Ageing related thyroid deficiency increases brain-targeted transport of liver-derived ApoE4-laden exosomes leading to cognitive impairments

Manman Zhang

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Wenliang Gong

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Beina Chen

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Ming Ji

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Binjie Chen

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Dianjun Zhang

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Xinyu Li

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Yuefei Zhou

Department of Orthopaedics, The First Hospital, China Medical University

Chengyi Dong

Department of Orthopaedics, The First Hospital, China Medical University

Gehua Wen

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Xiaoni Zhan

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Xiafang Wu

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Maosheng Xia

Department of Orthopaedics, The First Hospital, China Medical University

Baoman Li (✉ bmli@cmu.edu.cn)

Department of Forensic Analytical Toxicology, School of Forensic Medicine, China Medical University

Alexei Verkhratsky (✉ Alexej.Verkhatsky@manchester.ac.uk)

Faculty of Biology, Medicine and Health, The University of Manchester

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Abstract

Alzheimer's disease (AD) is the prevalent cause of dementia in the ageing world population. Apolipoprotein E4 (ApoE4) allele is the key genetic risk factor for AD, although the mechanisms linking APOE4 with neurocognitive impairments and aberrant metabolism remains to be fully characterised. We discovered significantly increase in the ApoE4 contents of serum exosomes in old healthy subjects and AD patients carrying ApoE4 allele as compared with adult healthy subjects. Elevated exosomal ApoE4 demonstrated significant inverse correlation with serum thyroid hormones level and cognitive function. We analysed effects of ApoE4 in peripheral exosomes on the neurological disorders in aged or thyroidectomized young mice. Ageing-associated hypothyroidism as well as acute thyroidectomy augmented transport of liver-derived ApoE4 reach exosomes into the brain, where ApoE4 activates NLRP3 inflammasome by increasing cholesterol level in neural cells. This, in turn, affects cognition, locomotion and mood. Our study reveals pathological potential of exosomes-mediated relocation of ApoE4 from the periphery to the brain, which process can represent potential therapeutic target.

Main

Alzheimer's disease (AD) is the leading cause of age-associated neurodegeneration, with no effective therapeutic management. Apolipoprotein E (ApoE) serves multiple functions including maintenance of the structure of the lipoprotein particles and regulation of the metabolism of several different lipoprotein¹. Human ApoE exists in three isoforms, ApoE2, ApoE3, and ApoE4, which differ only in two residues. The ApoE4 allele has been reported as the genetic risk factor for late-onset, sporadic AD almost three decades ago²⁻⁴, yet how ApoE4 predisposes to AD remains incompletely understood. Several reports have identified an association between dysregulation of thyroid hormones (THs) and AD⁵⁻⁷. Serum levels of THs (total triiodothyronine (TT3), free T3 (FT3), total thyroxine (TT4) and free thyroxine (FT4)) gradually decrease with age in humans and rodents⁸⁻¹⁰. Whether thyroid hormonal status is affected in ApoE4 allele carriers is unknown. In the body ApoE4 is mainly produced by hepatocytes, whereas in the brain ApoE4 is primarily expressed in and secreted by astrocytes¹¹⁻¹². The 34kDa ApoE4 cannot pass the blood brain barrier (BBB), however exosomes are reported to mediate transport of nucleic acid and proteins across BBB¹³. In this study, we demonstrate that age-dependent decline in thyroid hormones promotes the transport of liver-derived ApoE4 into the brain by accelerating exosomal delivery, this peripheral ApoE4 facilitates cognitive impairments.

Multiple correlations between THs level, cognitive abilities and ApoE4 -containing serum exosomes in cognitively preserved and cognitively compromised subjects

We selected 60 ApoE4 heterozygous subjects including 15 healthy adults (22 - 48 years old), 15 healthy elderly (68 - 85 years old) and 30 AD patients (69 - 83 years old). All participants were subjected to cognitive evaluation with mini-mental state examination (MMSE); their cognitive status was correlated with blood levels of THs including TT3, FT3, TT4 and FT4, and ApoE4 content of serum exosomes (Fig. 1A). The level of ApoE4 normalised to b-actin in serum exosomes was significantly increased in old and AD groups compared with adult group (Fig. 1B), whereas ApoE4 content of serum exosomes in AD group was significantly higher than in the old healthy subjects ($p=0.0112$). At the same time, the non-exosome profiles were extracted from the supernatant after exosomes removal after ultracentrifuge, in which ApoE4 did not differ between adult, old and AD subjects (Fig. 1C). Levels of TT3, FT3, TT4 and FT4 decreased in old and AD groups when compared to young adults; although there was no significant difference between old and AD subjects (Fig. 1D). All THs were inversely linearly correlated to the normalised ApoE4/b-actin level ($p < 0.01$; Fig. 1D-right inserts). In the cognitive related tests, the MMSE and MoCA scores were significantly decreased in AD group as compared with young and old healthy subjects (Fig. 1E). Correlations between MMSE or MoCA scores and the corresponding ApoE4 exosomal content similarly presented inverted linear relationship ($p<0.01$; Fig. 1E-right inserts). According to the value of R^2 , the most relevant indicators are FT3 levels and MoCA scores; the multiple correlation analysis demonstrated that exosomal ApoE4 content, presents significant inverse relationship with FT3 level and cognitive capacities (Fig. 1F). The increased exosomal ApoE4 was associated with the lower THs level and the impaired cognition, hence we may suggest that age-dependent thyroid insufficiency may promote peripheral exosome transportation of ApoE4 thus facilitating cognitive decline.

Ageing and hypothyroidism accelerated ApoE4 transport from the liver to the brain

To identify changes of serum exosomes ApoE4 transport with ageing, we set an adult (3 months-old) and old (18 months-old) wild type while 2 months-old mice were randomly separated into sham and thyroidectomy surgery groups. The experimental design is shown in Fig. 2A.

Lentiviral transfection of hepatocytes with human ApoE4 (h-ApoE4) under control of hepatocyte specific protein arginase-1(Arg1) promoter¹⁴⁻¹⁵ was achieved by microinjection through hepatic portal vein (Fig. 2B). Following this procedure hepatocytes selectively express h-ApoE4 as shown in Fig. 2C; mice injected with lentivirus without vector served as negative control. To characterise transport of h-ApoE4, liver and serum exosomes were extracted (Fig. 2D). Protein levels of h-ApoE4 in both exosomal pools were significantly increased in old compared to adult mice ($p<0.0001$). Adult mice in thyroidectomy group similarly demonstrated increased h-ApoE4 levels in exosomes extracted from liver ($p=0.0013$) and serum ($p<0.0001$) (Fig. 2E). Two weeks after injection of h-ApoE4 co-expression vectors the immunostaining for h-ApoE4 and AQP1 (the latter was used to stain epithelial cell) was performed in choroid plexus. The

immunoreactivity for liver-originated h-ApoE4 was readily detected in the choroid plexus of old mice, but was rarely observed in adult mice (Fig. 2F). The ApoE4 was detected in epithelial cell thus indicating transcellular transport. Significant amounts of liver derived h-ApoE4 ended up in cortex and hippocampus of old and thyroidectomied animals (Fig. 3A). Immunofluorescence intensity of h-ApoE4 in cortex and hippocampus was significantly increased in old and thyroidectomy animals as compared with adult group ($p < 0.0001$), whereas no significant difference between adult and sham group has been detected (Fig. 3B).

Levothyroxine decreases h-ApoE4 in exosomes of old ApoE4-KI mice

We used human APOE4 knock-in (ApoE4-KI) mice to characterise exosomal ApoE4 transport in association with age-dependent THs decline. Adult, old, sham and thyroidectomy male ApoE4-KI mice groups were set, as shown in Fig. 4A. Levels of TT3, FT3, TT4 and FT4 were decreased in old ($p < 0.0001$) and thyroidectomied mice ($p < 0.0001$). Administration of levothyroxine (L-thy, 20 mg/kg/day, intraperitoneal injection; i.p.) for 14 days effectively increased THs levels in old mice, as compared with old group (Fig. 4B). Liver and serum exosomal h-ApoE4 levels were significantly increased in old and thyroidectomy mice as compared with adult and group ($p < 0.0001$), and there was no significant difference in h-ApoE4 levels between adult and sham groups. Injection of L-thy effectively reduced h-ApoE4 content of liver and serum exosomes compared with untreated old mice ($p < 0.0001$) (Fig. 5A and 5B). Exosomal CD9 was significantly higher in old and thyroidectomy groups ($p < 0.0001$), being significantly decreased by L-thy injection ($p < 0.0001$) (Fig. 5C and 5D). Exosomal CD81 significantly decreased in old or thyroidectomy group as compared with adults ($p < 0.0001$), this decrease recovered after injection of L-thy ($p < 0.0001$) (Fig. 5C and 5E). Correlation between the h-ApoE4 levels and THS is shown in supplementary Fig. 1. Similarly to observations on human subjects, exosomal h-ApoE4 content demonstrated inverse correlation with THs levels in ApoE4-KI mice (supplementary Fig. 1A and 1B).

Ageing increases cerebral accumulation of ApoE4 triggered the elevation of ROS

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In cortex and hippocampus of ApoE4-KI mice, cholesterol levels were significantly increased in both old and thyroidectomied animals when compared to adult controls ($p < 0.0001$). Intraperitoneal injection of ApoE4 inhibitor PH-002¹⁶ at 200 mg/kg/day for 14 days, reduced elevated cholesterol in both cortex ($p = 0.0401$) and hippocampus ($p = 0.0367$) of old mice (Fig. 6A). Glutathione (GSH) mitochondrial content was significantly reduced in the cortex and hippocampus of old and thyroidectomied mice ($p < 0.0001$). Injection of PH-002 recovered reduced GSH in cortex ($p = 0.0161$) and hippocampus ($p = 0.0017$) of old

animals (Fig. 6B). Mitochondrial uncoupling protein UCP4 is expressed in the brain and can be regulated by cholesterol in diabetic rats¹⁷. The UCP4 acts as a cationic carrier protein in the mitochondrial inner membrane that reduces reactive oxygen species (ROS) production by reducing electron leakage in the respiratory chain¹⁸. As compared with adult group, the protein expression of UCP4 decreased in the cortex and hippocampus of old and thyroidectomized mice ($p < 0.0001$) (Fig. 3J and 3K). Similarly, UCP4 mRNA level was down-regulated in old and thyroidectomized mice as compared with adult group ($p < 0.0001$) (Fig. 6E). Injection of PH-002 increased protein and mRNA expression of UCP4 in cortex ($p = 0.0005$ and $p = 0.0014$) and hippocampus ($p < 0.0001$; $p = 0.0240$) as compared with old mice group (Fig. 6C-6E). Mitochondrial production of ROS in cortex and hippocampus was increased in old and thyroidectomized mice ($p < 0.0001$); this increase was effectively suppressed by PH-002 ($p = 0.0021$ for cortex and $p = 0.0425$ for hippocampus) (Fig. 6F).

High liver-derived ApoE4 impairs cognitive functions of aged animals by activating NLRP3 inflammasome

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Effects of liver-derived ApoE4 on the neural cells and behavioural performance was studied in adult, old, sham and thyroidectomy animals (Fig. 7A). In addition, L-thy and inhibitors (PH-002 and MCC950) were intraperitoneally injected one hour before the injection of lenti-Arg1-ApoE4-eGFP vector in old mice. Two weeks after liver vector transfection, protein expressions of NLRP3, caspase-1 and gasdermin (GSDMD) were significantly increased in the cortex and hippocampus of the old and thyroidectomized mice ($p < 0.0001$), while injection of PH-002 down-regulated their expression ($p < 0.0001$) (Fig. 7B-7E). Expression of associated pro-caspase-1 and apoptosis-associated speck-like protein containing a CARD (ASC) was not affected by ageing or dysfunctional thyroid hormone secretion (Fig. 7F and 7G). Pyroptosis related protein GSDMD was co-localised with astrocytic marker GFAP in cortex and in hippocampus (Fig. 7H). Immunofluorescence intensity of GSDMD staining increased in old or thyroidectomized mice ($p < 0.0001$). Injection of PH-002 significantly suppressed the GSDMD immunoreactivity of aged animals ($p < 0.0001$) (Fig. 7I). Images of brain tissues co-labelled with antibodies against GSDMD, microglial marker Iba1 and neuronal marker NeuN are presented in supplementary Fig. 2A. Intensity of GSDMD labelling in microglia and neurons increased in old or thyroidectomized mice while PH-002 reversed this increase (Supplementary Fig. 2B).

Liver-derived ApoE4 causes neurological impairments

In Morris water maze the time of escape latency was significantly increased in old or thyroidectomized wild type mice with liver transfection of lenti-Arg1-ApoE4-eGFP vector as compared with adult mice ($p<0.0001$). This increase was reversed by injections of 20 mg/kg/day L-thy, 200 mg/kg/day PH-002 or 50 mg/kg/day MCC950 (the selective inhibitor of NLRP3 inflammasome). Similarly, L-thy, PH-002 and MCC950 alleviated decreased time in target quadrant observed in old or thyroidectomized mice (Fig. 8A). Rotarod test and pole test were applied to characterise motor control in ApoE4-KI mice (Fig. 8B). The time on rod for old or thyroidectomized mice was significantly decreased ($p<0.0001$): while the T-LA time (turn downward from the top of pole (T-turn time) and descend to the floor) was significantly longer in old and thyroidectomized animals ($p<0.0001$). Administration of L-thy, PH-002 or MCC950 significantly improved motor function in old mice (Fig. 8B). In the open field test the travelled distance and the time spent in central area were decreased in old or thyroidectomized mice ($p<0.0001$); both parameters were normalised by L-thy, PH-002 or MCC950 (Fig. 8C). Tail suspension and forced swimming tests showed longer immobility times in old and thyroidectomized mice ($p<0.0001$); treatments with L-thy, PH-002 or MCC950 shortened the immobility time in old mice (Fig. 8D).

The ApoE4 allele is an acknowledged risk factor of age-associated cognitive impairments and AD. Here we demonstrate that in old animals ApoE4 produced in the liver is transported, with exosomes, to the brain. Increase in the brain-targeted exosomal transport is linked to the age-dependent decline in thyroid hormones secretion. Exosomes carrying ApoE4 can cross the blood-brain barrier, and can enter the cerebrospinal fluid through choroid plexus by transcellular route (Fig. 9A). Subsequently ApoE4 is distributed throughout the cerebral parenchyma, resulting in the accumulation of ApoE4 in brain (Fig. 9B). In neural cells, elevated ApoE4 increases intracellular cholesterol level that accelerates accumulation of ROS through suppressing GSH and UCP4 mitochondrial content. Elevated ROS triggers activation of NLRP3 inflammasome and instigates pyroptosis of neural cells, which contribute to cognition, motor and mood impairments (Fig. 9). Our clinical research supports the above statements, as the levels of ApoE4 in serum exosomes is significantly higher in patients with AD-related dementia, which is associating with the decline in thyroid hormones and cognitive abilities.

In postmenopausal women, low cognitive functions are associated with the presence of ApoE4 allele and lower levels of FT3 and TT3, although thyroid-stimulating hormone (TSH) levels are increased¹⁹. Lower serum TSH levels and ApoE4 allele influence with memory cognitive functions of elder Koreans²⁰. We studied 60 carriers of ApoE4 allele including 32 male and 28 female subjects, the related gender ratio was 1.14. We did not detect and gender-dependent differences in exosomal ApoE4 content. At the same time levels of ApoE4 in exosomes extracted from serum and liver demonstrate inverse correlation with cognitive function and levels of TT3, FT3, TT4 and FT4. Similar inverse correlation between the levels of four THs and exosomal ApoE4 content was found in ApoE4-KI mice (Supplementary Fig. 1). Furthermore, ApoE4 level in serum exosome was higher in cognitively compromised AD patients when compared to

healthy old subjects. Thus, increased level of exosomal ApoE4 may be used as biological marker for the early diagnosis of AD.

We subsequently found that the exosomes mediate the transport of ApoE4 from the liver to the brain. Decreased THs secretion in ageing affects expression of CD9 and CD81 in exosomes, which in turn modifies exosomal transport. Both CD9 and CD81 are the protein markers of exosomes²¹, they belong to tetraspanin family modulating exosomal adhesion strengthening²². In this work, we discovered that increase in CD9 and decrease in CD81 associated with ageing may promote exosomal transport by changing the exosomal adhesion function.

Intracellularly accumulated ApoE4 competes with ApoE2 for high density lipoprotein (HDL) to block the extracellular transportation of cholesterol²³. In addition, accumulation of ApoE4 in neural cells may impair the mitochondrial function²⁴⁻²⁵. We demonstrate that the age-related accumulation of h-ApoE4 significantly increases cholesterol and decreases GSH in cortex and hippocampus. We also found age-dependent up-regulation of UCP4 which may contribute to the production of mitochondrial ROS. Enhanced production of ROS in cortex and hippocampus triggers activation of nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome²⁶. NLRP3 inflammasome is a multiprotein complex of the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and inflammatory caspase 1. Activation of NLRP3 inflammasome activates caspase 1 and accelerates maturation and release of pro-inflammatory factors, such as IL-1 β and IL-18²⁶. During this process, gasdermin D (GSDMD), a physiological substrate of the canonical inflammasome pathway is recruited to the NLRP3 inflammasome causing pyroptosis and release of mature IL-1 β /18 through plasma membrane pores²⁷⁻²⁸. Here, we showed that liver-derived ApoE4 triggers cognitive impairments, motor and depressive- or anxiety-like behaviours by activating NLRP3 inflammasome.

In AD patients, cognitive and motor deficits are frequently associated with neuropsychiatric manifestations²⁹. Neuronal loss in the prefrontal cortex (PFC) and hippocampus³⁰⁻³¹, as well as an increased release of pro-inflammatory cytokines³², link AD with mood disorders. We demonstrate that increased exosomal transport of APOE4 from the liver to the brain associated with the age-related TH affects cognitive functions and evokes depressive- and anxiety-like behaviours. These changes occur together with ApoE4-induced activation of NLRP3 inflammasome and associated pyroptosis of neural cells. In conclusion, age-associated TH decline increases ApoE4 transport into the brain thus contributing to the high incidence of AD-related dementia and neuronal impairments in ApoE4 allele carriers.

Methods

Materials

Primary antibody of GSDMD (sc-393656), ASC (sc-365611), caspase-1 (sc-56036), CD9 (sc-13118) and AQP1 (sc-25287) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody of NLPR3 (ab214185), pro-caspase-1 (ab179515), GFAP (ab48050), UCP4 (ab183886), secondary antibody Alexa Fluor 555 donkey anti-rabbit (ab150074), Alexa Fluor 647 goat anti-chicken (ab150171), and TT3 ELISA kit (ab108685), FT3 ELISA kit (ab108663), TT4 ELISA kit (ab178664), FT4 ELISA kit (ab108686) were purchased from Abcam (Cambridge, MA, USA). Primary antibody of CD81 (MA5-32333), ApoE4 (MA5-16146), arginase-1 (PA5-85267), MAP2 (PA1-16751), NeuN (PA5-78693), secondary antibody Alexa Fluor 488 donkey anti-rat (A21208) and electrochemiluminescence (ECL) detection reagents (#32132) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibody of b-actin (E021020) and secondary antibody HRP-labeled goat anti mouse (E030110) and HRP-labeled goat anti rabbit (E030120) were purchased from Earthox (Millbrae, CA, USA). Primary antibody of GAPDH (60004-1-Ig) was purchased from Proteintech (Chicago, USA). Iba1 antibody used for immunofluorescence was purchased from Wako Chemicals (USA). The ROS assay kit (BB-470532) was purchased from BestBio (Shanghai, China). The glutathione assay kit (CS0260-1KT), cholesterol quantitation kit (MAK043-1KT), PH-002 (SML0461) and levothyroxine (T-073) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Co-expression lentivirus vectors of Arg1-ApoE4 (human)-eGFP (NM_007482-linker-NM_0000041) were designed and organised from Genechem (Shanghai, China). The quantitative PCR (RR820A and RR047A) was purchased from TaKaRa Bio (Kusatsu, Japanese). MCC950 (CP-456773) was purchased from MedChem Express (Shanghai, China). Mitochondria extraction Kit (G006-1-1) was purchased from Jiancheng Bioengineering (Nanjing, China).

Clinical Experiments

This study was performed on 60 ApoE4 heterozygous participants, including 15 healthy adult subjects, 15 healthy old subjects and 30 patients with clinical diagnosis of AD-related dementia. Participants were chosen from 93 healthy adult subjects (20-55 years old), 95 healthy old subjects (>51 years old) and 83 diagnosed AD-related dementia patients (>55 years old) by DNA sequencing of the extracted venous blood, which were carried out by TaKaRa biotechnology (Dalian, China). All participants have junior high school or above education background. Healthy adult and old subjects were selected from age-matched individuals, who did not have neurodegenerative diseases, stroke and brain tumour. AD-related dementia patients were diagnosed with Alzheimer's disease per the criteria of the National Institute on ageing-Alzheimer's Association and excluded vascular dementia³³⁻³⁴. This study was authorised and approved by Medical Ethics Committee of China Medical University, No. [2020]059 and registered on Chinese Clinical Trial Registry (registration number: ChiCTR2000029731).

Mini-Mental State Examination (MMSE) Scores

MMSE is the most commonly used dementia screening tool that can be performed in the relatively short time of 5 to 10 min. The MMSE consists of 30 questions with a maximum score of 30. The MMSE tests the following 7 cognitive domains: orientation in time and place, memory registration and recall, attention and calculation, and language. The MMSE score of 26 or higher is representative of both cognitive and physical health. Scores under 25 indicate cognitive impairment³⁵.

Montreal Cognitive Assessment (MoCA) Scores

MoCA is a screening method to detect cognitive function developed by Nasreddine et al. Administration of MoCA takes about 10 to 15 min. There are 12 items for cognitive domains; memory is tested by a short-term memory recall task (5 points); visuospatial ability is tested using a clock-drawing test (CDT; 3 points) and a 3-dimensional cube copy (1 point); executive function is tested using a trail-making test, part B (TMT-B; 1 point), a phonemic fluency task (1 point), and a 2-item verbal abstraction task (2 points); attention, concentration, and working memory is tested using a sustained attention task (1 point), a serial subtraction task (3 points), and digits forward and backward tasks (1 point each); language is tested using a 3-item confrontation naming task with low familiarity animals (lion, camel, rhinoceros; 3 points) and repetition of 2 syntactically complex sentences (2 points); orientation in time and place was also tested (6 points). The maximum score of MoCA is 30, and higher scores indicate better cognitive function. The score of MoCA ≥ 26 is representative of both cognitive and psychological healthy, score from 11 to 21 shows the cognitive disorders³⁶⁻³⁷.

Animals

Wild type C57BL/6 mice and human APOE4 replacement transgenic mice (B6.129-Apoe^{tm3(APOE*4)Mae}Ldlr^{tm1(LDLR)Mae}/J) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). In this study, the only male mice were used. Adult mice (3 months) were randomly divided into three groups: adult group, sham and thyroidectomy group; old mice (18 months) were randomly divided into five groups: old group, normal saline injection group, levothyroxine injection group, ApoE4 inhibitor (PH-002) injection group and NLRP3 inflammasome inhibitor MCC950 injection group. The mice were raised in standard housing conditions ($22 \pm 1^\circ\text{C}$; light/dark cycle of 12/12h), with water and food available *ad libitum*. All experiments were performed in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023) and its 1978 revision, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, No. [2021]117.

Thyroidectomy

The adult mice were anaesthetised with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and placed on their backs to expose the neck area. A midline skin incision was made along the length of the neck. The underlying tissues were cleared and the salivary glands were retracted laterally. The two halves of the sternohyoid muscle were separated and retracted laterally. Thyroid muscle was separated from the thyroid gland lobes and retracted along with the sternohyoid muscle. A midline cut was made in the isthmus and the thyroid glands were excised bilaterally. Extreme care was taken so as not to damage the laryngeal nerve. Sham group underwent the same surgical procedures without removal of the thyroid gland. Mice were monitored closely for at least 1 week after the surgery for complications and animals were used for experimentation 4 weeks later³⁸.

Microinjection into Hepatic Portal Vein

C57BL/6 mice of wild type were anaesthetised as above and place the mice in a supine position, on its back with abdomen exposed. Remove hair on the ventral left side of the rodent from the second rib space down to the 4th inguinal mammary gland nipple by wiping the area with chemical depilatory. Allow the depilatory to sit for 2 min and then remove completely with gauze and H₂O. Using sterile gloves and a sterilised scalpel with sterile blade, make a single 1-inch incision into the skin between the median and sagittal planes on the left side of the mouse, starting just below the ribs and ending just above the plane of the fourth inguinal mammary gland teat. Using autoclaved or bead sterilised scissors and forceps, make a similar 1-inch incision into the peritoneum. A 4 x 4" gauze pad soaked in sterile saline was placed on the left side of the mouse, where the incision was made, such that internal organs can be placed on the gauze and not come into contact with the surrounding skin or surgical area. Hold the median side of the incision, including skin and peritoneal lining, aside with the forceps and use a sterile cotton swab to carefully pull the large and small intestines out, placing them on the sterile gauze soaked in sterile saline. Pull out large and small intestines until the portal vein is visualised. Cover the internal organs in the saline soaked gauze to maintain internal moisture and sterility. Injection of the co-expression lentivirus vectors of Arg1-ApoE4 (human)-eGFP, the needle was inserted at about 10 mm below the liver and 3-5 mm into the portal vein with an angle < 5° and bevel facing up; 5 µl containing lentivirus at 4X10⁷ TU was slowly injected. The negative control group was only injected with lentivirus without the target vector. Mice were maintained body temperature and individually housed after full recovery from anaesthesia. After 2 weeks, the mice were used for the followed experiments³⁹.

Serum Exosome Extraction

Each mouse was anaesthetised and blood was collected by inserting a capillary glass tube into the medial cantus of the orbit while gently pushing and rotating it forward. We used EP tube to collect venous blood

from seven mice as one sample. After the collection of the blood samples, serum was separated from the blood by centrifugation at 3400 x g for 10 minutes. Serum samples were centrifuged at 800 x g for 5 minutes, then 2000 x g for 10 minutes. The supernatant was filtered using a 0.2 mm syringe filter (Corning or Whatman, USA). The filtrated serum was centrifuged in the ultracentrifugation (Beckman Coulter Optima L-90K) at 198,000 x g for 4 hours to collect exosomes. Exosome pellets were resuspended in 200 ml phosphate buffer saline (PBS) and stored at -80°C⁴⁰.

Liver Exosome Extraction

After anaesthesia as above, the mice were myocardial perfused with Hank's buffer. The liver tissue was taken out and placed in a clean beaker. The livers of five mice were collected as one sample. The liver was cut up, digested with 0.125% trypsin for 20 minutes, and then poured into the glass homogeniser. The homogenised liver tissues were filtered with a 40 µm screen. Then, the filtrated solution was transferred to a 15 ml centrifuge tube, and was centrifuged at 3000 x g for 10 minutes, then at 12,000 x g for 20 minutes. The collected supernatant was centrifuged by ultracentrifugation at 150,000 x g for 4 hours. Exosome pellets were resuspended in 200 ml PBS and stored at -80°C.

Immunofluorescence (IF)

The anesthetised mice were perfused through myocardium with 4% paraformaldehyde (PFA) for 15 minutes. The brain or liver tissue was cut into 60 µm slices. Brain or liver slices were permeabilised by incubation for 1 hour with donkey serum. Primary antibodies against ApoE4 or GSDMD were used at a 1:100 dilution, against arginase-1 (Arg-1), aquaporins 1 (AQP1), glial fibrillary acidic protein (GFAP), microtubule associate protein 2 (MAP2), NeuN and Iba1 used at 1:200 dilution. And nuclei were stained with marker 4', 6'-diamidino-2-phenylindole (DAPI) at 1:1000 dilution. The incubation with the primary antibodies were overnight at 4 °C and then donkey anti-rat or anti-rabbit Alexa Fluor 488/555, or goat anti-chicken Alexa Fluor 647 conjugated secondary antibody for 2 h at room temperature. Images were captured using a confocal scanning microscope (DMi8, Leica, Wetzlar, Germany)⁴¹.

Western Blotting

For quantifying expressions of h-ApoE4, CD9, CD81, NLRP3, pro-caspase-1, caspase-1, GSDMD, ASC, UCP4, β-actin and GAPDH, the samples containing 100 µg of protein were added to 10% SDS-polyacrylamide gel electrophoresis. After electrophoretic separation and the gels were transferred to PVDF membranes, the samples were blocked by 5% skimmed milk powder for 1 hour, and membranes were incubated overnight with the primary antibodies, specific to either human specific ApoE4 at 1:1000 dilution, CD9 at 1:1000 dilution, CD81 at 1:1000 dilution, NLRP3 at 1:1000 dilution, pro-caspase-1 at

1:1000 dilution, caspase-1 at 1:1000 dilution, GSDMD at 1:1000 dilution, ASC at 1:1000 dilution, UCP4 at 1:1000 dilution, β -actin at 1:5000 dilution and GAPDH at 1:3000 dilution. After washing, specific binding was detected by horseradish peroxidase-conjugated secondary antibodies. Staining was visualised by ECL detection reagents and was analysed with an Electrophoresis Gel Imaging Analysis System (MF-ChemiBIS 3.2, DNR Bio-Imaging Systems, Israel). Band density was measured with Window AlphaEaseTM FC 32-bit software⁴².

Real-time PCR

To measure the mRNA of UCP4, the RNA of cortex and hippocampus was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed to cDNA by using a reverse transcription reagent kit (Takara, Otsu, Shiga, Japan) in a Robo-cycler thermocycler. Real-time PCR was performed with the LightCycler 480 SYBR Green I Master kit (4887352001, purchased from Roche) and the products were detected using an a LightCyler 480 instrument (Roche, Mannheim, Germany). The sequences of Real-time PCR primers used for mRNA quantification were as follows: 5'-ATTGCGATTTCTGTTGTACAT-3' and 5'-CCATATTCACCAGTGCTGCTCTT-3' for mice UCP4 and 5'-TGTTGCCAAAAGGGTCATCTCC-3' and 5'-GCCAGCCCCAGCATCAAAGGTG-3' for mice GAPDH. Relative genomic expression was calculated by the $2^{-\Delta\Delta C_t}$ method⁴³⁻⁴⁴.

Mitochondria Extraction

Mitochondria isolation and fractionation was conducted by using the mitochondria extraction kit, according to the manufacturer's protocol. Fresh collected tissues of cortex and hippocampus were homogenised with an all-glass Dounce homogeniser in cold lysis buffer. Then, the homogenates were centrifugated at 800×g, 4 °C for 5 min. After centrifugation, the supernatant was slowly transferred to a new pre-cooled tube, which pre-added with 500 ml extraction reagent A. To centrifuge the sample tubes at 15,000×g, 4 °C for 10 minutes, the supernatant was obtained as cytoplasm extract. The sediment was suspended with 200 ml wash buffer, and then centrifuged at 15,000×g, 4 °C for 10 minutes again. To dispose the supernatant, the sediment was resuspended with 100 μ l storage buffer and kept under -80 °C.

ROS Level Detection

To assay the ROS level, a commercial ROS assay kit (BB-470532, BestBio, Shanghai, China) was used and operated as the protocols. The ROS assay kit based on the oxidant-sensitive fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The tissues of cortex and hippocampus were incubated with a 1 ml serum-free culture medium containing 10 μ M of DCFH-DA. After incubation for 30 minutes at 37 °C, the cells were washed with warm PBS three times to remove the excess dye. Finally, the

fluorescence intensity of each well was measured using a fluorescence microplate reader (Infinite M200 Pro, Hombrechtikon, Switzerland). The excitation wavelength was at 488 nm, and the emission peak was examined at 525 nm.

ELISA Assays

The concentrations of TT3, FT3, TT4 and FT4 in serum were determined using a commercial ELISA kit based on the manufacture instructions. The levels of cholesterol and GSH in tissue samples from the cortex and hippocampus were measured using a commercial ELISA kit based on the manufacturer's instructions. Cytokine levels in samples were determined in duplicate following assay instructions provided by the manufacturers. The optical density (OD) of each microwell was measured at 450 nm using a microplate reader.

Behavioural Tests

In behavioural tests, mice were randomly assigned to avoid interference from different test. Behavioural tests were recorded, stored, and analysed by using the SMART™ tracking software program (Panlab, SL, Barcelona, Spain). The apparatus was cleaned with 25% ethanol solution after each test.

Morris Maze Test

Morris water maze test is to check a spatial learning and memory. The mice were trained over five consecutive days in four different quadrants. The mice were trained to locate and escape onto the platform during this period. A different starting position for each mouse was used each time. Animals that failed to find the location within 90 second were guided to the platform and were allowed to remain on it for 20 second. The platform position was fixed throughout the test. In the place navigation test, the escape latencies for each mouse per day were calculated. In the memory retention session, a single test was conducted in the pool, in which the hidden platform was removed a 1-min probe trial was conducted to measure the time spent in the target quadrant by each mouse⁴⁵.

Rotating Rod Test

The rotating rod test is a test for assessing motion ability in mice. The individual mouse was placed on the rotating bar, which was set to a rotation speed at 20 rpm during the test. The time spent on the rotating bar was recorded as the latent period. The latency before falling was recorded using a stop-watch, with a maximum of 90 second. Time of staying on rotating rod indicates motion balance ability⁴⁶.

Pole Test

The pole test is also a test for assessing motion ability in mice. Each mouse was paced head-upward on the top of a vertical rough-surfaced pole (diameter 1 cm; height 55 cm). The turn downward from the top of pole (T-turn time) and the descend to the floor (T-LA) time was recorded⁴⁷.

Open Field Test

The open field test is an anxiety-based test. The mice were placed in the centre square of an open field box (60 × 60 × 40 cm) divided into nine squares, and behaviours were recorded for 6 minutes. The parameters used for analysis included the total travel distance and the time spent in the central area⁴⁶.

Forced Swimming Test

The forced swimming test is a despair-based behavioural test. Each mouse was trained to swim for 15 min before the formal measurement. Then the trained mouse was put into a glass cylinder that contained 30 cm deep water ($25 \pm 1^\circ\text{C}$) and left for 6 min. The immobility time was recorded during the last 4 min that followed 2 min of habituation⁴⁸.

Tail Suspension Test

The tail suspension test is also a despair-based behavioural test. Mice were suspended by their tails at 20 cm from ground for 6 min. The time of immobility was recorded to calculate percentage. The immobility time in tail suspension test reflects despair-like behaviour⁴⁷.

Statistics and Reproducibility

For statistical analysis we used one-way analysis of variance (ANOVA) followed by a Tukey's or Dunnett's post hoc multiple comparison test for unequal replications using GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA) and SPSS 24 software (International Business Machines Corp., NY, USA). One-way ANOVA for comparisons including more than two groups; unpaired two-tailed t-test for two group comparisons. All statistical data in the text are presented as the mean $\pm$ SD, the value of significance was set at $p < 0.05$.

Declarations

Data Availability

Source data underlying the main and supplementary figures are available in Supplementary Data. The data that support the findings of this study are available from the corresponding author Baoman Li upon reasonable request.

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Author Contributions

A.V. and B.L. designed and supervised the study; Y.Z. and M.Z. enrolled and filtrated the clinical participants; Y.D. and M.X. collected the data in clinic study; G.W., BN.C., BJ.C., M.J., X.W. and WL.G. collected the data in vivo and analysed the relevant data; C.D., X.Z., Y.Z., M.Z., D.Z. and X.L. performed the behavioural experiments in vivo; BN.C. and M.J. analysed the data; B.L. and A.V. wrote the manuscript.

Competing interests

The authors have no conflicts of interest to disclose.

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Figures

Figure 1

Correlation of ApoE4 level in serum exosomes with thyroid hormones levels and cognitive performance in human subjects. (A) Enrolled 60 carriers of ApoE4 allele included 15 adult healthy subjects, 15 old healthy subjects and 30 old AD patients. Collected serum was used to extract exosomes and measure TT3, FT3, TT4 and FT4. Participants cognitive abilities were assessed by MMSE and MoCA scoring. (B) Representative western blots of ApoE4 in the serum extracted exosomes and the the supernatant from

which which exosomes precipitate was removed after ultracentrifuge. (C) Normalised intensities of ApoE4 by β -actin. (D) TT3, FT3, TT4 and FT4 in adult, old and AD groups. Correlation analysis between the ApoE4 level in serum exosomes and TH levels are shown in inserts at the right. (E) MMSE and MoCA scores; correlation analysis between the ApoE4 level in serum exosomes and these scores are shown in inserts at the right. (F) Multiple correlation analysis of the normalised ApoE4 in serum exosomes with the normalised MoCA scores and serum FT3 in adult group. The individual measured value was plotted and shown as mean $\pm$ SD. One-way ANOVA for comparisons including more than two groups; unpaired two-tailed t-test for two group comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Ageing and thyroidectomy increased exosomal transport of liver-derived ApoE4 into the brain. (A) Experimental design: adult and old mice were injected with lenti-Arg1-ApoE4-eGFP vector through hepatic portal veins; 2 months old mice were randomly operated sham or thyroidectomy. 4 weeks after surgery mice were injected lenti-Arg1-ApoE4-eGFP vector through hepatic portal veins. (B) Injection of lentivirus vectors of human ApoE4 (h-ApoE4) and arginase-1 (Arg1) (Lenti-Arg1-ApoE4-eGFP) through hepatic

portal vein. (C) Immunofluorescence images taken from liver tissue of h-ApoE4 (green) and Arg1 (red) with nucleus marker DAPI (blue) performed 3 days after injection of lenti-Arg1-ApoE4-eGFP or lentivirus negative control vectors in adult mice. Scale bar = 50 μ m. (D) Electron microscope images of exosome extracted from liver and serum (left), and the representative Western blots for h-ApoE4 in exosomes extracted from liver and blood serum (right). (E) Relative expression ratio of h-ApoE4 and GAPDH is plotted as mean $\pm$ SD, n=6. One-way ANOVA for comparisons including more than two groups; unpaired two-tailed t-test for two group comparisons. Compared to adult group, *p<0.05, **p<0.01, ***p<0.001. (F) Immunofluorescence images of h-ApoE4 (green) co-stained with AQP1 (red) and DAPI (blue) in choroid plexus. Scale bar=50 μ m.

A

B

Figure 3

Ageing and thyroidectomy increased exosomal transport of liver-derived ApoE4 into the brain. (A) Immunofluorescence images of h-ApoE4 (green) co-stained with AQP1 (red) and DAPI (blue) in the whole cortex and hippocampus. Scale bar = 50 μ m. (B) Immunofluorescence intensity of h-ApoE4 normalised to adult group. The normalised intensity is plotted as mean $\pm$ SD, n=6. One-way ANOVA for comparisons including more than two groups; unpaired two-tailed t-test for two group comparisons. One-way ANOVA

A

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after surgery measurements were made. (B) TT3, FT3, TT4 and FT4 values are plotted as mean $\pm$ SD, $n=6$. One-way ANOVA for comparisons including more than two groups; unpaired two-tailed t-test for two group comparisons. As comparison with adult group, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 5

The effects of L-thy and ApoE4 inhibitor on the exosome transport of h-ApoE4 in ApoE4-KI mice. (A) Representative Western blots of h-ApoE4 in the exosomes extracted from liver and serum. (B) Expression

ratio of h-ApoE4 and GAPDH are plotted as mean $\pm$ SD, n = 6. (C) Representative Western blots of CD9 and CD81 in the exosomes extracted from liver and serum. (D and E) Ratio of CD9 or CD81 normalised to GAPDH is plotted as mean $\pm$ SD, n = 6. One-way ANOVA for comparisons including more than two groups; unpaired two-tailed t-test for two group comparisons. As comparison with adult group, *p<0.05, **p<0.01, ***p<0.001.

Figure 6

The effects of L-thy and ApoE4 inhibitor on the cerebral oxidative stress in ApoE4-KI mice. (A and B) Cholesterol and GSH levels in cortex and hippocampus measured by ELISA are plotted as mean $\pm$ SD, n = 6. (C) Representative Western blots of UCP4 in cortex and in hippocampus. (D) Ratio of UCP4 to β -actin is plotted as mean $\pm$ SD, n = 6. (E) mRNA expression of UCP4 measured by RT-PCR in cortex and in hippocampus, the relative ratio of UCP4 and GAPDH was normalised to adult group and plotted as mean $\pm$ SD, n = 6. (F) ROS level in cortex and hippocampus are plotted as mean $\pm$ SD, n = 6. One-way ANOVA for comparisons including more than two groups; unpaired two-tailed t-test for two group comparisons. As comparison with adult group, *p<0.05, **p<0.01, ***p<0.001.

Figure 7

Activation of NLRP3 inflammasome induced by liver-derived ApoE4. (A) Experimental design: 1 hour before the hepatic portal vein injection mice in old group were randomly intraperitoneal injected with normal saline (NS), 20 $\mu\text{g/kg/day}$ L-thy, 200 mg/kg/day ApoE4 inhibitor PH-002 or 50 mg/kg/day NLRP3 inflammasomes inhibitor MCC950, lasting for 2 weeks. 2 months old mice randomly underwent thyroidectomy or sham surgery. 4 weeks after surgery mice were injected with lenti-Arg1-ApoE4-eGFP

vector through hepatic portal veins for 2 weeks. (B) Representative Western blots of NLRP3, caspase-1, GSDMD, pro-caspase-1 and ASC collected from the proteins in cortex and hippocampus. (C-G) Expression ratios of NLRP3 (C), caspase-1 (D), GSDMD (E), pro-caspase-1, (F) and ASC (G) normalised to β -actin are plotted as mean $\pm$ SD, n = 6. (H) Immunofluorescence images of GSDMD (green) co-stained with GFAP (red) and DAPI (blue) in cortex (above two rows) and hippocampus (lower two rows). Scale bar = 50 μ m. (I) Immunofluorescence intensity of GSDMD normalised to adult group shown as mean $\pm$ SD, n = 6. One-way ANOVA for comparisons including more than two groups; unpaired two-tailed t-test for two group comparisons. As comparison with adult group, *p<0.05, **p<0.01, ***p<0.001.

Figure 8

Behavioural impairments induced by liver-derived ApoE4. (A) Cognitive function in Morris water maze test. Time of escape latency and time in target quadrant were recorded and shown as mean $\pm$ SD, $n = 6$. (B) Motor function in rotarod and pole tests. Time on rod and T-LA time are shown as mean $\pm$ SD, $n = 6$. (C) Anxiety-like behaviours in open field test. Total travel distance and time spent in the central area were shown as mean $\pm$ SD, $n = 6$. (D) Depressive-like behaviours in tail suspension and forced swimming tests.

Immobility time are shown as mean $\pm$ SD, $n = 6$. One-way ANOVA for comparisons including more than two groups; unpaired two-tailed t-test for two group comparisons. Compared to adult group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 9

Ageing related hypothyroidism promotes exosomal transport of liver-derived ApoE4 to the brain, thus activating NLRP3 inflammasome and cause neurological impairments. Ageing associated

hypothyroidism promotes liver-derived ApoE4 exosomal transport . ApoE4-laden exosomes exosomes to cross BBB entering the brain (A), liver-derived ApoE4-reach exosomes also enter CSF in choroid plexus (B). Both processes result in the accumulation of ApoE4 in the brain. Elevated ApoE4 increases the cholesterol level in neural cells, which suppresses mitochondrial content of GSH and UCP4 thus leading to an increase in ROS. Increased ROS activates NLRP3 inflammasome triggering pyroptosis, that contributes to neurocognitive decline and depression.

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