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Brain Isoprenoids Farnesyl Pyrophosphate and Geranylgeranyl Pyrophosphate are Increased in Aged Mice

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Abstract The mevalonate/isoprenoids/cholesterol pathway has a fundamental role in the brain. Increasing age could be associated with specific changes in mevalonate downstream products. Other than age differences in brain cholesterol and dolichol levels, there has been little if any evidence on the short-chain isoprenoids farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), as well as downstream lipid products. The purpose of the present study was to determine whether brain levels of FPP, GGPP and sterol precursors and metabolites would be altered in aged mice (23 months) as compared to middle-aged mice (12 months) and young mice (3 months). FPP and GGPP levels were found to be significantly higher in brain homogenates of 23-months-old mice. The ratio of FPP to GGPP did not differ among the three age groups suggesting that increasing age does not alter the relative distribution of the two isoprenoids. Gene expression of FPP synthase and GGPP synthase did not differ among the three age groups. Gene expression of HMG-CoA reductase was significantly increased with age but in contrast gene expression of squalene synthase was reduced with increasing age. Levels of squalene, lanosterol and lathosterol did not differ among the three age groups. Desmosterol and 7-dehydroxycholesterol, which are direct precursors in the final step of cholesterol biosynthesis were significantly lower in brains of aged mice. Levels of cholesterol and its metabolites 24S- and 25S-hydroxycholesterol were similar in all three age groups. Our novel find ings on increased FPP and GGPP levels in brains of aged mice may impact on protein prenylation and contribute to neuronal dysfunction observed in aging and certain neurodegenerative diseases.

Keywords Brain · Cholesterol · Isoprenoids · Mevalonate pathway · Ageing · Alzheimer

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Introduction

The mevalonate/isoprenoid/cholesterol (MVA) pathway is a key biochemical pathway in mammals (Fig. 1) [1]. Biosynthesis in that pathway mainly occurs in the endoplasmic reticulum but also in peroxisomes [2]. Initial steps of the MVA-isoprenoid pathway involve the synthesis of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) from acetyl-CoA and acetoacetyl-CoA (Fig. 1). Subsequently, HMG-CoA reductase (HMGR), the initial rate limiting step in this pathway catalyzes production of mevalonate [3]. Published work on the MVA pathway in the brain has largely focused on the impact of cholesterol on membrane structure and cell function [4–9]. The MVA-pathway has also attracted considerable attention in work on aging and in certain neurodegenerative diseases which has been described in two recent

comprehensive reviews [10, 11]. Cholesterol is one of the products of this pathway with approximately 25 % of this sterol residing in the central nervous system (CNS; [12, 13]). Cholesterol is a major component of the myelin sheath and it is an integral component of the plasma membrane [14, 15]. The blood brain barrier (BBB) effectively protects the CNS from peripheral cholesterol, thus cholesterol in the CNS originates from *de novo* biosynthesis [13, 16]. The MVA pathway provides brain cells with cholesterol and essential intermediates like the longer chain isoprenoids dolichol, ubiquinone or heme A which farnesylpyrophosphate (FPP) is the precursor [17, 18]. FPP and geranylgeranylpyrophosphate (GGPP) serve as substrates for prenylation of small GTPases (e.g. Rac1, RhoA, Cdc42, Rab3, etc.; Fig. 1; [17, 19, 20]). Attachment of FPP and GGPP to small GTPases facilitates the insertion of the proteins into cell membranes and is the initial step leading to proper protein function [17, 20].

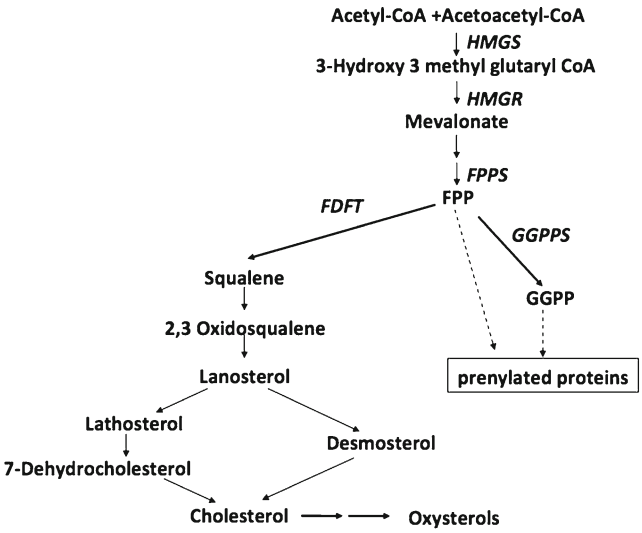

Fig. 1 Mevalonate/isoprenoid/cholesterol-pathway. Initial steps of the mevalonate (MVA)-isoprenoid pathway involve the synthesis of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) from acetyl-CoA and acetoacetyl-CoA. HMG-CoA reductase (*HMGR*), the rate limiting step of the entire pathway then forms mevalonate. After mevalonate production different enzymes catalyzes the production of farnesylpyrophosphate (*FPP*) and geranylgeranylpyrophosphate (*GGPP*). The synthesis of GGPP and FPP is catalyzed by synthases (*FPPS* and *GGPPS*). FPP and GGPP are substrates for transferases involved in post-translational prenylation of small GTPases (Rac1, RhoA, Cdc41, Rab3, etc.). Prenylation of these proteins is required for proper localization and function in cell membranes. FPP is a key branch point of the pathway leading to GGPP synthesis and to squalene that is catalyzed by farnesylpyrophosphate farnesyltransferase (*FDFT*), also known as squalenesynthase. From squalene two subsequent enzymatic reactions result in the production of lanosterol, which represents the structure of all steroids. Subsequent reactions result in the production of desmosterol and 7-dehydroxycholesterol and finally to cholesterol. Metabolism of cholesterol is catalyzed by hydroxylases leading to oxysterols, such as 24- and 24-OH-cholesterol

Aging and MVA-derived products have been widely discussed for several years [21, 22]. Most studies focused on age-related differences in cholesterol homeostasis [7, 14, 23, 24]. Although the majority of studies reported unchanged or even reduced cholesterol levels in total brain homogenates of aged rodents and humans, an age-related increase in cholesterol levels was observed in brain membranes [7, 25–28]. These age-related differences in cholesterol abundance are accompanied by changes in how cholesterol is distributed in the exofacial and cytofacial leaflets of synaptic plasma membranes [23].

Dolichol which is another product of the isoprenoid precursor FPP increases with age [29, 30]. There was approximately a 10-fold increase in dolichol in brain synaptic plasma membranes of 28-month-old mice as compared with 6-month-old mice [31]. Levels of dolichol were also markedly higher in human brain tissue of aged individuals [32, 33]. FPP and GGPP, like cholesterol and dolichol, are derived from mevalonate (Fig. 1) and a straightforward prediction derived from the aforementioned studies is that brain FPP and GGPP levels would be higher in aged as compared with younger animals. This hypothesis has not been tested and the absence of such data is in stark contrast to the interest in the role of isoprenoids in protein prenylation and cell function [17]. Impeding progress on an understanding of FPP and GGPP regulation and consequences on protein targets have been the analytical difficulties in the quantification of FPP and GGPP in brain tissue [34]. We have recently reported FPP and GGPP levels in human brain tissue and mouse brain using a newly developed and validated (ultra-) high-performance liquid chromatography ((U)HPLC) method coupled to a fluorescence (FLD) or tandem mass spectrometer (MS/MS) [34–36]. The purpose of the present study was to determine if levels of brain FPP, GGPP and sterol precursors and metabolites would be higher in aged mice as compared with younger mice.

Materials and Methods

C57Bl/6 Mice

Female mice (3, 12 and 23 months of age) were obtained from Charles River (Sulzfeld, Germany). The mice were maintained on a 12-h dark–light cycle with pelleted food and tap water ad libitum. All experiments were carried out according to the European Communities Council Directive (86/609/EEC) by individuals with appropriate training according to the requirements of the Federation of European Laboratory Animal Science Associations and appropriate experience. Mice were acclimated to the new environment and maintained under conditions as described above for 1 week prior to sample preparation.

Brain Tissue Preparation

Brains were dissected into two hemispheres. One hemisphere (without brain stem and cerebellum) was used for the determination of FPP and GGPP levels. Frontal cortices of the second hemisphere were used for the determination of cholesterol and its precursors and metabolites. Total protein concentrations were measured in purified homogenates of the cerebrum and the frontal cortex using the BCA Protein Assay Kit from ThermoFisher/Pierce (Bonn, Germany).

Quantification of FPP and GGPP Levels

Quantification of FPP and GGPP was performed as previously described [34, 35]. Due to the limit of quantification of the analytical method [34], a minimum quantity of brain tissue that refers to one hemisphere (cerebrum minus cerebellum and brain stem) had to be used for analysis. Briefly, FPP and GGPP brain tissue samples were homogenized in 100 mM Tris buffer (pH 8.5) containing 5 μL Halt® and 10 μL Phosstop® phosphatase inhibitors. The homogenate was vigorously mixed with 1 mL 100 mM Tris buffer (pH 8.5) and then spiked with 15 μL 2.8 μM solution of 5-(dimethylamino)naphthalene-1-(4-nonylphenol)-sulfonic acid ester used as the internal standard (IS). The mixture was loaded onto Merck Extrelut® NT1-columns (Darmstadt, Germany) and eluted with 1-butanol–ammonium hydroxide–water mixture. The filtrate was centrifuged and the supernatant was evaporated under reduced pressure. After sonication, the solution was applied to Oasis® HLB solid-phase extraction cartridges. The extract was washed with methanol and finally eluted with an ammonium hydroxide–propanol–*n*-hexane mixture. The filtrate was vacuum-dried and re-dissolved in an assay buffer for the enzymatic reaction. For pre-column dansyl-labeling, the dried residue was dissolved in Tris–HCl assay buffer and spiked with D*-GCVLS and D*-GCVLL (dansyl-labeled peptides) as well as with the coupling enzymes FTase and GGTase, respectively. The chromatographic separation was carried out on a Jasco HPLC-system (LG-980-02, PU-980, AS-950; Gross-Umstadt, Germany) with a gradient elution on an Ascentis® Express C-18 reversed-phase analytical column from Supelco (150× 2.1 mm, 2.7 μm; Munich, Germany) protected by a Phenomenex Security guard column (C-18, 4×2.0 mm; Aschaffenburg, Germany). Protein concentrations were measured using the BCA Protein Assay Kit from ThermoFisher/Pierce (Bonn, Germany). Samples were measured in triplicates.

Quantification of Cholesterol and its Precursors and Metabolites

Cholesterol and its precursors and metabolites were quantified using GC-MS as previously reported [37]. Lipid extraction was carried out using the Folch method with slight modifications [38]. Frontal cortices were homogenized at 4 °C with 0.75 mL PBS and 3.25 mL Folch organic solvent mixture (chloroform/methanol 2:1, containing 0.05 % butylated hydroxytoluene). The homogenates were centrifuged at 1,000×*g* for 10 min at 4 °C. The upper phase was discarded and the lower organic phase carefully transferred to a glass vial and evaporated under a stream of nitrogen. A 1.7 mL of 0.5 M KOH (in 100 % methanol) and 1 mL of water was added with a mixture of heavy isotopes, 40 ng of 7 alpha-hydroxycholesterol-d_7, 40 ng of 7 beta-hydroxycholesterol-d_7, 40 ng of 26 (27)-hydroxycholesterol-d_5, 80 ng of 7-ketocholesterol-d_7, 0.2 μg 5-alpha cholestane, 0.2 μg of lathosterol-d_4, 0.2 μg of campesterol-d_7, and 0.2 μg of beta-sitosterol-d_7 in 25 μL of ethanol were added to the sample and mixed. The tube was purged with argon gas and closed with a Teflon cap. After gentle mixing, samples were incubated for 15 h at 23 °C in order to measure the total (free + esterified) forms of cholesterol, cholesterol oxidation products, and oxysterols.

Mixed anion-exchange SPE columns were preconditioned with 2 ml methanol; followed by 2 mL of 20 mM formic acid (pH 4.5). A 0.5 mL of 0.4 M acetic acid was added to the hydrolysed samples and then neutralized with 0.85 ml of 1 M HCl. The hydrolysed samples (pH 4.5) were loaded onto the columns, and the columns were washed with 2 mL of 40 % methanol/formic acid (pH 4.5). After washing, 2 mL of hexane and 2 mL of ethyl acetate/hexane (30:70) were added to elute cholesterol and oxysterols. The eluted samples were evaporated under a stream of nitrogen. The aliquots were derivatized with 25 μL acetonitrile and 25 μL BSTFA+TMCS for an hour at room temperature (RT). For cholesterol and oxysterols measurement, the derivatized samples were analysed using Agilent 5975 inert XL mass selective detector. Helium was used as the carrier gas at a flow rate of 0.8 mL/min, derivatized samples (1 μL) were injected into the GC injection port (280 °C). Column temperature was increased from 160 to 300 °C at 40 °C/min after 1 min at 160 °C, and then kept constant at 300 °C for 6 min. Selective ion monitoring was performed using electron ionization mode at 70 eV (with ion source maintained at 230 °C and the quadrupole at 150 °C) to monitor one target ion and two qualifier ions selected from each compound's mass spectrum to optimize sensitivity and specificity. Quantification of cholesterol precursors and oxysterols was achieved by relating its peak area of target ion to its corresponding internal standard peak.

RNA Isolation, Reverse Transcription, Primer Design and qRT-PCR

RNA was isolated from brain frontal cortex using the Trizol method and purified with Invitrogen's ChargeSwitch® Total RNA Cell Kit procedure according to the manufacturer's instructions. RNA was resuspended in 50 to 100 μL of the ChargeSwitch® Elution Buffer depending on original pellet size. RNA concentration was determined by measuring the absorbance ratio of 260/280 and 260/230 nm using ThermoFisher Nanodrop 100 spectrophotometer. Five hundred nanograms of RNA was then reverse transcribed using Thermo scientific Verso cDNA synthesis Kit in a Bio-Rad's iQ5 multicolor Real Time PCR according to the manufacturer's instructions.

Internal primers were designed for each of the targets. The NCBI's Entrez Gene database was the source of the nucleotide sequences of each gene. Using the BLAST program, intron/exon borders were identified and primers were designed to amplify around those regions to eliminate amplification of genomic DNA rather than the desired cDNA. Primer sequences were generated using MIT's program "Primer3". The following primers were used:

HMGR: Left 5′-GAG GCA TTT GAC AGC ACT AGC-3′

Right 5′-TGC ATT TCA GGG AAA TAC TCG-3′

FPPS: Left 5′-GAA GAT CCT GCT GGA GAT GG-3′

Right 5′-GTT GTC CTG GAT GTC AGT GC-3′

GGPPS: Left 5′-CTA TCT GGG CAG TTC CAA GC-3′

Right 5′-GAC TTC AGT GAG GGT GTT TCC-3′

FDFT : Left 5′-ATG CCT GCC GTC AAA GCT AT-3′

Right 5′-GAT CCG GTG ATA AAT CTC TTC-3′

GAPDH: Left 5′-GAA ATC CCA TCA CCA TCT TCC-3′

Right 5′-ATG GTT CAC ACC CAT GAC G-3′

RNA primers and the master mix were purchased from Dharmacon. Relative abundance of mRNA was measured through real-time analysis using Solaris qPCR Master Mix. Thermal cycles were programmed according to the manufacturer's instructions. In each 25 μL reaction, there were 250 ng of cDNA template and a required amount of the company premix primer probes (forward and reverse primers). GAPDH was used as a reference gene to normalize the data for each experiment and therefore gene fold expression was determined using $\Delta\Delta C_T$ calculations.

Statistics

All data are expressed as means ± standard error of the mean (SEM) unless stated otherwise. For direct comparison of differences between two groups, students' *t* test was calculated. All calculations were performed with GraphPad Prism version 5.00 for Mac, GraphPad software, San Diego, USA.

Results and Discussion

Brain FPP, and GGPP Levels in Different Age Groups of Mice

Figure 2 shows data on FPP and GGPP in brain homogenates of mice of 3, 12 and 23 months of age. There was approximately a 44 % increase in FPP abundance in the 23-months-old mice as compared with the 3- and 12-months-old mice (Fig. 2, panel A), which was significantly different. GGPP levels were also significantly higher in the 23-months-old mice as compared with the two other age groups (Fig. 2, panel B). There was a 64 % increase in GGPP in the 23 months old when compared with the 3 and 12-months-old mice. While FPP and GGPP levels were significantly higher in the 23-months group as compared with the other groups, the ratio of FPP to GGPP did not differ among the three age groups (3 months 0.21; 12 months 0.20; and 23 months 0.18) suggesting that increasing age does not alter the relative distribution of the two isoprenoids. Regardless of age, there is substantially more GGPP as compared with FPP and this finding is in agreement with a recent report on FPP and GGPP in brain tissue of Alzheimer's patients and controls and in statin-treated mice [35].

Gene expression of FPP synthase and GGPP synthase did not differ among the three age groups (Table 2). The greater abundance of FPP and GGPP in the 23-month-old mice could be due to an increase in substrates and a decrease in use for downstream products. Accordingly, HMGR gene

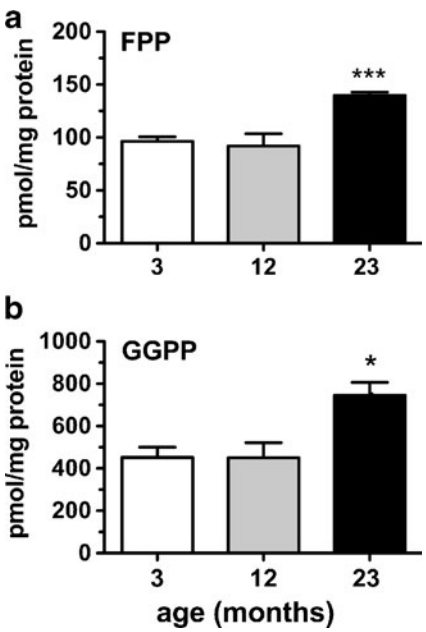

Fig. 2 Brain levels of short-chain isoprenoids FPP and GGPP. **a** Farnesylpyrophosphate (*FPP*) and **b** geranylgeranylpyrophosphate (*GGPP*) levels in cerebrum homogenates of 3, 12, and 23-month-old mice were determined using HPLC-FD as described in "Materials and Methods". Results are shown as means ± SEM; ***$p<0.001$ and* $p<0.05$; $n=6$

Table 1 Brain levels of cholesterol and its precursors and metablolites in young, middle-aged and aged mice

	3 Months	12 Months	23 Months
Cholesterol	50.963±8.831	43.900±6.468	60.937±3.314
Precursors			
Squalene	1.113±0.351	1.057±0.487	1.073±0.248
Lanosterol	9.311±3.795	8.242±2.472	7.110±2.218
Lathosterol	48.298±38.285	38.285±6.981	35.183±12.914
7-Dehydroxycholesterol	83.760±20.852	60.184±8.798*	58.527±12.256*
Desmosterol	70.622±25.990	39.863±13.045*	40.760±9.408*
Metabolites			
24-OH-Cholesterol	71.811±19.407	63.291±21.350	73.415±12.811
25-OH-Cholesterol	17.533±4.910	15.510±4.827	18.164±3.352

Brain levels of cholesterol (nanomoles per milligram of tissue) and its precursors and metabolites (picomoles per milligram of tissue) were determined in homogenates isolated from the frontal cortex of young (3 months), middle-aged (12 months), and aged (23 months) C57/BJ6 mice using GC/MS as described in "Materials and Methods" (refer also to Fig. 1). Mean ± SD, $n=6$

*$p<0.05$ vs. young (t test)

expression was significantly enhanced and levels of cholesterol precursors were decreased (Tables 1 and 2). Alternative explanations are elevated enzyme activity of the two synthases, reduced utilization particularly for GGPP in protein prenylation, or the use of different tissues.

Brain Levels of Cholesterol and Its Precursors and Metabolites in Different Age Groups of Mice

Gene expression of HMGR was significantly increased with age but in contrast gene expression of squalene synthase (FDFT) was reduced with increasing age (Table 2). The protein product of the *HMGR gene* is HMG-CoA reductase which is the initial rate-limiting step in cholesterol synthesis. The immediate lipid product of HMG-CoA reductase activity is mevalonate. Through a series of reactions, mevalonate is converted to isopentyl pyrophosphate which is the precursor for isoprenoid compounds and subsequently cholesterol [17]. FPP is the immediate precursor of squalene and GGPP (Fig. 1) and both FPP and GGPP levels were significantly higher in brain homogenates of 23-month-old mice as compared with the younger age groups (Fig. 2). However, levels of squalene, lanosterol and lathosterol did not differ among the three age groups as shown in Table 1. Although another study demonstrated significantly reduced squalene synthase activity in brains of aged rats [25], in our study, squalene and cholesterol levels were unaffected even though FPP is a precursor of squalene. FPP may be shunted away from squalene production and instead used for the production of dolichol and GGPP that both increase with age. We also found significantly lower desmosterol and 7-dehydroxycholesterol levels in brain homogenates of 12 and 23-months-old mice as compared with 3-months-old mice (Table 1). Both intermediates are direct precursors at the final step of cholesterol biosynthesis. Levels of cholesterol and its metabolites 24*S*- and 25*S*-hydroxycholesterol were similar among the three age groups (Table 1).This study showed for the first time that regulation of FPP and GGPP is altered in the central nervous system of aged mice. The targeting of FPP and GGPP in the aged brain is similar to that reported in Alzheimer's disease (AD) brain tissue [35]. FPP and GGPP levels of AD samples were substantially higher when compared with age-matched controls. Total cholesterol levels were similar in brain tissue of AD and control samples. Consistent with elevation of FPP and GGPP levels, it was shown in that study that gene expression of FPP synthase and GGPP synthase (protein products are enzymes directly involved in FPP and GGPP production) were also elevated in AD brain tissue. In the current

Table 2 Gene expression data of relevant enzymes

	3 Months	12 Months	23 Months
HMGR	2.6402±0.587	8.7897±2.917*	9.5814±1.153*
FPPS	0.6178±0.268	0.3513±0.040	0.262±0.088
GGPPS	0.8954±0.270	0.8059±0.090	1.2763±0.497
FDFT	1.1608±0.221	0.5003±0.062	0.2803±0.112*

mRNA levels were determined in cerebrum homogenates isolated from brains of young (3 months), middle-aged (12 months), and aged (23 months) C57/BJ6 mice using by qRT-PCR as described in "Materials and Methods"

HMGR HMG-CoA reductase, *FPPS* farnesly diphosphate synthase, *FDFT* farnesyldiphosphate farnesyltransferase 1 (also known as squalene synthase), *GGPPS* geranylgeranyl pyrophosphate synthase I (refer also to Fig. 1). Mean ± SEM, $n=6$

*$p<0.05$ vs. young (t test)

study, gene expression of FPP synthase and GGPP synthase did not differ among the three age groups (data not shown). FPP and GGPP regulation may differ in AD as compared with aging.

Elevated levels of FPP and GGPP whether in aging or AD may have pathological consequences on neuronal function. *In vitro* evidence indicates possible harmful effects of FPP, GGPP, and its alcohols [39–41]. FPP (10 μmol/l) regulates fatty acid synthesis in Caco-2 cells by a mechanism that is likely independent of the SREBP pathway [42], geranylgeraniol and farnesol (80 μmol/l) inhibited phosphatidylcholine biosynthesis and cell cycle arrest in A549 cells [40], and GGPP (29.8 μmol/l) enhanced γ-secretase processing of Alzheimer-related amyloid precursor protein, consequently leading to elevated beta-amyloid peptide levels (Aβ1-40) in HEK293 cells [41]. Accordingly, FPP and GGPP (10 μmol/l) enhanced Aβ1-42 levels in H4-cells [43]. However, harmful effects of FPP and GGPP have been reported exclusively in vitro and relatively high isoprenoid concentrations were applied mainly to non-neuronal cell lines. Thus, future investigations have to include neuronal cell lines and physiological concentrations in the picomolar range [44]. Moreover, it has not been established if elevated FPP and GGPP levels have an impact on protein prenylation in the brain. Clearly, studies are needed ranging from synthesis of FPP and GGPP to effects of these isoprenoids both on prenylation and effects independent of protein prenylation.

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