

A Study of the SORL1 Gene in the Pathogenesis of Late-onset Alzheimer's Disease by Affecting the Expression of BDNF

Mingri Zhao^{2#}, Jiangfeng Liu^{1#}, Jingli He¹, Xun Chen¹, Yanjin Feng¹, Chen Wang¹, Wanxi Liu², Xionghao Liu², Mujun Liu^{3*}, Deren Hou^{1*},

1 Department of Neurology, The Third Xiangya Hospital, Central South University, 138 Tongzipo Road, Changsha, Hunan 410013, China.

2 Center for Medical Genetics & Hunan Key Laboratory of Medical Genetics, School of Life Sciences, Central South University, Changsha, Hunan 410013, China.

3 Department of Cell Biology, School of Life Sciences, Central South University, Changsha, Hunan 410013, China.

[#]These authors have contributed equally to this work and share first authorship.

*Correspondence

Deren Hou, hou0718@126.com. Mujun Liu, liumujun03@csu.edu.cn.

Abstract background

Alzheimer's disease is a neurodegenerative disease characterized by progressive memory impairment and other cognitive disorders. It is divided into Familial Alzheimer's disease (FAD) and Sporadic Alzheimer's disease (SAD). SAD is also called delayed Late-onset Alzheimer's disease (LOAD). Sortilin Related Receptor 1 (SORL1) is a high-risk pathogenic gene of LOAD, which can participate in the occurrence and development of AD by affecting the transport and metabolism of intracellular β -amyloid precursor protein (APP). The expression of SORL1 is significantly downregulated in patients with LOAD.

Results

In the SORL1 knockout (SORL1 KO) mouse model constructed by CRISPR/cas9, we found that the expression of Brain Derived Neurotrophic Factor (BDNF) in the brain of SORL1 KO mice was significantly down-regulated and Amyloid β -protein ($A\beta$) deposition was found in the brain of

SORL1 KO mice. Through the SORL1 knockdown N2a cell model constructed by shRNA, we also found that when the SORL1 expression was knocked down, the BDNF expression was also downregulated and the cell viability decreased. The results of immunohistochemistry and in vitro cell model experiments suggest that the downregulation of BDNF caused by SORL1 knockdown may be mainly achieved by affecting the expression and distribution of N-Methyl-D-aspartate (NMDAR).

Conclusions

SORL1 knockout changes the expression and distribution of NMDAR in cells, downregulates the expression of BDNF, and thus affects the learning and memory of mice.

Keywords Late-onset Alzheimer's disease, SORL1, BDNF, NMDAR

1. Background

Alzheimer's disease (AD) is a central nervous system degenerative disease characterized by progressive cognitive impairment and behavioral changes, which is concealed in onset and affected by genetic and environmental factors. It is divided into Familial Alzheimer's disease (FAD) and Late-onset Alzheimer's disease (LOAD). Clinically, 90% of AD patients are LOAD [1]. AD mainly occurs in the elderly. The prevalence rate increases significantly with the increase of age. AD is the main and common disease of senile dementia. With the aging of the population, the prevalence of AD has increased significantly. According to the 2018 "World Alzheimer Report", one new dementia case occurs every 3 seconds worldwide. About 50 million people have dementia worldwide in 2018. By 2050, the number of people with dementia is expected to reach 152 million. Alzheimer's disease not only seriously affects the quality of life of patients and their families, but also brings a huge economic burden to the society.

However, at present, the pathogenesis of Alzheimer's disease is still unclear. For many years, drugs developed for characteristic pathological β -amyloid peptide ($A\beta$) deposition have ended in failure. It is urgent to further understand the mechanism of Alzheimer's disease [2-4].

FAD is inherited in an autosomal dominant manner. It is mainly caused by mutations of β -amyloid precursor protein (APP), Presenilin1 (PSEN1), and Presenilin2 (PSEN2) genes. APP, PSEN1, and PSEN2 are all involved in $A\beta$ deposition [5]. However, the pathogenesis of LOAD is unknown at present. and it is generally believed that LOAD is the result of the joint effect of environmental

factors and polygenic factors. In recent years, Genome-wide association study (GWAS) analysis and our previous case-control studies of more than 200 AD patients have shown that SORL1 is a high-risk susceptibility gene of LOAD [6,7].

SORL1 gene is located on chromosome 11q23.2-q24.2, which is about 177.49KB in length and encodes a membrane protein about 250kDa expressed in the neurons in the central and peripheral nervous systems. As a type I transmembrane protein, SORL1 is composed of seven domains.

SORL1 belongs not only to the Low density lipo protein receptor (LDLR) family, but also to the Vacuolar Protein Sorting (Vps10) domain receptor family [8]. SORL1 mainly exists in the early nuclear endosome and trans-Golgor matrix (TGN). SORL1 interacts with different cytoplasmic adaptors to make APP move forward and retrograde between TGN and early endosomes, and thereby restrict APP. APP is transported to endocytic compartments that are conducive to the formation of amyloid to reduce the deposition of A β protein [9, 10]. In addition, some studies have shown that knockout of SORL1 in human-induced pluripotent stem cell (hiPSC) leads to the enlargement of intima, which is a typical cytopathological change of AD. This pathological change is not affected by inhibiting β -secretase (BACE). SORL1 participates in the occurrence and development of AD not only by affecting the metabolism of APP, but also by affecting the intracellular transport of APP, tau, and other related proteins [11].

In addition to the classical pathological features of A β deposition and neurofibrillary tangles (NFTs), AD patients also have pathological manifestations of neuronal loss in the frontotemporal hippocampus [12]. Some studies have shown that the expression of brain derived neurotrophic factor (BDNF) in AD patients is significantly downregulated. Methods such as aerobic exercise to promote the expression of BDNF can significantly improve the behavioral cognitive impairment of AD [13, 14]. BDNF is a neurotrophic factor that plays an important role in synapse growth, myelination, neuron growth, differentiation, and survival. BDNF can promote the growth of neuronal synapses and regulate neuron long-term potentiation (LTP) and long-term inhibition (LTD). The normal expression and transport of BDNF play a vital role in learning and memory [15]. Some studies have shown that SORL1 may be the downstream target of BDNF. BDNF can increase SORL1 gene transcriptional activation more than 10 times faster through extracellular signal-regulated kinase (ERK) pathway than the control group without BDNF. In BDNF^{-/-} mouse model, a significant down-regulation of SORL1 expression activity was observed. The decrease of

BDNF expression causes down-regulation of neuroprotective effects and regulation of SORL1 expression, thus affects the transport and metabolism of APP, and participates in the occurrence and development of AD. RoheM et al have shown that when SORL1 is knocked-out, the function of BDNF to reduce A β deposition decreases, suggesting that BDNF may lose its function to improve cognitive and behavioral impairment in mice [16]. As an intracellular sorted receptor-related protein, SORL1 may regulate the expression and transport of BDNF. Through the construction of SORL1 knockout mice by CRISPR/Cas9, we found that the expression of BDNF in hippocampus and cortex of SORL1 knockout mice was significantly down-regulated. When shRNA was used to interfere with the expression of SORL1 in N2a cells, the expression of BDNF was also significantly down-regulated. Further studies of SORL1 knockout mice and SORL1 interference in the N2a cell model found that SORL1 knockout could participate in the occurrence and development of AD by down-regulating the expression of BDNF and inducing the deposition of A β . The regulatory effect of SORL1 on BDNF may be achieved by affecting the expression and distribution of N-methyl-D-aspartic acid receptor (NMDAR), which is a BDNF upstream molecule. SORL1 can down-regulate the expression of NMDAR and further turn off the transcriptional initiation of cAMP-response element binding protein (CREB), and ultimately affect the expression of BDNF. We found that SORL1 not only regulates the transport and metabolism of APP, but also affects the expression of BDNF by regulating the distribution of NMDAR expression, and thus participates in the occurrence and development of LOAD. The purpose of this study is to clarify the regulatory role of SORL1 on BDNF and in the development of LOAD, to provide new ideas for further understanding of the pathogenesis of LOAD, and to find new therapeutic targets.

2. Materials and Methods

Antibodies and Chemicals

Antibodies against SORL1, BDNF, and NMDAR2B were purchased from Abcam. β -actin, Amyloid-Beta were purchased from Thermo Scientific. Antibodies against Tubulin and CREB were obtained from Cell Signaling Technology. The protein quantitative BCA kit was purchased from Thermo Fisher Scientific. Protease inhibitors and phosphatase inhibitors were purchased from Selleck.

Sequences for shRNAs

The shRNA sequence of Negative Control was GTTCTCCGAACGTGTCACGT. The sense strand sequences of shRNA were GCACAACACCAATGACTTTGT (#1) and GCTAGCAACTCTACAGAAATA (#2) for SORL1.

Animals

SORL1^{-/-} mice were generated by using CRISPR/Cas9 technology. Cas9 mRNA and two guide RNAs (gRNA) targeting the upstream and downstream regions of the mouse SORL1 gene were injected into C57BL/6 mouse oocytes to obtain the first generation of SORL1^{+/-} mice. The female and male SORL1^{+/-} mice were raised in the same cage. SORL1^{-/-} mice were obtained after breeding. All mice were identified by Sanger sequencing technology. When the offspring mice were 3 weeks old, the mice were marked by toe-clipping. The tails of the mice were cut to extract DNA. DNA sequencing was performed after PCR amplification. At the same time, western blotting was used to detect the expression of SORL1 protein extracted from mouse brain tissue.

The primers for genotyping were as follows:

SORL1- forward: 5'-ATGGCGACACGGAGCAGCAGGAG-3'

SORL1- reverse: 5'-AAGATGTACCTCTTGTGTCAGCAGGGCTG-3'

APP/PS1 double transgenic mice were purchased from Cavens Biotechnology Company (Changzhou, China) by the Department of Zoology of Central South University. All mice were raised in the SPF mouse feeding room. All procedures regarding the care and use of animals were approved by the Institutional Animal Care and Use Committee of Central South University of China.

Immunofluorescence staining

Seven-micron-thick sections including the mouse cortex and hippocampus region were collected and incubated overnight with primary antibodies against SORL1 (diluted 1:700), BDNF (diluted 1:800), Amyloid-Beta (diluted 1:1000), and NMDAR-2B (diluted 1:500). The slices were then incubated in the dark with corresponding secondary antibodies. With 3,3N-Diaminobenzidine (DAB) coloring, nuclei were stained with hematoxylin. Slices were imaged using a Nikon fluorescent microscope. The Image average optical density value was measured and analyzed with the Image software.

Cell culture and transfection

N2a cell line was purchased from the American Type Culture Collection (ATCC, USA), cultured in MEM/opti-MEM (1:1) medium (Gibco, MA, USA) supplemented with 5% fetal bovine serum (Gibco, MA, USA), 100U/ml penicillin, and 100µg/ml streptomycin (Thermo Fisher Scientific, MA, USA) at 37 °C in a 5% CO₂ incubator. Plasmid and shRNA transfections were performed with Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) reagents according to the manufacturer's protocol.

CCK-8 assay

After transfection of 2µg shRNA-SORL1 plasmid into N2a cells for 48h, the proliferation activity of N2a cells was detected by CCK-8 assay. Briefly, cells were seeded into 96-well microdilution plates at a density of 5x10³ cells/well with 100µl DMEM/Opti-MEM (1:1) containing 5% FBS. Then 10µl CCK-8 (Bimake, Shanghai, China) solution was added to each hole and the plates were incubated in 37 °C incubator for 2-4 hours. Absorbance was measured at 450nm using a microplate reader.

Cell apoptosis assay

N2a cells were transfected with 2µg shRNA- SORL 1 plasmid for 48h. Then the cells were collected by trypsinization. Cell apoptosis was measured using an Annexin V-PE/7-AAD Apoptosis kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. Briefly, cells were harvested and washed with DPBS buffer and resuspended in 100µl binding buffer. Annexin V-PE and 7-AAD was then added and the cell suspension was incubated in the dark for 10 min. Fluorescence intensity was measured by flow cytometry (DxP Athena™).

RNA isolation, reverse transcription, and RT-qPCR

Cells were lysed with TRIzol® reagent according to the manufacturer's instructions. RNA quantification was done using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Next, cDNA was synthesized from 1µg total RNA using a Vazyme reverse transcription kit (Nanjing, China). Real-time PCR analysis was carried out with the SYBR Green Master Mix (Thermo Fisher Scientific, MA, USA). Primers used for RT-qPCR were the following: SORL1, forward: 5'-AGCAGGAGGGAGTCGAGAC-3'; reverse: 5'-GTTCCCTAGCCGGAGATCGC-3'; GAPDH, forward: 5'- ATCATCCCTGCATCCACT-3'; reverse: 5'- ATCCACGACGGACACATT-3' (Sangon Biotech, China).

Western blotting

Cells or tissue samples total protein lysates were prepared using RIPA buffer (Thermo Fisher Scientific) in the presence of a protease inhibitor and PhosStop (Selleck). Protein concentration was quantified using a BCA protein assay kit (Thermo Fisher Scientific). Proteins in lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes (PVDF), and immunoblotted with the corresponding antibodies overnight at 4°C. Membranes were then washed and incubated with horseradish peroxidase conjugate secondary antibodies. Blots were developed using the SuperSignal West Pico Substrate (Thermo Fisher Scientific) chemiluminescence kit and Gene Genius Bio-imaging System (Bio-Rad).

3. Results

3.1 Construction and identification of SORL1 KO mice

SORL1^{WT}, SORL1^{-/-}, SORL1^{+/-} mice were identified by Sanger sequencing technology and western blotting. It was seen that the four bases of CAAT at site 306-309 in the CDS sequence of SORL1 gene were knocked out by CRISPR/Cas9 technique. When there was no nested peak, the mice with four bases of CAAT were wild type (WT) mice. The mice with missing four bases of CAAT were SORL1^{-/-} mice. The mice with nested peaks were SORL1^{+/-} mice (Fig 1A). The protein extracted from the brain of the identified mice was detected by Western blot. It was found that the protein encoded by SORL1 gene could not be translated normally after the four bases of CAAT at site 306-309 being knocked out in the CDS sequence of the gene (Fig 1B).

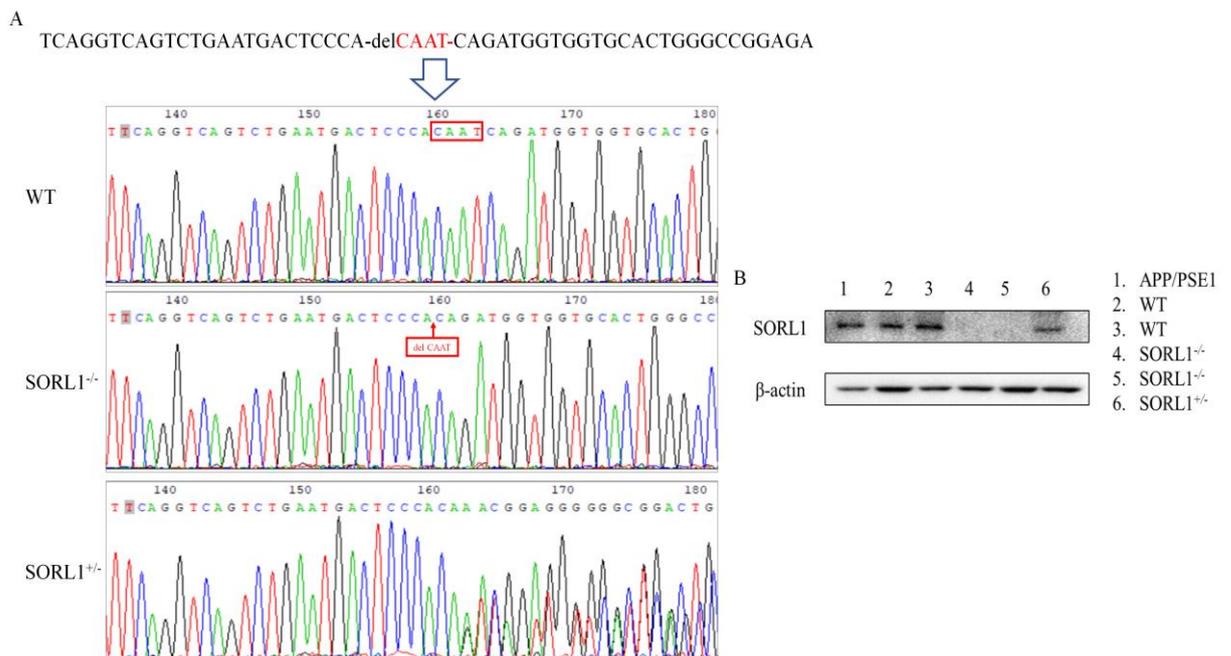
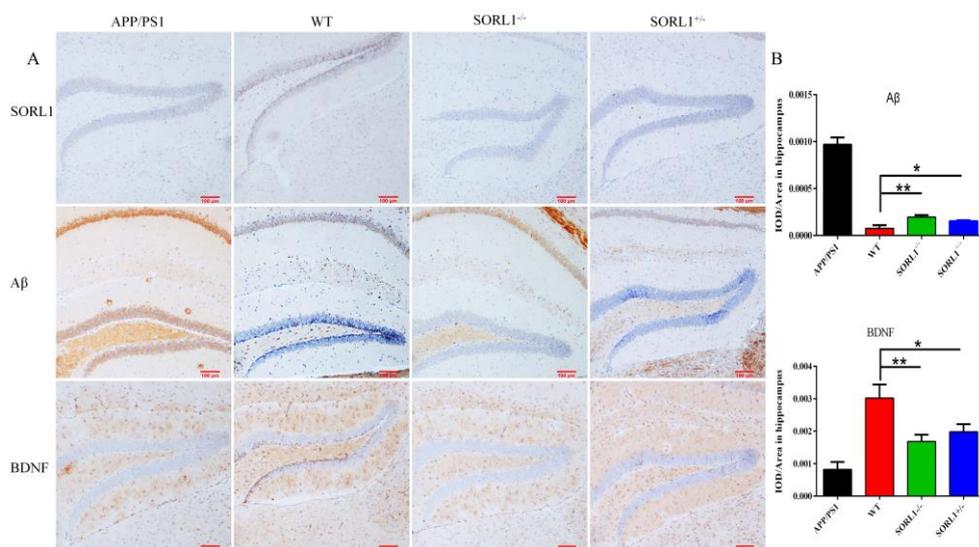


Figure 1. Construction and identification of SORL1 knockout mice. (A) Mouse DNA sequencing

genotype identification, (B) Detection of the expression of SORL1 in different groups of mouse brain tissue using Western blotting.

3.2 A β deposition and down-regulation of BDNF expression in cortex and hippocampus of SORL1 knockout mice

As a high-risk susceptibility gene of LOAD, SORL1 causes AD through a mechanism that may be different from that causing FAD. Studies have shown that SORL1 deletion leads to AD pathology by affecting its transport in endosome/lysosome without changing the process and expression of APP. This suggests that the pathology of AD caused by SORL1 deletion does not only depend on classical A β deposition. At early-stage AD, SORL1 protein dysfunction may affect the transcription and expression of downstream molecules and affect synaptic plasticity and Long-term potentiation (LTP) [11]. Our study found that when SORL1 is knocked out, A β deposition is found in the mouse cortex and hippocampus (Fig 2A, B). At the same time, the expression of BDNF was observed to decrease (Fig 2C, D). It is suggested that SORL1, as a high-risk susceptibility gene for LOAD, causes AD in a way that is related to not only A β decomposition, but also decreased BDNF expression. Our findings revealed that SORL1 may affect neuronal survival, synaptic plasticity, and the formation of learning and memory by regulating the expression of BDNF.



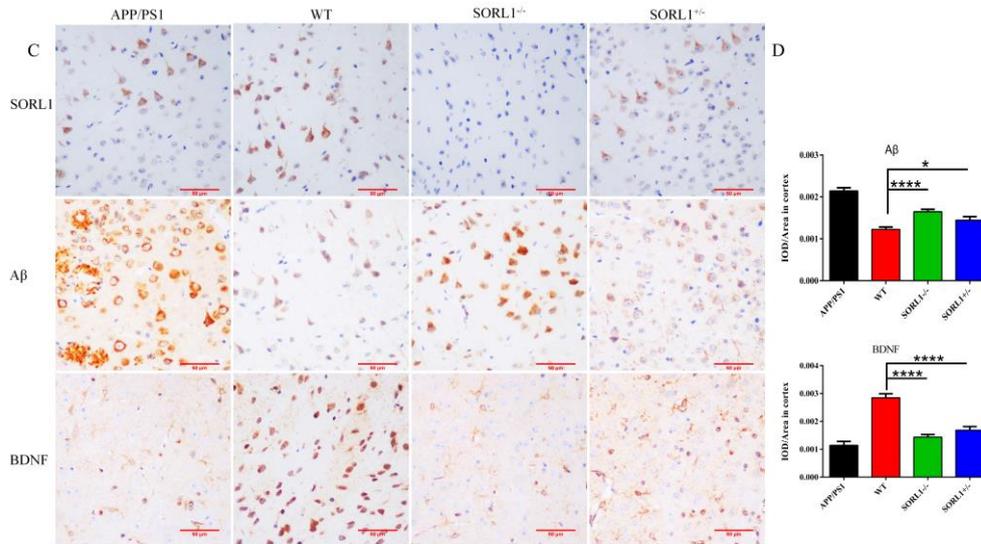


Figure 2. Immunohistochemical detection of the expression of A β and BDNF in mouse brain tissue. (A) Detection of A β and BDNF expression in mouse hippocampus, (B) Detection of A β and BDNF expression in mouse cortex, (C, D) statistical result of (A, B).

3.3 SORL1 regulates the expression of BDNF

In SORL1 knockout mice, the occurrence of A β deposition affected BDNF expression. Studies have shown that BDNF can reduce the production of A β by promoting the expression of SORL1. BDNF is an upstream transcriptional regulator of SORL1^[16]. Our study found that SORL1 also regulates the expression of BDNF. In N2a cells, after shRNA interference with SORL1 (Fig 3A, B), BDNF expression was significantly down-regulated (Fig 3C, E). Then apoptosis occurred (Fig 3F, G) following decreased cell viability (Fig 3D).

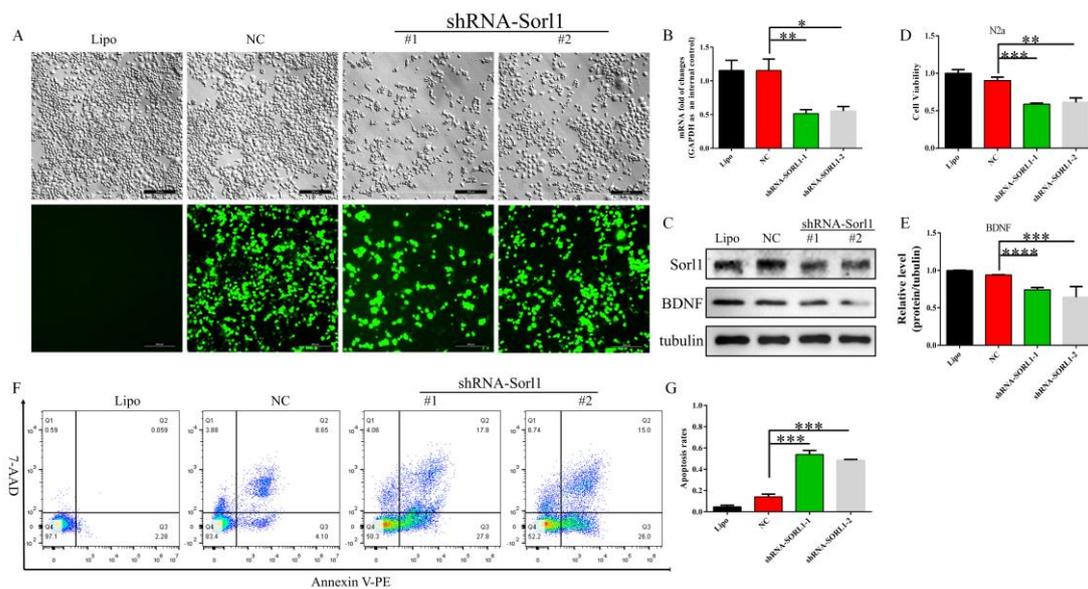


Figure 3. Expression of BDNF after interference with shRNA-SORL1 in N2a cells. (A) Observation of N2a cell morphology after interference with SORL1 expression, (B) qPCR to detect shRNA transfection efficiency, (C) Western blot to detect changes in BDNF expression after interference with SORL1, (D) CCK8 to detect changes in cell viability after shRNA interference with SORL1 for 48 hours. (F) Flow cytometric detection of cell apoptosis after shRNA interference with SORL1 for 48 hours, (E, G) Statistical results of (C, F) respectively.

3.4 SORL1 regulates the expression of BDNF by mediating the expression and distribution of NMDAR.

BDNF plays an important role in synaptic plasticity and neuronal survival. Some studies have shown that NMDAR can regulate the expression of BDNF by turning on/off the cAMP-response element binding protein (CREB). In shRNA-SORL1 cell model, we found that the expression of NMDAR-NR2B and CREB were inhibited by interference with SORL1 (Fig 4D-F). In SORL1 knockout mice, the expression of NMDAR-NR2B in the hippocampus and cortex of mice also significantly decreased (Fig 4A-C). It is suggested that SORL1 may affect the expression of BDNF by regulating the distribution of NMDAR-NR2B expression and participate in the occurrence and development of LOAD.

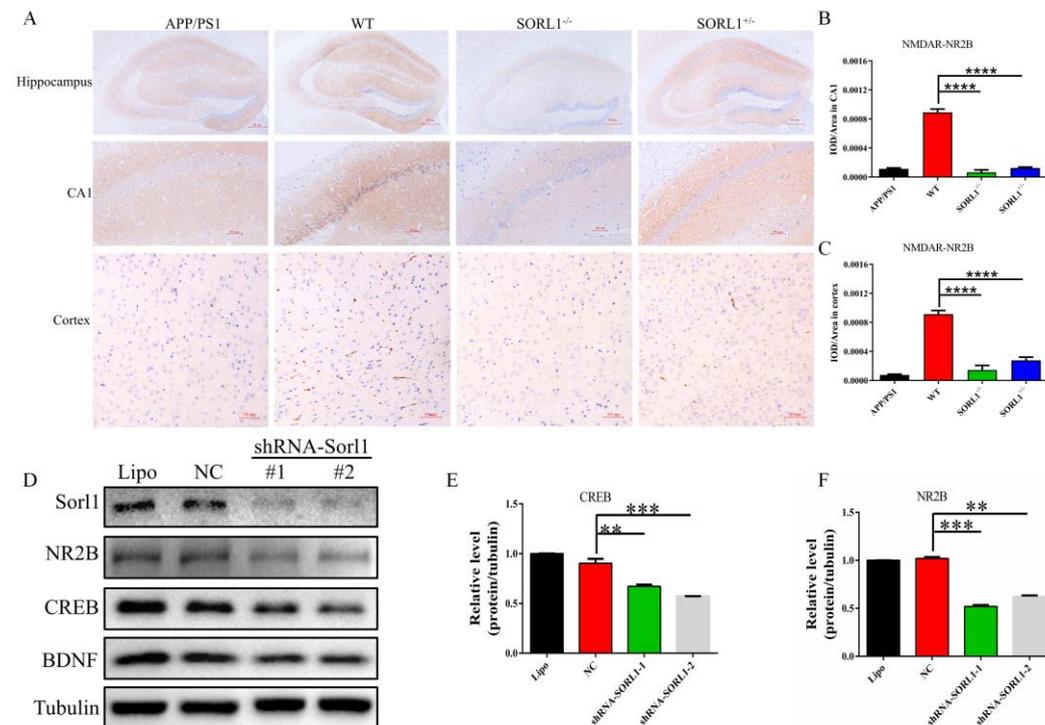


Figure 4. SORL1 regulates the expression of BDNF by mediating the expression and distribution of NMDAR. (A) Immunohistochemical detection of NMDAR-NR2B expression in mouse

hippocampus, hippocampal CA1 area, and cortex (D) Western blot detection of NMDAR-NR2B, CREB, and BDNF expression after shRNA interference with SORL1, (B) Statistical results of the expression of NMDAR-NR2B in the CA1 region of the hippocampus of mice, (C) Statistical results of the expression of NMDAR-NR2B in the cortical region of mice, (E, F) Statistical results of (D).

4. Discussion

LOAD is a kind of senile disease affected by both genetic and environmental factors. In recent years, GWAS has found that LOAD is related to more than 20 genes including SORL1 gene [17]. Our study found that SORL1, as a high-risk pathogenic gene of LOAD, participates in the occurrence and development of the disease by affecting the transport and metabolism of APP and the distribution of NMDAR expression in the synaptic/extrasynaptic. SORL1 also regulates the expression of BDNF by turning off the transcriptional translation of CREB. The abnormal closure of SORL1-NMDAR-CREB-BDNF signaling pathway and down-regulation of BDNF expression affect neuronal survival, synaptic plasticity, and memory formation and maintenance.

Studies have shown that the mRNA expression of SORL1 is significantly down-regulated in LOAD patients., The GWAS also reveals that SORL1 gene and its single nucleotide polymorphism (SNP) are associated with LOAD [18-20]. As a sorting protein-related receptor, SORL1 shuttles between Golgi matrix, endoplasmic reticulum, and cytoplasm. Therefore, it is related to the transport and metabolism of many substances in the cell. As a member of LDLR family, SORL1 participates in the occurrence and development of AD by regulating the transport and metabolism of APP. Studies have shown that SORL1 is involved in the processing and degradation of APP. When APP protein is degraded by γ -secretase and β -secretase to form toxic A β fragments, the presence of SORL1 can reduce the decomposition of APP by γ -secretase and reduce the production of toxic A β [21, 22]. However, removal of SORL1 gene in human pluripotent stem cell model can lead to enlarged endosome, affect the normal transport of APP in the cell, and induce the production of AD pathology [11].

As a high-risk pathogenic gene of LOAD, SORL1 may be involved in the progress of AD through many ways.

In early-stage AD patients, pathological changes of neuronal loss in the frontotemporal lobe hippocampus (such as nerve injury, synaptic loss and synaptic dysfunction) had been found before

the appearance of classical pathological features of A β deposition and neurofibrillary tangles (NFTs). These pathological changes are closely related to the brain-derived neurotrophic factor (BDNF) which supports the survival and differentiation of neurons. BDNF plays an important role in the formation of learning and memory. Down-regulation of BDNF expression is considered as the key factor leading to AD-related dementia. Compared with normal subjects, AD patients showed significantly decreased expression of BDNF in the peripheral blood system [23-26]. Some studies have shown that BDNF gene mutation is closely related to LOAD. RoheM et al found that BDNF can reduce the production of A β by promoting the expression of SORL1 [16]. However, exogenous use of BDNF did not reduce A β deposition in SORL1 knockout mice. This result suggests that SORL1 plays an important role in the function of BDNF. When SORL1 is deleted, the function of BDNF in improving cognitive and behavioral impairment in mice decreases [11, 27]. Through the SORL1 knockout mouse model, we found that after SORL1 was knocked out, BDNF was significantly down-regulated and the expression of A β in mouse cortex significantly increased, indicating that SORL1, as an intracellular sorter receptor-related protein, may regulate the expression and transport of BDNF. Furthermore, in the SORL1 knockdown N2a cell model constructed by shRNA, we found that the expression of BDNF was down-regulated when SORL1 was knocked out and the protein expression of upstream transcriptional regulator CREB and NMDAR was significantly down-regulated.

CREB is an important transcription factor regulating BDNF. Some studies have shown that the activation of CREB transcriptional activity can significantly up-regulate the expression of BDNF and play an important role in the maintenance of cognitive functions such as nerve cell growth, synaptic plasticity, and dendritic regeneration [28, 29]. The activation of CREB is related to the balance of synaptic/extrasynaptic NMDAR. NMDAR is a tetramer formed by multiple NR2/NR3 subunits and NR1. Like α -amino-3-hydroxy-5-methyl-4-isoxazolyl propionic acid receptor (AMPA), NMDAR is a subtype of ionic glutamate receptor located in the postsynaptic membrane of neurons. NMDAR is very important for the maintenance of synaptic plasticity and neuronal survival, and therefore a key receptor for the formation of learning and memory. It has been found that synaptic NMDAR activation can promote the expression of CREB and BDNF, which in turn plays an important role in maintaining cell growth and differentiation, synaptic plasticity, and synaptic LTP. Down-regulation of synaptic NMDAR expression or increase of

extrasynaptic NMDAR activity can induce overload Ca^{2+} inflow, dephosphorylation of CREB, and ineffective transcription of BDNF, which will then result in inhibition of neuronal growth and differentiation, synaptic loss, and abnormal LTP. The imbalance of synaptic/extrasynaptic NMDAR activity is closely related to neuronal dysfunction [30-32]. Some studies have shown that the expression of NMDAR subunit NR2B in rats decreased significantly after microwave irradiation, and the spatial learning and memory ability of rats also decreased [33]. Lin Z et al. found that treatment with xanthoceraside in AD transgenic mice can increase the expression level of NMDAR-NR2B and reduce learning and memory impairment in AD mice. Lin's study suggested that NMDAR-NR2B plays a key role in enhancing learning and memory [34]. Our study found that the expression of NMDAR-NR2B significantly decreased in the hippocampal CA1 and cortical regions of SORL1 knockout mice, and the down-regulated expression of NMDAR2B was also observed in N2a cells. This downregulated expression interfered with the expression of SORL1. These results suggest that SORL1 is involved in the occurrence and development of LOAD in multiple pathways. SORL1 participates in the progress of LOAD by affecting the transport and metabolism of APP and the expression of BDNF through the SORL1-NMDAR-CREB-BDNF signal axis. The expression of BDNF then reduces the body's neuroprotective function. The reduced function affects learning and memory. Then LOAD occurs. In conclusion, our research shows that SORL1 participates in the pathogenesis of LOAD through multiple pathways. In addition to its classic impact on APP transport and metabolism, the regulation of the NMDAR-CREB-BDNF signal axis is also an important mechanism involved in the occurrence and development of LOAD. When administering BDNF to treat AD, attention should be paid to determine whether the patient has SORL1 gene mutation and whether SORL1 can be expressed normally. How SORL1 regulates the transport and distribution of synaptic/extrasynaptic NMDAR and how it affects the expression, transport, and metabolism of BDNF requires further research.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Abbreviations

AD	Alzheimer's disease
LOAD	Late-onset Alzheimer's disease
A β	Amyloid β -protein
BDNF	Brain Derived Neurotrophic Factor
NMDAR	N-Methyl-D-aspartate
CREB	cAMP-response element binding protein
APP	β -amyloid precursor protein
Sorl1	Sortilin Related Receptor 1

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Author information

Mingri Zhao and Jiangfeng Liu contributed equally to this work.

Corresponding authors

Correspondence to Mujun Liu or Deren Hou.

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Ethics declarations

Ethics approval and consent to participate

The experiments were performed on animals as per the norms of the Institutional Ethics Committee, Central South University, China.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.