Am J Transl Res 2022;14(8):5343-5362
www.ajtr.org /ISSN:1943-8141/AJTR0143076

Original Article

Wnt5a protects motor neurons in amyotrophic lateral sclerosis by regulating the Wnt/Ca^{2+} signaling pathway

Jinmeng Liu[1*], Fenghua Zhou[2*], Yanchun Chen[3,4*], Yingjun Guan[3], Fandi Meng[3], Zhenhan Zhao[3], Xuemei Wang[3], Xueshuai Gao[3], Xin Jiang[3], Haoyun Zhang[4], Qing Wang[4], Shuanhu Zhou[5], Xin Wang[6]

[1]Laboratory of Biochemistry and Molecular Biology, Weifang Medical University, Weifang 261053, Shandong, PR China; [2]Department of Pathology, Weifang Medical University, Weifang 261053, Shandong, PR China; [3]Department of Histology and Embryology, Weifang Medical University, Weifang 261053, Shandong, PR China; [4]Neurologic Disorders and Regenerative Repair Laboratory, Weifang Medical University, Weifang 261053, Shandong, PR China; [5]Department of Orthopaedic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston 02115, MA, USA; [6]Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston 02115, MA, USA; [*]Equal contributors and co-first authors.

Received March 23, 2022; Accepted July 14, 2022; Epub August 15, 2022; Published August 30, 2022

Abstract: Objectives: We aimed to detect the expression profile of downstream signaling molecules of non-canonical Wnt pathway in $SOD1^{G93A}$ transgenic mice (ALS mice) and $SOD1^{G93A}$ mutant motor neuron-like hybrid (NSC-34) cells. Characterizing the molecular mechanism of the Wnt5a-mediated non-canonical Wnt/Ca^{2+} signaling pathway in motor neuron (MN) degeneration may provide a feasible approach to effective treatment of amyotrophic lateral sclerosis (ALS). Methods: The expressions of CaMKII-α, CaMKII-β and TAK1 in the spinal cord of $SOD1^{G93A}$ ALS transgenic mice at different ages were determined using western blotting and immunofluorescence. The level of Ca^{2+} and cell apoptosis were assessed with flow cytometry and cell viability was evaluated using MTS assay. Cell proliferation was analyzed by the EdU cell proliferation assay. Neurite length was measured after treatment with retinoic acid. Results: CaMKII-α, CaMKII-β, and TAK1 were down-regulated in the spinal cord of ALS mice. Ca^{2+} level and CaMKII-α, CaMKII-β, and TAK1 were down-regulated in $SOD1^{G93A}$ mutant NSC-34 cells. Expression of Ca^{2+}, CaMKII-α, CaMKII-β, and TAK1 were up-regulated in $SOD1^{G93A}$ mutant NSC-34 cells after Wnt5a overexpression and down-regulated after Wnt5a knockdown. Overexpression of Wnt5a promoted cell viability and proliferation but inhibited cell apoptosis. Contrastingly, Wnt5a knockdown inhibited cell viability and proliferation but promoted cell apoptosis. CaMKII inhibitor KN-93 and CaMKII activator oleic acid reversed changes in cell viability, proliferation, apoptosis, and neurite outgrowth induced by Wnt5a overexpression and knockdown. Conclusions: This study demonstrates that Wnt5a protects MNs in ALS by regulating cell viability, proliferation, apoptosis, and neurite growth through the Wnt/Ca^{2+} signaling pathway. Our data indicate that the non-canonical Wnt/Ca^{2+} signaling pathway regulated by Wnt5a is involved in MN degeneration in ALS.

Keywords: ALS, Wnt5a, Wnt/Ca^{2+} signaling, proliferation, apoptosis, neurite growth

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disease of motor neurons (MNs). This fatal neurodegenerative disease is characterized by decreased survival of MNs in the brain and spinal cord. Its main clinical manifestations are progressive muscle weakness and atrophy in the absence of effective treatment. When respiratory muscles are involved, most patients succumb to respiratory failure within three to five years after disease onset [1, 2]. At present, the exact mechanism of MN degeneration in ALS is unclear. However, some pathogenic mechanisms have been proposed, including disturbances in RNA metabolism, impaired protein homeostasis, nucleocytoplasmic transport defects, impaired DNA repair, excitotoxicity, mitochondrial dysfunction, oxidative stress, axonal transport disruption, neuroinflammation, oligodendrocyte dysfunction, and vesicular transport defects [3, 4]. Unfortunately, an effective treatment for ALS is presently lacking.

ALS cases can be divided into sporadic ALS (sALS) and familial ALS (fALS). The former clinical classification, which occurs randomly without a family history, accounts for 90% of ALS cases, whereas the latter accounts for about 10% of ALS cases [5]. To date, more than 25 gene variants have been profiled in ALS. The first ALS-related gene encoding copper/zinc superoxide dismutase 1 (SOD1) was identified in 1993 [6]. Research has shown that approximately 20% of fALS cases are caused by missense mutations in the *SOD1* gene [7]. Evidence has shown that SOD1 mutant transgenic mice develop muscle weakness and neuronal degeneration similar to that of ALS patients. Thus, it can be used as an ideal model to study the pathogenesis of ALS [8-11]. In recent years, mouse models expressing *SOD1* have been used to test ALS treatment in vivo. Results of such models have shown that virus-mediated gene therapy improves symptoms and motor function of *SOD1* mutants in ALS transgenic mice and increases the survival rate of MNs [12-14]. More research is needed to discover targeted therapies for ALS.

Wnt signaling is a conserved pathway in animals that regulates the occurrence and development of various neurodegenerative diseases, including the pathogenic mechanisms of ALS. Presently, more than 19 genes associated with Wnt signaling have been profiled in both mice and humans. Wnt genes encode Wnt proteins that activate the Wnt signaling pathway. The extracellular Wnt signal stimulates several intracellular signal transduction cascades, including the β-catenin-dependent pathway (Wnt/β-catenin pathway) for canonical and non-canonical β-catenin-independent pathway. The β-catenin-independent pathway comprises Wnt/Planar Cell Polarity (Wnt/PCP) and Wnt/Ca^{2+} pathways [15-17]. Understanding the Wnt pathway and the associated genes is a promising way to discover therapeutic targets to counteract harmful effects of neurodegenerative diseases [18]. Wnt ligands are well known to activate the canonical β-catenin pathway, which regulates acetylcholine receptor (AChR) aggregation as well as the formation and maintenance of neuromuscular junctions (NMJ). Previous findings suggested that β-catenin distribution could be an underlying factor affecting the onset of neurodegeneration in fALS [19]. Therefore, it may be an ideal target for novel ALS treatment targeting the Wnt/β-catenin pathway to maintain the stability of the NMJ [18]. At present, the involvement of non-canonical Wnt signaling pathways in neuronal survival and death processes is uncertain [20].

Wnt5a is an important non-canonical Wnt signaling pathway ligand. It uses Ca^{2+} as a second messenger to achieve its biological effect [21]. Wnt5a binds to the homologous frizzled (Fzd) receptor, which leads to an increase of intracellular Ca^{2+} concentration. Once released, Ca^{2+} activates calcium/calmodulin-dependent kinase II (CamKII) and then activates TGFβ activated kinase (TAK1) to regulate many biological processes such as cytoskeleton rearrangement, cell adhesion, and migration [16, 22, 23]. Two CaMKII subtypes occur in neurons (α and β subtypes), which regulate long-term potentiation, axon growth, and neurotransmitter release [24, 25]. Some research suggests that Wnt5a-activated Ca^{2+} signaling plays an important role in neurite outgrowth of various neuronal cell types [26]. It can also protect neurons in Alzheimer's disease (AD) through the Wnt/Ca^{2+} signaling pathway [27]. However, for ALS, there is no reliable evidence indicating whether Wnt5a can affect the function of MNs through the Wnt/Ca^{2+} signaling pathway.

Here, we detected the expression of CaMKII-α, CaMKII-β, and TAK1 in ALS mice and $SOD1^{G93A}$ mutant NSC-34 cells and explored the role of Wnt5a in degeneration of MNs. This study further gives insight into the molecular mechanism of ALS, providing a basis for development of new strategies to treat ALS.

Materials and methods

Animals and tissue preparation

Amyotrophic lateral sclerosis transgenic mice (ALS mice) with $SOD1^{G93A}$ gene mutation and wild-type mice (WT mice) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Male ALS mice aged 6-8 weeks were bred with adult female WT mice. At four weeks, 0.5 cm from the tail of young mice was collected and the genomic DNA isolated from the tail tissue was amplified using polymerase chain reaction (PCR) for genotyping. Adult mice were divided into ALS groups and WT groups. Mice in each

group were killed at the early stage (95 days), middle stage (108 days) and late stage (122 days). Each group of ALS mice was matched with a WT littermate of the same age as the control group. The spinal cord tissues were directly isolated from the same mice and stored at -80°C until protein and RNA extraction. Other mice were perfused with 4% paraformaldehyde, and their spinal cords were stripped out and prepared as frozen sections for immunofluorescence staining. All experiments were repeated four times. The study was conducted following the guidelines of the Declaration of Helsinki, and was approved by the Medical Ethics Committee of Weifang Medical University (protocol code 2018-No. 156).

Cell lines and cell treatment

NSC-34, a mouse embryonic spinal cord motor neuron × N18TG2 neuroblastoma hybrid cell line, was used in this study. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 2 mol/L of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in 5% CO_2 condition and incubated at 37°C. When the cells reached 90% confluence, cells were dissociated into a single cell suspension and seeded into 6-well plates. Upon reaching 60%-70% confluence, the cells were transfected with Lipofectamine 2000 (Invitrogen Life Technologies, USA). Plasmids pcDNA3.1-WT-SOD1 and pcDNA3.1-G93A-SOD1 were kindly gifted by Professor Angelo Poletti (University of Milan, Italy) [28]. Plasmids pcDNA3.1-Wnt5a and pcDNA3.1-Control were purchased from Genechem (Shanghai, China). The small interfering RNAs (siRNA) against Wnt5a (GGACAATACTTCTGTCTTT) and siRNA-control were purchased from RiboBio (Guangzhou, China). NSC-34 cells were transfected with pcDNA3.1-WT-SOD1 and pcDNA3.1-G93A-SOD1 plasmids at 2 μg/mL. pcDNA3.1-G93A-SOD1 plasmids and Wnt5a overexpressed plasmids or siRNA-Wnt5a were co-transfected into NSC-34 cells. After 48 h, total RNA and protein were extracted from the cells. CaMKII inhibitor KN-93 was purchased from MCE (USA), whereas CaMKII activator oleic acid (OA) was purchased from Santa Cruz (USA). Transfected NSC-34 cells were treated with KN-93 (5 μM) and OA (10 μM) for 24 h. All the procedures were performed in triplicate.

Western blotting

Western blotting was performed as previously described [29]. Protein concentration in the protein lysate collected was measured using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). The antibodies used for western blotting included rabbit polyclonal anti-CaMKII-α (1:1000, Abcam, Cambridge, MA, USA), rabbit polyclonal anti-CaMKII-β (1:1000, Proteintech, Wuhan, China), rabbit polyclonal anti-TAK1 (1:700, Proteintech, Wuhan, China), goat polyclonal anti-Wnt5a (1:500, R&D, Minnesota, USA), and mouse monoclonal anti-GAPDH (1:1500, Proteintech). ECL (Thermo Scientific, Waltham, MA, USA) was added to enhance luminescence reaction and the protein bands were detected using a chemiluminescence instrument. The optical density (OD) of protein bands was normalized to those of the internal control GAPDH to analyze western blotting.

Immunofluorescence

Immunofluorescence staining was performed as previously described [6]. The following primary antibodies were applied: rabbit anti-CaMKII-α, rabbit anti-CaMKII-β, rabbit anti-TAK1 and mouse anti-β-tubulin III (1:200, R&D Systems, MN, USA). The secondary antibodies used included Alexa Fluor 488-conjugated anti-mouse IgG (1:400, Jackson ImmunoResearch), and Cy3 conjugated anti-rabbit IgG (1:400, Jackson ImmunoResearch). PBS rather than the first antibody was used in the control group. No specific staining was used to stain cells. All images were observed and captured using a fluorescence microscope (Olympus, Tokyo, Japan). The number of double-positive cells was determined using ImageJ software.

Intracellular concentration of Ca^{2+} assay

Intracellular Ca^{2+} level was measured with Fluo-3 AM (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's instructions. Specifically, transfected cells were collected into a tube and incubated with 4 μM Fluo-3 AM for 30 min in D-Hanks at 37°C. The contents were then washed three times with D-Hanks and incubated for an additional 20 min without Fluo-3 AM to complete the de-esterification process of the dye. Finally, stained cells were washed with ice-cold D-Hanks

three times and then detected using flow cytometry.

MTS viability assay

Cell viability was measured using Cell Titer 96®AQueous One Solution Cell Proliferation Assay. Briefly, 1×10^4 transfected cells were dispensed into each well of a 96-well plate. The cells were cultured in a CO_2 incubator after transfection. MTS reagent and 10% FBS medium were added into each well in the ratio of 1:4 and the plate was incubated in a CO_2 incubator for 40 min. Cell viability was detected at 0, 24, 48, 72 and 96 h after transfection. Absorbance (OD) was measured at 490 nm. Finally, the cell proliferation curve was drawn at various time points.

EdU cell proliferation assay

Cell proliferation was assessed using 5-Ethynyl-2-deoxyuridine (EdU) DNA proliferation assay at 48 h after transfection. The number of cells in the S phase was assessed following the manual of Cell-Light TMEdU Apollo® 567 In Vitro Kit (RiboBio, Guangzhou, China). Briefly, 1×10^5 cells were cultured in each well of 96-well plates. Transfected cells were labeled with 0.1% reagent A. Then, 4% paraformaldehyde was added and incubated for 15 min at room temperature to fix the cells for microscopy. 1× Apollo staining reaction solution was added to each well before incubation in the dark for 30 min. After washing with PBS three times, DNA staining was performed with Hoechst 33342 reaction solution for 15 min. Cell images were observed under a fluorescence microscope (Olympus, Tokyo, Japan). The percentage of EdU-positive cells was calculated from five random fields.

Cell apoptosis assay

Apoptosis of NSC-34 cells was determined using the FITC Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, cells were plated in 6-well culture plates at a density of 1×10^6/well and then cultured for 48 h. After incubation, cells were collected into 1.5 ml EP tubes, rinsed with PBS, and re-suspended in 1× Annexin V binding buffer. Then, cells were stained with 5 μL annexin V-FITC and 5 μL PI in darkness for 20 min at room temperature. After staining, apoptotic cells in each treatment (1×10^5 cells) were analyzed using flow cytometry. All experiments were performed in triplicate.

Measurement of neurite outgrowth

The NSC-34 cells were cultured on coverslips coated with poly-L-lysine, followed by treatment with 10 μmol/L retinoic acid (RA, Sigma-Aldrich, MO, USA) for 48 h after transfection to induce the neurite outgrowth. Then, cells were photographed using the microscope (Leica Microsystems CMS GmbH, Wezlar, Germany). Neurite length was defined as the distance from the soma to the tip of the longest branch [30]. Five random fields were used for image analysis in each group. The data on neurite length were obtained in three independent experiments.

Statistical analysis

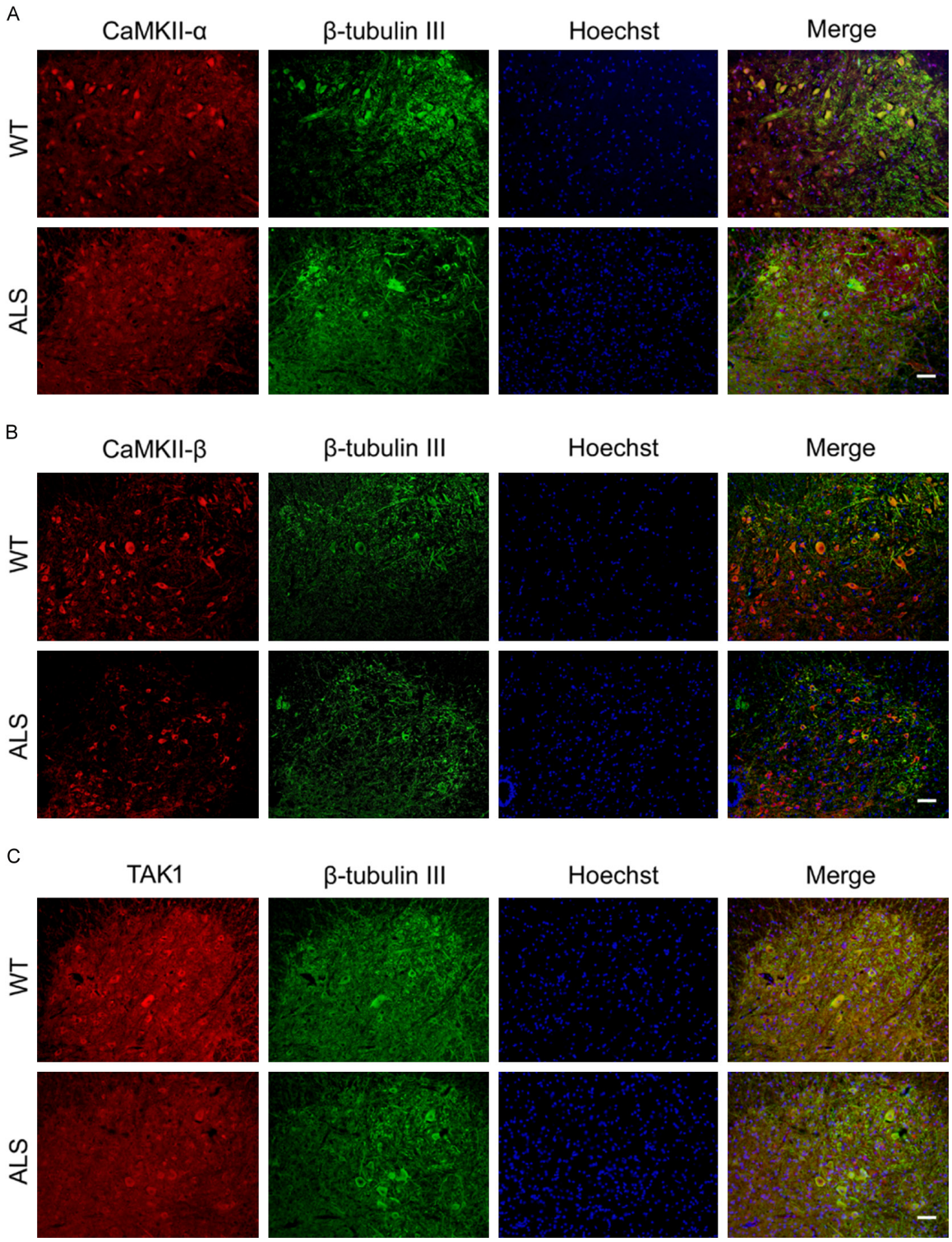
All data were statistically analyzed using SPSS software 22.0 and expressed as mean ± standard deviation (SD). Differences between groups were analyzed using two-tailed Student's t-test and were significant at $P < 0.05$. All graphs were generated using GraphPad Prism 7.0 software.

Results

CaMKII-α, CaMKII-β and TAK1 are down-regulated in the spinal cord of ALS mice

CaMKII and TAK1 are key regulatory molecules of the Wnt/Ca^{2+} signaling pathway. To determine the distribution and localization of CaMKII-α, CaMKII-β, and TAK1 in ALS, their expression levels were detected using double immunofluorescence staining. Our results showed that CaMKII-α, CaMKII-β, and TAK1 were expressed in the anterior horn of gray matter of spinal cord and co-expressed with neurons labeled with β-tubulin III. In addition, the number of CaMKII-α/β-tubulin III, CaMKII-β/β-tubulin III, and TAK1/β-tubulin III double-positive cells in ALS middle stage was 49.45%, 58.87% and 55.41% lower respectively compared to that in the ventral horn of the gray matter of WT mice (**Figure 1A-D**, $P < 0.01$, $P < 0.001$).

To investigate the expression of CaMKII-α, CaMKII-β, and TAK1 in ALS, western blotting technique was used to quantify the expression levels of CaMKII-α, CaMKII-β, and TAK1 in the


A
CaMKII-α
β-tubulin III
Hoechst
Merge
WT
ALS
B
CaMKII-β
β-tubulin III
Hoechst
Merge
WT
ALS
C
TAK1
β-tubulin III
Hoechst
Merge
WT
ALS

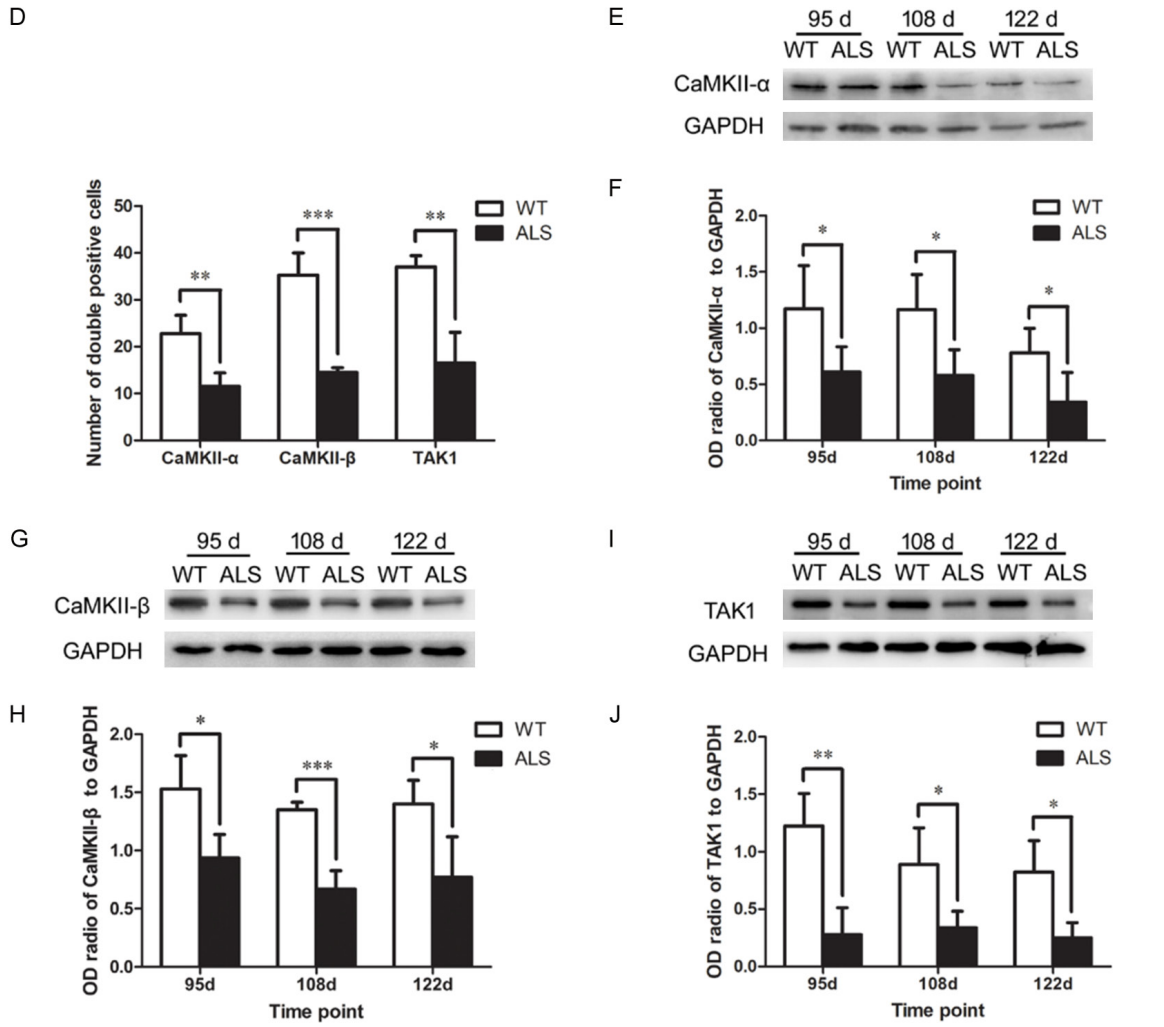


Figure 1. Expression of CaMKII-α, CaMKII-β and TAK1 in the spinal cord of ALS mice and WT mice. A-D. Co-localization of CaMKII-α, CaMKII-β and TAK1 as detected by immunofluorescence double staining in the anterior horn of the spinal cord gray matter of 108-day-old mice. Scale bar = 50 μm. E, G, I. Protein bands of CaMKII-α, CaMKII-β and TAK1 in the spinal cord. F, H, J. Bar chart showing CaMKII-α, CaMKII-β and TAK1 protein level as determined by western blotting assay. GAPDH was used as the internal control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs. WT.

spinal cord of ALS mice at different stages. The expression of CaMKII-α, CaMKII-β, and TAK1 proteins in ALS mice was 48.01%, 38.83%, and 77.30% lower at 95 d, 50.30%, 50.45%, and 61.99% lower at 108 d, 56.04%, 44.94%, and 69.63% lower at 122 d, compared to WT mice. The differences were statistically significant (**Figure 1E-J**, $P < 0.05$, $P < 0.01$, $P < 0.001$). These results show that ALS affected the expression of the 3 proteins.

The level of Ca^{2+} is decreased and CaMKII-α, CaMKII-β, and TAK1 are down-regulated in $SOD1^{G93A}$ mutant NSC-34 cells

To further verify the effect of *SOD1* gene mutation on key signal molecules in the Wnt/Ca^{2+} signaling pathway *in vitro*, a NSC-34 cell model was constructed by transfecting pcDNA3.1-G93A-SOD1 and pcDNA3.1-WT-SOD1 plasmids. The expression levels of CaMKII-α, CaMKII-β, and TAK1 as well as intracellular Ca^{2+} levels were detected at 48 h after transfection. Flow cytometry results demonstrated that the percent of Ca^{2+} was 16.02% lower in NSC-34 cells transfected with the pcDNA3.1-G93A-SOD1 plasmid than in cells transfected with the pcDNA3.1-WT-SOD1 plasmid (**Figure 2A**, **2B**, $P < 0.05$). Western blotting analysis showed that the expression of CaMKII-α, CaMKII-β and TAK1 proteins was 62.77%, 74.54% and 46.43% lower in NSC-34 cells carrying the pcDNA3.1-G93A-SOD1 plasmid than that of cells carrying the pcDNA3.1-WT-SOD1 plasmid

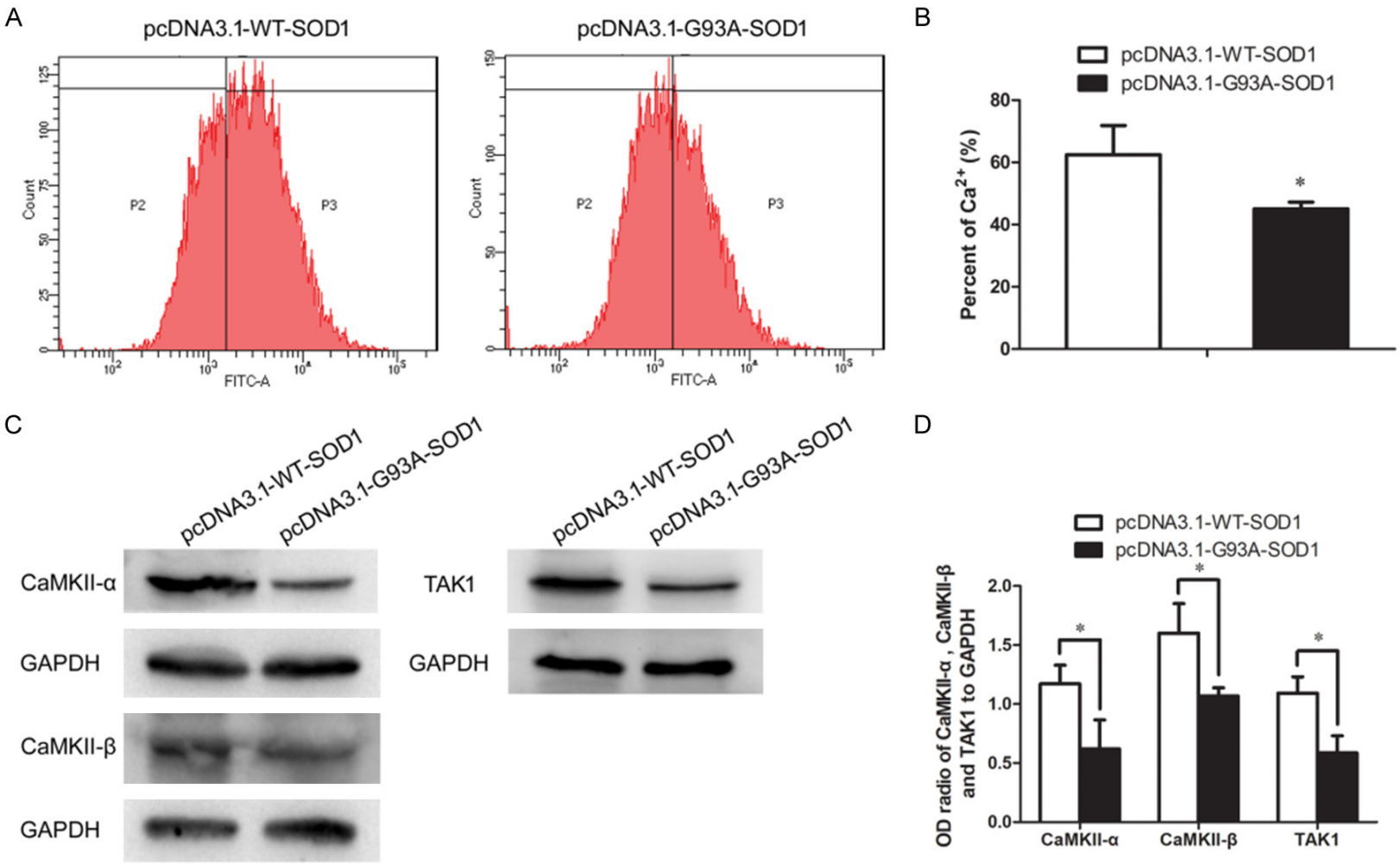


Figure 2. Changes in Ca^{2+}, CaMKII-α, CaMKII-β and TAK1 expression level in NSC-34 cells carrying SOD1-G93A mutation. A. Two-matrix chart showing the intracellular Ca^{2+} level in NSC-34 cells transfected with pcDNA3.1-WT-SOD1 and pcDNA3.1-G93A-SOD1 plasmid for 48 h. B. Bar chart showing the percentage of Ca^{2+} in NSC-34 cells transfected with pcDNA3.1-WT-SOD1 and pcDNA3.1-G93A-SOD1 plasmid for 48 h. C. Protein bands of CaMKII-α, CaMKII-β, and TAK1 in NSC-34 cells transfected with pcDNA3.1-WT-SOD1 and pcDNA3.1-G93A-SOD1 plasmids for 48 h. D. Bar chart showing CaMKII-α, CaMKII-β, and TAK1 protein level as analyzed by western blotting. GAPDH was used as the internal control. *$P < 0.05$, vs. pcDNA3.1-WT-SOD1.

(**Figure 2C**, **2D**, $P < 0.05$). These results indicated that changes in CaMKII-α, CaMKII-β, and TAK1 expression were consistent with those observed in animal models.

Modulation of Wnt5a can regulate Ca^{2+}, CaMKII-α, CaMKII-β, and TAK1 levels in $SOD1^{G93A}$ mutant NSC-34 cells

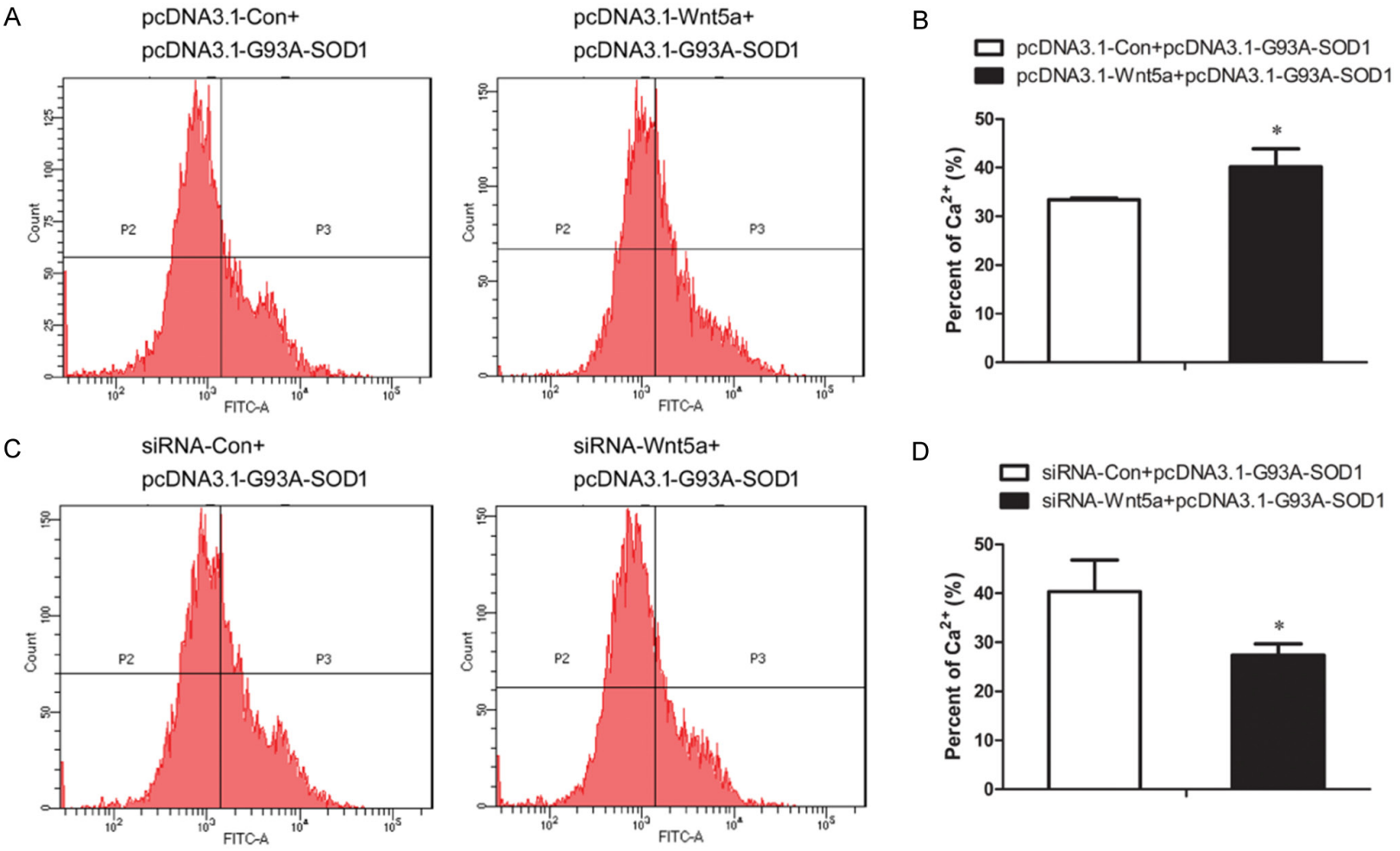
A previous study found that the expression of Wnt5a is abnormal in ALS [31]. To study the regulation of Wnt5a on Wnt/Ca^{2+} signaling molecules in ALS, the expression levels of CaMKII-α, CaMKII-β, and TAK1 as well as intracellular Ca^{2+} levels in $SOD1^{G93A}$ mutant NSC-34 cells were detected at 48 h after overexpressing and knocking down Wnt5a. A correct intracellular Ca^{2+} level is crucial for MNs function [32]. Flow cytometry results showed that Ca^{2+} in $SOD1^{G93A}$ mutant NSC-34 cells increased by 6.68% after overexpressing Wnt5a and decreased by 12.97% after knocking down Wnt5a (**Figure 3A-D**, $P < 0.05$). Western blotting analysis showed that the expression levels of CaMKII-α, CaMKII-β, and TAK1 proteins in $SOD1^{G93A}$ mutant NSC-34 cells increased 2.48-fold, 1.19-fold, and 1.04-fold, respectively, after Wnt5a overexpression, but decreased by 73.3%, 75.66%, and 45.70% after knocking down Wnt5a, respectively (**Figure 3E-H**, $P < 0.05$, $P < 0.01$, $P < 0.001$). These results suggest that the level of intracellular Ca^{2+} and the expression levels of CaMKII-α, CaMKII-β, and TAK1 are regulated by Wnt5a.

Modulation of Wnt5a affects cell viability, proliferation, and apoptosis by Wnt/Ca^{2+} signaling pathway

To evaluate the regulation of Wnt/Ca^{2+} signaling cascade by Wnt5a in ALS, we analyzed the expression levels of CaMKII-α, CaMKII-β, and TAK1 in the $SOD1^{G93A}$ mutant NSC-34. This was achieved after intervening Wnt5a with CaMKII inhibitor KN-93 (5 μM) and CaMKII activator oleic acid (OA) (10 μM) for 24 h using western blotting. The results showed that KN-93 decreased CaMKII-α, CaMKII-β, and TAK1 levels in $SOD1^{G93A}$ mutant NSC-34 cells by 34.74%, 30.59%, and 23.87%, respectively, compared to untreated KN-93 cells. In addition, OA induced a 51.16%, 37.26%, and 40.04% increase in CaMKII-α, CaMKII-β, and TAK1 level, respectively, in $SOD1^{G93A}$ mutant NSC-34 cells compared to cells untreated with OA (**Figure 4A-H**, $P < 0.05$, $P < 0.01$). These results demonstrated that KN-93 inhibited the expression of CaMKII-α, CaMKII-β, and TAK1 by overexpressing Wnt5a in $SOD1^{G93A}$ mutant NSC-34 cells. OA restored the expression of CaMKII-α, CaMKII-β, and TAK1 that were lost by knocking down Wnt5a in $SOD1^{G93A}$ mutant NSC-34 cells. This also suggests that Wnt5a signals may play a role in $SOD1^{G93A}$ mutant NSC-34 cells through the Wnt/Ca^{2+} pathway.

To further investigate the role of Wnt5a-regulated Wnt/Ca^{2+} signaling pathway in ALS, we treated $SOD1^{G93A}$ mutant NSC-34 cells after modulating Wnt5a expression with KN-93 and OA. We then carried out MTS assays to examine their effect on cell function. MTS results showed that cell viability in the pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1 group increased by 31.90%, 20.38%, 18.60% and 27.32% compared to that in the pcDNA3.1-Con+pcDNA3.1-G93A-SOD1 group at 24, 48, 72, and 96 h after transfection, respectively. On the other hand, cell viability in the pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1+KN-93 group significantly decreased by 37.3%, 29.86%, 30.14%, and 31.42% compared to the pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1 group at 24, 48, 72, and 96 h after transfection, respectively (**Figure 4I**, $P < 0.05$, $P < 0.01$, $P < 0.001$). Cell viability in the siRNA-Wnt5a+pcDNA3.1-G93A-SOD1 group decreased by 23.53%, 21.02%, and 27.69% compared to the siRNA-Con+pcDNA3.1-G93A-SOD1 group, respectively. In contrast, cell viability in the siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA group significantly increased by 13.40%, 15.02%, and 21.30% compared to the siRNA-Wnt5a+pcDNA3.1-G93A-SOD1 group at 48, 72, and 96 h after transfection, respectively (**Figure 4J**, $P < 0.05$, $P < 0.01$). These findings suggest that KN-93 inhibited cell growth caused by over-expressing Wnt5a whereas OA reversed low cell growth induced by knocking down Wnt5a.

EdU results showed that cell proliferation rate was increased by 13.37% in the pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1 group in comparison to the pcDNA3.1-Con+pcDNA3.1-G93A-SOD1 group and decreased by 21.02% with KN-93 treatment after transfection at 48 h (**Figure 5A**, **5C**, $P < 0.05$, $P < 0.01$). The proliferation rate decreased by 12.64% in the siRNA-Wnt5a+pcDNA3.1-G93A-SOD1 group in comparison to the siRNA-Con+pcDNA3.1-G93A-SOD1 group and increased by 8.57% with OA treatment after transfection at 48 h (**Figure 5B**, **5D**, $P < 0.05$). MNs in the spinal cord are


A
pcDNA3.1-Con+
pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+
pcDNA3.1-G93A-SOD1
Count
FITC-A
P2
P3
B
pcDNA3.1-Con+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1
Percent of Ca2+ (%)
C
siRNA-Con+
pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+
pcDNA3.1-G93A-SOD1
D
siRNA-Con+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1

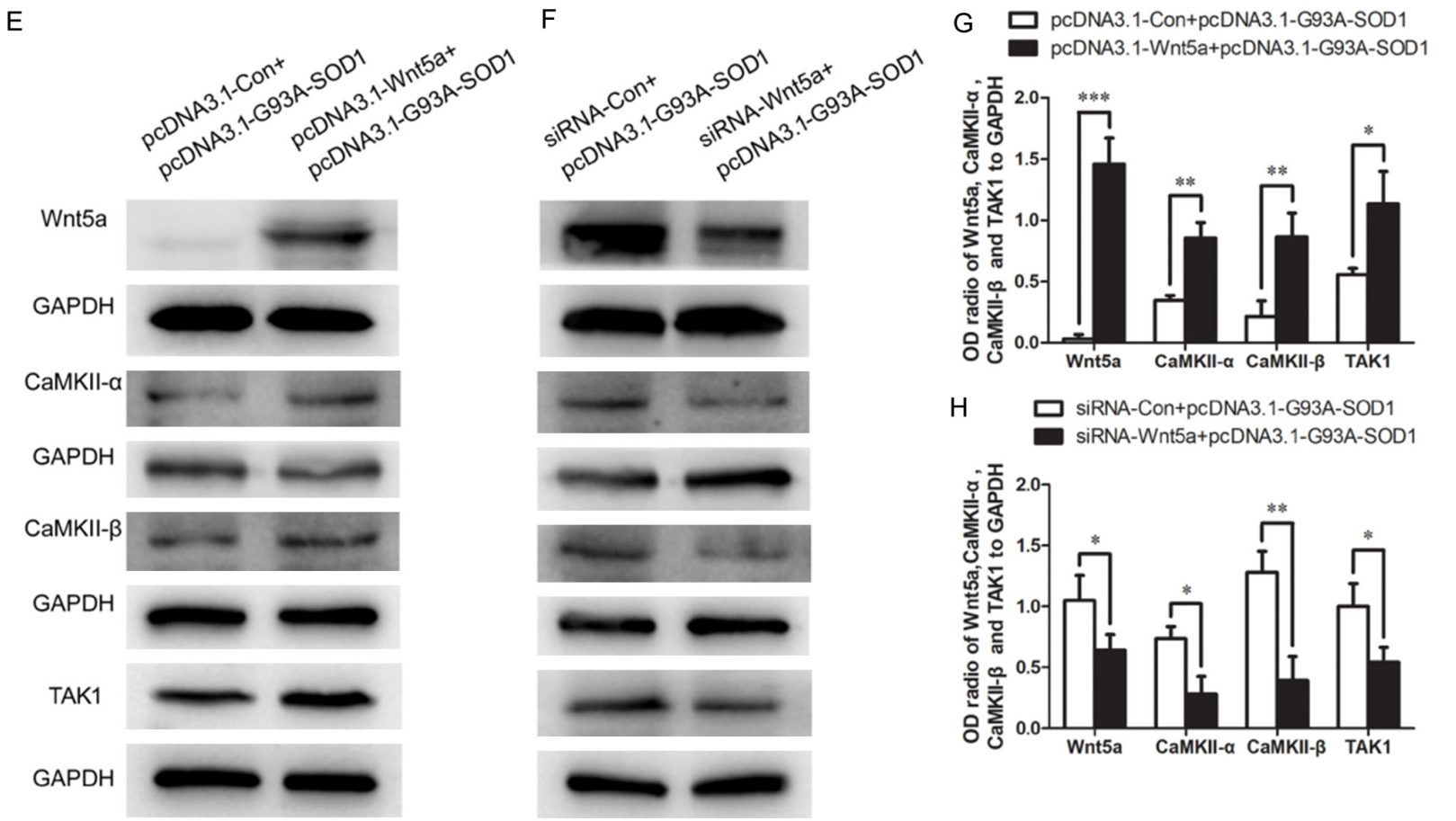
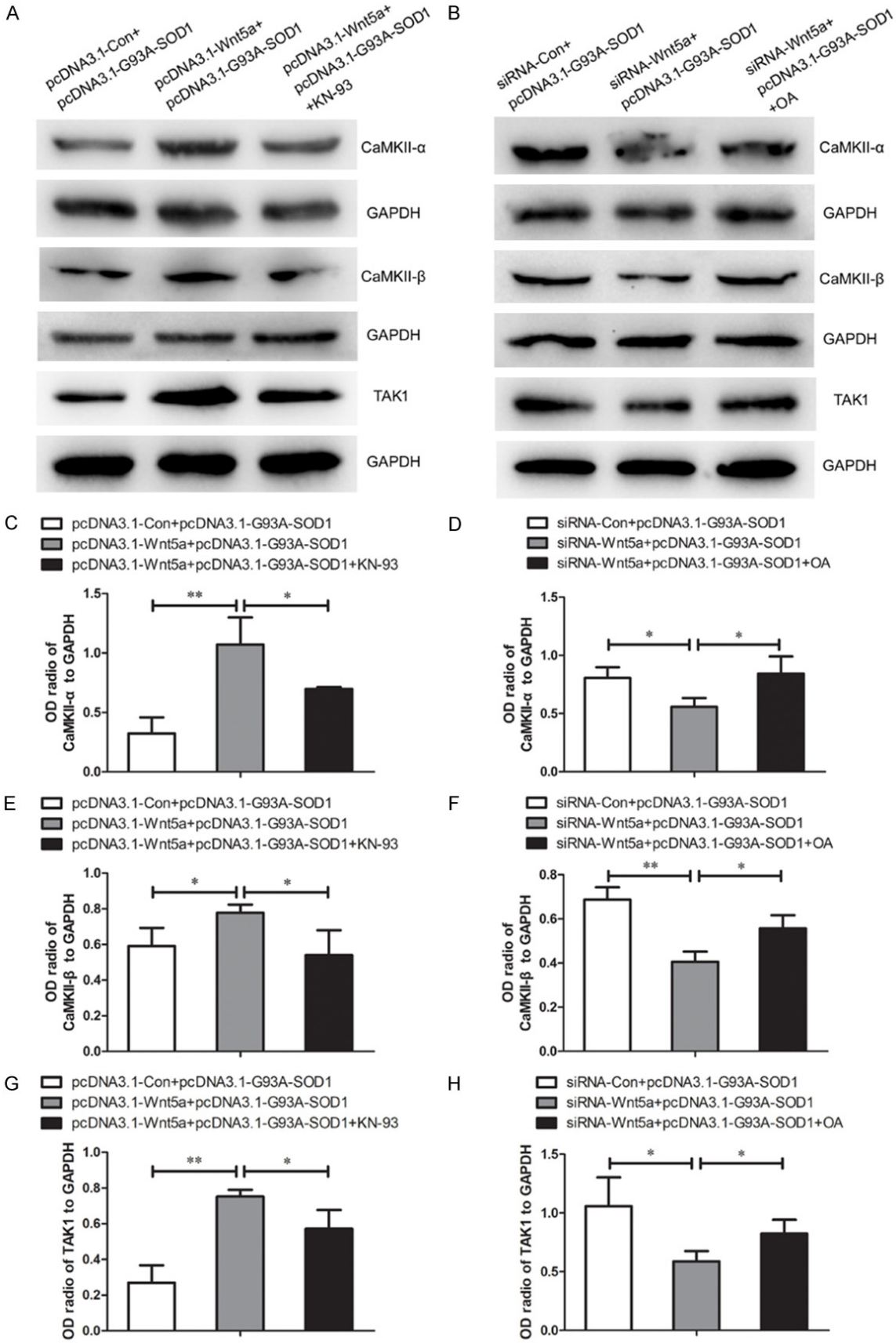


Figure 3. Overexpression and knockdown of Wnt5a up-regulated and down-regulated intracellular Ca^{2+} level, CaMKII-α, CaMKII-β, and TAK1 expression in $SOD1^{G93A}$ mutant NSC-34 cells. A, C. Two-matrix chart showing the intracellular concentration of Ca^{2+} in NSC-34 cells after transfection for 48 h. B, D. Bar chart displaying the percentage of Ca^{2+} in NSC-34 cells after transfection for 48 h. E, F. Protein bands of CaMKII-α, CaMKII-β, and TAK1 in NSC-34 cells after transfection for 48 h. G, H. Bar chart indicating CaMKII-α, CaMKII-β, and TAK1 protein level as analyzed by western blotting. GAPDH was used as the internal control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. pcDNA3.1-Con and pcDNA3.1-G93A-SOD1, siRNA-Con and pcDNA3.1-G93A-SOD1.


A
pcDNA3.1-Con+ pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+ pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+ pcDNA3.1-G93A-SOD1 +KN-93
CaMKII-α
GAPDH
CaMKII-β
GAPDH
TAK1
GAPDH
B
siRNA-Con+ pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+ pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+ pcDNA3.1-G93A-SOD1 +OA
CaMKII-α
GAPDH
CaMKII-β
GAPDH
TAK1
GAPDH
C
pcDNA3.1-Con+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1+KN-93
OD radio of CaMKII-α to GAPDH
D
siRNA-Con+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA
OD radio of CaMKII-α to GAPDH
E
pcDNA3.1-Con+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1+KN-93
OD radio of CaMKII-β to GAPDH
F
siRNA-Con+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA
OD radio of CaMKII-β to GAPDH
G
pcDNA3.1-Con+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1+KN-93
OD radio of TAK1 to GAPDH
H
siRNA-Con+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA
OD radio of TAK1 to GAPDH

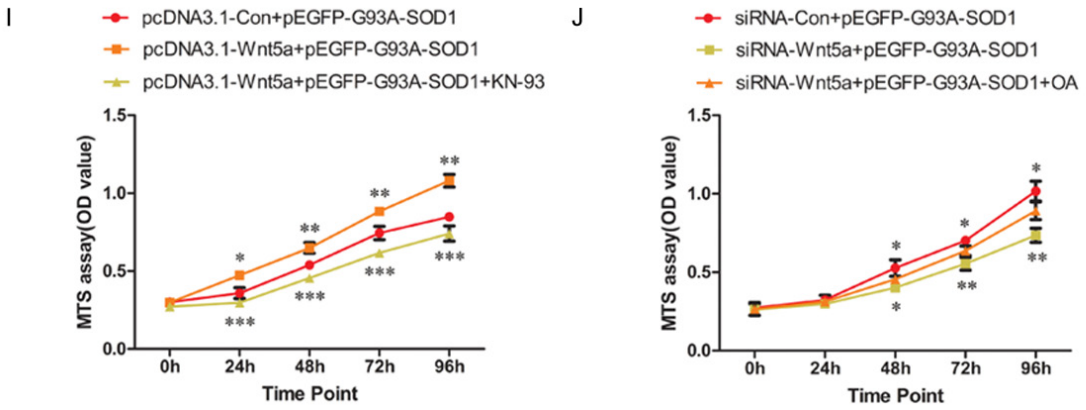


Figure 4. Effect of KN-93 and oleic acid (OA) on viability of $SOD1^{G93A}$ mutant NSC-34 cells after overexpression and knockdown of Wnt5a. A-H. CaMKII-α, CaMKII-β and TAK1 protein level were analyzed by western blotting. GAPDH served as the internal control. I, J. Line chart showing the viability of NSC-34 cells after transfection for different durations as determined by MTS assay. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. vs. pcDNA3.1-Con and pcDNA3.1-G93A-SOD1, siRNA-Con and pcDNA3.1-G93A-SOD1.

the main targets of degenerative neurons in ALS. High concentration of SOD1 may lead to the death of MNs [33]. Therefore, the present study sought to examine the impact of Wnt5a on $SOD1^{G93A}$ mutant NSC-34 cell apoptosis. As seen in **Figure 6A**, **6C**, the proportions of apoptotic cells was 24.76 ± 2.84%, 15.08 ± 1.25% and 19.59 ± 1.59% in the pcDNA3.1-Con+pcDNA3.1-G93A-SOD1, pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1 and pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1+KN-93 group, respectively ($P < 0.05$, $P < 0.01$). In contrast, the proportions of apoptotic cells was 13.57 ± 1.55%, 19.97 ± 1.59%, and 16.89 ± 0.30% in the siRNA-Con+pcDNA3.1-G93A-SOD1, siRNA-Wnt5a+pcDNA3.1-G93A-SOD1, and siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA group, respectively (**Figure 6B**, **6D**, $P < 0.05$, $P < 0.01$). These results suggested that KN-93 and OA could reverse the change in cell viability, proliferation, and apoptosis induced by over-expressing and knocking down Wnt5a. Furthermore, Wnt5a could improve $SOD1^{G93A}$ mutant NSC-34 cell viability and proliferation. Finally, our results suggest that the expression level of Wnt5a can affect $SOD1^{G93A}$ mutant NSC-34 cell viability, proliferation, and apoptosis by regulating the Wnt/Ca^{2+} pathway.

CaMKII inhibitor KN-93 and CaMKII activator oleic acid reversed the changes of neurite outgrowth induced by overexpressing and knocking down Wnt5a

A recent study linked Wnt5a-CaMKII signaling pathway with the elongation of neurites [34]. To determine whether the Wnt/Ca^{2+} signaling pathway was associated with neurite outgrowth, the number of cells with neurites was counted and the length of neuritis was measured. The percentages of cells with one or more neurites were 23.43% ± 0.66%, 30.66% ± 3.82%, and 21.11% ± 4.44% in pcDNA3.1-Con+pcDNA3.1-G93A-SOD1, pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1, and pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1+KN-93 groups, with an average length of NSC-34 cell's longest neurite of 17.25 ± 0.48 μm, 25.73 ± 4.21 μm, 18.22 ± 1.32 μm, respectively (**Figure 7A**, **7C**, **7E**, $P < 0.05$). KN-93 reversed a Wnt5a-mediated decrease of neurite outgrowth in $SOD1^{G93A}$ mutant NSC-34 cells. There was a 9.55% and 29.19% decrease in the number and length of cells with neurites in the pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1+KN-93 group, respectively, compared to that in the pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1 group. Our data showed that KN-93 inhibits neurite outgrowth induced by overexpression of Wnt5a. On the contrary, the percent of cells with one or more neurites was 26.73% ± 4.12%, 18.64% ± 1.98%, and 28.21% ± 3.04 in siRNA-Con+pcDNA3.1-G93A-SOD1, siRNA-Wnt5a+pcDNA3.1-G93A-SOD1, and siRNA-Con+pcDNA3.1-G93A-SOD1+OA groups with an average length of the NSC-34 cell's longest neurite of 25.9 ± 3.43 μm, 17.68 ± 3.64 μm, and 25.97 ± 1.95 μm respectively (**Figure 7B**, **7D**, **7F**, $P < 0.05$). Therefore, $SOD1^{G93A}$ mutant NSC-34 cells reversed a Wnt5a-mediated decrease in neurite outgrowth, as evidenced by a 9.57% and 46.89% increase in the number of cells with neurites and the length of neu-

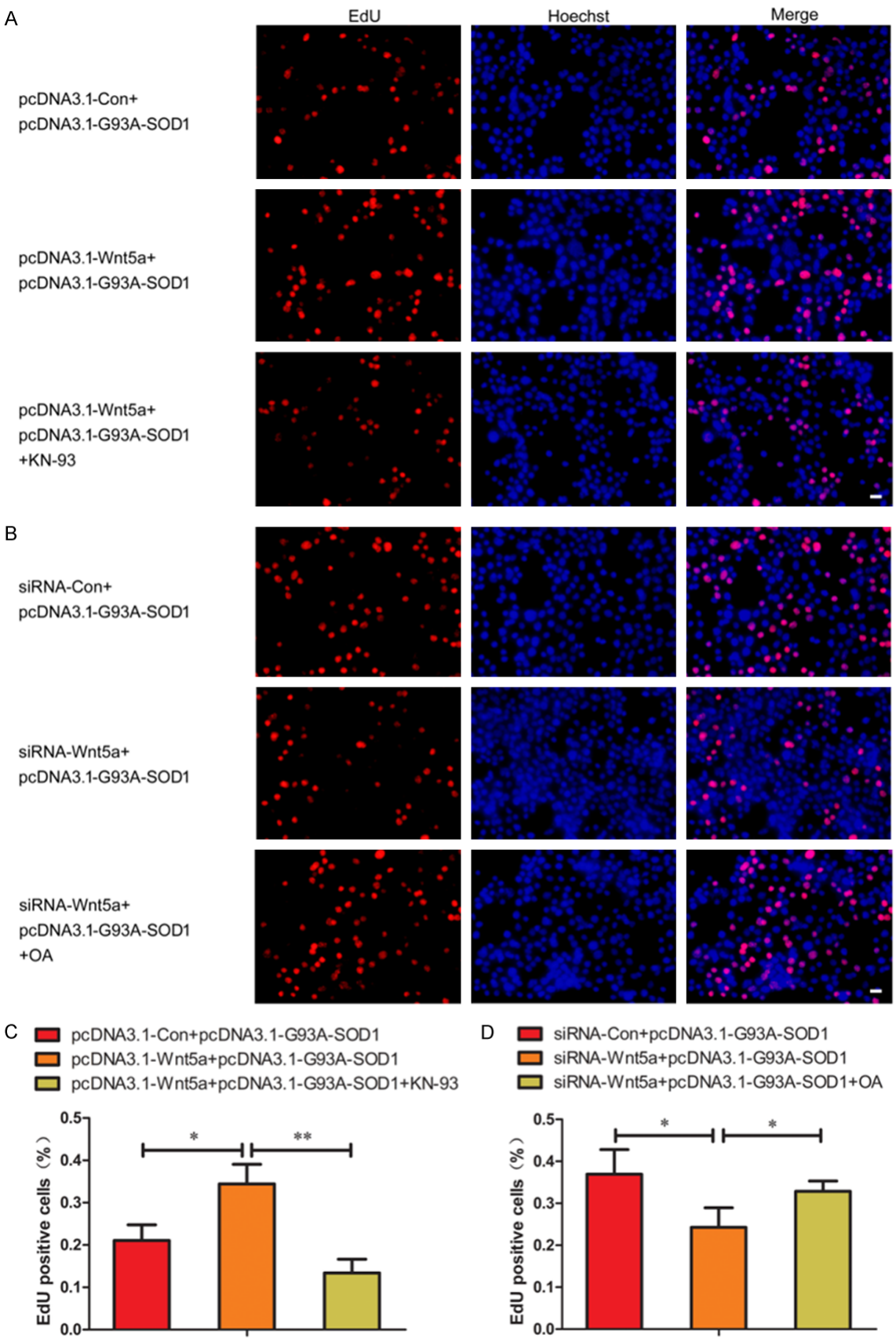

A
EdU
Hoechst
Merge
pcDNA3.1-Con+
pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+
pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+
pcDNA3.1-G93A-SOD1
+KN-93
B
siRNA-Con+
pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+
pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+
pcDNA3.1-G93A-SOD1
+OA
C
pcDNA3.1-Con+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1+KN-93
EdU positive cells（%）
0.5
0.4
0.3
0.2
0.1
0.0
*
**
D
siRNA-Con+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA
EdU positive cells（%）
0.5
0.4
0.3
0.2
0.1
0.0
*
*

Figure 5. Effect of KN-93 and OA on the proliferation of $SOD1^{G93A}$ mutant NSC-34 cells after overexpression and knockdown of Wnt5a. A, B. Representative images of EdU-positive cells obtained under a fluorescence microscope. Scale bar = 20 μm. C. Bar chart showing that KN-93 treatment for 48 h reversed the raised proliferation of NSC-34 cells after co-transfecting pcDNA3.1-Wnt5a and pcDNA3.1-G93A-SOD1 plasmids. D. Bar chart showing that OA treatment for 48 h reversed the decreased proliferation of NSC-34 cells after co-transfecting siRNA-Wnt5a and pcDNA3.1-G93A-SOD1 plasmids. *$P < 0.05$, **$P < 0.01$ vs. pcDNA3.1-Con and pcDNA3.1-G93A-SOD1, siRNA-Con and pcDNA3.1-G93A-SOD1.

rites, respectively, in siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA group compared to the siRNA-Wnt5a+pcDNA3.1-G93A-SOD1 group. These results indicate that Wnt5a improved the neurite outgrowth by regulating the Wnt/Ca^{2+} pathway.

Discussion

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease of MNs in the cortex, brainstem, and spinal cord [5]. It was first described by French neurologist Charcot in 1869 as a condition affecting the upper and lower MNs [35]. Advanced stage of the disease may lead to the loss or death of MNs with the loss of motor function [36]. The ALS pathogenic genes include *C9ORF72*, *SOD1*, *TARDBP*, *FUS*, *OPTN, PFN1*, *MATR3*, *TUBA4A*, and *TBK1* among others [37]. *SOD1* gene was the first gene associated with ALS to be identified, in 1993 [4, 38]. A previous study showed that many MNs died in the ventral horn and myelinated axons were lost in the ventral motor roots of the spinal cord in the $SOD1^{G93A}$ animal model [39].

Wnt signaling pathway regulates multiple cell functions and controls many aspects of development, including cell proliferation, apoptosis, migration, and cell polarity [22]. Wnt signaling pathway mediated by Wnt3a, Wnt5a, and Wnt7a can antagonize neurotoxicity of AD β-amyloid protein, protect hippocampal neurons, and improve cognitive function of patients. Activation of Wnt/β-catenin signaling pathway is associated with the viability of PD dopaminergic neurons, which maintains the integrity of dopaminergic neurons. Hence, targeting Wnt signaling molecules may confer beneficial therapeutic effects in neurodegenerative diseases [40-42].

Previously, differentially expressed canonical Wnt signal ligands (Wnt1, Wnt2, Wnt3a, and Wnt10a) and receptors (Fzd2, Fzd4, Fzd7 and LRP5) in the spinal cords of ALS mice were examined at different stages using transcriptional microarray analysis. The expression of non-canonical Wnt ligand Wnt5a is also abnormal in ALS mice [31, 43]. In the current study, we confirmed that canonical Wnt signaling pathway was activated in the pathogenesis of ALS. Furthermore, silencing RNA helicase DDX3 inhibited the proliferation of $SOD1^{G93A}$ mutant NSC-34 cells, promoted cell apoptosis and affected neurite outgrowth through Wnt signaling molecule CK1ε [44]. These results indicate that the Wnt signaling pathway regulates MNs degeneration in ALS.

Studies have found that canonical and non-canonical Wnt signaling pathways modulate the occurrence and development of various diseases [45, 46]. Wnt5a is a key signaling molecule that participates in the non-canonical Wnt signaling pathway. In our previous study, abnormal Wnt5a expression was detected in the spinal cord of ALS mice [43]. Wnt5a-mediated non-canonical signaling pathway plays an important role in neuronal damage [33, 47]. In mammals, Wnt5a activates intracellular Ca^{2+} release and CaMKII, whih regulates neurite outgrowth and cell function by the Wnt/Ca^{2+} pathway [48]. Spalloni *et al.* [49] found a decrease in CaMKII-α autophosphorylation at threonine-286 in cortical M1 region of mice overexpressing the $SOD1^{G93A}$ gene. West *et al.* [25] identified TAK1 as a regulator of synaptic growth responses during neurodegenerative processes. In the present study, the number of CaMKII-α/β-tubulin III, CaMKII-β/β-tubulin III and TAK1/β-tubulin III double-positive cells in ALS middle stage were less than that in the ventral horn of gray matter of WT mice indicated that CaMKII-α, CaMKII-β, and TAK1 expression were associaed with the degeneration of MNs in ALS. The results of western blotting also showed that the expression of CaMKII-α, CaMKII-β, and TAK1 was low in the onset period, demonstrating that CaMKII-α, CaMKII-β, and TAK1 are involved in the development of ALS.

Intracellular Ca^{2+} homeostasis is disrupted in many neurodegenerative diseases. Overstimulation of glutamate receptor in MNs and subse-

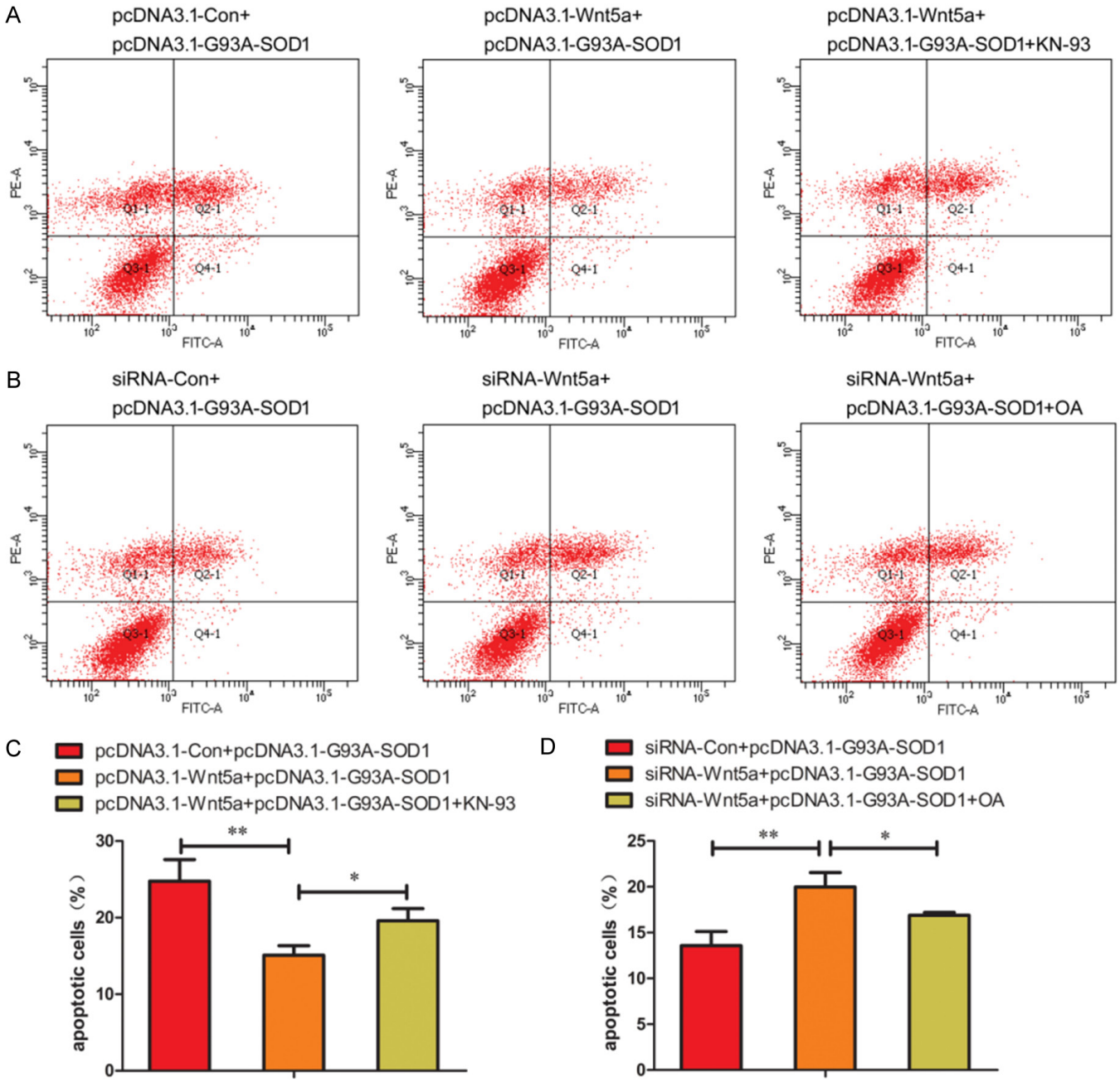

A
pcDNA3.1-Con+
pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+
pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+
pcDNA3.1-G93A-SOD1+KN-93
B
siRNA-Con+
pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+
pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+
pcDNA3.1-G93A-SOD1+OA
PE-A
FITC-A
Q1-1
Q2-1
Q3-1
Q4-1
C
pcDNA3.1-Con+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1
pcDNA3.1-Wnt5a+pcDNA3.1-G93A-SOD1+KN-93
apoptotic cells (%)
D
siRNA-Con+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1
siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA
apoptotic cells (%)

Figure 6. Effect of KN-93 and oleic acid (OA) on apoptosis of $SOD1^{G93A}$ mutant NSC-34 cells after overexpression and knockdown of Wnt5a. A, B. Four-matrix chart showing apoptosis of cells determined by flow cytometry assay. C. Bar chart showing the percentage of apoptotic cell death after co-transfecting pcDNA3.1-Wnt5a and pcDNA3.1-G93A-SOD1 plasmid for 48 h. D. Bar chart showing the percentage of apoptotic cell death after co-transfecting siRNA-Wnt5a and pcDNA3.1-G93A-SOD1 plasmids. *$P < 0.05$, **$P < 0.01$. vs. pcDNA3.1-Con and pcDNA3.1-G93A-SOD1, siRNA-Con and pcDNA3.1-G93A-SOD1.

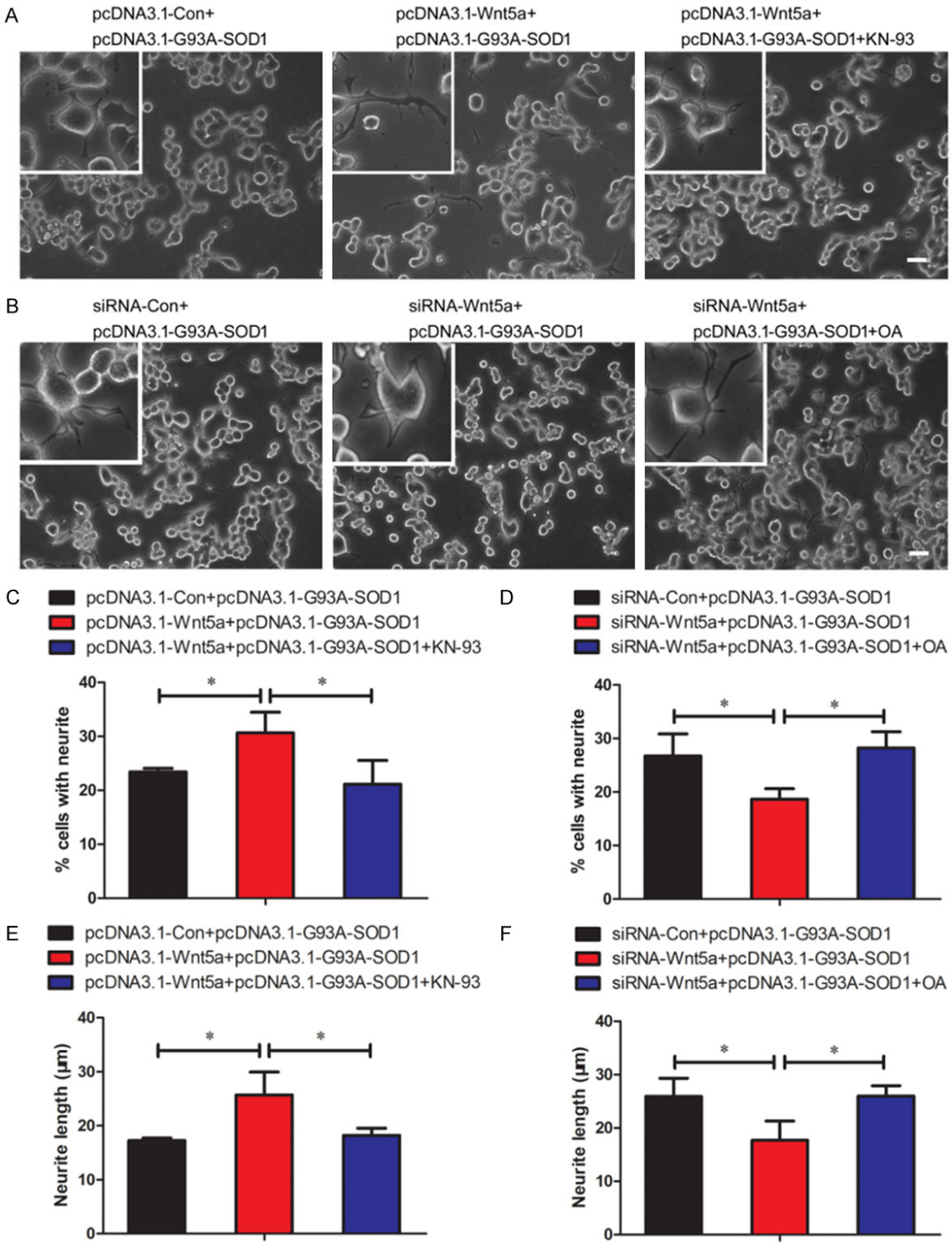


Figure 7. Effect of KN-93 and oleic acid (OA) on neurite outgrowth in $SOD1^{G93A}$ mutant NSC-34 cells after overexpression and knockdown of Wnt5a. A, B. Representative images showing neurite outgrowth in NSC-34 cells. Scale bar = 50 μm. C, D. Bar chart showing the percentages of cells with one or more neurites in NSC-34 cells after co-transfecting pcDNA3.1-Wnt5a and pcDNA3.1-G93A-SOD1 plasmid for 48 h. E, F. Bar chart showing the average length of longest neurite in NSC-34 cells after co-transfecting siRNA-Wnt5a and pcDNA3.1-G93A-SOD1 plasmids for 48 h. *$P < 0.05$. vs. pcDNA3.1-Con and pcDNA3.1-G93A-SOD1, siRNA-Con and pcDNA3.1-G93A-SOD1.

quent excitotoxicity induced by high calcium ion implantation may lead to MN degeneration in ALS patients and SOD1 mutant mice model [50]. This study found a lower intracellular Ca^{2+} level in NSC-34 cells carrying the pcDNA3.1-G93A-SOD1 plasmid compared toth NSC-34 cells transfected with pcDNA3.1-WT-SOD1 plasmid at 48 h. CaMKII is a key molecule in the pathological cascade downstream of abnormal Ca^{2+} signaling [51-53]. Our results showed that mRNA and protein levels of CaMKII-α, CaMKII-β, and TAK1 at 48 h in NSC-34 cells carrying the pcDNA3.1-G93A-SOD1 plasmid were lower than in NSC-34 cells transfected with the pcDNA3.1-WT-SOD1 plasmid. These results suggested that the mutation in *SOD1* gene reduced the expression of CaMKII-α, CaMKII-β, and TAK1, indicating that Wnt/Ca^{2+} signaling pathway was involved in the pathogenesis of ALS.

To determine whether Wnt5a regulated the Wnt/Ca^{2+} signaling pathway in the pathogenesis of ALS, NSC-34 cells were respectively transfected with pcDNA3.1-G93A-SOD1 plasmid and pcDNA3.1-Wnt5a plasmid or siRNA-Wnt5a. We found that overexpression of Wnt5a up-regulated the intracellular Ca^{2+} level and CaMKII-α, CaMKII-β and TAK1 expression, whereas knockdown of Wnt5a downregulated them. These results suggested that Wnt5a positively regulates the intracellular Ca^{2+} level and expression of CaMKII-α, CaMKII-β, and TAK1 in ALS.

The loss of MNs has also been linked to the pathogenesis of ALS. Martin [54] found that apoptosis was activated in ALS. *SOD1* is an antioxidant enzyme that can protect neurons from the damage from free superoxide radicals. The mutation of *SOD1* can stimulate protein aggregation and lead to apoptosis [55]. Han et al. [56] found that higher Wnt5a levels promoted cell proliferation whereas Wnt5a knockdown reduced cell proliferation. This study showed that overexpression of Wnt5a promoted proliferation of $SOD1^{G93A}$ mutant NSC-34 cells whereas knockdown of Wnt5a reduced proliferation of $SOD1^{G93A}$ mutant NSC-34 cells. However, over-expressing Wnt5a reduced apoptosis of $SOD1^{G93A}$ mutant NSC-34 cells whereas knockdown of Wnt5a promoted it. These findings imply that Wnt5a is important for cell growth and exerts a neuroprotective effect through the Wnt/Ca^{2+} pathway on $SOD1^{G93A}$ mutant NSC-34 cells.

Neurons are made up of three distinct sections: the soma, which contains the nucleus and the majority of the cellular organelles, a long axonal process that transmits information, and a complex dendritic arbor that receives information from neighboring neurons [57, 58]. Mature neurites include axons and dendrites, which are supported by microtubule cytoskeleton composed of bundles of microtubules. Studies have shown that the degeneration of ALS MNs is related to the changes in the cytoskeleton. $SOD1^{G93A}$ mutation regulates the neurite outgrowth of MNs by affecting cytoskeleton status [59]. CaMKII is a cytoskeleton-related protein, which, when overexpressed leads to neurite outgrowth [60]. Lund et al. [24] confirmed a change in the expression of CaMKII after axonal injury in sciatic MNs. To determine whether Wnt5a regulates neurite outgrowth to affect the function of ALS MNs, the present study detected the neurite outgrowth of $SOD1^{G93A}$ mutant NSC-34 cells using RA. Overexpression of Wnt5a increased the length of $SOD1^{G93A}$ mutant NSC-34 cells neurites and the percentages of cells with one or more neurites. Similarly, knocking down Wnt5a inhibited neurite outgrowth in $SOD1^{G93A}$ mutant NSC-34 cells. This suggests that Wnt5a may participate in neuronal degeneration by regulating neurite outgrowth in ALS.

Finally, specific CaMKII inhibitor KN-93 and CaMKII activator OA were used to verify the role of the Wnt5a-mediated Wnt/Ca^{2+} pathway. The results showed that KN-93 and OA reversed the $SOD1^{G93A}$ mutant NSC-34 cell viability, proliferation, apoptosis, and neurite outgrowth after over-expressing and knocking down Wnt5a. These results suggested that Wnt5a

regulates cell function by affecting neuron motor degeneration through the Wnt/Ca^{2+} signaling pathway. In Arredondo's study, the researchers determined that Wnt5a signals induce neurogenesis through CaMKII and promote dendritic development of newborn neurons by activating Wnt/JNK and Wnt/CaMKII signaling using specific inhibitors [61]. These results are consistent with our findings. Wnt5a has significance and value waranting additional research, development, and clinical application in ALS.

However, whether Wnt5a regulates canonical and non-canonical Wnt signaling pathways by binding different cell receptors involved in development of ALS needs further research.

Conclusions

CaMKII and TAK1 play an important roles in the degeneration of MNs in ALS. Wnt5a can protect $SOD1^{G93A}$ mutant NSC-34 cells from death by regulating cell viability, proliferation, apoptosis and neurite outgrowth through the Wnt/Ca^{2+} signaling pathway. This indicates that Wnt5a regulates neuronal degeneration in ALS.

Acknowledgements

This work was supported by grants from the Natural Science Foundation of China (Grant No. 81871006), Shandong Province Natural Science Foundation of China (Grant No. ZR2020MH150 and ZR2020MH149), Support Program for Youth Innovation Technology in Colleges and Universities of Shandong Province of China (Grant No. 2019KJK004), Key Project of Shandong Province Higher Educational Science and Technology Program of China (Grant No. J18KZ013), Grant from the Brigham and Women's Hospital BRI Fund to Sustain Research Excellence (to X.W.) and Shandong medical and health science and technology development plan project (Grant No. 2019WS606).

Disclosure of conflict of interest

None.

Address correspondence to: Yingjun Guan, Department of Histology and Embryology, Weifang Medical University, Weifang 261053, Shandong, PR China. Tel: +86-536-2602076; E-mail: guanyj@wfmc.edu.cn; Xin Wang, Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston 02115, Massachusetts, USA. Tel: 1-617-732-4186; E-mail: xwang@rics.bwh.harvard.edu

References

[1] Taylor JP, Brown RJ and Cleveland DW. Decoding ALS: from genes to mechanism. Nature 2016; 539: 197-206.

[2] Madruga E, Maestro I and Martinez A. Mitophagy modulation, a new player in the race against ALS. Int J Mol Sci 2021; 22: 740.

[3] Liu J, Zhou F, Guan Y, Meng F, Zhao Z, Su Q, Bao W, Wang X, Zhao J, Huo Z, Zhang L, Zhou S, Chen Y and Wang X. The Biogenesis of miRNAs and their role in the development of amyotrophic lateral sclerosis. Cells 2022; 11: 572.

[4] Mejzini R, Flynn LL, Pitout IL, Fletcher S, Wilton SD and Akkari PA. ALS genetics, mechanisms, and therapeutics: where are we now? Front Neurosci 2019; 13: 1310.

[5] Mathew B, Ruiz P, Pathak V and Suto MJ. Development of novel small molecules for the treatment of ALS. Bioorg Med Chem Lett 2020; 30: 126950.

[6] Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993; 362: 59-62.

[7] Chen Y, Guan Y, Liu H, Wu X, Yu L, Wang S, Zhao C, Du H and Wang X. Activation of the Wnt/beta-catenin signaling pathway is associated with glial proliferation in the adult spinal cord of ALS transgenic mice. Biochem Biophys Res Commun 2012; 420: 397-403.

[8] Tokuda E and Furukawa Y. Copper homeostasis as a therapeutic target in amyotrophic lateral sclerosis with SOD1 mutations. Int J Mol Sci 2016; 17: 636.

[9] Li W, Fotinos A, Wu Q, Chen Y, Zhu Y, Baranov S, Tu Y, Zhou EW, Sinha B, Kristal BS and Wang X. N-acetyl-L-tryptophan delays disease onset and extends survival in an amyotrophic lateral sclerosis transgenic mouse model. Neurobiol Dis 2015; 80: 93-103.

[10] Blasco H, Patin F, Andres CR, Corcia P and Gordon PH. Amyotrophic lateral sclerosis, 2016: existing therapies and the ongoing search for neuroprotection. Expert Opin Pharmacother 2016; 17: 1669-1682.

[11] Sironi F, Vallarola A, Violatto MB, Talamini L, Freschi M, De Gioia R, Capelli C, Agostini A, Moscatelli D, Tortarolo M, Bigini P, Introna M and Bendotti C. Multiple intracerebroventricular injections of human umbilical cord mesenchymal stem cells delay motor neurons loss but not disease progression of SOD1G93A mice. Stem Cell Res 2017; 25: 166-178.

[12] Modol-Caballero G, Garcia-Lareu B, Verdes S, Ariza L, Sánchez-Brualla I, Brocard F, Bosch A, Navarro X and Herrando-Grabulosa M. Therapeutic role of neuregulin 1 type III in SOD1-linked amyotrophic lateral sclerosis. Neurotherapeutics 2020; 17: 1048-1060.
[13] Keeler AM, Zieger M, Semple C, Pucci L, Veinbachs A, Brown RH Jr, Mueller C and ElMallah MK. Intralingual and intrapleural AAV gene therapy prolongs survival in a SOD1 ALS mouse model. Mol Ther Methods Clin Dev 2020; 17: 246-257.
[14] Lim CKW, Gapinske M, Brooks AK, Woods WS, Powell JE, Zeballos C MA, Winter J, Perez-Pinera P and Gaj T. Treatment of a mouse model of ALS by in vivo base editing. Mol Ther 2020; 28: 1177-1189.
[15] Huelsken J and Behrens J. The Wnt signalling pathway. J Cell Sci 2002; 115: 3977-3978.
[16] Komiya Y and Habas R. Wnt signal transduction pathways. Organogenesis 2008; 4: 68-75.
[17] Jiang X, Guan Y, Zhao Z, Meng F, Wang X, Gao X, Liu J, Chen Y, Zhou F, Zhou S and Wang X. Potential roles of the WNT signaling pathway in amyotrophic lateral sclerosis. Cells 2021; 10: 839.
[18] Serafino A, Giovannini D, Rossi S and Cozzolino M. Targeting the Wnt/beta-catenin pathway in neurodegenerative diseases: recent approaches and current challenges. Expert Opin Drug Discov 2020; 15: 803-822.
[19] Pinto C, Medinas DB, Fuentes-Villalobos F, Maripillán J, Castro AF, Martínez AD, Osses N, Hetz C and Henríquez JP. β-catenin aggregation in models of ALS motor neurons: GSK3β inhibition effect and neuronal differentiation. Neurobiol Dis 2019; 130: 104497.
[20] Gonzalez-Fernandez C, Gonzalez P and Rodriguez FJ. New insights into Wnt signaling alterations in amyotrophic lateral sclerosis: a potential therapeutic target? Neural Regen Res 2020; 15: 1580-1589.
[21] Kohn AD and Moon RT. Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium 2005; 38: 439-446.
[22] Ng LF, Kaur P, Bunnag N, Suresh J, Sung ICH, Tan QH, Gruber J and Tolwinski NS. WNT signaling in disease. Cells 2019; 8: 826.
[23] De A. Wnt/Ca^{2+} signaling pathway: a brief overview. Acta Biochim Biophys Sin 2011; 43: 745-756.
[24] Lund LM and McQuarrie IG. Calcium/calmodulin-dependent protein kinase IIbeta isoform is expressed in motor neurons during axon outgrowth and is part of slow axonal transport. J Neurosci Res 2002; 67: 720-728.
[25] West RJ, Lu Y, Marie B, Gao FB and Sweeney ST. Rab8, POSH, and TAK1 regulate synaptic growth in a Drosophila model of frontotemporal dementia. J Cell Biol 2015; 208: 931-947.
[26] Horigane S, Ageta-Ishihara N, Kamijo S, Fujii H, Okamura M, Kinoshita M, Takemoto-Kimura S and Bito H. Facilitation of axon outgrowth via a Wnt5a-CaMKK-CaMKIalpha pathway during neuronal polarization. Mol Brain 2016; 9: 8.
[27] Arrazola MS, Silva-Alvarez C and Inestrosa NC. How the Wnt signaling pathway protects from neurodegeneration: the mitochondrial scenario. Front Cell Neurosci 2015; 9: 166.
[28] Poletti A, Cattaneo E and Taroni F. The neurotoxicity of mutant proteins 20 years after the discovery of the first mutant gene involved in neurodegeneration. Foreword. Prog Neurobiol 2012; 97: 53.
[29] Chen Y, Guan Y, Zhang Z, Liu H, Wang S, Yu L, Wu X and Wang X. Wnt signaling pathway is involved in the pathogenesis of amyotrophic lateral sclerosis in adult transgenic mice. Neurol Res 2012; 34: 390-399.
[30] Zhang P, Luo X, Guo Z, Xiong A, Dong H, Zhang Q, Liu C, Zhu J, Wang H, Yu N, Zhang J, Hong Y, Yang L and Huang J. Neuritin inhibits notch signaling through interacted with neuralized to promote the neurite growth. Front Mol Neurosci 2017; 10: 179.
[31] Li X, Guan Y, Chen Y, Zhang C, Shi C, Zhou F, Yu L, Juan J and Wang X. Expression of Wnt5a and its receptor Fzd2 is changed in the spinal cord of adult amyotrophic lateral sclerosis transgenic mice. Int J Clin Exp Pathol 2013; 6: 1245-1260.
[32] Nijssen J, Comley LH and Hedlund E. Motor neuron vulnerability and resistance in amyotrophic lateral sclerosis. Acta Neuropathol 2017; 133: 863-885.
[33] Subramaniam S. Selective neuronal death in neurodegenerative diseases: the ongoing mystery. Yale J Biol Med 2019; 92: 695-705.
[34] Coullery RP, Ferrari ME and Rosso SB. Neuronal development and axon growth are altered by glyphosate through a WNT non-canonical signaling pathway. Neurotoxicology 2016; 52: 150-161.
[35] Oskarsson B, Gendron TF and Staff NP. Amyotrophic lateral sclerosis: an update for 2018. Mayo Clin Proc 2018; 93: 1617-1628.
[36] Kanning KC, Kaplan A and Henderson CE. Motor neuron diversity in development and disease. Annu Rev Neurosci 2010; 33: 409-440.
[37] Lattante S, Ciura S, Rouleau GA and Kabashi E. Defining the genetic connection linking amyotrophic lateral sclerosis (ALS) with frontotemporal dementia (FTD). Trends Genet 2015; 31: 263-273.
[38] Kim G, Gautier O, Tassoni-Tsuchida E, Ma XR and Gitler AD. ALS genetics: gains, losses, and implications for future therapies. Neuron 2020; 108: 822-842.
[39] Bendotti C and Carrì MT. Lessons from models of SOD1-linked familial ALS. Trends Mol Med 2004; 10: 393-400.

[40] Vallee A and Lecarpentier Y. Alzheimer disease: crosstalk between the canonical Wnt/Beta-Catenin pathway and PPARs alpha and gamma. Front Neurosci 2016; 10: 459.
[41] Serafino A, Sferrazza G, Colini Baldeschi A, Nicotera G, Andreola F, Pittaluga E and Pierimarchi P. Developing drugs that target the Wnt pathway: recent approaches in cancer and neurodegenerative diseases. Expert Opin Drug Discov 2017; 12: 169-186.
[42] Marchetti B. Wnt/β-Catenin signaling pathway governs a full program for dopaminergic neuron survival, neurorescue and regeneration in the MPTP mouse model of Parkinson's disease. Int J Mol Sci 2018; 19: 3743.
[43] Yu L, Guan Y, Wu X, Chen Y, Liu Z, Du H and Wang X. Wnt signaling is altered by spinal cord neuronal dysfunction in amyotrophic lateral sclerosis transgenic mice. Neurochem Res 2013; 38: 1904-1913.
[44] Chen Y, Wang Q, Wang Q, Liu H, Zhou F, Zhang Y, Yuan M, Zhao C, Guan Y and Wang X. DDX3 binding with CK1ε was closely related to motor neuron degeneration of ALS by affecting neurite outgrowth. Am J Transl Res 2017; 9: 4627.
[45] Aznar N, Sun N, Dunkel Y, Ear J, Buschman MD and Ghosh P. A Daple-Akt feed-forward loop enhances noncanonical Wnt signals by compartmentalizing β-catenin. Mol Biol Cell 2017; 28: 3709-3723.
[46] Mikels AJ and Nusse R. Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. PLoS Biol 2006; 4: e115.
[47] Chen CM, Orefice LL, Chiu SL, LeGates TA, Hattar S, Huganir RL, Zhao H, Xu B and Kuruvilla R. Wnt5a is essential for hippocampal dendritic maintenance and spatial learning and memory in adult mice. Proc Natl Acad Sci U S A 2017; 114: E619-E628.
[48] Kumawat K and Gosens R. WNT-5A: signaling and functions in health and disease. Cell Mol Life Sci 2016; 73: 567-587.
[49] Spalloni A, Origlia N, Sgobio C, Trabalza A, Nutini M, Berretta N, Bernardi G, Domenici L, Ammassari-Teule M and Longone P. Postsynaptic alteration of NR2A subunit and defective autophosphorylation of alphaCaMKII at threonine-286 contribute to abnormal plasticity and morphology of upper motor neurons in presymptomatic SOD1G93A mice, a murine model for amyotrophic lateral sclerosis. Cereb Cortex 2011; 21: 796-805.
[50] Van Den Bosch L, Van Damme P, Bogaert E and Robberecht W. The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis. Biochim Biophys Acta 2006; 1762: 1068-1082.
[51] Oka M, Fujisaki N, Maruko-Otake A, Ohtake Y, Shimizu S, Saito T, Hisanaga SI, Iijima KM and Ando K. Ca^{2+}/calmodulin-dependent protein kinase II promotes neurodegeneration caused by tau phosphorylated at Ser262/356 in a transgenic Drosophila model of tauopathy. J Biochem 2017; 162: 335-342.
[52] LaFerla FM. Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. Nat Rev Neurosci 2002; 3: 862-872.
[53] Ghosh A and Giese KP. Calcium/calmodulin-dependent kinase II and Alzheimer's disease. Mol Brain 2015; 8: 78.
[54] Martin LJ. Neuronal death in amyotrophic lateral sclerosis is apoptosis: possible contribution of a programmed cell death mechanism. J Neuropathol Exp Neurol 1999; 58: 459-471.
[55] Ghavami S, Shojaei S, Yeganeh B, Ande SR, Jangamreddy JR, Mehrpour M, Christoffersson J, Chaabane W, Moghadam AR, Kashani HH, Hashemi M, Owji AA and Łos MJ. Autophagy and apoptosis dysfunction in neurodegenerative disorders. Prog Neurobiol 2014; 112: 24-49.
[56] Han C, Li J, Wang C, Ouyang H, Ding X, Liu Y, Chen S and Luo L. Wnt5a contributes to the differentiation of human embryonic stem cells into lentoid bodies through the noncanonical Wnt/JNK signaling pathway. Invest Ophthalmol Vis Sci 2018; 59: 3449-3460.
[57] Tahirovic S and Bradke F. Neuronal polarity. Cold Spring Harb Perspect Biol 2009; 1: a1644.
[58] Gilbert J and Man HY. Fundamental elements in autism: from neurogenesis and neurite growth to synaptic plasticity. Front Cell Neurosci 2017; 11: 359.
[59] Osking Z, Ayers JI, Hildebrandt R, Skruber K, Brown H, Ryu D, Eukovich AR, Golde TE, Borchelt DR, Read TA and Vitriol EA. ALS-linked SOD1 mutants enhance neurite outgrowth and branching in adult motor neurons. iScience 2019; 19: 448-449.
[60] Goshima Y, Ohsako S and Yamauchi T. Overexpression of Ca^{2+}/calmodulin-dependent protein kinase II in Neuro2a and NG108-15 neuroblastoma cell lines promotes neurite outgrowth and growth cone motility. J Neurosci 1993; 13: 559-567.
[61] Arredondo SB, Guerrero FG, Herrera-Soto A, Jensen-Flores J, Bustamante DB, Oñate-Ponce A, Henny P, Varas-Godoy M, Inestrosa NC and Varela-Nallar L. Wnt5a promotes differentiation and development of adult-born neurons in the hippocampus by noncanonical Wnt signaling. Stem Cells 2020; 38: 422-436.