanosterol and lathosterol in the hippocampus at 12 months, while the level of desmosterol was elevated in the hippocampus at 24 months. The concentrations of cholesterol and 24(S)-hydroxycholesterol remained unaffected. Food-derived phytosterols were significantly lower after dietary restriction in both the cortex and hippocampus at 12 and 24 months. These findings provide new insight into the effects of dietary restriction on cholesterol metabolism in the brain, lending further support to its neuroprotective effect.

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Introduction

The beneficial effects of dietary restriction (DR) on health and general well-being have been recognized for decades. Dietary restriction is capable of prolonging the lifespan in different species and has positive effects in retarding aging and preventing

and/or delaying the onset of numerous age-associated diseases (reviewed in Cutler and Mattson 2006). The cortex and hippocampus are brain regions particularly vulnerable to aging and to neurodegenerative conditions commonly associated with aging (Jessberger and Gage 2008; Asha Devi 2009). Increased oxidative stress, changes in different neurotransmitter systems (Mora et al. 2007), and decrease of spine densities and neurogenesis (Burke and Barnes 2006; Segovia et al. 2006) are some of the underlying causes of general neuronal dysfunction with advancing age (reviewed in Fontán-Lozano et al. 2008). Both of these brain regions exhibit high responsiveness to the beneficial effects of DR. Studies on the rodent central nervous system (CNS) have revealed that long-term DR can improve learning and memory, and diminish age-related behavioral impairments and cognitive decline (Means et al. 1993; Halagappa et al. 2007; Adams et al. 2008) caused by disruptions of the pivotal brain functions associated with the cortex and hippocampus. Furthermore, DR alleviates the decline in hippocampal neurogenesis (Park et al. 2013) and counteracts the age-related decrease of synaptic plasticity (Fontán-Lozano et al. 2008; Mladenovic Djordjevic et al. 2010).

Cholesterol is an essential component of synapses that, aside from directly enhancing presynaptic differentiation, enables continuous synaptogenesis and provides stability of evoked transmitter release (Goritz et al. 2005). The formation, function, and stability of synapses are sensitive to changes in cholesterol metabolism (Pfrieger 2003). Cholesterol is ubiquitously distributed throughout the brain where it is produced in situ (Dietschy and Turley 2001), and since it does not pass the blood–brain barrier (BBB), the maintenance of steady levels of cholesterol is crucial for proper CNS functioning. Cholesterol homeostasis is accomplished through a sophisticated regulation of synthesis, transport, and elimination of excessive cholesterol from the brain. Cholesterol biosynthesis (Fig. 1) is a multistep process that starts with acetyl coenzyme A which is processed to the cholesterol precursor lanosterol. From this point onwards, two alternative pathways lead to cholesterol formation: the Bloch pathway via the direct cholesterol precursor desmosterol, and the Kandutsch–Russell pathway via lathosterol as featuring intermediate (Goldstein and Brown 1990). Evidence for the

alternative use of these pathways during aging has been reported. In young rodents, cerebral cholesterol is mainly synthesized by the Bloch pathway, while the Kandutsch–Russell pathway prevails in older rodents (Lutjohann et al. 2002; Smiljanic et al. 2013). The rate-limiting enzyme in cholesterol synthesis is the endoplasmic reticulum-bound 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (HMGCR) which is responsible for the conversion of 3-hydroxy-3-methylglutaryl-coenzyme-A into mevalonate (Siperstein and Fagan 1966). Recycling and intercellular transport of cholesterol in the brain are efficiently maintained by apolipoprotein E (ApoE) (Mauch et al. 2001; Pfrieger 2002). Excess cholesterol is mainly eliminated following its conversion into 24(S)-hydroxycholesterol (24S-OHC) by the enzyme cholesterol 24-hydroxylase (Cyp46a1) (Lund et al. 1999). Thereby, the transformed cholesterol passes the BBB, enters the circulation, and is eliminated by the liver (Li-Hawkins et al. 2000; Bjorkhem et al. 2001).

Phytosterols are plant-derived steroid compounds similar to cholesterol. Phytosterol-enriched foods and dietary supplements have been marketed for decades, and scientific evidence supports a relationship between phytosterol consumption and lower blood cholesterol levels (Peterson 1958; Plat et al. 2000). In contrast to cholesterol, the phytosterols can pass the BBB (Jansen et al. 2006; Vanmierlo et al. 2012) and accumulate in the brain, preferentially within the lipid rafts of brain cells, altering membrane fluidity and modulating the lipid raft-dependent protein signaling cascade (Vanmierlo et al. 2012; Hac-Wydro et al. 2009). Despite the beneficial role of phytosterols in reducing soluble amyloid beta 42 (A β 42) levels, they also may manifest unfavorable effects on neuroinflammation and production of reactive oxygen species (ROS) (Koivisto et al. 2014). Recent findings showed that high phytosterol concentrations in the brains of ATP-binding cassette g5 (Abcg5)-deficient mice affected brain cholesterol metabolism, particularly in the hippocampus, but did not have major effects either on memory functions, anxiety, or mood-related behavior (Vanmierlo et al. 2011). Altogether, the exact role and further metabolism of phytosterols in the brain remain to be fully clarified.

Although numerous studies have dealt with the impact of aging on brain cholesterol metabolism, the published data are inconsistent (reviewed in Ledesma et al. 2012). We previously showed that aging decreases cholesterol synthesis in the brain while the overall

Fig. 1 Schematic representation of cholesterol synthetic pathways

cholesterol levels remain relatively stable (Smiljanic et al. 2013). While it is well documented how DR modulates cholesterol metabolism peripherally (Martini et al. 2007, 2008), little is known on how it affects cholesterol levels and metabolism in the aging brain. Although a few studies have addressed this issue (Pallottini et al. 2003; Fon Tacer et al. 2010; Mulas et al. 2005), the effects of long-term DR on both cholesterol and its intermediates in the aging brain have not been examined. Pallottini et al. (2003) followed the impact of both DR and every-other-day feeding on dolichol and cholesterol accumulation, while Mulas et al. (2005) used the same dietary regime to analyze its effects on cholesterol and cholesterol ester levels in the whole brains of aging rats. Although the study of Fon Tacer et al. (2010) focused on the metabolism of cholesterol intermediates, the authors studied these changes only during short-term (20 h) fasting in the adult rat brain. Moreover, although cholesterol metabolism in the brain is mostly autonomous, recent studies have revealed comparable changes in the serum and brain lipid profiles (Stranahan et al. 2011; Camargo et al. 2012). Various mechanisms have been suggested to promote lipid transport across the BBB. They involve increased angiogenesis of brain capillaries (Saito et al. 2009) and/or increased

expression of lipoprotein receptors in brain capillary cells (Karasinska et al. 2009), although the connection between cholesterol metabolism in the serum and brain needs to be investigated further.

Given the importance of cholesterol metabolites as biologically active molecules, we aimed to elucidate the effects of long-term DR on cholesterol metabolism in the aging brain. To that end, we measured the concentrations of cholesterol, its intermediates and metabolites in the cortex and hippocampus, as well as in sera of middle-aged (12 months) and aged (24 months) rats. We also examined the levels of the key proteins involved in cholesterol synthesis, transport, and degradation in the brain. Finally, we sought to understand how the levels of phytosterols as the sole food-derived sterols were affected by the same DR regimen.

Materials and methods

Animals and treatments

Male Wistar rats were used in this study. All animal procedures complied with the EEC Directive (86/609/EEC) on the protection of animals used for experimental

and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research “Sinisa Stankovic,” University of Belgrade. The animals were housed under standard conditions (23 ± 2 °C, relative humidity 60–70 %, 12-h light/dark cycle), and their health status was routinely checked. Food (standard laboratory chow pellets containing 8.34 % water, 21.61 % crude protein, 2.36 % crude fat, 6.68 % crude fiber, 6.55 % crude ash, 1.95 % minerals) was available ad libitum (AL) until 3 months of age. At that time, the average daily food consumption was determined and rats were divided into two groups: one group continued to receive food ad libitum (AL group), while the other group (DR group) was allowed 100 % of the determined mean daily intake every other day (EOD). All experimental groups ($n=5$ per group) were maintained on this dietary regimen up to 12 and 24 months of age when they were euthanized by decapitation. Their brains were quickly removed, and the cortex and hippocampus were dissected on ice, collected for subsequent sterol and protein analysis, and stored on -80 °C until further use. Blood was collected from the trunk, and the serum was isolated and frozen.

Sterol profile determination

Prior to analysis, the cortex and hippocampus samples (~20 mg each) were spun in a speed vacuum dryer (12 mbar; Savant AES 1000) for 24 h in order to relate the individual sterol concentrations to dry weight. The sterols were extracted from the dried tissue by placing in a 1.5-ml mixture of chloroform/methanol (2:1) for 24 h at 4 °C. Sterol levels were determined as described previously (Lutjohann et al. 2002; Jansen et al. 2006). In brief, 50 µg 5 α -cholestane (Serva) (50 µl from a stock solution of 5 α -cholestane in cyclohexane; 1 mg/ml) and 1 µg epicoprostanol (Sigma) (10 µl from a stock solution epicoprostanol in cyclohexane; 100 µg/ml) were added to 100 µl serum and 1.125 ml chloroform/methanol brain extract. One milliliter of NaOH (1 M) in 80 % ethanol was added for alkaline hydrolysis over 60 min at 61 °C. Sterols were subsequently extracted with 3 ml of cyclohexane twice. The organic solvents were evaporated, and the residual sterols were dissolved in 160 µl *n*-decane. Next, 80 µl of the *n*-decane samples were transferred into micro-vials for GC/MS quantification. The sterols were derivatized to trimethylsilyl (TMSi) ethers by adding 10 µl TMSi reagent (pyridine/hexamethylsilazane/

trimethylchlorosilane; 9:3:1, by volume; all reagents were applied from Merck) and incubated for 1 h at 64 °C. The residual 80 µl of *n*-decane samples were diluted with 300 µl *n*-decane and derivatized with 30 µl TMSi reagent preceding analysis of cholesterol by gas chromatography–flame ionization detection (GC/FID).

The food sterol content compared to the total chow composition was determined by the same procedure and is shown in Table 1.

Western blot analysis

For Western blot analysis, the tissues were homogenized and sonicated in 10 volumes of RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 % NP-40, 0.5 % Triton X-100, 0.1 % SDS, 1 mM EDTA, 1 mM EGTA) containing a complete protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (25 mM NaF, 5 mM Na₄P₂O₇, 2 mM Na₃VO₄). Protein concentrations were determined using the Micro BCA Protein Assay Kit (Pierce Biotechnology) and bovine serum albumin (BSA) as standard. Fifteen micrograms of proteins per lane for Cyp46a1 and ApoE, and 25 µg per lane for HMGCR were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were blocked in 5 % non-fat dry milk/Tris-buffered saline with 0.05 % Tween 20 (TBST) (150 mM NaCl, 50 mM Tris, pH 7.4, and 0.05 % Tween 20) for 1 h at room temperature (RT) and incubated with the following primary antibodies for the indicated times: goat anti-ApoE (1:5,000; Calbiochem) for 1 h/RT, rabbit anti-CYP46A1 antibody T623 (1:1,000; a generous gift from Dr. David Russell, University of Texas Southwestern Medical Center, Dallas, USA) for 2 h/RT, and rabbit anti-HMGCR (1:300, Millipore) overnight at +4 °C. The antibodies were diluted in TBST, and milk was added to a final concentration of 3 % only for the anti-HMGCR antibody. Following several rinses in TBST, the membranes were incubated for 1 h at RT with the appropriate horse radish peroxidase (HRP)-

Table 1 The sterol content in the food

Food	Content (ng/mg)	Weight (%)
Cholesterol	25.83	0.11
Sitosterol	405.48	1.67
Campesterol	111.00	0.46
Stigmasterol	31.19	0.13

conjugated secondary antibodies (bovine anti-rabbit, 1:2,000; bovine anti-goat, 1:5,000; both from Santa Cruz, sc-2370 and sc-2350, respectively) diluted in TBST. HRP-immunoreactive bands were visualized by enhanced chemiluminescence (ECL, GE Healthcare) and film (Kodak Biomax) exposure. Each blot was re-probed with rabbit anti-actin antibody (1:10,000; Sigma) diluted in TBST. Signals were quantified densitometrically using Image Quant software (v. 5.2, GE Healthcare) and expressed as relative values (i.e., normalized to the corresponding β -actin signals). Changes in the levels of analyzed proteins in each DR group were expressed as ratios (fold changes) relative to the appropriate age-matched control group.

Statistical analysis

All values were expressed as the mean $\pm$ SEM. Differences between the experimental groups were tested using nonparametric Mann–Whitney's *U* test (STATISTICA v. 6.0, StatSoft, Tulsa, OK). Statistical significance was set at $P < 0.05$.

Results

Long-term DR affects the level of cholesterol precursors and phytosterols in the serum

The effects of DR on sterol concentrations in serum from 12- and 24-month-old animals are presented in Table 2. DR had no influence on serum cholesterol levels in either of the age groups analyzed. However, the levels of lanosterol were significantly reduced in 12-month-old animals exposed to DR (34 %). Similarly, the concentration of lathosterol, the cholesterol precursor in the Kandutsch–Russel pathway, was decreased significantly in both 12- and 24-month-old rats under DR (51 and 34 %, respectively). In contrast, the concentration of desmosterol, the product in Bloch cholesterol synthesis pathway, was dramatically increased in 24-month-old animals under DR (177 %), while no changes were detected in the 12-month-old animals. Furthermore, DR had no influence on the levels of 24S-OHC in either age group.

The effect of DR on the concentrations of different phytosterols in the serum of 12- and 24-month-old animals is presented in Table 2. Dietary restriction decreased serum phytosterol levels in both age groups.

Compared to age-matched controls, reduced concentrations of phytosterols sitosterol (58 and 74 %), campesterol (54 and 65 %), as well as stigmasterol (42 and 61 %) were detected in 12- and 24-month-old DR-treated animals, respectively.

Long-term DR affects the levels of cholesterol precursors but not cholesterol homeostasis in the cortex and hippocampus of aging rats

We next sought to understand how the levels of cholesterol and its precursors are changing in the brain of aging rats under DR, specifically, how the cortex and hippocampus are affected. Therefore, we measured the levels of cholesterol, its precursors (lanosterol, lathosterol, and desmosterol), and degradation sterol (24S-OHC) in these regions in control and long-term DR-treated animals (Fig. 2).

The levels of cholesterol in each age group analyzed did not change under DR (Fig. 2a). However, DR did decrease the concentration of the main cholesterol precursor lanosterol in 12-month-old animals in both the cortex and hippocampus (33 and 32 %, respectively; Fig. 2b) without any effect in 24-month-old animals. It is of interest to note that the concentration of lanosterol decreases in the cortex of 24-month-old control animals when compared to the 12-month-old control group (44 %). The same is true for the lanosterol concentration in the hippocampus during aging (30 %). Therefore, the decrease of lanosterol in DR-treated animals consequently leads to the steady levels of lanosterol in both 12- and 24-month DR-fed age groups, in both the cortex and hippocampus (Fig. 2b).

DR affected differently the concentrations of lathosterol and desmosterol, precursors of cholesterol in the Kandutsch–Russell and Bloch pathway, respectively, in different brain regions (Fig. 2c, d). The levels of both lathosterol and desmosterol remained unchanged in the cortex of control and DR-fed animals in both age groups. However, DR decreased the levels of lathosterol (35 %) in the hippocampus in the 12-month-old age group and, in contrast, increased the levels of desmosterol (37 %) in the hippocampus of DR-fed 24-month-old animals. Levels of lathosterol in the hippocampus of 24-month-old DR-fed animals remained the same as in the control (Fig. 2c). Similarly, the levels of desmosterol in the hippocampus of 12-month-old animals were unaffected with DR (Fig. 2d).

Table 2 Sterol concentrations in the rat serum

Serum	Units	12 months		24 months	
		AL	DR	AL	DR
Sterols					
Cholesterol	mg/dl	90.59±8.85	72.36±3.73	112.82±17.51	97.32±3.66
Lanosterol	µg/dl	10.30±0.98	6.76±0.25*	8.44±1.32	6.59±0.21
Lathosterol	mg/dl	0.086±0.011	0.042±0.002*	0.077±0.015	0.051±0.005*
Desmosterol	mg/dl	0.100±0.007	0.130±0.019	0.124±0.026	0.343±0.050*
24S-hydroxycholesterol	ng/ml	27.17±2.10	27.10±0.93	31.33±3.52	28.44±0.99
Phytosterols					
Sitosterol	mg/dl	4.65±0.42	1.94±0.31*	3.94±0.54	1.04±0.14*
Campesterol	mg/dl	3.88±0.34	1.77±0.23*	2.96±0.36	1.05±0.13*
Stigmasterol	µg/dl	38.26±2.77	22.08±3.38*	33.19±1.48	12.9±1.57*

Data represent the mean ± SEM (five rats per group)

* $P<0.05$ vs. the age-matched control

Considering that the levels of cholesterol remain unchanged in our experimental paradigm, we sought to understand if the levels of cholesterol degradation product, 24S-OHC, were affected by DR. The levels of 24S-OHC were not affected by exposure to DR in either of the examined brain regions in each age group (Fig. 2e).

Long-term DR increases desmosterol/cholesterol ratio in the serum and hippocampus of aging rats

The levels of desmosterol increase in the serum and hippocampus of aging rats under DR, but the cholesterol levels remain the same. Given that the ratio between desmosterol and cholesterol in the serum was recently proposed to be a useful marker for determining AD-related degenerative changes (Sato et al. 2012), we sought to understand how DR is affecting the ratio of desmosterol vs. cholesterol in serum and brain structures analyzed. The results are presented in Table 3. Significant increase in the ratio of desmosterol/cholesterol was observed in the serum of DR-treated animals in both age groups. Similarly, significant increase of desmosterol/cholesterol was observed in the hippocampus of 24-month-old rats under DR when compared to control. DR did not have any effect on the desmosterol/cholesterol ratio in the cortex in either of age groups analyzed.

Long-term DR selectively affects the expression of proteins involved in cholesterol metabolism

Although cholesterol homeostasis remained unchanged during DR in both the cortex and hippocampus, the concentration of some of the precursors was significantly altered in the structures analyzed (Fig. 2). We sought to understand if these changes were compensated with the altered levels of proteins involved in biosynthesis (HMGCR), recycling (ApoE), and degradation (Cyp46a1) of cholesterol. We performed Western blot analysis of the expression levels of these proteins in the cortex and hippocampus of control and DR-fed, 12- and 24-month-old animals. The results are presented as fold of control in Fig. 3.

Firstly, we analyzed the expression of HMGCR, the rate-limiting enzyme in cholesterol synthesis. The expression level of this enzyme in the cortex was not affected under DR in neither of the age groups analyzed (Fig. 3a). However, DR significantly lowered the expression of HMGCR (41 %) in the hippocampus of 24-month-old rats when compared to age-matched control (Fig. 3b). Considering that recycling and intercellular transport of cholesterol are very important in maintaining cholesterol levels, we sought to understand if ApoE, a protein that is responsible for these processes, is affected with long-term DR. We showed that long-term DR caused a significant decrease (24 %) of ApoE levels in the cortex of 12-month-old rats (Fig. 3c). In contrast, in the hippocampus of 24-month-old rats, DR

Fig. 2 The effect of long-term DR on the concentrations of cholesterol and cholesterol precursors in the cortex and hippocampus. Gas chromatography/mass spectrometry analysis of the levels of cholesterol (a); its precursors lanosterol (b), lathosterol (c), and desmosterol (d); and its metabolites 24S-hydroxycholesterol (24S-OHC) (e) and cholestanol (f) in the cortex (left columns) and hippocampus (right columns) of rats fed ad libitum (AL light bars) and rats exposed to dietary restriction (DR dark bars) for 12 and 24 months. The data are presented as the mean $\pm$ SEM (five rats per group). * $P < 0.05$ vs. age-matched control; # $P < 0.05$ vs. 12 months

significantly increased (34 %) the levels of ApoE when compared to age-matched control (Fig. 3d). Finally, we

analyzed how DR affects expression levels of the enzyme Cyp46a1 responsible for the conversion of cholesterol into 24S-OHC, cholesterol degradation product. Western blot analysis did not reveal any effect of DR on the levels of this protein either in the cortex or in hippocampus in both age groups analyzed (Fig. 3e, f).

Long-term DR lowers the levels of phytosterols in the cortex and hippocampus

Considering that phytosterol levels in the body depend solely on their intake from the food, we analyzed how long-term DR affects their concentrations in the cortex and hippocampus of aging animals. The DR-induced changes in the levels of the phytosterols analyzed are presented in Fig. 4.

In the rat cortex, DR induced reduction in the concentrations of phytosterols. When compared to control values in 12- and 24-month-old animals, sitosterol concentration was reduced 51 and 47 % (Fig. 4a), campesterol 54 and 49 % (Fig. 4b), and stigmasterol 43 and 44 % (Fig. 4c), respectively. Following DR in the hippocampus, a significant decrease in the concentrations of sitosterol and campesterol was observed only in 24-month-old animals (48 and 50 %, respectively; Fig. 4a, b), while stigmasterol was decreased in both 12- and 24-month-old animals (40 and 45 %, respectively; Fig. 4c). Stigmasterol concentrations in all experimental groups were much lower compared to sitosterol and campesterol.

Discussion

Maintenance of brain cholesterol homeostasis is a prerequisite for the proper functioning of the CNS (reviewed in Pfrieger and Ungerer 2011). Although previous studies have demonstrated that cholesterol homeostasis in the brain remains unaffected under different dietary manipulations (Pallottini et al. 2003; Mulas et al. 2005; Hayakawa et al. 2007; Fon Tacer et al. 2010), a significant decrease in the levels of cholesterol precursors was detected even after short-term fasting (20 h). We hypothesized that long-term DR modulates brain cholesterol metabolism and accordingly analyzed its metabolites and the proteins involved in cholesterol synthesis, recycling, and catabolism in the cortex and hippocampus during aging.

Table 3 Desmosterol/cholesterol ratio in rat serum, cortex and hippocampus

Data are expressed $\mu\text{g}/\text{mg}$ cholesterol (serum) and $\text{ng}/\mu\text{g}$ cholesterol (brain)

* $P<0.05$ vs. the age-matched control

Desmosterol/cholesterol	12 months		24 months	
	AL	DR	AL	DR
Serum	1.13 $\pm$ 0.13	1.79 $\pm$ 0.23*	1.08 $\pm$ 0.08	3.56 $\pm$ 0.56*
Cortex	4.75 $\pm$ 0.51	3.9 $\pm$ 0.31	3.68 $\pm$ 0.30	3.88 $\pm$ 0.30
Hippocampus	6.98 $\pm$ 0.77	9.70 $\pm$ 0.48	6.05 $\pm$ 0.35	8.03 $\pm$ 0.51*

In middle-aged rats (12 months), long-term DR significantly decreased the concentration of lanosterol in the cortex, and of both lanosterol and

lathosterol in the hippocampus. At the same time, the expression levels of HMGCR, the limiting enzyme in cholesterol synthesis, remained unaffected in both

Fig. 3 Levels of the proteins involved in cholesterol metabolism in the cortex and hippocampus following long-term DR. Western blot analysis of HMGCR, ApoE, and CYP46A1 in the rat cortex (a–c, respectively) and hippocampus (d–f, respectively) of rats fed ad libitum (AL light bars) and rats exposed to dietary restriction (DR dark bars) for 12 and 24 months. Graphs are accompanied by

representative immunoblots, with actin bands as the loading control. The protein levels are expressed as fold changes relative to the control values (age-matched control animals). The data are presented as the mean $\pm$ SEM (five rats per group). * $P<0.05$ vs. age-matched control

Fig. 4 Levels of phytosterols in the cortex and hippocampus following long-term DR. Gas chromatography/mass spectrometry analysis of the levels of phytosterols in the cortex (*left columns*) and hippocampus (*right columns*) of rats fed ad libitum (AL *light bars*) and rats exposed to dietary restriction (DR *dark bars*) for 12 and 24 months. The data are presented as the mean $\pm$ SEM (five rats per group). * $P < 0.05$ vs. age-matched control; # $P < 0.05$ vs. 12 months

brain regions, indicating that DR affects the amount of available precursors. Moreover, the observed unaltered levels of the enzyme Cyp46a1 and the product of its activity (24S-OHC) implied that cholesterol catabolism remained unchanged.

In the cortex of old rats (24 months), DR had no effect on the concentrations of the examined cholesterol precursors or on the expression levels of the

analyzed proteins. However, in the hippocampus of old rats, DR promoted a pronounced increase in desmosterol concentration and ApoE level while lowering HMGCR. The increase in the amount of desmosterol could point to an age-related adaptive mechanism under DR in order to increase the membrane-active pool of sterols in the brain for several reasons: (i) desmosterol cannot be hydroxylated to generate 24(S)-hydroxycholesterol, a brain-derived secretory sterol; (ii) desmosterol has a reduced propensity to be esterified as compared to cholesterol; and (iii) desmosterol can activate LXR to stimulate sterol secretion by astrocytes (Jansen et al. 2013). The fact that desmosterol is a direct cholesterol precursor produced in the Bloch cholesterol synthesis pathway suggests a switch to a less energy-consuming sterol synthesis pathway in the old animals exposed to DR. Indeed, DR induced a significant decrease in HMGCR, a rate-limiting enzyme in cholesterol synthesis, in the hippocampus of 24-month-old rats, thus supporting the paradigm according to which the increased level of desmosterol could be a result of energy-saving mechanism. Cholesterol synthesis decreases during normal aging, but the levels of total cholesterol do not change (Thelen et al. 2006; Smiljanic et al. 2013).

In the light of recent findings that prolonged exposure to DR increases both the total number of dividing and progenitor cells in the dentate gyrus, the neurogenesis niche in adult rats (Couillard-Despres et al. 2011; Park et al. 2013), the abovementioned properties of desmosterol may suffice to increase the pool of membrane-active brain sterols. Furthermore, in $DHCR24^{-/-}$ (24-dehydrocholesterol reductase) mice lacking cholesterol, desmosterol accounts for 99 % of the total sterols, indicating that this precursor takes over the function of cholesterol despite differences in their biophysical and functional properties (Wechsler et al. 2003; Vainio et al. 2006).

The dietary regimen applied in the present study impacted ApoE protein levels in both brain regions. However, the finding that the decrease in cholesterol synthesis with age was accompanied by unchanged levels of total cholesterol indicates that under normal conditions, the apolipoprotein trafficking processes are not impaired and that they provide a constant supply of cholesterol where needed (Thelen et al. 2006). Interestingly, the increased levels of ApoE in the hippocampus of aged (24 months) rats under DR, together

with the unchanged levels of 24S-OHC, point to a further enhancement of cholesterol reutilization and energy preservation.

In our study, as in the work by Stranahan et al. (2011), changes in the serum lipid profile mirror the lipid profile changes in the hippocampus, although a clear link has not yet been established. The DR-induced increase of desmosterol in the serum of old animals is more significant in the light of the recent findings of Sato et al. (2012). The authors showed that the plasma levels of desmosterol are an accurate estimate of the levels of desmosterol in the brain. In our study, the elevated desmosterol levels in the plasma are in correlation with its increase in the hippocampus detected under DR. Moreover, they demonstrate that the ratio of desmosterol and cholesterol in the serum could serve as a valuable novel specific biomarker for the early diagnosis of Alzheimer's disease (AD); a lower ratio correlates with more severe AD. Our data show that long-term DR increases the desmosterol/cholesterol ratio in the serum, indicating that the DR-induced increase of desmosterol could have a protective role in the aging brain, especially because aging per se induces a decrease in desmosterol levels in the hippocampus (Smiljanic et al. 2013).

Exposing the animals to long-term DR resulted in a decrease of phytosterol concentrations in both brain regions and in the serum, regardless of the animals' age. Given that the phytosterols originated solely from food, the observed reduction correlated with the amount of provided food. The abundance ratio of the examined phytosterols differs in the brain and serum. Campesterol and sitosterol prevailed over stigmasterol in the brain. Furthermore, in contrast to the serum, in the brain, campesterol was more abundant than sitosterol, suggesting that some phytosterols crossed the BBB more efficiently than others, as was shown previously (Jansen et al. 2006; Vanmierlo et al. 2012; Smiljanic et al. 2013). The trans-endothelial flux efficiency of plant sterols is determined by the molecular complexity of the sterol side chain (Vanmierlo et al. 2012). It was demonstrated that the magnitude of the foregoing effect depends on the geometry of the sterol molecule which is determined by the structure of its side chain (cholesterol > campesterol > sitosterol > stigmasterol) (Hac-Wydro et al. 2009), probably reflecting the difference in esterification rate within the endothelial cells.

A region-specific study of cholesterol homeostasis is of considerable importance, since each brain region

possesses a specific energy balance, cytoarchitecture, function, and myelin composition, and all of these distinctive features could be influenced by cholesterol metabolism. For example, although desmosterol predominates in both brain regions during aging, its level in the hippocampus is much higher than in the cortex (Smiljanic et al. 2013). This could be attributed, at least in part, to neurogenesis that takes place in the adult dentate gyrus (Couillard-Despres et al. 2011). Accordingly, the DR-induced increase in desmosterol concentration in the aging hippocampus could at least in part correlate with the increased number of progenitor cells differentiating into neurons following DR (Park et al. 2013). Furthermore, the different responses of the key proteins involved in cholesterol homeostasis in the cortex and hippocampus to DR reported herein could be attributed to the specific requirements for cholesterol in different brain regions (De Marinis et al. 2008; Trapani and Pallottini 2010; Segatto et al. 2012; Perovic et al. 2009).

Our study revealed that long-term exposure to DR influences the concentrations of cholesterol precursors and the turnover rate of cholesterol, but that it does not impair cholesterol homeostasis in either the cortex or the hippocampus of middle-aged and aged rats. This work also showed that old animals exposed to DR have elevated levels of desmosterol in the hippocampus, pointing to the switch to low-energy sterol synthesis which could have a potentially protective role against neurodegenerative diseases, such as AD. This study provides new insight into the protective effect of DR on the brain.

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