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Norboldine improves cognitive impairment and pathological features in Alzheimer's disease by activating AMPK/GSK3β/Nrf2 signaling pathway

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ABSTRACT

Ethnopharmacological relevance: Lindera aggregata (Sims) Kosterm is a common traditional herb that has multiple bioactivities. Radix Linderae (LR), the dry roots of *Lindera aggregata* (Sims) Kosterm, is a traditional Chinese herbal medicine with antioxidant, anti-inflammatory and immunomodulatory properties, first found in Kaibao Era. Norboldine (Nor) is an alkaloid extracted from LR and is one of the primary active ingredients of LR. However, the pharmacological functions and mechanism of Nor in Alzheimer's disease (AD) are still unknown.

Aim of the study: This study aims to investigate the effect and mechanism of Nor therapy in improving the cognitive impairment and pathological features of 3 × Tg mice.

Materials and methods: 3 × Tg mice were treated with two concentrations of Nor for one month and then the memory and cognitive abilities of mice were assessed by novel object recognition experiment and Morris water maze. The impact of Nor on the pathology of ADwere examined in PC12 cells and animal tissues using western blotting and immunofluorescence. Finally, western blotting was used to verify the anti-apoptotic effect of Nor by activating AMPK/GSK3β/Nrf2 signaling pathway at animal and cellular levels.

Results: In this study, we showed that Nor treatment improved the capacity of the learning and memory of 3 × Tg mice and alleviated AD pathology such as Aβ deposition. In addition, Nor restored the abnormalities of mitochondrial membrane potential, significantly reduced the production of intracellular ROS and neuronal cell apoptosis. Mechanistically, we combined network pharmacology and experimental verification to show that Nor may exert antioxidant stress and anti-apoptotic through the AMPK/GSK3β/Nrf2 signaling pathway.

Conclusion: Our data provide some evidence that Nor exerts a neuroprotective effect through the AMPK/GSK3β/Nrf2 pathway, thereby improving cognitive impairment in AD model mice. Natural products derived from traditional Chinese medicines are becoming increasingly popular in the process of new drug development and discovery, and our findings provide new perspectives for the discovery of improved treatment strategies for AD.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia, characterized by memory loss and cognitive impairment (Lane et al., 2018). Latest statistics showed that the prevalence of dementia will triple worldwide by 2050 (Scheltens et al., 2021). Therefore, there is an

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urgent need to explore and discover new drugs.

The dominant features of AD include the loss of neurons and abnormal accumulation of Aβ. Neuron loss is widely considered to be the primary driver of cognitive decline in patients with AD (Mecca and van Dyck, 2021). Studies have shown that neuronal loss occurs in the core regions of Aβ deposition, and the deposition of Aβ around neurons leads to an increase in ROS and thus causes neuronal mitochondrial dysfunction (Shin et al., 1989; Behrouz et al., 1991; Zhang et al., 2021). In addition, it also activates the apoptotic signaling pathway and induces neuronal cell death. Importantly, impaired mitochondrial function increases the production of ROS, and the two reinforce each other in a vicious cycle that ultimately leads to neuron death and impaired cognitive function (de la Monte et al., 2000; Hirai et al., 2001; Coskun et al., 2004; Cai and Tammineni, 2016). On the other hand, previous studies have found that there are mitochondrial breaks and loss of membrane potential in brain tissues of patients with AD, as well as significant changes in biomarkers and mediators of oxidative stress (Bonda et al., 2010; Han et al., 2021). Therefore, the search for drugs that inhibit mitochondrial dysfunction induced neuronal cell apoptosis may be a potential method to treat AD.

Clinical studies have proved that Chinese medicine can improve cognition and physical function of early AD patients, and the material basis for its efficacy is the chemical components contained in Chinese medicine (Feng et al., 2018; Ding et al., 2022). The search for natural products with high efficiency and low side effects has been one of the hot spots in drug research, and some natural products based on traditional Chinese medicine have unique advantages in anti-AD. Linderae Radix (LR), which has been used in Chinese medicine for thousands of years, is the dried tuberous root of *Lindera aggregate* (Sims) Kosterm in the *Lauraceae* family and its main producing areas are Zhejiang, Jiangsu and other places in China (Wagner et al., 2017). In local area, it is also known as "wuyao". The Qing Dynasty 《*Ben cao xin bian*》 and 《*Ben cao cong xin*》 recorded the warm in property, non-toxic, with dispersing "qi", dispelling cold, removing pain and other effects. AD is defined as senile dementia in Chinese medicine. It is recorded in 《*Jingyue Quanshu*》 that dementia can be treated by promoting "qi" circulation and alleviating depression (Liu et al., 2024). In addition, the traditional bibliographic describe the use of LR in the treatment of some brain disorders, such as the use of LR in the treatment of stroke in 《*Ben cao bei yao*》 and 《*Ben cao cong xin*》; 《*Yifang Jijie*》 records that LR relieves depression (Huang et al., 2023). LR has pharmacological activities such as anti-inflammatory, analgesic, and anti-oxidant, and it is widely used to treat neurological disorders (Li et al., 2019). For example, LR has effects on neuroinflammation, synaptic loss, and subsequent memory impairment in Aβ-induced AD models, and has neuroprotective effects (Kim et al., 2023). In addition, LR increased cell viability in corticosterone treated PC12 cells, and decreased the mRNA expresstion of caspase-3and Bax/Bcl-2 ratio (Choi et al., 2017). Recent years, more and more research has been carried out on the active ingredients of LR, some of which have been reported to have multiple pharmacological effects, but most of the research only stops at cells (Gonzalez et al., 2022). For example, Isolinderalactone is anti-inflammatory, antioxidant, and anti-apoptotic in several cancer cell models (Hwang et al., 2019; Kwak et al., 2022; Shen et al., 2023); Total alkaloids reduce inflammation in several cell lines (Wang et al., 2007; Luo et al., 2009). Norboldine (Nor) belongs to the alkaloid class isoquinolines, which are natural products extracted from the root extract of LR. In this study, we investigated the effects of Nor on cognitive function and AD pathology in PC12 cells and 3 × Tg mice, and elucidated the mechanism of Nor in AD by combining network pharmacology and biological experimental methods.

2. Methods

2.1. Network pharmacology research

The candidate genes of Nor were acquired from Traditional Chinese Medicine Systems Pharmacology Datebase (TCMSP), Swiss Target Prediction and TargetNet. A search for "AD" was performed in five databases of Genecards, OMIM, drugbank, TTD and Disgenet, to identify potential targets. Next, AD-related disease targets were mapped with potential pharmacologic targets of Nor in the Venny 2.1.0 to obtain intersection targets. The PPI protein interaction network was obtained by importing the cross targets into the string database. Meanwhile, "CytoNCA" in Cytoscape 3.9.0 was used to screen the interaction network and explore the hub gene.

2.2. Reagents

Norboldine (purity: HPLC≥98%) was purchased from Chengdu DeSiTe Biological Technology. Linderae Radix (LR) is the dried root of *Lindera aggregate* (Sims) Kosterm (also known as wuyao), and this plant name is well recognized (http://www.theplantlist.org). High-glucose DMEM medium and Fetal bovine serum (FBS) were derived from Biochannel and Inviogent, respectively. Penicillin (100 U/mL)/treptomycin (100 μg/mL) (15,140-122) was obtained from Gibco. DMSO (Cellular level, D2650), Bovine serum albumin (BSA) and MTT powders were gained from Sigma Corporation. The Annexin V-FITC/PI apoptosis detection kit, DCFH-DA kit, JC-1 kit and RIPA lysis buffer were all purchased from Beyotime. The phosphatase inhibitors cocktail and protease inhibitors were gained from MCE. Polyvinylidene difluoride membranes (PVDF) were obtained from Bio-Rad. Aβ 1–42 (catalog number PA4391, molecular mass of 4514.10), NH2-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVG GVVIA-COOH, was purchased from WuHan Dan Gang Biological Technology Co., Ltd. The list of primers and antibody information are shown in Table S1 and Table S2.

2.3. Cell culture and treatment

PC12 cells were acquired from Wuhan Sunncell Biotech. PC12 cells were cultured in 10% DMEM high-glucose medium. After 24 h, when the cells reached 70% on the pore plate, the cells were first incubated with Nor and then stimulated with Aβ1-42. Nor and Aβ1-42 were dissolved in DMSO to obtain storage solution (100 mM for Nor and 10 mM for Aβ1-42. In all the cell experiments, cells acclimated in the pore plate for 24 h before any treatments.

2.4. MTT assay

The cell viability was determined by thiazolyl bluet tetrazolium bromide (MTT) assay. PC12 cells in 96 well plates were treated with different concentrations of Nor for 2.5 h and then incubated with Aβ1-42 for 24 h. Subsequently, MTT was applied to incubated seeded cells for an additional 3 h. Comparison of light absorption values (OD) at 570 nm between treatment and control groups.

2.5. Reactive oxygen species (ROS) determination

DCFH-DA kit was used to determine the intracellular ROS levels. Briefly, PC12 cells were seeded in 96-well plates and cultured for 24 h. After treatment with Nor and Aβ1-42, the cells in each group were exposed to 10 μM DCFH-DA and incubated at 37 °C for 30 min, then washed twice with phosphate buffered saline (PBS) solution. The fluorescence was measured using an Infinite M200 PRO Multimode Microplate at an emission wavelength of 665 nm and an excitation wavelength of 640 nm.

2.6. Determination of mitochondrial membrane potential (△ψm)

The JC-1 kit was used to measure the effect of Nor on mitochondrial membrane potential. Similar to the pre-treatment for ROS detection, PC12 cells were treated with 1 × JC-1 staining solution (10 μg/ml) at

37 °C. Afterward, the PC12 cells of each group were washed 3 times with PBS and observed under inverted fluorescent microscope (Leica).

2.7. Flow cytometry

PC12 cells were seeded in a 6-well plate and cultured for 24h. Afterward, cells were pretreated with Nor and then incubated with Aβ1-42 for 24 h. PC12 cells were digested with 0.25% trypsin (without EDTA) and centrifuged at 1000 rpm for 5min. The percentage of apoptosis was detected using flow cytometry assay. Briefly, Annexin V-FITC (5 μl) and Propidium Iodide (PI,10 μl) were added to the cells and incubated with the cells for 30 min at room temperature in dark. The apoptotic cells was analyzed using Flow cytometer (C6 pus, DB).

2.8. Western blotting

Brain tissue or cells samples were fully lysed on ice with RIPA buffer (adding protease and phosphatase inhibitors). Proteins were separated on 10% or 12.5% Tris-HCL polyacrylamide gels and transferred to PVDF membrane. And then it was blocked by 3%BSA for 1 h and then incubated at 4 °C overnight with specific primary antibody. The next day, the corresponding enzyme-labeled secondary antibody was incubated at room temperature for 2 h. Ultimately, the protein bands were quantified by Image J gel analysis software.

2.9. Animals and treatment

The 7-month-old 3 × Tg mice and C57 mice were purchased from Zhejiang Experimental Animal Center. Feed and water were sufficient and the mice were kept at an indoor temperature of 25 °C. All drug stock solutions were obtained by dissolving the drug with DMSO and the mice were intraperitoneally injected once a day according to body weight for 1 month. After the behavioral assessment, the experimental mice were sacrificed by cervical dislocation after anesthesia with 5% chloral hydrate. Brain tissues were dissected and some samples were placed into a −80 °C refrigerator. The remaining brain tissue specimens were fixed and dehydrated, then embedded with OCT and frozen at −80 °C.The operating procedures adheres to Hangzhou Medical College Animal Ethics Committee.

2.10. Morris water maze (MWM) test

Briefly, the laboratory mice were first trained for five days of place navigation experiment, and on the sixth day for space exploration experiments. In the first 5 days, an escape platform was placed in the designated quadrant, and the time the mice spent searching for the platform was used as an evaluation index of their learning ability. On the sixth day, after the platform was removed, the time the mice swam in the target quadrant and the number of times they crossed the platform were recorded, which reflected the mice's memory ability. The VisuTrack MWM image analysis system is used for data acquisition and analysis.

2.11. New object recognition test (NORT)

The new object recognition experiment was conducted over 2 consecutive days. After the mice acclimated to the experimental environment, we began the first day of the experiment, observing and recording the exposure of each group of mice to two identical objects. After the next 24 h, one of the two identical objects was replaced with a different object, and the same indicator was observed and recorded. Each mouse was given 5 min to explore objects.

2.12. Elisa assay

The protein levels of Aβ40 and Aβ42 in serum were detected by enzyme-linked immunosorbent assay (ELISA). The Aβ40 (EM0863) and Aβ42 (EM0864) ELISA kits were purchased from FineTest (Wuhan, China). Briefly, whole blood was collected from mice through the heart and left at room temperature for 1 h. The serum was collected after 10000g centrifugation for 15 min and tested according to the instructions provided by the manufacturer. The content of serum Aβ was measured at 450 nm by a Multi-function microplate reader.

2.13. Immunofluorescence (IF)

Oct-embedded mouse brain tissue was cut into 20 μM slices and then dried. After permeability, the brain sections were blocked with 10%BSA buffer (diluted with PBS) at room temperature for 1h. Slices were dropped into primary antibody and incubated at 4 °C overnight. Fluorescent secondary antibody staining was performed for 2 h. SlowFade® Anti-fading DAPI drops onto a slide to stain the nucleus, covers the slide and captures the image using an inverted microscope.

2.14. Statistical analysis

All the above date were collected using GraphPad Prism statistical software (version 8) and were expressed as the means ± SEM. One-way or two-factor analysis of variance (ANOVA) was used for comparison among the experimental groups, and Tukey postmortem test was performed. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Nor attenuates the apoptosis of PC12 cells induced by Aβ1-42

To evaluate protective effect of Nor (Fig. 1A) in PC12 cells induced by Aβ1-42, we performed MTT assay. To do this, the toxicty of different concentrations of Aβ1-42 on PC12 cells were assessed. MTT results showed that incubation with Aβ1-42 at a concentration of 10 μM had a significant cytotoxic effect, ultimately resulting in 60% of PC12 cell death (Fig. 1B). Meanwhile, the toxicity of Nor was evaluated, and the results suggested that there is no toxicity to the drug concentration of our choice (Fig. S1A). Cell viability results demonstrated that Nor had a protective effect on Aβ-induced cell damage, especially when the concentration of Nor was 2.5 μM (Fig. 1C). For another thing, flow cytometry results also indicated that Nor was able to reverse the changes in the number of apoptosis induced by Aβ1-42 and prevent the damage of nerve cells (Fig. 1D and E). Western blotting results showed that Nor increased Bcl2 proteins levels and decreased the levels of Cleaved-caspase3 proteins, which were affected by Aβ1-42 (Fig. 1F–H). In conclusion, these simulation results confirmed that Nor can inhibit the apoptosis of PC12 cells induced by Aβ1-42.

3.2. Nor improves oxidative stress induced by Aβ1-42

In the previous studies, oxidative stress has been identified as an important mediator of neuronal cell death (Ratan and Baraban, 1995). Therefore, the intracellular ROS content was determined by DCFH-DA staining. The results revealed that Aβ1-42 increase ROS content while Nor decreased (Fig. 2A and C). In addition, the JC-1 experiment results showed that Aβ1-42 had a promoting effect on mitochondrial function damage, and Nor treatment prevented the reduction of mitochondrial membrane potential, which was demonstrated by changes in the ratio of red and green fluorescence (Fig. 2A and B).

3.3. Nor reduced neuronal damage induced by Aβ1-42 through the AMPK/GSK3β/Nrf2 pathway in PC12 cells

Based on the TCMSP and several datebases, 104 Nor targets were found, 4526 AD-related targets were screening, and a total of 56 overlapping targets were identifiedFig. S2A. The core target was retrieved using CytoNCA in Cytoscape software (3.9.0), resulting in the first

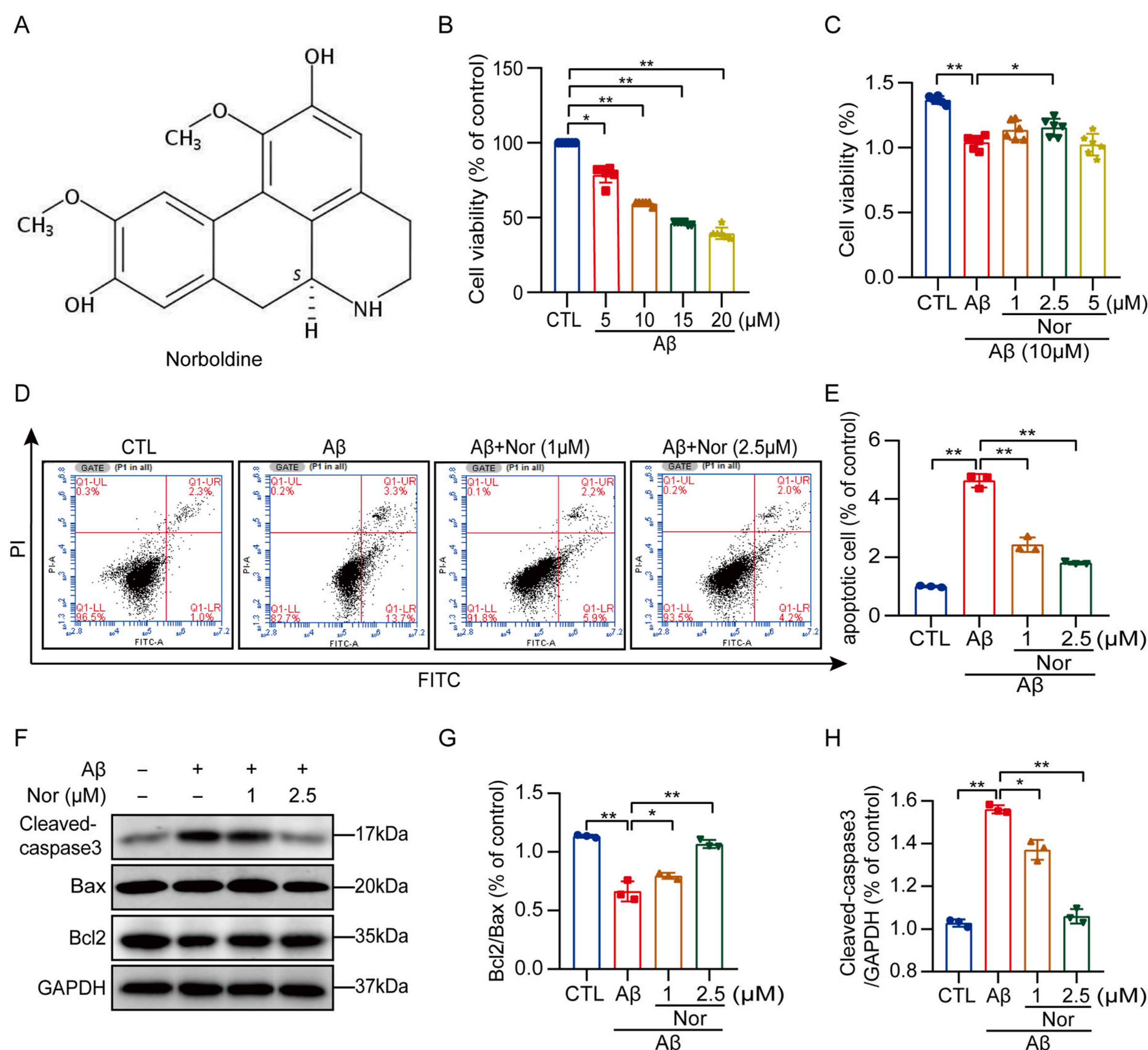

Fig. 1. Nor attenuates the apoptosis of PC12 cells induced by Aβ1-42 in vitro. (A) The chemical structure of Norboldine (Nor). (B) Concentration screening of Aβ. (C) Effect of Nor on cell viability of PC12 cells. (D) Apoptosis was measured by flow cytometry. (E) Quantitative analysis of (D). (F) Western blotting analysis of the expression levels of cleaved-caspase3, Bax, and Bcl2 in PC12 cells. (G) and (H) Statistical analysis of (F). The data are expressed as the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01.

ranking of GSK3β, followed by visualization (56 nodes and 189 edges) (Fig. 3A). LR active ingredients and intersection target date are shown in Table S3 and Table S4.

AKT, ERK, and AMPK are classical upstream of GSK3β, in which Nor treatment has little effect on phosphorylation of AKT and ERK (Fig. S2A-C). Nrf2 is closely related to antioxidant activity, and AMPK/GSK3β has been found to be involved in the activation of Nrf2. Therefore, in order to further study the mechanism of Nor in treating neuronal apoptosis, the protein levels of AMPK, GSK3β and nuclear Nrf2 were detected by Western blotting. Our investigations unveiled that Nor induced slightly up-regulation of phosphorylated AMPK, phosphorylated GSK3β and nuclear Nrf2 (Fig. 3B–E). Meanwhile, mRNA levels of Nrf2 downstream genes HO-1 and Nqo-1 were elevated under the effect of Nor (Fig. 3F). The above results discovered that Nor might exert its neuroprotective role in alleviating nerve injury through AMPK/GSK3β/Nrf2 signaling pathway.

3.4. Inhibition of the AMPK/GSK3β/Nrf2 pathway reversed the protective effect of nor in PC12 cells

AMPK is a key protein involved in a variety of signal transduction pathways, and AMPK signaling pathway is involved in the occurrence and development of AD by regulating oxidative stress and mitochondrial function (Zhao et al., 2024). To further corroborate whether the AMPK/GSK3β/Nrf2 pathway was involved in the neuroprotective effects of Nor, we blocked AMPK signaling pathway by using AMPK inhibitor (Compound C) and si-AMPK. MTT result showed that pretreatment with Compound C for 30min could reverse the neuroprotective effect of Nor in PC12 cells (Fig. 4A). Meanwhile, compared with Nor treatment group, compound C pretreatment decreased the mRNA level and protein level of Bcl2/Bax ratio, increased the protein level of cleaved-caspase3 (Fig. 4B–D). In addition, Western blotting results showed that the elevated expression levels of P-AMPK, P-GSK3β and Nrf2 in the Nor treatment group were reversed by the Compound C (Fig. 4E and F). Next, we found that the effect of Nor on oxidative stress (Fig. 4G, I, 4J) and cell apoptosis (Fig. 4H and K) were decreased by blocking AMPK pathway.

The result were further verified by knocking down AMPK using si-AMPK. As expected, AMPK knockdown decreased the activity of PC12 cells (Fig. 5A), reduced the ratio of Bcl2/Bax protein and increased the protein level of Cleaved-caspase3 (Fig. 5B and C). JC-1 staining and DCFA-DA staining showed that the effect of Nor on mitochondrial

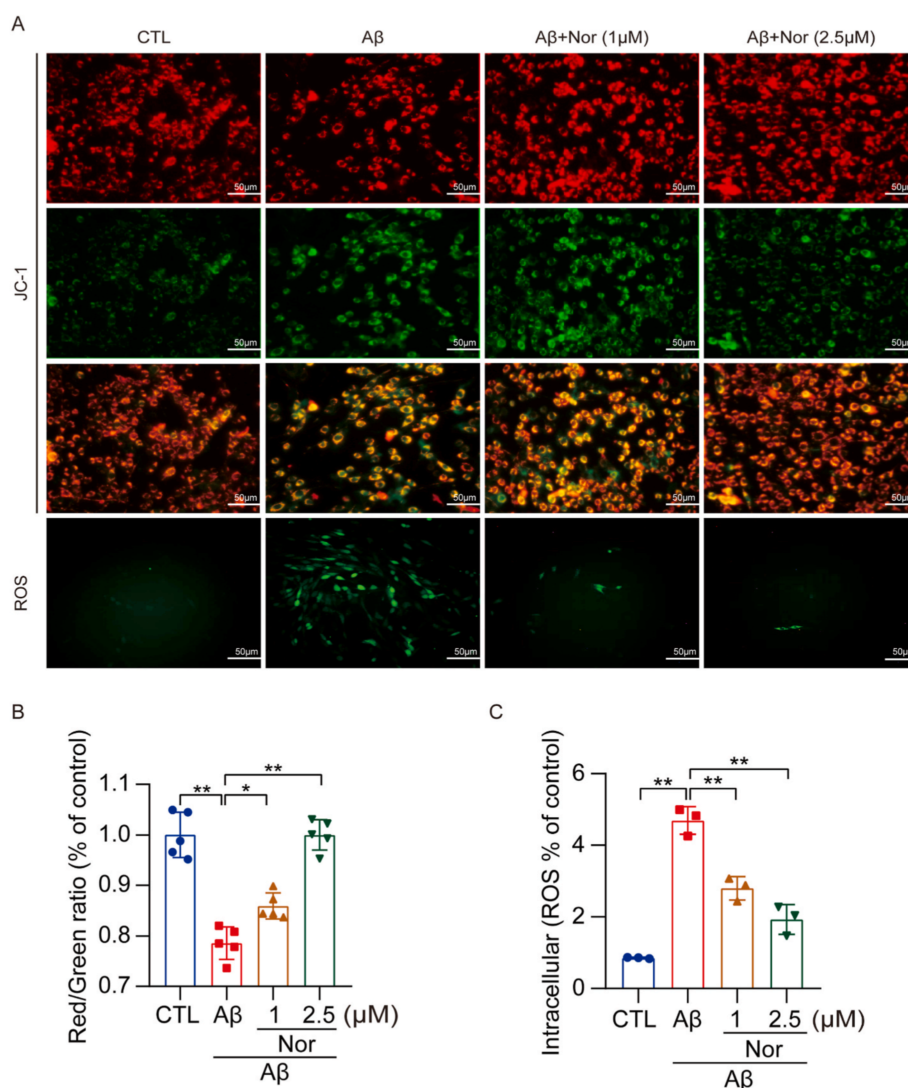

Fig. 2. Nor improves oxidative stress induced by Aβ1-42. (A) Representative fluorescent pictures of JC-1 and ROS staining. (B) and (C) Quantitative analysis of (A). The data are expressed as the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01.

membrane potential and intracellular ROS production were reversed in the si-AMPK group (Fig. 5D, F, 5G). Similarly, flow cytometry showed that the number of apoptosis cells was increased in the si-AMPK group (Fig. 5E and H). Together, these results indicated that the antioxidant and anti-apoptotic effects of Nor in PC12 cells were reduced by inhibition of AMPK/GSK3β/Nrf2 signaling pathway.

3.5. Nor ameliorated cognitive deficits of 3 × Tg mice

Behavioral tests were used to further validate the therapeutic of Nor on cognitive impairment in vivo. After a one-month treatment period, NORT was performed to record the movement trajectories of mice in each group (Fig. 6A). Donepezil (5 mg/kg) is already approved for clinical treatment of AD, so it is used as a positive control in this article (Adlimoghaddam et al., 2018). There was no difference in average speed between treated and untreated 3 × Tg mice (Fig. 6B). Notably, the Nor treatment group increased the number of times the mice explored new objects (object B) (Fig. 6C). There was no difference in Total distance between treated and untreated 3 × Tg mice (Fig. 6D). Moreover, the 6-day MWM test further supported that the Nor group exhibited better learning and memory abilities than the 3 × Tg mice. The results showed a decrease in the latency to find the target platform in the Nor groups, compared with the 3 × Tg mice (Fig. 6E). In the results of statistical analysis, it was found that the escape latency of the Nor group to find the platform on the 4th day was shorter, and there was statistical difference (Fig. 6F and G). The mice injected with Nor performed better in a subsequent spatial probe test. Specifically, animals pretreated with Nor showed significant increases in the number of platform crossings and the percentage of time in the target quadrant (Fig. 6H and I). There was no statistical difference in average speed (Fig. 6J). The results of both behavioral studies indicated that Nor improved impaired cognitive function in 3 × Tg mice.

3.6. Nor treatment improves the pathology of AD

Studies have report that the pathological features of AD are the accumulation of Aβ in the brain and neuroinflammation, which is mainly characterized by the activation of glial cells (Cai et al., 2022). In this study, immunofluorescence (IF) and ELISA was used to verify whether Nor improved the pathology of AD. Primarily, we examined brain tissue markers of microglial cells (Iba1) and astrocyte markers (GFAP), which are the main glial cells in the brain (Fig. 7A). At the same time, mice treated with Nor showed fewer Aβ deposits (Fig. 7A). Statistical analysis also proved that the levels of Iba1, GFAP and APP in the Nor treatment group were lower than those in the 3 × Tg group (Fig. 7B–D). ELISA results showed that Nor significantly reduced the

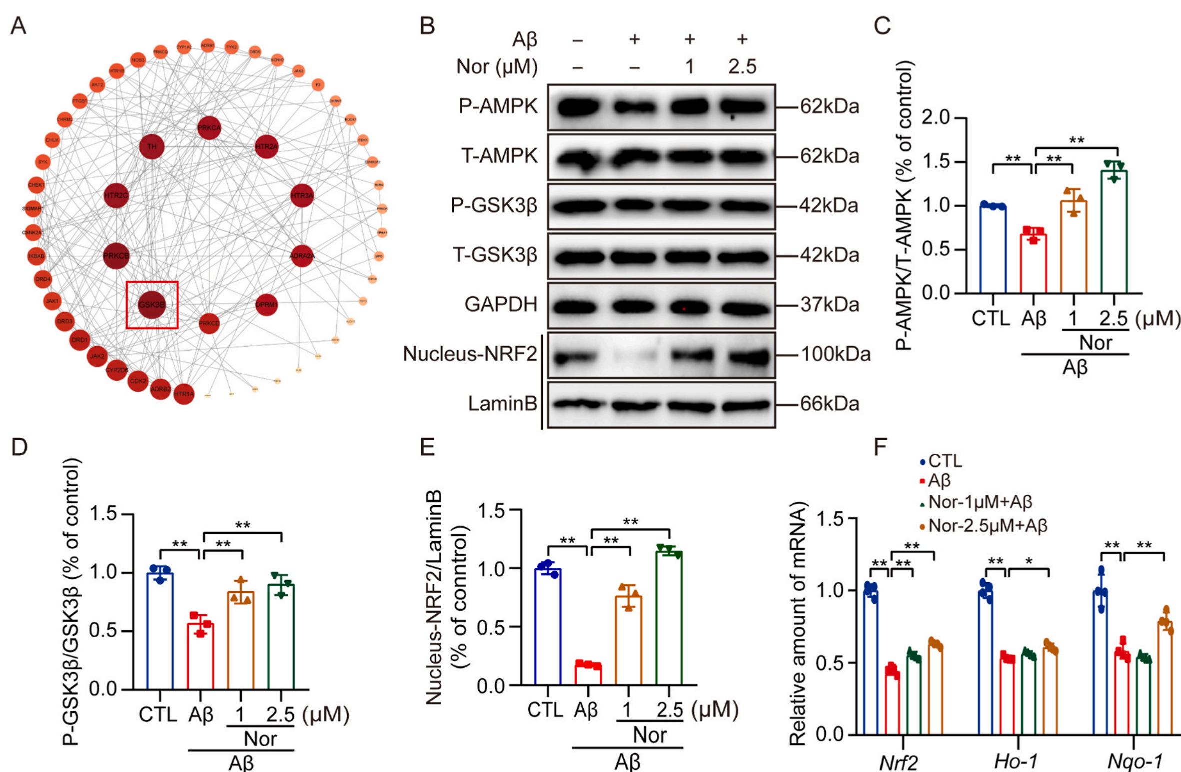

Fig. 3. Nor reduced neuronal damage induced by Aβ1-42 through the AMPK/GSK3β/Nrf2 pathway in PC12 cells. The expression levels of total protein and phosphorylated protein were detected by Western blotting. (A) Identification of Nor anti-AD core targets. (B) Western blotting results of the expression levels of P-AMPK, P-GSK3β, and nuclear Nrf2 in PC12 cells. (C–E) Quantitative data of Western blotting intensity of protein in (B). (F) Statistical analysis of mRNA expression levels of Nrf2, HO-1, and NQO-1 in PC12 cells by RT-qPCR. The data are expressed as the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$.

level of toxic proteins Aβ40 and Aβ42 (Fig. 7E and F). These results demonstrated that Nor treatment alleviated the pathology of AD in 3 × Tg mice.

3.7. *Nor reduces neuronal cell apoptosis via activation of the AMPK/GSK3β/Nrf2 pathway in 3 × Tg mice*

Hippocampal neuron apoptosis is one of the dominant causes of AD. As shown in Fig. 8A and B, both Nor and donepezil increased the number of positive cells of hippocampal neurons, indicating that Nor attenuated neuronal apoptosis compared with the 3 × Tg group. Meanwhile, the expression levels of apoptosis-related proteins in brain tissues was determined by Western blotting experiments. Nor increased Bcl2 protein levels and decreased cleaved-caspase3 and Bax protein levels, as expected (Fig. 8C–E). We further explored the anti-AD potential of Nor and its mechanism through in vivo studies. The results showed that Nor boost the phosphorylation levels of AMPK, GSK3β and the expression levels of Nrf2 in the 3 × Tg mice's brain (Fig. 8F and G). Taken together, these in vivo data further illustrate that Nor could play a neuroprotective role by activating the AMPK/GSK3β/Nrf2 pathway.

4. Discussion

In this study, we investigated the protective effects of Nor in both in vivo and in vitro models of neuronal injury and its possible mechanisms. Our data showed that Nor restored mitochondrial membrane potential, decreased ROS concentration, and further improved the apoptosis of PC12 cells induced by Aβ1-42. In terms of mechanism, Nor inhibited neuronal apoptosis by activating the AMPK/GSK3β/Nrf2 pathway, and the use of the AMPK inhibitor Compound C and si-AMPK could reduce the anti-apoptotic activity of Nor. Similar results were also obtained in vivo, we found that Nor effectively reduced cognitive impairment and pathological features in 3 × Tg mice.

LR has good antioxidant, anti-inflammatory and anti-apoptotic effects. However, the active ingredients and mechanism of action are still uncertain. Nor is an important active component of LR and our study provides a new idea for the screening and mechanism exploration of active components of LR.

Apoptosis is a physiological death process regulated by genes to maintain tissue stability, and is closely associated with the onset of AD. Caspase3 is the biomarker of apoptosis. In our experiment, we used Aβ1-42 to induce apoptosis of PC12 cells, and the optimal therapeutic concentration of Nor we screened could effectively reduce such apoptosis. Oxidative stress is one of the dominant ways to induce neuronal apoptosis and the brains of people with AD show oxidative damage, which indicated that oxidative stress exerts a crucial role in AD (Cheignon et al., 2018). In addition, mitochondrial damage in AD is mainly caused by cytochrome oxidase (Bolin et al., 2006) deficiency (Wang et al., 2020). COX abnormalities and its mediated mitochondrial damage can increase ROS content and selectively damage neurons in patients with AD (Davis et al., 1997). Based on this, we evaluated the effects of Nor on ROS production and mitochondrial membrane potential, and the results were in line with our expectations. AD induces abnormal apoptotic cascade reaction in the cortex and hippocampus, in which apoptotic participants affect signal transduction across multiple pathways, ultimately leading to neuronal loss (Sharma et al., 2021). Loss of neurons is thought to be one of the causes of cognitive impairment. Therefore, we investigated whether Nor could improve the behavior and cognitive ability of 3 × Tg AD model mice through animal experiments. We were pleased to find that Nor not only improved cognitive deficits in AD model mice, but also reduced the pathological features of AD, with an effect comparable to Donepezil. This suggests that Nor has a neuroprotective effect and potential for the treatment of AD. However, the mechanism and target of Nor on AD are still unclear.

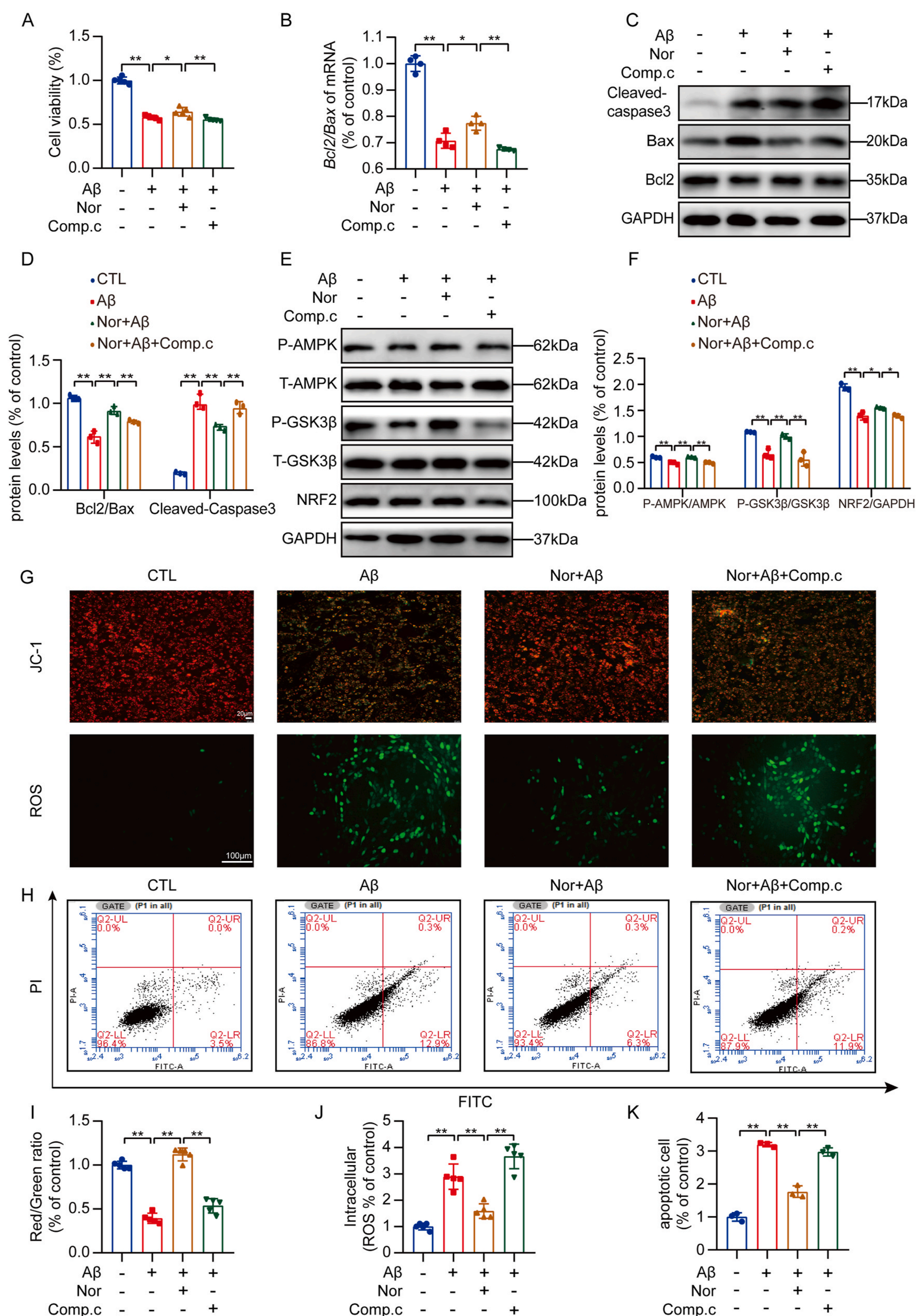

Fig. 4. Inhibition of the AMPK/GSK3β/Nrf2 pathway reversed the protective effect of Nor on nerve cells. (A) Effect of Nor in **PC12 cell viability after AMPK inhibition.** (B) Statistical analysis of mRNA expression of Bcl2/Bax ratio in PC12 cells. (C) (D) Western blotting analysis of the expression of cleaved-caspase3, Bax, and Bcl2 in PC12 cells. (E) (F) Western blotting results of the expression of P-AMPK, P-GSK3β, and Nrf2 in PC12 cells. (G) Representative fluorescent images of PC12 cells stained with JC-1 and ROS. (H) Apoptosis was detected by flow cytometry. (I–K) Quantitative analysis of (G–H). The data are expressed as the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01.

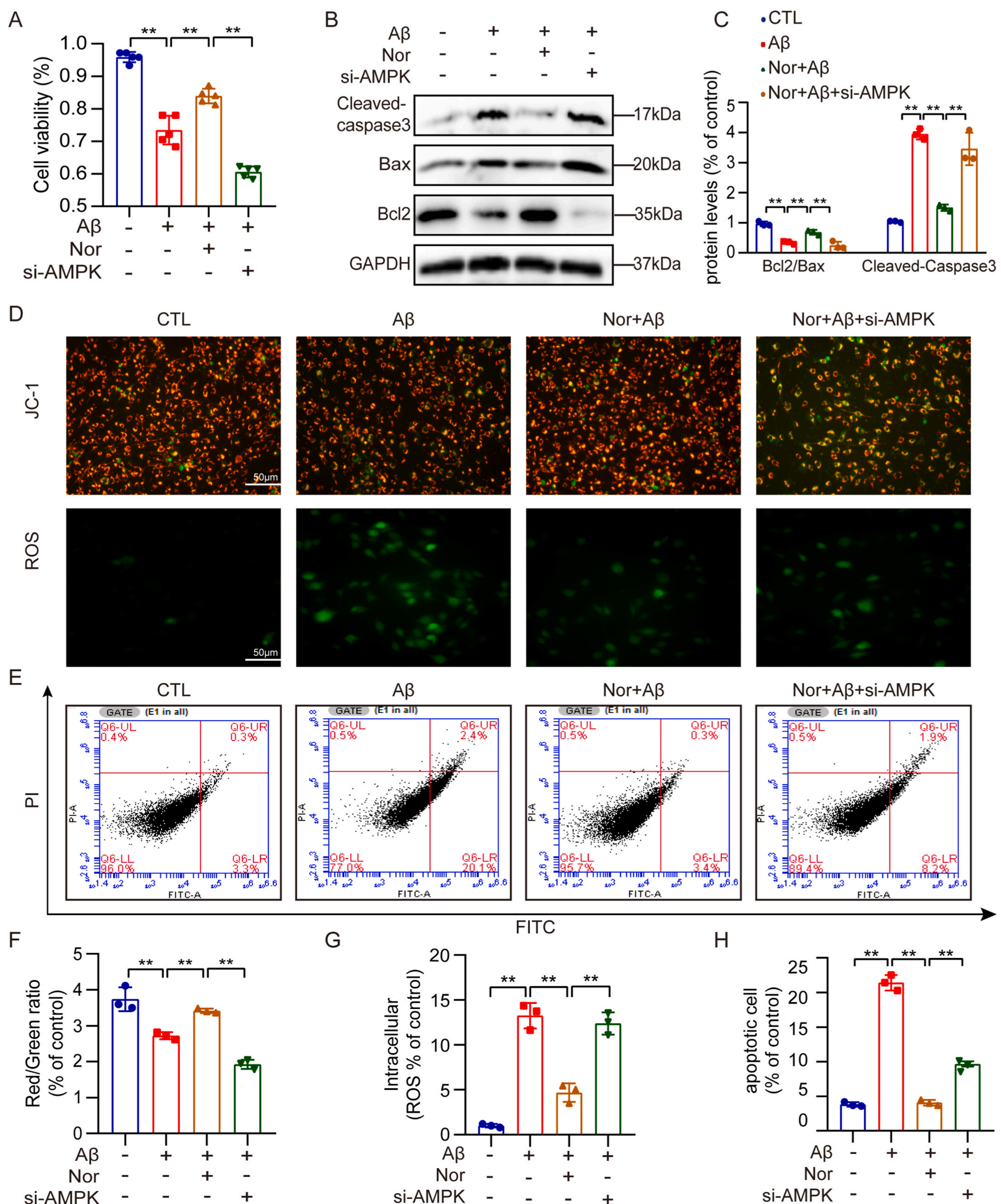

Fig. 5. Knocking down AMPK also reversed the anti-AD effect of Nor. (A) Effect of Nor in PC12 cell viability after AMPK knockdown. (B) Western blotting analysis of the expression of cleaved-caspase3, Bax, and Bcl2. (C) Quantitative analysis of (B). (D) Representative fluorescent images of JC-1 and ROS. (E) Apoptosis was detected by flow cytometry. (F) Quantitative analysis of JC-1 staining. (G) Quantitative analysis of ROS staining. The data are expressed as the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01.

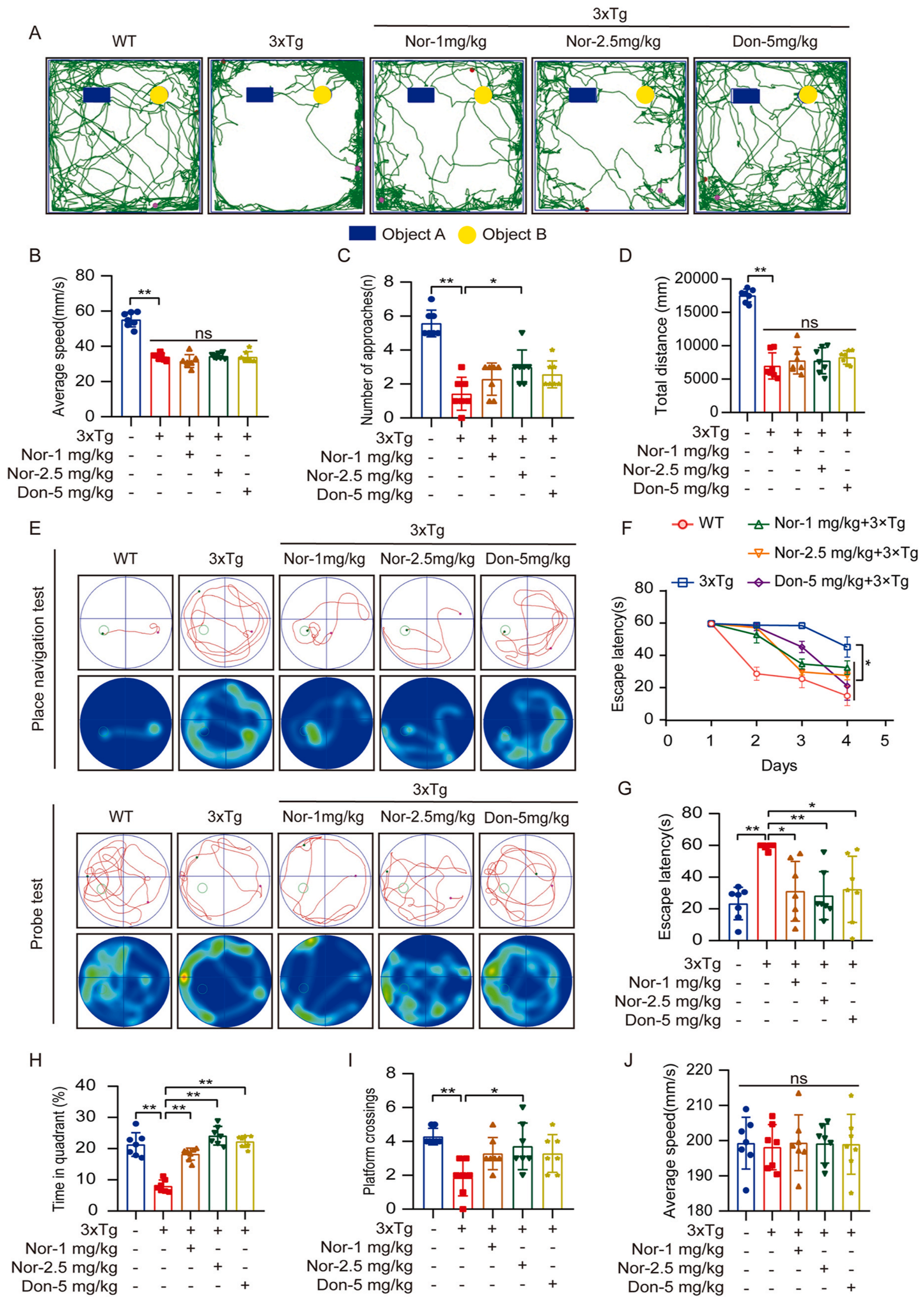

Fig. 6. Norboldine ameliorated cognitive deficits of 3xTg AD mice. (A) The representative curve of NORT. (B) Average speed on the second day of the NORT. (C) The number of approaches to the novel object. (D) Total distance on the second day of the new object recognition test. (E) Representative curve of place navigation test and probe test. (F) Escape latency of mice on days 1–4. (G) The time it takes to find the hidden platform (escape latency) on day 6. (H) Time spent in the target quadrant where the platform had been located for the first 5 days. (I) The average crossing platform times of each group of mice within 60 s. (J) Average swimming speed of mice. The data are presented as mean ± SEM of n = 7 mice per group. *P < 0.05, **P < 0.01.

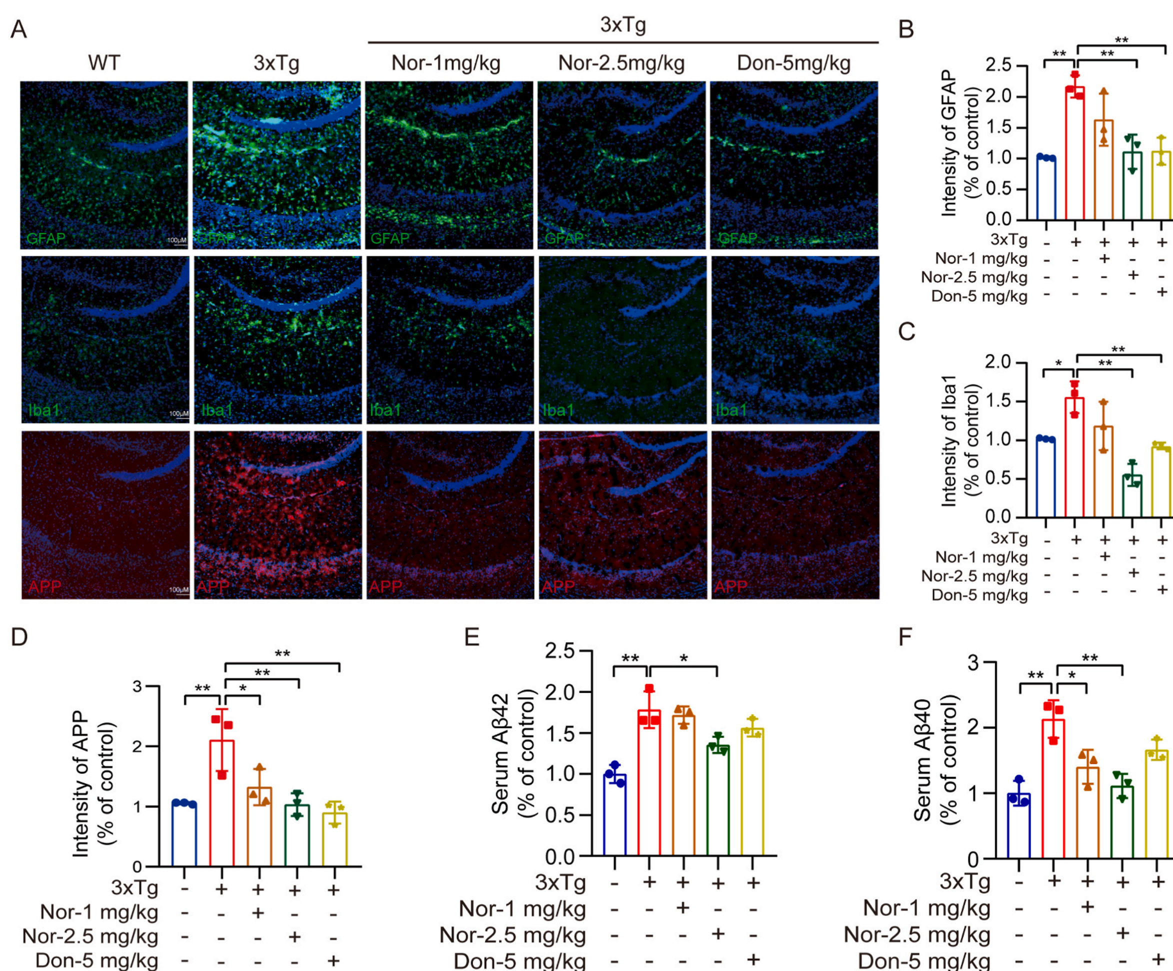

Fig. 7. Norboldine treatment improves the pathology of AD. (A) Representative immunofluorescence images of hippocampus. Scale:100 μm. (B–D) Quantitative analysis of (A). (E) (F) relative content of serum Aβ42 and Aβ40 in each group. The data are expressed as the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01.

We used network pharmacology to sort the crossover targets between Nor and AD according to the Degree value, and identified the core targets GSK3β where Nor may play an anti-AD role. Several studies have shown that GSK3β is pathologically correlated with Aβ in AD (Patel and Woodgett, 2017). The key proteins upstream of GSK3β include AMPK, ERK and AKT (Lv et al., 2017; Rai et al., 2019; Bian et al., 2021). In this study, we found that Nor regulates the phosphorylation of AMPK, but does not significantly regulate ERK and AKT. AMPK is closely related to cell metabolism and is involved in metabolic stress such as neurodegeneration and oxidative stress (Carling et al., 2012). In addition, Nrf2 is a critical transcription factor that regulates the body's anti-oxidative stress. Several studies have shown that Nrf2 activation can delay cognitive dysfunction in various AD models by affecting mitochondrial function (Bhatia and Sharma, 2021; Villavicencio Tejo and Quintanilla, 2021; Saha et al., 2022). Notably, expression level of GSK3β were elevated in Nrf2 knockdown tissues and cells, while inhibition of GSK3β led to Nrf2 activation under oxidative stress in AD (Espada et al., 2009; Zou et al., 2013; Gameiro et al., 2017; Ma et al., 2023). Overall, this reveals that targeting AMPK/GSK3β/Nrf2 balance might be a promising therapeutic strategy. We found that Nor upregulated expression of p-AMPK, p-GSK3β and Nrf2 proteins induced by Aβ1-42. Besides, the expression levels of Nrf2 and its driver genes NQO1 and HO-1 decreased in brain tissues of elderly and AD patients (Patel and Woodgett, 2017), which supports increased mRNA levels of HO-1 and NQO-1 after Nor treatment. To further investigate the mechanism, we observed whether AMPK inhibitor Compound C and AMPK knockdown could reverse the neuroprotective effects of Nor. Western blotting analysis showed that Compound C could inhibit the up-regulation of P-AMPK, P-GSK3β and Nrf2 proteins induced by Nor. Meanwhile, the effects of Nor on neuronal apoptosis and mitochondrial membrane potential were reversed after treatment with Compound C and si-AMPK. Suggesting that Nor exerts neuroprotective effects through the AMPK/GSK3β/Nrf2 signaling pathway.

In recent years, natural products for the treatment of neurodegenerative diseases, especially those derived from traditional Chinese medicine, have received increasing attention. Researchers have already made certain notable breakthroughs, and our study suggests that Nor may be a novel candidate for alleviating symptoms of AD.

5. Conclusion

In conclusion, our study shows that Nor reduces neuronal cell apoptosis and improves cognitive disorder in 3 × Tg mice through AMPK/GSK3β/Nrf2 pathway. Network pharmacology show that Nor has the potential of patent medicine, which indicates that Nor is promising to develop into a new anti-AD drug.

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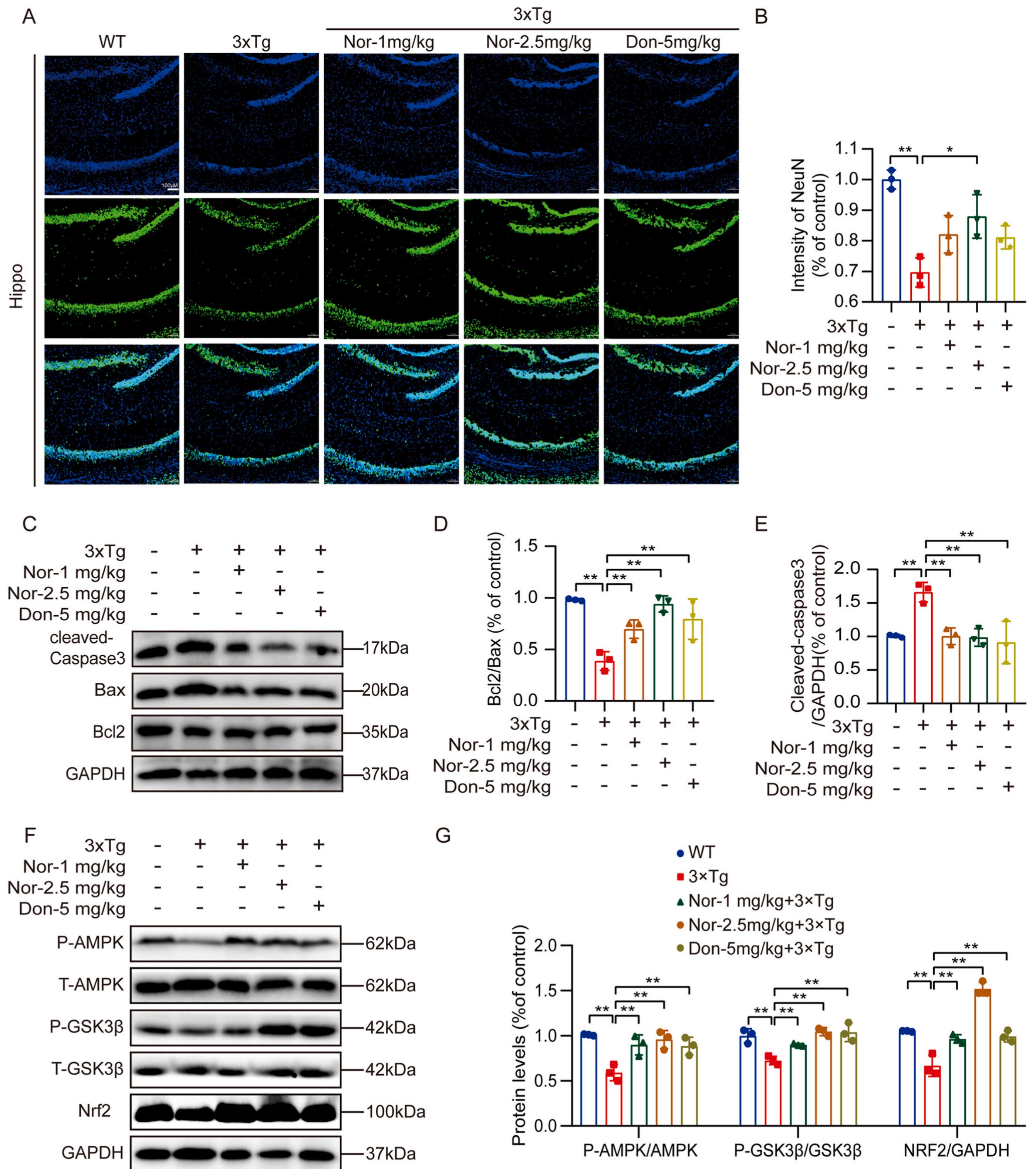

Fig. 8. Nor reduces neuronal cell apoptosis via activation of the AMPK/GSK3β/Nrf2 pathway in 3×Tg AD mice. (A) Representative images of immunofluorescence of neurons in the hippocampus. (B) Quantitative analysis of (A). (C–E) quantification of cleaved-caspase3, Bax and Bcl2 in hippocampal homogenates. (F) and (G) The expression of P-AMPK, P-GSK3β and Nrf2 were determined and quantitative analysis by Western blotting. The data are expressed as the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$.

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Ethics statement

All experiments and procedures were performed in accordance with the Hangzhou Medical College Animal Care Guidelines and approved by the Ethics Committee of the School of Pharmacy, Hangzhou Medical College (No. 2023-052).

CRediT authorship contribution statement

Yuqing Zeng: Writing – original draft, Validation. **Li Xiong:** Validation. **Hao Tang:** Validation. **Linjie Chen:** Validation. **Qin Yu:** Validation. **Liwei Li:** Validation. **Fan Chen:** Data curation. **Luyao Li:** Data curation. **Yanyan Zheng:** Data curation. **Jinfeng Sun:** Data curation. **Lingyu She:** Validation. **Wei Wang:** Methodology. **Guang Liang:** Writing – review & editing. **Xia Zhao:** Writing – review & editing.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

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Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid β-peptide
3 × Tg	$PS1_{M146V}/APP_{Swe}/tau_{P301L}$
AMPK	AMP-activated protein kinase
C57	C57BL/6
GSK3β	glycogen synthase kinase
HO-1	heme oxygenase 1
NQO1	Quinone Oxidoreductase 1
Nrf2	nuclear factor erythroid-2-related factor 2
APP	Amyloid precursor protein
ROS	Reactive oxygen species
PVDF	Poly vinylidene fluoride
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Comp.C	Compound C

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2024.118498.

References

Adlimoghaddam, A., Neuendorff, M., Roy, B., Albensi, B.C., 2018. A review of clinical treatment considerations of donepezil in severe Alzheimer's disease. CNS Neurosci. Ther. 24 (10), 876–888.

Behrouz, N., Defossez, A., Delacourte, A., Mazzuca, M., 1991. The immunohistochemical evidence of amyloid diffuse deposits as a pathological hallmark in Alzheimer's disease. J. Gerontol. 46 (6), B209–B212.

Bhatia, V., Sharma, S., 2021. Role of mitochondrial dysfunction, oxidative stress and autophagy in progression of Alzheimer's disease. J. Neurol. Sci. 421, 117253.

Bian, Y., Chen, Y., Wang, X., Cui, G., Ung, C.O.L., Lu, J.H., Cong, W., Tang, B., Lee, S.M., 2021. Oxyphylla A ameliorates cognitive deficits and alleviates neuropathology via the Akt-GSK3β and Nrf2-Keap1-HO-1 pathways in vitro and in vivo murine models of Alzheimer's disease. J. Adv. Res. 34, 1–12.

Bolin, C.M., Basha, R., Cox, D., Zawia, N.H., Maloney, B., Lahiri, D.K., Cardozo-Pelaez, F., 2006. Exposure to lead and the developmental origin of oxidative DNA damage in the aging brain. Faseb. J. 20 (6), 788–790.

Bonda, D.J., Wang, X., Perry, G., Nunomura, A., Tabaton, M., Zhu, X., Smith, M.A., 2010. Oxidative stress in Alzheimer disease: a possibility for prevention. Neuropharmacology 59 (4–5), 290–294.

Cai, Q., Tammineni, P., 2016. Alterations in Mitochondrial Quality Control in Alzheimer's Disease, p. 10.

Cai, Y., Liu, J., Wang, B., Sun, M., Yang, H., 2022. Microglia in the neuroinflammatory pathogenesis of Alzheimer's disease and related therapeutic targets. Front. Immunol. 13, 856376.

Carling, D., Thornton, C., Woods, A., Sanders, M.J., 2012. AMP-activated protein kinase: new regulation, new roles? Biochem. J. 445 (1), 11–27.

Cheignon, C., Tomas, M., Bonnefont-Rousselot, D., Faller, P., Hureau, C., Collin, F., 2018. Oxidative stress and the amyloid beta peptide in Alzheimer's disease. Redox Biol. 14, 450–464.

Choi, J.E., Park, D.-M., Chun, E., Choi, J.J., Seo, J.H., Kim, S., Son, J., Do, M., Kim, S.Y., Park, Y.-C., Jung, I.C., Jin, M., 2017. Control of stress-induced depressive disorders by So-ochim-tang-gamibang, a Korean herbal medicine. J. Ethnopharmacol. 196, 141–150.

Coskun, P.E., Beal, M.F., Wallace, D.C., 2004. Alzheimer's brains harbor somatic mtDNA control-region mutations that suppress mitochondrial transcription and replication. Proc. Natl. Acad. Sci. U.S.A. 101 (29), 10726–10731.

Davis, R.E., Miller, S., Herrnstadt, C., Ghosh, S.S., Fahy, E., Shinobu, L.A., Galasko, D., Thal, L.J., Beal, M.F., Howell, N., Parker Jr., W.D., 1997. Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. Proc. Natl. Acad. Sci. U.S.A. 94 (9), 4526–4531.

de la Monte, S.M., Luong, T., Neely, T.R., Robinson, D., Wands, J.R., 2000. Mitochondrial DNA damage as a mechanism of cell loss in Alzheimer's disease. Lab. Invest. 80 (8), 1323–1335.

Ding, M.R., Qu, Y.J., Hu, B., An, H.M., 2022. Signal pathways in the treatment of Alzheimer's disease with traditional Chinese medicine. Biomed. Pharmacother. 152, 113208.

Espada, S., Rojo, A.I., Salinas, M., Cuadrado, A., 2009. The muscarinic M1 receptor activates Nrf2 through a signaling cascade that involves protein kinase C and inhibition of GSK-3beta: connecting neurotransmission with neuroprotection. J. Neurochem. 110 (3), 1107–1119.

Feng, L.D., Tian, Y., Wang, X., Dai, R., Cai, S., Cao, Y.J., Si, Y.C., 2018. Therapy of dredging the bowels enhanced the neuroprotective effect of nourishing kidney herbs on hippocampal cholinergic system in Alzheimer's disease model rat induced by Aβ 1-42. Evid Based Complement Alternat Med 2018, 3282385.

Gameiro, I., Michalska, P., Tenti, G., Cores, Á., Buendia, I., Rojo, A.I., Georgakopoulos, N. D., Hernández-Guijo, J.M., Teresa Ramos, M., Wells, G., López, M.G., Cuadrado, A., Menéndez, J.C., León, R., 2017. Discovery of the first dual GSK3β inhibitor/Nrf2 inducer. A new multitarget therapeutic strategy for Alzheimer's disease. Sci. Rep. 7, 45701.

Gonzalez, B.L., de Oliveira, N.C., Ritter, M.R., Tonin, F.S., Melo, E.B., Sanches, A.C.C., Fernandez-Llimos, F., Petruco, M.V., de Mello, J.C.P., Chierrito, D., de Medeiros Araújo, D.C., 2022. The naturally-derived alkaloids as a potential treatment for COVID-19: a scoping review. Phytother Res. 36 (7), 2686–2709.

Han, J., Park, H., Maharana, C., Gwon, A.R., Park, J., Baek, S.H., Bae, H.G., Cho, Y., Kim, H.K., Sul, J.H., Lee, J., Kim, E., Kim, J., Cho, Y., Park, S., Palomera, L.F., Arumugam, T.V., Mattson, M.P., Jo, D.G., 2021. Alzheimer's disease-causing presenilin-1 mutations have deleterious effects on mitochondrial function. Theranostics 11 (18), 8855–8873.

Hirai, K., Aliev, G., Nunomura, A., Fujioka, H., Russell, R.L., Atwood, C.S., Johnson, A.B., Kress, Y., Vinters, H.V., Tabaton, M., Shimohama, S., Cash, A.D., Siedlak, S.L., Harris, P.L., Jones, P.K., Petersen, R.B., Perry, G., Smith, M.A., 2001. Mitochondrial abnormalities in Alzheimer's disease. J. Neurosci. 21 (9), 3017–3023.

Huang, Q., Liu, K., Qin, L., Zhu, B., 2023. Lindera aggregata (Sims) Kosterm: a systematic review of its traditional applications, phytochemical and pharmacological properties, and quality control. Medicinal Plant Biology 2 (1).

Hwang, J.Y., Park, J.H., Kim, M.J., Kim, W.J., Ha, K.T., Choi, B.T., Lee, S.Y., Shin, H.K., 2019. Isolinderalactone regulates the BCL-2/caspase-3/PARP pathway and suppresses tumor growth in a human glioblastoma multiforme xenograft mouse model. Cancer Lett. 443, 25–33.

Kim, S.H., Ju, I.G., Kim, J.H., Eo, H., Son, S.R., Jang, D.S., Oh, M.S., 2023. Linderae radix ameliorates cognitive dysfunction by inhibiting neuroinflammation and synaptic damage in Alzheimer's disease models. Mol. Neurobiol. 60 (12), 7196–7207.

Kwak, A.W., Park, J.W., Lee, S.O., Lee, J.Y., Seo, J.H., Yoon, G., Lee, M.H., Choi, J.S., Shim, J.H., 2022. Isolinderalactone sensitizes oxaliplatin-resistance colorectal cancer cells through JNK/p38 MAPK signaling pathways. Phytomedicine 105, 154383.

Lane, C.A., Hardy, J., Schott, J.M., 2018. Alzheimer's disease. Eur. J. Neurol. 25 (1), 59–70.

Li, S., Li, R., Zeng, Y., Meng, X.L., Wen, C.B., Zheng, S.C., 2019. [Chemical components and pharmacological action of Aconiti Radix]. Zhongguo Zhongyao Zazhi 44 (12), 2433–2443.

Liu, S., Xu, L., Shen, Y., Wang, L., Lai, X., Hu, H., 2024. Qingxin Kaiqiao Fang decreases Tau hyperphosphorylation in Alzheimer's disease via the PI3K/Akt/GSK3β pathway in vitro and in vivo. J. Ethnopharmacol. 318, 117031.
Luo, Y., Liu, M., Yao, X., Xia, Y., Dai, Y., Chou, G., Wang, Z., 2009. Total alkaloids from Radix Linderae prevent the production of inflammatory mediators in lipopolysaccharide-stimulated RAW 264.7 cells by suppressing NF-κB and MAPKs activation. Cytokine 46 (1), 104–110.
Lv, H., Liu, Q., Wen, Z., Feng, H., Deng, X., Ci, X., 2017. Xanthohumol ameliorates lipopolysaccharide (LPS)-induced acute lung injury via induction of AMPK/GSK3β-Nrf2 signal axis. Redox Biol. 12, 311–324.
Ma, D.Y., Liu, J.X., Wang, L.D., Zhi, X.Y., Luo, L., Zhao, J.Y., Qin, Y., 2023. GSK-3β-dependent Nrf2 antioxidant response modulates ferroptosis of lens epithelial cells in age-related cataract. Free Radic. Biol. Med. 204, 161–176.
Mecca, A.P., van Dyck, C.H., 2021. Alzheimer's & dementia: the journal of the Alzheimer's association. Alzheimers Dement 17 (2), 316–317.
Patel, P., Woodgett, J.R., 2017. Glycogen synthase kinase 3: a kinase for all pathways? Curr. Top. Dev. Biol. 123, 277–302.
Rai, S.N., Dilnashin, H., Birla, H., Singh, S.S., Zahra, W., Rathore, A.S., Singh, B.K., Singh, S.P., 2019. The role of PI3K/akt and ERK in neurodegenerative disorders. Neurotox. Res. 35 (3), 775–795.
Ratan, R.R., Baraban, J.M., 1995. Apoptotic death in an in vitro model of neuronal oxidative stress. Clin. Exp. Pharmacol. Physiol. 22 (4), 309–310.
Saha, S., Buttari, B., Profumo, E., Tucci, P., Saso, L., 2022. A Perspective on Nrf2 Signaling Pathway for Neuroinflammation: A Potential Therapeutic Target in Alzheimer's and Parkinson's Diseases, p. 15.
Scheltens, P., De Strooper, B., Kivipelto, M., Holstege, H., Chételat, G., Teunissen, C.E., Cummings, J., van der Flier, W.M., 2021. Alzheimer's disease. Lancet 397 (10284), 1577–1590.
Sharma, V.K., Singh, T.G., Singh, S., Garg, N., Dhiman, S., 2021. Apoptotic pathways and Alzheimer's disease: probing therapeutic potential. Neurochem. Res. 46 (12), 3103–3122.
Shen, X., Chen, H., Zhang, H., Luo, L., Wen, T., Liu, L., Hu, Q., Wang, L., 2023. A natural sesquiterpene lactone isolinderalactone attenuates lipopolysaccharide-induced inflammatory response and acute lung injury through inhibition of NF-κB pathway and activation Nrf2 pathway in macrophages. Int. Immunopharm. 124 (Pt B), 110965.
Shin, R.W., Ogomori, K., Kitamoto, T., Tateishi, J., 1989. Increased tau accumulation in senile plaques as a hallmark in Alzheimer's disease. Am. J. Pathol. 134 (6), 1365–1371.
Villavicencio Tejo, F., Quintanilla, R.A., 2021. Contribution of the Nrf2 pathway on oxidative damage and mitochondrial failure in Parkinson and Alzheimer's disease. Antioxidants 10 (7).
Wagner, H., Püls, S., Barghouti, T., Staudinger, A., Melchart, D., 2017. Radix linderae – wuyao. Chromatographic Fingerprint Analysis of Herbal Medicines Volume V: Thin-Layer and High Performance Liquid Chromatography of Chinese Drugs. Springer International Publishing, Cham, pp. 95–102. H. Wagner, S. Püls, T. Barghouti, A. Staudinger and D. Melchart.
Wang, C., Dai, Y., Yang, J., Chou, G., Wang, C., Wang, Z., 2007. Treatment with total alkaloids from Radix Linderae reduces inflammation and joint destruction in type II collagen-induced model for rheumatoid arthritis. J. Ethnopharmacol. 111 (2), 322–328.
Wang, W., Zhao, F., Ma, X., Perry, G., Zhu, X., 2020. Mitochondria dysfunction in the pathogenesis of Alzheimer's disease: recent advances. Mol. Neurodegener. 15 (1), 30.
Zhang, J., Wu, N., Wang, S., Yao, Z., Xiao, F., Lu, J., Chen, B., 2021. Neuronal loss and microgliosis are restricted to the core of Aβ deposits in mouse models of Alzheimer's disease. Aging Cell 20 (6), e13380.
Zhao, Z., Yan, J., Huang, L., Yang, X., 2024. Phytochemicals targeting Alzheimer's disease via the AMP-activated protein kinase pathway, effects, and mechanisms of action. Biomed. Pharmacother. 173, 116373.
Zou, Y., Hong, B., Fan, L., Zhou, L., Liu, Y., Wu, Q., Zhang, X., Dong, M., 2013. Protective effect of puerarin against beta-amyloid-induced oxidative stress in neuronal cultures from rat hippocampus: involvement of the GSK-3β/Nrf2 signaling pathway. Free Radic. Res. 47 (1), 55–63.