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REGULAR ARTICLE

High fat diet induces brain injury and neuronal apoptosis via down-regulating 3-β hydroxycholesterol 24 reductase (DHCR24)

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Abstract

Hyperlipidemia (HLP) is one of the risk factors for memory impairment and cognitive impairment. However, its pathological molecular mechanism remained unclear. 3β-hydroxysterol Δ24- reductase (DHCR24) is a key enzyme in cholesterol synthesis and has been reported to decrease in the affected areas in the brain of neurodegenerative disorders. In this study, hyperlipidemic mouse model was established to study the effect of high blood lipid on brain. The data obtained from HPLC analysis demonstrated that the cholesterol level in the brain of mice with hyperlipidemia was significantly elevated compared to the control group. While the pathological damages were observed in both cerebral cortex and hippocampus in the brain of hyperlipidemic mice. Furthermore, the protein level of DHCR24 was downregulated accompanied by elevated ubiquitination level in the hyperlipidemic mice brain. The mouse neuroblastoma cells N2a were exposed to the excess cholesterol loading, the cells underwent apoptosis and the mRNA and protein of DHCR24 in cholesterol-loaded N2a cells were significantly reduced. In addition, the expression level of endoplasmic reticulum stress marker protein (Bip and Chop) was markedly increased in response to the cholesterol loading. More importantly, overexpression of DHCR24 in N2a reversed neuronal apoptosis induced by the cholesterol loading. Conclusively, these findings suggested that hyperlipidemia could cause brain tissue injuries via down-regulating DHCR24, and overexpression of DHCR24 may alleviate hyperlipidemia-induced neuronal cells damage by reversing the endoplasmic reticulum stress-mediated apoptosis.

Keywords DHCR24 · Hyperlipidemia · Cholesterol · High fat diet

Introduction

Cholesterol, a crucial lipid component of mammalian cell membranes, plays a vital role in the formation of cell barriers and physiological signaling. Although cholesterol deficiency is rare, it can have profound consequences. Conversely, excessive cholesterol levels can decrease membrane fluidity, disrupt membrane micro-domains, alter membrane protein function, and ultimately lead to cell dysfunction and death, indicating its toxic effects. (Song et al. 2021). Furthermore, imbalances in cholesterol levels are associated with diseases such as dyslipidemia, diabetes, and fatty liver. Dyslipidemia diabetes, a metabolic disorder characterized by elevated levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and/or decreased high-density lipoprotein cholesterol (HDL-C), has been extensively studied. (Liu et al. 2019; Zhou et al. 2015). Moreover, dyslipidemia, including hyperlipidemia and hypercholesterolemia, is considered a risk factor for memory impairment and cognitive decline, drawing significant attention in research (Chen and Zissimopoulos 2018; Lourida et al. 2019).

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Alzheimer's disease (AD) is a neurodegenerative disease, and one of the most important public health problems in the world with high incidence rate and mortality. There are many causes and pathogenic factors, which lead to the dysfunction of neurotransmitter system and the decline of cognitive ability that accounts for the mechanism of the pathogenesis of AD. In the last thirty years, great progress has been made in the research of AD. However, the pathological mechanism of AD is still unclear. Among them, the two most classical hypotheses are amyloid β-protein (Aβ) cascade and tau protein hyperphosphorylation. Currently, cholesterol metabolism has emerged as a prominent factor in the development of Alzheimer's disease (AD), supported by robust evidence substantiating the cholesterol pathogenesis hypothesis. Furthermore, compelling findings from animal studies have indicated that hypercholesterolemia could impair the integrity of the blood–brain barrier (de Oliveira et al. 2020). On the one hand cholesterol not only participates in the formation and degradation of Aβ in the brain tissue of AD patients, but also regulates the function and aggregation of Aβ and affects its neurotoxic effect (Maulik et al. 2013). On the other hand, as a metabolite of oxysterol, oxidized cholesterol can enter the brain through the blood–brain barrier from the blood circulation, and vice versa. It has been found that oxysterol and the associated catalytic enzymes are changed in the AD brain and have been proven to be the influencing factors of disease progression (Loera-Valencia et al. 2019). Many years of research suggests that patients taking cholesterol lowering drugs can reduce the probability of secondary AD. Nevertheless, it is important to note that the metabolic pathways for cholesterol in the brain differ from those in the body. Therefore, the potential correlation between brain cholesterol and serum cholesterol levels requires further investigation to elucidate the intricacies of cholesterol metabolism in the context of Alzheimer's disease.

Cholesterol regulation in the body is primarily achieved through post-transcriptional control mechanisms. Studies have revealed that the degradation of cholesterol by the downstream enzyme squalene monooxygenase also plays a pivotal role in modulating its flux (Gill 2011). DHCR24, a crucial enzyme in the final step of cholesterol synthesis pathway, has emerged as a promising candidate for feedback regulation due to its ability to precisely modulate cholesterol production. Recent findings from research have revealed that DHCR24 is also subject to post-translational regulation, further underscoring its significance as a potential target for regulating cholesterol synthesis (Zerenturk et al. 2012; Jansen et al. 2013). DHCR24, which is also referred to as Seladin-1, holds particular significance as it has been identified as one of the selective indicators associated with AD. This noteworthy observation further underscores the potential importance of DHCR24 in the pathogenesis of AD. It is not only involved in the de novo synthesis of cholesterol, but also involved in the neuroprotection of oxidative stress, endoplasmic reticulum stress, Aβ toxicity and apoptosis (Martiskainen et al. 2017). Downregulation of DHCR24 expression was observed in brain lesions of AD patients (Iivonen et al. 2002). It is also found that DHCR24 gene polymorphism can regulate the risk of AD, also suggesting that DHCR24 may be related to AD (Lämsä et al. 2007). Furthermore, in vitro experiments have elucidated that the reduction in DHCR24 levels correlates with increased stability of BACE1, thereby promoting the hydrolysis of amyloid-beta (Aβ) by amyloid precursor protein (APP). These findings provide additional evidence for the potential involvement of DHCR24 in the complex interplay of AD pathogenesis. Therefore, increasing the expression level of DHCR24 in the affected area of brain may provide a potential therapy for the intervention of the pathogenesis of AD.

The increase of lipid intake is the main reason for the increase of the proportion of patients with dyslipidemia. However, it is still controversial whether peripheral hyperlipidemia can cause the increase of cholesterol concentration in the brain, and whether the DHCR24 level in the brain would be affected in response to the elevated serum cholesterol level. We postulate that peripheral hyperlipidemia may trigger brain damage and result in elevated cholesterol levels in the brain, subsequently leading to downregulation of DHCR24 expression. This speculative hypothesis suggests a potential link between peripheral hyperlipidemia and DHCR24-mediated cholesterol metabolism in the brain, which warrants further investigation to better understand the intricate mechanisms underlying the pathogenesis of Alzheimer's disease. Therefore, in the present study, we examined the effect of excess cholesterol loading on the neuron damage and expression level of DHCR24 at both animal and cellular levels.

Materials and methods

Ethical statement

The present study was conducted in accordance with Laboratory Animal-Guideline for ethical review of animal welfare (GB/T 35,892–2018, National Standards of the People's Republic of China).

Reagents and chemicals

The detection kits for four items of blood lipid (TG, TC, LDL-C and HDL-C) were purchased from the Nanjing

Jiancheng Institute of Biotechnology (Nanjing, China). TRIzol reagent was obtained from Life Science Technologies Corporation (Cal., USA). Gibco BRL (Gaithersburg, MD, USA) provided foetal bovine serum (FBS). Dulbecco's modified Eagle medium (DMEM) was from Hyclone (Beijing, China). Eosin, and haematoxylin dye was purchased from Nanjing Chemical Reagent Factory (Nanjing, China). The recombinant adenovirus Ad-DHCR24 was purchased from Obio Technology (Shanghai) Corp., Ltd. 4, 6- Dianmidino-2-phenylindole (DAPI) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). PI Assay Kit was obtained from Beijing Sizhengbai Biotechnology Co. Ltd. (Beijing, China).

Primary antibodies against DHCR24, Akt, chop and bip were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-b-actin Rabbit mAb and secondary antibodies (HRP-labeled Goat Anti-Rabbit IgG) were obtained from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China).

Animals and feeding

Animal management procedures and handling methods strictly abide by the "Guidelines for the Management and Use of Laboratory Animal Feeding". Male C57BL/6 mouse were purchased from the Liaoning Changsheng Biological Company. Twenty-four C57/BL6 mouse were separated randomly into two groups and fed with either normal diet or high fat diet for 4 weeks and body weights were monitored every week.

Preparation of CLCD (Cholesterol-loaded Cyclodextrin)

Cholesterol (200 mg, Sigma) dissolved in 1 mL chloroform. β-Cyclodextrin (CD, 1 g) dissolved in 2 mL methanol. 0.45 mL aliquot of sterol solution was combined to CD solution, stirred, and dried under N2 gas. Resultant crystals dried for 24 h and stored in glass at room temperature. CLCD working solution: 50 mg crystals/1 mL serum free medium: warm to 37 °C and vortexed briefly.

Cell culture

N2a cells were obtained American Type Culture Collection, Manassas, VA, USA. The cells were cultured at 37 °C 10% FBS/DMEM in 5% CO_2 atmosphere in an incubator. For cholesterol treatment, CLCD working solution were incubated for 48 h with N2a cells.

Transfection and analysis

N2a cells were seeded onto 6-well plates at a density of 5×10^5 cells per well for 24 h until the confluence reached 60–80%. Lipofectamine 2000TM reagent was used for transfection according to the manufacturer's instructions. For each transfection, the recombinant adenovirus Ad-DHCR24/Ad-lacZ was prepared and overlaid onto the cells separately in DMEM medium and incubated for 6 h at 37 °C in a CO_2 incubator. Then, the medium was removed and replaced with 10% FBS/DMEM and after 42 h, the DHCR24 overexpression efficiency was evaluated by western blot. The control and Ad-lacZ group were used as negative controls.

Measurement of blood indexes in serum

Samples of blood serum were collected from all groups of mice and the levels of TG, TC, LDL-C, HDL-C were detected in accordance with the kit manual operation. The indicators were calculated following the instruction of the kit.

Lipid extraction and total cholesterol assay

To extract lipids for the total cholesterol measurement, the brain of mouse was homogenized in chloroform: methanol (2:1, 0.1 g brain/2 ml solvent) mix and centrifuged for 5 min at 2000 × g. After collecting the supernatant and added ddH_2O, mix and centrifuged for 10 min at 2000 × g. Collect the lipid-containing lower layer liquid (chloroform layer) and dry the liquid with N2 gas to obtain the lipid. High Performance Liquid Chromatography (HPLC) was used to measure the total cholesterol levels in the brain of mouse. In brief, make a standard curve of cholesterol (range 0–10 mg/mL). Dissolve the obtained total lipids in 1 mL of chromatographic grade absolute ethanol and filter the sample using a 0.22 μm filter. Set the sample detection conditions and methods, use the treated chromatographic grade methanol as the mobile phase, and the C18 column as the stationary phase, and detect at UV 210 nm.

Histopathological examination

Histopathological examination was carried out as described earlier with some modifications (Ishfaq et al. 2019). Briefly, brain samples were fixed in 10% formalin overnight, dehydrated in a series of alcohol solution and embedded in wax. After embedding, samples were cut into Sects. (5 μm), stained with H&E and observed under light microscope (Nikon E100, 40X magnification, Tokyo, Japan).

TUNEL assays

The cells were cultured in six-well plate for at 37 °C for 12 h. Then, the cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Subsequently, apoptosis of cells was determined with TUNEL

reaction mixture at 37 °C for 1 h. Cells were washed three-time in TBST and inverted on the glass slide with tablet containing DAPI. TUNEL assays were conducted using a TUNEL fluorescence FITC kit (Vazyme Nanjing, Jiangsu, China) according to the manufacturer's instructions. Finally, the slides were counterstained with hematoxylin, examined under a fluorescence microscope (Olympus, Tokyo, Japan), and the percentage of positive cells to the total cells was calculated in each sample.

Duolink™ assay system

Duolink™ fluorescence method was employed as per manufacturer's recommendations (O link Biosciences). N2a cells (1×10^4 cells) were seeded and differentiated in chamber slides. N2a cells were serum-starved overnight (DMEM and 2% BSA) and treated in microvesicle-free DMEM with 0.5% BSA for 24 h. Mouse DHCR24 antibody (Danvers, MA, USA) and Rabbit MDM2 antibody (Santa Cruz Technology, Inc) were used to assess DHCR24/MDM2 interactions. Images were taken with Zeiss LSM 780 confocal microscope.

Filipin staining assays

The cholesterol level of cells was detected by Filipin (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, cells were fixed with 3% paraformaldehyde for 30 min and three-time washed in TBST. Cells were incubated with Glycine solution (1.5 mg/mL) for 10 min. 1 mL of 5 mg/mL Filipin reagent was added in six-well plate. After incubation for 2 h at 37 °C in the dark, invert the cell slides on the glass slide with tablet containing PI. The sample fluorescence was measured.

Quantitative real-time RT-PCR

The Quantitative real-time RT-PCR analysis was performed in order to confirm the efficiency of DHCR24 expression on mRNA- level in the brain of mouse and N2a cells. Total RNA was extracted from the brain of mouse after feeding high-fat diet and normal diet for 4 weeks. Besides, total RNA was extracted from N2a cells after loading CLCD working solution for 48 h. RNAiso Plus (Total RNA extraction reagent, catalog no.9108/9109) was used to extract RNA from brain tissue and cells. Total RNA (500 ng) was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (catalog no. RR047A). Amplification of cDNAs was performed with Applied Biosystems 7500 Fast Real-Time PCR System, using the SYBR® Premix Ex Taq™ II (catalog no. RR820A). All reagents were purchased from TAKARA Biotechnology. All the procedures were carried out according to manufacturer's protocol. The comparative $^{\Delta\Delta}$Ct method was used to calculate GAPDH-normalized expression levels of DHCR24 mRNAs.

The primers used in the RT–PCR were:

DHCR24 sense 5' -GCCGCTCTCGCTTATCTTCG-3',

DHCR24 antisense 5' -GTCTTGCTACCCTGCTCCTT-3',

GAPDH sense 5'-GCACCGTCAAGGCTCAGAAC-3',

GAPDH antisense 5'-TGGTGAAGACGCCAGTGGA-3'.

Western blot

The western blotting analysis was performed in order to confirm the efficiency of DHCR24 expression on protein level in the brain of mouse and N2a cells. Total protein was extracted from the brain of mouse after feeding high-fat diet and normal diet for 4 weeks. While the total protein was extracted from N2a cells after loading CLCD working solution for 48 h. In brief, samples were mixed with equal volumes of 2 × SDS loading buffer and subjected to

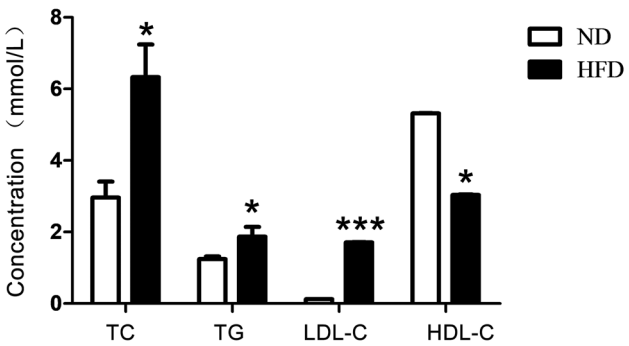
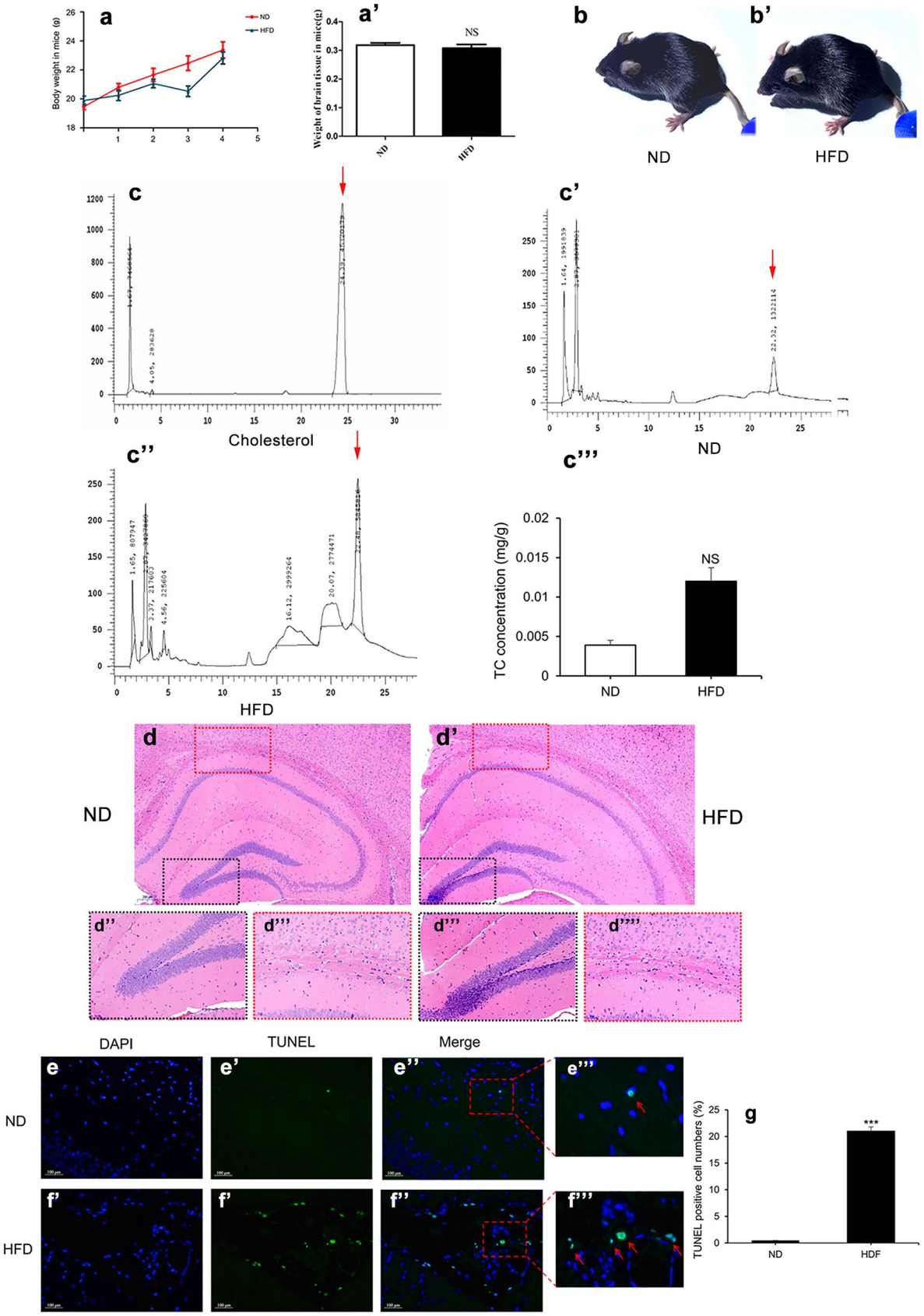
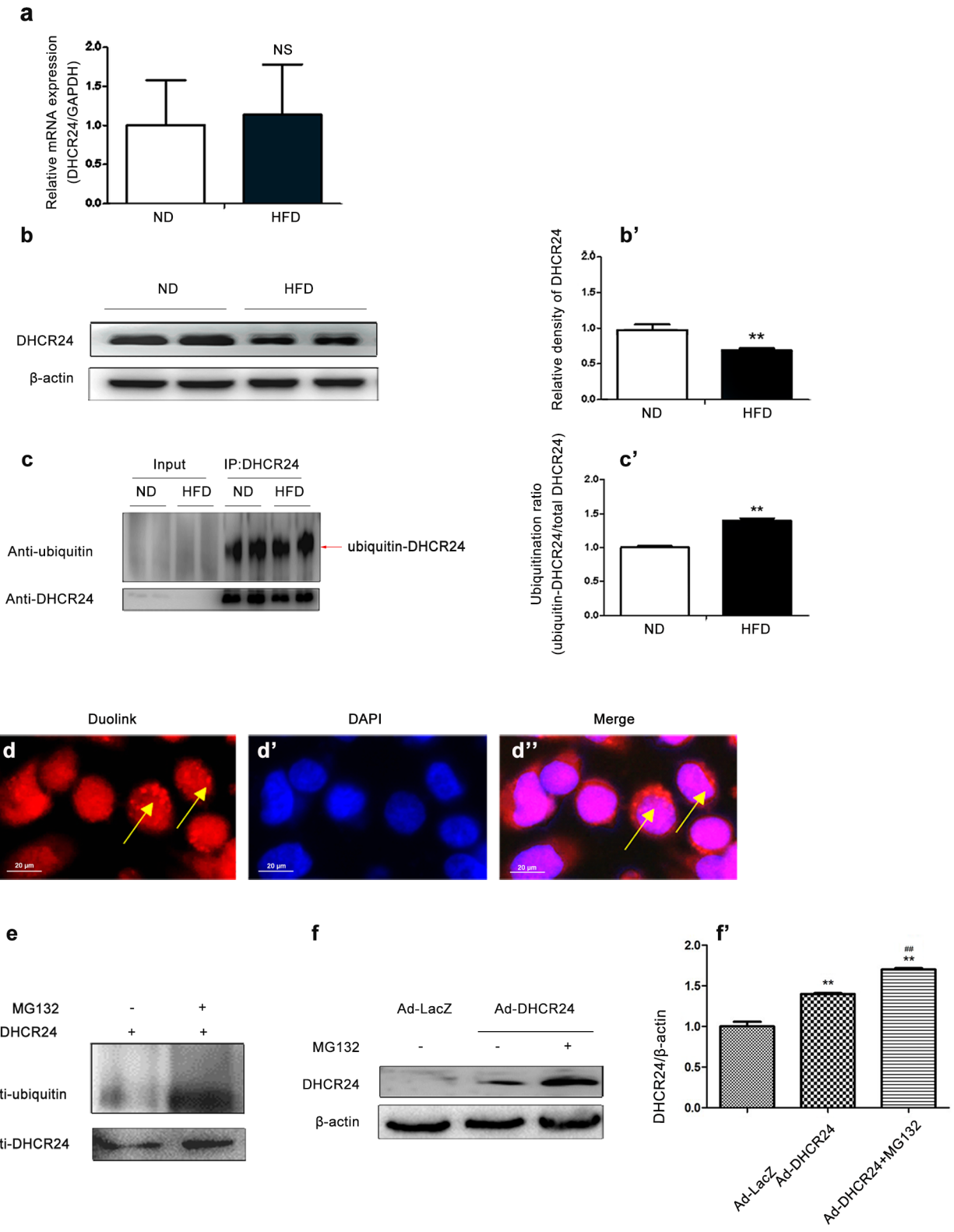


Fig. 1 Peripheral blood lipid is displayed in this figure. The experimental groups included the ND (Normal diet) and HFD group (Hight-fat diet). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus the ND group. The results are expressed as the mean ± SD, n = 5

Fig. 2 Hyperlipidemia causes brain damage in mice. **a**-**b**' The body weight, brain tissue weight and appearance change of mice were recorded within 4 weeks. **c**–**c**''' HPLC was used to detect the cholesterol content in the brain of the mice and calculate the cholesterol concentration per unit tissue. **d**-**d**'''' The photomicrographs by HE staining showed chromatin concentration (red arrows), loose structure and chaotic (red arrows). Scale bars, 200 μm. **e**–**g** TUNEL assays were performed on mice brain slices. Nuclei are stained with DAPI. Green fluorescence indicates TUNEL positive cells. Bar graph represents mean ± SD, n = 5. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus the ND group


a
ND
HFD
Body weight in mice (g)
a'
NS
Weight of brain tissue in mice(g)
ND
HFD
b
b'
ND
HFD
c
Cholesterol
c'
ND
c''
HFD
c'''
TC concentration (mg/g)
NS
ND
HFD
d
d'
ND
HFD
d''
d'''
d''''
DAPI
TUNEL
Merge
e
e'
e''
e'''
ND
f'
f'
f''
f'''
HFD
g
TUNEL positive cell numbers (%)
***
ND
HDF


a
Relative mRNA expression (DHCR24/GAPDH)
NS
ND
HFD
b
ND
HFD
DHCR24
β-actin
b'
Relative density of DHCR24
**
ND
HFD
c
Input
IP:DHCR24
ND
HFD
ND
HFD
Anti-ubiquitin
ubiquitin-DHCR24
Anti-DHCR24
c'
Ubiquitination ratio (ubiquitin-DHCR24/total DHCR24)
**
ND
HFD
Duolink
DAPI
Merge
d
d'
d''
20 μm
e
MG132
IP:DHCR24
Anti-ubiquitin
Anti-DHCR24
f
Ad-LacZ
Ad-DHCR24
MG132
DHCR24
β-actin
f'
DHCR24/β-actin
**
##
**
Ad-LacZ
Ad-DHCR24
Ad-DHCR24+MG132

◂**Fig. 3** Effect of hyperlipidemia on the regulation of DHCR24 expression in the brain of mice. **a** The expression levels of DHCR24 gene were determined by RT-PCR. The experimental groups included the ND group and HFD group. **b**, **b'** Hyperlipidemia induces down-regulation of DHCR24 protein expression in the brain of mice. The protein of brain was extracted, and the concentration was measured. Western blot assaying on the levels of DHCR24. The β-actin level was used as the internal standard. **c**, **c'** The ubiquitination level of DHCR24 was analyzed by co-immunoprecipitation. **d**-**d**'' DHCR24/MDM2 interactions were assessed by Duolink assay in N2a cells (yellow arrows). Scale bars, 20 μm. **e** N2a cells were treated with the absence and presence of MG132 (20 μM) for 6 h, respectively. The interaction of DHCR24 with MDM2 was assessed by co-immunoprecipitation. **f**, **f'** N2a cells transfected with Ad-lacZ/Ad-DHCR24 adenoviruses, untreated or treated MG132 for 6 h and the levels of DHCR24 were quantified by western blotting. The antibody was anti-DHCR24 and anti-ubiquitin body. Bar graph represents mean ± SD, n = 5. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus the ND group

10% SDS-PAGE. For each sample, a constant amount of protein (20 μg) was loaded on the gel. After electrophoretic separation, the proteins were transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in TBST for 1.5 h at room temperature. The protein blots were incubated with primary antibody (DHCR24, Bip or Chop) overnight at 4 °C, then probed with HRP-conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature. The membrane was then washed and used via enhanced chemiluminescence (ECL) detection (Amersham-Pharmacia Biotech). The level of immunoreactivity was assessed as a peak intensity using an image capture and analysis system (GeneGnome, Syngene, UK). The anti-β-actin antibody was used to control the protein quality and ensure equal loading.

Immunoprecipitation assays

Immunoprecipitation assays were performed in order to confirm the expression of ubiquitinated-DHCR24 in the brain of mouse. In brief, total protein was extracted from the brain of mouse. DHCR24 was immunoprecipitated with anti-DHCR24 monoclonal antibody (Santa). Immunoprecipitation assays were conducted using a Beaver-Beads™ Protein A/G Immunoprecipitation Kit (Beaver Suzhou, Jiangsu, China) according to the manufacturer's instructions. Immunoprecipitates were separated by electrophoresis and transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in TBST for 1.5 h at room temperature. The protein blots were incubated with primary anti-DHCR24 or anti-ubiquitin antibodies (MedChemExpress, Monmouth Junction, NJ, USA) overnight at 4 °C, then probed with HRP-conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature. The membrane was then washed and used via enhanced chemiluminescence (ECL) detection.

Statistical analysis

All the data were expressed as the mean ± SD. Statistical significance was determined using a student t-test or one-way analysis of variance (ANOVA) in the case of comparisons among more than two groups following Dunnett's T3 test. A value of $p < 0.05$ were considered as statistically significant. All the graphs were made by GraphPad Prism (Version 8.0.1).

Results

Establishment of hyperlipidemia model of mouse

To explore the effect of hypercholesterolemia on brain tissue, mice were fed with high-fat diet for 4 weeks to establish hyperlipidemia model. The levels of blood lipid, including LDL-C, TC, TG and HDL-C were examined in peripheral blood samples of mouse for control (Normal diet, ND) and Hyperlipidemic model (High-fat diet, HFD) groups. As shown in Fig. 1, TC, TG and HDL-C were increased in HFD group compared to the ND group ($p = 0.036$). Meanwhile, the LDL-C level was remarkably increased ($p = 0.00067$). The finding showed that Hyperlipidemia Model of mouse was successfully constructed. Therefore, the model was used for subsequent experiments.

Hyperlipidemia increases the cholesterol level and causes tissue damage of mice brains

To determine whether hyperlipidemia damage the brain of mouse, hyperlipidemia model was established by feeding mice with high-fat diet. The Body weight was recorded and monitored for four weeks, and brain tissue weight was recorded after dissection. Results are shown in Fig. 2a, there was no significant change in the body weight of the two groups of mice monitored at different time points, indicating that four weeks of high-fat diet did not cause a significant increase in the body weight and brain tissue weight of the mice. However, it can be observed that the fur of the model group is shiny, greasy and the oil secretion was significantly increased (Fig. 2b'). HPLC technology was performed to assess the cholesterol concentration in the brain tissues of mice. The brain cholesterol concentration in HFD group was significantly higher relative to ND group (Fig. 2c-c'''). Compared to the ND group, HE staining results showed that the pathological changes of the brain caused by hyperlipidemia was observed in HFD group. The nuclear chromatin in the DG area in the HFD group was significantly concentrated in the Hippocampus DG mouse, and the structure of

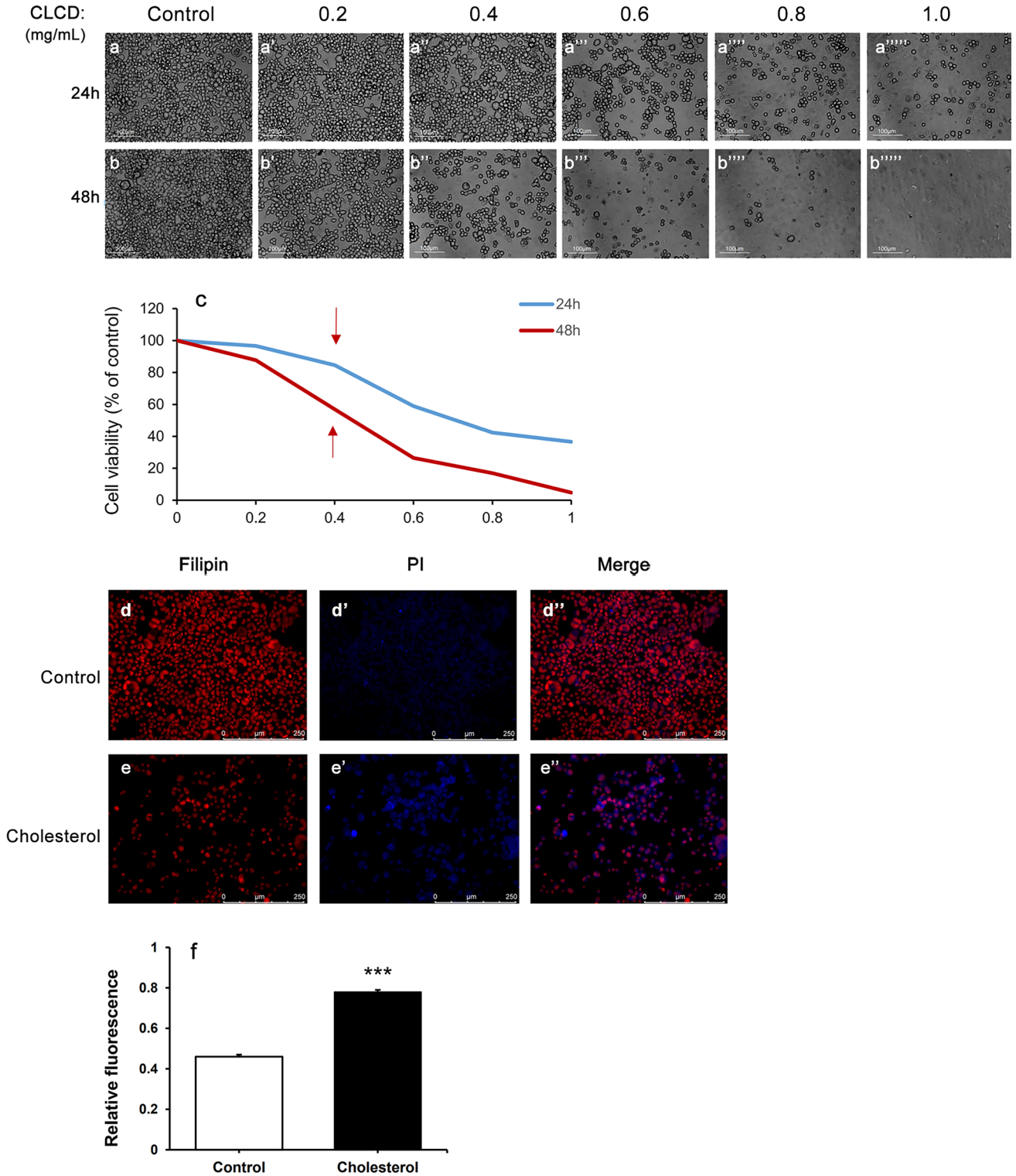


Fig. 4 Simulation of hypercholesterolemia in vitro by cholesterol loading. **a**-**c** Determination of cell viability in N2a cells following CLCD overload by inverted microscope. Different concentrations of CLCD (0, 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) were applied to N2a cells after 24 h and 48 h. Scale bars, 100 μm. Values are expressed as mean ± SD (n = 5). *$p < 0.05$ as compared with control group; **$p < 0.01$ as compared with control group. (d-f) After loading N2a with 0.4 mg/mL CLCD for 24 h, Filipin fluorescent staining was performed. Nuclei are stained with PI. Blue fluorescence indicates intracellular cholesterol levels. Scale bars, 250 μm

nerve cells in the cerebral cortex was loose and disordered (Fig. 2d-d''''). TUNEL assays of mouse brain slices showed that the HFD group significantly increased apoptosis in the hippocampus (Fig. 2e-g).

Hyperlipidemia downregulated the protein level of DHCR24 while increased the ubiquitination levels of DHCR24 protein in mice brain

To elucidate the relationship between hyperlipidemia-induced brain damage in mouse and DHCR24 expression, we employed RT-PCR and western blot to determine DHCR24 genes and protein expression levels, respectively. Compared with ND group, the mRNA expression level of DHCR24 in the hippocampus region of the brain in HFD group was not significantly changed (Fig. 3a). However, the western blot results showed that the expression of DHCR24 protein in the hippocampus region of the brain was significantly down-regulated (Fig. 3b, b'; $p = 0.0074$).

To further explore the molecular mechanism of downregulation of DHCR24 proteins, we performed co-immunoprecipitation to evaluate the modification of DHCR24 by ubiquitination. The results showed that ubiquitin modified DHCR24 of HFD group was significantly more than that of ND group in the IP-treated cell lysates while total levels of DHCR24 were significantly reduced of HFD group in both input and IP -treated cells (Fig. 3c, c'). This result suggested that elevated cholesterol level in brain induced by hyperlipidemia might down-regulated DHCR24 expression via increasing its ubiquitination-modified level.

To this end, this study further verified the ubiquitination of DHCR24 at the neuronal level using the mouse neuroblastoma cell line N2a cells. A previous study demonstrated that DHCR24 could interact with MDM2, a E3 ligase (Wu et al. 2004). Here we first performed the interaction between DHCR24 and MDM2 in our experimental system by a very powerful tool for detecting in situ protein–protein interaction by Duolink PLA assay. The Duolink assay showed increasing DHCR24/MDM2 interactions, characterized by red punctate dots in N2a cells (Fig. 3d-d''). Furthermore, after treated with proteasome inhibitor MG132 in N2a cells, the diffuse bands in the experimental group treated with 20 um MG132 were significantly increased, indicating that the ubiquitination level of DHCR24 was enhanced, supporting that DHCR24 is also modified by ubiquitination and therefore undergoes post-translational regulation at neuronal level (Fig. 3e). To further confirm the ubiquitin-modification of DHCR24, we overexpressed DHCR24 by adenovirus driving and performed directly Western Blotting analysis to study the effect of MG132 on the protein level change of DHCR24. N2a cells were transfected with Ad-lacZ and ad-DHCR24 recombinant adenovirus for 48 h, and one group of cells transfected with ad-DHCR24 recombinant adenovirus was treated with MG132 for 6 h. Western blotting results showed that compared with the control group without MG132 stimulation, the protein level of DHCR24 was significantly increased after MG132 stimulation (Fig. 3f, f'). Taken together, these results strongly demonstrated that DHCR24 could undergo the post-translational regulation through proteasome degradation pathway of DHCR24.

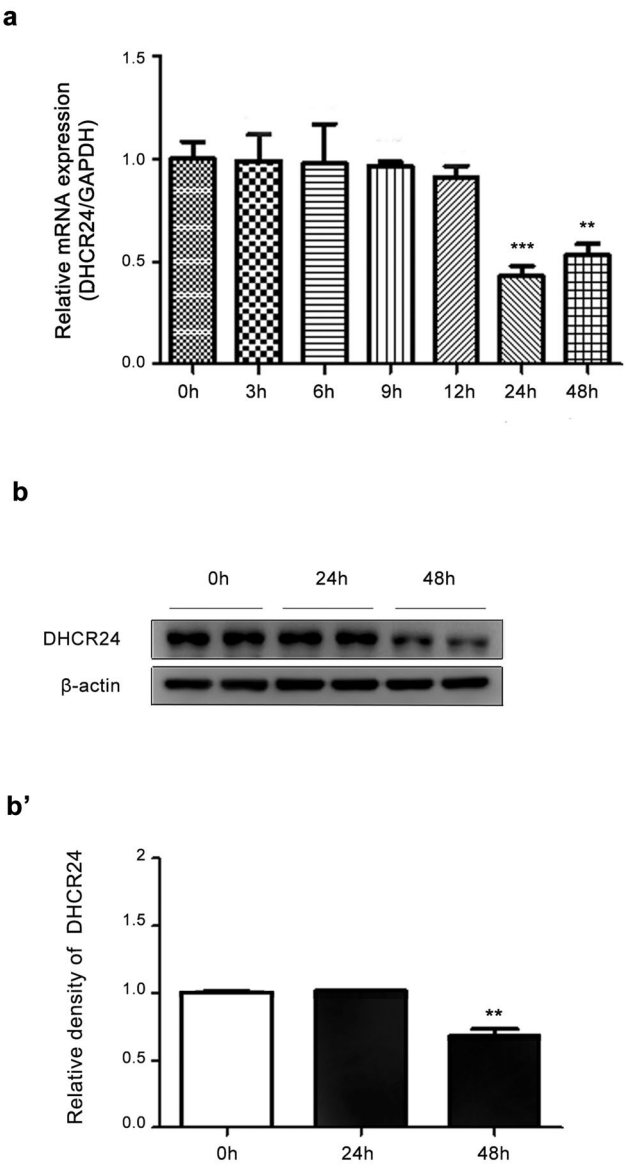


Fig. 5 Effect of cholesterol overload on DHCR24 expression in N2a cells. This figure shows a mRNA expression and b protein expression of DHCR24 in CLCD overload N2a cells of difference time groups. **a** The N2a cells was applied with 0.4 mg/mL CLCD load for up to 48 h by RT-PCR. **b**, **b'** CLCD overload induced ERS in N2a cells by western blotting. Bar graph represents mean ± SD, n = 3. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus the control group

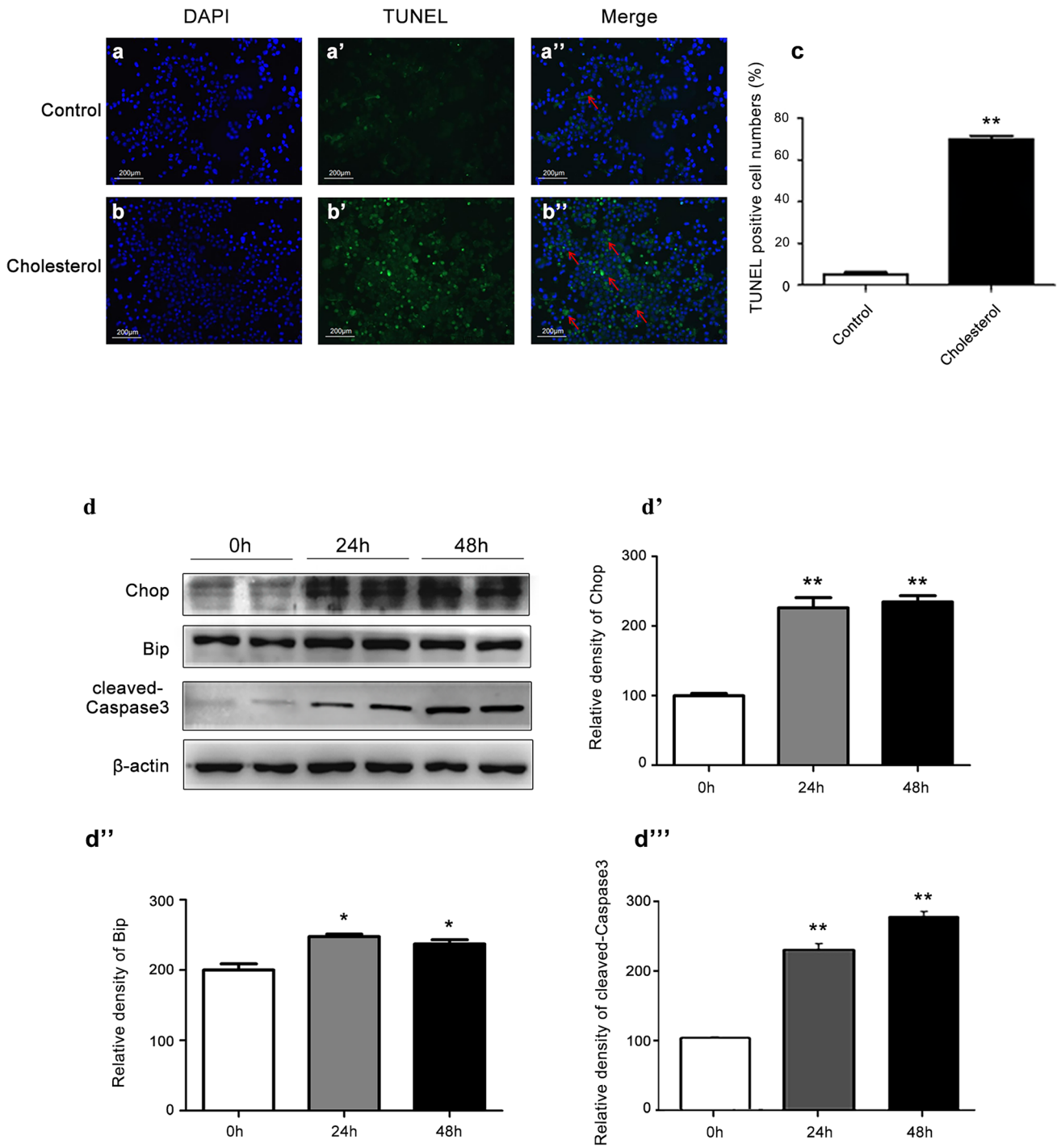


Fig. 6 Endoplasmic reticulum stress may mediate apoptosis upon cholesterol overload in N2a cells. **a**-**c** TUNEL assays of the control N2a cells and N2a overloaded with CLCD for 48 h. Nuclei are stained with DAPI. Green fluorescence indicates TUNEL positive cells (White arrows). Scale bars, 200 μm. **d**, **d**''' Expression analysis of chop, Bip in N2a cells overloaded with CLCD for 0, 24 and 48 h by western blotting. Bar graph represents mean ± SD, n = 3. *$p < 0.05$; **$p < 0.01$ versus the control group

Mouse neuroblastoma cells N2a is also sensitive to apoptosis induced by excess cholesterol loading

To study the effect of cholesterol loading directly on the neuronal cells at cellular level, we performed the subsequent experiments utilizing the N2a cells. To obtain an appropriate concentration of CLCD (Cholesterol-loaded Cyclodextrin, CLCD) overload in nerve cells, N2a cells were stimulated for 24 h and 48 h at a range of CLCD (0–1 mg/mL). The result showed that the numbers of adherent cells were significantly decreased in a dose and time-dependent manner in response to the different treatment of CLCD. A noticeable decrease in the number of adherent cells was observed in the group treated with 0.4 mg/mL of CLCD for 48 h when compared to the control group (Fig. 4a-c).

To explore whether the CLCD-loading in culture medium could increase the cellular cholesterol level, we performed the free cholesterol fluorescent staining by utilizing the cholesterol fluorescent probe called Filipin. As shown in Fig. 4d-f, the intensity of blue-fluorescent signal in CLCD-loading groups (Cholesterol group) was significantly stronger compared to control. This data demonstrated that the CLCD-loading in the culture medium could increase the cellular cholesterol level in N2a cells.

Excess cholesterol loading down-regulates both mRNA and protein expressions of DHCR24 in N2a cells

To further verify the effect of cholesterol overloading on DHCR24 mRNA and protein expression of N2a at the cellular level, we used RT-PCR and Western blot analysis to detect the expression level of DHCR24 after CLCD overloading at 0 h, 24 h, and 48 h. As shown in Fig. 5a, the mRNA expression of DHCR24 by RT-PCR was significantly reduced at 24 h ($p = 0.00032$) and 48 h ($p = 0.0066$) by CLCD overload compared to the control group (at 0 h). Meanwhile, DHCR24 proteins were also significantly decreased after 48 h of CLCD-overload (Fig. 5b, b').

Endoplasmic reticulum stress may mediate apoptosis upon cholesterol overload in N2a cells

In order to examine whether CLCD overload induces apoptosis in N2a cells, the TUNEL assay was performed to assess the apoptosis in CLCD-overloaded N2a cells. Compared with the control group, the green-fluorescent signals representing TUNEL-positive cell were markedly increased in CLCD-overloading group (Fig. 6a-c), suggesting that the CLCD-overloading induced the apoptosis of N2a cells.

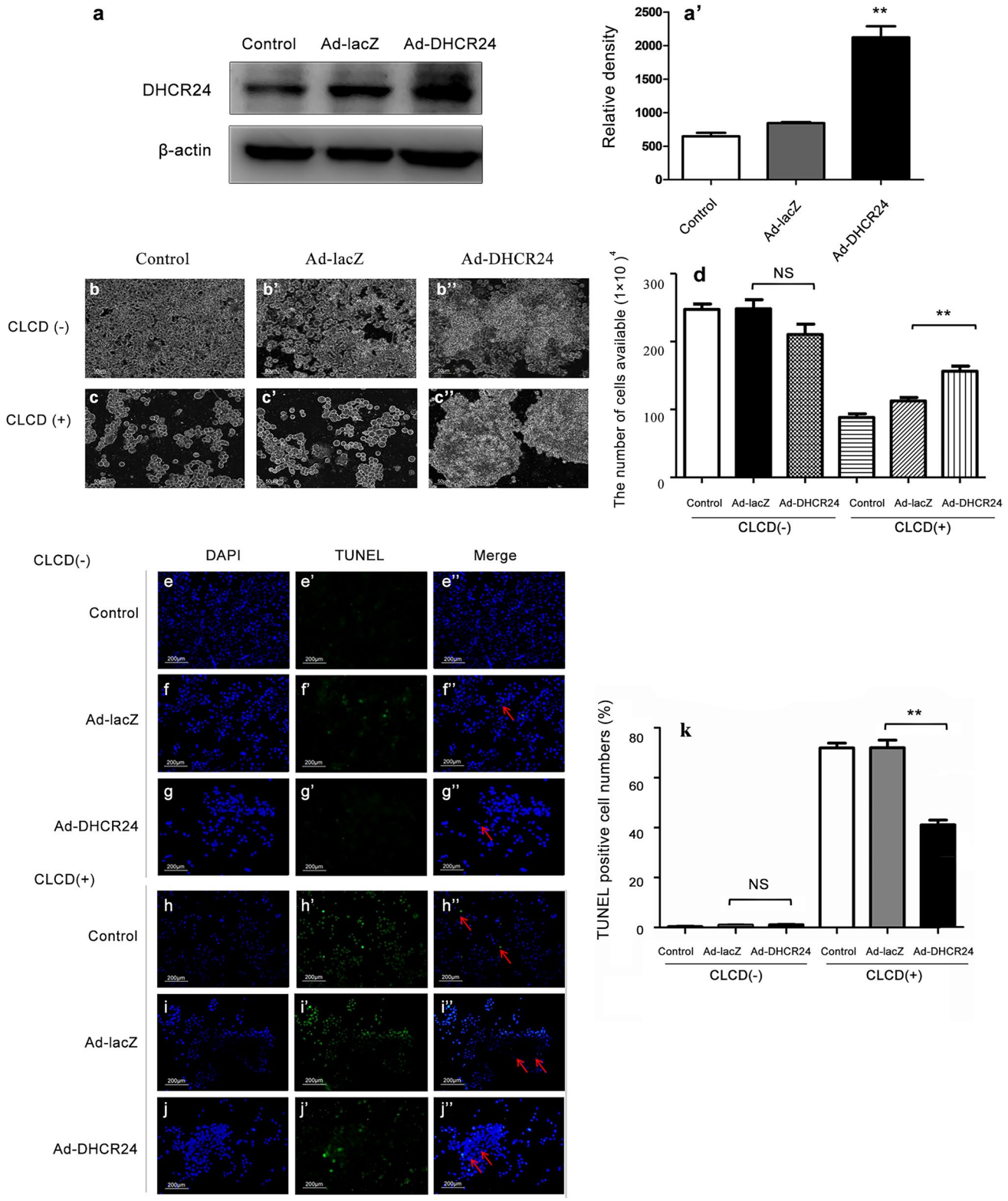
Some studies have confirmed that the excessive activated endoplasmic reticulum stress signaling pathway may not only contribute to the occurrence of apoptosis but induce protein ubiquitination and degradation (Chaudhari et al. 2014; Cybulsky 2013). We therefore used western blot to detect the endoplasmic reticulum stress (ERS) response iconic protein (Bip and Chop) and apoptosis iconic protein (Caspase-3). As illustrated in Fig. 6d-d''', Bip, Chop and cleaved-Caspase-3 expression were up-regulated after CLCD load, suggesting that CLCD load could induce ERS and apoptosis.

Ad-DHCR24 reversed cholesterol overloaded induced apoptosis in N2a cells

Finally, we used the manipulated genetic approach to gain further insight into the neuron-protective function of DHCR24 during cholesterol overloading-induced apoptosis in N2a cells. In the present study, the overexpression of DHCR24 in N2a was achieved by infection of the recombinant adenovirus vector Ad-DHCR24, and the adenovirus Ad-lacZ as a control group. As depicted in Fig. 7a, a', the Western blotting analysis confirmed the significant upregulation of DHCR24 expression induced by Ad-DHCR24, providing robust evidence of successful DHCR24 overexpression. Moreover, the number of adherent cells in the Ad-DHCR24-infected group was significantly higher after CLCD overloading (Fig. 7b-d). TUNEL assays showed that the number of green fluorescence dots reduced in CLCD overloaded N2a cells infected by Ad-DHCR24 in comparison to that tin Ad-lacZ-infected group (Fig. 7e-k). This data strongly demonstrated that the overexpression of DHCR24 could rescue neuronal cells from excess cholesterol loading-induced apoptosis.

Discussion

Hyperlipidemia, a systemic disorder of lipid metabolism caused by various factors, has emerged as a global health crisis due to its chronic nature. Experimental studies have demonstrated that high-fat diet (HFD)-induced midlife obesity has been implicated in the development of dementia, highlighting the potential association between hyperlipidemia and cognitive decline (Bandosz et al. 2020); promotes Aβ protein expression and memory loss in the brain of transgenic mice (Matthews et al. 2016), and Xiong et al. proposed that high-fat diet (HFD) may exacerbate tau pathology in P301L transgenic mice, underscoring the potential contribution of HFD to the progression of tau-related pathology (Xiong et al. 2022). However, increased production of Aβ-amyloid and hyperphosphorylated tau protein are


a
Control
Ad-lacZ
Ad-DHCR24
DHCR24
β-actin
a'
Relative density
2500
2000
1500
1000
500
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Control
Ad-lacZ
Ad-DHCR24
Control
Ad-lacZ
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CLCD (-)
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300
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Control
Ad-lacZ
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Ad-DHCR24
CLCD(-)
CLCD(+)
CLCD(-)
DAPI
TUNEL
Merge
Control
Ad-lacZ
Ad-DHCR24
CLCD(+)
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Ad-lacZ
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e
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h'
h''
i
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j
j'
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200μm
k
TUNEL positive cell numbers (%)
80
60
40
20
0
NS
**
Control
Ad-lacZ
Ad-DHCR24
Control
Ad-lacZ
Ad-DHCR24
CLCD(-)
CLCD(+)

◂**Fig. 7** CLCD-overload-induced apoptosis was reversed by overexpression of DHCR24 in N2a cells. **a**, **a**' Ad-DHCR24 up-regulates DHCR24 expression in N2a cells. N2a cells transfected with Ad-lacZ /Ad-DHCR24 adenoviruses, and the levels of DHCR24 were quantified by western blotting. Quantification of the protein levels following transfection with Ad-DHCR24, $^{**}p < 0.01$ versus Ad-lacZ group. Gels were representative of three different experiments. **b**-**d** N2a-Ad-DHCR24 cells were overloaded with CLCD for 48 h, then these were observed by morphology light microscope. Scale bars, 50 μm. **e**-**k** Adenovirus Ad-DHCR24 reverses CLCD load induced apoptosis of N2a cells. N2a cells were infected with adenovirus for 48 h and then loaded with CLCD for 48 h. N2a cells were infected with Ad-DHCR24 adenovirus for 48 h and then loaded with CLCD for 48 h by TUNEL assays. Nuclei are stained with DAPI. Green fluorescence indicates TUNEL positive cells. Scale bars, 200 μm

significant pathological hallmarks of AD, and are strongly associated with the severity of dementia. A longitudinal 13-year follow-up study revealed that elevated levels of total cholesterol and low-density lipoprotein cholesterol are associated with an increased risk of AD, further underscoring the potential role of cholesterol metabolism in the pathogenesis of AD (Schilling et al. 2017). As a result, researchers advocate for the adoption of preventive measures and risk reduction strategies for Alzheimer's disease and related dementias (ADRD), which include maintaining a healthy lifestyle and effectively managing diseases such as dyslipidemia. These proactive approaches may hold promise in mitigating the risk of AD and related cognitive impairments, highlighting the importance of comprehensive disease management strategies for brain health (Chen et al. 2018; Lourida et al. 2019; Organization 2019). Several clinical studies provide evidence of a bidirectional relationship between metabolic disease and cognitive impairments forming a vicious circle (Xiong et al. 2022). Nevertheless, the precise mechanisms by which increased peripheral blood lipids resulting from a high-fat diet exert deleterious effects on the brain remain incompletely understood. For instance, it is not yet clear whether hyperlipidemia, coupled with hypercholesterolemia, can effectively traverse the blood–brain barrier and induce pathological changes in brain tissue. Further research is needed to elucidate the intricate interplay between peripheral lipid metabolism and brain health, shedding light on the potential impact of hyperlipidemia on brain function and neurodegenerative diseases. To this end, this study established a C57/BL6 high-fat model mouse, and HPLC confirmed that hyperlipidemic mouse were accompanied by the increase of brain cholesterol level (Fig. 2c''). In addition,

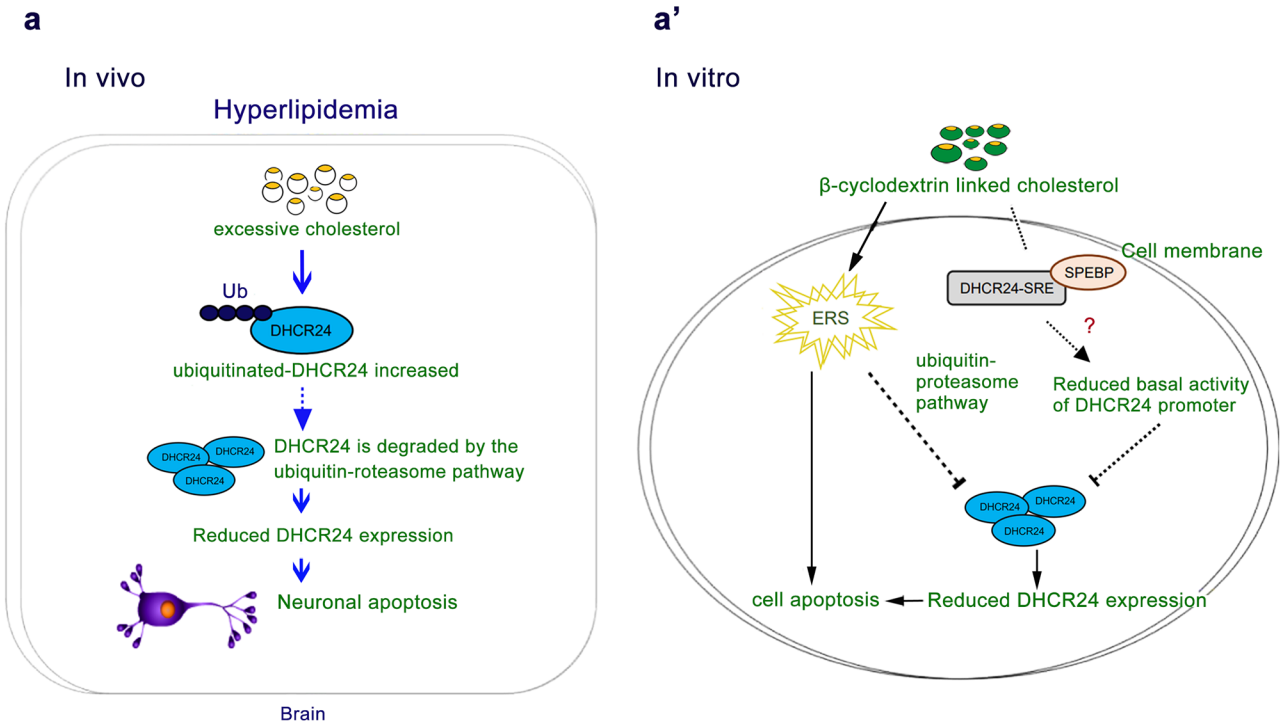

Fig. 8 The underlying mechanism of hyperlipidemia induced in brain tissue and DHCR24 expression changed in mouse. **a** Importantly, peripheral hyperlipidemia could be accompanied by the increase of cholesterol content in the brain of mouse, and the increase of ubiquitination modification of DHCR24 leads to the down regulation of protein expression, thus weakening its neuroprotective effect. **a**' In vitro results showed that extracellular cholesterol-loading could increase the intracellular cholesterol level, and the protein and mRNA levels of DHCR24 were down-regulated that could lead to apoptosis

we assessed the histopathological alterations in the hippocampal formation of mouse brains using HE staining and TUNEL apoptosis detection. Remarkably, we observed significant apoptosis phenomenon, indicating the presence of apoptotic cell death in the hippocampal region (Fig. 2d', f'), suggesting that hyperlipidemia accompanied by hypercholesterolemia that could cause the brain damage. HE staining and TUNEL results revealed prominent chromatin condensation in the nucleus of the hippocampal region, disordered arrangement of neurons in the cerebral cortex region, and disrupted tissue architecture, accompanied by evident apoptosis (as depicted in Fig. 2d', f'). Based on our findings, it can be inferred that hyperlipidemia may trigger cholesterol accumulation in the brain, resulting in detrimental effects on brain tissue.

As per existing knowledge, brain cholesterol is an essential component of nerve cells and is primarily synthesized in situ by astrocytes, given that lipoproteins are unable to cross the intact blood–brain barrier (Dietschy and Turley 2001). While plasma cholesterol does not directly impact neurons, research conducted on animals has demonstrated that hypercholesterolemia may indeed compromise the integrity of the blood–brain barrier (de Oliveira et al. 2020;). Furthermore, studies have shown that cholesterol accumulation has been observed in distinct brain regions, such as the cortex, striatum, hippocampus, and substantia nigra, in chronic mouse models of hypercholesterolemia. These findings suggest that hypercholesterolemia may significantly compromise the blood–brain barrier in mice, leading to increased cholesterol levels in the brain (Paul and Borah 2017). Based on the results of this study, it is proposed that hyperlipidemia induced by a high-fat diet may result in cholesterol accumulation in the brain by compromising the integrity of the blood–brain barrier, thereby leading to pathological changes in the hippocampus.

Cholesterol stable metabolism plays a crucial role in neuronal information transmission (Kacher et al. 2019). Several studies have found that DHCR24, a key enzyme that catalyses the final step of endogenous cholesterol synthesis, is present in almost all neurons, and its gene mutation (E191K, N294T, K306N, and Y471S) and can cause elevated plasma and tissue cholesterol levels, leading to strepsteremia (Andersson et al. 2002). Furthermore, our related studies on virtual screening of DHCR24 inhibitors for reducing cholesterol have yielded promising results (Wang et al. 2023). DHCR24 is localized in the endoplasmic reticulum and directly involved in oxidative stress response (Greeve et al. 2000a, b). At the same time, several studies and previous experiments of our research group have confirmed that DHCR24 play a neuroprotective role by antagonizing apoptosis induced by oxidative stress and endoplasmic reticulum stress, or by maintaining cell survival signaling pathway that was related to growth factor receptor in caveolae (Lu et al. 2014). However, the impact of hyperlipidemia-induced increase in peripheral blood cholesterol on the expression of DHCR24 in the brain remains a secondary issue to be addressed. The expression levels of DHCR24 gene and protein in the brain tissue of mice were detected. It was found that the high-fat diet had no significant effect on the mRNA expression of DHCR24, but the protein expression was significantly downregulated (Fig. 3a and b). Excessive cholesterol has been reported to form oxysterol or lanosterol, which can induce insulin-induced gene protein-1 (insig-1) to promote the recruitment of HMGCR membrane domain by ubiquitin ligase AMFR/gp78. This leads to ubiquitination and dissociation of HMGCR from the membrane, followed by degradation through the endoplasmic reticulum-related degradation pathway. As a result, cholesterol synthesis is inhibited and cholesterol content is rapidly reduced (Jansen et al. 2013; Zerenturk et al. 2013). This post-transcriptional regulation represents an important and rapid feedback mechanism for cholesterol homeostasis, complementing the downregulation of cholesterol synthesis-related enzymes at the transcriptional level. It is reported that DHCR24 can interact with MDM2, the E3 ligase so that it might be ubiquitinated (Wu et al. 2004). We also confirmed that DHCR24 could interact with E3 ubiquitin ligase MDM2 through Duolink experiment (Fig. 3d-d''), while DHCR24 can be modified by ubiquitination at both animal and cellular level (Fig. 3e, f). Therefore, we speculate that the mechanism of downregulation of DHCR24 protein may be related to the increase of cerebral cholesterol. In the present study, IP experiment was used to elucidate the hypothesis that the increased ubiquitination of DHCR24 protein led to the downregulation of DHCR24 protein expression in the brain tissue of mouse with hyperlipidemia (Fig. 3c). These data suggested that hypercholesterolemia induced by high-fat diet promotes the ubiquitination and degradation of DHCR24 protein, which weakens its protective effect on the brain neurons. Moreover, in our follow-up studies, we used recombinant adenovirus Ad-DHCR24 to drive the overexpression of DHCR24 protein in N2a cells, confirming that up-regulation of DHCR24 protein expression can reverse the apoptosis of N2a nerve cells induced by cholesterol loading (Fig. 7e-j''). Hence, our data present the first evidence that DHCR24 overexpression can safeguard neuronal cells from apoptosis induced by cholesterol-loading, underscoring the potential of targeting DHCR24 as a promising strategy for developing novel therapeutic interventions against AD, particularly in the context of hyperlipidemia. For the first time, our findings demonstrate that overexpression of DHCR24 can protect neuronal cells from cholesterol-induced apoptosis, indicating that targeting DHCR24 overexpression could be a promising strategy for mitigating pathological symptoms such as memory decline and cognitive impairment associated with hypercholesterolemia.

A balanced metabolism of cholesterol plays a crucial role in various neurobiological processes (Kacher et al. 2019). Previous research has indicated that brain cholesterol levels decrease during both Alzheimer's disease (AD) and normal aging (Cecchi et al. 2008). DHCR24, a key player in de novo cholesterol synthesis, has been extensively proven to play a role in the protection of neuronal cells during stressful conditions (Wu et al. 2004; Lu et al. 2014; Cecchi et al. 2008). Through in vivo experiments, we have shed light on the protective mechanism of DHCR24 on neuronal damage caused by hypercholesterolemia, as demonstrated by our cell-level experiments. In our study, the effect of cholesterol loading on the expression of DHCR24 protein and the survival of nerve cells, we first confirmed that cholesterol loading induced the increase of intracellular cholesterol level in nerve cells (Fig. 4d-f), and found that the expression of DHCR24 protein was down regulated after cholesterol loading (Fig. 5b), which was consistent with the experimental results in vivo. It is noteworthy that the apoptosis increased significantly in N2a cells after cholesterol-loading (Fig. 6a-c), and the endoplasmic reticulum stress response iconic protein (Bip and chop) was significantly upregulated (Fig. 6d), suggesting that cholesterol-loading could lead to activation of endoplasmic reticulum stress pathway. We speculate that this result may be related to the increased ubiquitination modification of DHCR24 and the downregulation of protein levels, and induces the occurrence of apoptosis. RT-PCR showed that the mRNA expression of DHCR24 gene was also down regulated at 24 h and 48 h after cholesterol loading (Fig. 5a). This may be because extracellular cholesterol-loading may destroy cholesterol homeostasis, which leads to the negative feedback regulation of DHCR24 and the decrease of mRNA expression at the transcriptional level at cellular level. In HepG2 cells where LDLR is the sole source of cholesterol, sterol regulatory element binding proteins (SREBPs) can regulate the sterol regulatory elements (SRE) in the proximal promoter of DHCR24 according to the increase of cholesterol concentration. Thus, reducing the basal activity of DHCR24 promoter resulting in the decrease of DHCR24 mRNA expression (Daimiel et al. 2012). The present study may suggest that similar regulatory mechanisms may also exist in N2a nerve cells, of course further studies are needed to confirm. Cholesterol in brain tissue is mainly synthesized by glial cells, and apolipoprotein E is responsible for transporting it to neurons. Therefore, the downregulation of DHCR24 protein expression associated with increased cholesterol may be mainly due to protein degradation mediated by ubiquitin proteasome pathway both in vivo and in vitro, while the regulation of mRNA expression of DHCR24 on the transcription level in response to cholesterol loading at cellular level was also observed although its mechanism needs to be further studied.

The results of this study are summarized in Fig. 8. Peripheral hyperlipidemia could be accompanied by the increase of cholesterol content in the brain of mouse, and the increase of ubiquitination modification of DHCR24 leads to the down regulation of protein expression, thus weakening its neuroprotective effect in vivo. While, extracellular cholesterol-loading increased the intracellular cholesterol level in vitro, and the protein and mRNA levels of DHCR24 were both down-regulated, leading to apoptosis. The down regulation of DHCR24 protein expression may be related to the activation of endoplasmic reticulum stress and the degradation of ubiquitinated proteasome pathway of DHCR24. Overexpression of DHCR24 could protect the nerve cells from cholesterol-overloading.

Conclusion

Hyperlipidemia, as a chronic disease, has posed a significant global health crisis. In recent years, the association between hyperlipidemia accompanied by hypercholesterolemia with memory impairment and cognitive decline has gained widespread recognition as a major contributing factor, garnering significant attention in research and clinical settings alike. This study suggests that the increase in cholesterol levels in brain accompanied by hyperlipidemia may lead to endoplasmic reticulum stress, resulting in increased ubiquitination of DHCR24 protein and down-regulated expression, thus weakening the neuroprotective effect. The overexpression of DHCR24 driven by recombinant adenovirus, could reverse the damage of cholesterol loaded neurons. In summary, our study provides a possible molecular explanation for hyperlipidemia induced brain injury and cognitive impairment, and suggests that upregulating the expression of DHCR24 protein may become a new method for preventing and treating hyperlipidemia induced AD.

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Author's contribution Xiuli Lu and Bing Gao supervised the whole experiments. Ziyin Lu and Haozhen Wang, designed this study and contributed to the paper writing. Xiujin Zhang, Xiuting Huang, Shan Jiang, Chen Lu, Yang Li, and Ting Liu performed the practical work and completed the experiments. All authors have read and approved the final version of this manuscript to be published.

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Data availability Please contact author for data requests.

Declarations

Ethics approval and consent to participate The present study was conducted in accordance with Laboratory Animal-Guideline for ethical review of animal welfare (GB/T 35,892–2018, National Standards of the People's Republic of China).

Consent for publication Not applicable.

Competing interest The authors declare that there are no conflicts of interest.

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