Molecular Nutrition & Food Research
www.mnf-journal.com

A Short-Term Western Diet Impairs Cholesterol Homeostasis and Key Players of Beta Amyloid Metabolism in Brain of Middle Aged Rats

*Maria Stefania Spagnuolo, Valentina Pallottini, Arianna Mazzoli, Lucia Iannotta, Claudia Tonini, Barbara Morone, Marcus Ståhlman, Raffaella Crescenzo, Maria Strazzullo, Susanna Iossa, and Luisa Cigliano**

Scope: Cholesterol homeostasis is crucial for brain functioning. Unhealthy nutrition can influence cerebral physiology, but the effect of western diets on brain cholesterol homeostasis, particularly at middle age, is unknown. Given the link between brain cholesterol alteration and beta amyloid production, the aim is to evaluate whether a diet rich in fat and fructose affects the protein network implicated in cholesterol synthesis and shuttling between glial cells and neurons, as well as crucial markers of beta amyloid metabolism.
Methods and results: Middle aged rats are fed a high fat–high fructose (HFF) or a control diet for 4 weeks. Inflammatory markers and cholesterol levels significantly increase in hippocampus of HFF rats. A higher activation of 3-hydroxy 3-methylglutaryl coenzyme-A reductase, coupled with lower levels of apolipoprotein E, LXR-beta, and lipoproteins receptors is measured in hippocampus from HFF rats. The alteration of critical players of cholesterol homeostasis is associated with increased level of amyloid precursor protein, presenilin 1, and nicastrin, and decreased level of insulin degrading enzyme.
Conclusions: Overall these data show that a western diet is associated with perturbation of cholesterol homeostasis in middle aged rats, mostly in hippocampus. This might trigger molecular events involved in the onset of neurodegenerative diseases.

1. Introduction

Nutrition can represent a powerful environmental factor in modulating aging, particularly during early middle age (that is the mid of the lifespan and corresponds to about 40–50 years in humans), when many physiological functions begin to gradually decline. Western dietary patterns are characterized by high intakes of unhealthy foods and sugar-sweetened beverages, which contain high amounts of sugar such as sucrose and fructose and saturated fat. Notably, epidemiological human studies about high fructose and/or high fat diet clearly show that increased intake of these nutrients affects cognitive impairment and memory.[1–4] At a mechanistic level, it has been reported that rats fed a high fat–high fructose (HFF) diet exhibited impaired spatial learning ability and reduced hippocampal dendritic spine density.[5] In addition, increased memory errors and inflammation were observed in the hippocampus of middle aged rats fed with

Dr. M. S. Spagnuolo
Institute for the Animal Production System
National Research Council
Naples 80147, Italy
Prof. V. Pallottini, Dr. C. Tonini
Department of Science
Biomedical and Technology Science Section
University Roma Tre
Rome 00146, Italy
Dr. A. Mazzoli, Dr. L. Iannotta, Prof. R. Crescenzo, Prof. S. Iossa, Dr. L. Cigliano
Department of Biology
University of Naples Federico II
Naples 80126, Italy
E-mail: luisa.cigliano@unina.it
Dr. B. Morone, Dr. M. Strazzullo
Institute of Genetics and Biophysics "A. Buzzati-Traverso"
National Research Council
Naples 80131, Italy
Dr. M. Ståhlman
Wallenberg Laboratory
Department of Molecular and Clinical Medicine
Sahlgrenska Academy
University of Gothenburg
Gothenburg 413 45, Sweden

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202000541

DOI: 10.1002/mnfr.202000541

diets enriched in saturated fats and cholesterol.[6] Our research group has recently shown that HFF diet, in middle aged rats, induces insulin resistance in the liver,[7] but interestingly, no diet-associated alteration in insulin signaling was observed in hippocampus and frontal cortex,[8] notwithstanding the condition of oxidative stress found in both brain areas.[9] This diet is also associated with a significant increase of both plasma and hepatic levels of cholesterol and triglycerides.[7]

In the central nervous system (CNS), cholesterol plays critical roles by modulating membrane fluidity, cellular function, and synaptic integrity.[10,11] Although the brain represents only 2% of total body weight, it has a concentration of cholesterol tenfold higher than other tissues.[12] As the blood–brain barrier (BBB) does not allow the passage of plasma lipoproteins, almost all cholesterol in the brain derives from de novo synthesis,[13] and its homeostasis requires a complex interplay between critical players involved in its cellular biosynthesis, its trafficking between astrocytes and neurons, and its disposal from the brain.

Cellular cholesterol synthesis is a complex process that includes more than 30 enzymatic steps, with 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) as the rate-limiting enzyme. HMGCR activity depends on short-term regulation, operated by phosphorylation and dephosphorylation,[14,15] as well as on long-term modulation due to transcriptional and degradative events,[16] whose efficiency requires the expression of critical modulators, such as the transcription factor sterol regulatory element protein 2 (SREBP2).[11] During development and myelinogenesis, both astrocytes and neurons produce cholesterol autonomously, while when the brain matures, neurons reduce the expression of many genes implicated in cholesterol synthesis.[17] Therefore, adult neurons get the cholesterol they need from astrocytes,[17] which release cholesterol-rich lipoproteins containing the apolipoprotein E (ApoE), the major lipid transport protein in the brain. These HDL-like particles are then uptaken by neurons via endocytosis mediated by LDL receptor (LDL-R) and LDL receptor–related protein 1 (LRP-1).[18,19] Through this exchange, cholesterol is shuttled from astrocytes to neurons.

Impaired brain cholesterol homeostasis has been linked to the pathogenesis of Alzheimer's disease (AD), and other neurodegenerative diseases,[10,20–22] but most of the previous studies focused on the effects of various dietary treatments on cholesterol metabolism in peripheral organs, while the information regarding dietary effects in the brain is scarce. Therefore, in this study we sought to focus on the consequences of a typical western diet on key players of cholesterol metabolism in hippocampus and frontal cortex of middle aged rats. HFF diet was chosen, since it closely resembles the human dietary habits[23] and it is linked to increased risk of AD.[24] This study has focused on a small window of time (4 weeks of treatment) in order to observe early effects of the HFF diet on the protein network of cholesterol metabolism, in terms of cholesterol synthesis (HMGCR total level and its phosphorylated form) and trafficking, namely, LXR-β, SREBP2, ApoE, cholesterol 24-hydroxylase (CYP46A1), and the receptors LDL-R and LRP-1. In addition, as the alteration of cholesterol homeostasis can be closely connected with inflammation and changes of beta amyloid (Aβ) metabolism, the effect of the HFF diet on crucial markers of inflammation and Aβ metabolism (Amyloid precursor protein, APP; nicastrin, presenilin 1, insulin degrading enzyme, IDE) was investigated.

Layman's summary of our work

1) Unhealthy nutrition can influence brain physiology. Given the link between brain cholesterol alteration and neurodegeneration, this study evaluated whether a typical western diet, rich in fat and fructose, can affect, during middle age, crucial players implicated in brain cholesterol homeostasis and in beta amyloid metabolism.
2) Middle aged rats were fed a high fat–high fructose (HFF) diet or a control diet for 4 weeks. Inflammatory markers and cholesterol levels were found increased in hippocampus of HFF rats, together with the alteration of critical players of cholesterol homeostasis and increased level of amyloid precursor protein, presenilin 1, and nicastrin, suggesting also a perturbation of beta amyloid metabolism.
3) Overall the results show that a western diet is associated with perturbation of cholesterol homeostasis in middle aged rats, mostly in hippocampus, and this alteration might trigger molecular events involved in the onset of neurodegenerative diseases, such as Alzheimer's disease.

2. Results

2.1. Animal Data and Cholesterol Levels

The dietary treatment with western diet elicited a significant increase in body weight in HFF rats (**Figure 1**A). In addition, plasma concentrations of total cholesterol were significantly higher in HFF rats compared to controls (control rats = 90 ± 3 mg dL^{-1}, HFF rats = 130 ± 5 mg dL^{-1}, $p < 0.05$). Further, cholesterol level was found to be significantly higher ($p < 0.05$) in hippocampus of HFF rats (Figure 1B), while no difference between the experimental groups was observed in frontal cortex (control rats = 19.2 ± 1.8 nmol per mg dry tissue; HFF rats = 19.5 ± 2.6 nmol per mg dry tissue).

2.2. High Fat–High Fructose Diet Induces Hippocampal Inflammation

We previously reported that long-term high fat diet-induced hippocampal inflammation,[25] and that a short-term fructose feeding was associated with inflammation in hippocampus of young and adult rats.[26] We therefore wondered whether a 4 week HFF feeding might alter brain inflammatory markers in middle aged rats. To this aim, the amount of TNF-alpha, glial fibrillary acidic protein (GFAP), a marker of astrogliosis, and ionized calcium binding adapter 1 (Iba 1), a marker of microglial activation, was assessed. TNF-alpha level was significantly higher in hippocampus of HFF fed rats ($p < 0.001$, **Figure 2**A), while no changes were detected in frontal cortex (Figure 2B). In agreement with results from TNF-alpha titration, we found a diet-associated increase of GFAP ($p = 0.01$; Figure 2C) and Iba1 level ($p = 0.0005$; Figure 2E)

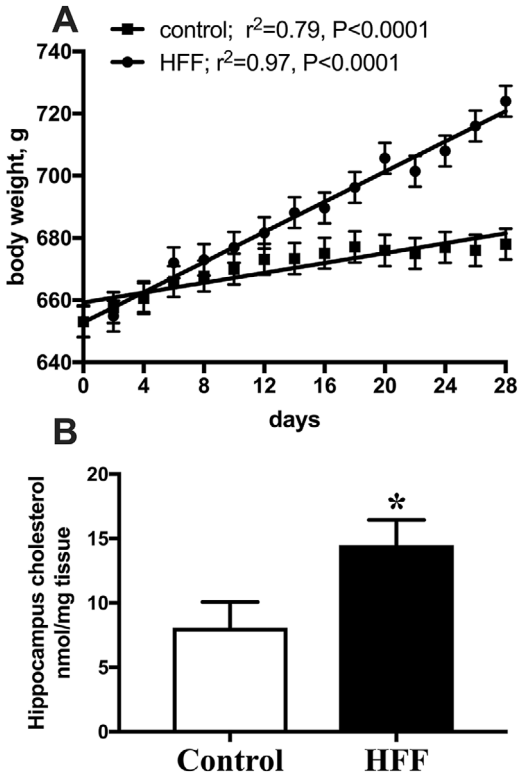

Figure 1. Animal data. A) Middle aged rats (11 months old) were fed a control (full square) or HFF diet (full circle) for 4 weeks. During the treatment, body weight was monitored every alternate day. Data are reported as means ± SEM of eight rats. Linear regression analysis was carried out using GraphPad Prism and difference between slopes was statistically significant ($p < 0.0001$). B) Hippocampal free cholesterol was quantified using straight-phase HPLC coupled to evaporative light-scattering detection. Each sample was analyzed in triplicate, and the average value was calculated. Data are referred to dry tissue weight and reported as means ± SEM of 8 rats. *$p < 0.05$ versus control rats.

in hippocampus of treated rats, while they were not affected in the frontal cortex (Figure 2D,F). Overall, these results show that HFF diet is associated with the increase of inflammation markers in hippocampus and suggest that this brain area is more susceptible than frontal cortex to the nutritional insult induced by diet.

2.3. Effect of HFF Diet on HMGCR Activation State, nSREBP2 Level, and CYP46A1 Amount

To investigate whether the diet-associated changes in brain cholesterol levels might be linked to the rate-limiting enzyme of cholesterol biosynthesis, the amount of HMGCR and its phosphorylation state have been measured. As shown in **Figure 3**A, the phosphorylation state of the enzyme was significantly lower in hippocampus ($p < 0.001$) which means a higher activation of HMCGR[27] in HFF fed rats respect to controls. No change was observed in the cortex (Figure 3B). The amount of total HMGCR was lower in both hippocampus ($p < 0.001$) and cortex ($p < 0.05$) of HFF fed rats.

We further measured nuclear amount of SREBP2, a key player of the feedback system that guarantees brain cholesterol homeostasis. As shown in Figure 3C, the hippocampal level of this protein did not differ between control and treated rats, while it was reduced ($p < 0.05$) in the cortex of HFF rats (Figure 3D).

We further assessed mRNA and protein level of CYP46A1, a brain-specific enzyme responsible for the elimination of cholesterol from the brain through its conversion into 24(S)-hydroxycholesterol (24-OHC).[28] We did not find diet-dependent changes in CYP46A1 protein level in hippocampus (Figure 3E), but a significant decrease was observed in the cortex of HFF rats ($p < 0.05$; Figure 3F). Interestingly, mRNA level was found significantly higher ($p < 0.01$, Figure S1A, Supporting Information) in hippocampus of HFF rats but did not differ between the two groups in frontal cortex (Figure S1B, Supporting Information).

2.4. Effect of HFF Diet on BBB Integrity

Changes in cholesterol homeostasis, as well as high fat or cholesterol enriched diets have been shown to disrupt BBB.[29,30] We therefore investigated whether the HFF diet affects brain concentration of occludin and zonula occludens-1 (ZO-1), two tight junction proteins of the BBB,[31,32] as well as the amount of cerebral IgG, whose leakage into the brain represents a marker of BBB permeability alteration.[33] We found no diet-induced alteration in BBB, as the amounts of occludin, ZO-1 and IgG were not significantly different in control and treated rats, in both hippocampus and cortex (**Figure 4**).

2.5. Effect of HFF Diet on ApoE and LXR-Beta

ApoE plays a crucial role in cholesterol homeostasis in the CNS, as it redistributes cholesterol and phospholipids for membrane repair and remodeling[19] and is also critical in modulation of Aβ homeostasis.[34] We therefore investigated whether HFF diet affects the apolipoprotein level in middle aged rats. ApoE amount was lower in both hippocampus ($p = 0.01$, **Figure 5**A) and frontal cortex ($p < 0.001$, Figure 5B) of HFF rats compared to control rats. In agreement with these results we also observed a reduction of LXR-beta, which directly regulates ApoE expression,[35] in both hippocampus ($p < 0.05$, Figure 5C) and cortex ($p = 0.01$, Figure 5D) of treated rats.

2.6. Effect of HFF Diet on ApoE Receptors Level

Cholesterol uptake into neurons depends on the presence of specific receptors of HDL-like lipoproteins, such as the transmembrane glycoprotein LDL-R.[36] The amount of mature LDL-R (the glycosylated membrane form of about 160 kDa) was found decreased of 2.5-fold ($p = 0.02$), while the level of LDL-R precursor, a form of about 110 kDa, was decreased to about 1.4-fold ($p = 0.045$) in hippocampus of HFF treated rats (**Figure 6**A). Conversely, no changes in the amount of both precursor and mature LDL-R were observed in frontal cortex (Figure 6B). Also, the amount of LRP-1, a critical receptor for both cholesterol uptake and Aβ clearance in glial cells, was measured. A significant decrease of its amount ($p = 0.0007$) was found in hippocampus of HFF fed rats

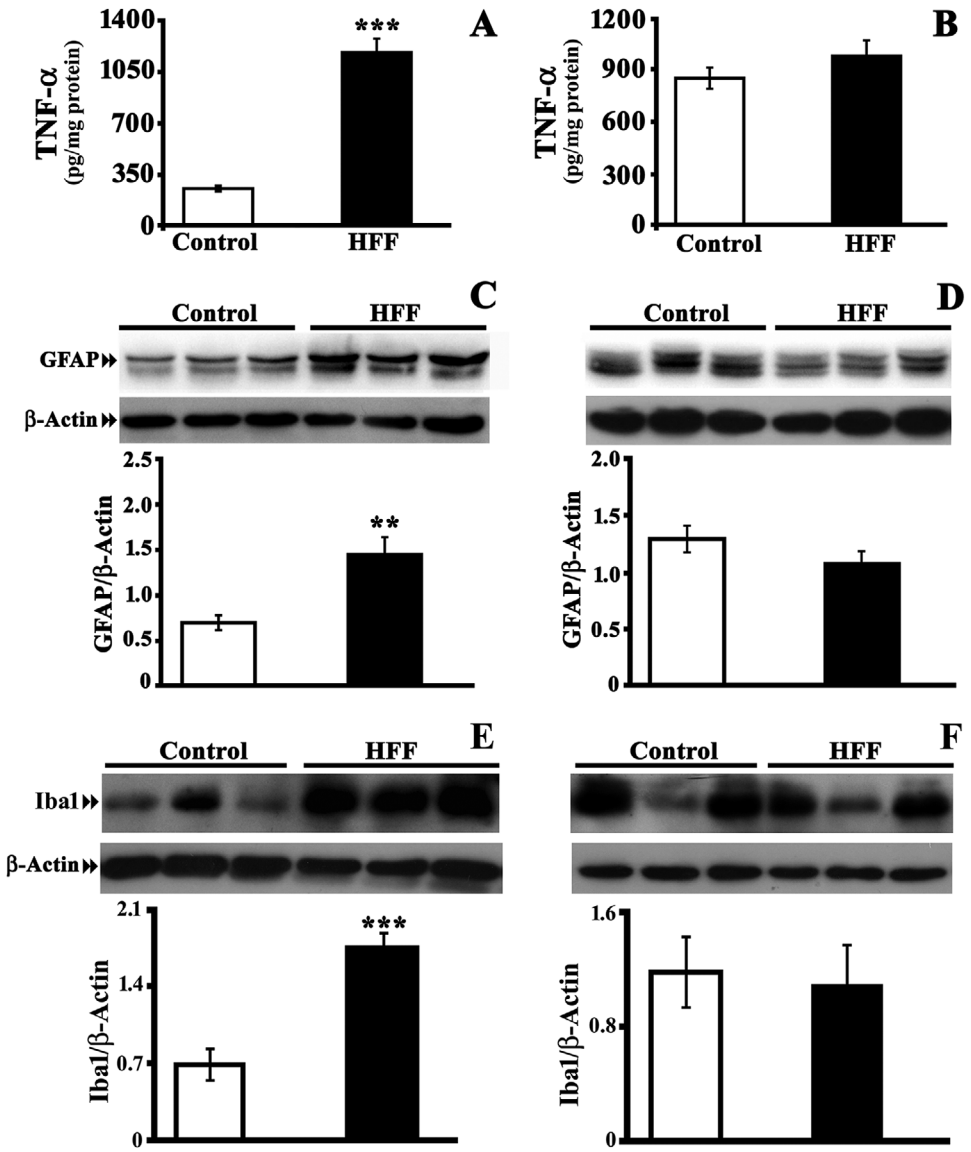

Figure 2. TNF-alpha, GFAP, and Iba1 amount. TNF-alpha was titrated in protein extracts from A) hippocampus or B) frontal cortex of rats fed a control or HFF diet for 4 weeks. Data are reported as means ± SEM of eight rats. ***$p < 0.001$ versus control rats. GFAP level in protein extracts from C) hippocampus or D) frontal cortex of rats fed a control or HFF diet. Iba1 level in protein extracts from E) hippocampus or F) frontal cortex of rats fed a control or HFF diet. Representative western blots are shown. Quantitative densitometry was carried out, and protein amounts were expressed relative to β-actin level. Data are reported as means ± SEM of eight rats. **$p < 0.01$, ***$p < 0.001$ versus control rats.

(Figure 6C), while no change was observed in frontal cortex (Figure 6D).

2.7. Effect of HFF diet on APP, IDE, Presenilin 1, and Nicastrin

APP is produced in large amounts in neurons, and it is also rapidly degraded.[37] When overexpressed, it can lead to a dangerous increase in Aβ, whose accumulation and aggregation represents a hallmark of AD. As it was previously reported that high fat, high sucrose, as well as high fat high cholesterol diets are associated with enhanced expression of this protein,[38,39] we evaluated whether HFF may influence APP amount in middle aged rats. Protein amount was higher in both hippocampus ($p = 0.01$; **Figure 7**A) and frontal cortex ($p = 0.01$; Figure 7B) of treated rats with respect to the controls. Gene expression was also evaluated, and no significant change was detected in the transcript levels in both hippocampus and cortex (Figure S2A,B, Supporting Information).

Further the level of IDE, one of the key enzymes involved in Aβ degradation in the brain,[40] was found lower in hippocampus ($p = 0.004$; Figure 7C) and cortex ($p = 0.01$; Figure 7D) of HFF fed animals.

As γ-secretase is the final protease involved in Aβ production,[41] both mRNA and protein level of presenilin 1

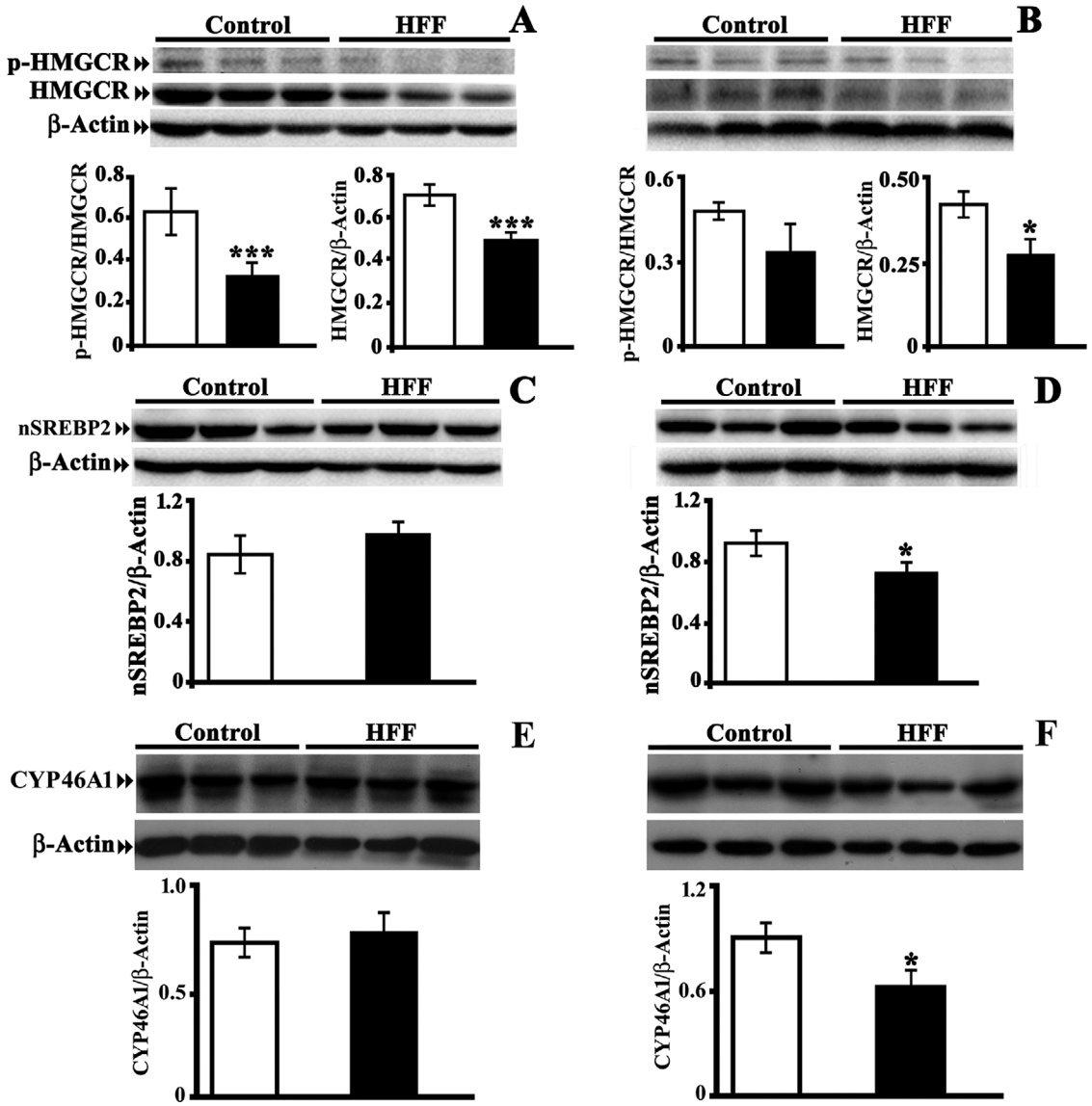

Figure 3. HMGCR, nSREBP2, and CYP46A1 amount. The phosphorylation state of HMGCR (expressed as P-HMGCR/HMGCR ratio) and total amount of HMGCR were assessed in protein extracts from A) hippocampus or B) cortex of rats fed a control or HFF diet. nSREBP2 level in protein extracts from C) hippocampus or D) cortex of rats fed a control or HFF diet. CYP46A1 amount in protein extracts from E) hippocampus or F) cortex of rats fed a control or HFF diet. Representative western blots are shown. Quantitative densitometry was carried out, and concentrations were expressed relative to β-actin level. Data are reported as means ± SEM of eight rats. $^{*}p < 0.05$, $^{***}p < 0.001$ versus control rats.

and nicastrin, two key components of γ-secretase complex, were evaluated. The protein amount of presenilin 1 was significantly higher ($p < 0.05$) in both hippocampus (**Figure 8**A) and cortex (Figure 8B) of HFF rats, while mRNA level was found to be unchanged (Figure S2C,D, supporting Information). Nicastrin protein level was significantly higher in hippocampus ($p < 0.01$, Figure 8C) of treated rats, while did not change in cortex (Figure 8D). Conversely mRNA level did not differ between control and HFF rats in hippocampus (Figure S2E, Supporting Information), while it was lower ($p < 0.01$) in cortex of HFF rats (Figure S2F, Supporting Information).

3. Discussion

Cholesterol plays key structural and physiological roles for proper brain functioning, and disturbances in its homeostasis underlie different neurodegenerative diseases.[10,11] Although clinical and epidemiological studies have emphasized the adverse influence of dietary fat and sugars on the brain, particularly during aging,[42] the underlying molecular mechanisms are still unknown. As cholesterol biosynthesis and metabolism are differently modulated in different brain regions,[43] we evaluated in middle aged rats the effect of a short-term western diet on both hippocampus and frontal cortex, which are critical areas for

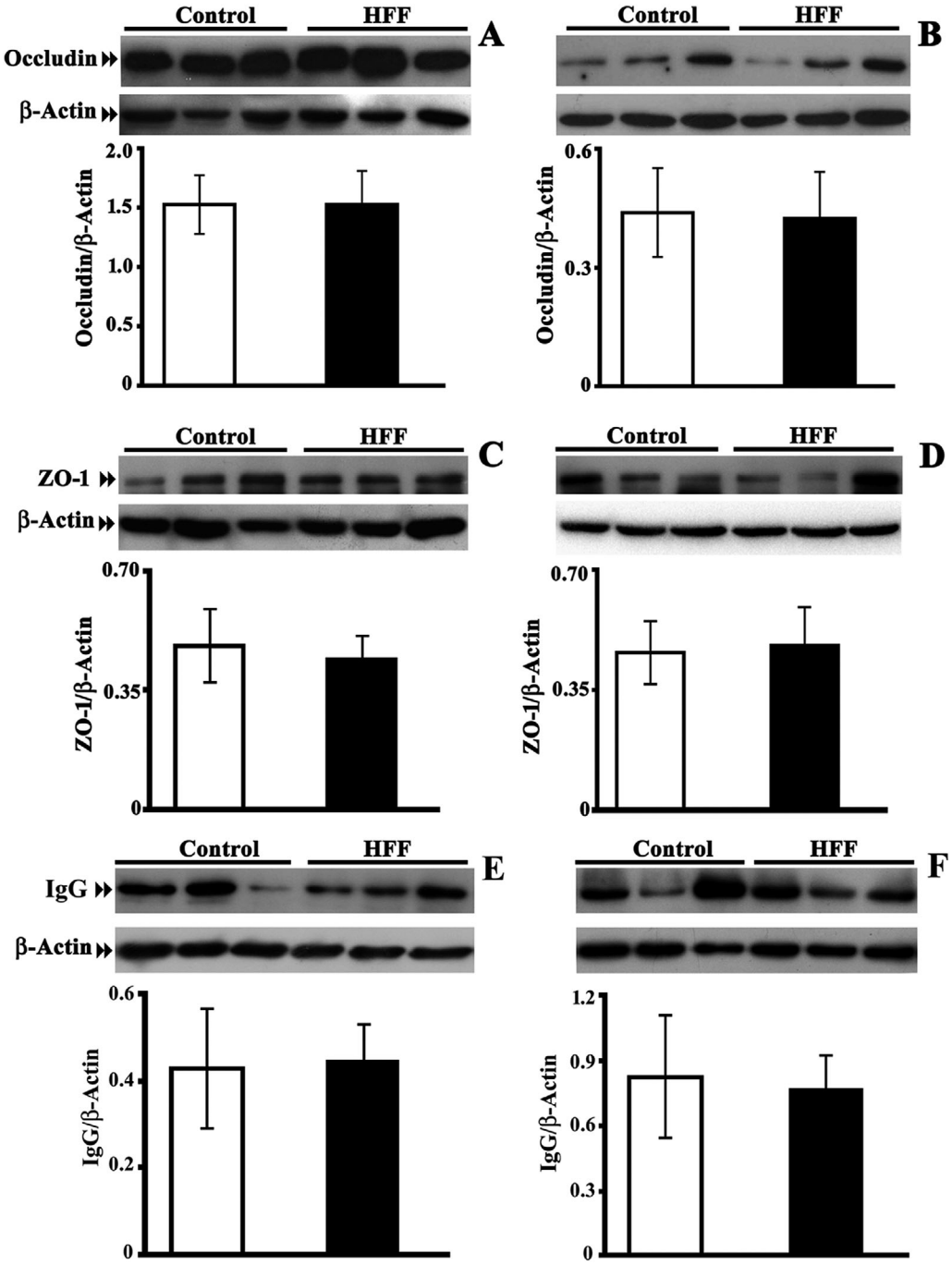

Figure 4. Occludin, ZO-1, and IgG amount. Occludin amount in protein extracts from A) hippocampus or B) frontal cortex of rats fed a control or HFF diet. ZO-1 level in protein extracts from C) hippocampus or D) frontal cortex of rats fed a control or HFF diet. IgG amount in protein extracts from E) hippocampus or F) frontal cortex of rats fed a control or HFF diet. Representative western blots are shown. Quantitative densitometry was carried out, and protein amounts were expressed relative to β-actin level. Data are reported as means ± SEM of eight rats.

learning and memory. Our results show that the HFF feeding caused systemic hypercholesterolemia and an increase in body weight. This suggests that middle aged rats are more sensitive than adults to diet-induced metabolic disturbance, since we previously found that a similar diet did not induce changes in body weight and plasma cholesterol when administered to adult rats.[44,45] We recently reported that this type of dietary treatment induces in middle aged rats a condition of oxidative stress more pronounced in the hippocampus compared to frontal cortex.[9] Interestingly the data presented here show that the concentration of TNF-alpha, as well as GFAP and Iba1, proteins reflecting astrocytes and microglial activation, respectively, was higher in hippocampus but not in frontal cortex of HFF rats, thus suggesting a diet-induced inflammation in this specific area,

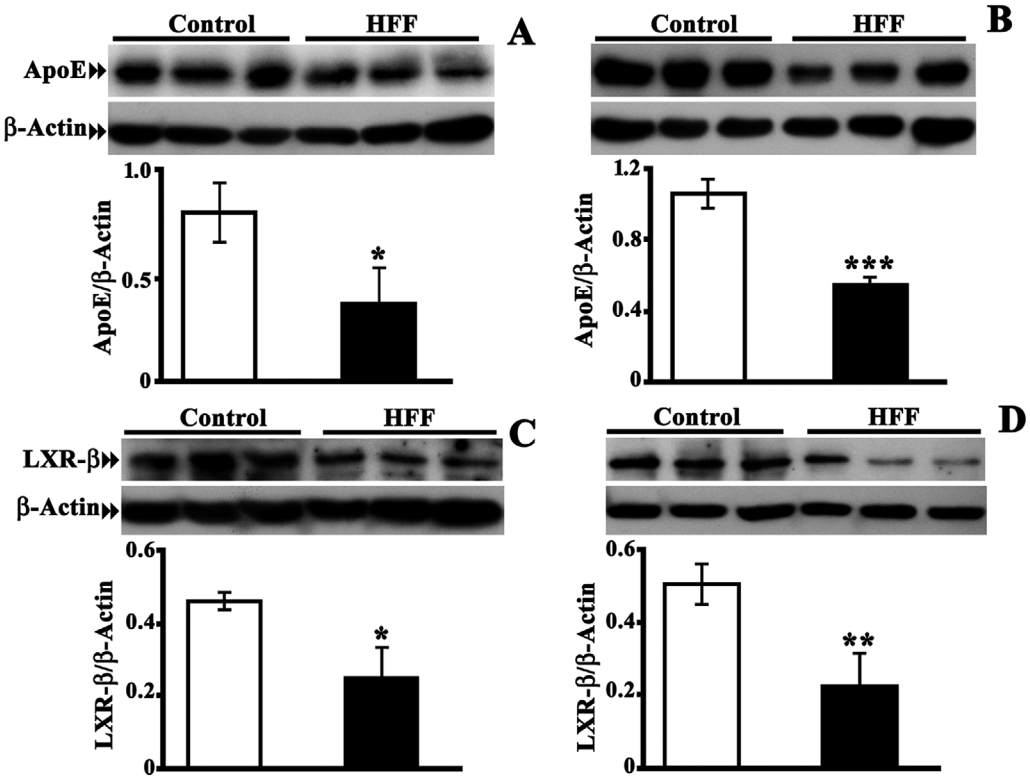

Figure 5. ApoE and LXR-β amount. ApoE amount in protein extracts from A) hippocampus or B) frontal cortex of rats fed a control or HFF diet. LXR-β level in protein extracts from C) hippocampus or D) frontal cortex of rats fed a control or HFF diet. Representative western blots are shown. Quantitative densitometry was carried out, and protein amounts were expressed relative to β-actin level. Data are reported as means ± SEM of eight rats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control rats.

which might contribute to oxidative brain injury. Our results in middle aged rats are in line with previous reports showing increased neuroinflammation in the hippocampus following western diet consumption,[46] and activation of astrocyte and microglia associated with high dietary cholesterol.[47]

Notably, we found that the short-term diet enriched with fat and fructose induces an increase in free cholesterol concentration in hippocampus. These results are in agreement with data obtained in adult rats fed with hypercholesterolemic diet for 4 weeks,[47] and in young rats fed for 3 months with a saturated fats and simple sugars enriched diet,[48] strongly supporting the hypothesis of a cross-talk between central and peripheral metabolism. No changes in cholesterol levels were observed in frontal cortex, although we cannot exclude variations in the levels of cholesterol precursors, as dietary treatments were previously reported to reduce their levels.[15,49] Overall our results suggest that hippocampus, in middle aged rats, could be particularly susceptible to the western diet used. Brain cholesterol levels are not related to those in the periphery since the BBB prevents the entry of cholesterol lipoproteins from the circulation,[12] hence almost all the cholesterol found in the CNS is produced from local biosynthesis.[17] However, since long-term high fat or western diets have been previously shown to alter BBB permeability,[29,50,51] we evaluated the level of occludin, ZO-1 and IgG in our experimental model, and no changes were observed between HFF treated and control rats, in line with data showing that western diet increased BBB permeability only when rats are treated for 90 days.[30] In the absence of diet-induced BBB damage, we hypothesized that the alteration in brain cholesterol level might depend on changes in the enzymes involved in cholesterol homeostasis. Notably in hippocampus, where a significant increase in free cholesterol level was detected, HFF diet was associated with a significant decrease in the HMGCR phosphorylation, namely an increase in the enzyme ability to synthesize cholesterol. Indeed, HMGCR results less phosphorylated and in turn more active in the presence of a redox imbalance,[52,53] a condition found in hippocampus of middle aged rats following the HFF diet.[9] The change in HMGCR activation state seems not to be related to changes in SREBP2. Another factor that could contribute to the increase of hippocampus cholesterol may be the lack of increase in protein content of CYP46A1, a first defense mechanism against cholesterol excess. This could be likely due to the short-term duration of the HFF diet. On the other hand, the diet-associated increase of CYP46A1 mRNA content in hippocampus, concurrently with no change in the protein level, leads to hypothesize either a defect in mRNA translation or an increase in protein degradation. The lack of cholesterol-induced canonical modulations of key players (i.e., CYP46A1) suggests that the HFF diet can alter molecular processes underlying the homeostasis of this important lipid.

A body of evidence report that hypercholesterolemia is associated with an increased brain influx of 27-hydroxycholesterol,[28]

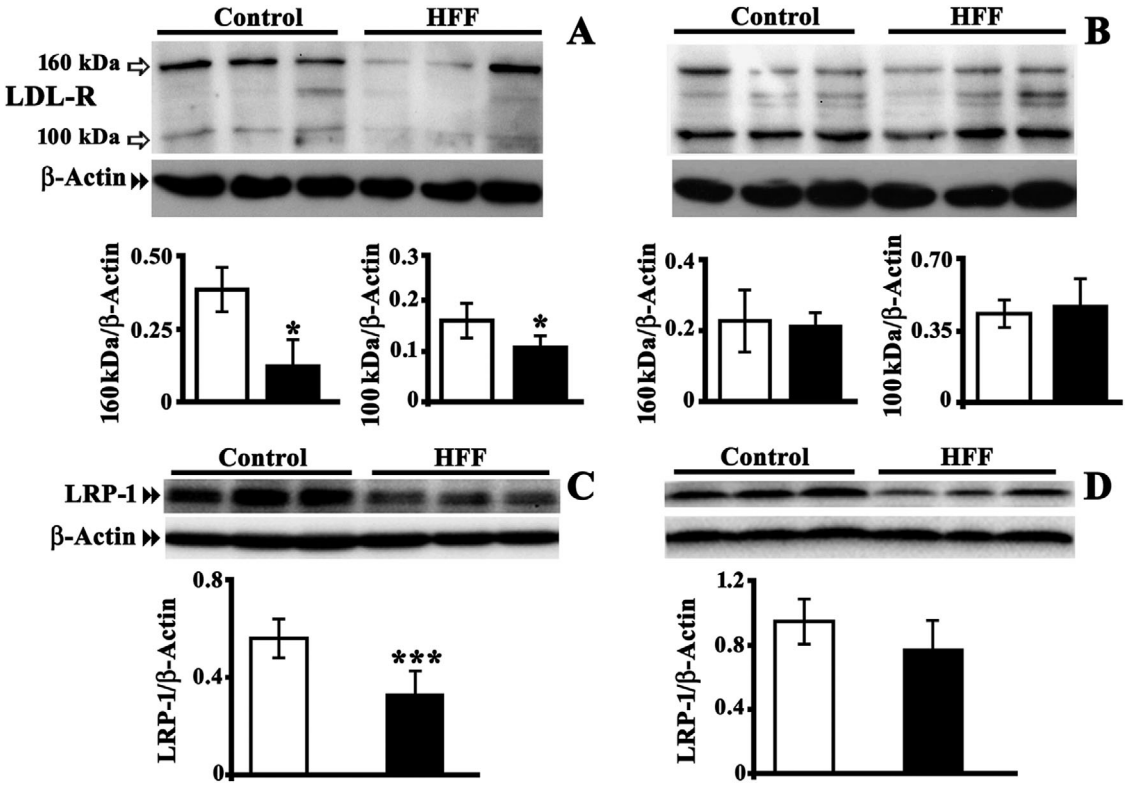

Figure 6. LDL-R and LRP-1 amount. LDL-R (mature form, 160 kDa; precursor, 100 kDa) amount in protein extracts from A) hippocampus or B) frontal cortex of rats fed a control or HFF diet. LRP-1 amount in protein extracts from C) hippocampus or D) frontal cortex of rats fed a control or HFF diet. Representative western blots are shown. Quantitative densitometry was carried out, and protein amounts were expressed relative to β-actin level. Data are reported as means ± SEM of eight rats. $^{*}p < 0.05$, $^{***}p < 0.001$ versus control rats.

which in turn regulates brain cholesterol synthesis. However, based on the data on higher levels of cholesterol in hippocampus, we can speculate that in our condition the regulatory role exerted by 27-hydroxycholesterol is bypassed by the onset of oxidative stress in this area.[9]

In the context of brain cholesterol homeostasis, ApoE plays a key role in regulating the lipoprotein transport system that very efficiently recycles cholesterol.[19] Astrocytes release cholesterol and ApoE that are assembled with phospholipids into lipoprotein particles, which are similar in size to plasma HDL.[17] These lipoproteins interact, via ApoE, with LDL-R and with LRP-1, thus allowing cholesterol internalization in neurons. The finding of lower LXR and ApoE level in brain of HFF rats might suggest an impairment in cholesterol efflux from glial cells, and a possible perturbation of the mechanisms involved in cholesterol trafficking, although more efforts have to be done to better clarify this aspect. Nevertheless, the concomitant decrease of LDL-R and LRP-1 in hippocampus of treated rats might represent a physiological compensative response to limit excessive internalization of cholesterol in neuronal cells, which is known to promote apoptosis.[54] The decrease of ApoE, in both hippocampus and frontal cortex of HFF rats, is of profound relevance if interpreted in light of its role in mediating not only the distribution of lipids, guarantying cholesterol homeostasis, but also in modulating Aβ production, aggregation, and clearance.[18] Interestingly, the ApoE decrease following the HFF treatment is associated with the lowering of both LDL-R and LRP-1 levels, known to participate in Aβ uptake by glial cells for intracellular degradation.[55,56] Hence the impairment in cholesterol homeostasis might also predispose to an alteration of Aβ metabolism. In this context, it is worth mentioning that brain cholesterol homeostasis is directly implied in the regulation of Aβ production, by modulating secretase activities and amyloidogenic processing of APP, as well as its clearance.[57,58] Indeed, we found that APP protein was upregulated in the brain of HFF rats. To further assess whether HFF diet affects Aβ homeostasis as well, we evaluated the level of IDE, an endopeptidase that cleaves insulin and is also implicated in brain Aβ degradation.[40] Our results show that the amount of IDE significantly decreased after 4 weeks of HFF diet treatment. Further we analyzed the amount of presenilin 1 and nicastrin, two components of γ-secretase, an intramembrane aspartyl protease, which participates in APP proteolysis, contributing to Aβ1-42 production.[41] A diet-associated increase of protein level of presenilin 1, the central catalytic moiety of the complex,[59] was observed in both hippocampus and cortex. Conversely, protein level of nicastrin, which promotes the maturation and proper trafficking of other components of γ-secretase, was found increased only in hippocampus of treated rats. These results led us to hypothesize that the here used western diet, as determining an alteration of brain cholesterol, might also interfere with Aβ metabolism, in line with previous results obtained with different dietary treatments,[47,60]

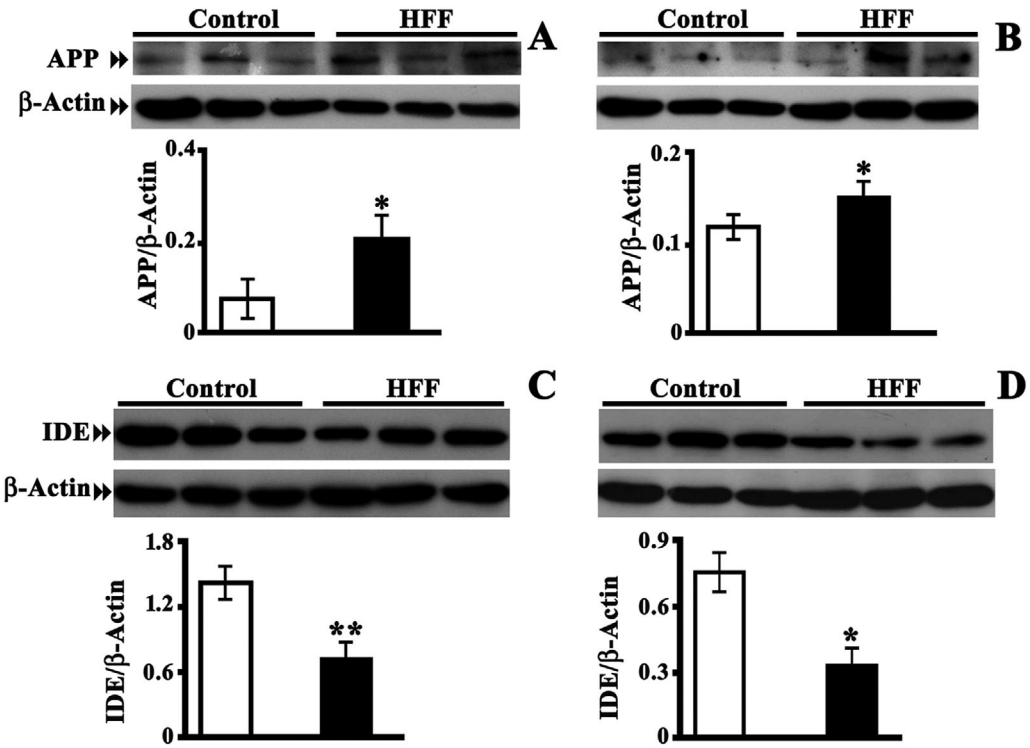

Figure 7. APP and IDE expression. APP level in protein extracts from A) hippocampus or B) cortex of rats fed a control or HFF diet. IDE amount in protein extracts from C) hippocampus or D) cortex of rats fed a control or HFF diet. Representative western blots are shown. Quantitative densitometry was carried out, and protein amounts were expressed relative to β-actin level. Data are reported as means ± SEM of eight rats. *$p < 0.05$, **$p < 0.01$ versus control rats.

particularly by affecting APP and presenilin 1 protein level. In conclusion, our data highlights for the first time a perturbation of cholesterol homeostasis in middle aged rats already after a short-term western diet, particularly in hippocampus (**Figure 9**). The increase in cholesterol concentration, associated with the alteration in the levels of crucial players of both cholesterol and Aβ homeostasis, outlines a picture which is a prelude to early molecular events that could predispose or favor the onset and the progression of neurodegenerative diseases.

4. Experimental Section

Materials: BSA fraction V, salts, and buffers were purchased from Sigma-Aldrich (St. Louis, MO, USA). The dye reagent for protein titration, and the PVDF membrane were from Bio-Rad (Bio-Rad, Hercules, CA).

Animals and Treatments: The rats (Male Sprague-Dawley rats; 11 months old, middle aged) were purchased from Charles River (Calco, Como, Italy), and were caged as previously described.[9] Treatment, housing, and euthanasia of animals were carried out in accordance with national and international policies (EU Directive 2010/63/EU). All experimental procedures were approved by "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University of Naples Federico II and authorized by the Italian Ministry of Health (n. 260/2015-PR).

Rats were allowed to adapt to animal house for 14 days before beginning the treatment. During this period all the rats were fed a control diet. Animals were then randomly assigned to the control group ($n = 8$; fed a control diet for 4 weeks) or to the treated group ($n = 8$; fed a HFF diet). Diet compositions are shown in Table S1, Supporting Information. Body weight, food, and water intake were monitored daily. At the end of the experimental period, rats were euthanized by decapitation, plasma samples were collected and stored at −20 °C, and brains were removed to dissect hippocampus and frontal cortex, as previously published.[9] Samples were then snap frozen in liquid nitrogen and stored at −80 °C for subsequent analyses.

Cholesterol Measurement: A commercial kit (SGM Italia, Rome, Italy) adopting colorimetric enzymatic method was used to measure the concentrations of total cholesterol in plasma.

Brain lipids were extracted using the BUME method.[61] The lipid extract was evaporated under a stream of nitrogen and reconstituted in chloroform: methanol (2:1, v/v). The free cholesterol was quantified using straight-phase HPLC coupled to evaporative light-scattering detection according to previous publication.[61,62] Data were given for dry brain weight.

Protein Extraction: Proteins were extracted from both hippocampus and frontal cortex by homogenizing aliquots (about 100 mg) of frozen tissues in six volumes w/v of cold RIPA buffer, as previously published.[63] The content of selected markers of cholesterol homeostasis, inflammation, Aβ metabolism, and BBB integrity was then evaluated by western blotting.

Analysis of TNF-Alpha Concentration: Slices of hippocampus or frontal cortex were homogenized as previously described.[25] TNF-alpha concentration was evaluated by sandwich ELISA, using the TNF-alpha Duo-Set kit (R&D, DBA Italia), diluting each homogenate 1:20. Data were reported as pg of TNF-alpha per mg of proteins.

Western Blotting: Aliquots (30 μg of protein) of hippocampus or frontal cortex homogenates were fractionated by electrophoresis on 12.5% (to quantify Iba 1; presenilin 1; CYP46A1) or 10% (GFAP; occludin, IDE; nicastrin; LXR-β; ApoE) or 8% (LDL-R; APP; ZO-1), or 7% (HMGCR, P-HMGCR, LRP-1, nSREBP2) polyacrylamide gel. Proteins were blotted onto PVDF membrane,[64] and the membranes were then incubated with primary antibodies, followed by the appropriate peroxidase-conjugated secondary antibody, as described in Supporting Information.

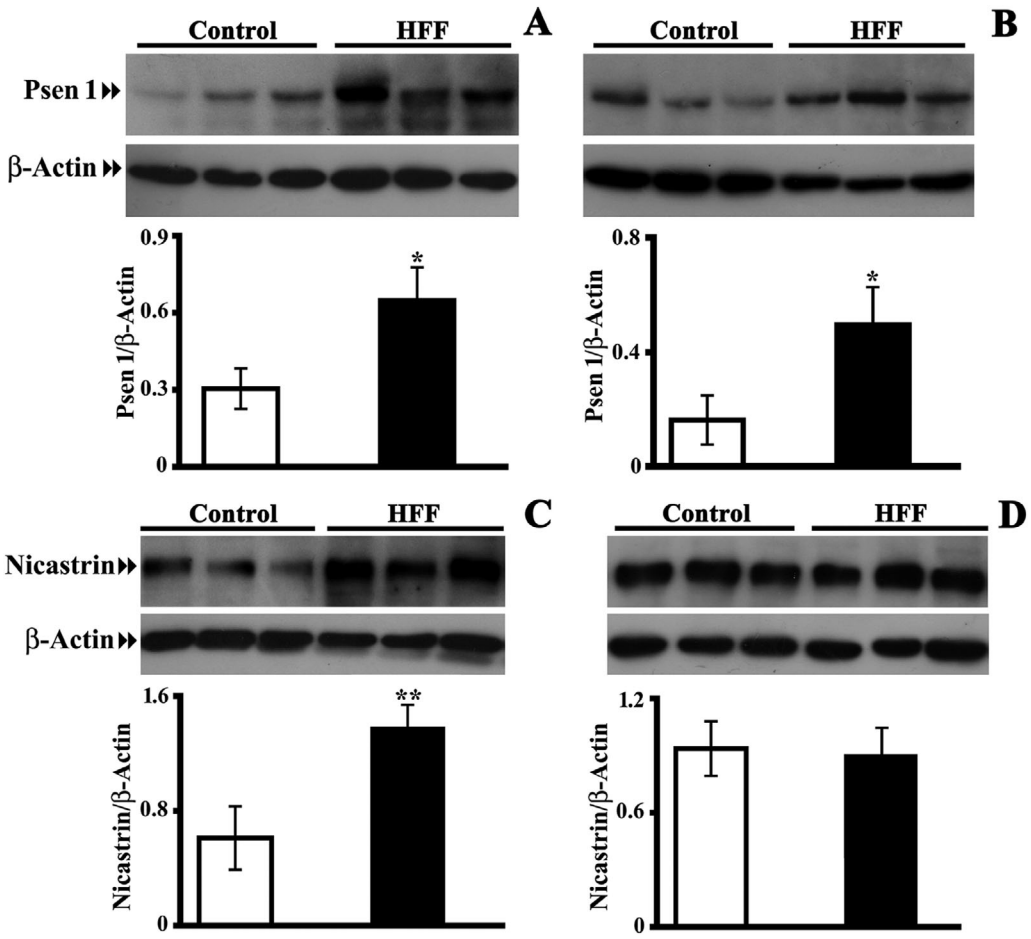

Figure 8. Presenilin 1 and nicastrin amount. Presenilin 1 (Psen 1) amount in protein extracts from A) hippocampus or B) cortex of rats fed a control or HFF diet. Nicastrin amount in protein extracts from C) hippocampus or D) cortex of rats fed a control or HFF diet. Representative western blots are shown. Quantitative densitometry was carried out, and protein amounts were expressed relative to β-actin level. Data are reported as means ± SEM of eight rats. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control rats.

After detection of each marker, for loading control, the membranes were stripped,[54] and then incubated with mouse anti-β-actin IgG (diluted 1000 in 0.25% non-fat milk) followed by goat anti-mouse-horseradish peroxidase-conjugated IgG (1:300 000 in 0.25% non-fat milk; 1 h, 37 °C). Detection was carried out using the Excellent Chemiluminescent Detection Kit (ElabScience, Microtech, Naples, Italy), and quantitative densitometry of the bands was performed by analyzing chemidoc or digital images of X-ray films exposed to immunostained membranes. Signal quantification was carried out by Un-Scan-It gel software (Silk Scientific, UT, USA).

Total RNA Extraction and Quantitative Real-Time PCR: Methods for total RNA extraction and quantitative real-time PCR are detailed in Supporting Information.

Statistical Analysis: Data were expressed as mean values ± SEM. The program GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used to perform two-tailed, unpaired, Student's *t* test. $p < 0.05$ was considered significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This study was funded by FIRB-Futuro in Ricerca-Italian Ministry of Education, University and Research (MIUR) (awarded to L.C., Department of Biology, University of Naples Federico II) and by PRIN-Italian Ministry of Education, University and Research (MIUR) (awarded to V.P., Department of Science Roma Tre). The authors thank Dr. Emilia de Santis for skillful management of animal house.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.S.S. and L.C. conceived and designed the research, performed ELISA and WB experiments, analyzed, and interpreted results, wrote the manuscript. L.I., S.I., R.C., and A.M. contributed to the planning and execution of experiments with rats, analyzed data, and critically revised the manuscript. M.S. analyzed cholesterol data and critically revised the manuscript. C.T. and V.P. contributed to the planning and execution of WB experiments, analyzed data, and critically revised the manuscript. B.M. and M.Str. contributed to the planning and execution of real-time PCR experiments, analyzed results, and critically revised the manuscript.

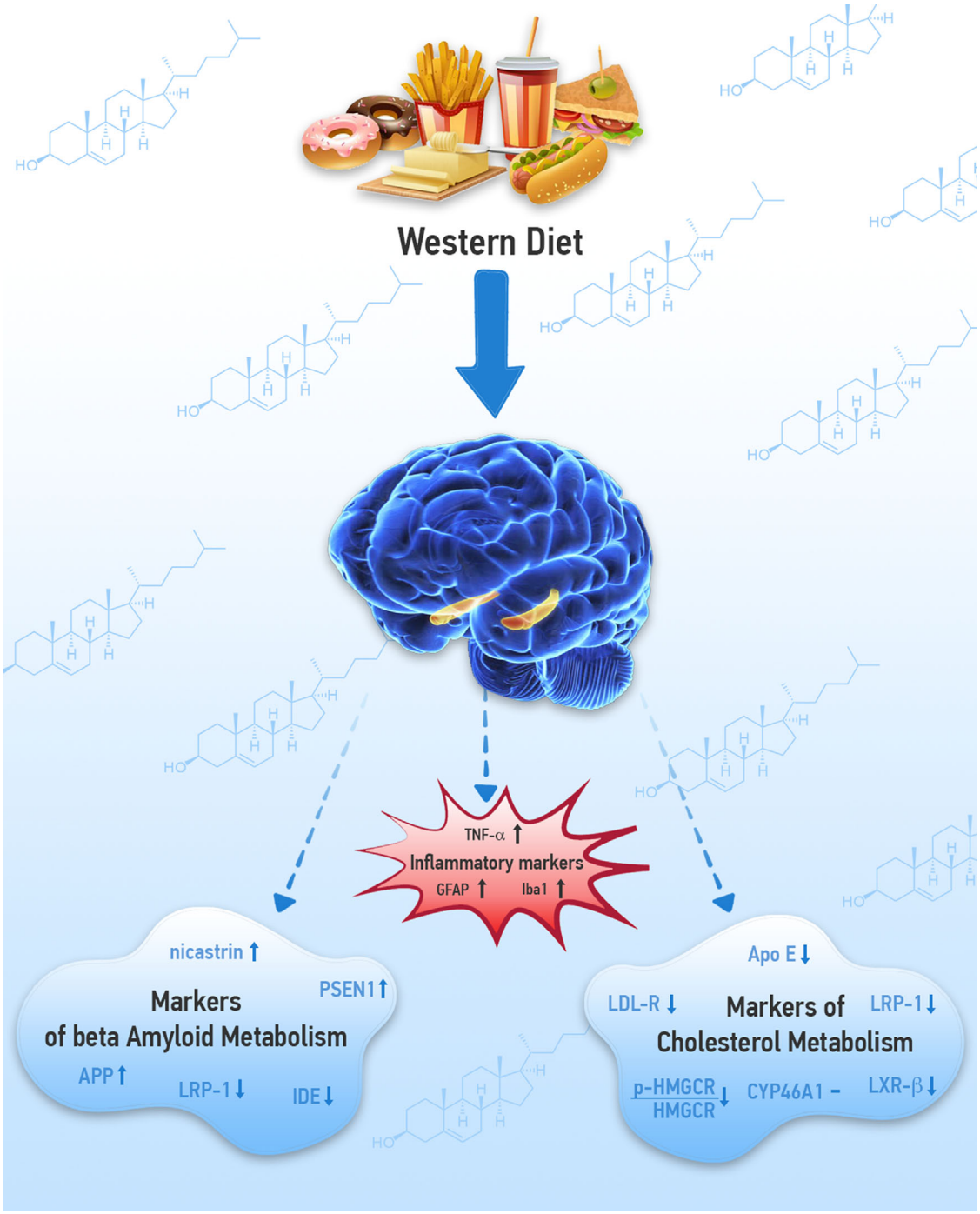

Figure 9. HFF diet modulates cholesterol homeostasis in brain of middle aged rats. A short-term western diet is associated with early alterations of brain cholesterol homeostasis, of markers of Aβ metabolism, and with the increase of molecular markers of inflammation, in middle aged rats.

Keywords

apolipoprotein E, cholesterol, high fat–high fructose diet, hippocampus, middle age

Received: June 5, 2020
Published online:

[1] M. R. Yeomans, *Proc. Nutr. Soc.* **2017**, *76*, 455.
[2] X. Ye, X. Gao, T. Scott, K. L. Tucker, *Br. J. Nutr.* **2011**, *106*, 1423.
[3] C. P. Chong, S. Shahar, H. Haron, N. C. Din, *Clin. Interventions Aging.* **2019**, *14*, 1331.

[4] G. Y. Cao, M. Li, L. Han, F. Tayie, S. S. Yao, Z. Huang, P. Ai, Y. Z. Liu, Y. H. Hu, B. Xu, *J. Prev. Alzheimers Dis.* **2019**, *6*, 204.
[5] A. M. Stranahan, E. D. Norman, K. Lee, R. G. Cutler, R. S. Telljohann, J. M. Egan, M. P. Mattson, *Hippocampus* **2008**, *18*, 1085.
[6] A. C. Granholm, H. A. Bimonte-Nelson, A. B. Moore, M. E. Nelson, L. R. Freeman, K. Sambamurti, *J. Alzheimers Dis.* **2008**, *14*, 133.
[7] A. Mazzoli, R. Crescenzo, L. Cigliano, M. S. Spagnuolo, R. Cancelliere, C. Gatto, S. Iossa, *Nutrients* **2019**, *11*, 2670.
[8] A. Mazzoli, M. S. Spagnuolo, C. Gatto, M. Nazzaro, R. Cancelliere, R. Crescenzo, S. Iossa, L. Cigliano, *Int. J. Mol. Sci.* **2020**, *21*, 786.
[9] R. Crescenzo, M. S. Spagnuolo, R. Cancelliere, L. Iannotta, A. Mazzoli, C. Gatto, S. Iossa, L. Cigliano, *Mol. Neurobiol.* **2019**, *56*, 7651.
[10] V. Cartocci, M. Servadio, V. Trezza, V. Pallottini, *J. Cell. Physiol.* **2017**, *232*, 281.
[11] F. Arenas, C. Garcia-Ruiz, J. C. Fernandez-Checa, *Front. Mol. Neurosci.* **2017**, *10*, 382.
[12] J. M. Dietschy, S. D. Turley, *J. Lipid Res.* **2004**, *45*, 1375.
[13] J. Zhang, Q. Liu, *Protein Cell* **2015**, *6*, 254.
[14] P. J. Espenshade, A. L. Hughes, *Annu. Rev. Genet.* **2007**, *41*, 401.
[15] K. Smiljanic, T. Vanmierlo, A. M. Djordjevic, M. Perovic, S. Ivkovic, D. Lütjohann, S. Kanazir, *AGE* **2014**, *36*, 9654.
[16] C. Martini, V. Pallottini, *Genes Nutr.* **2007**, *2*, 181.
[17] F. W. Pfrieger, N. Ungerer, *Prog. Lipid Res.* **2011**, *50*, 357.
[18] Y. Huang, R. W. Mahley, *Neurobiol. Dis.* **2014**, *72*, 3.
[19] R. W. Mahley, *Arterioscler., Thromb., Vasc. Biol.* **2016**, *36*, 1305.
[20] M. Segatto, L. Leboffe, L. Trapani, V. Pallottini, *Curr. Med. Chem.* **2014**, *21*, 2788.
[21] M. Segatto, C. Tonini, F. Pfrieger, V. Trezza, V. Pallottini, *Int. J. Mol. Sci.* **2019**, *20*, 3317.
[22] J. E. Vance, *Dis. Models Mech.* **2012**, *5*, 746.
[23] R. C. Bortolin, A. R. Vargas, J. Gasparotto, P. R. Chaves, C. E. Schnorr, K. B. Martinello, A. K. Silveira, T. K. Rabelo, D. P. Gelain, J. C. F. Moreira, *Int. J. Obes.* **2018**, *42*, 525.
[24] P. M. Sullivan, *Neurobiol. Dis.* **2020**, *138*, 104790.
[25] M. S. Spagnuolo, M. P. Mollica, B. Maresca, G. Cavaliere, C. Cefaliello, G. Trinchese, R. Scudiero, M. Crispino, L. Cigliano, *Front. Cell. Neurosci.* **2015**, *15*, 9.
[26] L. Cigliano, M. S. Spagnuolo, R. Crescenzo, R. Cancelliere, L. Iannotta, A. Mazzoli, G. Liverini, S. Iossa, *Mol. Neurobiol.* **2018**, *55*, 2869.
[27] L. Trapani, M. Segatto, S. Incerpi, V. Pallottini, *Curr. Mol. Med.* **2011**, *11*, 790.
[28] P. Gamba, E. Staurenghi, G. Testa, S. Giannelli, B. Sottero, G. Leonarduzzi, *Front. Neurosci.* **2019**, *13*, 556.
[29] H. C. Chang, Y. T. Tai, Y. G. Cherng, J. W. Lin, S. H. Liu, T. L. Chen, R. M. Chen, *J. Agric. Food Chem.* **2014**, *62*, 3466.
[30] S. L. Hargrave, T. L. Davidson, W. Zheng, K. P. Kinzig, *Behav. Neurosci.* **2016**, *130*, 123.
[31] H. Wolburg, A. Lippoldt, *Vasc. Pharmacol.* **2002**, *38*, 323.
[32] Y. L. Lin, H. C. Chang, T. L. Chen, J. H. Chang, W. T. Chiu, J. W. Lin, R. M. Chen, *J. Nutr.* **2010**, *140*, 2187.
[33] R. D. Bell, E. A. Winkler, A. P. Sagare, I. Singh, B. LaRue, R. Deane, B. V. Zlokovic, *Neuron* **2010**, *68*, 409.
[34] M. S. Spagnuolo, B. Maresca, V. La Marca, A. Carrizzo, C. Veronesi, C. Cupidi, T. Piccoli, R. G. Maletta, A. C. Bruni, P. Abrescia, L. Cigliano, *ACS Chem. Neurosci.* **2014**, *5*, 837.
[35] R. Courtney, G. E. Landreth, *Trends Endocrinol. Metab.* **2016**, *27*, 404.
[36] J. L. Goldstein, M. S. Brown, *J. Lipid Res.* **1984**, *25*, 1450.
[37] J. Lee, C. Retamal, L. Cuitiño, A. Caruano-Yzermans, J. E. Shin, P. van Kerkhof, M. P. Marzolo, G. Bu, *J. Biol. Chem.* **2008**, *283*, 11501.
[38] K. L. Puig, A. M. Floden, R. Adhikari, M. Y. Golovko, C. K. Combs, *PLoS One* **2012**, *7*, e30378.
[39] Y. Selvi, H. S. Gergerlioglu, N. Akbaba, M. Oz, A. Kandeger, E. A. Demir, F. H. Yerlikaya, K. E. Nurullahoglu-Atalik, *Acta Neuropsychiatr.* **2017**, *29*, 291.
[40] W. Farris S. Mansourian, Y. Chang, L. Lindsley, E. A. Eckman, M. P. Frosch, C. B. Eckman, R. E. Tanzi, D. J. Selkoe, S. Guenette, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4162.
[41] M. R Hass, C. Sato, R. Kopan, G. Zhao, *Semin. Cell Dev. Biol.* **2009**, *20*, 201.
[42] R. Loera-Valencia, J. Goikolea, C. Parrado-Fernandez, P. Merino-Serrais, S. Maioli, *J. Steroid Biochem. Mol. Biol.* **2019**, *190*, 104.
[43] M. Segatto, L. Trapani, C. Lecis, V. Pallottini, *Acta Physiol.* **2012**, *206*, 62.
[44] R. Crescenzo, F. Bianco, P. Coppola, A. Mazzoli, M. Tussellino, R. Carotenuto, G. Liverini, S. Iossa, *Exp. Physiol.* **2014**, *99*, 1203.
[45] R. Crescenzo, F. Bianco, P. Coppola, A. Mazzoli, L. Cigliano, G. Liverini, S. Iossa, *Eur. J. Nutr.* **2015**, *54*, 183.
[46] P. J. Pistell, C. D. Morrison, S. Gupta, A. G. Knight, J. N. Keller, D. K. Ingram, A. J. Bruce-Keller, *J. Neuroimmunol.* **2010**, *219*, 25.
[47] W. Yang, H. Shi, J. Zhang, Z. Shen, G. Zhou, M. Hu, *Lipids Health Dis.* **2017**, *16*, 26.
[48] A. M. Stranahan, R. G. Cutler, C. Button, R. Telljohann, M. P. Mattson, *J. Neurochem.* **2011**, *118*, 611.
[49] K. Smiljanic, S. Todorovic, A. M. Djordjevic, T. Vanmierlo, D. Lütjohann, S. Ivkovic, S. Kanazir, *Biogerontology* **2018**, *19*, 121.
[50] S. E. Kanoski, Y. Zhang, W. Zheng, T. L. Davidson, *J. Alzheimers Dis.* **2010**, *21*, 207.
[51] T. L. Davidson, A. Monnot, A. U. Neal, A. A. Martin, J. J. Horton, W. Zheng, *Physiol. Behav.* **2012**, *107*, 26.
[52] V. Pallottini, C. Martini, G. Cavallini, E. Bergamini, K. J. Mustard, D. G. Hardie, A. Trentalance, *Mech. Ageing Dev.* **2007**, *128*, 688.
[53] L. Trapani, M. Segatto, V. Simeoni, V. Balducci, A. Dhawan, V. S. Parmar, A. K. Prasad, L. Saso, S. Incerpi, V. Pallottini, *Biochimie* **2011**, *93*, 1165.
[54] M. S. Spagnuolo, A. Donizetti, L. Iannotta, V. Aliperti, C. Cupidi, A. C. Bruni, L. Cigliano, *J. Cell. Physiol.* **2018**, *233*, 6925.
[55] J. Herz, D. K. Strickland, *J. Clin. Invest.* **2001**, *108*, 779.
[56] J. M. Basak, P. B. Verghese, H. Yoon, J. Kim, D. M. Holtzman, *J. Biol. Chem.* **2012**, *287*, 13959.
[57] G. Di Paolo, T. W. Kim, *Nat. Rev. Neurosci.* **2011**, *12*, 284.
[58] M. G. Martin, F. Pfrieger, C. G. Dotti, *EMBO Rep.* **2014**, *15*, 1036.
[59] M. Smolarkiewicz, T. Skrzypczak, P. Wojtaszek, *Protoplasma* **2013**, *250*, 997.
[60] D. Luo, X. Hou, L. Hou, M. Wang, S. Xu, C. Dong, X. Liu, *Eur. J. Pharmacol.* **2011**, *664*, 14.
[61] L. Lofgren, G. B. Forsberg, M. Stahlman, *Sci. Rep.* **2016**, *6*, 27688.
[62] R. Homan, M. K. Anderson, *J. Chromatogr. B Biomed. Sci. Appl.* **1998**, *708*, 21.
[63] M. S. Spagnuolo, B. Maresca, M. P. Mollica, G. Cavaliere, C. Cefaliello, G. Trinchese, M. G. Esposito, R. Scudiero, M. Crispino, P. Abrescia, L. Cigliano, *Front. Cell. Neurosci.* **2014**, *8*, 212.
[64] M. S. Spagnuolo, P. Bergamo, R. Crescenzo, L. Iannotta, L. Treppiccione, S. Iossa, L. Cigliano, *Nutr. Neurosci.* **2018**, *24*, 1.