

The Gut-Brain Axis in Alzheimer's: From Microbiota Genetics to Stigmasterol's Neuroprotection Mechanism

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Objective: This study aimed to identify novel therapeutic targets for Alzheimer's disease (AD) by investigating the role of the intestinal flora (IF) via the gut-brain axis, and to predict a potential natural compound for AD treatment and elucidate its underlying mechanism.

Methods: Following a primary analytical axis, we first employed Mendelian randomization (MR) to infer causal relationships between gut microbiota and AD. To pinpoint molecular targets, we integrated Summary-data-based MR (SMR) with single-cell and spatial transcriptomics. Subsequently, network pharmacology and molecular docking were used to identify stigmasterol as a candidate compound targeting the causal pathway. Finally, the neuroprotective effects and the STIM1/Orai1-mediated mechanism were experimentally validated in vitro using A β ₁₋₄₂ exposed SH-SY5Y cells.

Results: MR-based causal inference identified *Desulfovibrio* as a risk factor for AD, while *Slackia* and the *Lachnospiraceae* NK4A136 group were protective factors. Seven key AD-related genes were identified by combining MR results with databases, which were highly druggable. SMR analysis and multi-omics integration pinpointed STIM1-mediated calcium signaling as the core causal pathway. Following the identification of stigmasterol via network pharmacology and molecular docking, in vitro experimental validation confirmed that stigmasterol significantly inhibited A β ₁₋₄₂ induced neuronal apoptosis and calcium overload by specifically modulating the STIM1/Orai1 pathway and the Bcl-2/Bax ratio.

Conclusion: This study decodes the gut-brain axis by establishing the specific causal pathway. We demonstrate that Stigmasterol exerts neuroprotective effects by inhibiting apoptosis through a IF-associated mechanism involving the STIM1/Orai1 pathway, providing novel insights into AD pathogenesis and offering a promising therapeutic strategy based on natural compounds.

Keywords: intestinal flora, Alzheimer's disease, mendelian randomization, bioinformatic analysis, network pharmacology, stigmasterol

Introduction

Alzheimer's disease (AD) is an irreversible neurodegenerative disease; currently, about 50 million people worldwide suffer from AD, and it is estimated that its global prevalence will increase to twice the current level by 2050.^{1,2} Studies have shown that intestinal flora (IF) and its metabolites can modulate neuroimmune responses and glial cell activity, thereby playing a crucial role in Alzheimer's disease (AD) pathogenesis.³⁻⁵ Despite these associations, what remains unknown is whether specific gut microbiota exert a direct causal influence on AD and the precise molecular pathways

involved, as current evidence is largely confined by the limitations of observational studies. Specifically, the knowledge gap regarding the causal mechanisms linking gut dysbiosis to neurodegeneration needs to be bridged.

In this study, we explicitly hypothesized that calcium signaling, particularly the STIM1-mediated pathway, serves as the central causal bridge within the gut-brain axis. To test this, we will use a genome-wide Mendelian randomization (MR) method, using single nucleotide polymorphisms (SNPs) as instrumental variables (IV),⁶ to explore the causal relationship between IF and AD. It will screen for AD targets influenced by IF, and combine single-cell RNA sequencing (scRNA-seq) data from AD patients and hippocampal spatial transcriptome sequencing (Stereo-seq) to parse brain cell heterogeneity and visualize their expression in cell subsets and spatial localization. Summary-data-based Mendelian Randomization (SMR) will be used to determine the causal association of core pathways with AD, Phenome-wide association study (PheWAS) will analyze the medicinal potential of core targets, and reverse network pharmacology will be used to predict traditional Chinese medicines and their active components with potential therapeutic effects. Their efficacy and medication safety will be systematically evaluated through molecular docking and in vitro experiments. The conduct of this study will not only mine the causal relationship between IF and AD, deeply analyze the pathogenesis of AD, but also provide new ideas and an important theoretical basis for the TCM treatment of AD. This integrated approach—combining Mendelian randomization (MR), multi-omics, and experimental validation—is necessary to overcome the limitations of reverse causation, pinpoint high-resolution therapeutic targets, and provide robust biological evidence for the neuroprotective efficacy of natural compounds like stigmaterol.

Materials and Methods

Study Design

All summary data used in this study were obtained from legal, publicly available data sources. The specific study flowchart is shown in [Figure 1](#).

Computational Methods

MR Data Sources and Analysis

This Mendelian randomization (MR) analysis was designed to elucidate the potential causal relationship between intestinal flora (IF) and Alzheimer's disease (AD), adhering to the STROBE-MR reporting guidelines⁷ and established methodological principles.⁸ The MR framework rests on three core assumptions: (1) the genetic variants used as instrumental variables (IVs) are significantly associated with specific taxa of IF; (2) the association between the genetic variants and AD is not influenced by unmeasured confounding factors; (3) the effect of the genetic variants on AD is mediated exclusively through the IF taxa. A reverse MR analysis was also performed to assess the potential causal effect of AD on IFs.

Exposure summary statistics for IF were derived from the MiBioGen consortium GWAS ($n = 18,340$; ~85% European ancestry), which profiled microbial taxa using 16S rRNA sequencing across six taxonomic levels.⁹ Alzheimer's disease (AD) outcome data were obtained from the IEU OpenGWAS dataset (ID: ieu-b-5067).¹⁰ To secure sufficient genetic instruments from gut microbiota GWAS,¹¹ instrumental variables (IVs) were selected at a widely accepted threshold of $P < 1 \times 10^{-5}$, pruned for linkage disequilibrium ($r^2 < 0.01$, European 1000 Genomes reference), and filtered by effect allele frequency > 0.01 , exclusion of palindromic SNPs, and F-statistic ≥ 10 .

The inverse-variance weighted (IVW) method was employed as the primary approach to estimate the causal effects of intestinal flora on Alzheimer's disease. Sensitivity analyses were conducted using MR-Egger regression, weighted median, and weighted mode methods, and causal estimates for individual SNPs were calculated with the Wald ratio.

Identify the Key Targets and Predict Potential Chinese Herbs

Significant SNPs were annotated to their nearest genes using SNPnexus,¹² and the corresponding exposure genes were analyzed by MR. Expression quantitative trait locus (eQTL) data from peripheral blood transcriptomes of European populations were incorporated, retaining independent loci with $P < 0.05$.¹³ AD-related genes were obtained from DisGeNET,¹⁴ and their intersection with SNP-mapped genes was determined using jvenn to identify key molecular

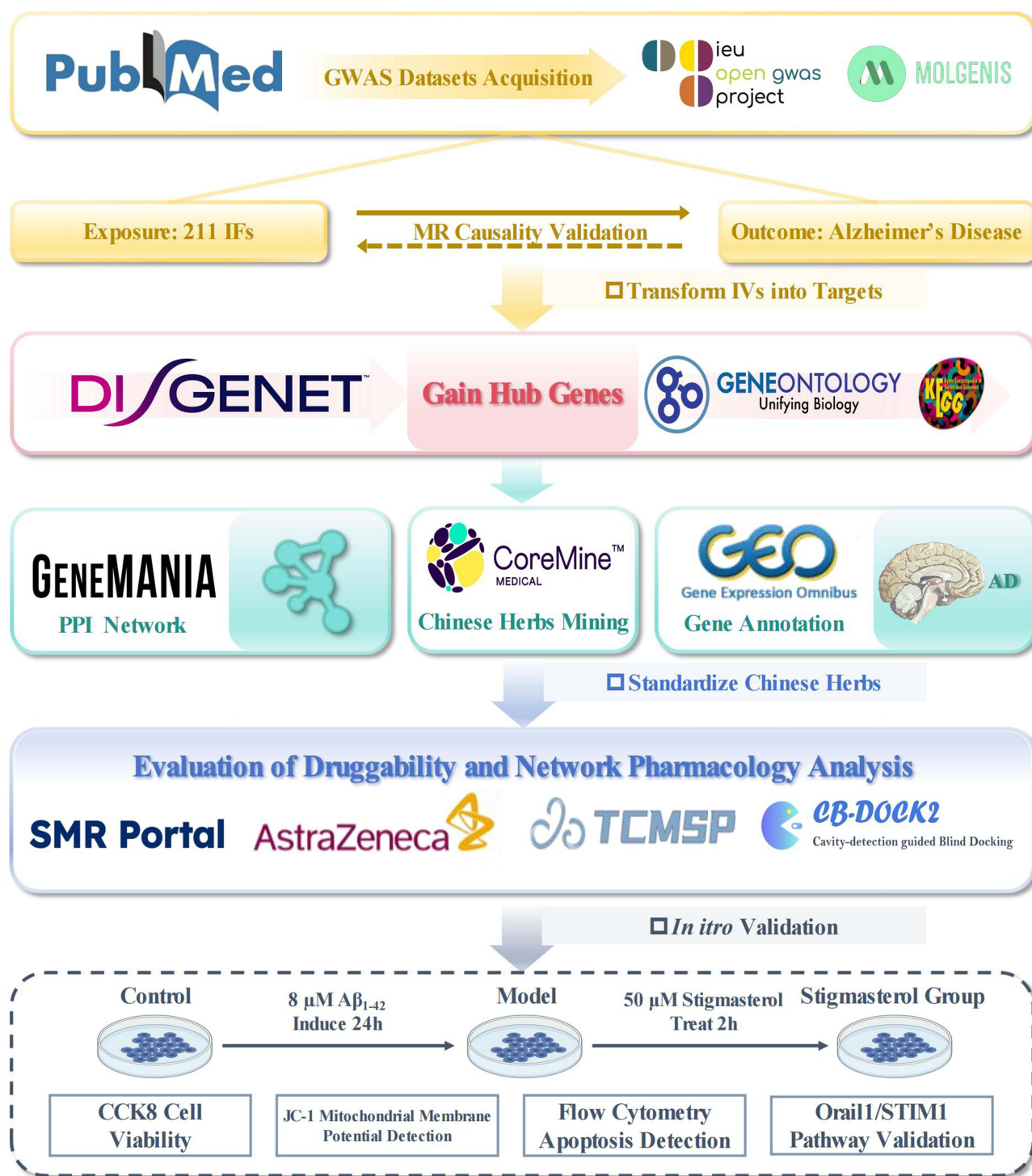


Figure 1 Study Flowchart.

targets. Finally, Coremine was employed to predict Chinese herbs potentially interacting with these targets, with a relevance score threshold > 2.

Enrichment Analysis and Determination of Core Pathway-Disease Causality

Protein–protein interaction (PPI) networks of key targets were constructed using GeneMANIA (<https://genemania.org/>). Functional enrichment was conducted via KEGG and GO analyses. SMR was applied to assess causal links between core

pathway-related genes and AD across multi-omic levels, including methylation (mQTL), eQTL, splicing (sQTL), and circulating proteins (pQTL).¹⁵ The cis-xQTL threshold was set at $P < 5 \times 10^{-9}$ within a ± 1000 kb window around target genes. The Heterogeneity in Dependent Instruments (HEIDI) test was used to exclude linkage effects. All analyses were performed in SMR v1.4.0, with significance defined as $P_{\text{smr}} < 0.05$ and $P_{\text{HEIDI}} \geq 0.05$.

Single-Cell RNA Sequencing Data Analysis of Key Targets

Single-cell and spatial transcriptomic analyses were performed to delineate the cellular and spatial expression patterns of key genes. Single-cell RNA-sequencing data (GSE175814) from AD patients were obtained from the Gene Expression Omnibus, encompassing brain regions including the auditory association cortex (BA41/42), prefrontal cortex (BA6/8), and anterior hippocampal cortex.¹⁶ Low-quality cells expressing <200 or $>4,500$ genes or exhibiting $>20\%$ mitochondrial transcripts were excluded. Gene expression matrices were log-normalized, and highly variable genes were identified for principal component analysis (PCA) and clustering. Uniform Manifold Approximation and Projection (UMAP) and t-distributed stochastic neighbor embedding (t-SNE) were applied for dimensionality reduction and visualization of cellular heterogeneity. Cell types were annotated using scMayoMap.

Spatial Transcriptome Sequencing Data Analysis of Key Targets

A hippocampal spatial transcriptomic dataset (CNP0005077) from AD patients was obtained from the China National GeneBank (CNCB, <https://db.cngb.org/>).¹⁷ The dataset comprises hippocampal sections from eight patients with moderate-to-advanced AD and eight age- and sex-matched controls, totaling 32 spatial sections spanning 2,130 genes and 5,166 unique molecular identifiers across 17 cell types.

Spatial expression profiling of seven key target genes was performed to delineate their anatomical localization and expression abundance within hippocampal structures, providing spatial references for subsequent cellular-level validation. Raw spatial transcriptomic data were processed and visualized to delineate gene coordinates and spatial expression landscapes, highlighting the distribution patterns of target genes across hippocampal subregions.

Druggability Analysis Based on PheWAS

To assess horizontal pleiotropy and potential adverse effects of candidate drug targets, a phenome-wide association study (PheWAS) was performed using the AstraZeneca PheWAS Portal (<https://azphewas.com/>). The portal contains data from $\sim 450,000$ exome-sequenced UK Biobank participants, encompassing $\sim 15,500$ binary and $\sim 1,500$ continuous phenotypes.¹⁸ Multiple testing correction was applied, with significance defined at $P < 2 \times 10^{-9}$. Manhattan plots were generated in R using the qqman package to visualize the PheWAS outcomes.

Network Pharmacology and Molecular Docking

To elucidate the potential pharmacological effects of the predicted Chinese herbs, a network pharmacology and molecular docking analysis was conducted. Active compounds were retrieved from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) using the herb keywords *Prunus mume* (Meihua), *Cornus officinalis* (Shanzhuyu), *Centipeda minima* (Ebushicao), *Macrocytis pyrifera* (Haizao), *Calculus bovis* (Niu Huang), *Rhinoceros horn* (Xijiao), *Lycium barbarum* (Gouqizi), *Sesamum indicum* (Heizhima), *Alpinia officinarum* (Gaoliangjiang), and *Prunus mume* var. *mume* (Wumei). Screening criteria were set as oral bioavailability (OB) $\geq 30\%$, drug-likeness (DL) ≥ 0.18 , blood–brain barrier (BBB) permeability ≥ -0.3 , and half-life (HL) ≥ 4 . For herbs not available in TCMSP, compounds were supplemented from the HERB database according to Lipinski's Rule of Five and OB $\geq 30\%$.

Predicted compound–target interactions were identified via SwissTargetPrediction, with TargetNet used for supplementary mapping. The compound–target network was constructed, and key components were prioritized based on degree, betweenness, and closeness centralities calculated by the Network Analyzer plugin. The top five nodes by degree value were designated as potential bioactive compounds for AD therapy. Protein structures were obtained from the Protein Data Bank (PDB). The binding conformation with the lowest energy was selected for subsequent interaction analysis.

Experimental Methods

Experimental Cells and Main Instruments

The human neuroblastoma cell line SH-SY5Y was obtained from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. This cell line was selected as our *in vitro* model because it exhibits human neuronal characteristics and is a widely validated, standard model for investigating A β -induced neurotoxicity, calcium dyshomeostasis, and apoptosis in Alzheimer's disease research. Cells were cultured in a humidified incubator (Thermo Fisher Scientific, USA). Major instruments included an Alliance 6.7 gel imaging system (UVITEC, UK), an inverted phase-contrast microscope (Olympus, Japan), and a Western blot electrophoresis system (Bio-Rad, USA).

Experimental Reagents

Reagents included Dulbecco's Modified Eagle Medium (DMEM, high glucose; Gibco), penicillin–streptomycin (Waltham), fetal bovine serum (Gibco), PBS (Mianlun, Dalian), β -amyloid 1–42 (A β 1–42; Mianlun, Dalian; cat. no. MB10425), CCK8 assay kit (Beyotime; cat. no. C0037), JC-1 mitochondrial membrane potential assay (Solarbio; cat. no. C1086), and apoptosis detection kit (Beyotime; cat. no. C1086).

Primary antibodies included anti- β -actin (mouse monoclonal, CST; cat. no. 3700), anti-STIM1 (Thermo Fisher Scientific; cat. no. RT1596), anti-Orai1 (Haodi Huatuo, Shenzhen; cat. no. PL0402498), anti-Bax (Thermo Fisher Scientific; cat. no. ER0907), and anti-Bcl-2 (Sanying, Wuhan; cat. no. 80313-1-RR). HRP-conjugated secondary antibodies were used for detection.

Determination of Safe Intervention Concentration of Stigmasterol and Cell Viability

Cells were maintained at 37 °C in a humidified incubator with 5% CO₂. Once stabilized, SH-SY5Y cells were seeded into 96-well plates (1×10^5 cells/well). To determine the safe intervention range of stigmasterol, cells were exposed to different concentrations (10, 30, 50, and 100 μ M) for 24 h. Cell viability was assessed using the CCK-8 assay, in which 10 μ L of reagent was added per well and incubated for 2 h before measuring absorbance at 450 nm with a microplate reader to calculate the IC₅₀.

For AD modeling, cells in the model and treatment groups were exposed to 8 μ M A β 1–42 for 24 h. The stigmasterol groups received concurrent stigmasterol treatment at sub-IC₅₀ concentrations. Cell viability was then evaluated using the same CCK-8 protocol.

Detection of Mitochondrial Membrane Potential and Cell Apoptosis Level

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using the JC-1 assay. Cells were stained according to the manufacturer's instructions, and fluorescence shifts from red to green indicated $\Delta\Psi_m$ loss.

To quantify apoptosis, cells from the control, A β model, and stigmasterol treatment groups (5×10^6 per group) were collected, washed with PBS, and stained with Annexin V-APC/PI for 15 min in the dark. After resuspension (1×10^6 cells/mL), apoptotic cells were quantified by flow cytometry. Early and late apoptotic populations were identified as Annexin V⁺/PI[−] and Annexin V⁺/PI⁺, respectively.

Detection of Target Protein Expression Levels in the STIM1/Orai1 Signaling Pathway and Apoptotic Proteins via Western Blot

Protein expression of STIM1, Orai1, Bax, Bcl-2, and β -actin was determined by Western blotting. After transfer to nitrocellulose membranes, blots were incubated with primary antibodies at 4 °C overnight and HRP-conjugated secondary antibodies for 1 h at room temperature. Signals were visualized using enhanced chemiluminescence, and band intensities were quantified with image analysis software. Protein levels were normalized to β -actin.

Statistical Analysis

All statistical and computational analyses were performed using RStudio (version 4.3.2; R Foundation for Statistical Computing, Vienna, Austria). Network visualization and topological analyses were conducted in Cytoscape (version 3.9.1). Mendelian randomization (MR) was primarily performed using the inverse-variance weighted (IVW) method, with MR-Egger, weighted median, and weighted mode approaches applied for sensitivity validation. Multi-omic causal

inference was conducted using SMR software (version 1.4.0), and molecular docking simulations were executed in AutoDock Tools employing the Lamarckian genetic algorithm.

Data conforming to a normal distribution were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, whereas non-parametric data were evaluated using the Kruskal–Wallis test. Two-tailed $P < 0.05$ were considered statistically significant after appropriate corrections for multiple testing. All graphs and statistical visualizations were generated in R or GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA).

Results

Mendelian Randomization Results

The range of genetic variants exposed by each IV included 3–22 SNPs, with F-statistics ranging from 17 to 29, indicating the absence of weak instrument bias. Results from MR-PRESSO showed no horizontal pleiotropy ($P > 0.05$). Through weighted median analysis, three IFs with causal associations with AD were identified. Among these, *Desulfovibrio* was positively correlated with AD risk (OR = 1.001, 95% CI = 1.000–1.002, $P = 0.014$), while *Slackia* (OR = 0.999, 95% CI = 0.998–0.999, $P = 0.026$) and the *Lachnospiraceae NK4A136* group (OR = 0.999, 95% CI = 0.998–0.999, $P = 0.018$) were negatively correlated with AD risk (see Figure 2A). The intercept of MR Egger regression did not deviate from 0, further supporting the absence of horizontal pleiotropy (see Figure 2B and D). Leave-one-out analysis revealed no significant differences in the causal relationship between AD across different IV groups, indicating that the associations were not driven by a single IV (see Figure 2E and G). Funnel plot analysis showed no heterogeneity among the genetic IVs of these bacterial taxa (see Figure 2H and Figure 2J). Additionally, reverse MR analysis found no evidence of a causal relationship between AD and these IFs. These MR findings established robust genetically predicted causal links between three specific gut microbiota taxa and the risk of AD.

Acquisition of Core Targets and Prediction of Potential Chinese Herbs

A total of 24 genes were screened by mapping SNP-associated neighboring genes using the SNPnexus database combined with MR analysis (See Figure 3A). A search in the DisGeNET database using “Alzheimer’s Disease” as the keyword yielded 3,397 potential targets related to AD. Subsequently, the jvenn software was used to perform intersection analysis between the 24 genes and the 3,397 AD targets, resulting in the identification of 7 key targets (SUCLG2, STIM1, CYP46A1, ANK3, CSMD1, RYR3, and RBFOX1). Based on these targets, further Chinese herbs association prediction was conducted in the database, resulting in 44 potentially relevant Chinese herbs (no associated Chinese herbs were found for the RYR3 target). By setting an association threshold (> 2) and considering inclusion in the Chinese Pharmacopoeia (2020 edition), 10 representative Chinese herbs with potential benefits for AD treatment were finally screened out: *Prunus mume* (Meihua), *Cornus officinalis* (Shanzhuyu), *Centipeda minima* (Ebushicao), *Macrocytis pyrifera* (Haizao), *Calculus bovis* (Niu Huang), *Rhinoceros horn* (Xijiao), *Lycium barbarum* (Gouqizi), *Sesamum indicum* (Heizhima), *Alpinia officinarum* (Gaoliangjiang), and *Prunus mume* var. *mume* (Wumei). (see Figure 3B and C). This integrative target mining strategy pinpointed seven core AD-related genes and identified ten Chinese herbs with potential therapeutic value.

Determination of Causality Between Core Pathways, Biological Processes, and Disease

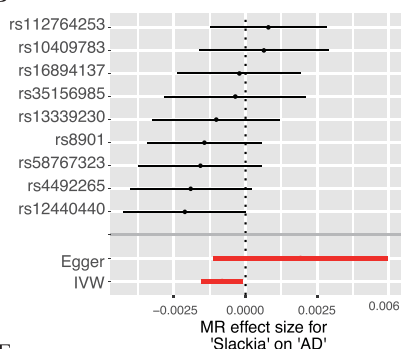
To explore the interaction relationships among key genes, a PPI interaction model was constructed (see Figure 3D). This network consisted of 27 protein nodes and multiple interaction edges; the connections between nodes represented various association types, including physical interactions, co-expression, genetic interactions, and pathway co-activation, presenting complex regulatory relationships overall. Proteins such as STIM1, ANK3, RYR3, and SUCLG2 exhibited high connectivity and occupied core positions in the network.

To investigate the biological functions of core genes in disease occurrence and progression, the R package clusterProfiler was used to perform KEGG pathway and GO functional enrichment analyses for the core genes. KEGG pathway enrichment analysis results (see Figure 3E) showed that the most critical pathway was the “Calcium signaling

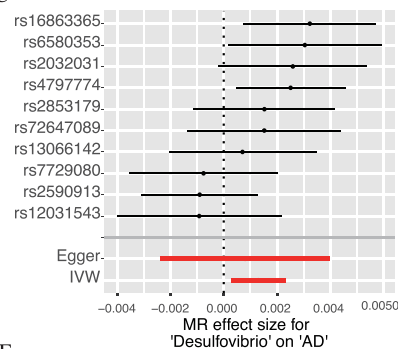
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exposure	outcome	nsnp	method	pval	OR(95% CI)
Desulfovibrio	AD	10	MR Egger	0.635	1.001 (0.998 to 1.004)
		10	Weighted median	0.012	1.002 (1.000 to 1.003)
		10	Inverse variance weighted	0.014	1.001 (1.000 to 1.002)
		10	Simple mode	0.103	1.002 (1.000 to 1.005)
		10	Weighted mode	0.113	1.002 (1.000 to 1.005)
LachnospiraceaeNK4A136group	AD	16	MR Egger	0.399	0.999 (0.997 to 1.001)
		16	Weighted median	0.221	0.999 (0.998 to 1.000)
		16	Inverse variance weighted	0.018	0.999 (0.998 to 1.000)
		16	Simple mode	0.560	0.999 (0.998 to 1.001)
		16	Weighted mode	0.403	0.999 (0.998 to 1.001)
Slackia	AD	9	MR Egger	0.257	1.002 (0.999 to 1.005)
		9	Weighted median	0.049	0.999 (0.998 to 1.000)
		9	Inverse variance weighted	0.026	0.999 (0.998 to 1.000)
		9	Simple mode	0.121	0.999 (0.997 to 1.000)
		9	Weighted mode	0.134	0.999 (0.997 to 1.000)

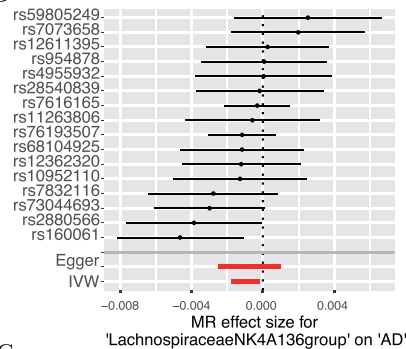
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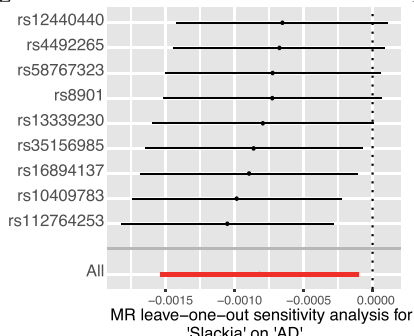
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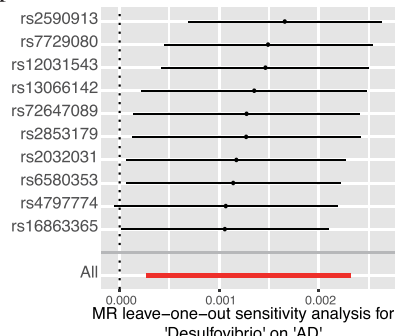
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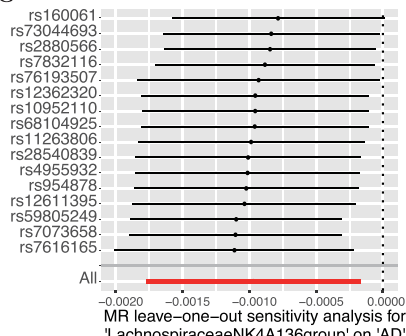
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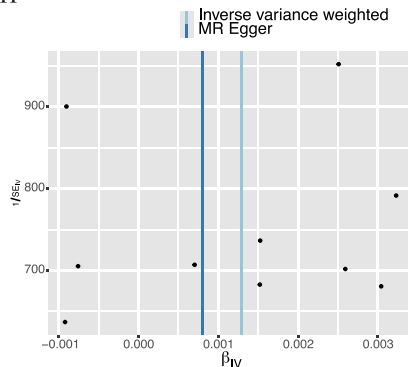
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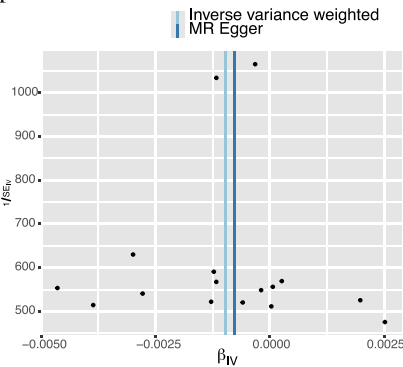
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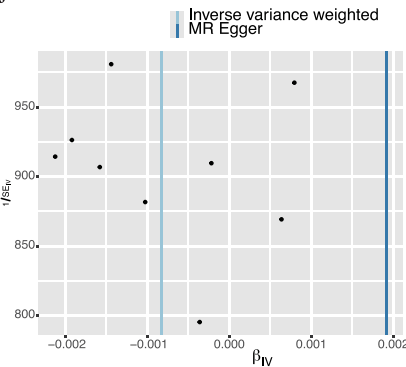


Figure 2 Results of MR Analysis: (A) Three IFs associated with AD; (B) Analysis results of *Slackia* and AD; (C) Analysis results of *Desulfovibrio* and AD; (D) MR analysis results of the *Lachnospiraceae NK4A136 group* and AD; (E) Leave-one-out analysis results of *Slackia* and AD; (F) Leave-one-out analysis results of *Desulfovibrio* and AD; (G) Leave-one-out analysis results of the *Lachnospiraceae NK4A136 group* and AD; (H) Funnel plot analysis results of *Slackia*; (I) Funnel plot analysis results of *Desulfovibrio*; (J) Funnel plot analysis results of the *Lachnospiraceae NK4A136 group*. Bold text indicates $P < 0.05$.

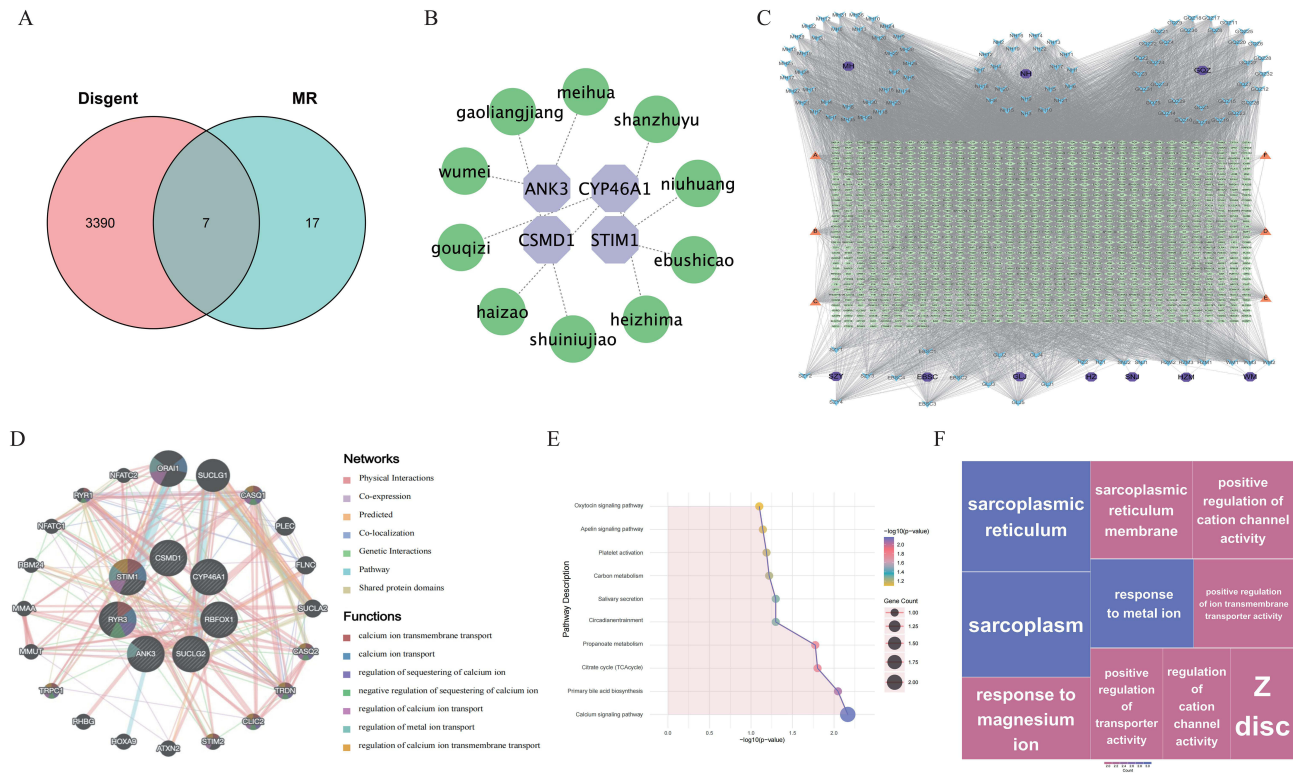


Figure 3 Results of Chinese Herbs Mining, Network Pharmacology and Enrichment Analysis. **(A)** Intersection Targets of SNP-adjacent Genes and AD; **(B)** Chinese Herbs Mined from Key Genes; **(C)** Chinese Herbs-Compound-Target Network; **(D)** Result of PPI Analysis; **(E)** KEGG pathway enrichment results; **(F)** GO functional enrichment results.

pathway”; additional enriched metabolic pathways included “Primary bile acid biosynthesis” and “Citrate cycle (TCA cycle)”. GO functional enrichment analysis results revealed that core target genes were involved in 82 biological processes (see Figure 3F). These genes were mainly enriched in biological processes such as “response to metal ion”, “response to magnesium ion”, “positive regulation of cation channel activity”, “calcium ion homeostasis”, and “regulation of monoatomic cation transmembrane transport”.

SMR analysis was performed on genes related to the calcium signaling pathway retrieved from KEGG (see Figure 4A). The results showed that the calcium signaling pathway had extensive causal associations with AD occurrence, and these associations exhibited different strengths across various molecular regulatory levels (see Figure 4B and E). At the transcriptional level, the expression levels of genes such as CAMK1, STIM2, GRIN3A, and ATP2A1 within the pathway showed significant causal relationships with AD risk. At the DNA methylation level, the signal enrichment degree was much higher than that at the other three levels. At the protein abundance and alternative splicing levels, relatively fewer pathway genes showed causal relationships with AD. Through multi-omics SMR analysis, genetic evidence confirmed the causal relationship between calcium signaling pathway dysfunction and AD pathogenesis; DNA methylation and gene transcription are key links driving the pathogenic effects of this pathway.

Results of Single-Cell Sequencing Analysis

In-depth analysis was conducted on scRNA-seq data from AD patients, and visualization analysis was performed using Uniform Manifold Approximation and Projection (UMAP) based on the first 15 principal components. Clustering analysis successfully distinguished and annotated 14 cell subpopulations with significant differences. The SingleR R package was used for systematic annotation of these cell populations, identifying multiple cell types including B cells and endothelial cells. UMAP visualization results showed the distribution characteristics of these cell types in the two-dimensional reduced space of UMAP_1 and UMAP_2; meanwhile, t-distributed Stochastic Neighbor Embedding (tSNE) provided supplementary verification in the tSNE_1 and tSNE_2 dimensions, and the results of the two analysis

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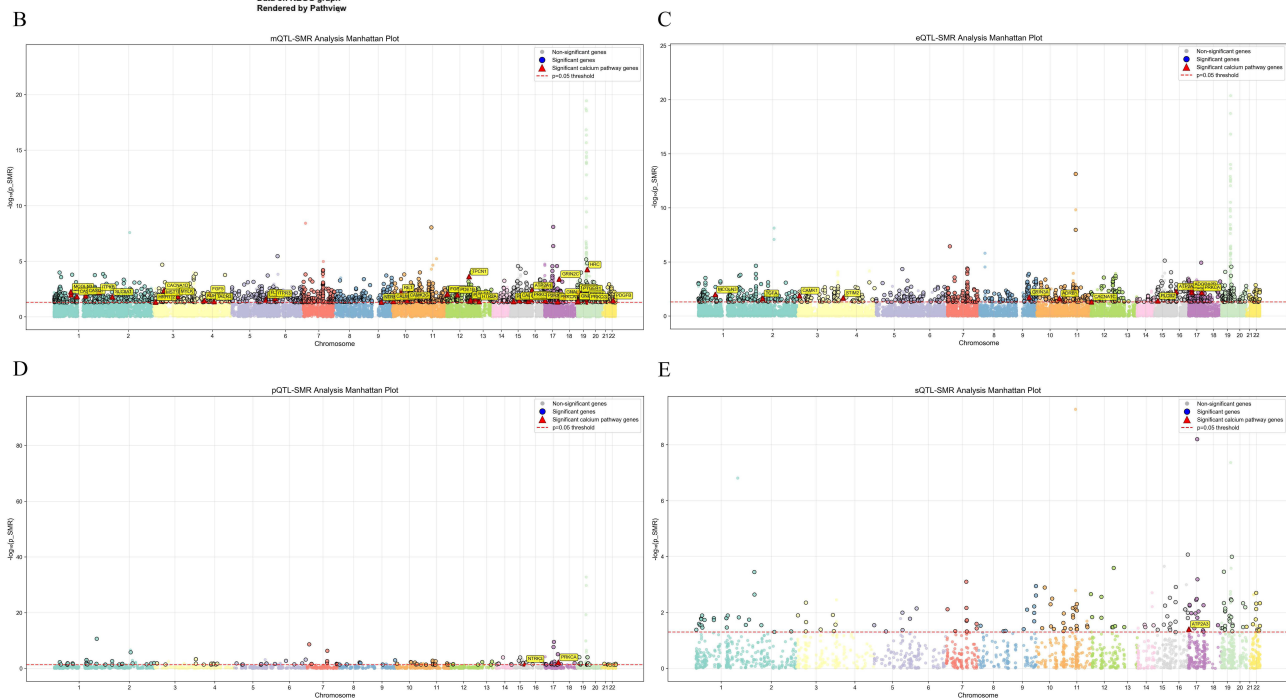
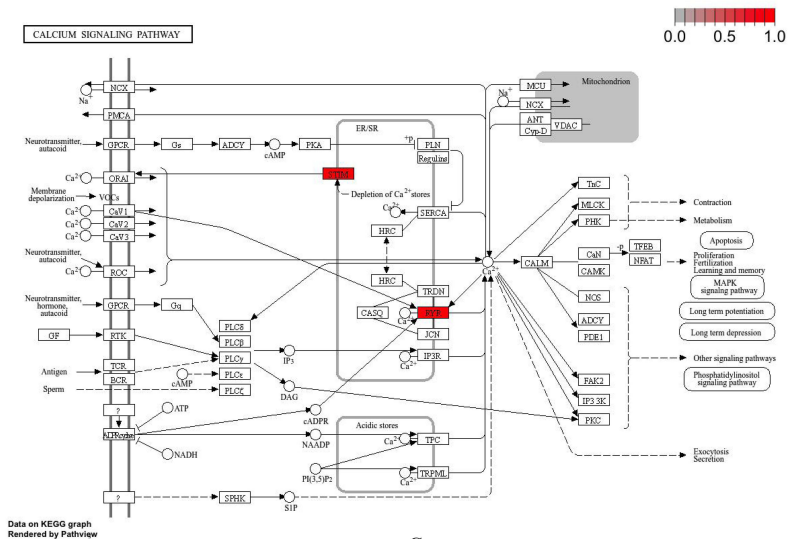


Figure 4 Multi-Omics SMR Analysis of Core Pathways: (A) Calcium signaling pathway; (B) mQTL-SMR analysis; (C) eQTL-SMR analysis; (D) pQTL-SMR analysis; (E) sQTL-SMR analysis.

methods showed high consistency (Figure 5A). Further analysis revealed that among the 7 key intersection genes, only ANK3, STIM1, and SUCLG2 showed significant expression in the cell populations (Figure 5B). The SUCLG2 gene showed highly specific and strong expression in Mural cells, with its expression cell ratio and average expression level significantly higher than all other cells. The STIM1 gene was also relatively active in Mural cells and macrophages, while ANK3 was expressed at a low ratio but high level in a variety of cells. Single-cell profiling revealed distinct, cell-type-specific expression patterns for the core target genes within the AD brain microenvironment.

Results of Spatial Transcriptomics Analysis

Unsupervised clustering was performed on spatial loci in the dataset, accurately dividing the hippocampal tissue into multiple anatomical subregions including the Alveus, CA1, CA2, CA3-4, dentate gyrus (DG), and Subiculum (Sub) (see Figure 5C). Multiple cell types were identified, such as astrocytes (Astro), excitatory neurons (EX), inhibitory neurons

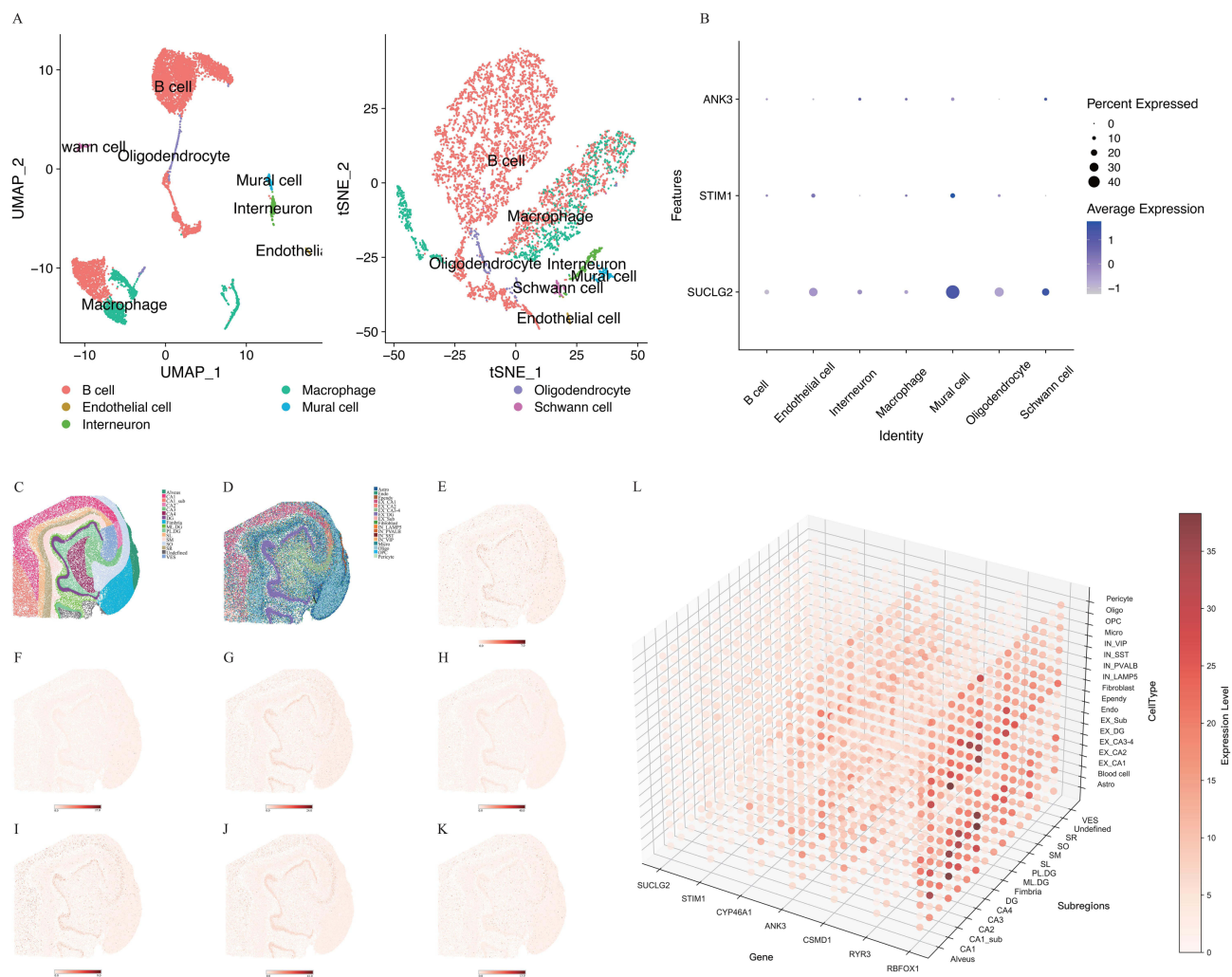


Figure 5 Results of Single-Cell Sequencing Analysis & Spatial Transcriptomics Analysis: **(A)** Uniform Manifold Approximation and Projection (UMAP) clustering plot and t-distributed Stochastic Neighbor Embedding (tSNE) clustering plot; **(B)** Expression patterns of potential therapeutic genes in different cell types from AD samples; **(C)** Anatomical subregions of the hippocampus; **(D)** Spatial distribution of various cell types in the hippocampus; **(E–K)** Spatial expression distribution of STIM1, SUCLG2, ANK3, CSMD1, CYP46A1, RBFOX1, and RYR3 genes in tissue sections; **(L)** Heatmap of key Gene-Subregions-Cell Type Expression Level.

(IN), and oligodendrocytes (Oligo) (see Figure 5D). Spatial expression pattern analysis of the 7 key genes revealed unique distribution characteristics for each gene (see Figure 5C–L): RBFOX1, ANK3, and RYR3 were widely expressed in the neuronal layers of the hippocampus, with high enrichment particularly in excitatory neurons of regions such as CA1, CA3-4, and DG. The expression of SUCLG2 and CSMD1 showed stronger regional specificity, with high expression signals mainly restricted to the dentate gyrus (DG) and hippocampal CA3-4 region. The expression of STIM1 was relatively diffuse, but with relatively concentrated signals in the CA1 and Subiculum regions. The expression signal of CYP46A1 was widely distributed throughout the tissue section and not limited to specific neuronal layers.

To confirm the spatial distribution of gene expression, quantitative analysis was performed (see Figure 5L). As a pan-neuronal expression gene, RBFOX1 showed an expression level of 14.58 in LAMP5-positive interneurons. SUCLG2 and CSMD1 were specifically enriched in excitatory neurons of the dentate gyrus, with average expression levels of 4.12 and 5.21, respectively. Although the expression of STIM1 was relatively diffuse, it also showed high expression in excitatory neurons of the CA1 and Subiculum regions. These findings revealed highly heterogeneous spatial expression profiles of different key genes across hippocampal subregions and cell types, providing important localization references for subsequent functional studies.

Druggability Analysis Based on PheWAS

To identify potential adverse reactions of druggable genes targeted for AD, the PheWAS Portal database was used to systematically examine the genetic associations between the 7 core genes and a wide range of clinical phenotypes. A genome-wide significance threshold ($P < 5 \times 10^{-8}$) was adopted as the criterion for determining whether an association was statistically significant. The results showed that none of the 7 genes exhibited a significant statistical association with any disease phenotype in the database; modulating the function of these genes may not significantly increase the risk of common diseases, implying a low likelihood of systemic off-target effects caused by drugs targeting these genes (see Figure 6A–N). PheWAS analysis confirmed that modulating these seven core genes carries a low risk of systemic pleiotropic side effects, supporting their safety as therapeutic targets.

Results of Network Pharmacology and Molecular Docking

A total of 119 active components were obtained after screening according to the screening criteria. In the “drug-component-target” network analysis, with node degree value as the primary criterion and betweenness centrality/closeness centrality as secondary criteria, the top 5 components were screened out: mandenol, stigmasterol, CLR, 1-[(Z)-2-(3,5-dimethoxyphenyl)ethenyl]-3,5-dimethoxybenzene, and butyl-2-ethylhexyl phthalate. Combined with highly

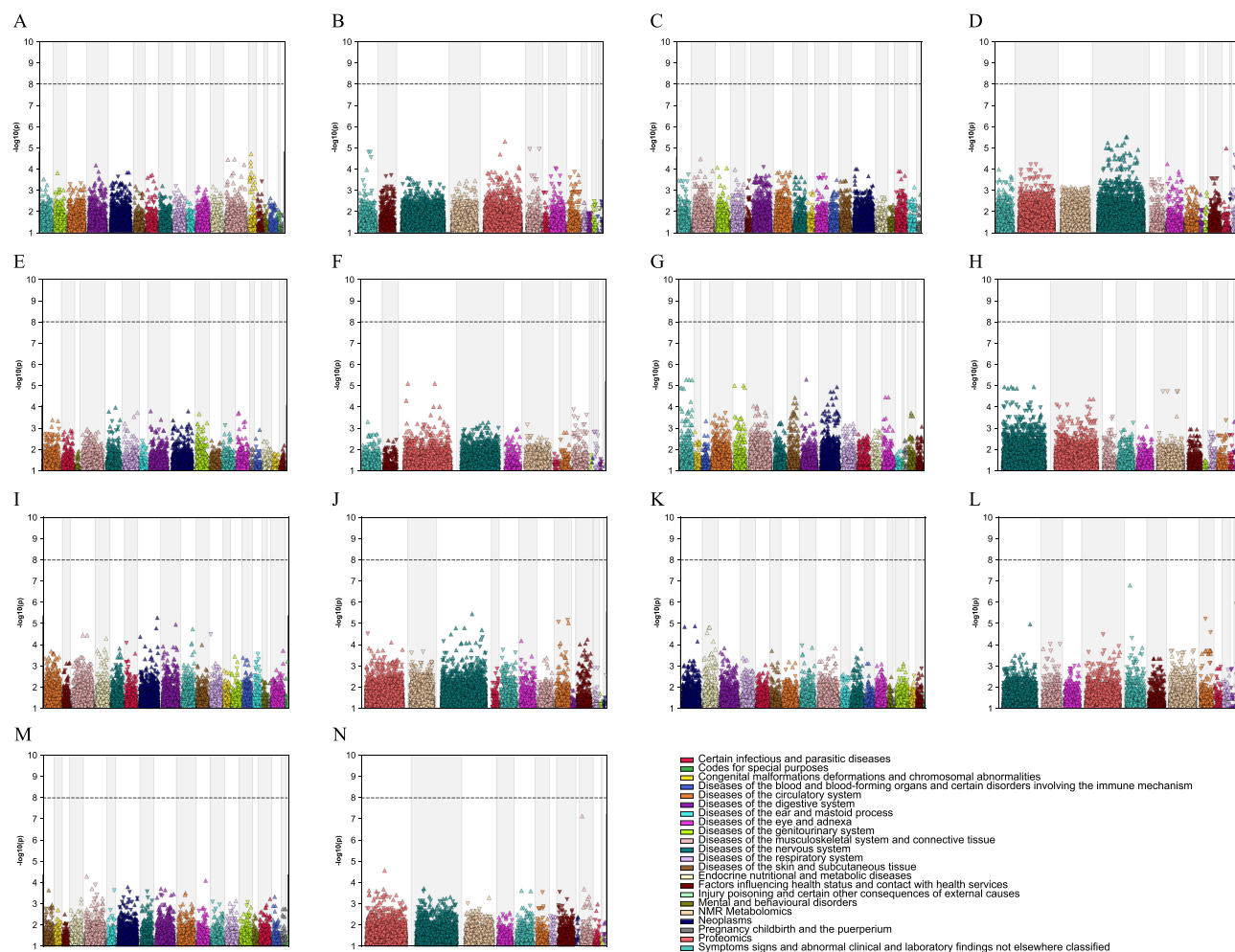


Figure 6 Results of PheWAS Analysis for Core Genes: (A) ANK3 - Binary Logistic Regression; (B) ANK3 - Linear Regression; (C) CSMD1 - Binary Logistic Regression; (D) CSMD1 - Linear Regression; (E) CYP46A1 - Binary Logistic Regression; (F) CYP46A1 - Linear Regression; (G) RBMFOX1 - Binary Logistic Regression; (H) RBMFOX1 - Linear Regression; (I) RYR3 - Binary Logistic Regression; (J) RYR3 - Linear Regression; (K) STIM1 - Binary Logistic Regression; (L) STIM1 - Linear Regression; (M) SUCLG2 - Binary Logistic Regression; (N) SUCLG2 - Linear Regression.

relevant targets identified in single-cell analysis and traditional TCM prediction, ANK3 and STIM1 were determined as candidate proteins for molecular docking.

Molecular docking results showed that the binding energies of ANK3-CLR, ANK3-stigmasterol, STIM1-butyl -2-ethylhexyl phthalate, STIM1-mandenol, and STIM1-1-[(Z)-2-(3,5-dimethoxyphenyl)ethenyl]-3,5-dimethoxybenzene were all below -5 kcal/mol, indicating good binding affinity. Meanwhile, the binding energies of STIM1-CLR and STIM1-stigmasterol were below -7 kcal/mol, showing strong interactions. The docking results were visualized in three dimensions using PyMOL software to display the interactions (see Figure 7A–K). Molecular docking identified stigmasterol as a highly promising bioactive candidate demonstrating strong binding affinity for the core targets STIM1.

Results of Cellular Experiments

Detection of Cell Proliferation Activity by CCK8 Assay

To determine the safe intervention concentration, a toxicity curve of SH-SY5Y cells treated with different concentrations of stigmasterol was generated using the CCK8 assay. The half-maximal inhibitory concentration (IC₅₀) value showed that stigmasterol at a concentration of 67.78 μ M could inhibit 50% of cell proliferation; therefore, 50 μ M stigmasterol was selected for subsequent experiments (see Figure 8A). The cell viability assay revealed that compared with the control group, A β ₁₋₄₂ treatment significantly reduced the cell viability of the model group. However, after stigmasterol

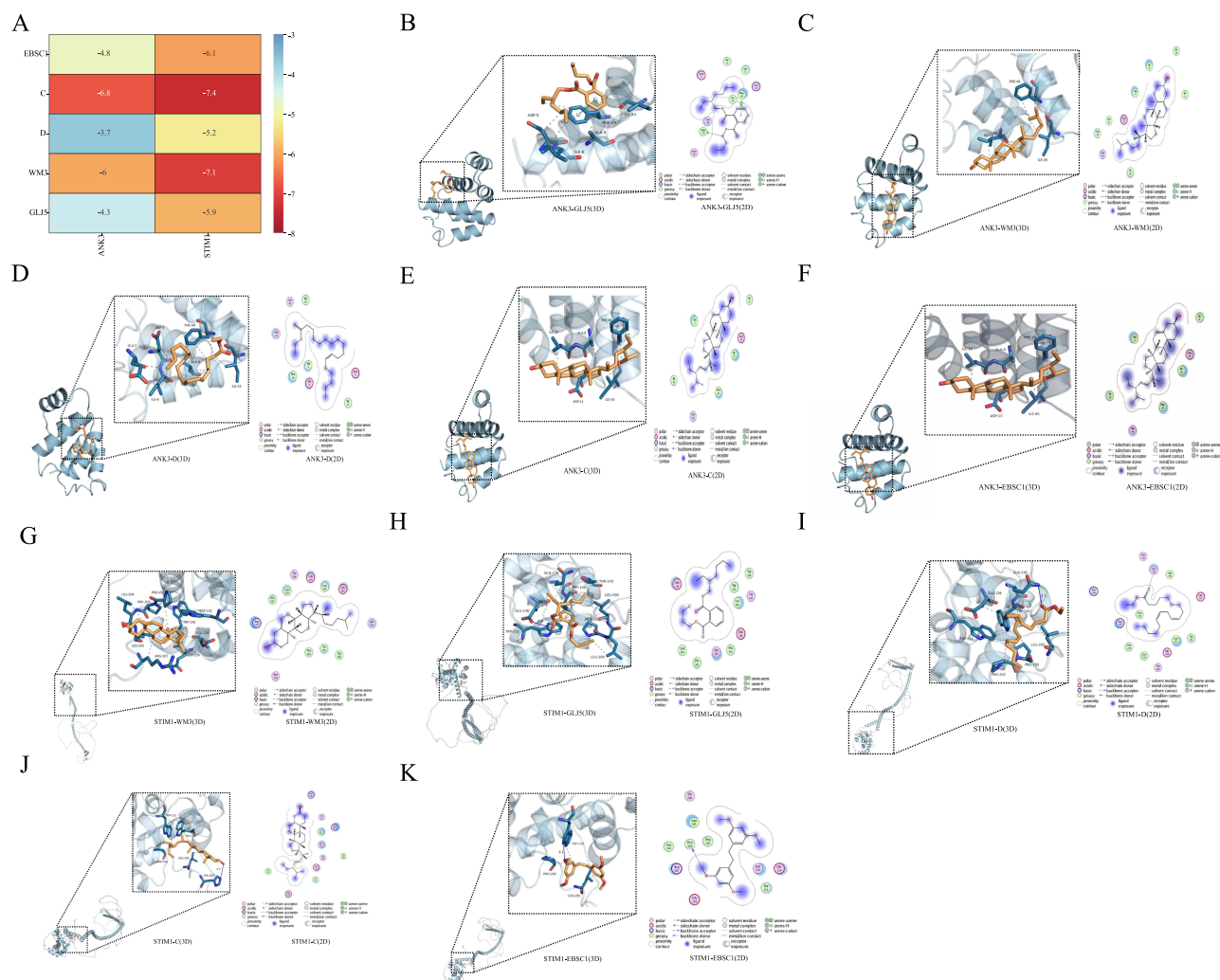


Figure 7 Results of Molecular Docking: (A) Heatmap; (B) ANK3-GLJ5; (C) ANK3-WM3; (D) ANK3-D; (E) ANK3-C; (F) ANK3-EBSCI; (G) STIM1-WM3; (H) STIM1-GLJ5; (I) STIM1-D; (J) STIM1-C; (K) STIM1-EBSCI.

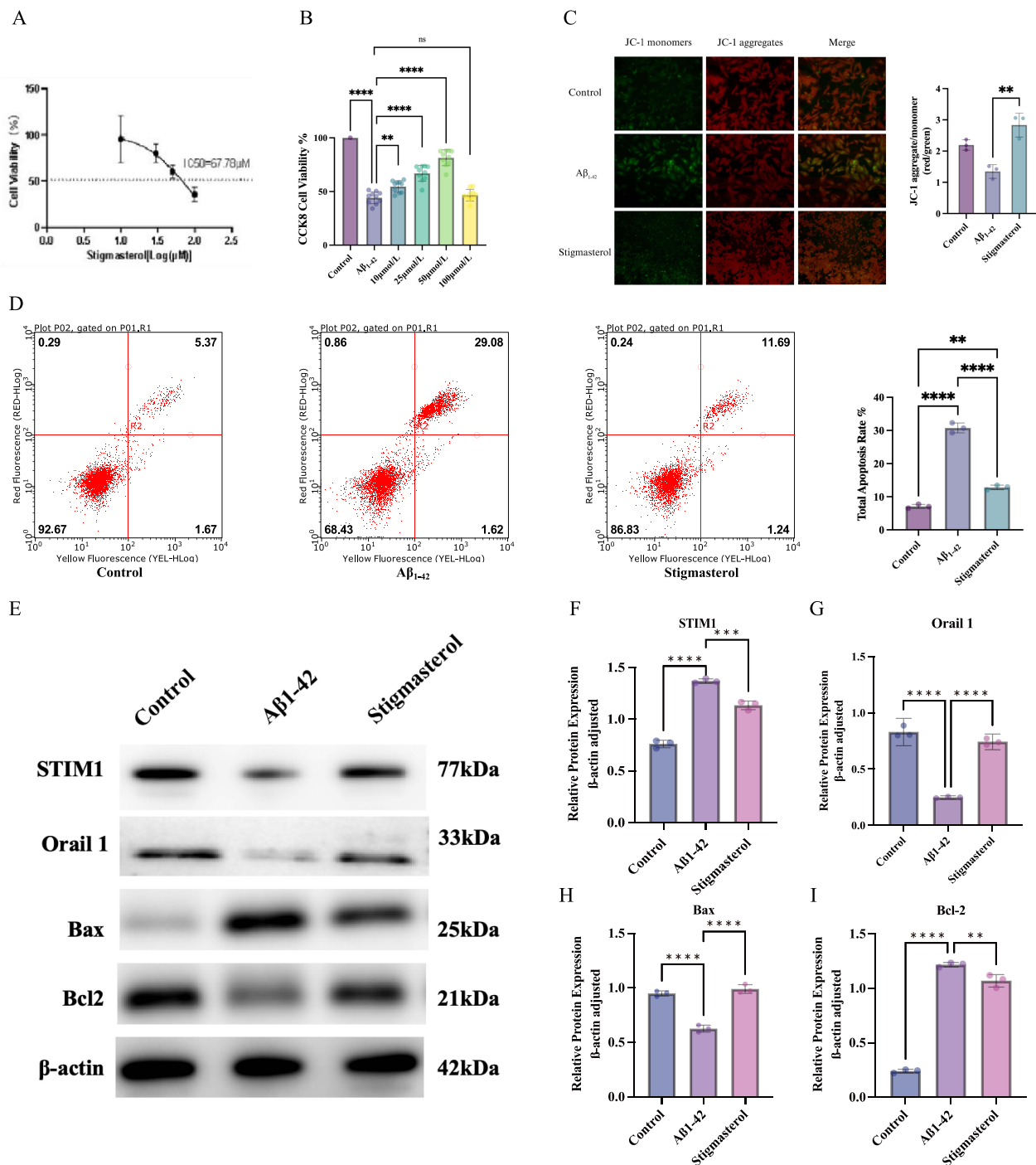


Figure 8 Determination of Optimal Intervention Concentration of Stigmasterol and Verification of Anti-Apoptotic Effect: **(A)** CCK8 toxicity curve; **(B)** Cell viability; **(C)** JC-1 immunofluorescence; **(D)** Flow cytometry; **(E)** Western blot protein bands; **(F)** Quantitative analysis of STIM1; **(G)** Quantitative analysis of Orail 1; **(H)** Quantitative analysis of Bax; **(I)** Quantitative analysis of Bcl-2. ns, not significant; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

intervention, the cell viability was significantly higher than that of the model group, indicating that stigmasterol exerts a protective effect against Aβ₁₋₄₂ induced cell damage (see Figure 8B).

Detection of Mitochondrial Membrane Potential and Cell Apoptosis

In this study, the JC-1 detection kit was used to evaluate changes in mitochondrial membrane potential. The results showed that compared with the blank control group, the membrane potential of the Aβ₁₋₄₂ treated group was significantly

reduced, accompanied by mitochondrial depolarization, which suggested mitochondrial function damage. However, compared with the $A\beta_{1-42}$ group, stigmasterol intervention significantly increased the membrane potential and markedly decreased the number of cells with collapsed membrane potential. This indicated that stigmasterol may protect against the collapse of membrane potential in the early stage of $A\beta_{1-42}$ induced SH-SY5Y cell apoptosis (see Figure 8C). Flow cytometry results showed that $A\beta_{1-42}$ intervention significantly increased the level of cell apoptosis, while stigmasterol intervention could inhibit cell apoptosis and exert a protective effect against AD (see Figure 8D).

Detection of Expression Levels of Target Proteins in the STIM1/Orai1 Signaling Pathway and Apoptotic Proteins by Western Blot

The expression levels of STIM1, Orai1, and apoptosis markers (Bax and Bcl-2) after stigmasterol treatment were detected using Western blot (see Figure 8E–I). Compared with the control group, the expression of STIM1 and Orai1 in the $A\beta_{1-42}$ group was significantly inhibited. Meanwhile, the expression level of the pro-apoptotic protein Bax was significantly upregulated in the model group, whereas the anti-apoptotic protein Bcl-2 was significantly downregulated. Compared with the model group, stigmasterol treatment significantly upregulated the protein expression levels of STIM1 and Orai1, and simultaneously reversed the changes in the expression of apoptosis-related proteins—specifically, the expression of Bax was decreased while the expression of Bcl-2 was significantly increased. These results indicated that stigmasterol may activate the STIM1/Orai1 signaling pathway, thereby regulating the expression of key proteins in the Bcl-2/Bax apoptotic pathway, and ultimately effectively inhibiting $A\beta_{1-42}$ induced cell apoptosis.

Discussion

AD is the leading global neurodegenerative disease characterized by complex pathological mechanisms and extremely limited clinical intervention options. Its classic pathological features include $A\beta$ deposition and intraneuronal neurofibrillary tangles formed by the hyperphosphorylation of Tau protein.¹⁹ Evidence suggests that the pathogenesis of AD is a multifactorial process driven by both genetic and environmental factors.²⁰ The proposal of the “IF-gut-brain axis” theory has provided a new perspective for examining the pathogenesis of AD.²¹ Studies have found that the composition of IF in AD patients undergoes significant changes.²² However, whether such changes are the “cause” or “effect” of the disease remains a key unresolved scientific question in the field.²³ This study adopted a strategy combining integrated multi-omics analysis and experimental validation to construct a complete evidence chain, spanning from population genetic etiology to core pathological pathways, then to potential targets and drugs, and finally to active component verification. Specifically, this study not only established an MR-supported causal inference for the role of specific IF in AD pathogenesis, moving beyond observational associations, but also provided a hypothesis-driven interpretation that these IFs influence AD progression by regulating host calcium signaling homeostasis. Furthermore, in vitro experimental validation verified the neuroprotective effects of TCM active components, providing a solid theoretical foundation and experimental basis for understanding the gut-brain axis pathological mechanism of AD and developing new prevention and treatment strategies.

Genetic Causality: Specific Gut Microbiota as Drivers or Protectors in AD Pathology

Using MR analysis, this study provided strong evidence distinguishing true genetically supported causal inferences from mere epidemiological associations between IF and AD at the genetic level. The results clearly indicated that the abundance of *Desulfovibrio* exhibits a positive causal association with AD risk, while the genus *Slackia* and the *Lachnospiraceae* NK4A136 group show a negative causal association with AD risk. As a Gram-negative sulfate-reducing bacterium, the pro-inflammatory and neurotoxic potential of *Desulfovibrio* has attracted attention.²⁴ *Desulfovibrio* can metabolically produce excessive H_2S , although H_2S is an important gaseous signaling molecule, at pathological concentrations, it inhibits mitochondrial respiratory chain complex IV, leading to energy metabolism disorders and oxidative stress, thereby inducing neuronal damage.²⁵ As a Gram-negative bacterium, its cell wall contains lipopolysaccharide (LPS), a potent endotoxin.²⁶ IF dysbiosis is often accompanied by impaired intestinal barrier function, which allows the translocation of microbial components like LPS into the systemic circulation, triggering persistent low-grade systemic inflammation.²⁷ These peripheral inflammatory factors can penetrate or disrupt the blood-brain barrier,

activate microglia and astrocytes in the central nervous system, and induce or exacerbate neuroinflammation, which is a key driver throughout the entire pathological process of AD.²⁸ In contrast, certain species within the genus *Slackia* have been confirmed to participate in the metabolism of soy isoflavones, capable of converting dietary daidzein into equol, a metabolite with greater biological activity.²⁹ The ability to produce equol is associated with better cognitive function and a lower risk of dementia, as equol possesses strong antioxidant properties and exhibits a higher affinity for estrogen receptor β , a receptor implicated in cognitive processes, potentially exerting neuroprotective effects through this pathway.³⁰ Lachnospiraceae is one of the main bacterial groups in the gut that produce short-chain fatty acids (SCFAs), particularly butyrate.³¹ SCFAs are key metabolites produced by IF through the fermentation of dietary fiber and play a central role in maintaining gut-brain axis homeostasis.³² They not only serve as an energy source for colonic epithelial cells but also enhance the integrity of the intestinal barrier and inhibit intestinal inflammation, thereby reducing the entry of harmful substances into the bloodstream.³³ More importantly, SCFAs can enter the circulatory system and cross the blood–brain barrier, where they regulate microglial activation, promote neurotrophic factor expression, and modulate neurotransmitter synthesis, ultimately exerting anti-neuroinflammatory and direct neuroprotective effects.³⁴

Molecular Mechanism: Calcium Signaling Dyshomeostasis as the Gut-Brain Bridge

After confirming the causal role of IF, this study further explored the molecular mechanisms by which IF influence the host. By intersecting genes adjacent to IF-associated SNPs with a database of AD-related disease genes, we screened out 7 core genes: *SUCLG2*, *STIM1*, *CYP46A1*, *ANK3*, *CSMD1*, *RYR3*, and *RBFOX1*. Both the core gene PPI network analysis and functional enrichment analysis indicated a core pathophysiological process—dysregulation of the calcium signaling pathway—linking IF to the calcium dyshomeostasis hypothesis of AD. The calcium dyshomeostasis hypothesis posits that long-term, chronic dysregulation of intracellular calcium concentration in neurons is an early key event triggering synaptic dysfunction, A β production, hyperphosphorylation of Tau protein, and even neuronal death.³⁵ *STIM1*, *RYR3*, and *ANK3* constitute the core hub for calcium signal regulation. Among them, *STIM1* encodes stromal interaction molecule 1, a key sensor of the endoplasmic reticulum (ER) calcium store.³⁶ When ER calcium is depleted, *STIM1* undergoes a conformational change, translocates to the near-plasma membrane region, and couples with *Orai1* channels on the cell membrane to mediate store-operated calcium entry (SOCE)—a critical mechanism for replenishing the ER calcium store and maintaining cellular calcium homeostasis.³⁷ *RYR3* encodes ryanodine receptor 3, one of the main calcium release channels on the ER, which releases calcium into the cytoplasm in response to physiological signals and is involved in processes such as excitation-contraction coupling and neurotransmitter release.^{38,39} *ANK3* encodes ankyrin-G, a key scaffold protein responsible for anchoring and assembling various ion channels, including voltage-gated sodium and potassium channels, at critical functional domains of neurons.^{40,41} This determines neuronal excitability and signal transduction patterns, thereby indirectly and profoundly affecting the dynamic balance of calcium ions.⁴² The joint targeting of these three genes implies that the impact of IF on AD may be achieved by disrupting three core processes: calcium sensing, calcium release, and channel organization in neurons.⁴³

The enriched metabolic pathways are also closely related to calcium signaling, pointing to the ER-mitochondria axis as a key site for intracellular pathology.⁴⁴ Based on our multi-omics mechanistic inference, abnormal ER calcium signaling drives mitochondrial calcium overload,⁴⁵ which directly inhibits TCA cycle enzymes such as *SUCLG2*.^{46,47} This disruption reduces ATP production and triggers oxidative stress and apoptosis.⁴⁸ *CYP46A1* is a key rate-limiting enzyme in brain cholesterol metabolism.⁴⁹ Its product, 24S-hydroxycholesterol, is crucial for maintaining synaptic plasticity and neuronal function, while disorders of cholesterol metabolism are also associated with mitochondrial dysfunction and calcium homeostasis imbalance. The primary bile acid biosynthesis pathway identified in enrichment analysis also deserves attention: as downstream metabolites of cholesterol, bile acids are profoundly regulated by IF, forming another potential pathway for gut-brain communication.⁵⁰

Multi-Omics Validation: Epigenetic Regulation and Spatial Specificity of Key Targets

To further confirm the core role of the calcium signaling pathway in AD pathogenesis, SMR analysis was used to systematically evaluate the causal relationship between this pathway and AD across multiple molecular levels. The results showed extensive and profound genetically supported causal associations, distinct from mere observational

correlations, between calcium signaling pathway dysfunction and AD pathogenesis. The degree of causal association at the DNA methylation level was much higher than that at other levels, strongly suggesting that epigenetic modifications—particularly DNA methylation—may be a key regulatory mechanism mediating calcium signaling pathway dysfunction and driving the pathological progression of AD. This finding provides a potential molecular mechanism for understanding how IF remotely regulate brain gene function: metabolites produced by IF act as regulators of epigenetic modifications, affect the methylation status of calcium signaling pathway genes in the brain via the bloodstream, thereby altering their expression levels, and ultimately leading to neuronal dysfunction.^{51–54}

To clarify the spatiotemporal expression specificity of core genes, single-cell sequencing and spatial transcriptomic annotation were performed. The hippocampus is not a homogeneous structure but consists of subregions with distinct functions and cytoarchitectures (eg., CA1, CA2, CA3–4, Dentate Gyrus [DG], Subiculum).⁵⁵ Early pathological changes and neuronal loss in AD exhibit distinct regional selectivity, with the entorhinal cortex, hippocampal CA1 region, and Subiculum being among the earliest affected regions.⁵⁶ The high expression of ANK3 and RYR3 in excitatory neurons is consistent with their core roles in regulating neuronal excitability, synaptic transmission, and plasticity.⁵⁷ STIM1 is expressed in both excitatory neurons and astrocytes, reflecting its universal importance in maintaining calcium homeostasis in both neurons and glial cells.^{58,59} Abnormal calcium signaling in astrocytes is closely associated with the occurrence of neuroinflammation and the loss of synaptic support function, and is an important component of AD pathology.⁶⁰

Therapeutic Strategy: Stigmasterol Rescues Neuronal Survival via the STIM1/Orai1 Axis

To translate the bioinformatics analysis results into therapeutic strategies with practical application potential, based on the 7 core genes screened earlier, reverse network pharmacology was used to predict potential Chinese herbs that may exert therapeutic effects by regulating these targets. Numerous previous studies have indicated that the predicted Chinese herbs, including *Prunus mume* (Meihua), *Cornus officinalis* (Shanzhuyu), *Centipeda minima* (Ebushicao), *Macrocytis pyrifera* (Haizao), *Calculus bovis* (Niuhuang), *Rhinoceros horn* (Xijiao), *Lycium barbarum* (Gouqizi), *Sesamum indicum* (Heizhima), *Alpinia officinarum* (Gaoliangjiang), and *Prunus mume* var. *mume* (Wumei), exhibit pharmacological effects such as antioxidant, anti-inflammatory, anti-apoptotic, and anti-aging properties, along with potential neuroprotective effects.^{61–74} Prior to drug development, PheWAS was used to evaluate the safety of key targets. Combining network pharmacology, molecular docking, and single-cell expression profile data, the study focused on the core targets STIM1 and ANK3, as well as the active component stigmasterol. Building on bioinformatics findings, robust in vitro experimental validation strongly confirmed the neuroprotective effect of stigmasterol. Stigmasterol exhibits antioxidant, anti-inflammatory, and anti-apoptotic biological activities.⁷⁵ Notably, stigmasterol can cross the blood-brain barrier—providing a critical pharmacokinetic basis for its use as a therapeutic drug for central nervous system diseases.⁷⁶

Molecular-level experiments validated our scientific hypothesis. The results showed that A β 1–42 treatment significantly downregulated the protein expression levels of STIM1 and its downstream effector molecule Orai1 in cells, while stigmasterol intervention significantly upregulated their expression. First, this confirms the association between A β toxicity and the inhibition of STIM1/Orai1 pathway function. Previous studies have shown that A β oligomers can directly interact with STIM1, inhibit SOCE function, and lead to calcium homeostasis disorders.⁷⁷ Second, it reveals the core mechanism by which stigmasterol exerts neuroprotective effects: stigmasterol does not exert a general anti-apoptotic effect but specifically acts on the STIM1/Orai1 pathway to restore SOCE function inhibited by A β . SOCE mediated by STIM1/Orai1 is the basis for replenishing the ER calcium store and maintaining normal calcium signal oscillations.⁷⁸ Following stigmasterol treatment, Bax expression decreased while Bcl-2 increased. Our targeted validation demonstrates that by mitigating cytoplasmic calcium overload, stigmasterol prevents Bax activation at the mitochondrial membrane, effectively halting the apoptotic cascade.⁷⁹ The toxic effect of A β can be regarded as a type of “gain-of-loss function” on the SOCE pathway,^{80,81} while stigmasterol acts as a pathway function restorer—consistent with the view in some studies that moderate upregulation of SOCE may be beneficial in the context of AD pathology,⁸² opening up a new direction for the development of novel therapeutic drugs for AD.

Limitations and Future Directions

Despite the robustness of our multi-tier approach, several caveats remain. First, MR-supported causal inferences rely predominantly on European-ancestry GWAS, necessitating cross-ethnic validation. Second, while our *in vitro* assays elucidated STIM1/Orai1-mediated mechanisms, they cannot fully recapitulate *in vivo* neuro-glial interactions, requiring future validation in transgenic AD models. Finally, biological validation was limited to stigmaterol. Looking forward, the integrative multi-omics framework applied herein provides a biologically grounded feature space highly compatible with explainable AI models. Bridging biology with interpretable machine learning will enable mechanistic, rather than purely statistical, interpretations of AD patterns and accelerate TCM drug discovery.^{83,84}

Conclusions

In conclusion, this study advances the literature by establishing MR-supported causal links between specific gut microbiota and AD, identifying STIM1/Orai1-mediated calcium signaling as a mechanistic bridge, and validating the neuroprotective efficacy of stigmaterol. While our *in vitro* data robustly identify novel targets along the gut-brain axis, their clinical translation requires cautious interpretation due to the reliance on European-ancestry GWAS cohorts and the absence of *in vivo* validation. Ultimately, this integrative multi-omics framework highlights the great potential of modernizing TCM for complex diseases.

Institutional Review Board Statement

Ethical review and approval were waived for this study. The research utilizes anonymized, publicly available datasets and does not involve direct interaction with human subjects. Thus, it is exempt from ethical approval in accordance with items 1 and 2 of Article 32 of the Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects (dated February 18, 2023, China).

Abbreviations

AD, Alzheimer's Disease; A β 1-42, β -amyloid 1-42; BBB, Blood-Brain Barrier; CNGB, China National GeneBank; DG, Dentate Gyrus; DL, Drug-Likeness; EAF, Effect Allele Frequency; eQTL, Expression Quantitative Trait Locus; ER, Endoplasmic Reticulum; GEO, Gene Expression Omnibus; GO, Gene Ontology; GWAS, Genome-Wide Association Study; HEIDI, Heterogeneity in Dependent Instruments; HL, Half-Life; HRP, Horseradish Peroxidase; IF, Intestinal Flora; IV, Instrumental Variables; IVW, Inverse-Variance Weighted; KEGG, Kyoto Encyclopedia of Genes and Genomes; LD, Linkage Disequilibrium; LPS, Lipopolysaccharide; MR, Mendelian Randomization; mQTL, Methylation Quantitative Trait Loci; NMDA, N-methyl-D-aspartate; OB, Oral Bioavailability; PDB, Protein Data Bank; PheWAS, Phenome-Wide Association Study; PPI, Protein-Protein Interaction; pQTL, Protein Quantitative Trait Loci; scRNA-seq, Single-Cell RNA Sequencing; SCFAs Short-Chain Fatty Acids; SMR, Summary-data-based Mendelian Randomization; SNPs, Single Nucleotide Polymorphisms; SOCE, Store-Operated Calcium Entry; sQTL, Splicing Quantitative Trait Loci; Stereo-seq, Spatial Transcriptome Sequencing; Sub Subiculum; TCA, Tricarboxylic Acid; TCM, Traditional Chinese Medicines; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform.

Data Sharing Statement

The original contributions and experimental data presented in the study are included in the article; further inquiries can be directed to the corresponding author upon reasonable request. The publicly available datasets analyzed in this study can be found in the following repositories: Alzheimer's disease GWAS data are available from the IEU OpenGWAS project (<https://gwas.mrcieu.ac.uk/>, ID: ieu-b-5067); gut microbiota GWAS data are available from the MiBioGen consortium via the MOLGENIS platform (<https://mibiogen.gcc.rug.nl/>); single-cell RNA sequencing data are available from the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>, accession: GSE175814); and spatial transcriptomics data are available from the China National GeneBank DataBase (CNGBdb, <https://db.cngb.org/>, accession: CNP0005077).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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