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Long-term omega-3 fatty acid supplementation prevents expression changes in cochlear homocysteine metabolism and ameliorates progressive hearing loss in C57BL/6J mice

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Abstract

Omega-3 polyunsaturated fatty acids (PUFAs) are essential nutrients well known for their beneficial effects, among others on cognitive development and maintenance, inflammation and oxidative stress. Previous studies have shown an inverse association between high plasma levels of PUFAs and age-related hearing loss, and the relationship between low serum folate and elevated plasma homocysteine levels and hearing loss. Therefore, we used C57BL/6J mice and long-term omega-3 supplementation to evaluate the impact on hearing by analyzing their auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) thresholds. The omega-3 group showed significantly lower ABR hearing thresholds (~25 dB sound pressure level) and higher DPOAE amplitudes in mid–high frequencies when compared to the control group. These changes did not correlate with alterations between groups in plasma homocysteine or serum folate levels as measured by high-performance liquid chromatography and a microbiological method, respectively. Aging in the control group was associated with imbalanced cytokine expression toward increased proinflammatory cytokines as determined by quantitative reverse transcriptase polymerase chain reaction; these changes were prevented by omega-3 supplementation. Genes involved in homocysteine metabolism showed decreased expression during aging of control animals, and only alterations in *Bhmt* and *Cbs* were significantly prevented by omega-3 feeding. Western blotting showed that omega-3 supplementation precluded the CBS protein increase detected in 10-month-old controls but also produced an increase in BHMT protein levels. Altogether, the results obtained suggest a long-term protective role of omega-3 supplementation on cochlear metabolism and progression of hearing loss.

Keywords: Eicosapentaenoic acid; Homocysteine metabolism; Inflammation; Omega-3 fatty acids; Oxidative stress; Presbycusis

1. Introduction

The functional decline of the organism during aging is associated with the onset of a variety of chronic illnesses, including cancer, diabetes, atherosclerosis, osteoporosis and cardiovascular disease [1,2]. Aging is also associated with a progressive sensory impairment that is often concomitant with cognitive decline [1,3,4]. Most chronic diseases share common biochemical alterations leading to cellular degeneration and organ malfunction [2]. Treatment of all these disorders represents a key health challenge but also a major socioeconomic problem because, for example, the population over 65 years will presumably double by 2050 in the European Union [5]. Therefore, biomedical research is focusing in the understanding of the genetic and molecular mechanisms underlying aging and in the design of new strategies to promote active and healthy aging, including nutritional intervention.

Hearing loss is one of the fields in which nutrition intervention studies may have more preventive potential, especially age-related hearing loss (ARHL). Approximately 30% of the population over 65 years suffers ARHL, its incidence increasing exponentially with age [6,7]. Worldwide epidemiological studies have shown an association between deficiencies in several essential nutrients and hearing loss [8–12]. Moreover, other studies also provided evidences of its putative prevention by dietary supplementation with folic acid [13] or due to different levels of n-3 polyunsaturated (ω3) fatty acids (PUFAs) in the diet [14–17]. The general basis for this protection seems to rely on the relationship between vascular disease and hearing loss, which was suggested initially by the lack of presbycusis and cardiovascular disease in the Mabaan tribe and further supported by several studies in search of a correlation between cardiovascular events and hearing loss

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[18–22]. These studies showed microvascular disease as the underlying cause for the atrophy of the stria vascularis [20,23], which is the structure within the cochlea that maintains its metabolic and ionic homeostasis [24]. Animal models of AHRL have provided further information on its etiology. Thus, for example, the gerbil also presents progressive blood flow reduction and microvascular alterations at the stria vascularis and spiral ligament associated with hearing loss and aging [25,26]. Altogether, these data suggest that a reduction in the blood supply of essential nutrients into the cochlea, and the subsequent metabolic alterations, could be among the main triggers of hearing loss progression.

High levels of plasma homocysteine (tHcy), a key metabolite of the folate and methionine cycles [27], are considered an independent risk factor for cardiovascular disease [28] and, more recently, also of human hearing loss [12,13]. Vascular diseases are also closely related to inflammation, a condition ameliorated by supplementation with ω3 fatty acids [29,30]. These relationships were the basis for the limited number of studies on the effects of ω3 supplementation on hearing loss and Hcy metabolism carried out to date. These studies showed an inverse correlation between the ingested levels of long-chain ω3 fatty acids and hearing loss [15,16], but opposite effects on tHcy resulted from fish oil supplementation [31,32]. Dissimilar results on tHcy and/or auditory brainstem response (ABR) were also obtained from animal studies of ω3 supplementation carried out for limited time periods in adults or during pregnancy and lactation [33–37]. Therefore, the urgent need of a better knowledge of hearing loss progression and the potential of dietary components to prevent and/or delay its onset led us to use a classical model of early hearing loss, the C57BL/6J mice, to carry out a detailed follow-up of the auditory capacity in long-term ω3 supplementation. Altogether, our results show that ω3 supplementation attenuated progression of hearing loss, this maintenance of function associating with differences in cochlear Hcy metabolism and in the balanced ratio of anti- and proinflammatory biomarkers.

2. Materials and methods

2.1. Mouse handling and experimental design

Seven-week-old C57BL/6J female mice [38,39] were purchased from Charles River Laboratories (Hollister, CA, USA) and housed under standard conditions. After an acclimatization period of 1 week, these 2-month-old mice were randomly divided into two experimental groups that were fed for 8 months either control (C-10M; $n=15$) or ω3-supplemented (ω3-10M; $n=9$) diets *ad libitum*. A group of mice within those receiving the control diet was sacrificed at 4 months of age for comparative purposes (C-4M; $n=4$). The composition of both semisynthetic diets was adjusted to mice requirements in full accordance with National Research Council directives [40], with modifications affecting only the fat content (Table 1). The AIN-93M-MX mineral mix (TD.94049) and the AIN-93-VX vitamin mix (TD.94047) used for diet preparation were purchased from Harlan Teklad (Indianapolis, IN, USA), whereas the fish oil Eupoly-3™ eicosapentaenoic acid (EPA) was gently supplied by Biosearch SA (Granada, Spain). Diet pellets were freshly made at the onset of the experiment and stored at −20°C in portions of the appropriate size until use; thawing was carried out at 4°C, and new pellets were provided every other day. Animals were weighted weekly and maintained on a 12-h:12-h dark/light cycle under controlled temperature and humidity conditions at the facilities of the Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM). All experiments were approved by the CSIC Bioethics Committee and carried out in full accordance with the European Union (2010/63/EU) and Spanish regulations (RD 53/2013) for the use of laboratory animals.

Table 1
Diet composition

	Control diet (g/kg)	ω-3 diet (g/kg)
Cornstarch	620.7	620.7
Casein (≥85%)	140.0	140.0
Cellulose	50.0	50.0
Saccharose	100.0	100.0
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
L-Cysteine	2.5	2.5
Choline	2.5	2.5
tert-Butylhydroquinone	0.008	0.008
Soybean oil	40.0	38.4
Fish oil Eupoly-3™ EPA	–	1.6

2.2. Hearing assessment

Hearing was evaluated at the beginning of the experiment (2 months of age) and monthly from 4 months of age onward (Fig. 1). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and the ABR was recorded for hearing assessment as previously described [41,42]. Briefly, broadband click and pure tone frequencies were recorded at 4, 8, 16, 20, 28 and 40 kHz at an intensity range from 90 to 10 dB sound pressure level (SPL) in 5–10-dB steps. The electrical responses were amplified and averaged to determine hearing thresholds for each stimulus. Peak and interpeak latencies were analyzed at 15–20-dB SPL above hearing threshold after click stimulation. Animals were kept thermostatized and monitored during both anesthesia and the following period of recovery. The anesthetics used in the present work are known to be metabolized in the liver and kidney within 24 h.

The recording of distortion product otoacoustic emissions (DPOAEs) was performed after stimulation with f1 and f2 primary tones, with a ratio f2/f1=1.2. An Etymotic ER-10B+™ probe was used for the generation of f1 and f2 and sound capturing. The calibration of the probe for determining the degree of attenuation of the primary frequencies was performed using a sound-attenuating cylinder with known volume (i.e., 1-ml syringe) to simulate the mouse ear canal with the aid of the TDT equipment, as described previously [43,44]. Primary tones for 8-, 10-, 14-, 18- and 22-kHz frequencies were tested. Decreasing intensity steps of 5–10 dB were performed from 80 to 30 dB SPL for each pair of frequencies (150 averages per intensity). Simultaneously, the probe was connected to a 100–50,000-μV amplifier, and the signal processing and subsequent analysis were carried out with the help of software BioSigRP™. The 2f1-f2 DPOAE component was considered positive when exceeding 5 dB SPL above background noise, this being calculated as the average amplitude of five randomly chosen points on both sides of the 2f1-f2 component. All the frequencies used were generated by SigGenRP™ (Tucker Davis Technologies TDT, Alachua, FL, USA).

2.3. Cochlea extraction and processing

Mice were sacrificed by CO_2 asphyxiation for tissue removal at 4 and 10 months of age. Tissues were immediately frozen in liquid nitrogen for protein or RNA extraction. Blood samples were collected by cardiac puncture and placed in regular or heparin-coated tubes (Laboratorios Farmacéuticos Rovi, Madrid, Spain). After centrifugation at 2500×*g* for 10 min, the corresponding serum or plasma fractions were isolated and stored at −80°C until use.

2.4. Metabolite determinations

Total plasma Hcy (tHcy) was determined after derivatization using the Reagent kit for the high-performance liquid chromatography analysis of Hcy in plasma/serum (Chromsystems Instruments & Chemicals GMBH, Munich, Germany) following manufacturer's instructions. Serum folate levels were analyzed by a microbiological method using *Lactobacillus casei* (American Type Culture Collection; ATCC 7469) [45], as modified by Tamura [46].

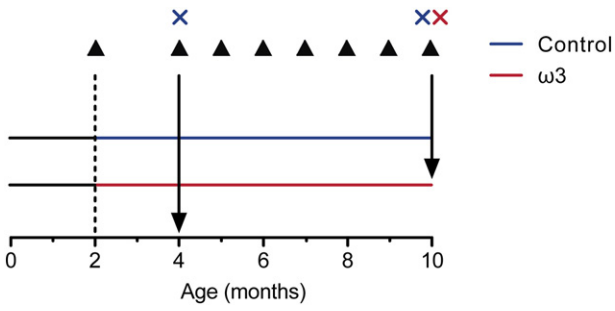

Fig. 1. Scheme of the experimental procedure. Mice were fed a standard diet until they became 2 months old ($n=24$). At this age, ABR analysis was performed, and mice were randomly divided into two experimental groups receiving the standard (control; $n=15$) or the ω3-supplemented diets (ω3; $n=9$). From 4 months of age onward, ABR was performed monthly, and sampling was carried out at 4 (only in the control group, C-4M; $n=4$) and 10 months of age (C-10M and ω3-10M). ABR analysis is represented by triangles, and sampling is indicated by a blue (control) or red "X" symbol (ω3 supplemented); points of detailed biochemical analysis are indicated by arrows.

2.5. Protein extraction and Western blotting

Whole cochlear protein was prepared using the ReadyPrep™ Protein Extraction kit for total protein (Bio-Rad, Hercules, CA, USA). Protein concentration was determined using the RC DC™ Protein Assay kit (Bio-Rad) and bovine serum albumin as standard. Samples (100 μg) were loaded into Mini-PROTEAN TGX™ 10% precast polyacrylamide gels (Bio-Rad) and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad) with the Trans-Blot Turbo™ transfer starter system (Bio-Rad) for incubation with the appropriate antibodies (Table 2). Signals were developed using Clarity™ Western ECL Substrate (Bio-Rad), captured using ImageQuant™ LAS4000 mini system (GE Healthcare, Buckinghamshire, UK) and quantified with the ImageQuant™ TL software (GE Healthcare) as previously reported [47]. Values were normalized against β-actin signals, and mean values for the C-4M controls were considered as 100%.

2.6. Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total cochlear RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany), its quality and quantity determined on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription and PCR were done as previously described [48,49]. cDNAs (10 ng) were amplified in triplicate using gene-specific primers (Table 3) and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) or TaqMan probes (*Cat, Gss, O3fa1, Il6, Il1b, Tnfa, Il10, Igf1, Igf1r, Tgfb1* and *Tgfb1r*) using the ABI 7900HT-Real-Time PCR System (Applied Biosystems). Relative expression ratios were normalized to the geometric mean of the *Rn18s* or the *Rplp0* genes and the fold changes calculated [50,51].

2.7. Statistical analysis

The IBM statistics SPSS v22 software package (Chicago, IL, USA) was used for statistical analysis. Statistical significance was determined by Student's *t* test for unpaired samples (C-10M vs. ω3-10M) or by one-way analysis of variance with Bonferroni *post hoc* test when the C-4M control set was also included in the analysis. All results are expressed as the mean±standard error of the mean (S.E.M.), and differences were considered significant when $P \leq .05$.

3. Results

3.1. Body weight and food intake

Initially, the average daily food intake was significantly lower in the ω3-supplemented group than in the control group (4.12±0.11g vs. 4.70±0.22 g; $P=.03$), but this parameter became similar from 10 weeks onward (4.52±0.15 g vs. 4.80±0.04 g; $P>.05$). No differences in weight gain were observed along the study between control and ω3-supplemented groups. At the end point of the study, animals in the two dietary groups, C-10M and ω3-10M, showed normal weight values (24.6±0.9 g vs. 23.3±0.6 g; $P>.05$) according to the strain characteristics [52].

3.2. Progression of sensorineural hearing loss

Hearing was assessed along the study, the final end point being 10 months of age (Fig. 1). ABR thresholds, wave amplitudes and latencies remained similar at 7 months of age in control and ω3-supplemented groups (Fig. 2A). Nonetheless, DPOAE analysis showed a significant decrease in the *2f1-f2* amplitude at 8 kHz for the control group when measured at intensities of 20 dB SPL above threshold (Fig. 2B).

Damage was more evident at 10 months of age, where ω3-10M mice showed significant decreases in the ABR thresholds at several frequencies as compared to the C-10M group (Fig. 3A). Precisely, ABR thresholds in response to 4-, 8- and 40-kHz frequencies declined 27 dB SPL ($P<.001$), 16 dB SPL ($P=.02$) and 17 dB SPL ($P=.027$), respectively (Fig. 3A). Age-related changes in the ABR waves include a decrease in their amplitudes together with an increase on their latencies. There were no statistical differences between both groups in the click ABR wave analysis of latencies and interpeak latencies. However, ω3-10M mice showed a smaller decrease in wave I amplitude along with a decrease in its latency (Fig. 3B–C) when compared to the C-10M group. Moreover, a mild and nonsignificant progressive delay in wave IV latency was shown in the C-10M group compared with the ω3-10M mice (Fig. 3B–D).

3.3. Effects in cytokine expression and stress biomarkers

Quantitative RT-PCR (RT-qPCR) was used to determine putative changes in several genes involved in inflammation and stress pathways (Fig. 4). Comparison of C-4M and C-10M cochleae showed significant decreases in expression levels of the anti-inflammatory

Table 2
Antibodies used in this study

Primary antibody	Source	Dilution (v/v)	Secondary antibody	Source	Dilution (v/v)	Technique
Mouse anti-phospho-SAPK/JNK	Cell Signaling (9255)	1:2000	IgG sheep anti-mouse HRP conjugated	GE Healthcare (NA931)	1:3000	WB
Rabbit anti-SAPK/JNK	Cell Signaling (9252)	1:1000	IgG goat anti-rabbit HRP conjugated	Bio-Rad (170-6515)	1:3000	WB
Rabbit anti-phospho-p42/44 MAPK (ERK 1/2)	Cell Signaling (9101)	1:1000	IgG goat anti-rabbit HRP conjugated	Bio-Rad (170-6515)	1:3000	WB
Rabbit anti- p42/44 MAPK (ERK 1/2)	Cell Signaling (9102)	1:1000	IgG goat anti-rabbit HRP conjugated	Bio-Rad (170-6515)	1:3000	WB
Rabbit anti-p38α	Santa Cruz (sc-535)	1:1000	IgG goat anti-rabbit HRP conjugated	Bio-Rad (170-6515)	1:3000	WB
Rabbit anti-phospho-p38 MAP kinase	Cell Signaling (9211)	1:1000	IgG goat anti-rabbit HRP conjugated	Bio-Rad (170-6515)	1:3000	WB
Mouse anti-β-actin	Sigma (A5441)	1:5000	IgG sheep anti-mouse HRP conjugated	GE Healthcare (NA931)	1:20000	WB
Mouse anti-CBS	Abnova (H00000875-A01)	1:3000	IgG sheep anti-mouse HRP conjugated	GE Healthcare (NA931)	1:2500	WB
Rabbit anti-BHMT	González et al.[a]	1:5000	IgG goat anti-rabbit HRP conjugated	Bio-Rad (170-6515)	1:5000	WB
Rabbit anti-ADK	Abcam (ab88903)	1:1000	IgG goat anti-rabbit HRP conjugated	Bio-Rad (170-6515)	1:10000	WB
Rabbit anti-Hcy	Chemicon-Millipore (AB5512)	1:1000	IgG goat anti-rabbit HRP conjugated	Bio-Rad (170-6515)	1:3000	WB
Goat anti-AHCY	Santa Cruz (sc-55759)	1:500	IgG donkey anti-goat HRP conjugated	Santa-Cruz (sc-2020)	1:3000	WB
Goat anti-MTR	Abnova (PAB6022)	1:100	IgG donkey anti-goat HRP conjugated	Santa-Cruz (sc-2020)	1:3000	WB

WB, Western blotting.
[a] Gonzalez B, Campillo N, Garrido F, Gasset M, Sanz-Aparicio J, Pajares MA. Active-site-mutagenesis study of rat liver betaine-homocysteine S-methyltransferase. Biochem J 2003;370:945–52.

Table 3
SYBR Green primers used in real-time RT-PCR experiments

Gene	Bases[a]	Forward primer (5'-3')[b]	Bases	Reverse primer (5'-3')	nM[d]
Ada	641–663	GGGATGAGACCATTGAAGGAAGT	709–689	TCTTTACTGCGCCCTCATAGG	300
Adk[c]	883–903	AGAGGCAGAGGACCGTGATCT	946–925	TCATTCTCTGCAGCCACTATGG	300
Bhmt	672–692	CAGAATTCCCCTTTGGATTGG	742–721	GGCCTCTCTGGCATATTTTTGA	300
Cbs[c]	1274–1292	GCAGCGCTGTGTGGTCATC	1337–1312	GTCACTCAGGAACTTGGACATGTAGT	300
Ahcy	1531–1554	TCGAAGTGTCCAATGTTACAGACA	1592–1575	CTTGGCCGGCACTTTGAG	300
Mtr	2006–2026	CGCGATCAAGTTTGGTATGGA	2080–2057	TCCTTGTGGATAGCATCATACACA	300
Cth[c]	190–212	CAGTCCTCGGGTTTTGAATACAG	260–241	GCAGCCACTGCTTTTTCCAA	300
Rn18s	1645–1666	CCAGTAAGTGCGGGTCATAAGC	1737–1716	CCTCACTAAACCATCCAATCGG	100

[a] Base numbers indicate the location of the primer sequences in the corresponding mRNA.
[b] Primers were previously described by Martínez-Vega et al. [49] except for *Cth* primers that were designed for this study using the Program Primer Express 3.0 and the mouse gene sequence with reference NC_000069.6.
[c] Primers for *Cbs*, *Cth* and *Adk* were designed in regions common to the several splicing forms reported.
[d] Primer concentrations used for SYBR Green detection.

cytokine *Il10*, as well as in *Igf1* and in the *Igf1r* receptor ($P<.05$; Fig. 4A). On the other hand, proinflammatory cytokines *Il1b* and *Il6* exhibited higher expression levels in C-10M than in C-4M cochleae ($P<.05$; Fig. 4A). These expression changes were partially prevented in ω3-10M because *Igf1* and *Igf1r* mRNA levels increased significantly, whereas *Il1b* and *Il6* expression levels decreased as compared to C-10M cochleae ($P<.05$). In fact, expression values in ω3-10M were comparable to those obtained in C-4M cochleae. Additionally, the supplementation produced a significant increase in *Tgf1br* expression as compared to matched C-10M cochleae ($P<.05$), whereas no changes

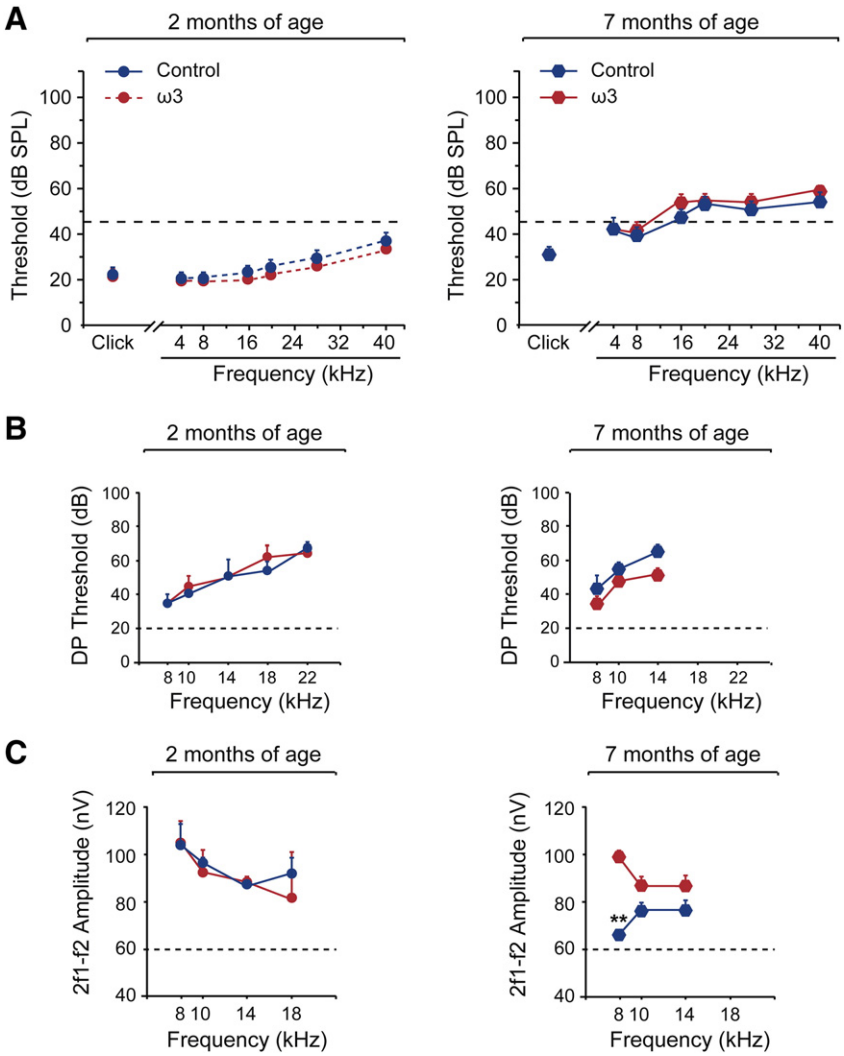

Fig. 2. Hearing evaluation in 7-month-old mice. (A) ABR thresholds in response to click and tone burst stimuli in control (blue) and ω3-supplemented (red) mice at 2 (beginning of the experiment) and 7 months of age. (B) DP thresholds (dB SPL) in response to 8-, 10-, 14-, 18- and 22-kHz primary tones in control (blue) and ω3-supplemented (red) mice at 2 and 7 months of age. (C) *2f1-f2* amplitudes (nV) in response to 8-, 10-, 14-, 18- and 22-kHz primary tones in control (blue) and ω3-supplemented (red) mice at 2 and 7 months of age. Results are shown as the mean±S.E.M. Statistical significance: $^{**}P<.01$.

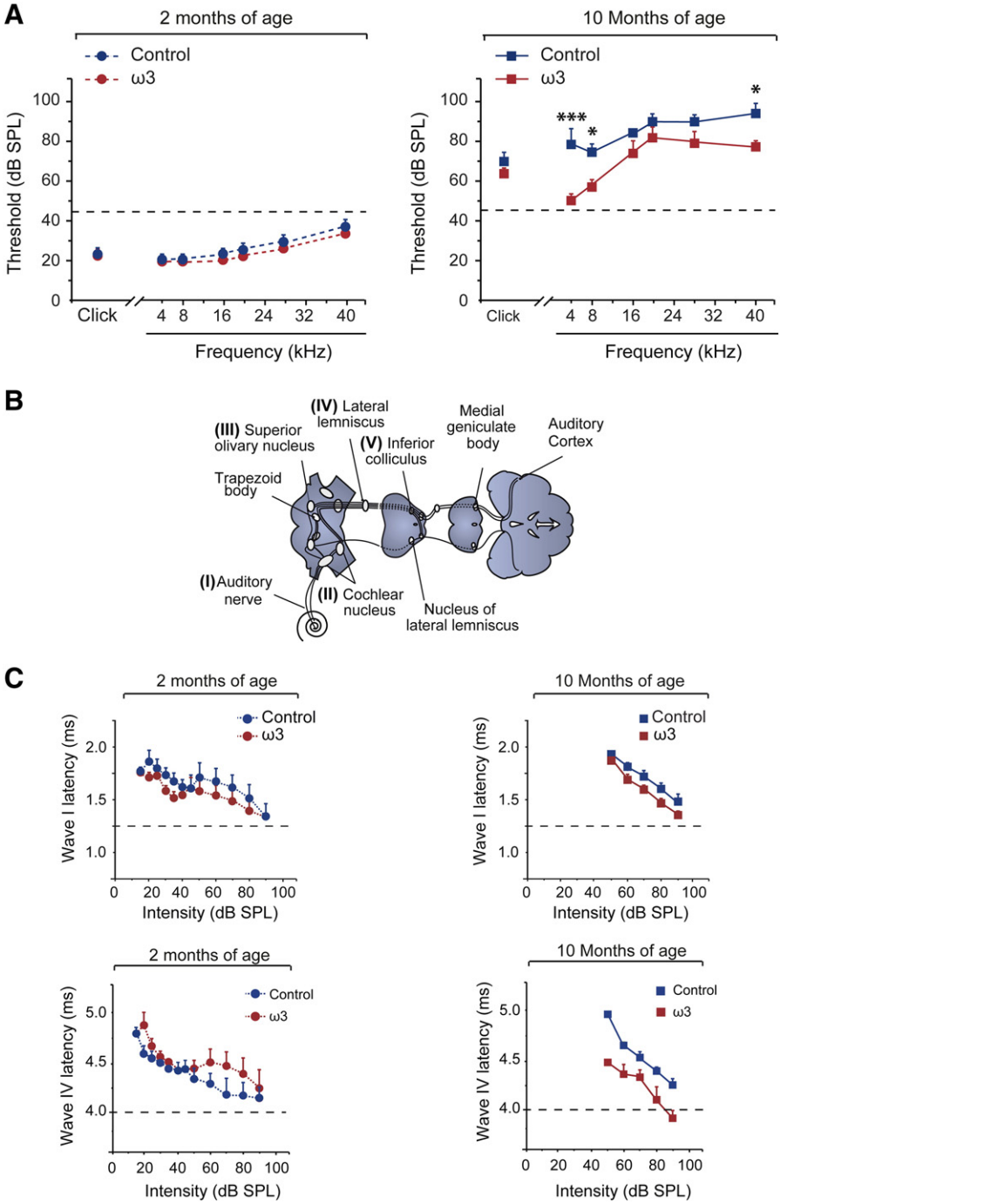

Fig. 3. Hearing evaluation in 10 month-old mice. (A) ABR thresholds in response to click and tone burst stimuli in control (blue; $n=5$) and ω3-supplemented (red; $n=6$) mice at 2 (beginning of the experiment) and 10 months of age. (B) Scheme of the auditory pathway adapted from Murillo-Cuesta et al. [42] indicating the main structures and their corresponding ABR waves: (I) auditory nerve, (II) cochlear nucleus, (III) superior olivary nucleus, (IV) lateral lemniscus and (V) inferior colliculus. (C) Wave I and wave IV latency–intensity functions at 2 and 10 months of age. Data are shown as the mean±S.E.M. Statistical significance: $^{**}P<.05$, $^{***}P<.001$.

in expression of *Tnfa* and *Tgf1b* were observed (Fig. 4A). In parallel, the cochlear expression levels of the fatty acid transporter *O3fa1* were evaluated along the study, but no differences between groups were observed.

Key elements involved in stress signaling pathways were also studied by Western blotting (Fig. 4B). No significant changes in ERK phosphorylation were detected among groups, whereas JNK phosphorylation levels were lower in ω3-10M than in the C-10M cochleae ($P=.017$). Both C-10M and ω3-10M cochleae exhibited increased levels of p38α phosphorylation as compared to the C-4M samples ($P=.024$). Additionally, signs of oxidative stress were found when C-4M and C-10M cochleae were compared, as judged by the changes detected by RT-qPCR in appropriate biomarkers (Fig. 4C). *Cat* expression levels were reduced ~40% in C-10M as compared to C-4M control cochleae and a similar decrease was detected in *Gss* expression levels. These changes were partially prevented in ω3-10M cochleae, which exhibited values of *Cat* and *Gss* expression approaching those of C-4M mice.

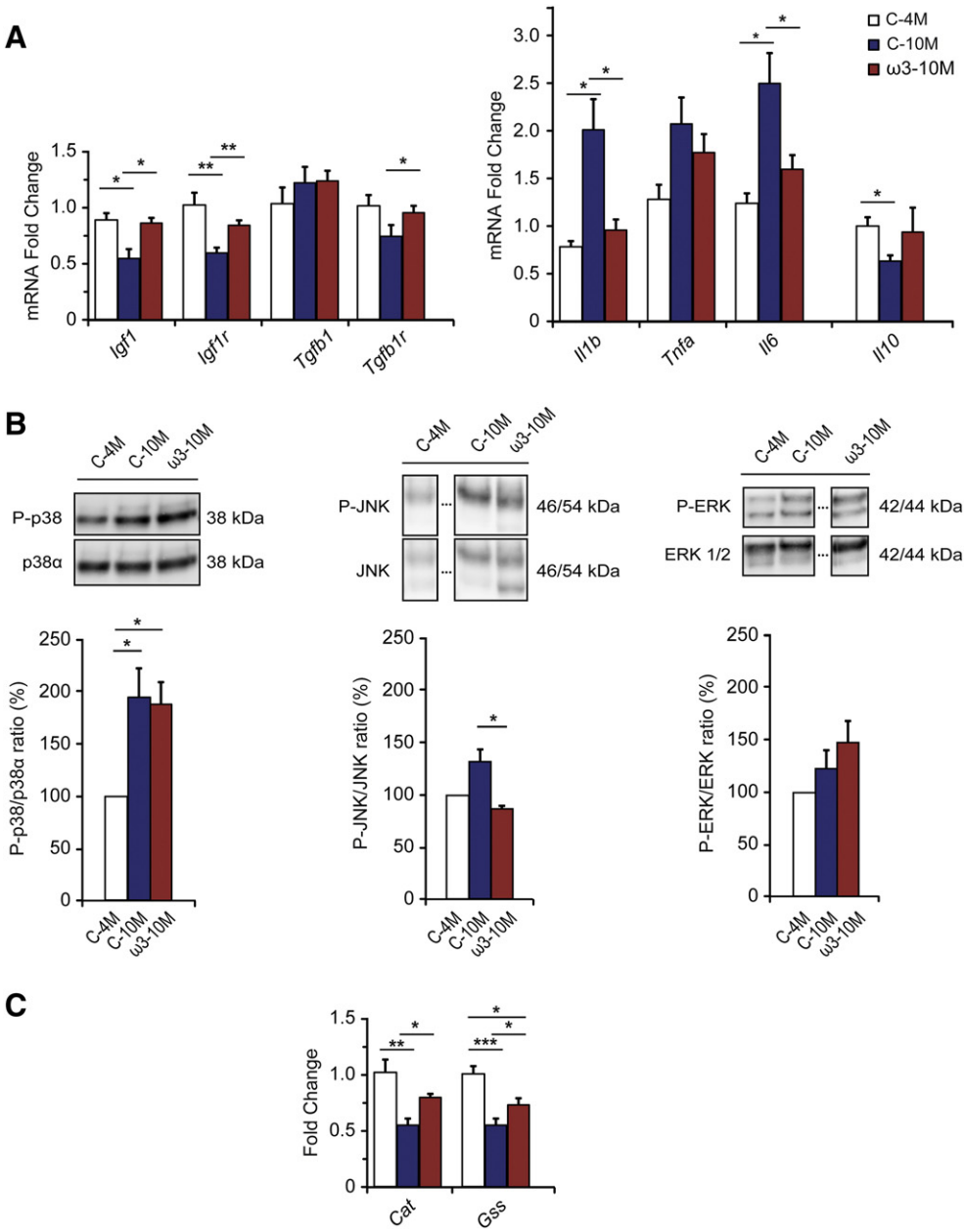

Fig. 4. Evaluation of inflammation and oxidative stress markers. (A) Cochlear expression levels of genes related to inflammation and insulin signaling in mice receiving control (C-4M and C-10M) or ω3-supplemented diets (ω3-10M) at 4 (C-4M) or 10 months of age (C-10M and ω3-10M). *Rn18s* and *Rplp0* genes were used as the endogenous housekeeping controls. (B) Protein levels of phospho-p38 (P-p38), phospho-JNK (P-JNK) and phospho-ERK (P-ERK) stress kinases measured by Western blotting using p38α, ERK 1/2 and JNK as total protein controls. (C) Evaluation of cochlear expression levels for *Cat* and *Gss* genes in mice fed control (C-4M and C-10M) or ω3-supplemented diets (ω3-10M) using *Rn18s* and *Rplp0* genes as endogenous housekeeping controls. Mean data of the C-4M group are presented as 100% for graphical purposes. Values are presented as the mean±S.E.M. for each group: C-4M (n=4), C-10M (n=6), ω3-10M (n=6). Statistical significance: $^{*}P<.05$, $^{**}P<.01$.

3.4. Alterations in cochlear homocysteine metabolism

At the end point of the study, no significant changes in tHcy [3.56± 0.71 (n=4) vs. 3.22±0.53 μM (n=6); $P>.05$] or serum folate levels [45.02±9.95 (n=5) vs. 32.36±2.83 μg/L (n=6); $P>.05$] were detected between C-10M and ω3-10M groups. Systemic levels may not reflect changes taking place in cochlear Hcy metabolism given the small size of the organ and the low expression levels of cochlear Hcy metabolism enzymes. Hence, putative alterations in expression of genes involved in this metabolic pathway were analyzed by RT-qPCR (Fig. 5A). Comparison between the C-4M and C-10M cochleae showed that aging induced significant reductions in the expression of *Ahcy*, *Adk*, *Bhmt*, *Mtr*, *Cbs* and *Cth* genes ($P<.05$), whereas *Ada* expression was not affected. Only the decreased levels of *Bhmt* and *Cbs* transcripts were significantly prevented in ω3-10M cochleae ($P<.05$); the expression values of these genes remained similar to those in the C-4M cochleae. Moreover, supplementation with ω3 also led to a significant decrease in *Ada* expression as compared to that shown in C-10M cochleae. In general, a tendency towards preservation of mRNA levels similar to those in Hcy metabolism of C-4M cochleae was detected after 8 months of ω3-supplementation.

Effects on cochlear Hcy metabolism were also measured by Western blotting (Fig. 5B–C). Comparison between C-4M and C-10M cochleae showed no significant changes in BHMT (68 kDa in this tissue

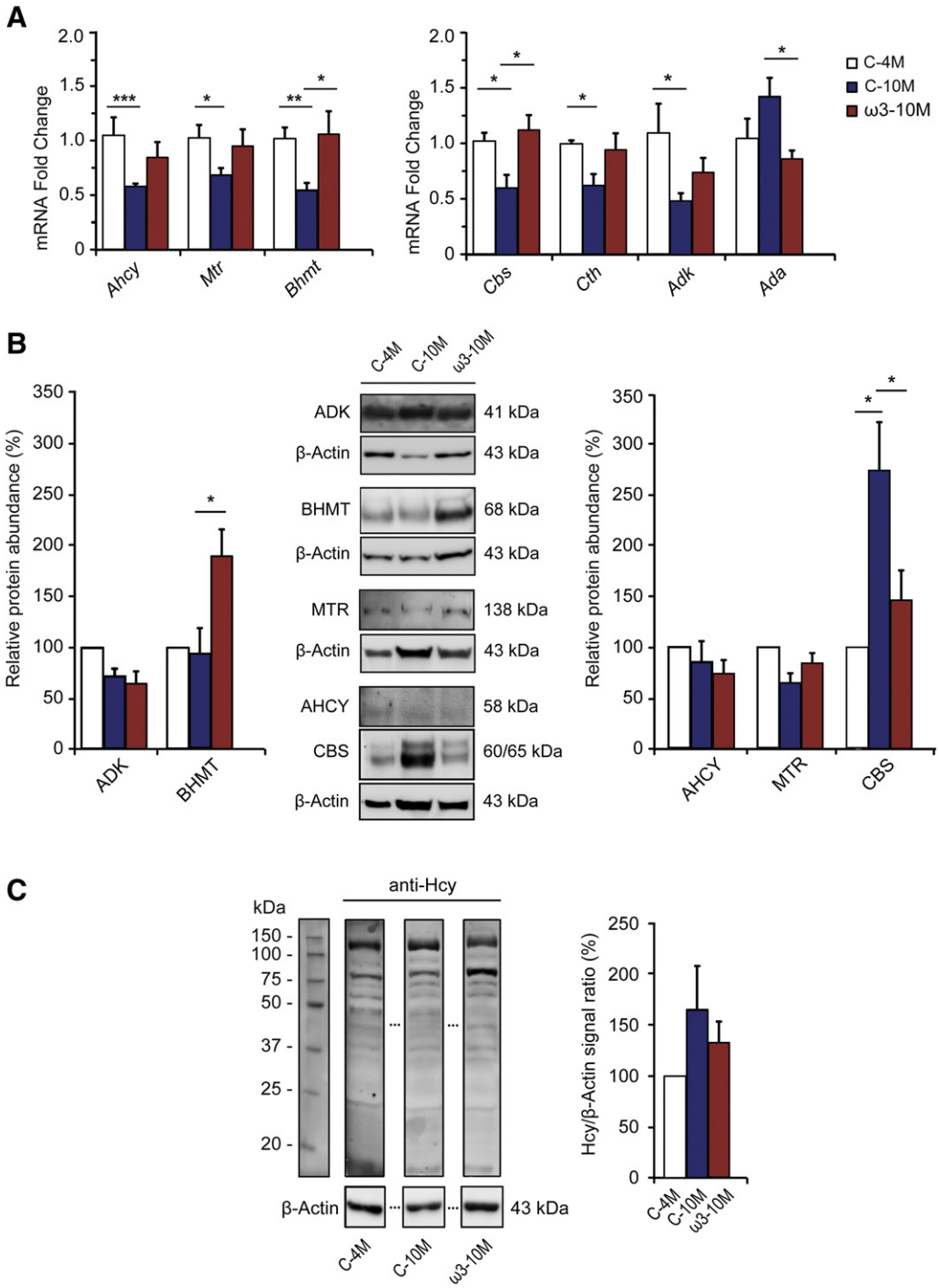

Fig. 5. Analysis of changes in cochlear homocysteine metabolism. (A) Alterations in mRNA expression levels of *Ahcy*, *Mtr*, *Bhmt*, *Cbs*, *Cth*, *Adk* and *Ada* analyzed by RT-qPCR using the *Rn18s* gene as the endogenous control. Sample groups are shown according to the diet received and the mice age as 4-month-old controls (C-4M, $n=4$), 10-month-old controls (C-10M, $n=8$) and ω3 supplemented (ω3-10M, $n=6$). (B) Representative images of ADK, BHMT, MTR, AHCY and CBS protein levels measured by Western blotting using β-actin as the loading control; histograms show the densitometric analysis of the data. (C) Representative images of antihomocysteine Western blotting and the corresponding densitometric analysis of the results. Mean data of the C-4M group are presented as 100% for graphical purposes. Values are presented as the mean±S.E.M. for each condition: C-4M ($n=4$), C-10M ($n=6$), ω3-10M ($n=6$). Statistical significance: $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$.

[49]), MTR, AHCY (58 kDa in the cochlea [49]) and ADK protein levels due to the aging process, whereas the joint levels of both CBS splicing forms increased significantly in the C-10M cochleae. Fatty acid supplementation did not alter MTR, AHCY and ADK protein levels in ω3-10M cochleae as compared to both C-4M and C-10M groups but induced a significant increase in BHMT levels as compared to C-10M cochleae. In contrast, ω3-10M showed CBS protein levels similar to those in C-4M cochleae and significantly lower than those detected in C-10M samples. These data suggested a putative enhancement of Hcy intracellular levels and hence in protein homocysteinylation.

However, examination by Western blotting of cochlear extracts using anti-Hcy showed no significant changes in global protein homocysteinylation levels between cochleae of the different animal groups (Fig. 5C).

4. Discussion

Progressive hearing loss is a major part of the age-related sensory impairment, which shares common molecular mechanisms with many other age-related chronic diseases, including diabetes and cardiovascular disorders [1,2,53,54]. Among them, increased inflammation, impairment of insulin signaling, abnormal proteostasis, oxidative stress and alterations in intermediary metabolism have been reported [41,49,53–56], and hence targeting these traits may offer opportunities to slow down progression of hearing loss. Long-chain ω3 PUFAs, which have demonstrated anti-inflammatory properties, may provide this type of intervention through changes in nutritional patterns as deduced from epidemiological studies [15]. In fact, the loss of hearing acuity observed in C-10M mice was partially prevented in ω3-10M animals, this reproducible effect being however of limited magnitude. One potential reason may rely on the use of EPA-rich fish oil, instead of a more equilibrated formulation of EPA and docosahexaenoic acid (DHA). However, EPA is known to be more effective than DHA in reducing inflammation [57], and indeed *Il1b*, *Il6* and *Il10* expression levels in ω3-10M were similar to those in C-4M cochleae. Detection of increased expression and/or levels of proinflammatory cytokines during aging is a change not unique to the C57BL/6J strain but also detected in, for example, senescence-accelerated resistant (SAMR) mice in which hearing loss and inflammation are shown at similar ages [58]. However, SAMR mice show increased levels of TNFα, whereas *Tnfa* expression in C-10M is not significantly enhanced or modified by ω3-supplementation. Such difference may in turn rely on the moderate signs of cochlear oxidative stress observed in C-10M, which is a key determinant for the production of proinflammatory cytokines.

Circulating levels of IGF-1 are reduced with age in mammals [55,59], and its low levels correlate with different types of hearing loss and presbycusis [56]. *Igf1*-null mice develop hearing loss affecting the number of cochlear neurons, the degree of innervation of the hair cells and also the stria vascularis, in turn, the main metabolic structure of the cochlea [55]. Reduced expression of *Igf1* and its receptor *Igf1r* was therefore expected, and confirmed, in C-10M cochleae. Maintenance or recovery of IGF-1 levels by ω3-supplementation, especially EPA, has been demonstrated in several epidemiological studies and animal models of acute or chronic pathologies [60–62]. In this line of evidence, our study shows that ω3-10M cochleae preserve the *Igf1* and *Igf1r* expression levels shown in C-4M cochleae and hence no signs of impairment in insulin signaling. IGF-1 deficiency in the mouse causes an all-frequency neurosensorial deafness and neural loss [41,63,64]. In contrast, here we report that ω3-10M mice show lower thresholds than C-10M mice only at specific scattered frequencies. This result suggests that there is a general improvement in hearing upon ω3 supplementation, with differences registered in some frequencies reaching statistical significance. Cochlear stress pathways are activated in response to proinflammatory cytokines and oxidative stress, among others in noise-induced hearing loss, as a consequence of aging, and in *Igf1*-null mice [56,65–67]. This behavior was partially shared by C-10M mice, which presented with increased phospho-p38/p38 ratios but lack significant activation of JNK or ERK. Prevention of p38 activation was not achieved in ω3-10M cochleae, which in contrast showed decreased phospho-JNK/JNK levels. Although ω3 supplementation has proved its ability to decrease IL-6 and IL-1β levels together with the activation of stress pathways in several experimental settings [68,69], this does not seem to be the case in our model of hearing loss progression, the difference relying possibly in the trigger of hearing loss, cisplatin [69] versus age in our study. Activation of the p38 signaling pathway has also been described in cortical neurons grown in the presence of Hcy, a metabolite whose increased levels are considered an independent risk factor for both neurological disorders and cardiovascular disease and whose metabolism is altered in hearing loss [49,70]. This Hcy-dependent activation of p38 involves stimulation of *N*-methyl-D-aspartate receptors and ERK activation [71], this last pathway remaining essentially unaffected in C-10M and ω3-10M cochleae in which the contribution of the neuronal population is limited by the presence of other cell types. Inflammation is generally secondary to oxidative stress, as a consequence of increased free oxygen and nitrogen radical generation in the sensory cells, neurons and stria vascularis. Accumulative cellular damage contributes to ARHL that typically shows the strongest alterations in the cochlear basal sensory epithelium responsible for the perception of high frequencies [49,72]. In contrast, treatments aimed to decrease the oxidative stress and inflammatory response, secondary to cochlear injury, have shown all-frequency protection patterns [73–75].

Alterations in Hcy metabolism due to aging seem limited in our model. First, no significant changes in systemic tHcy or folate levels are detected in C-10M mice as compared to those previously reported for 4-month-old animals on a control diet [49]. Second, although reductions in expression levels of genes involved in cochlear Hcy metabolism were detected at 10 months of age, these changes had a limited effect in protein levels. In fact, only CBS protein levels increased in C-10M cochleae, suggesting an enhanced flux through the transsulfuration pathway. These differences between steady-state levels of several proteins and their mRNAs can be ascribed to changes in their half-lives that take place, for example, during oxidative stress, as demonstrated previously in hepatoma cell lines [76]. Increased levels of CBS might be required to synthesize higher levels of H_2S rather than for cysteine production given the ability of the former to differentially regulate the synthesis of anti- and pro-oxidant enzymes and anti- and proinflammatory mediators [77–79]. In fact, several synthetic drugs with capacity to act as H_2S providers have been used for the treatment of hearing loss with diverse results [80,81].

The supplementation with ω3 fatty acids used in the present study did not induce changes in tHcy or folate levels, as previously reported in short-term supplementation studies in rats [36,37]. However, the supplementation was able to reduce *Ada* expression in C-10M cochleae, a protein with undetectable levels in 4-month-old mice [49], hence further favoring adenosine elimination through ADK, the main enzyme responsible for this role in the cochlea [82]. Moreover, the supplementation prevented the decrease in *Bhmt* and *Cbs* expression in ω3-10M cochleae, but this change led to opposite effects at the protein level. Thus, while the specific 68-kDa BHMT band detected in cochlea [49] increased its levels dramatically, a strong decrease in CBS protein towards levels of C-4M controls was detected. No comparison with other ω3 supplementation studies is possible given that the few published reports analyzing effects on hearing loss did not evaluate cochlear Hcy metabolism [33,34]. Hence, the results available refer to rat liver or human hepatoma HepG2 cells, where no changes in *Cbs, Ahcy* or *Mtr* expression, or in CBS and AHCY activities, were observed after supplementation with fish oil [83] or culture in the presence of DHA, EPA or α-linolenic acid [84]. Other short-term studies did report elevations in hepatic expression of *Bhmt* and *Cbs*, but not in *Ahcy*, upon supplementation with several long-chain ω3 fatty acids, including EPA [36]. Some of these studies also indicated the ability of ω3 fatty acids to regulate expression and identified fatty acid responsive elements in the promoters of *Mtr* and *Cbs*, among other genes of the methionine cycle [84]. This fact may also contribute to explain the opposite effects observed in *Cbs* expression and protein levels in C-10M cochleae.

Changes induced by the long-term ω3 supplementation are directed towards enhanced Hcy remethylation through methionine

synthesis using betaine, a product of choline oxidation [27,85]. An increased need of methionine can be explained by a higher requirement of *S*-adenosylmethionine for methylation reactions, including several epigenetic modifications. Such an effect has been already observed in liver of ω3-supplemented animals, where increased levels of methionine adenosyltransferase have been detected [86]. This enhanced remethylation may also derive from the incorporation of dietary ω3 fatty acids into phospholipids, their hydrolysis to produce anti-inflammatory eicosanoids and related molecules, and the resulting increase in phosphatidylcholine turnover. This process could render a choline excess, which, in turn, would be oxidized to betaine, the methyl donor for BHMT [27]. The increased expression of hepatic *Gnmt* and *Pemt* encountered in rat liver after short-term supplementation with several ω3 fatty acids, including EPA, points to this direction [36]. However, cochlear expression and protein levels of these two methyltransferases, which are among the highest consumers of *S*-adenosylmethionine in the liver [87], have not been described. Phosphatidylcholine species synthesized by PEMT are especially rich in arachidonic acid, and hence their cochlear levels may be reduced in EPA supplementation toward EPA phosphatidylcholine, as detected in measurements carried out in plasmatic phosphatidylcholine [88].

In summary, long-term ω3 supplementation partially prevents progression of hearing loss in C57BL/6J mice. This effect is mainly exerted through maintenance of IGF-1 signaling and the balance between proinflammatory and anti-inflammatory cytokines shown in C-4M mice. Additionally, the increased Hcy elimination through transsulfuration observed in C-10M cochleae is compensated by the ω3 diet through increased BHMT remethylation to preserve the flux through the methionine cycle and the protein homocysteinylation levels within normality. However, age-related activation of the p38 stress pathway is not prevented, and hence, changes in protein expression leading to hearing loss are still observed.

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