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Elevated TMPRSS6 affects cognition and learning in Alzheimer's disease through neuronal iron dysregulation

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Abstract

Aim: TMPRSS6, a type II transmembrane serine protease predominantly expressed in the liver, plays a crucial role in regulating systemic iron homeostasis. However, the expression and function of TMPRSS6 in the central nervous system remain poorly understood.

Methods: Adeno-associated viruses (AAVs) carrying plasmids with neuron-specific promoters for TMPRSS6 knockdown were stereotactically injected into the hippocampus of 6-month-old wild-type (WT) and amyloid precursor protein (APP)/PS1 male mice. Three months later, the mice underwent a water maze test, and hippocampal tissues were collected for subsequent experiments.



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Results: The analysis of Alzheimer's disease (AD) databases we describe here identified a significant increase in *TMPRSS6* mRNA levels in the hippocampus of AD patients, a finding corroborated by elevated *TMPRSS6* expression in the hippocampus of APP/PS1 transgenic mice, which exhibit an AD phenotype. Knockdown of *TMPRSS6* in the hippocampus of these mice led to a significant enhancement in cognitive and learning abilities, accompanied by a reduction in the accumulation of APP and amyloid-beta (A β) plaques. Further experiments revealed that *TMPRSS6* knockdown decreased iron and reactive oxygen species (ROS) levels in the hippocampus, upregulated glutathione peroxidase 4 (GPX4), downregulated acyl-CoA synthetase long-chain family member 4 (ACSL4), ameliorated mitochondrial cristae damage, and inhibited ferroptosis, which might be associated with the bone morphogenic protein/Smad signaling pathway.

Conclusion: Our findings shed light on the role of *TMPRSS6* in the central nervous system, which may offer valuable insights for the development of therapeutic strategies for AD or other disorders associated with brain iron accumulation.

Keywords: *TMPRSS6*, Alzheimer's disease, iron metabolism, ferroptosis, Hepcidin

INTRODUCTION

TMPRSS6, also known as matriptase-2 (MT-2), is encoded by the *TMPRSS6* gene, which contains 18 exons and 17 introns. *TMPRSS6* is a type II transmembrane serine protease that influences bone morphogenic protein (BMP) binding to BMP receptors (BMPRs) by cleaving Hemojuvelin (HJV), thereby inhibiting intracellular signaling cascades, reducing the phosphorylation of Smad1, Smad5, and Smad8, forming the Smad1/5/8 complex, and suppressing transcription of the iron regulatory hormone, Hepcidin. For some mutations in *TMPRSS6*, HJV can stabilize the binding of BMP and BMPRs, promoting Hepcidin expression^[1-3]. The biological function of Hepcidin is to bind to the membrane iron transport protein, ferroportin 1 (FPN1), leading to its degradation and a decrease in cellular iron release, thus regulating systemic iron homeostasis, as FPN1 exports iron to the circulation from enterocytes and macrophages^[4]. Thus, mutations in the *TMPRSS6* gene and abnormal expression can lead to anemia and iron-refractory iron deficiency anemia (IRIDA)^[5].

In human peripheral tissues, the *TMPRSS6* gene is mainly expressed in the liver^[6], regulating systemic iron homeostasis through hepatic synthesis of Hepcidin. Recent studies have found that *TMPRSS6* may also be involved in Hepcidin regulation in the BV2 murine microglial cell line^[7]. Our previous study revealed that *TMPRSS6* significantly inhibits the proliferation and tumor growth of neuroblastoma neuro-2a cells. In that work, we found *TMPRSS6* to inhibit Smad1/5/8 phosphorylation by cleaving the HJV, making *TMPRSS6* a potential candidate target for inhibiting neuronal tumor growth^[8]. In sequencing alignment and SNP analysis studies on high or low levels of feather pecking behavior in chickens, three differentially fixed alleles of the *TMPRSS6* gene were identified in the promoter and intergenic regions. We hypothesize that there is a relationship between *TMPRSS6* and the control of behavior in animals^[9], with *TMPRSS6* playing a significant regulatory role in nervous system function.

In the early stages of Alzheimer's disease (AD), the hippocampus is typically among the first areas to be affected, becoming one of the most severely impacted brain regions, leading to significant impairments in the patient's memory, learning abilities, and spatial cognition. Based on previous studies, we believe that *TMPRSS6* plays an important regulatory role in the functioning of the nervous system. Therefore, does *TMPRSS6* have a role in the progression of AD? Can the progression of AD be alleviated by regulating the expression of *TMPRSS6*?

In the present study, we first analyzed the changes in expression of *TMPRSS6* in different brain regions of AD patients, finding that *TMPRSS6* mRNA levels were significantly increased in the hippocampus but unchanged in the prefrontal cortex. Further research revealed that *TMPRSS6* expression was also elevated in the hippocampus of AD mice. Knockdown of *TMPRSS6* significantly improved the cognitive impairments in AD mice, possibly due to decreased brain iron levels, oxidative stress, and ferroptosis.

METHODS

Animal models and treatments

Amyloid precursor protein (APP)/PS1 double transgenic mice served as the primary AD model. Both APP/PS1 transgenic and C57BL/6 wild-type (WT) cohorts were sourced from Beijing XiNuoyin Technology Co., Ltd. Animals were housed individually in plastic cages under controlled conditions (22 ± 1 °C, 40% humidity, 12-hour light/dark cycle), with ad libitum access to standard chow and water. All procedures strictly followed the National Institutes of Health (NIH) guidelines for laboratory animal care and use and were approved by the Institutional Animal Ethics Committee of Hebei Normal University.

Male APP/PS1 transgenic mice at six months of age, along with WT counterparts, were randomly allocated to three experimental cohorts: WT-hSyn-Con (wild-type controls), APP/PS1-hSyn-Con (APP/PS1 controls), and APP/PS1+hSyn-sh*TMPRSS6* (APP/PS1 *TMPRSS6* knockdown). The hSyn is a neuron-specific promoter. Using a stereotaxic instrument, 0.5 μ L adeno-associated virus (AAV)-hSyn-Con or AAV-hSyn-sh*TMPRSS6* was injected into the hippocampus of the mice. The APP/PS1+hSyn-sh*TMPRSS6* group received AAV9-packaged plasmids expressing shRNA targeting *TMPRSS6* (sh-*TMPRSS6* sequence: 5'-GGTATTCCTAGGGTACAAAG-3'), while the WT-hSyn-Con and APP/PS1-hSyn-Con groups received AAV9-packaged plasmids containing scrambled shRNA sequences as controls (AAV9, purchased from Beijing Weizhen Co., Ltd.). Stereotaxic coordinates for viral delivery were defined as 1.8 mm dorsal to bregma, 1.5 mm lateral from the midline, and 2.0 mm ventral to the cranial surface.

At 9 months of age, the mice underwent behavioral testing. Following the tests, the mice were euthanized via transcardiac perfusion under anesthesia. Brain tissues were promptly collected for subsequent analyses, including western blot analysis and quantitative reverse transcription PCR (qRT-PCR). After perfusion, mouse brain tissues were fixed with formaldehyde for 12 h for frozen tissue sectioning.

Morris water maze test

Spatial learning and memory capacities were evaluated using the standardized Morris water maze (MWM) paradigm. The testing apparatus comprised a circular stainless steel tank (diameter: 120 cm; height: 50 cm) partitioned into four equal zones. Water opacity was achieved through titanium dioxide suspension, with thermal regulation maintaining a constant 21 ± 1 °C.

During acquisition trials, animals were sequentially released from randomized entry points across all quadrants. Escape latency (time required to reach the visible platform) was quantified, with trials terminated at 120 s. Failed attempts were assigned the maximal latency value. Daily performance metrics were collected over five consecutive days to assess learning progression.

On postoperative day 6, the platform was submerged below the water level for spatial memory assessment. Two parameters were analyzed within a 120-second observation window: (1) platform crossing frequency within the original target zone; and (2) cumulative duration spent in the designated quadrant.

Western blot analysis

The hippocampus and frontal cortex were meticulously excised and subsequently stored at a temperature of -80 °C to facilitate subsequent western blotting procedures for protein analysis. Tissue samples were subjected to homogenization using RIPA buffer supplemented with a protease inhibitor cocktail tablet mixture, followed by sonication to facilitate cellular disruption. Following centrifugation at 12,000 × g for 20 min at 4 °C, the resultant supernatants were carefully harvested, and protein concentration was quantified using a bicinchoninic acid (BCA) assay kit. Protein samples underwent electrophoretic separation on 10% or 12% SDS-polyacrylamide gels, followed by semi-dry transfer to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in TBS-T (0.05% Tween-20 in Tris-buffered saline) for 1.5 h at room temperature, then probed with primary antibodies at 4 °C overnight. After three 5-minute TBS-T washes, membranes were incubated for 90 min at ambient temperature with species-matched HRP-conjugated secondary antibodies (anti-rabbit/mouse IgG). Immunoreactive bands were visualized via enhanced chemiluminescence (ECL) detection. Detailed antibody specifications are provided in [Table 1](#).

qRT-PCR

Total RNA was isolated from homogenized brain tissue using TRIzol reagent through phase separation with chloroform, followed by isopropanol precipitation. The resulting RNA pellet underwent two 70% ethanol washes before dissolution in RNase-free aqueous solution. First-strand cDNA synthesis was carried out with a commercial reverse transcription system (R223, Vazyme). Quantitative PCR amplification employed SYBR Green chemistry (Master Mix Q321, Vazyme) under manufacturer-specified conditions.

For data normalization, target gene cycle threshold (Ct) values were referenced to β -actin endogenous controls within corresponding samples. Relative expression differences between experimental groups were calculated via the $2^{-\Delta\Delta C_t}$ method, with results expressed as fold changes relative to control values. Gene-specific primer sequences utilized in this study are detailed below:

TMPRSS6 forward: 5'-TCTGTGCTGGCTACCGCAAG-3'

TMPRSS6 reverse: 5'-GCAACAGATACCACACTGGGAA-3'

Hepcidin forward: 5'-AAGCAGGGCAGACATTGCGAT-3'

Hepcidin reverse: 5'-CAGGATGTGGCTCTAGGCTATGT-3'

β -actin forward: 5'-AGGCCCAGAGCAAGAGAGGTA-3'

β -actin reverse: 5'-TCTCCATGTCGTCCCAGTTG-3'

DAB-enhanced perls iron histochemistry

To quench endogenous peroxidase activity, brain sections were treated with 3% hydrogen peroxide (H₂O₂) for 20 min at room temperature. Tissue samples were then immersed in Perls' staining solution (2% potassium ferrocyanide/2% HCl) for 14 h. After thorough PBS washing, sections were briefly incubated with DAB chromogen (ZLI-9018, Zhongshan Jinqiao) for 0.1 min to intensify the iron signal, followed by distilled water rinsing to halt the reaction. Finally, graded ethanol dehydration and xylene clearing were performed for permanent mounting.

Immunofluorescence

Following humane euthanasia via transcardiac perfusion under general anesthesia, brain tissues were immersion-fixed in 4% paraformaldehyde for 12 h. Coronal cryosections (15 μ m thickness) were generated using a cryostat. For immunofluorescence processing, sections were sequentially subjected to: (1) blocking buffer pretreatment; (2) overnight primary antibody incubation; (3) PBS washing; and (4) 1-hour incubation at 37 °C with species-specific secondary antibodies (DyLight 488-conjugated anti-rabbit IgG or DyLight 594-conjugated anti-mouse IgG). Nuclear staining was achieved using DAPI for a 5-minute interval, followed by washing and mounting of the samples. The stained sections were then imaged utilizing

Table 1. List of antibodies used

Antibody	Catalog number	Manufacturer
TMPRSS6	ABT317	Sigma-Aldrich
APP/A β	25524-1-AP	Proteintech
GPX4	AB125066	Abcam
ACSL4	AB155282	Abcam
FtL	AB109373	Abcam
TfR1	13-6800	Invitrogen
HJV	11758-1-AP	Proteintech
P-Smad1/5	9516	Cell Signaling Tech
Smad1	6944	Cell Signaling Tech
Smad4	46535	Cell Signaling Tech
FPN1	MTP11-S	Alpha Diagnostic Intl
GAPDH	60004-1-Ig	Proteintech
β -actin	CW0096M	CWBIO

APP: Amyloid precursor protein; A β : amyloid-beta; GPX4: glutathione peroxidase 4; ACSL4: acyl-CoA synthetase long-chain family member 4; FtL: ferritin light chain; TfR1: transferrin receptor 1; HJV: Hemojuvelin; FPN1: ferroportin 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

a confocal microscope (Olympus FV3000, Japan). The specific antibodies employed in this experimental protocol are detailed in [Table 2](#).

Reactive oxygen species assay

Hippocampal reactive oxygen species (ROS) levels were assayed using a commercial detection kit (E004-1, Nanjing Jiancheng Bioengineering Institute). The cell-permeant fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) undergoes intracellular esterase-mediated hydrolysis to form membrane-impermeable DCFH. In the presence of ROS, DCFH is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF), exhibiting excitation/emission maxima at 488/525 nm, with signal intensity proportional to intracellular oxidative stress levels.

The experimental procedure began with tissue weighing. The tissue was homogenized and filtered through a 100 μ m aperture filter. After centrifugation at 1,000 $\times g$ for 10 min, the supernatant was discarded, and the cell pellet was collected. The cells were then washed twice with PBS and prepared for fluorescence detection following the kit's instructions. The fluorescence values were normalized to the control group and expressed per unit mass for statistical analysis.

Thioflavin-S staining

Following three sequential 5-minute PBS (0.1 M) rinses, tissue sections were stained with 0.01% Thioflavin S (ThS, T1892, Sigma-Aldrich) in 50% ethanol for 8 min. Post-staining ethanol washes (50% concentration, 5 cycles $\times$ 5 min each) were implemented to remove unbound dye. Fluorescence signal acquisition and quantitative analysis were conducted using an Olympus FV3000 confocal laser scanning microscopy system.

Transmission electron microscopy

The rodents were anesthetized and subsequently subjected to transcardial perfusion with saline solution, followed by fixation using a 3% glutaraldehyde solution. The brain specimens were expeditiously excised, and the hippocampal region was subsequently dissected into cubes measuring 1 millimeter in each dimension. The specimens were subjected to fixation using a 3% glutaraldehyde solution for a duration of 24 h at a temperature of 4 $^{\circ}$ C. Subsequently, they were treated with 1% osmic acid, diluted in a 0.1 M phosphate buffer, for a period of 30 min. Following this, dehydration procedures were carried out,

Table 2. List of antibodies used

Antibody	Catalog number	Manufacturer
TMPRSS6	AB28287	Abcam
NeuN	MAB377	Sigma-Aldrich

culminating in embedding the samples in Araldite resin. Ultrathin sections, each with a nominal thickness of 200 nm, were meticulously prepared utilizing an ultramicrotome. Subsequent to sectioning, these samples were stained using uranyl acetate and lead citrate before undergoing examination under a high-resolution electron microscope (model H-7800, manufactured by Hitachi, Tokyo, Japan).

Statistical analysis

Quantitative data are presented as mean $\pm$ SEM. Normality was verified using Shapiro-Wilk tests. Parametric datasets underwent one-way ANOVA with Tukey's multiple comparisons test for post hoc analysis. Statistical significance was defined as $P < 0.05$. All analyses were executed using Image Lab (v6.1), Microsoft Excel 2019, and GraphPad Prism 9.0.

RESULTS

APP/PS1 mice exhibit increased hippocampal TMPRSS6 expression

Our initial analysis of the AD database (<http://www.alzdata.org/>) revealed that TMPRSS6 mRNA expression is significantly increased in the hippocampus of AD patients, whereas it exhibited a non-significant downward trend in the frontal cortex [Figure 1A]. Our qRT-PCR experimental results from APP/PS1 mice are consistent with the AD database [Figure 1B and C]. Further, western blot analysis showed that TMPRSS6 protein levels also significantly increased in the hippocampus, while exhibiting a non-significant downward trend in the frontal cortex [Figure 1D-G]. Based on these findings, we proceeded to perform additional analyses of mouse hippocampal tissue in the AD model mice.

Knockdown of *TMPRSS6* in neurons significantly improves the learning and cognitive impairment of APP/PS1 mice

Aging APP/PS1 transgenic mice display cognitive dysfunction similar to AD^[10]. To assess the participation of TMPRSS6 in this process, we disrupted hippocampal TMPRSS6 via AAV shRNA knockdown in 6-month-old AD model mice. WT and APP/PS1 mice injected with AAV containing scrambled shRNA were used as controls. After 3 months, we subjected the mice to a water maze behavioral test [Figure 2A]. Compared with the APP/PS1 control group, the escape latency in the knockdown group was significantly shortened, approaching the level of the WT control group [Figure 2B]. The spend time in the target quadrant of *TMPRSS6* knockdown group had not significant improvement [Figure 2C]; the number of crossings in the target platform increased significantly [Figure 2D]. These findings suggest that TMPRSS6 inhibition in nerve cells can significantly improve learning and memory dysfunction in APP/PS1 transgenic mice.

Decreased expression of neuronal *TMPRSS6* in AAV-shRNA knockdown mice

We validated that AAV-mediated shRNA delivery reduced TMPRSS6 expression in the treated mice. *TMPRSS6* mRNA levels were significantly decreased after injection with AAV loaded with *TMPRSS6*-targeting shRNA [Figure 3A], leading to decreased TMPRSS6 protein expression [Figure 3B and C]; the immunofluorescence double labeling results also showed a significant decrease in TMPRSS6 in neurons of the CA3 and DG regions [Figure 3D-G].

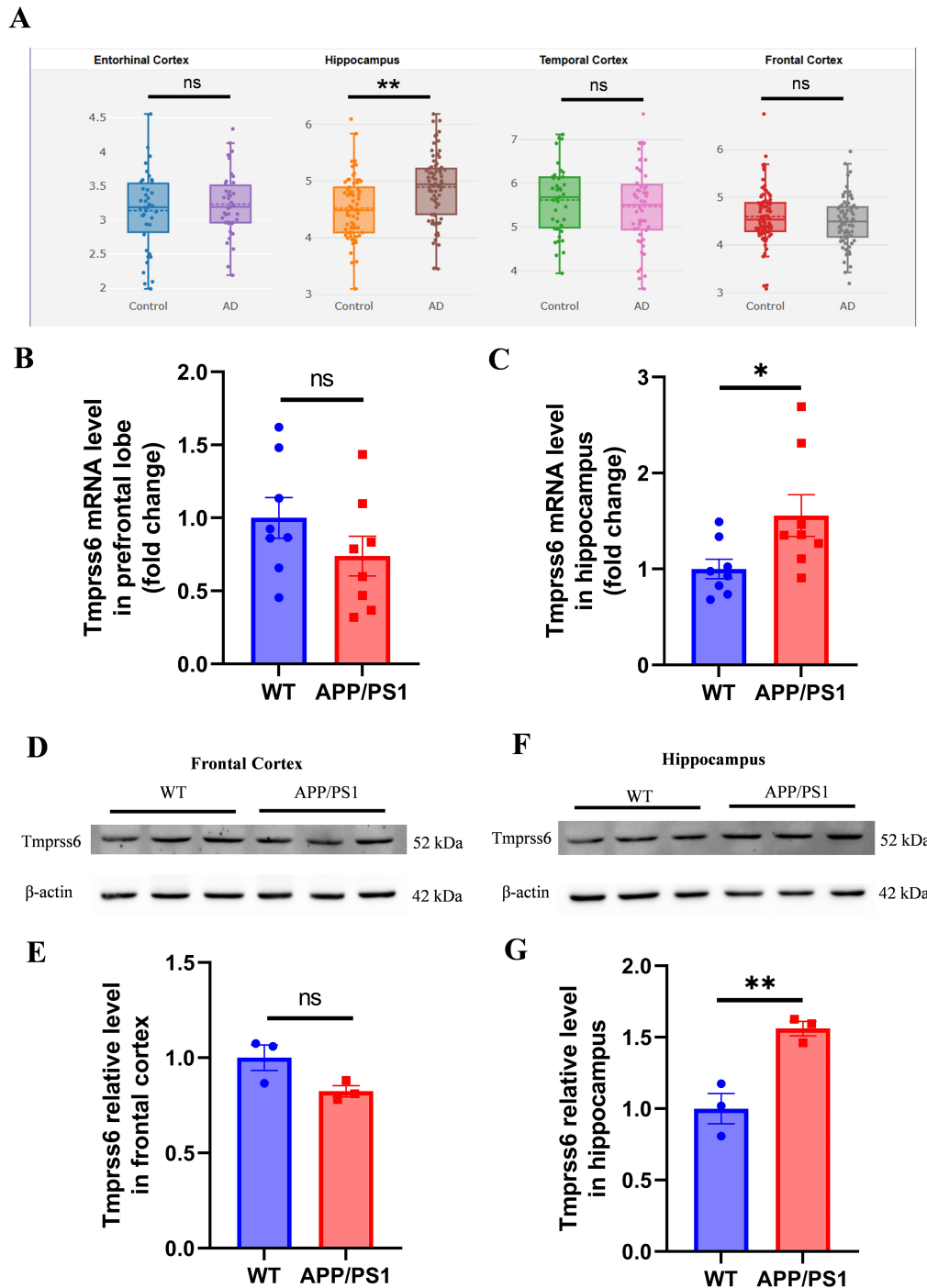


Figure 1. Expression levels of TMPRSS6 in the hippocampus. (A) Cross-platform normalized expression of TMPRSS6 from <http://www.alzdata.org/> data; (B and C) qRT-PCR detection of *TMPPRS6* mRNA in the frontal cortex and hippocampus of mice; (D and E) Western blot analysis of TMPRSS6 in the frontal cortex of mice, and quantification; (F and G) Western blot analysis of TMPRSS6 in the hippocampus of mice, and quantification. The data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, ns: not significant. qRT-PCR: Quantitative reverse transcription polymerase chain reaction; SEM: standard error of the mean.

Knockdown of *TMPPRS6* in neurons reduces amyloid-beta plaque aggregation in the brains of APP/PS1 mice

A distinctive hallmark of AD is the buildup of amyloid-beta ($A\beta$) plaques in the brain^[11]. $A\beta$ plaques are

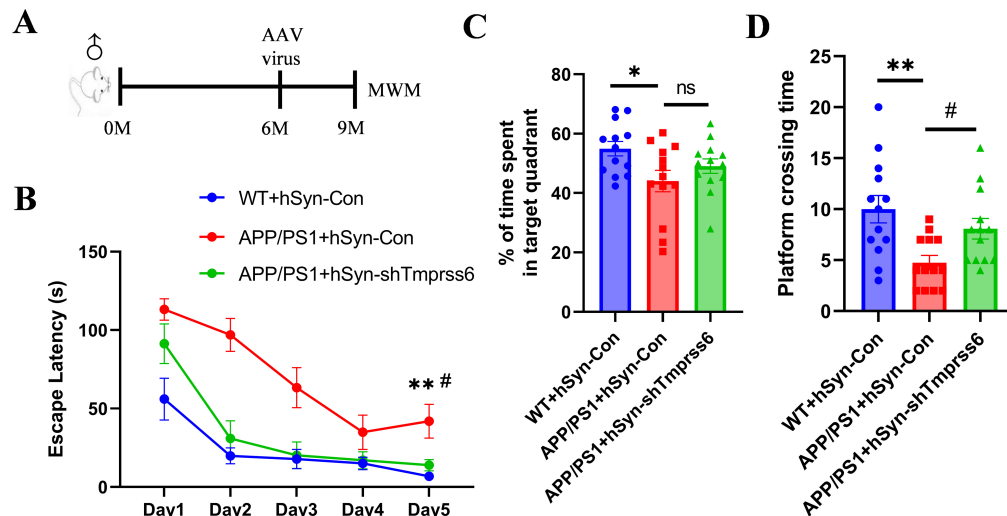


Figure 2. Mouse treatment schedule and water maze data results. (A) Stereotaxic injection diagram; (B) Escape latency time during the training stage; (C) Percentage of time spent in the target quadrant; (D) Number of times crossing over the platform area on the sixth day. The data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, APP/PS1+hSyn-Con vs. WT-hSyn-Con; # $P < 0.05$, APP/PS1+hSyn-shTMPRSS6 vs. APP/PS1+hSyn-Con, ns: not significant. SEM: Standard error of the mean; APP: amyloid precursor protein; WT: wild-type.

generated by the enzymatic cleavage of APP^[12]. We observed a significant decrease in APP levels in the hippocampus of mice in the knockdown group through western blot analysis [Figure 4A and B]. Next, we evaluated the A β plaque levels in the hippocampus of mice, and found that A β levels in the knockdown group showed a significant decrease [Figure 4C and D]. Thioflavin S binds to A β plaques and can be observed by fluorescence microscopy. We further found that A β plaque was significantly reduced in the hippocampus by staining murine brain sections with Thioflavin S^[13] [Figure 4E-H]. Based on these results, we conclude that decreased expression of TMPRSS6 in APP/PS1 mouse neurons significantly diminishes APP protein levels, which ultimately relieves the A β plaque burden. Importantly, this change is consistent with the improved cognitive function we observed in the APP/PS1 mice with depleted TMPRSS6.

Knockdown of *TMPRSS6* in neurons alleviates neuronal ferroptosis

Glutathione peroxidase 4 (GPX4), a pivotal antioxidant enzyme, mitigates lipid peroxidation by neutralizing reactive lipid species and suppressing polyunsaturated fatty acid-derived free radical formation, thereby conferring cellular protection against ferroptosis^[14]. Western blot analyses revealed substantial downregulation of hippocampal GPX4 expression in APP/PS1 transgenic mice relative to WT counterparts. Remarkably, neuronal TMPRSS6 knockdown effectively rescued GPX4 levels in the AD model hippocampus [Figure 5A and B]. Conversely, acyl-CoA synthetase long-chain family member 4 (ACSL4), a central regulator of lipid remodeling, demonstrates pro-ferroptotic activity through its metabolic functions^[15]. Our intervention with TMPRSS6 silencing in neurons significantly attenuated ACSL4 overexpression in the hippocampal region of APP/PS1 mutants [Figure 5C and D]. Another characteristic of ferroptosis is a reduction in the number of inner mitochondrial ridges and the occurrence of cavitation. Transmission electron microscopy revealed that *TMPRSS6* knockdown significantly improved the cavitation state of neuronal mitochondria [Figure 5E].

Neuronal *TMPRSS6* knockdown alleviates iron deposition

In the brains of AD patients, pathological iron accumulation in the hippocampus and cerebral cortex has been well-documented^[16]. Western blot analysis revealed significantly decreased expression of the iron storage protein subunit, ferritin light chain (FtL), in the hippocampal region of APP/PS1 mice in the

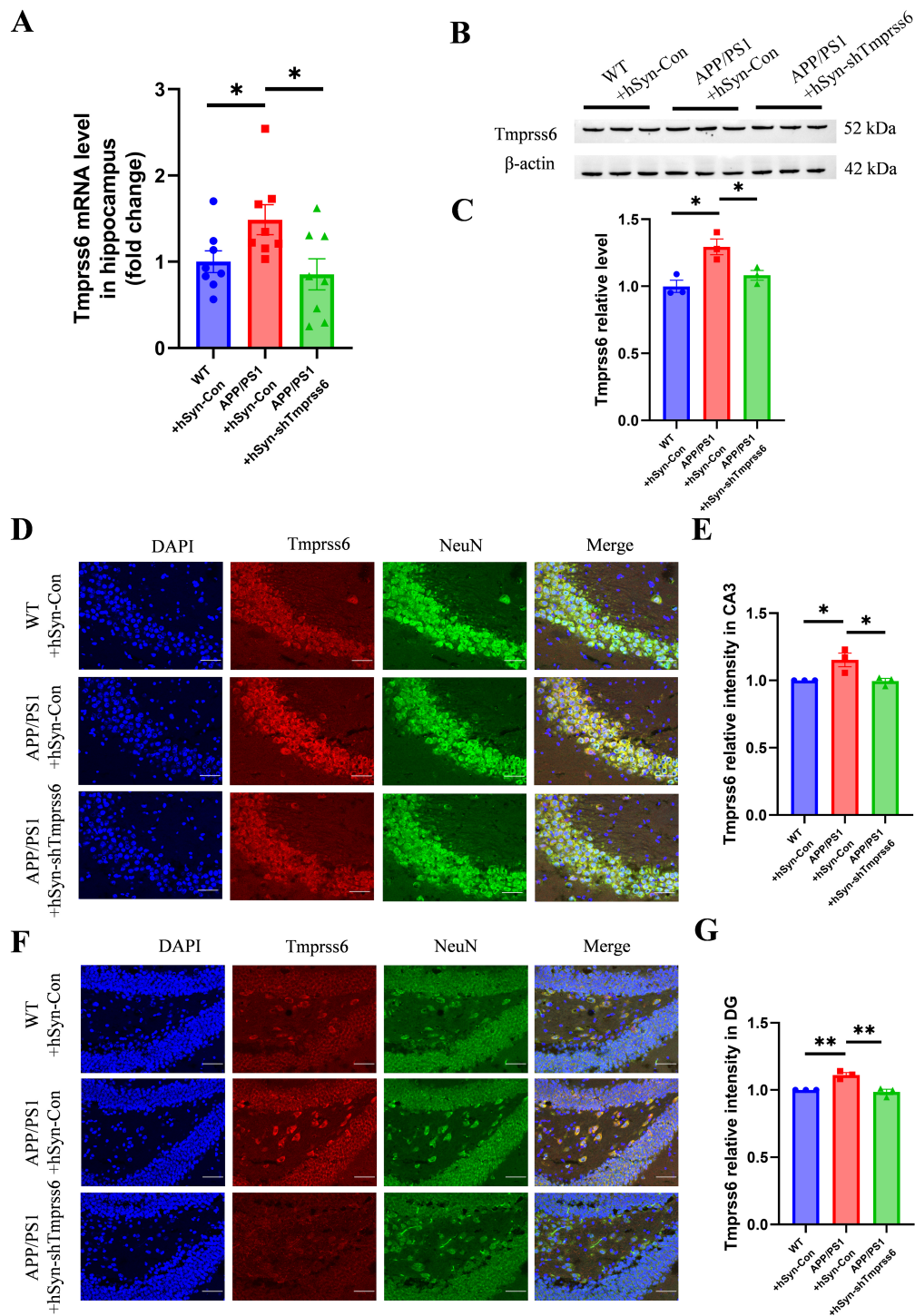


Figure 3. Validation of neuronal knockdown of TMPRSS6. (A) qRT-PCR analysis of *TMPPRS6* mRNA in the hippocampus; (B and C) Western blot images of TMPRSS6 in the hippocampus and its quantification; (D-G) Immunofluorescence of TMPRSS6 protein in the CA3 (D) and DG (F) regions and its quantification. (scale bar: 50 μ m). NeuN: Label neurons; DAPI: Label nucleus. The data are presented as the mean $\pm$ SEM. * P < 0.05, ** P < 0.01. qRT-PCR: Quantitative reverse transcription polymerase chain reaction; SEM: standard error of the mean.

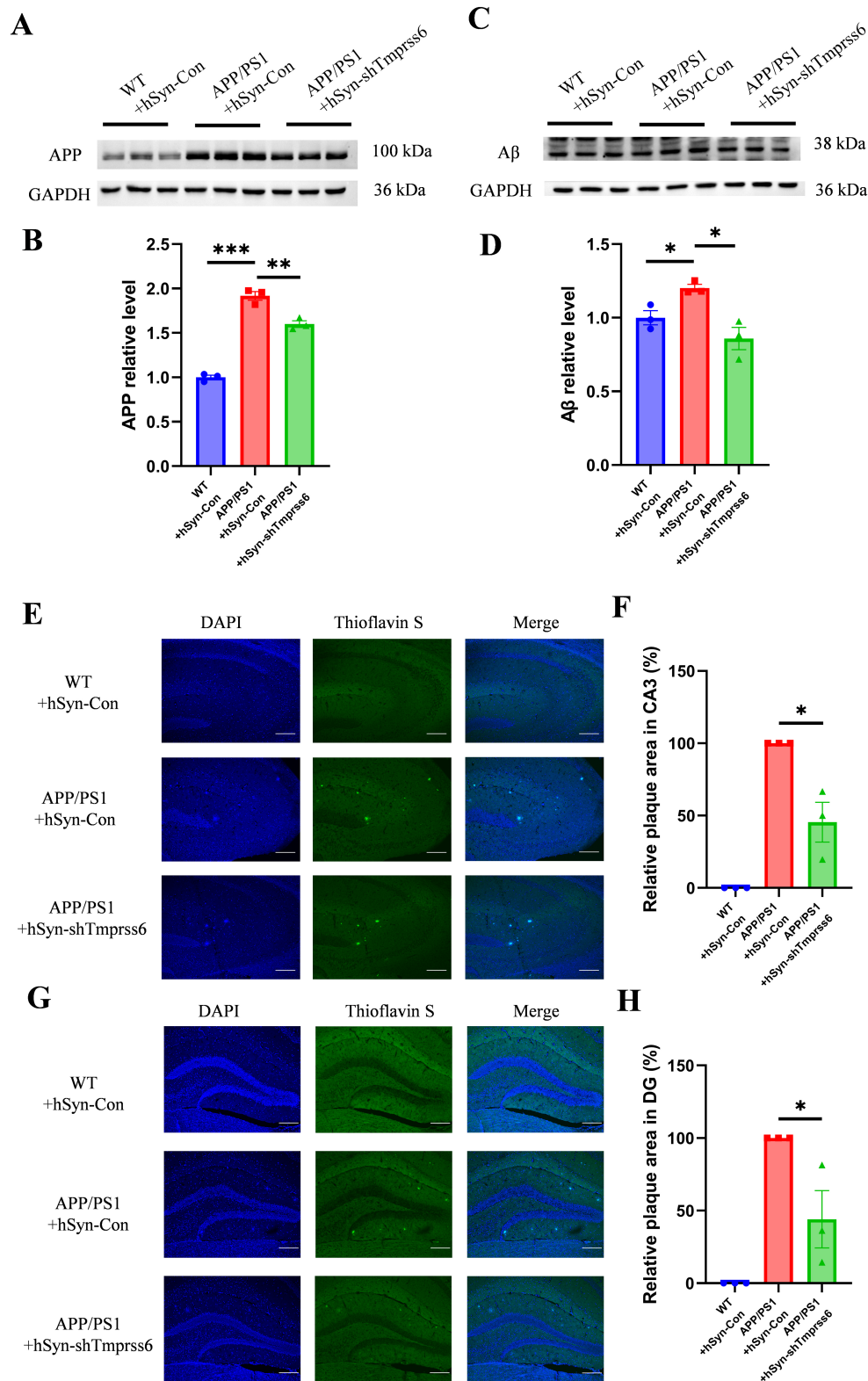


Figure 4. Levels of APP/Aβ in the hippocampus. (A and B) Representative western blot images of APP in the hippocampus, and quantification; (C and D) Representative western blot images of Aβ in the hippocampus, and quantification; (E-H) Thioflavin S staining in the CA3 (E) and DG (G) regions of the hippocampus (scale bar: 200 μm) and quantification. The data are presented as the mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. APP: Amyloid precursor protein; Aβ: amyloid-beta; SEM: standard error of the mean.

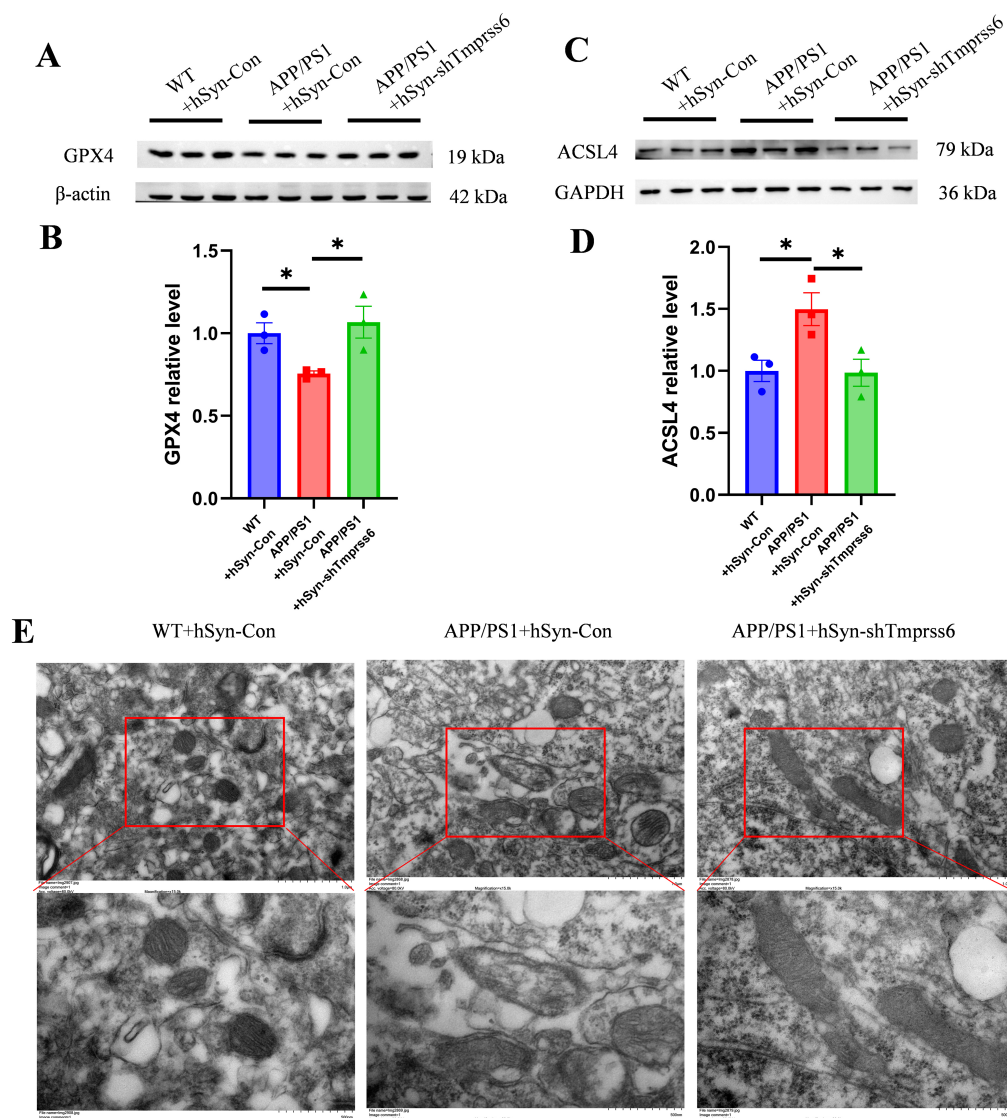


Figure 5. Neuronal ferroptosis and mitochondrial morphology. (A and B) Western blot images of GPX4 in the hippocampus, and quantification; (C and D) Western blot images of ACSL4 in the hippocampus, and quantification; (E) Transmission electron microscope images of neuronal mitochondria. The data are presented as the mean $\pm$ SEM. * $P < 0.05$. GPX4: Glutathione peroxidase 4; ACSL4: acyl-CoA synthetase long-chain family member 4; SEM: standard error of the mean.

TMPRSS6 knockdown group [Figure 6A and B]. The transferrin receptor 1 (TfR1), which binds to the iron carrier protein, transferrin (TF), is the rate-limiting factor in cellular iron acquisition^[17]. Knockdown of the *TMPRSS6* gene significantly decreased intracellular iron deposition and promoted TfR1 level recovery [Figure 6C and D]. These reciprocal changes in FtL (iron storage) and TfR1 (iron acquisition) are congruent with a shift in the neurons to a lowered iron status. Perl's iron staining further confirmed that *TMPRSS6* knockout significantly decreased iron deposition and iron plaque formation in the hippocampus of APP/PS1 mice [Figure 6E]. Excessive iron accumulation or imbalance may trigger oxidative stress and ROS production^[18]. We confirmed this experimentally by examining neuronal ROS levels [Figure 6F].

TMPRSS6 regulates iron metabolism through the BMP/Smad signaling pathway in the murine hippocampus

As part of systemic iron homeostasis, *TMPRSS6* protein regulates the BMP/Smad signaling pathway by

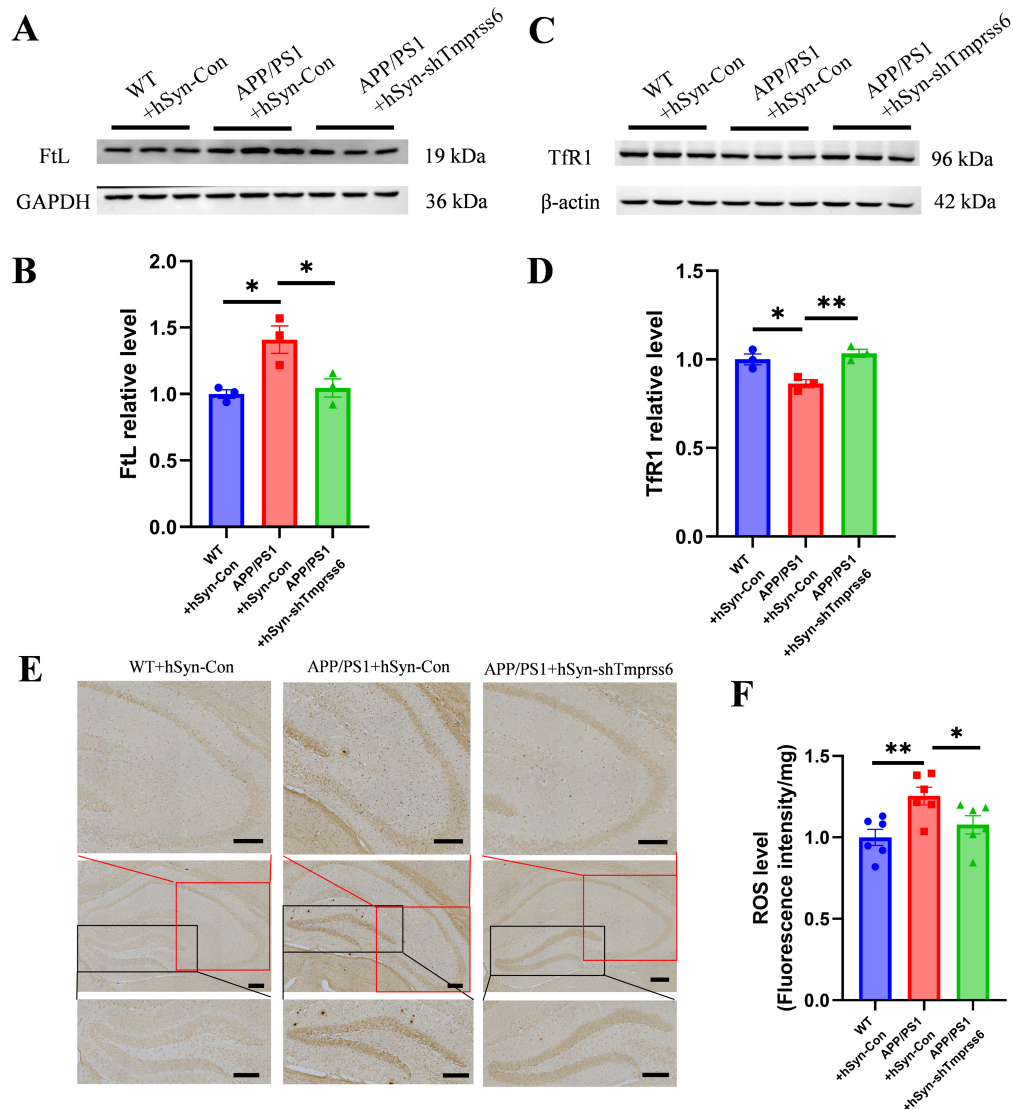


Figure 6. Determination of iron and related molecules, as well as ROS in the hippocampus. (A and B) Western blot images of Ftl in the hippocampus, and quantification; (C and D) Western blot images of TfR1 in the hippocampus, and quantification; (E) DAB-enhanced Perls iron staining of the hippocampus (scale bar: 200 μ m); (F) ROS levels in the hippocampus. The data are expressed as the mean $\pm$ SEM. * P < 0.05, ** P < 0.01. ROS: Reactive oxygen species; Ftl: ferritin light chain; TfR1: transferrin receptor 1; SEM: standard error of the mean.

cleaving HJV, thereby inhibiting hepatic synthesis of Hepcidin^[19]. We examined HJV levels in the mouse hippocampus by western blot analysis, whereby we observed that *TMPRSS6* knockdown increased HJV protein levels [Figure 7A and B]. In the knockdown group, the phosphorylation level of Smad1/5 increases [Figure 7C and D]. In contrast, the level of Smad1 decreases [Figure 7E and F]. Smad4 binding to phosphorylated Smad1 in the nucleus mediates *Hepcidin* transcription. We also observed increased Smad4 [Figure 7G and H] as a result of *TMPRSS6* knockdown. Importantly, decreased *TMPRSS6* gene expression significantly increased *Hepcidin* transcription [Figure 7I]. The results of immunohistochemistry also confirmed this finding [Supplementary Figure 1], which is consistent with the decreased FPN1 expression shown in Figure 7J and K.

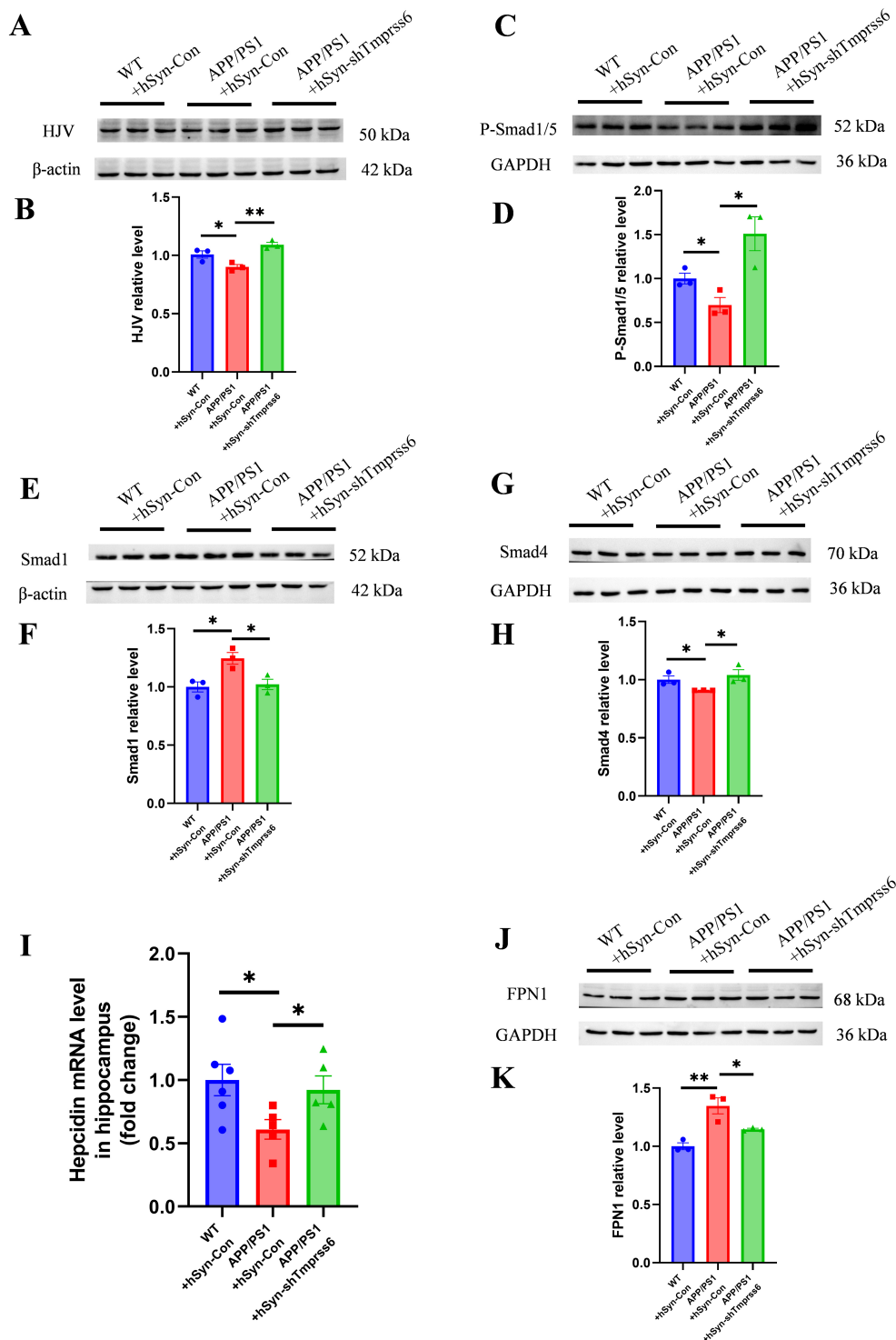


Figure 7. Effects of neuronal *TMPRSS6* knockdown on the BMP/Smad signaling pathway. (A and B) Western blot images of HJV in the hippocampus, and quantification; (C and D) Western blot images of p-Smad1/5 in the hippocampus, and quantification; (E and F) Western blot images of Smad1 in the hippocampus, and quantification; (G and H) Western blot images of Smad4 in the hippocampus, and quantification; (I) qRT-PCR evaluation of *Hepcidin* mRNA levels in the hippocampus; (J and K) Western blot images of FPN1 in the hippocampus, and quantification. The data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. BMP: Bone morphogenic protein; HJV: Hemojuvelin; qRT-PCR: quantitative reverse transcription polymerase chain reaction; FPN1: ferroportin 1; SEM: standard error of the mean.

DISCUSSION

AD is the most common age-related disorder and accounts for the highest proportion of neurodegenerative diseases. Common pathological features of AD patients include A β deposition, hyperphosphorylation of tau protein, and neurofibrillary tangles. Behaviorally, the disease manifests as a severe decline in spatial learning and memory^[20]. Cerebral iron deposition has been recognized as a well-established neuropathological hallmark of AD since the mid-20th century. Excessive iron concentrations promote APP overexpression, driving A β plaque formation. Furthermore, iron dyshomeostasis exacerbates oxidative damage and facilitates pathological Tau hyperphosphorylation^[21]. Our transcriptomic and proteomic analyses identified pronounced TMPRSS6 upregulation in the hippocampi of AD patients, a phenomenon recapitulated in APP/PS1 transgenic mice at both transcriptional and translational levels, indicating a potential pathogenic role of TMPRSS6 overexpression in AD progression. Targeted hippocampal TMPRSS6 silencing in murine models markedly attenuated AD-associated behavioral deficits. Mechanistically, TMPRSS6 knockdown substantially reduced hippocampal APP expression and A β deposition, suggesting that TMPRSS6-mediated iron regulation critically contributes to cognitive preservation in this AD paradigm.

Previous studies have shown that iron deposition in the brain of AD patients accelerates neuronal ferroptosis^[22] and apoptosis^[23], promoting disease progression. Inhibiting ferroptosis and decreasing iron-induced apoptosis may become important strategies for treating AD^[21,22]. With this in mind, we investigated the effects of TMPRSS6 knockdown on ferroptosis-related markers in the hippocampus and found that TMPRSS6 knockdown significantly decreased iron levels and reactive ROS levels in the hippocampus of APP/PS1 transgenic mice, while increasing GPX4 levels and decreasing ACSL4 levels. By transmission electron microscopy, we also found that TMPRSS6 knockdown significantly improved mitochondrial cristae disruption in hippocampal cells. Taken together, these results suggest that TMPRSS6 knockdown inhibited ferroptosis in hippocampal neurons.

TMPSRS6 cleaves the HJV protein, thereby inhibiting the Smad4-regulated Hepcidin expression pathway. We found that TMPRSS6 knockdown increased Smad4 expression, possibly enhancing Hepcidin expression and regulating FPN1. Our results also indicate that knocking down TMPRSS6 suppressed the expression of FPN1, which may be the result of increased Hepcidin acting on it, or it may be due to the decreased brain iron response caused by knocking down the TMPRSS6 gene. The latter mechanism would be under the control of the iron-responsive elements (IRE)/iron-regulatory proteins (IRPs), which link cellular iron levels to the expression of proteins related to cellular iron metabolism, such as FtL and TfR^[24].

Previous reports have suggested that Hepcidin ameliorates A β -mediated neurotoxicity and oxidative stress *in vitro*, particularly within astrocytic and microglial cultures^[25]. Preclinical studies reveal that central administration of Hepcidin attenuates A β -triggered neuroinflammation and mitigates neuronal degeneration in murine models. Mechanistically, cerebral Hepcidin overexpression suppresses transendothelial iron flux at the blood-brain barrier interface, effectively reducing parenchymal iron accumulation^[26,27]. These findings collectively propose that therapeutic upregulation of brain-derived Hepcidin could exhibit neuroprotective potential against AD pathogenesis. The role of TMPRSS6 in iron metabolism is to cleave HJV, so depletion of TMPRSS6 can stabilize the binding of HJV with BMPRs on the cell membrane, facilitating the binding of BMPs to their cognate receptors and increasing the expression of Hepcidin by promoting the BMP/Smad pathway. The decrease in iron levels and ROS may then prevent ferroptosis, providing protection from neuronal damage. Our findings not only suggest TMPRSS6 as a pathogenic factor in AD, but also introduce the protein as a potential target for AD and other diseases with pathogenic brain iron accumulation.

Although this study has advanced the understanding of the *TMPRSS6* gene's function and mechanisms, several limitations should be noted. The local hippocampal injection of *TMPRSS6*-targeting shRNA plasmids resulted in limited knockdown efficiency, which may be insufficient to reflect the gene's role in AD pathogenesis. Additionally, the lack of cell-type specificity in *TMPRSS6* knockdown may obscure its neuronal role, thereby reducing sensitivity in detecting relevant effects. To address these constraints, future studies employing AD model mice with neuron-specific *TMPRSS6* knockdown may offer clearer insights into the gene's precise mechanistic contributions to AD progression.

DECLARATIONS

Authors' contributions

Completed most of the experimental results: Sun H
Guided the experiment throughout: Zuo Y, Bai J
Helped with experiments and data processing: Kang S, Li C
Provided material support and supervision: Dong L, Yu P, Gao G, Zhao J
Conceived the study and edited the manuscript: Chang Y
All authors have read and approved the final manuscript.

Availability of data and materials

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All the animal experimental procedures in this study were approved by the experimental animal ethics committee of Hebei Normal University (Ethical Review Consent number: 2020LLSC18).

Consent for publication

Not applicable.

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