Citrulline diet supplementation improves specific age-related raft changes in wild-type rodent hippocampus

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Abstract The levels of molecules crucial for signal transduction processing change in the brain with aging. Lipid rafts are membrane microdomains involved in cell signaling. We describe here substantial

biophysical and biochemical changes occurring within the rafts in hippocampus neurons from aging wild-type rats and mice. Using continuous sucrose density gradients, we observed light-, medium-, and heavy raft subpopulations in young adult rodent hippocampus neurons containing very low levels of amyloid precursor protein (APP) and almost no caveolin-1 (CAV-1). By contrast, old rodents had a homogeneous age-specific high-density caveolar raft subpopulation containing significantly more cholesterol (CHOL), CAV-1, and APP. C99-APP-Cter fragment detection demonstrates that the first step of amyloidogenic APP processing takes place in this caveolar structure during physiological aging of the rat brain. In this age-specific caveolar raft subpopulation, levels of the C99-APP-Cter fragment are exponentially correlated with those of APP, suggesting that high APP concentrations may be associated with a risk of large increases in beta-amyloid peptide levels. Citrulline (an intermediate amino acid of the urea cycle) supplementation in the diet of aged rats for 3 months reduced these age-related hippocampus raft changes, resulting in raft patterns tightly close to those in young animals: CHOL, CAV-1, and APP concentrations were significantly lower and the C99-APP-Cter fragment was less abundant in the heavy raft subpopulation than in controls. Thus, we report substantial changes in raft structures during the aging of rodent hippocampus and describe new and promising areas of investigation concerning the possible protective effect of citrulline on brain function during aging.

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Introduction

A number of metabolic changes related to cell physiology and signaling occur during normal aging, and the accumulation or exacerbation of these modifications may lead to the development of age-related diseases. In the brain, changes in the amount of cholesterol (CHOL), an essential membrane component, and imbalances due to oxidative stress may lead to the development of neurodegenerative diseases, such as Alzheimer disease (AD).

The blood–brain barrier is impermeable to serum lipoproteins, and human brain cholesterol is locally synthesized *de novo*. Neurons mostly obtain cholesterol by uptake of apolipoprotein E/CHOL complexes via endocytosis, through the low-density lipoprotein receptor-related protein. CHOL distribution in the membrane is not uniform and lipid rafts are dynamic CHOL- and glycosphingolipid-enriched membrane microdomains that serve as platforms for intracellular cell signaling. They promote protein–protein and protein–lipid interactions (Simons and Ikonen 1997) and are involved in endocytosis. Flotillin-1 (FLOT), an integral membrane protein, is commonly used as an internal raft marker. Caveolae, a subpopulation of rafts, are plasma membrane invaginations caused by polymerization of structural caveolin proteins, including caveolin-1 (CAV-1). CAV-1 is a CHOL-binding protein with a scaffolding domain that interacts with many signaling molecules (Sargiacomo et al. 1993; Lisanti et al. 1994; Song et al. 1996; Kurzchalia and Parton 1999), abolishing their activity (Park et al. 2000; Sato et al. 2004). CHOL regulates the formation of caveolae by affecting CAV-1 transcription: increase in CHOL level is associated with increase in CAV-1 production. Increases in CAV-1 levels have been observed in aging rodent tissues, including the brain (Park et al. 2000; Kang et al. 2006), and in the brains of patients with AD (Gaudreault et al. 2004).

Aging alters both raft CHOL distribution in synaptic plasma membranes (Igbavboa et al. 1996, 2005) and membrane fluidity (Larbi et al. 2004). In synaptic lipid rafts, consistent differences in raft protein levels have been observed between young and aged rat

brains (Jiang et al. 2010). These differences may be associated with differences in signal transduction, resulting in severe consequences of aging, such as an increase in the risk of neurodegenerative disease in elderly individuals. It is now well established that rafts are involved in amyloid precursor protein (APP) cleavage to generate beta-amyloid peptide (A β), a key event in AD (Vetrivel and Thinakaran 2010). The enzyme cleaving the APP β -site and presenilins are both involved in A β generation and are highly enriched in CHOL-rich raft microdomains, whereas α -secretase is located in phospholipid-rich domains (Vetrivel and Thinakaran 2010).

The vulnerability of neuronal cell lines to oxidative stress depends on their cell membrane composition (Clement et al. 2009) and fluidity (Clement et al. 2010). Moreover, oxidative stress directly promotes A β production in the lipid rafts of a neuronal cell line (Oda et al. 2010). These observations strongly suggest that lipid raft composition is tightly linked to oxidative stress.

Citrulline (CIT), first isolated from *Citrullus vulgaris* (watermelon), is associated with arginine metabolism. CIT possesses antioxidant properties and is one of the most potent scavengers of hydroxyl radicals (Yokota et al. 2002). It can also cross the blood–brain barrier. In old malnourished rats, CIT decreases the carbonylation of cerebral proteins (Moinard et al. 2007). We recently observed that long-term CIT supplementation in healthy aged rats increased lean body mass and decreased cutaneous and intra-abdominal fat mass (unpublished data). Thus, CIT possesses strong antioxidative properties (Cynober et al. 2010) and appears to have a major effect on the regulation of lipid and protein metabolism.

The aim of this study was to evaluate the differences in hippocampal raft composition between healthy old and young adult mice and rats. Using continuous linear sucrose gradients with detergent for raft purification, we demonstrated the presence of several raft subpopulations in the hippocampus of healthy 2-month-old rodents. In 23-month-old rodents, a single raft population predominated and was enriched in CAV-1, APP, and the amyloidogenic C99-APP-Cter fragment resulting from the first step of A β generation. After the inclusion of CIT in the diet for 12 weeks, this raft subpopulation contained less CAV-1, APP, CHOL, and C99-APP-Cter than that in the controls. This suggests that CIT may modify the

biochemical properties of rafts, thereby having a protective effect on brain function during aging.

Materials and methods

Materials

The various primary antibodies were purchased and diluted as follows: anti-calnexin, rabbit polyclonal antibody (SPA-860) (Stressgen, Victoria, Canada) diluted to 1/5,000; anti-Golgi 58 K, mouse monoclonal antibody (G2404) (Sigma-Aldrich, Steinheim, Germany) diluted to 1/5,000; anti-FLOT, mouse monoclonal antibody (F65020) (BD Transduction Laboratories, Biosciences) diluted to 1/1,000; anti-APP-Nter, mouse anti-Alzheimer Precursor Protein A4 monoclonal antibody (MAB 348) (Chemicon, Millipore Corporation) diluted to 1/1,000; anti-CAV-1, monoclonal 7C8 antibody (ab37141) (Abcam) diluted to 1/1,000 for immunoblotting and anti-CAV-1, monoclonal antibody (610406) (BD Transduction Laboratories, Biosciences) diluted to 1/100 for immunocytochemistry; anti-APP-Cter, a polyclonal antibody generated in rabbit (Langui et al. 2004) with the complete cytoplasmic sequence of APP, was diluted to 1/10,000; anti-A β -Nter, a purified rabbit polyclonal anti-rodent A β antibody (SIG-39153) (Covance, Eurogentec) diluted to 1/25; anti-mouse-HRP, ECL anti-mouse IgG (NA9310V) and anti-rabbit-HRP, ECL anti-rabbit IgG (NA9340V) (GE Healthcare, Buckinghamshire, UK) diluted to 1/1,000; biotinylated donkey anti-mouse IgG (Vector Laboratories, USA) diluted to 1/200; AF488 streptavidin (Jackson ImmunoResearch West Grove) diluted to 1/200; rabbit anti-glial fibrillary acidic protein (GFAP) (Sigma) diluted to 1/500; rabbit anti-neuron-specific enolase (NSE) (Chemicon) diluted to 1/500; and CY3 anti-rabbit antibody (Jackson ImmunoResearch West Grove) diluted to 1/500. Sucrose, 2-(*N*-morpholino)ethane sulfonic acid (MES), sodium citrate, H₂O₂, diaminobenzidine (DAB), bovine serum albumin (BSA), sodium pentobarbital, and Triton X-100 were obtained from Sigma. Sodium carbonate, paraformaldehyde and NaCl were obtained from VWR, and xylene was obtained from Carlo Erba. EDTA-free, complete protease-inhibitor cocktail tablets were obtained from Roche. CIT was a gift from Kyowa Hakko (Tokyo, Japan).

Experimental design

Male C57BL6J mice (2 and 24 months old), purchased from Janvier (France), were used for the biochemical and immunohistochemical analyses. For immunohistochemical analyses, mice were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused through the ascending aorta with 10 ml of saline (0.9 % NaCl), followed by 100 ml of ice-cold 3 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, post-fixed for 2 h in 3 % paraformaldehyde and stored in 10 % sucrose in phosphate-buffered saline (PBS) overnight. Brains were then frozen by immersion in isopentane (3 min, -40°C) and stored at -80°C until sections were cut. Frozen sagittal brain sections (10- μm thick) were cut from each brain using a freezing cryostat. The sections were collected on Superfrost slides (CML, France) and stored at -20°C until immunohistochemical processing.

Twenty-month-old male Sprague–Dawley rats were randomized into two groups and were fed ad libitum for 12 weeks with a chow diet enriched with CIT (1 g. $\text{kg}^{-1} \text{ day}^{-1}$) (CIT group, $n=8$) or with a control diet without CIT (NEAA group, $n=7$). The two diets were isonitrogenous and isocaloric. We previously observed that this dose of CIT resulted in a large increase in CIT concentration in both plasma and brain (Moinard, unpublished data). Animals were killed at the age of 23 months. Two-month-old- (YNG group, $n=6$) and 20-month-old (20-CTRL group, $n=3$) Sprague–Dawley rats were also used.

Ethics statement

All procedures were carried out in accordance with the recommendations of the European Economic Community (86/609/EEC) and approved by the regional animal ethics committee (Comité Régional d’Ethique pour l’Expérimentation Animale Ile-de-France) under authorization no. P2.CM.058.08.

Isolation of lipid rafts

Continuous 5–30 % sucrose gradients were used for mouse brain extracts, and continuous 12–30 % sucrose gradients were used for rat hippocampus extracts. Brain tissues were homogenized with a tissue grinder (Kontes Glass CO) in either 25 mM MES, 0.15 M

NaCl, 1 % Triton X-100, and pH 6.5 buffer, with protease inhibitors, according to Sargiacomo et al. (1993) or in 500 mM sodium carbonate, pH 11, with protease inhibitors, as described by Song et al. (1996). Extracts (2 mg of proteins per gradient), adjusted to 45 % sucrose in MES buffer (final volume of 1.2 ml), were placed at the bottom of ultracentrifuge tubes. Continuous sucrose gradients in MES buffer, containing either 250 mM sodium carbonate or 1 % Triton X-100, were layered above the homogenate using a gradient maker (Hoefer) (total volume 11 ml). All steps were carried out on ice. Gradients were then subjected to equilibrium flotation ultracentrifugation in a Beckman SW41 rotor (39,000 rpm, 18 h, 4 °C), and 12 fractions (“total” fractions, ~1 ml) were collected from the top to the bottom of each tube. The refractive index of each fraction was measured with a refractometer (Atago, Japan). The fractions were then harvested by centrifugation in MES buffer (39,000 rpm, 40 min, 4 °C): the resulting pellets, corresponding to washed and concentrated lipid rafts, were suspended in 100 µl of distilled water (“pellet” fractions). All or part of this suspension was immediately mixed with Laemmli buffer and boiled for Western blot analyses, and the remainder was frozen at –20 °C for cholesterol assays.

Western blotting

The pellet gradient fractions were subjected to standard SDS-PAGE Western blotting in 12.5 % Tris-HCl precast Criterion gels (Bio-Rad) to detect CAV-1, FLOT, full-length APP, calnexin, and Golgi 58 K or were loaded on 10–20 % Tris-HCl gels to detect full-length APP, C99-APP-Cter fragment, and FLOT. The proteins were then transferred onto PVDF membranes. The membranes were incubated overnight at 4 °C with primary antibodies (see “Materials”) and then with species-specific peroxidase-conjugated secondary antibodies. The peroxidase signal was visualized by enhanced chemiluminescence (ECL Western Blotting Detection Reagents from GE Healthcare).

For each animal, APP and CAV-1 were analyzed on the same membrane and with the same film. APP and the C99-APP-Cter fragment were analyzed on one membrane, but not that used for CAV-1, and with the same film. Immunoblot films were scanned with a GS-800 Calibrated Densitometer (Bio-Rad), and band intensity was quantified by densitometry using Image J software. The internal raft marker protein FLOT was

used to normalize the values for CAV-1, APP, and C99-APP-Cter fragment. Results are expressed in arbitrary units (AU).

Cholesterol assay

Due to limited material quantity available, CHOL contents of the pellet gradient fractions (5 µl) were evaluated from only four NEAA, three YNG and six CIT rats, by colorimetric assays carried out according to the manufacturer’s kit instructions (cholesterol/cholesterol Ester Quantification Kit [ab65359], Abcam). The amounts, in micrograms of CHOL, were then normalized to the internal FLOT marker and are expressed in arbitrary units.

Protein assay

Protein concentrations were determined using a Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Immunohistochemistry

Microwave antigen unmasking was performed on mouse brain slices in 10 mM citrate buffer (pH 6.0) for 5 min at 440 W. Slides were allowed to cool to room temperature (RT) for 20 min and were then washed in PBS for 15 min. The sections were incubated with 3 % H₂O₂ in PBS for 10 min at RT, to inactivate the endogenous peroxidase. They were rinsed in ice-cold PBS (5 × 5 min) and: (1) incubated in a blocking solution consisting of 2 % BSA in PBS for 1 h at RT and in primary antibody solution, containing a mouse monoclonal anti-CAV-1 antibody in PBS with 1 % BSA, for 16 h at 4 °C; (2) rinsed in ice-cold PBS (5 × 5 min) and incubated for 90 min with biotinylated donkey anti-mouse IgG in PBS with 3 % donkey serum; (3) rinsed in ice-cold PBS (5 × 5 min) and incubated for 1 h with avidin–biotinyl–peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA); (4) rinsed in PBS (5 × 5 min) and incubated for 10 min in 0.05 % DAB with 0.01 % H₂O₂ (Sigma); and (5) rinsed in PBS (5 × 5 min) to stop the reaction, then in distilled water (5 × 5 min) and dehydrated with ethanol, cleared in xylene, cover-slipped, and examined under a microscope. Some sections were also stained for Nissl, with 0.1 % cresyl violet. Control slices were subjected to the same procedures, but without primary antibody.

Double immunofluorescence was performed for CAV-1 and GFAP, and for CAV-1 and NSE. Microwave antigen retrieval and saturation in blocking buffer were as described above, and the sections were then incubated with the two primary antibodies (CAV-1 and GFAP, or CAV-1 and NSE) for 16 h at 4 °C. The sections were rinsed and incubated with biotinylated anti-mouse secondary antibody for 1 h at 37 °C, rinsed in PBS, and incubated with AF488-streptavidin for 1 h at 37 °C for the detection of CAV-1. The sections were again rinsed in PBS and incubated for 1 h at 37 °C with the CY3-conjugated anti-rabbit antibody.

To minimize lipofuscin autofluorescence, the sections were incubated in 1 % Sudan black in 70 % alcohol for 1 h, quickly washed in 70 % ethanol and in TBS, and mounted in 80 % glycerol. Sections incubated without primary antibody served as controls. Images were acquired with a Zeiss Axiophot Microscope. Single confocal images or z-stacks were acquired with the TCS SP2 confocal imaging system (Leica Microsystems, Heidelberg, Germany). For each optical section, double fluorescence images were acquired in sequential mode, to avoid potential contamination due to linkage-specific fluorescence-emission cross-talk, with an argon laser adjusted to 488 nm for AF-488 excitation, and a helium/neon laser adjusted to 543 nm for Cy3 excitation.

Statistical analyses

Three different statistical analyses were used, according to the number of samples. First, we used Student's *t* test ("unpaired") to compare means $\pm$ SEM values of APP and CAV-1 protein concentrations in total rafts and in heavy raft subpopulations in the three groups of rats (YNG, $n=6$; NEAA, $n=7$; CIT, $n=8$). The significance of the differences between means was determined with a type 1 error rate of 5 % (differences were considered significant if $p<0.05$).

Second, we used ANOVA to create a set of confidence intervals for the differences between the mean values for each factor, with a specified family-wise probability of coverage. The intervals were based on the studentized range statistic and Tukey's "honest significant difference" method. This statistical analysis was used only to compare CHOL contents between well-defined heavy raft subpopulations in the young (YNG, $n=3$), old control (NEAA, $n=4$), and old citrulline-fed (CIT, $n=6$) rat groups. Significant results

were obtained for a global type 1 error rate of 5 % (adjusted *p* value (*p* adj) <0.05).

Third, all regression analyses for CAV-1, APP, CHOL, and C99-APP-Cter values were modeled in a Bayesian context, which is appropriate for small sample sizes (Robert 2001; Gelman et al. 2004; Congdon 2006); Winbugs software was used. Bayesian modeling combines two sources of information, one from the data and one from prior knowledge, the result being generating a posterior distribution of parameters conditionally on the data set. This posterior distribution is an "updated information" corresponding to the current state of knowledge about the parameters. It is usually summarized as the posterior median and a 95 % credibility interval defined by 2.5 % and 97.5 % quantiles of this distribution. For regression, if zero is not included in the 95 % credibility interval of the slope, a null slope is not probable (case denoted as "significant" slope). The choice between models was based on the deviance information criterion (DIC), corresponding to a penalized deviance of the model's complexity (Spiegelhalter et al. 2002). Based on the minimization of DIC, relationships between APP, CAV-1, and C99-APP-Cter were modeled via a log-linear relationship, but associations with CHOL were modeled via a linear relationship. Bayesian regression models with a group effect (NEAA/CIT) were implemented, to allow comparison between the associations in each group. Marko chain Monte Carlo algorithms with 7,000 iterations and 1,000 iterations for burn-in were used for Bayesian inferences. Convergence was assessed by checking a good mix between iterations and accurate approximations with an MC error.

Results

Validation of the lipid raft isolation method

One widely used method for isolating lipid rafts from cells or tissues is based on the insolubility of raft structures in the cold non-ionic detergent, Triton X-100. Discontinuous sucrose gradients constituted of two sucrose layers (often 30 and 5 % sucrose) above the extract, adjusted to ~45 % sucrose, are generally used. After ultracentrifugation, the rafts float at the interface between the two layers. In these conditions, it is not possible to separate raft subpopulations with different densities (and properties). Furthermore, we

have observed non-negligible amounts of specific lipid raft structures floating in a diffuse manner in the 30 % sucrose layer (data not shown). This observation is clearly illustrated in the study by Head et al., who used this layer method to investigate CAV-1 (Head et al. 2010). We therefore chose to use a continuous sucrose gradient for lipid raft isolation. Continuous 5–30 % sucrose gradients were first tested with mouse brain tissue (see below). As no raft material was detected between the 5 and 15 % sucrose parts of the gradient, we then used 12–30 % continuous sucrose gradients to improve the separation of the various raft subpopulations in rat hippocampi.

Using this continuous 12–30 % gradient (which extends from 15 to 40 % sucrose after ultracentrifugation, as checked by refractive index), we then compared two of the most popular extraction methods: the Triton X-100 detergent method versus the sodium carbonate non-detergent method (Fig. 1A). FLOT, a specific raft marker (Bickel et al. 1997) (a–d in Fig. 1A), calnexin, a specific endoplasmic reticulum marker (e–h in Fig. 1A), Golgi 58 K (i–l in Fig. 1A), and APP (m–p in Fig. 1A) distributions in each total fraction (crude fractions harvested directly from the gradient) and the lipid raft-enriched pellet fractions (obtained after MES washing of the total fraction, see “Materials and methods”) from both gradients were analyzed by Western blotting.

The concentrated raft pellet fractions obtained following extraction with Triton X-100 were uncontaminated with either endoplasmic reticulum or Golgi proteins. We therefore used continuous 12–30 % sucrose gradients in 1 % Triton X-100 at 4 °C for subsequent experiments. We checked that the raft subpopulations isolated from Triton X-100 gradients were enriched in the lipid raft marker GM1 and in the raft-specific glypiated glycoprotein F3 (Fig. 1S) (Bouillot et al. 1996). The continuous sucrose gradients gave highly reproducible results (Fig. 1B).

No method has become established as the reference method for raft isolation. Detergent-based methods have some potential drawbacks, but we believe that the combined use of a detergent and a continuous sucrose gradient is probably the best approach to studying rafts. Indeed, potential changes in raft profile along the gradient may be indicative of related changes in the physical and chemical properties of the raft membranes.

Fig. 1 Isolation of rafts using continuous 12–30 % sucrose gradients containing 1 % Triton X-100 (detergent) or 500 mM sodium carbonate (non-detergent). **A** Young adult rat hippocampal extract, adjusted to 45 % sucrose was applied to the two types of sucrose gradient (2 mg of protein per gradient). The 12–30 % continuous sucrose gradient extended from 15–40 % after ultracentrifugation, as determined by the refractive index of each of the 12 fractions collected from the top to the bottom of the tube. TF represents the “total” fractions (crude fractions harvested directly from the gradient) and PF, the “pellet” fractions obtained after washing the TF fractions with MES, corresponding to concentrated lipid rafts. Comparison of the FLOT (a–d), calnexin (e–h), Golgi-58 K (i–l), and APP (m–p) distributions in each total and pellet fraction of the gradients, in Triton X-100 and in carbonate. The total and pellet fractions from the carbonate gradient were highly contaminated with calnexin (e,f) and, therefore, with endoplasmic reticulum proteins. There was more APP in the pellet fractions from the carbonate gradient (n), than in those from the Triton X-100 gradient (p), confirming this contamination. In the Triton X-100 gradient, most of the APP was present in the bottom total fractions (o), corresponding to soluble non-raft material, consistent with previous reports (Bouillot et al. 1996; Brouillet et al. 1999). Small amounts of Golgi material were detected in the pellet fraction of the carbonate gradient (j). Consequently, the 12–30 % sucrose continuous gradient in 1 % Triton X-100 at 4 °C was used for subsequent experiments because the raft pellet fractions seemed to be uncontaminated with either endoplasmic reticulum or Golgi proteins. **B** Reproducibility of the 12–30 % continuous sucrose gradient in 1 % Triton X-100. Refractive indexes were measured in 20 µl aliquots of each total gradient fraction (12 per gradient), harvested from the top (corresponding to the total gradient volume of about 12 ml) to the bottom of the gradient, for each animal from each group (young adult rats, YNG, *n*=6; old control rats, NEAA, *n*=7; and old rats on a CIT-enriched diet, CIT, *n*=8). Values obtained from single animals are shown. The refractive indexes along the different gradients were very similar, indicating a high reproducibility of gradient patterns between animals from the same and from different groups

Changes in brain raft subpopulations in mice during aging

Using a continuous 5–30 % sucrose gradient in 1 % Triton X-100 at 4 °C for raft purification, we previously detected the presence of two F3-enriched raft subpopulations in primary neurons (Bouillot et al. 1996), in a study addressing principally the predominant and heaviest raft subpopulation containing APP. Here, we also identified light- and heavy raft subpopulations in the cortex and hippocampus of 2-month-old mice (Fig. 2A, B). The heavy raft subpopulation was the most abundant in the cortex and hippocampus of 24-month-old mice, suggesting that raft composition had changed during aging (Fig. 2A, B). CAV-1 was present in the heavy raft subpopulation of the cortex and hippocampus of old mice but was not detected in young adult mice (Fig. 2A, B). Immunohistochemical

(A)

(B)

analyses confirmed this result (Fig. 2C). By double immunostaining of CAV-1 and GFAP for astrocytes and of CAV-1 and NSE for neurons, we observed that the increase in CAV-1 levels in old mice occurred in neurons (Fig. 2D, E).

Changes in hippocampus raft subpopulations in rats during aging and modulation by a CIT diet

Rats were used for these experiments, as they provide larger amounts of tissue than mice. We focused on the

hippocampus, which plays an early role in age-related memory deficiencies.

Rafts were isolated on continuous 12–30 % sucrose gradients in 1 % Triton X-100 at 4 °C from the hippocampus of rats from the three groups (young adult rats, YNG, $n=6$; old control rats, NEAA, $n=7$; and old rats fed a CIT-enriched diet, CIT, $n=8$). Raft subpopulations were defined using maximal FLOT protein enrichment along the continuous sucrose gradient, as determined by immunoblotting (Fig. 3A). The subpopulations were delimited by mean framing the sucrose values (Fig. 3B).

In the YNG group, we identified two or three raft subpopulations that appeared relatively continuous (Fig. 3A, B). By contrast, in the NEAA rat group, as in mice (Fig. 2A), there was only one abundant and well-defined heavy raft subpopulation (Fig. 3A, B).

All CIT samples contained the heavy raft subpopulation (Fig. 3A), but five of the eight rats also had a light raft subpopulation and three had a medium raft subpopulation. Furthermore, the subpopulations were relatively continuously distributed along the gradients, as for

Fig. 2 CAV-1 appears in the neurons of old mice and is present in the single heavy raft subpopulation at age 24 months. **A, B** FLOT and CAV-1 immunoblots of the cortex (**A**) and hippocampus (**B**) of young adult (2-month-old) and old (24-month-old) mice. A 5–30 % sucrose continuous gradient was used. The top fractions, 1 and 2, and the bottom pellet (fraction 12) are not shown. FLOT immunoblots show changes to raft subpopulation distributions during aging. **C–E** Immunohistochemistry to detect CAV-1 was performed on brain sections from young adult and old mice, either with peroxidase followed by Nissl staining (**C**) or fluorescence (**D, E**). **C** CAV-1 was clearly detected in the brain vessels of young adult mice. CAV-1 was not detected in the hippocampus (hipp.) of young adult mice, but it was present in old mice. **D** Double immunostaining for CAV-1 and GFAP clearly shows an absence of colocalization. **E** Double immunostaining for CAV-1 and NSE in the hippocampus of mice shows clear superimposition, suggesting that the increase in the abundance of CAV-1 in old mice occurred in neurons. Scale bars: 20 μ m (**C, D**), 150 μ m (**E**)

YNG samples (Fig. 3A, B). Thus, 3 months of CIT supplementation in old rats was associated with an pattern intermediate between those of young and old animals, indicating that CIT had influenced the biophysical properties of the rafts during aging and, consequently, the distribution of raft subpopulations along the sucrose gradient.

CAV-1 and APP protein levels in the hippocampus rafts from YNG, NEAA, and CIT rats

We investigated the abundance of the CAV-1 and APP proteins in the rafts of the three rat groups, by immunoblotting and quantitative analysis of band intensities. Total CAV-1 levels were 9.51 ± 3.60 AU ($n=6$),

47.34 ± 14.52 AU ($n=7$), and 12.69 ± 2.49 AU ($n=8$) for YNG, NEAA, and CIT rats, respectively, with statistically significant differences between the YNG and NEAA groups ($p=0.039$) and between the CIT and NEAA groups ($p=0.026$) but not between the CIT and YNG groups (Fig. 4A, E). In YNG samples, CAV-1 levels were low in light-, medium-, and heavy raft subpopulations (Fig. 4B). This contrasts with hippocampal tissues from young mice, in which no CAV-1 was detected (Fig. 2B). CAV-1 was detected in the heavy raft subpopulation of NEAA rats, consistent with findings for the cortex and hippocampus rafts in old mice (Fig. 2B). CAV-1 was preferentially detected in the heavy- and medium raft subpopulations of CIT rats (Fig. 4B, E). CAV-1 levels in heavy raft subpopulations differed significantly between YNG (1.47 ± 1.47 AU; $n=6$) and NEAA (45.41 ± 11.92 AU; $n=7$) rats ($p=0.006$), between CIT (16.91 ± 2.90 AU; $n=8$) and NEAA rats ($p=0.028$), and between YNG and CIT rats ($p=0.001$) (Fig. 4B).

Total APP levels were 5.17 ± 2.68 AU ($n=6$), 42.33 ± 10.88 AU ($n=7$), and 13.50 ± 4.28 AU ($n=8$) for the YNG, NEAA, and CIT groups, respectively. There were statistically significant differences between the YNG and NEAA groups ($p=0.011$) and between the CIT and NEAA groups ($p=0.022$), but not between the CIT and YNG groups (Fig. 4C, E). APP was detected in the three raft subpopulations of the YNG group, exclusively in the heavy raft subpopulation of the NEAA group, and was present in both the heavy- and medium raft subpopulations of the CIT group (Fig. 4D). APP levels in heavy rafts differed significantly between YNG (2.03 ± 1.09 AU; $n=6$) and NEAA rats ($46.09 \pm$

Fig. 3 Identification and localization by FLOT of different raft subpopulations in YNG, NEAA, and CIT rat hippocampus. **A** Representative immunoblots of FLOT. A 12–30 % continuous sucrose gradient was used. The maximum FLOT protein (asterisk) defines a raft subpopulation. **B** The raft distribution pattern was

averaged for each animal group along the gradient. The limits of heavy (circle), medium (triangle), and light (diamond) subpopulations are indicated. The ratio indicated above each subpopulation corresponds to the number of animals with this subpopulation divided by the total number of animals in the group

Fig. 4 CAV-1 and APP levels in total rafts and in raft subpopulations in the hippocampi of YNG, NEAA, and CIT rats. Data are expressed in arbitrary units (AU) and are presented as mean values $\pm$ SEMs. **A, C** The total levels of CAV-1 and APP, respectively, in all pellet fractions, are expressed relative to total FLOT levels detected along the same gradient (total CAV-1/total FLOT and total APP/total FLOT). **B, D** The levels of CAV-1 and APP,

respectively, are expressed for each raft subpopulation relative to FLOT levels for the same subpopulation (CAV-1/FLOT and APP/FLOT). Significant differences are indicated by an *asterisk*. **E** Representative immunoblots for each rat group. **F** Statistical analysis using a mixed Bayesian model (see “Materials and methods”) of the significant APP/CAV-1 correlation in the heavy raft subpopulation of each rat group

11.53 AU; $n=7$; $p=0.005$), between CIT (17.28 ± 4.96 AU; $n=8$) and NEAA rats ($p=0.032$), and between YNG and CIT rats ($p=0.023$) (Fig. 4D).

Three months of CIT supplementation in old rats resulted in a status intermediate between young and old animals for hippocampal tissue. CIT significantly reduced the increase in total CAV-1 and APP levels observed in old (NEAA) rats, resulting in total CAV-1 and APP levels comparable to those in YNG rafts.

Using a statistical Bayesian group effect model (see “Materials and methods”), we found a significant positive correlation between the values for APP and CAV-1 in the heavy raft subpopulations of both old rats (median posterior slope value of 1.41 [0.63; 2.22]) and CIT-fed old rats (median posterior value of 1.09 [0.04; 2.00]) (Fig. 4F).

CHOL content in the hippocampus rafts from YNG, NEAA, and CIT rats

Rafts are CHOL-enriched structures and, as expected, raft subpopulations defined according to peaks in FLOT content along the gradient matched those defined by high levels of CHOL. However, colorimetry for CHOL evaluation is less sensitive than immunoblotting with the anti-FLOT antibody. Thus, the light raft subpopulation of YNG rats and the medium raft subpopulation of CIT rats (Fig. 5A) were not detected by CHOL colorimetry.

The concentration and distribution of CHOL in the rafts changed with age (Fig. 5A). If we focus on the heavy raft subpopulation (fractions 9 and 10 of the gradients), CHOL levels differed significantly between YNG (0.21 ± 0.14 AU; $n=3$) and NEAA (7.33 ± 0.29 AU; $n=4$) rats (p adj=0.042) and between NEAA and CIT (1.47 ± 0.05 AU; $n=6$) rats (p adj=0.046), but not between YNG and CIT rats (Fig. 5A).

Using a Bayesian group effect model, we showed that the correlations between APP and CHOL in the heavy raft subpopulations were significant for both the NEAA and CIT groups, with median posterior slope values of 18.16 [13.31; 23.06] and 18.62 [10.91; 29.9], respectively (Fig. 5B). The CAV-1–CHOL correlation in the heavy raft subpopulation was significant for the NEAA group, with a median posterior slope value of 4.22 [0.65; 7.70] but was not significant for the CIT group, with a median posterior slope value of 4.82 [−0.41; 12.31] (Fig. 5C). CIT supplementation of the diet thus seems to modify the association between CAV-1 and CHOL in the heavy raft

subpopulation but had no effect on the association between APP and CHOL.

Levels of APP and amyloidogenic C99-APP-Cter in the heavy raft subpopulation of YNG, NEAA, and CIT rats

The abundance of APP in the heavy raft subpopulation increased substantially with age (Fig. 4D). β -Secretase is present in rafts. We therefore tested for the amyloidogenic C99-APP-Cter fragment (generated by the β -secretase cleavage of APP) in fraction 9 of the gradients (representative of the heavy raft subpopulation). We identified C99-APP-Cter with two antibodies: one directed against the specific N-terminal sequence of rodent β -amyloid peptide (Fig. 6A) and the other directed against the C-terminal end of APP (Fig. 6B).

C99-APP-Cter was observed in all NEAA ($n=6$) and CIT ($n=7$) rats (Fig. 6A) but was not detected in YNG rats ($n=4$). All CIT rats had low levels of APP and low levels of C99-APP-Cter. Surprisingly, C99-APP-Cter levels were exponentially correlated with APP levels (Fig. 6C). Bayesian statistical analysis for all the samples ($n=13$) indicated a significant global correlation between APP and C99-APP-Cter levels, with a median posterior slope value of 0.897 [0.555; 1.233]. The slope was also significant when the association was modeled linearly but had a substantially higher DIC (DIC=76.0 versus DIC=59.8 for linear and log-linear relationships, respectively).

Discussion

In this study, we demonstrated, for the first time, the biophysical and biochemical changes occurring within rodent hippocampal rafts during aging. The aging process is associated with the loss of the lightest raft subpopulations observed in young adults and with significant increases in the amounts of CHOL, CAV-1, and APP in the heavy raft caveolar structure. We confirmed, by immunohistochemistry, that CAV-1 was undetectable in the neurons of young adult mouse hippocampi but was present in old neurons. The amyloidogenic C99-APP-Cter fragment was also present in the heavy raft subpopulation of hippocampal tissue from old rats, and there was an exponential correlation between the amounts of APP and this C99-APP-Cter fragment. The administration of citrulline supplementation diet to

Fig. 5 CHOL level in different hippocampal raft subpopulations from YNG, NEAA, and CIT rats. Data are expressed in arbitrary units (AU) and are presented as mean values $\pm$ SEMs. **A** CHOL levels in the various raft subpopulations. Significant differences are indicated by an *asterisk*. **B, C** Bayesian statistical analysis. The correlation between APP and CHOL (**B**) was significant for the two groups (see “Results”). The correlation between CAV-1 and CHOL (**C**) was significant for the NEAA group, but not for the CIT group (see “Results”)

Fig. 6 The amyloidogenic C99-APP-Cter fragment in the heavy raft subpopulation of hippocampi from YNG, NEAA, and CIT rats. **A** Immunoblots of fraction 9 of the gradients (the fraction representative of the heavy raft subpopulation) for YNG ($n=3$), NEAA ($n=3$), and CIT ($n=3$) rats, probed for the APP, FLOT, and C99-APP-Cter proteins. C99-APP-Cter was detected with anti-rodent A β -Nter rabbit polyclonal antibody. **B** C99-APP-Cter fragment was detected with rabbit polyclonal anti-APP-Cter for the whole cytoplasmic sequence of APP. **C** Exponential correlation between APP and the C99-APP-Cter fragment in the old rat controls (NEAA, $n=6$) and in old rats on citrulline supplementation (CIT, $n=7$)

old rats for a period of 3 months was associated with an attenuation of this age-related pattern. All the results obtained are summarized in Fig. 7.

The heterogeneity of lipid rafts in various cells has already been described by several morphological, immunological and biochemical studies, suggesting the existence of different raft domains, with different specific protein/lipid compositions and different roles in the cell (Pike 2004). Along our continuous 12–30 % sucrose gradients in Triton X-100 at 4 °C, we identified two or three separate raft subpopulations with different flotation densities in young adult rodent cortex and hippocampal tissues. By contrast, only the heaviest raft subpopulation was found in old rats. There seems to be a dynamic change from the presence of heterogeneous raft structures, with almost no CAV-1 and low levels of APP and CHOL, in young rat hippocampal tissues, to a homogeneous single heavy raft structure, containing significantly larger amounts of CAV-1, APP, and CHOL, in old tissue. The heavy raft subpopulation, which was highly enriched in CAV-1, could therefore be considered as a marker of aged neurons.

CHOL and CAV-1 seem to be the main actors in this dynamic physiological phenomenon. CHOL homeostasis in the brain is maintained by highly complex and efficient mechanisms (Russell et al. 2009). The significant local age-associated increase in the CHOL content of the heavy raft subpopulation may result from a dynamic redistribution of CHOL within the membranes. An increase in CHOL concentration in the exofacial leaflet of the synaptic plasma membranes of aged C57/BL/6J mice has been reported (Igbavboa et al. 1996). Any change in exofacial leaflet composition may cause changes in rafts. CAV-1 is a CHOL-binding protein and CHOL promotes CAV-1 oligomer formation (Murata et al. 1995). We report a significant increase in CHOL abundance with aging, associated with a significant increase in CAV-1 levels, in the heavy raft subpopulation of rat hippocampi. CHOL directly affects CAV-1 production, so factors decreasing CHOL concentration also decrease CAV-1 levels. CAV-1 functions as a scaffolding protein and interacts with many signaling molecules, abolishing their activities.

Aging causes a decline and dysregulation of membrane-mediated signal transduction in many types of cells and organs. However, a senescent phenotype can be reversed by decreasing CAV-1 levels (Park et al. 2000; Yeo and Park 2002; Cho et al. 2003). These observations, along with our results, strongly suggest that a facet of the neuronal aging process is initiated at membrane rafts and depends upon increases in CHOL and CAV-1 abundance.

Fig. 7 Schematic representation of the physiological changes to rat hippocampal rafts during aging, and improvement after 3 months on dietary CIT supplementation (CIT diet). Three non-caveolar raft subpopulations are present in young adult rats (mostly light and medium rafts), whereas a single, specific, caveolar heavy raft subpopulation is found in aging control rats (NEAA), with significantly higher concentrations of CHOL, CAV-1, and APP. The amyloidogenic C99-APP-Cter fragment within these aged caveolae suggests that this structure leads to A β -peptide formation during

physiological aging. The high levels of CAV-1 may be associated with impaired cell signaling and the inhibition of NO synthase (see “Discussion”). In animals receiving the CIT diet, the heavy caveolar raft subpopulation continued to predominate, but two other lighter (mostly the lightest) raft subpopulations, similar to those observed in young adult rats, were present. Interestingly, the amounts of CHOL, CAV-1, APP, and C99-APP-Cter were much smaller in this caveolar raft subpopulation in CIT-fed rats than in control rats, suggesting that CIT may decrease A β -peptide formation

Some APP is present in rafts (Bouillot et al. 1996; Ehehalt et al. 2003), and the cytoplasmic domain of this protein associates with signal transduction molecules (Brouillet et al. 1999), FLOT (Chen et al. 2006), and CAV-1 (Ikezu et al. 1998). We observed that the amount of APP in rat hippocampus rafts increases significantly with age. This increase was significantly correlated with increases of CAV-1 and CHOL levels in the heavy raft subpopulation. One elegant recent study (Marquer et al. 2011) demonstrated that, in primary cultured neurons, membrane loading with CHOL is associated with the relocalization of APP from non-raft to raft domains, thus increasing β -secretase access to APP.

In all our hippocampal samples from old rats, we detected the endogenous amyloidogenic C99-APP-Cter fragment, the product of physiological β -secretase cleavage. The presence of C99-APP-Cter thus demonstrates that the first step of the amyloidogenic APP process can take place in lipid rafts and seems to be associated with physiological aging of the rat

hippocampus. Furthermore, an exponential correlation was observed between APP and C99-APP-Cter levels in this heavy raft subpopulation, suggesting that either β -secretase activity was differentially regulated as a function of APP levels within the raft or that the C99-APP-Cter fragment was sequestered in less functional caveolae, in which the accumulation of CHOL and CAV-1 may sterically hinder signal transduction and the endocytosis mediated by caveolae (Le et al. 2002; Nichols 2003). We cannot exclude the possibility that changes in raft profile and content during aging are compensatory events. However, if the exponential correlation observed between APP and the amyloidogenic C99-APP-Cter fragment also exists in humans, then high levels of APP in the heavy raft subpopulation may be an important factor in the development of AD.

We found that feeding aged rats with a CIT-supplemented diet resulted in significantly lower levels of CHOL, CAV-1, and APP and much lower level of the C99-APP-Cter fragment in the heavy raft subpopulation

of hippocampi than in control NEAA old rats. CIT supplementation thus seems to make the rafts more similar to those in young adult rats. The levels of CAV-1 and APP proteins in CIT-supplemented old animals were close to those in young adult rats. The heaviest raft subpopulation was still present, but lighter rafts were also present, as in young rats but not in control old rats. Indeed, in three control animals at the age of 20 months (20-CTRL rats), the age at which CIT supplementation was initiated, the raft profile was heterogeneous but the heavy subpopulation was clearly the most abundant (Fig. 2S, A). Total CAV-1 and APP levels in the total raft population in 20-month-old animals were intermediate between those in young adults and in 23-month-old animals (Fig. 2S, B). Thus, CIT supplementation seems to fix the raft profile and to stabilize the CAV-1 and APP contents of the rafts, possibly even causing a slight decrease in CAV-1 levels. These preliminary data suggest that CIT protects against the effects of aging. In this rat model, CIT supplementation is associated with an increase in lean body mass and decreases in cutaneous and intra-abdominal fat mass (Moinard, unpublished data). CIT, which crosses the blood–brain barrier, not only has a major effect on lipid and protein metabolism in peripheral tissues but also on the biochemical and biophysical properties of rat hippocampal raft structures, including their CHOL levels in particular, with a loss of the correlation between CAV-1 and CHOL observed in NEAA rats. Dietary modifications affecting the composition of rafts, including modifications to dietary fatty acid composition in particular, have already been reported (Yaqoob 2009).

Oxidative stress in the brain increases during aging, and lipid peroxidation has substantial effects on biological membranes, altering their fluidity and modifying the distribution of CHOL and sphingomyelin between raft and non-raft structures. In neuronal cells, resistance to oxidative stress depends on cell membrane composition and fluidity (Clement et al. 2009), and age-dependent increases in CHOL content are associated with a decrease in membrane fluidity (Larbi et al. 2004). We suggest that CIT, which can cross the blood–brain barrier and possesses strong antioxidant properties, may locally and directly limit deleterious membrane lipid peroxidation.

Dietary L-arginine is metabolized by nitric oxide synthase, to generate L-citrulline and nitric oxide (NO). There is a direct interaction between the scaffolding domain of CAV-1 and neuronal NOS (nNOS), resulting

in the inhibition of NO formation (Sato et al. 2004). We describe here a significant age-related increase in CAV-1 levels in the heavy raft subpopulation of rats, and several studies have reported a decrease in nNOS activity in the aging rat brain (Law et al. 2002; Necchi et al. 2002). nNOS is involved in long-term potentiation (LTP), synaptic plasticity, memory formation, and in spatial reference memory (Bohme et al. 1993; Noda et al. 1997). The significantly lower level of CAV-1 in the heavy raft subpopulation of the hippocampus of aged rats supplemented with CIT is consistent with a lower level of nNOS inhibition. CIT may act by totally or partially restoring NO synthesis and its protective effects on synaptic plasticity (Paul and Ekambaram 2011).

In the brain, CHOL degradation to 24-S-hydroxycholesterol, which is catalyzed by cholesterol 24-hydroxylase, an enzyme present only in neurons (Russell et al. 2009), is counterbalanced by its de novo synthesis by glial cells. With aging, 24-hydroxylase activity decreases, and the resulting increase in CHOL levels is regulated by negative feedback from its de novo synthesis. This reduces the flow of metabolites through the CHOL biosynthesis pathway. One of these metabolites, geranylgeraniol diphosphate, is required for learning in animals and for synaptic plasticity (LTP) *in vitro* (Russell et al. 2009). The decrease in CHOL levels in the rafts of the rat hippocampus associated with CIT supplementation may result from the activation of cholesterol 24-hydroxylase by CIT, restoring the de novo synthesis of CHOL and preserving synaptic plasticity. CIT may also decrease the age-related changes in the transbilayer distribution of CHOL, thereby decreasing the abundance of CHOL in rafts.

Evaluation of the expression and activity of both cholesterol 24-S-hydrolase and nNOS would provide information about the mechanisms involved in this strong effect of CIT on rafts during aging. Memory tests and analyses of LTP and LTD in old rats supplemented with CIT are required to confirm the protective effect of CIT on synaptic plasticity. In preliminary experiments in an ongoing collaborative work, we found that old rats given with CIT supplementation for 3 months had NMDA receptor-dependent synaptic potentials higher than those of the controls, suggesting that CIT may attenuate the synaptic deficits associated with aging. Other preliminary experiments with old wild-type mice fed with CIT for 3 months have indicated an improvement in performance in Y-maze spatial memory tests. On the basis of these preliminary

electrophysiological and cognitive test data, we can speculate that the decrease in CAV-1, APP, CHOL, and C99-APP-Cter levels in the rafts of aged rats given with CIT supplementation are consistent with CIT decreasing the deleterious effects of aging. It would also be interesting to assess the effects of CIT in young animals, to determine whether cognitive performances are affected and the content of rafts modified by CIT, as observed in aged animals.

CHOL plays an important role in the pathogenesis of AD, as CHOL concentrations are high in the brains of AD patients (Heverin et al. 2004; Gyls et al. 2007) and there is a positive correlation between the levels of CHOL and the amount of A β generated (Simons et al. 1998; Refolo et al. 2000; Wahrle et al. 2002; Eehalt et al. 2003; Hudry et al. 2010). It would therefore be interesting to assess the effects of dietary CIT supplementation in an Alzheimer mouse model.

In conclusion, citrulline supplementation in the diet of physiologically aging rats leads to a substantial improvement in the composition of membrane rafts in the hippocampus. These effects are potentially of great significance because rafts are specialized in signal transduction. Moreover, although these rats cannot serve as a model of AD, the CIT-associated improvement in APP status is of particular interest with respect to AD and may provide a basis for a simple and cheap preventive intervention that we are now assessing in a transgenic mouse model of AD development. Further studies are required to determine the probably multiple mechanisms underlying the effects of citrulline.

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