Impaired adult hippocampal neurogenesis in a mouse model of familial hypercholesterolemia: A role for the LDL receptor and cholesterol metabolism in adult neural precursor cells

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ABSTRACT

Objective: In familial hypercholesterolemia (FH), mutations in the low-density lipoprotein (LDL) receptor (LDLR) gene result in increased plasma LDL cholesterol. Clinical and preclinical studies have revealed an association between FH and hippocampus-related memory and mood impairment. We here asked whether hippocampal pathology in FH might be a consequence of compromised adult hippocampal neurogenesis.

Methods: We evaluated hippocampus-dependent behavior and neurogenesis in adult C57BL/6JRj and LDLr−/− mice. We investigated the effects of elevated cholesterol and the function of LDLr in neural precursor cells (NPC) isolated from adult C57BL/6JRj mice in vitro.

Results: Behavioral tests revealed that adult LDLr−/− mice showed reduced performance in a dentate gyrus (DG)-dependent metric change task. This phenotype was accompanied by a reduction in cell proliferation and adult neurogenesis in the DG of LDLr−/− mice, suggesting a potential direct impact of LDLr mutation on NPC. Exposure of NPC to LDL as well as LDLr gene knockdown reduced proliferation and disrupted transcriptional activity of genes involved in endogenous cholesterol synthesis and metabolism. The LDL treatment also induced an increase in intracellular lipid storage. Functional analysis of differentially expressed genes revealed parallel modulation of distinct regulatory networks upon LDL treatment and LDLr knockdown.

Conclusions: Together, these results suggest that high LDL levels and a loss of LDLr function, which are characteristic to individuals with FH, might contribute to a disease-related impairment in adult hippocampal neurogenesis and, consequently, cognitive functions.

Keywords Familial hypercholesterolemia; Adult neurogenesis; LDLr; Dentate gyrus; Neural precursor cell; Cholesterol

1. INTRODUCTION

Hypercholesterolemia is an important risk factor for the development of neurodegenerative diseases [1,2]. Particularly, altered cholesterol metabolism is considered a critical factor in the pathogenesis of Alzheimer’s disease [3,4]. Compared to individuals with the sporadic form, those with familial hypercholesterolemia (FH) present a higher incidence of mild cognitive impairment in later life [5]. Ariza and co-workers [6] reported that even young FH subjects already showed neuropsychological deficits.

FH is caused by inherited genetic abnormalities, predominantly in the low-density lipoprotein (LDL) receptor (LDLR) gene, resulting in an ineffective metabolism of LDL particles. Defective uptake of LDL by the liver leads to elevated plasma LDL cholesterol from birth and development of premature atherosclerosis and cardiovascular disease [7].

Despite the increasing number of clinical and preclinical evidence of FH association with cognitive impairment, it remains unclear whether the chronic exposure to high circulating cholesterol levels or the dysfunction of the LDLr as such contribute to the altered central nervous system (CNS) function. Cholesterol-carrying lipoproteins, such as LDL, cannot readily cross the blood—brain barrier (BBB). Instead, the majority of cholesterol in the brain is synthesized de novo within the brain tissue [8]. However, recent data suggest that hypercholesterolemia might weaken BBB function, disrupting cholesterol balance between the brain and the periphery, and this could favor pathological processes in the CNS [9–11]. The decrease in the LDLr activity in FH individuals might also bear consequences on neuronal development and function. Neurons in the adult brain take up ApoE-cholesterol complexes, produced and released by astrocytes, via endocytosis through the LRP1 and LDLr receptors [12]. Besides the cholesterol
uptake, however, the physiological and pathological roles of LDL in the brain remain unclear. Although LDLr<sup>-/-</sup> mice have normal brain morphology, they exhibit impairment in learning and memory and a depressive-like phenotype [13—19]. Together, these findings suggest that FH is accompanied by hippocampal dysfunction that is reflected at the onset of cognitive deficit.

Adult hippocampal neurogenesis, the process that leads to the addition of new granule neurons in the dentate gyrus (DG), is believed to contribute to hippocampal functions such as cognition and emotional behavior [20,21]. The new neurons originate from a pool of stem cells, located in the subgranular zone of the DG, that proliferate and give rise to precursor cells, which can potentially mature into functional glia or neurons past a number of defined stages [22]. There is a rising interest in how lipid metabolism can influence adult neural progenitors. Recent studies manipulated key components of fatty acids and cholesterol metabolism and found that, besides their requirement for new membrane production upon cell proliferation and differentiation, their availability can influence cell energetic states; moreover, they might act as signaling entities in adult neural precursor cells (NPC) [23]. Mulder and colleagues [13] have demonstrated that 14 months old LDLr<sup>-/-</sup> mice had reduced numbers of proliferating cells and synaptic connections in the DG compared to wild type controls. However, it remained unclear whether hippocampal cell proliferation and neurogenesis would be affected in younger animals, which already show cognitive impairment.

To address this question, we designed a set of experiments to explore if adult hippocampal neurogenesis might be involved in cognitive dysfunction associated with FH. We also investigated whether exposure to LDL and LDLr knock-down have a direct influence on cell proliferation and differentiation of hippocampal precursor cells <em>in vitro</em>.

2. MATERIALS AND METHODS

2.1. In vivo study

2.1.1. Animals

The progenitors of low-density lipoprotein receptor knockout mice (LDLr<sup>-/-</sup>; B6.129S7- Ldlr<sup>tm1Her/J</sup>) were obtained from Universidade Estadual de Campinas (UNICAMP, Sao Paulo, Brazil), originally purchased from Jackson Laboratory (JAX stock #002207; [24]) and bred at the same conditions as wild type C57BL/6J mice (JAX stock #000664) in our own breeding colony. Animals were maintained in groups of four to five animals per cage, under controlled temperature (23 ± 1 °C) and 12 h light cycle, with free access to food and water. The LDLr<sup>-/-</sup> mice display constitutive increased plasma cholesterol levels (2—3 times higher) in standard feeding conditions [24], as used in this study (UNIVLAB, Nuvital, Paraná, Brazil). All efforts were made to minimize the number and suffering of animals. The procedures used in the present study complied with the guidelines on animal care of the UFSC Ethics Committee on the Use of animals (PP00948), which follows the Principles of Laboratory Animal Care from National Institutes of Health.

2.1.2. BrdU injections

We used 5- bromo-2'-deoxyuridine (BrdU) to evaluate cell proliferation and overall neurogenesis. BrdU is a thymidine analogue that is incorporated into the DNA double-helix during the S-phase of the cell cycle, and thus marks actively proliferating cells [25]. C57BL/6J and LDLr<sup>-/-</sup> male mice at 3 months of age (n = 7/group) received i.p. injections of BrdU (100 mg/kg, in 0.1 M PBS, pH = 7.2; Sigma—Aldrich) every 12 h for 3 consecutive days. The same mice were then submitted to behavioral testing on the hippocampal sub-region dependent behavioral tasks and were euthanized 1 or 28 days later (Figure 1A) to assay proliferation or survival of new cells, respectively.

2.1.3. Hippocampal-dependent behavioral tasks

2.1.3.1. Behavioral apparatus and acclimation. The behavioral apparatus used for the metric and temporal ordering of visual objects consisted of a rectangular wooden box measuring 40 × 60 m × 50 cm high. Five sets of objects were used (one set for acclimation, one set for metric change, and three sets for temporal order). Objects included large Lego blocks, small glass bottles, and plastic toys and ranged in size between 2.5 and 5 cm at the base and between 5 and 15 cm tall. These objects were chosen as they are texturally and visually unique, and easily distinguishable for the mice. Between the habitation and test sessions, the mice were placed in a clean cage. The experimental box was wiped down with 70% ethanol after testing of each mouse to remove olfactory cues that could influence object exploration by subsequent mice. A video camera (Logitech, Newark, CA, USA) was mounted on the roof above the arena, and all test sessions were recorded using the ANY-maze software (Stoeling Co., Wooddale, IL, USA).

All animals (10—15 per group) underwent seven days of acclimation to the arena. On all days the animals were brought to the behavior room two hours prior to testing to allow them to acclimate to the room. On the first 3 days, animals were picked up by tails and placed in apparatus for 1 min with no objects present and then returned to their home cage. On the following 2 days, the animals were acclimated to the arena for 5 min with no objects present and then returned to their home cage. Finally, in the last 2 days of acclimation two objects were placed 40 cm apart in the center of the arena (these objects were randomly selected and were different from the ones used for either of the behavioral tests) and each animal was given a 10 min period to explore the arena and the objects before being placed back into its home cage. Following the 7 days of acclimation, the animals were subjected to the metric change task on day 8 and to the temporal order task on day 9 (Figure 1A).

2.1.3.2. Metric change task. The metric change task was conducted as initially described by Goodrich-Hunsaker [26—28] and composed of one habituation session and one test session. Mice were placed in the experimental box with two objects placed 40 cm apart. The mouse was allowed to freely explore the arena and stimulus objects for 15 min. After this habituation session, the mouse was removed to a clean cage for 3 min. During this interesson interval, the objects were moved closer to each other so that the objects were 20 cm apart. The mouse was then placed again in the experimental box and given 5 min to re-explore the objects during the test session. For the 15 min habituation session, total object exploration time (s) was calculated individually for the first, middle, and last 5 min time-blocks to facilitate comparison between the last 5 min of the exploration session and the 5 min test session.

When the time spent exploring objects through the three 5 min habituation periods did not decrease, it was taken as an indication that the animal did not habituate to the objects, and the mouse should be removed from the study. A decrease of less than 10% of the original exploration time is used as a criterion for elimination. In this study, all animals showed proper habituation behavior.

2.1.3.3. Temporal ordering task. The temporal order task was also conducted as initially described by Goodrich-Hunsaker [27,29] and composed of 3 habituation sessions and 1 test session. Prior to the first habituation session, 2 identical copies of the first object (object A) were
placed in the extremities of the experimental box, 10 cm from the end walls and centered between the long walls. For session 1, the mouse was placed in the center of the experimental box and given 5 min to freely explore the objects. Afterwards, the mouse was removed to a clean cage for 3 min. During this time, the first objects were replaced with 2 duplicates of a second object (object B). The procedure for the habituation session was then repeated once more, with 2 duplicates of a new object (object C). After session 3, the mouse was removed into a clean cage for 3 min and a copy of the first object and a copy of the third object (i.e., object A and object C) were placed in the box. The mouse was again allowed to explore the two objects during a 5 min test session. When a clear preference for one of the objects was detected during sessions 1–3 (i.e., difference in exploration time greater than 15 s), the animal was removed from the study (5 out of 13, for the C57BL/6J group and none for the LDLr−/− mice).

2.1.3.4. Behavioral analysis. A differentiation index for the metric spatial change task was calculated to facilitate the comparison between the test session and the last 5 min of the exploration session, as previously described [26]. Briefly, the index was calculated as: [(exploration time during the 5 min test session)/exploration time during the 5 min test session + exploration time during the last 5 min of the exploration session)]. This constrained all the values between 0 and 1. With this index, increased exploration during the 5 min test session compared to the last 5 min of the habituation is reflected as a value > 0.5, while decreased exploration (or continued habituation) is reflected as a value < 0.5. Exploration during the temporal ordering test sessions was also converted into a differentiation index to constrain the values between −1 and 1. The index was calculated as follows: [(exploration of object A − exploration of object C)/exploration of object A + exploration of object C].

2.1.4. Brain dissection and tissue processing for immunohistochemistry

Mice were deeply anesthetized with chloral hydrate (40%, i.p.) and perfused through the left cardiac ventricle with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. After perfusion, the brains were removed, post-fixed in the same fixative solution for 24 h at room temperature (RT) and immersed in a 30% sucrose solution in PBS at 4 °C. Serial coronal sections (30 μm) of hippocampi were obtained with a vibratome (Vibratome®, Series 1000, St. Louis, MO, USA) and stored in 0.1 M PBS at 4 °C.

To analyze cell proliferation and survival in the subgranular cell layer of the DG, a series of one-in-six free-floating sections were processed for detection of the BrdU and Ki67 immunoreactivity. Ki67 is a nuclear protein that is expressed during all active phases of the cell cycle, but protein that is expressed during all active phases of the cell cycle, but is not expressed during quiescence. The BrdU antibody recognizes a substitution of thymine by uracil in the DNA of proliferating cells. To detect BrdU and Ki67 immunoreactivity, the sections were incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG secondary antibody (1:200; Jackson Immunoresearch Laboratories) and Alexa 488 donkey anti-rabbit IgG secondary antibody (1:400; Jackson Immunoresearch Laboratories) for 2 h at RT.

2.1.5. Morphological quantification

All morphological analyses were performed on coded slides, with the experimenter blind to the experimental group, using a Leica microscope (Leica DM5500 B). The total numbers of BrdU and Ki-67 immunopositive cells in the DG of the hippocampus were estimated by manually counting all positive cells present within two—three nuclear diameters below the granule cell layer (GCL). All sections containing the DG region of the hippocampus, from 1.34 mm posterior to Bregma to 3.52 mm posterior to Bregma [32], were used for the analysis. Thus, the results were expressed as the total number of labeled cells in the DG sub-region of the hippocampus by multiplying the average number of labeled cells/DG section by the total number of 30 μm-thick sections that contain the DG (estimated as 73 sections).

2.1.6. Phenotypic analysis

Newly-formed neurons in the DG of adult mice were analyzed in one-in-six series of sections by immunofluorescent double staining [33]. A maximum of 50 BrdU-labeled cells per mouse were randomly selected for analysis of co labeling with NeuN. Analysis was performed by confocal microscopy (Leica DMi6000 B) in sequential scanning mode to avoid cross-bleeding between channels. Double-labeling was confirmed by three-dimensional reconstruction of z-series covering the entire nucleus (or cell) of interest.

2.2. In vitro study

We used two complementary in vitro models to dissect direct roles of elevated LDL levels and LDLr downregulation in survival, proliferation and fate decisions of adult NPC: i) primary neurospheres, which provide a good read-out for effects of extrinsic factors (here serum LDL) on proliferative and differentiation potential of NPC isolated directly from the DG of wild type mice; and ii) adherent monolayers, in which cells remain more isolated and homogeneous, thus eliminating the heterogeneous nature of the neurospheres, since cells inside the neurosphere have the tendency to differentiate [34–36].

2.2.1. Animals

C57BL/6J mice were bred by Jackson Laboratory and purchased from Janvier, Germany. Animal handling and all procedures during the experiment were conducted in accordance with the applicable European and national regulations (Tierschutzgesetz) and were approved by the responsible authority (Landesdirektion Sachsen, TVA 24-9168.24.1-2013-16a). All animals were 8 weeks of age at the time of dissection. Animals were maintained on a 12 h light/dark cycle with food and water provided ad libitum.

2.2.2. Culture of adherent adult hippocampal precursor cells

Primary mouse dentate gyrus NPC were isolated and the adherent monolayer cultures prepared as previously reported [34,35]. Cells were maintained in neurobasal growth medium (Gibco, Life Technologies) supplemented with 2% B27 (Invitrogen), 1X GlutaMAX (Life Technologies) and 50 units/ml penicillin/streptomycin (Life Technologies), 20 ng/mL human basic fibroblast growth factor (bFGF, PeproTech, Rocky Hill, NJ), and 20 ng/mL epidermal growth factor (EGF;
PeproTech). Every 2 days, 75% of the culture medium was replaced with fresh medium. Precursor cells were passaged at approximately 80% confluence by treating with Accutase (PAA Laboratories, Linz, Austria) for 2 min, and reseeded in PDL/laminin-coated dishes at a density of $1 \times 10^5$ cells per cm$^2$.

For the time-course differentiation analysis of gene expression, NPC were seeded in PDL/laminin-coated 60 × 15mm Petri dishes. Differentiation was induced by growth factor withdrawal whereby cells growing under standard proliferation conditions (20 ng/ml each of bFGF and EGF) were switched to medium without any growth factors $[34]$. Cells harvested before removal of growth factors were considered time 0, while the following dishes were harvested at 24--96 h after growth factor withdrawal.

2.2.3. Gene silencing

To knockdown the LDLr gene expression, adherent NPC were transfected with a pool of four different small interfering RNAs (siRNAs) targeting mouse LDLr (ON-TARGETplus SMARTpool, Cat. No. L-048572-00-0005, Dharmacon). Cells were cultured for 48 h in an antibiotic-free medium before the addition of 50 nM siRNA in culture medium containing 0.001% transfection agent (DharmaFECT, Dharmacon). After 24 h, the transfection media was replaced by regular medium containing 0.001% transfection agent (DharmaFECT, Dharmacon). Cells were cultured for 48 h in an Adherent cells were cultured in 6 or 24-well plates in growth medium

2.2.4. LDL and 27-HC treatment

Adherent cells were cultured in 6 or 24-well plates in growth medium for 2 days followed by 2 additional days in the presence of human LDL (0, 25, 50, 100 or 200 µg/mL) or 27-hydroxycholesterol (27-HC; 0, 1, 5 or 10 µM; Santa Cruz). LDL was purchased from Sigma or prepared as described previously $[37]$. For neurospheres assays, the freshly obtained cells were plated into 48 wells of a 96-well plate in the presence of LDL (0, 25, 50, 100 or 200 µg/mL) and grown as described above.

2.2.5. Immunocytochemistry

To assess cell proliferation, adherent monolayers were cultured on coverslips coated with PDL/laminin in 24-well plates. After treatments, adherent cells were incubated with BrdU (10 µM) for 2 h, then fixed with 4% PFA in 0.1 M PBS for 10 min at RT. For BrdU staining, cells were washed with PBS, and DNA was denatured with 1N HCl for 30 min at 37 °C. For all stainings, cells were blocked for 1 h in a blocking solution containing 10% donkey serum and 0.2% Triton X-100 in PBS, followed by an incubation with a primary (rat monoclonal anti-BrdU; 1:500; AbD Serotec); and secondary (Cy3; 1:1000 Jackson ImmunoResearch) antibodies prepared in blocking solution for 2 and 1 h, respectively. The nuclei were labeled with Hoechst and coverslips were mounted onto glass slides for Zeiss Apotome microscope imaging.

To assess apoptosis induction the number of pyknotic nuclei, average nucleus size and integrated density (fluorescence intensity normalized by cell size) were quantified per image using the ImageJ software.

2.2.6. CFSE cell proliferation assay

The influence of treatments on proliferation kinetics of NPC was estimated by cell division tracing with the fluorescein compound CFSE (Carboxyfluorescein succinimidyl ester). Before seeding, $1 \times 10^5$ cells were loaded with 10 µM CFSE for 10 min at 37 °C. The addition of media at 4 °C for 5 min stopped the reaction. After washing and resuspension, cells were seeded in 6-well plates, grown for 2 days and treated with LDL (100 µg/mL) or transfected with siRNAs for 48 h as described above. Finally, cells were washed, harvested and fixed in 2% PFA for 20 min at RT. Cell suspension was analyzed in BD FACSCanto II flow cytometer (BD Biosciences).

2.2.7. Quantitative RT-PCR

Total RNA was prepared using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). The adherent NPC cultures were washed once with PBS and then harvested by on-plate lysis with RLT buffer. Lysate was collected using a cell scraper and RNA extracted following the manufacturer’s protocol, including the optional DNase step. The concentration and the purity of RNA were analyzed using a NanoDrop 1000 spectrophotometer (Thermo Scientific). For reverse transcription, 1 µg of RNA was used. The cDNA was prepared using SuperScript II kit (Invitrogen) using oligo(dT) primers following the manufacturer’s protocol. A cDNA negative reaction was also set up without addition of reverse transcriptase enzyme to ensure no genomic DNA contamination.

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) for LRP1, LDLR, CYP27A1, CYP43A1, FDFT1, and HMGR was performed using a SYBR Green PCR Mix (Qiagen) following the manufacturer’s protocol. Template cDNA was prepared as above and each reaction contained cDNA from 0.1 µg total RNA. Thermal cycling and fluorescence detection were carried on a CFX96 Real Time PCR detection system (Bio-Rad, California, USA). Quantitation was done using the $\Delta\Delta$Ct method after normalizing to the TATA box binding protein (TBP) housekeeping gene. Primer sequences for LRP1 were 5’- TCTTCTGTCGCCTCTGGTG- 3’ and 5’- TCGGGCTTCACCTCTCATA-3’; for LDLR 5’- GGAGCAGACCATG-GATGTA- 3’ and 5’- ATGTTCTTCAGCCGCAAGT- 3’; for CYP27A1 5’- GGTATCTGGCCTACCTGCAC-3’ and 5’- CTCTGTAGATCTGGCTCT- 3’; for CYP46A1 5’- GTGGTGATGAGTGTGCTGGT- 3’ and 5’- TCAAGGTTCTCTCTCAGC-3’; for FDFT1 5’- AACATGCTGCTCCGAAAG- 3’ and 5’- GGAGTATGTCCTCGGAGA- 3’; for HMGR 5’- AAGC-CAGTGTGCCACAAAT- 3’ and 5’- TTGATGCTGCTCAGACCC- 3’ and TBP 5’- CCGAACAACAGCCTCCAC- 3’ and 5’- GGAG-TAAGCTCTGTCCGCT- 3’.

2.2.8. Lipid droplets staining

Treated and control cells grown on glass coverslips in 24-well plates were fixed in 4% PFA in 0.1 M PBS for 10 min at RT, washed with PBS and incubated with a solution of 0.1 µg/mL LDS40 (generously donated by Dr. Uenal Coskun) in PBS for 15 min $[38]$. Afterwards, the nuclei were labeled with Hoechst, coverslips were washed three times with PBS and mounted with fluorescence mounting medium without glycerol (Dako). Images were acquired with an SD spinning disk confocalscope (Carl Zeiss), excitation at 530 nm. The number of lipid droplets were quantified per image, and normalized by cell number on the field, using the ImageJ software.

2.2.9. Fluorescent LDL internalization assay

48 h after treatment, adherent cells plated on PDL/laminin-coated coverslips in 24-well plates were washed twice with media and incubated for 45 min at 37 °C. The first LDL pulse (10 µg/mL; BOD-IPY™ FL LDL, Invitrogen) was given 60 min before fixation. After 10 min of incubation with LDL-Bodipy, wells were washed twice with media and returned to the incubator until the second LDL pulse. LDL-Dil (10 µg/mL; Dil LDL, Invitrogen) was given 20 min before fixation. After 10 min of incubation wells were washed twice with media and kept at 37 °C5% CO$_2$ until the end of the protocol (fixation). Cells were washed with cold PBS and fixed in 4% PFA for 10 min at RT. The coverslips were washed three times with PBS and mounted with fluorescence mounting medium. Images were acquired with a SD spinning disk confocalscope (Carl Zeiss).
2.2.10 Microarray processing

Cells grown in 6-well plates were treated with 100 μg/mL LDL or saline as a control or transfected with LDLr or non-target siRNAs. Total RNA from three replicate cultures for each condition was extracted as described above. For each sample, 100 ng total RNA was used to synthesize Cy-3 labeled target cRNA with the Low Input Quick Amp Labeling Kit (Agilent Technologies, Germany), followed by hybridization to SurePrint G3 Mouse GE 8 × 60K one-colour microarrays (AMADID 028005; Agilent Technologies). Raw signal intensities were obtained with Feature Extraction software (protocol 028005_D_F_20150604; Agilent Technologies). The data were further analyzed in R using the limma package, which fits to each gene a linear model with treatment as a factor and computes moderated t-statistics applying the empirical Bayes approach [39]. Raw intensities were background corrected (subtraction method) and normalized between arrays applying the cyclic loess normalization. Only probes which were at least 10% brighter than the negative control (the 95th percentile of negative probes intensities for each array) on at least 3 arrays were retained. Replicated probes were averaged. A 10% FDR (false discovery rate) cut-off and an absolute value of fold change of 1.5 were used to identify differentially expressed genes. Functional enrichment analysis of canonical pathways and potential upstream regulators was performed with Ingenuity Pathway Analysis software (IPA; Qiagen) at default settings [40]. Array data are available online at the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo; access: GSE136798).

2.3 Statistics analysis

Data were analyzed using GraphPad Prism and R. The statistical analyses were carried out using unpaired two-tailed Student’s t-test, one-way analysis of variance (ANOVA) or ANOVA with repeated measures when appropriate. Post hoc comparisons were performed using the Dunnett’s test. Data are presented as means ± standard error of the mean (SEM). A p value < 0.05 was considered to be statistically significant. Full results of statistical tests are available in the Supplementary Table 1.

3. RESULTS

3.1. LDLr−/− mice perform poorly in the DG-dependent metric change task

It has been demonstrated that LDLr−/− mice perform poorly in a spatial memory task, suggesting a hippocampus related cognitive impairment [14]. In this study, seeking a DG-specific contribution to the spatial memory deficit in the LDLr−/− mice, we used the metric spatial change task (Figure 1A) to examine spatial pattern processing [26,27]. During the task, mice are allowed to explore a pair of objects placed 40 cm apart in a 15 min long habituation session (Figure 1B). In the test session the same objects are replaced only 20 cm apart, and the mice are allowed to explore for 5 min. An increased interest to re-explore the objects in the test session indicates that the mice were able to notice the metric novelty [27,41]. The habituation of mice during the 15 min session was evaluated by comparing the time that mice spent exploring the objects in three 5 min periods. Successful habituation was observed in both groups, as evidenced by a decrease in object exploration after repositioning of the objects, wild type mice, but not LDLr−/− mice, showed an increase in object exploration compared to the final 5 min of the habituation session [Figure 1D; t-test; t (18) = 3.62, p < 0.01].

3.2 Performance in the temporal ordering task is not affected by the absence of LDLr

The next day, we tested the mice in the temporal ordering task (Figure 1A), which is primarily dependent on the intact CA region of the hippocampus [42,43]. In this task, mice are presented three different pairs of identical objects in three consecutive sample sessions (Figure 1E). In the test trial, mice are exposed to one object from the first session and one object from the last session. Intact animals show preference for the object more distant in time. To exclude object preference during acquisition sessions, for each genotype we performed a one-way ANOVA of the object exploration time with the session as a factor. We did not detect an effect of the session for the C57BL/6J [F (2, 36) = 1.46, p = 0.25] or the LDLr−/− mice [F (2, 42) = 1.96, p = 0.15], suggesting that all mice similarly explored different objects across sessions 1–3. To determine group differences in temporal ordering, the differentiation scores of the exploration time of each of the objects during the test session were calculated (Figure 1G). The LDLr−/− group showed similar preference towards temporally distant object as the wild type group [t-test; t (21) = 0.61, p = 0.55].

3.3. Hippocampal cell proliferation and adult neurogenesis are decreased in LDLr−/− mice

To assess the impact of the LDLr knock-out on adult hippocampal neurogenesis, we first compared the rates of cellular proliferation between 3 months old C57BL/6J and LDLr−/− mice. Both groups of animals were injected with the thymidine analogue BrdU and euthanized 24 h later (Figure 1A). As shown in Figure 2B, the LDLr−/− mice had reduced numbers of BrdU labeled cells compared to wild-type mice [t-test; t (12) = 2.51, p = 0.0273], which indicates lower numbers of proliferating precursor cells. We corroborated this finding using immunohistochemistry against an endogenous antigen Ki-67 present on a nuclear protein that is expressed during all active phases of the cell cycle but is absent from cells in G0 [44]. This marker also indicated that cell proliferation was reduced in the LDLr−/− mice when compared to the wild type group [t-test; t (11) = 2.52; p = 0.028; Figure 2D].

Next, we assayed the number and neuronal differentiation of newly generated cells that survived 4 weeks after incorporation of BrdU to estimate net neurogenesis (Figure 1A). We observed a decline in the total number of 4 weeks old BrdU-labeled cells in the LDLr−/− animals (Figure 2F; t-test; t (12) = 2.71, p = 0.02). The proportion of new neurons among the cells that incorporated BrdU was determined using the mature neuronal marker NeuN and confocal microscopy (Figure 2G). The net neurogenesis, estimated by multiplying the number of BrdU-positive cells that survived the 28 day period by the proportion of BrdU-positive cells that co-expressed NeuN, was also significantly reduced in the hypercholesterolemic mice (Figure 2H; one-tailed t-test; t (8) = 2.22, p = 0.028).

3.4. LDL treatment and LDLr downregulation affect the proliferation of adult hippocampal precursor cells in vitro

Varying concentrations of LDL did not affect cell viability of adherent hippocampal NPC after 24 h [one-way ANOVA; F (4,10) = 2.27; p = 0.13; Fig. S1A], neither did the treatment increase pyknosis, a sign of apoptosis, after 48 h [one-way ANOVA; F (4,10) = 0.38,
To downregulate the expression of the SDRP mRNA in adherent cultures, cells were transfected with a pool of four siRNAs targeting LDLR or a pool of non-target control siRNAs. A reduction of 73% in LDLR mRNA was confirmed by RT-qPCR [t-test; t(4) = 22.79, p < 0.0001; Figure 3D]. The LDLR siRNA transfection did not affect cell survival after 24 h [t-test; t(4) = 0.30, p = 0.78; Figure S1D] or pyknosis at 48 h [t-test; t(4) = 1.96, p = 0.12; Figure 3E] but it decreased cell proliferation assessed by BrdU incorporation [t-test; t(4) = 17.27, p < 0.0001; Figure 3F]. The increased CFSE retention after the cell tracing also indicated a lower number of cell divisions [t-test; t(4) = 5.98, p = 0.004; Figure 3G].

In hypercholesterolemia, there is an excessive formation of the cholesterol oxidized metabolite 27-HC, which has been linked to pathogenesis of neurodegenerative diseases [45]. Therefore, we tested whether this compound could impair survival and proliferation of precursor cells. 27-HC decreased cell survival after 24 h [one-way ANOVA: F (3, 8) = 14.16, p = 0.0015; Figure S1G], and increased pyknosis at 48 h [one-way ANOVA: F (3, 8) = 34.97, p < 0.001; Figure 3H] without affecting cell proliferation [one-way ANOVA: F (3, 8) = 1.11, p = 0.4015; Figure 3I].

Together, our data show that the presence of high concentrations of LDL in the culture medium as well as lowering of LDLR expression reduced cell proliferation of adult hippocampal NPC.
3.5. LDL treatment increased intracellular lipid storage in precursor cells with no effect on LDL trafficking

Neurons in the adult brain take up the ApoE-cholesterol complexes via endocytosis through the LRP1 and LDLr receptors and cholesterol-loaded endocytic vesicles are hydrolyzed in lysosomes [46]. To check if the exogenously added LDL cholesterol would accumulate intracellularly, we estimated cholesterol storage by staining lipid droplets with the LD540 dye. Since the loss of LDLr might have altered cellular lipid homeostasis, we also investigated lipid droplets in siRNA transfected cells. We found that the LDL treatment of adult hippocampal NPC in vitro induced an increase in the number of lipid droplets (t-test; t(4) = 9.37, p = 0.0007; Figure 4B). On the other hand, the knock-down of the LDLr by siRNA did not cause any alteration (t-test; t(4) = 0.44, p = 0.68; Figure 4C).

To estimate if endocytosis of cholesterol in NPC is perturbed when LDLr is down-regulated or when cells are overloaded with LDL, we investigated the dynamics of LDL intracellular transport by pulse labeling with two different LDL fluorescent conjugates, LDL-Bodipy and LDL-DiI, at 60 and 20 min before fixation (Figure 4D). The qualitative evaluation suggested that after 48 h of LDL treatment, cells were still able to uptake fluorescent LDL, despite intracellular cholesterol overload. Additionally, knock-down of LDLr using siRNA did not prevent the uptake of LDL in adherent hippocampal NPC, because they were still able to internalize the dyes. Finally, the lack of co-localization of the dyes administered at different time points suggested that the intracellular trafficking of LDL cargo through subsequent endocytic compartments was not disrupted.

3.6. Cell differentiation, LDL treatment and LDLr knock-down modulate cholesterol metabolism-related genes

To better understand the roles of LDL cholesterol and LDLr in proliferation and differentiation of hippocampal NPC, we analyzed the dynamics of mRNA expression of genes related to cholesterol metabolism...
in NPC in vitro in proliferative conditions and at 24, 48 and 96 h after growth factor withdrawal, which induces cell cycle exit and differentiation [34] (Figure 5). Gene expression levels of key enzymes in the cholesterol synthesis pathway, HMG-CoA reductase (HMGCR; Figure 5A) and squalene synthase (FDFT1; Figure 5B), were decreased already after 24 h from the onset of differentiation [one-way ANOVA; HMGCR: F (3,16) = 79.51; p < 0.0001; FDFT1: F (3,16) = 29.06; p < 0.0001, respectively]. The LDLR mRNA expression was dramatically decreased in differentiated cells [one-way ANOVA; F (3,16) = 302.3; p < 0.0001] at all time points analyzed, while the LRP1 mRNA levels were increased [one-way ANOVA; F (3,16) = 8.88; p = 0.0011], with a significant difference from 48 h of differentiation (Figure 5C,D). The mRNA levels of enzymes that catalyze oxidation of cholesterol to oxysterols, CYP46A1 and CYP27A1, were increased after 24 h in differentiation conditions [CYP46A1: F (3,16) = 22.87; p < 0.0001; one-way ANOVA; and CYP27A1: F (3,16) = 4.34; p = 0.02; Figure 5E,F]. While the CYP27A1 mRNA returned to levels similar to the proliferating cells after 96 h of differentiation, CYP46A1 remained upregulated. In conclusion, transition from proliferation to differentiation in adult hippocampal NPC in vitro was associated with remodelling of cholesterol synthesis, uptake and catabolism.

We next examined the effects of LDL treatment and LDLr knock-down on the expression of genes related to cholesterol metabolism in NPC under proliferative conditions. As shown in Figure 6, 48 h of treatment with LDL at a concentration of 100 μg/mL decreased the expression of mRNAs encoding for HMG-CoA reductase and squalene synthase enzymes, as well as of LDLR and LRP1 (HMGCR: F (4) = 9.38; p = 0.007; FDFT1: F (4) = 17.43; p < 0.0001; LDLR: F (4) = 11.80; p = 0.0003; LRP1: F (4) = 0.60, p = 0.58). Cells transfected with siRNA against LDLR again showed a 70% reduction in the LDLR

Figure 3: Effects of LDL treatment and siRNA-mediated LDLr knock-down on survival and proliferation of adult hippocampal precursor cells in vitro. Precursor cells isolated from C57BL/6J mice were propagated as adherent monolayers and treated with: (A–C) human plasma LDL (0–200 μg/mL); (D–G) transfected with a pool of siRNAs targeting the LDLR gene or a pool of non-targeting siRNAs as a control; (H–I) or 27-HC (0–10 μM). (D) LDLR transcript expression after siRNA transfection was evaluated by qRT-PCR. (A, E, H) 48 h after each treatment, the number of pyknotic nuclei assessed by morphological analysis of Hoechst stainings. To assess cell proliferation, cultures were analyzed 48 h after addition of LDL (B–C), transfection with siRNAs (F–G) or 27-HC treatment (I). (B, F; I) Precursor cells were incubated for 2 h with BrdU. Proportion of BrdU-immunoreactive, actively dividing cells decreased after exposure to LDL (B) LDLR knock-down (F) but not 27-HC treatment (I). (C, G) Fluorescence of CFSE-loaded cells increased after addition of LDL (100 μg/mL; C) or transfection with LDLR siRNA (G) indicating fewer cell divisions compared to control cells. Data are presented as means ± SEM. N = 3 per group. *p < 0.05, t-test (C, D, E, F, G), one-way ANOVA followed by Dunnnett’s post hoc test (A, B, H, I).
expression ($t(4) = 8.07, p = 0.0013$). This downregulation was associated with reduced HMGCR and FDFT1 (HMGCR: $t(4) = 7.89, p = 0.0014$; FDFT1: $t(4) = 9.65, p = 0.0006$, respectively). Surprisingly, knock-down of LDLr also resulted in downregulation of the LRP1 receptor gene expression ($t(4) = 5.52, p = 0.0053$). The mRNA levels of CYP46A1 and CYP27A1 were not affected by either LDL treatment ($CYP46A1: t(4) = 0.72, p = 0.51$; CYP27A1: $t(4) = 1.83, p = 0.14$) or LDLr siRNA transfection ($CYP46A1: t(4) = 0.03, p = 0.98$; CYP27A1: $t(4) = 0.06, p = 0.95$).

3.7. Gene expression analysis revealed cell biological pathways affected by LDL treatment and LDLr downregulation

To get an insight into molecular changes that might underlie altered behavior of NPC upon LDL exposure and LDLr downregulation, we performed gene expression profiling using microarrays. We detected 42 and 1151 differentially expressed transcripts in LDL treated (100 µg/mL) and LDLr siRNA transfected cells, respectively (Figure 6A). We next carried out a functional annotation of differentially expressed genes using Ingenuity Pathway Analysis (IPA). IPA is
considering directionality and magnitude of gene expression changes to predict activation or inhibition of each enriched biological pathway. Furthermore, it predicts changes in activity of upstream regulators that are consistent with differences in gene expression [40]. Upstream regulator analysis revealed that the LDL treatment inhibited transcriptional networks dependent on sterol regulatory element-binding proteins (SREBPs) with top enriched canonical pathways related to cholesterol biosynthesis (Figure 6B). Although the knockdown of LDLR induced broad changes in gene expression, the enrichment analysis did not lead to strong conclusions (Supplementary Table 2). In agreement with slower proliferation of LDLR siRNA transfected NPC, several of the top enriched ingenuity canonical pathways were related to cell cycle regulation (Figure 6C and Supplementary Table 3). In both treatments, IPA also identified numerous putative upstream regulators (Table 1 and Supplementary Table 3).

4. DISCUSSION

In this study we have shown that a mutation in the LDLr gene, as found in FH, might have direct effects on the biology of adult neural stem cells and adult neurogenesis, which in turn might be responsible for cognitive impairments observed in cases of hypercholesterolemia [1–3]. Adult hippocampal neurogenesis is believed to functionally contribute to neural plasticity and cognition throughout life, while its decay might be linked to impaired cognitive and emotional resilience in humans [47–49]. Our findings show that 3 months old LDLr−/− mice performed poorly in the DG-dependent metric change task, which is in line with previous studies reporting spatial memory deficits in this rodent model of hypercholesterolemia [14,17,50]. Additionally, as previously reported, the LDLr−/− mice present hyperlocomotion [51] and, since they cover a

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**Figure 5:** Expression of cholesterol metabolism-related genes in precursor cells in vitro. (A–F) Time-course evaluation of mRNA levels in proliferating precursor cells (G) and at 24, 48 or 96 h of differentiation. (G–J) Effect of LDL treatment (100 μg/mL) and LDLR siRNA transfection on gene expression in proliferating precursor cells. mRNA levels were measured by qRT-PCR. HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; FDFT1: squalene synthase; LDLR: low-density lipoprotein receptor; LRP1: Low-density lipoprotein receptor-related protein 1; CYP46A1: cholesterol 24-hydroxylase; CYP27A1: sterol 27-hydroxylase; NT: non-target control. Data are presented as means ± SEM. N = 5 per group, time-course assay, N = 3 for treatments. *p < 0.05. One-way ANOVA followed by Dunnett’s post hoc (time-course assay) and t-test (treatments).
greater distance, it is reasonable that they probably find the objects more frequently during the training session, resulting in increased exploration values. Lack of definiteness in the CA-related temporal ordering task might be an indication that the previously observed synaptic loss in the CA of LDLr/C211/C211 mice at 6 and 14 months does not affect younger animals, or is not compromising its function [13,14]. In our study, the DG-related behavioral alterations were accompanied by decreased cell proliferation and neurogenesis in this structure. Notably, ablation of adult neurogenesis is well known to impair performance in behavioral tasks that require resolution of similar inputs, such as a subtle metric novelty [52].

**Figure 6:** Gene expression profiling in precursor cells reveals distinct biological effects of LDL and LDLr downregulation. Proliferating adherent precursor cells were treated with 100 μg/mL of LDL and LDLR siRNA, and after treatments gene expression was analyzed by microarrays. (A) Volcano plots showing differentially regulated genes at 10% false discovery rate and an absolute fold change cut-off of 1.5 (blue). Red points indicate genes from the cholesterol biosynthetic pathway changed in LDL treated cells. LDLR is highlighted in purple. (B, C) Selected top ingenuity canonical pathways enriched in LDL treated (B) and LDLR siRNA transfected (C) NPCs. Blue indicates pathways that are predicted to be inhibited in treated cells, while activated pathways are shown in orange. The strength of change (Z score) is indicated by the colour intensity. For pathways shown in grey, insufficient data exist to predict the activity change. Purple vertical line indicates significance of enrichment (p < 0.05). Full lists of differentially expressed genes and enriched pathways are available in the extended data Supplementary Tables 2–4.
attention to inherited factors, such as mutations in the LDLr gene. In the LDLr−/− mice, high plasma cholesterol levels and the absence of LDLr might both have a role in impairing the neurogenic process. High levels of cholesterol in the blood of the LDLr−/− mice are associated with increased BBB permeability. Recent studies that used dietary strategies, resembling the sporadic form of the disease, enforce the hypothesis of hypercholesterolemia disturbing neurogenesis and hippocampus-dependent behaviors in adult mice. However, the present study demonstrates for the first time an impairment in adult neurogenesis in the LDLr−/− model of FH.

We showed here that plasma cholesterol exposure and LDLr down-regulation can individually affect proliferation of NPC in vitro. Primary NPC challenged with high concentration of plasma LDL decreased proliferation and reduced differentiation towards the neuronal lineage (Fig. S2). Moreover, we observed a prominent effect on astrocytic morphology after LDL treatment. This observation might be linked to previously shown astrocytic alterations in the LDLr−/− mice. Additionally, it was recently reported that astrocytes are set with a very distinct transcriptome to manage lipid particles in CNS, and such LDL overload might be disrupting this machinery. We found that the exogenous LDL is taken up by the proliferating cells and stored intracellularly as lipid droplets. It was shown before that primary neurons treated with LDL exhibited increased cholesterol accumulation in endolysosomes and concomitantly decreased levels of synaptophysin. A recent study suggested that accumulation of lipids in cells from the neurogenic niche of the lateral ventricles, as found in brains from Alzheimer’s disease patients, could contribute to reduced NPC proliferation. In our study, it is conceivable that the increase in intracellular cholesterol content contributed to the effect on cell proliferation and led to the observed down regulation of the expression of sterol metabolism related genes (Table 1 and Supplementary Table 1). Specifically, the down-regulated genes contain the sterol regulatory element (SRE) promoter region, activated by the SRE-binding proteins (SREBPs), which are expressed by high intracellular sterol content. In contrast, the expression of LRPP gene, which doesn’t have the SRE promoter region, was not altered. In agreement, IPA upstream analysis of differentially expressed genes suggested inhibition of both SREBF1 and SREBF2. Inhibition of SREBPs and downregulation of HMGR may result in decreased supply of mevalonate pathway end products, like isoprenoids, which are added to proteins involved in signaling to modify their localization and function and consequently cell differentiation and proliferation. Finally, the increase in lipid droplet content per se could reshape intracellular signaling. These organelles play active roles in maturation, sequestration and turnover of many proteins, and even in transcriptional regulation.

De novo lipogenesis and fatty acid oxidation have a regulatory role in cell proliferation and maintenance of quiescence of adult NPC [65]. Several studies have demonstrated the important role of cholesterol metabolism in the development of the CNS and dependence of mature neurons on astrocytic cholesterol supply. Although there is a scarcity of information about cholesterol metabolism in adult neurogenic niches, regarding the LDLr family, interesting studies demonstrated the expression of LRP2 and LRP1 by the precursor cells from the subventricular zone of the lateral ventricles. In addition, the LRP6 protein was found to be expressed in the hippocampal adult NPC and manipulation of its expression affected the proliferative capacity of cells in vitro.

Herein we showed that the gene expression of cholesterol biosynthesis enzymes and LDLr are higher in proliferating than in the differentiating cells, resembling the high cholesterol requirement during CNS development, when both synthesis and uptake are highly stimulated. Accordingly, the downregulation of the LDLr expression reduced NPC proliferation. The LDLr knock-down reduced mRNA levels of LRPP as well. These two proteins are the main receptors for ApoE, which in turn is required for maintenance of the DG adult progenitor pool. Fluorescent LDL pulse-chase suggested that the internalization and intracellular dynamics dependent of the LDL receptors are intact, so it is unlikely that the cells are experiencing a restriction in extracellular cholesterol supply or disruption of endocytosis. It is important to note that none of the treatments completely eliminated LDLr. Even after siRNA transfection, cells maintained 30% of LDLr mRNA. LDLr downregulation, rather than knock-out, is concurrent with the phenotype expressed by most of the FH patients (heterozygous form of the disease, loss in receptor activity up to 50%). Remarkably, the LDLr treatment also strongly inhibited the LDLr expression. One could suggest that lack of the LDLr downstream signaling due to its down regulation is a convergent mechanism between the effect of the two treatments. Supporting LDLr importance in proliferative cells, cancer studies have shown that the LDLr inhibition
potentiates tumor regression induced by chemotherapy [74,75]. LDLR knock-down resulted in extensive changes in gene expression, distinct from the gene expression profiles after LDL treatment, indicative of some specificity of molecular regulation in both paradigms. IPA exploratory analysis implied modulation of several pathways and upstream regulators related to cell proliferation, including GADD45 signaling, important in adult hippocampal NPC proliferation and growth [76]. It is noteworthy that LDL treatment led to very few changes at the mRNA level, limited to the cholesterol synthesis pathway. This suggests that metabolic signaling triggered by intracellular accumulation of cholesterol induced post-transcriptional modifications in activity of regulatory proteins. In agreement, IPA upstream analysis pointed to changes in activation status of proteins and complexes known as hubs in orchestration of cellular metabolism and fate decisions, such as PTEN, MTORC1 or PPARG. Alternatively, the robust effect of LDL treatment over sterol related pathways might have mitigated the statistical highlight of other regulatory systems, involved in the cell cycle control, for example. In this regard, it was previously reported that in vitro exposure to LDL induces chromosome instability and mis-segregation resulting in aneuploidy [77,78], and that it can affect cell-cycle progression via STAT5B mediated induction of cell-cycle arrest [79]. Although we did not find important alterations in glycolysis or OXPHOS after LDLR siRNA and LDL treatments (Fig. S3), a redox imbalance in the first hours of LDL treatment [19] might be conditioning the mitochondria to deal with produced oxidants (observed as a shift in the maximal respiratory capacity; Fig. S3C). From a number of studies, mitochondria bioenergetics emerges as an important regulator of NPC quiescence, proliferation, cell cycle exit, and neuronal differentiation [80–82].

5. CONCLUSIONS

In summary, the present study suggests that impairment in hippocampal neurogenesis might contribute to the behavioral phenotype in the Ldlr−/− mice. Although we do not exclude niche-derived or systemic factors in the cognitive deterioration or negative regulation of adult neurogenesis, our in vitro data showed that high LDL levels and the downregulation of LDLr might directly contribute to a disruption of adult hippocampal NPC proliferation. The present findings also draw attention to the critical role of cholesterol metabolism in these processes and add to the accumulating evidence that complex metabolic networks orchestrate adult neural stem cell behavior and, accordingly, perturbation of the metabolic balance may have profound consequences for the cognitive capacity of the organism, in extreme cases leading to neuropathology.

AUTHOR CONTRIBUTIONS

Conceptualization, D.F.E., A.F.B., P.S.B., G.K., A.N.G., and T.L.W.; Methodology, D.F.E., A.F.B., P.S.B., G.K., A.N.G., and T.L.W.; In vivo experiments, D.F.E., J.O., and P.S.B.; In vitro experiments, D.F.E., A.F.B., K.S., A.P.; Analyses, D.F.E., A.F.B., P.S.B., G.K., and A.N.G.; Writing, D.F.E., A.F.B., P.S.B., G.K., and A.N.G.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.09.002.

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