Wnt5a Protects Motor Neuron in Amyotrophic Lateral Sclerosis by Regulating Wnt/Ca2+ Signaling Pathway

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Research

Keywords: ALS, Wnt5a, Wnt/Ca2+ signaling, proliferation, apoptosis, neurite growth

Posted Date: October 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-968570/v1

Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that lead to the degeneration and death of motor neurons (MNs). Wnt signaling participates in multiple neurodegenerative processes. Wnt5a is a critical signaling molecule in Wnt signaling pathway. Intracellular Ca^{2+} homeostasis is disrupted in a number of neurodegenerative diseases, and Ca^{2+}/Calmodulin dependent protein kinase II (CaMKII) involved in various diseases associated with Ca^{2+} signaling abnormalities. Here, we found that Wnt5a modulated MNs degeneration during ALS. CaMKII is a key effector of signals derived from Wnt/Ca^{2+} signaling pathway. Its main subtypes CaMKII-α and CaMKII-β were down-regulated in the spinal cord of $SOD1^{G93A}$ transgenic mice (ALS mice) and SOD1 mutant motor neuron like hybrid (NSC-34) cells. In addition, results showed that CaMKII-α and CaMKII-β were positively regulated by Wnt5a *in vitro*. Using specific CaMKII inhibitor and activator, we found that Wnt5a promoted NSC-34 cells viability and proliferation, inhibited cells apoptosis and promoted cells neurite outgrowth via the Wnt/Ca^{2+} signaling pathway. These results indicate that Wnt5a confers neuroprotection by promoting neuronal proliferation, inhibiting cell apoptosis and promoting neurite growth through Wnt/Ca^{2+} signaling pathway. Therefore, targeting Wnt5a can be an effective strategy to treat ALS.

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. The main clinical manifestations are progressive muscle weakness and atrophy without effective treatment. When respiratory muscles are involved, most patients succumb to respiratory failure within 3 to 5 years after the onset of the disease [1, 2]. In addition to degenerated MNs, glial cells also undergo pathological changes, and their dysfunction produces a toxic environment in the central nervous system (CNS). At present, the exact mechanism of MNs degeneration in ALS is still unclear. Some of the pathogenic mechanisms proposed include 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]. Unfortunately, there is no effective treatment for ALS.

ALS cases can be divided into sporadic ALS (SALS) and familial ALS (fALS). The former, which occurs randomly without a family history, represents about 90% of ALS cases, while the latter accounts for about 10% of ALS cases [4]. To date, more than 25 mutated genes have been profiled in SALS and fALS. The ALS related gene that encodes copper/zinc superoxide dismutase 1 (SOD1) was the first to be reported in 1993 [5]. Research has shown that approximately 20% of fALS cases are caused by missense mutations in the SOD1 gene [6]. Evidence has shown that SOD1 mutant transgenic mice develop 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 [7-10]. In recent years, the mouse models have been used to explore treatment of ALS. Results of such models have shown that virus-mediated gene therapy

enhances improved symptoms and motor function of SOD1 mutants in ALS transgenic mice and increases the survival rate of MNs [11-13]. But there is still a lot of research to be done on targeted therapy for ALS.

Wnt signaling is a conserved pathway in animal development and is highly associated with the occurrence and development of a variety of 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. The Wnt genes encode Wnt proteins to activate the Wnt signaling pathway. The extra-cellular Wnt signal stimulates several intracellular signal transduction cascades, including the β–catenin–dependent pathway (Wnt/β–catenin pathway) for canonical and non canonical for β–catenin–independent pathway. It is the β–catenin–independent pathway that comprise the Wnt/Planar Cell Polarity (Wnt/PCP) and the Wnt/Ca^{2+} pathways [14-16]. Knowledge of the Wnt pathway and the involved genes is a promising method to search for possible targets to counteract the harmful effects of neurodegenerative diseases. Wnt ligands are well known for activating the canonical β-catenin pathway, which regulates acetylcholine receptor (AChR) aggregation as well as the formation and maintenance of neuromuscular junctions (NMJ). The findings suggested that β-catenin distribution could be an underlying factor affecting the onset of neurodegeneration in fALS [17]. Therefore, it may provide a feasible method for the treatment of ALS by targeting the Wnt/β-catenin pathway to maintain the stability of NMJ [18]. At present, the involvement of non-canonical Wnt signaling pathways in neuronal survival and death processes remains unclear [19].

Wnt5a is an important non-canonical Wnt signaling pathway ligand. It uses Ca^{2+} as a second messenger to achieve its biological effect [20]. Wnt5a binds to the homologous frizzled (Fzd) receptor, which leads to an increase in the intracellular Ca^{2+} concentration. The released Ca^{2+} then activates CaMKII to regulate many biological processes such as cytoskeleton rearrangement, cell adhesion and migration [21,22]. CaMKII is mainly composed of α and β subtypes in neurons and plays a role in the regulation of long-term potentiation, axon growth and neurotransmitter release [23]. Some research suggests that Wnt5a-activated Ca^{2+} signaling plays an important role in neurite outgrowth of various neuronal cell types [24]. It can also protect neurons in Alzheimer's disease (AD) by regulating the Wnt/Ca^{2+} signaling pathway [25]. However, for ALS, there is no reliable evidence to indicate whether Wnt5a can affect the function of MNs through the Wnt/Ca^{2+} signaling pathway.

Here we detect the expression of CaMKII-α and CaMKII-β in ALS mice and SOD1 mutant NSC-34 cells and explore the role of Wnt5a in degeneration of MNs. This study further gives insights into the molecular mechanism of ALS, which may provide new strategies for treating ALS.

Materials And Methods

Animals and tissue preparation

Amyotrophic lateral sclerosis transgenic mice (ALS mice) with $SOD1^{G93A}$ gene mutation and Wide-type mice (WT mice) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Male ALS mice aged 6-8 weeks were mated with adult female WT mice. The tail of newborn mice was cut after four weeks and the genomic DNA of the tail tissue was amplified using polymerase chain reaction (PCR) for genotyping. Adult ALS mice were randomly divided into three groups based on the early stage (95 days), middle stage (108 days) and late stage (122 days). Each group of ALS mice was matched with WT Littermate of the same age as the control group. The spinal cord tissues were directly isolated from some mice and stored in -80°C refrigerator to be used for extracting protein and RNA. Other mice were perfused with 4% paraformaldehyde, and their spinal cords were stripped out and frozen sections were prepared for immunofluorescence staining.

Cell lines and cell treatment

NSC-34, a mouse neuroblastoma N18TG2 and mouse embryonic spinal cord motor neuron hybrid cell line were 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 a 5% CO_2 incubator at 37°C. Cells were cover the bottom of culture bottle and up to 90% and were mechanically dissociated into 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). The pcDNA3.1-WT-SOD1 and pcDNA3.1-G93A-SOD1 plasmids were the kind gift from Professor Angelo Poletti (University of Milan, Italy) [26]. The pcDNA3.1-Wnt5a and pcDNA3.1-Control plasmids were purchased from Genechem (Shanghai, China). The small interfering RNA (siRNA) of 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 plasmid or siRNA-Wnt5a were co-transfected into NSC-34 cells. After 48 h, the cells RNA and protein were extracted. CaMKII inhibitor KN-93 was purchased from MCE, while the 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 [27]. The protein lysate was collected to measure protein concentration using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). The antibodies used for western blot were as follows: rabbit polyclonal anti-CaMKII-α (1:1000, Abcam, Cambridge, MA, USA), rabbit polyclonal anti-CaMKII-β (1:1000, Proteintech, Wuhan, China), goat polyclonal anti-Wnt5a (1:500, R□D, Minnesota, USA), mouse Monoclonal anti-GAPDH (1:1500, Proteintech). ECL (Thermo Scientific, Waltham, MA, USA) was added for luminescence reaction and the protein bands were exposed using chemiluminescence instrument. The optical density (OD) of protein bands were normalized to those of GAPDH as internal control during the western blot analysis.

Immunofluorescence

The method was performed as previously described [6]. The following primary antibodies was applied: rabbit anti-CaMKII-α, rabbit anti-CaMKII-β and mouse anti-β-tubulin III (1:200, R&D Systems, MN, USA). The secondary antibodies used include Alexa Fluor 488-conjugated anti-mouse IgG (1:400, Jackson ImmunoResearch) and Cy3 conjugated anti-rabbit IgG (1:400, Jackson ImmunoResearch). PBS was used instead of the first antibody in the control group and there was no specific staining identified. The images were observed and obtained using fluorescence microscope (Olympus, Tokyo, Japan). The numbers of double positive cells were measured using the ImageJ software.

Intracellular concentration of Ca^{2+} determination assay

Intracellular Ca^{2+} level was measured with Fluo-3 AM (Beyotime Institute of Biotechnology, Shanghai, China) according to manufacturers' instructions. Specifically, transfected cells were collected into the tube and thereafter incubated with 4 μM Fluo-3AM 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 in the absence of Fluo-3AM to complete the de-esterification process of the dye. Finally, stained cells were washed with ice-cold D-Hanks 3 times and then detected via flow cytometry.

MTS viability assay

The cells viability was measured using Cell Titer 96®AQueous One Solution Cell Proliferation Assay. Briefly, 1×10^4 transfected cells were plated into each well of a 96-well plate. The cells were cultured in 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 then incubated in CO_2 incubator for 40 min. The cell viability was detected at 0, 24, 48, 72 and 96 h after transfection. Absorbance (OD) was measured at 490 nm. Finally, the cells 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 according to the manual of Cell-Light TMEdU Apollo® 567 In Vitro Kit (RiboBio). Briefly, 1×10^5 cells were cultured in each well of 96-well plates. The transfected cells were labeled with 0.1% reagent A. Then 4% paraformaldehyde was incubated for 15 min at room temperature to fix the cells. 1×Apollo staining reaction solution was added to each well, and incubated in dark for 30 min. After being washed by PBS for 3 times, DNA staining was performed using Hoechst 33342 reaction solution for 15 min. The cells images were observed under a fluorescence microscope (Olympus). The percentage of EdU-positive cells was calculated from five random fields.

Cell apoptosis assay

The apoptosis of the NSC-34 cells were determined using FITC Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, the cells were plated in 6-well culture plates at a density of 1×10^6 /well, then cultured with 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, the cells were stained with 5 μl annexin V-FITC and 5 μl PI in darkness for 20 min at room temperature. After staining, apoptotic cell in each treatment (1×10^5 cells) was analyzed using a flow cytometry. All the 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) for 48 h after transfection to induce the neurite outgrowth. Then, cells were photographed using the microscope (Leica Microsystems CMS GmbH). The length of neurite was defined as the distance from the soma to the tip of the longest branch [28]. Five random fields were used for image analysis in each group. The data on neurite length were obtained from three independent experiments.

Statistical Analysis

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

Results

The expression of CaMKII-α and CaMKII-β were down-regulated in the spinal cord of ALS mice

CaMKII is the key molecule of Wnt/Ca^{2+} signaling pathway. To indicate the distribution and localization of CaMKII-α and CaMKII-β in ALS, their expression was detected using double immunofluorescence staining technique. The results showed that CaMKII-α and CaMKII-β were expressed in the anterior horn of gray matter of spinal cord and co-expressed with neurons labeled with β-tubulin III. Meanwhile, the number of CaMKII-α/β-tubulin III and CaMKII-β/β-tubulin III double-positive cells in ALS middle stage were reduced by 49.45% and 58.87% compared to WT mice in the ventral horn of gray matter ($p = 0.0037$, $p = 0.0001$, Fig. 1a-c).

To investigate the expression of CaMKII-α and CaMKII-β in ALS, western blotting technique was used to quantify the expression of CaMKII-α and CaMKII-β in the spinal cord of ALS mice at different stages. The expression of CaMKII-α proteins was decreased by 56.01% (at 95 d), 47.23% (at 108 d,) 54.91% (at 122 d), compared with WT mice. The differences were statistically significant ($p = 0.045$, $p = 0.023$, $p = 0.043$, Fig. 1d-g). The expression of CaMKII-β proteins was decreased by 35.95% (at 95 d), 48.40% (at 108 d,) 45.41% (at 122 d), compared with WT mice. The differences were statistically significant ($p = 0.022$, $p =$

0.001, $p = 0.035$, Fig. 1d-g). These results show that the pathogenesis of ALS affected the expression of CaMKII-α and CaMKII-β.

The level of Ca^{2+} and the expression of CaMKII-α and CaMKII-β were down-regulated in SOD1 mutant NSC-34 cells model

To further verify the effect of SOD1 gene mutation on the key signal molecules in Wnt/Ca^{2+} signaling pathway *in vitro*, a NSC-34 cell model was constructed by transfecting the pcDNA3.1-G93A-SOD1 and pcDNA3.1-WT-SOD1 plasmids. The expression levels of CaMKII-α and CaMKII-β as well as intracellular Ca^{2+} were detected at 48 h after transfection. The flow cytometry results demonstrated that the percent of Ca^{2+} was decreased by 16.02% in NSC-34 cells transfected with the pcDNA3.1-G93A-SOD1 plasmid, compared with that transfected with the pcDNA3.1-WT-SOD1 plasmid ($p = 0.048$, Fig. 2a, b). Western blot analysis showed that the expression of CaMKII-α and CaMKII-β proteins were decreased by 62.77% and 74.54% in NSC-34 cells that carried the pcDNA3.1-G93A-SOD1 plasmid than those that carried the pcDNA3.1-WT-SOD1 plasmid ($p = 0.022$, $p = 0.001$, Fig. 2c, d). These results indicate that the changes of CaMKII-α and CaMKII-β expression were consistent with those of animal model.

Overexpression and knockdown of Wnt5a up-regulated and down-regulated Ca^{2+}, CaMKII-α and CaMKII-β levels in SOD1 mutant NSC-34 cells

To study the regulation of Wnt5a on Wnt/Ca^{2+} signaling molecules in ALS, the expression levels of CaMKII-α and CaMKII-β as well as intracellular Ca^{2+} (level of SOD1 mutant NSC-34 cells) were detected at 48 h after over-expressing and knocking down Wnt5a. A correct intracellular Ca^{2+} level is crucial for MNs function [29]. The flow cytometry results showed the percent of Ca^{2+}in SOD1 mutant NSC-34 cells were increased by 6.68% after overexpressing Wnt5a and decreased by 12.97% after knocking down Wnt5a ($p = 0.036$, $p = 0.031$, Fig.3a-d). Western blot analysis showed that the expression levels of CaMKII-α and CaMKII-β proteins in SOD1 mutant NSC-34 cells increased 2.48 fold and 1.19-fold after overexpression of Wnt5a, while they decreased by 73.32% and 75.66% after knocking down Wnt5a, respectively ($p = 0.0003$, $p = 0.0017$, $p = 0.015$, $p = 0.03$, $p = 0.0003$, $p = 0.006$, Fig.3e-h). These results suggest that the level of intracellular Ca^{2+} and the expression levels of CaMKII-α and CaMKII-β are manipulated by overexpression and knockdown of Wnt5a.

Wnt5a overexpression promoted cells viability, proliferation and inhibited cells apoptosis, while Wnt5a silencing inhibited cells viability, proliferation and promoted cells apoptosis

To determine the role of Wnt/Ca^{2+} signaling pathway regulated by Wnt5a in ALS, we examined the cells viability, proliferation, apoptosis using the CaMKII inhibitor KN-93 (5 μM) and activator Oleic acid (10 μM) for 24 h ($p = 0.008$, $p = 0.048$, $p = 0.044$, $p = 0.049$, $p = 0.023$, $p = 0.041$, $p = 0.0026$, $p = 0.025$, Fig. 5a, b).

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 with that in the pcDNA3.1-Con+pcDNA3.1-G93A-SOD1

group. On the other hand, the 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% at 24, 48, 72 and 96 h after transfection, respectively (p = 0.011, p = 0.0006, p = 0.0078, p = 0.0009, p = 0.006, p = 0.0001, p = 0.0012, p = 0.0008, Fig.4c). The cell viability in the siRNA-Wnt5a+pcDNA3.1-G93A-SOD1 group decreased by 23.53%, 21.02%, and 27.69% compared with that in the siRNA-Con+pcDNA3.1-G93A-SOD1 group, whereas the cell viability in the siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA group significantly increased by 13.40%, 15.02% and 21.30% at 48, 72 and 96 h after transfection, respectively (p = 0.025, p = 0.049, p = 0.007, p = 0.047, p = 0.003, p = 0.02, Fig.4d). These findings propose that KN-93 blocked the increased cells growth caused by over-expressing of Wnt5a.

The EdU results showed that proliferation rate was increased 13.37% in pcDNA3.1-Wnt5a+pcDNA3.1-

G93A-SOD1 group in comparison with pcDNA3.1-Con+pcDNA3.1-G93A-SOD1 group and decreased 21.02% with KN-93 treatment after transfection at 48 h (p = 0.018, p = 0.003, Fig.5a, c). The proliferation rate was decreased 12.64% in siRNA-Wnt5a+pcDNA3.1-G93A-SOD1 group when compared with siRNA-Con+pcDNA3.1-G93A-SOD1 group and decreased 8.57% with OA treatment after transfection at 48 h (p = 0.043, p = 0.048, Fig.5b, d). MNs in the spinal cord are the main targets of degenerative neurons in ALS. High concentration of SOD1 may lead to the death of MNs [30].

Therefore, this study sought to examine the impact of Wnt5a on SOD1 mutant NSC-34 cells apoptosis. As seen in Fig 6a, c, the proportions of apoptotic cells were 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 = 0.006, p = 0.018). The proportions of apoptotic cells were 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-SOD1and siRNA-Wnt5a+pcDNA3.1-G93A-SOD1+OA group, respectively (p = 0.0075, p = 0.03, Fig.6b, d). These results suggested that KN-93 can reverse and OA can rescue the change of cells viability, proliferation and apoptosis induced by Wnt5a overexpression. Furthermore, the results indicate that Wnt5a helped improve SOD1 mutant NSC-34 cells viability, proliferation and apoptosis. Finally, it can be deduced from the results that the expression level of Wnt5a can affect SOD1 mutant NSC-34 cells viability, proliferation and apoptosis by regulating the Wnt/Ca^{2+} pathway.

All the above results supported that KN-93 can block the Wnt/Ca^{2+} pathway, while OA can activate the Wnt/Ca^{2+} pathway in ALS. Wnt5a may mitigate neurodegeneration and protect MNs through the Wnt/Ca^{2+} pathway in ALS.

Discussion

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease of MNs in the cortex, brainstem and spinal cord [5]. It was described by French neurologist Charcot in 1869 that, affects the upper and lower MNs [32]. Advanced stage of the disease may lead to the loss or death of MNs with the loss of

motor function [33]. The ALS pathogenic genes include C9ORF72, SOD1, TARDBP, FUS, OPTN, PFN1, MATR3, TUBA4A, and TBK1 among others [34]. SOD1 gene was the first gene associated with ALS, in 1993 [3,35]. Previous researches showed that massive MNs died in the ventral horn, as well as the loss of myelinated axons in the ventral motor roots of the spinal cord in $SOD1^{G93A}$ animal model [36].

Wnt signaling pathway regulates multiple cell functions and controls many aspects of development, including cell proliferation, apoptosis, migration and cell polarity [21]. 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. The activation of Wnt/β-catenin signaling pathway is related to the viability of Parkinson's Diseases (PD) dopaminergic neurons, which is conducive to maintain the integrity of dopaminergic neurons. Hence, targeting Wnt signaling molecules may confer therapeutic effects in neurodegenerative diseases [37-39].

Previously, the differential expression of 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. Meanwhile, the expression of non-canonical Wnt ligand Wnt5a is also abnormal in ALS mice [40, 41]. In the current study, it was confirmed that canonical Wnt signaling pathway can be activated in the pathogenesis of ALS [4]. Furthermore, it was found that Wnt1, a canonical Wnt signaling molecule, can promote the proliferation and viability of SOD1 mutant NSC-34 cells, inhibit cell apoptosis and protect MNs. In addition, silencing RNA helicase DDX3 was found to inhibit the proliferation of SOD1 mutant NSC-34 cells, promote cell apoptosis and affect the outgrowth of neurite through Wnt signaling molecule CK1ε [42]. These results indicate that 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 [43,44]. Wnt5a is a key signaling molecule that participates in the non-canonical Wnt signaling pathway. In our previous study, Wnt5a abnormal expression was detected in the spinal cord of ALS mice [41]. Wnt5a mediated non-canonical signaling pathway plays an important role in neuronal damage [31,45]. In mammals, Wnt5a activates intracellular Ca^{2+} release and further activates CaMKII to regulate neurite outgrowth and cell function mediated by Wnt/Ca^{2+} pathway [46]. Spalloni *et al.* [47] found a decrease of the CaMKII-α autophosphorylation at threonine-286 in cortical M1 region of mice overexpressing the $SOD1^{G93A}$ gene. In this study, it was found that the number of CaMKII-α/β-tubulin III and CaMKII-β/β-tubulin III double-positive cells in ALS middle stage were less than WT mice in the ventral horn of gray matter, which indicated that CaMKII-α, and CaMKII-β expression were related to the degeneration of MNs in ALS. The results of qRT-PCR and western blotting also showed that the expression of CaMKII-α and CaMKII-β were decreased in the onset period, which demonstrated that CaMKII-α and CaMKII-β were involved in the development of ALS.

Intracellular Ca^{2+} homeostasis is disrupted in many neurodegenerative diseases. High stimulation of glutamate receptor in MNs and subsequent excitotoxicity induced by increased calcium ion implantation may lead to MNs degeneration in ALS patients and SOD1 mutant mice model [48]. This study detected

that the intracellular Ca^{2+} level of NSC-34 cells carrying the pcDNA3.1-G93A-SOD1 plasmid was lower than those of NSC-34 cells transfecting the pcDNA3.1-WT-SOD1 plasmid at 48 h. CaMKII is a key molecule in the pathological cascade downstream of abnormal Ca^{2+} signaling [49-51]. Our results showed that mRNA and protein levels of CaMKII-α and CaMKII-β 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 of SOD1 reduced the expression of CaMKII-α and CaMKII-β, indicating that Wnt/Ca^{2+} signaling pathway was involved in the pathogenesis of ALS.

To determine whether Wnt5a regulate 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 and CaMKII-β expression, whereas knockdown of Wnt5a down-regulated them. These results suggested that Wnt5a positively regulated the intracellular Ca^{2+} level and expression of CaMKII-α and CaMKII-β in ALS.

The loss of MNs was also linked to the pathogenesis of ALS. Martin [52] found that apoptosis was activated in ALS. SOD1 is an antioxidant enzyme, which can protect neurons from the damage of free superoxide radicals. The mutation of SOD1 can stimulate protein aggregation and lead to apoptosis [53]. Han et al. [54] found that the increase of Wnt5a promoted cell proliferation. On the contrary, knocking down Wnt5a reduced cell proliferation. This study supports that overexpression of Wnt5a promoted the cell proliferation of SOD1 mutant NSC-34 cells and knockdown of Wnt5a reduced the cell proliferation of SOD1 mutant NSC-34 cells. Meanwhile, over-expressing Wnt5a reduced the cell apoptosis of SOD1 mutant NSC-34 cells and knockdown of Wnt5a promoted the cell apoptosis of SOD1 mutant NSC-34 cells. These findings imply that Wnt5a is important for cell growth and exerted neuroprotective effect through Wnt/Ca^{2+} pathway on SOD1 mutant NSC-34 cells.

Neurons are made of three distinct sections: the soma, which contains the nucleus and the majority of the cellular organelles, a long axonal process to transmit information, and a complex dendritic arbor that receives information from neighboring neurons [55, 56]. 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 of cytoskeleton. $SOD1^{G93A}$ mutation can affect the cytoskeleton state to regulate the neurite outgrowth of MNs [57]. CaMKII is a cytoskeleton-related protein. Overexpression of CaMKII-α leads to neurite outgrowth [58]. Lund et al. [23] confirmed that the expression of CaMKII was changed after axonal injury in the sciatic MNs. To clarify whether Wnt5a regulates the neurite outgrowth to affect the function of ALS MNs, this study detected the neurite outgrowth of SOD1 mutant NSC-34 cells using RA. Overexpression of Wnt5a increased the length of SOD1 mutant NSC-34 cells and the percentages of cells with one or more neurites. Similarly, knocking down Wnt5a inhibited the neurite outgrowth of SOD1 mutant NSC-34 cells. It is suggested that Wnt5a may participate in neuronal degeneration by regulating the neurite outgrowth in ALS.

Finally, specific CaMKII inhibitor KN-93 and activator OA were used to verify the role of Wnt/Ca^{2+} pathway mediated by Wnt5a. The results showed that KN-93 reversed the NSC-34 cells viability, proliferation, apoptosis and neurite outgrowth after overexpression of Wnt5a and OA rescued the NSC-34 cells viability, proliferation, apoptosis and neurite outgrowth after knocking down Wnt5a. These results suggested that Wnt5a regulate the cells function to affect neuron motor degeneration through Wnt/Ca^{2+} signaling pathway. In Arredondo's study, they determined that Wnt5a signals induce neurogenesis through CaMKII, and promotes dendritic development of newborn neurons through activating Wnt/JNK and Wnt/CaMKII signaling using specific inhibitors [59]. These results are in line with those results of us. Wnt5a has certain significance and value for the continuous research, development and clinical application of ALS. However, whether Wnt5a regulates canonical and non-canonical Wnt signaling pathways through binding different cell receptors in development of ALS is still unclear and needs further research.

Conclusions

CaMKII plays an important role in the degeneration of MNs in ALS. Wnt5a can protect SOD1 mutant NSC-34 cells from death by regulating cells viability, proliferation, apoptosis and neurites outgrowth through the Wnt/Ca^{2+} signaling pathway. This indicates that Wnt5a participates in the regulation of neuronal degeneration in ALS.

Abbreviations

ALS: Amyotrophic lateral sclerosis; MNs: Motor neurons; CaMKII: Ca^{2+}/Calmodulin dependent protein kinase II;

CNS: central nervous system; SOD1: superoxide dismutase 1; WT: wild-type; AD: Alzheimer's disease; FBS: fetal bovine serum; DMEM: Dulbecco's Modified Eagle Medium; PCR: Polymerase Chain Reaction; siRNA: small interfering RNA; OD: Optical Density; AD: Alzheimer's disease; PD: Parkinson's Diseases

Declarations

Acknowledgments

Not applicable.

Authors' contributions

Y.G. and Y.C. designed the study and drafted the manuscript. Y.C. and H.Z. supervised the study. J.L., F.Z., Z.Z., F.M., X.W., X.J., Q.W. and L.Z. performed experiments and data analysis. All authors read and approved the final manuscript.

Funding

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 (2019KJK004), Key Project of Shandong Province Higher Educational Science and Technology Program of China (Grant No. J18KZ013).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Medical Ethics Committee of Weifang medical university (protocol code2018-No.156 and date of approval 26 February 2018).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

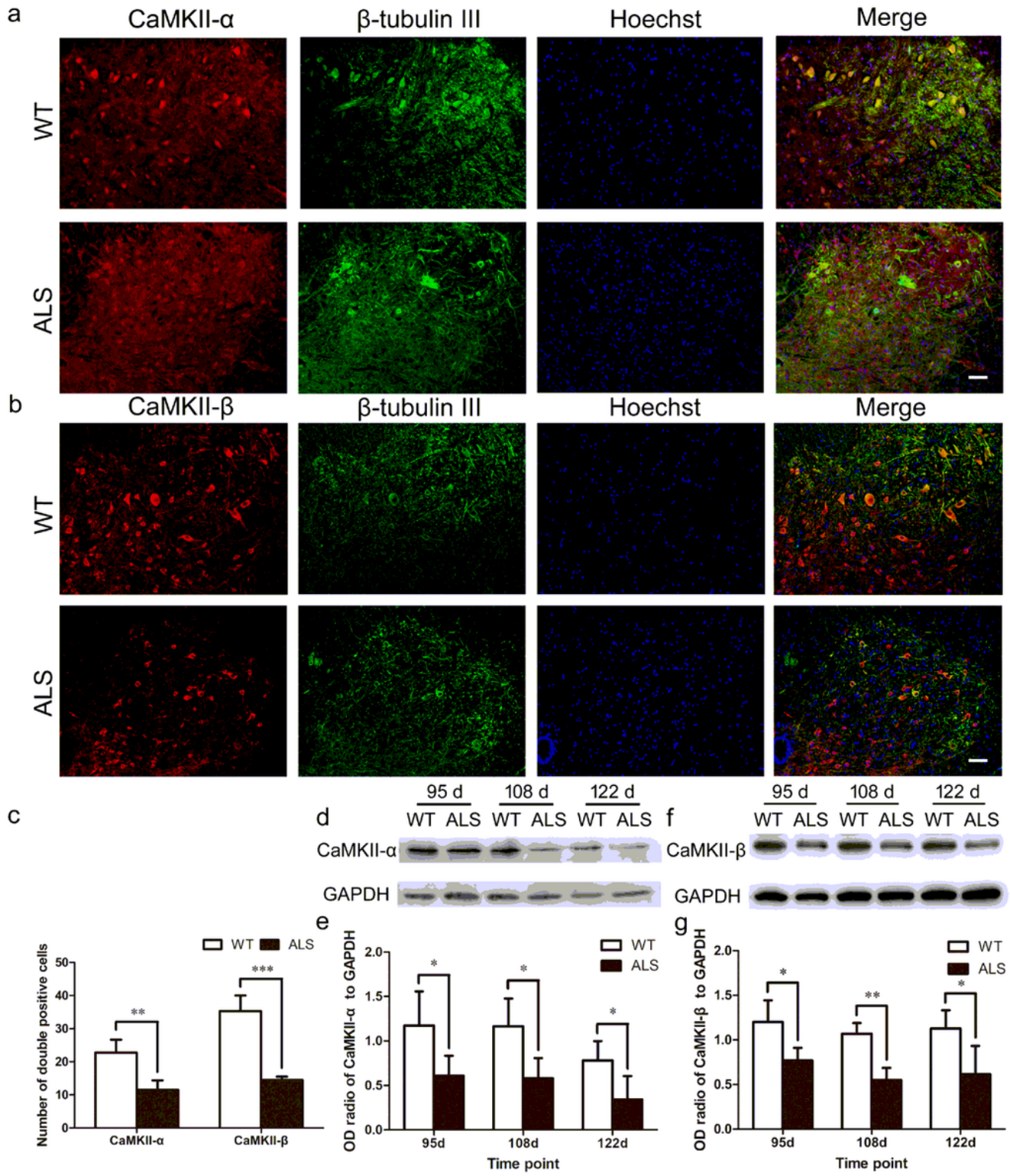

Figure 1

Expression of CaMKII-α and CaMKII-β in the spinal cord of ALS mice and WT mice. a-b Co-localization of CaMKII-α and CaMKII-β as detected by immunofluorescence double staining in the anterior horn of the spinal cords gray matter of 108-day-old mice. Scale bar = 100 μm. c Bar chart showing the number of CaMKII-α/β-tubulin III and CaMKII-β/β-tubulin III double-positive cells as measured by ImageJ software. d, f Protein bands of CaMKII-α and CaMKII-β in the spinal cord. e, g Bar chart showing the protein level of

CaMKII-α and CaMKII-β as determined by western blotting assay. GAPDH was used as the internal control. *p < 0.05, **p < 0.01, ***p < 0.001 vs. WT

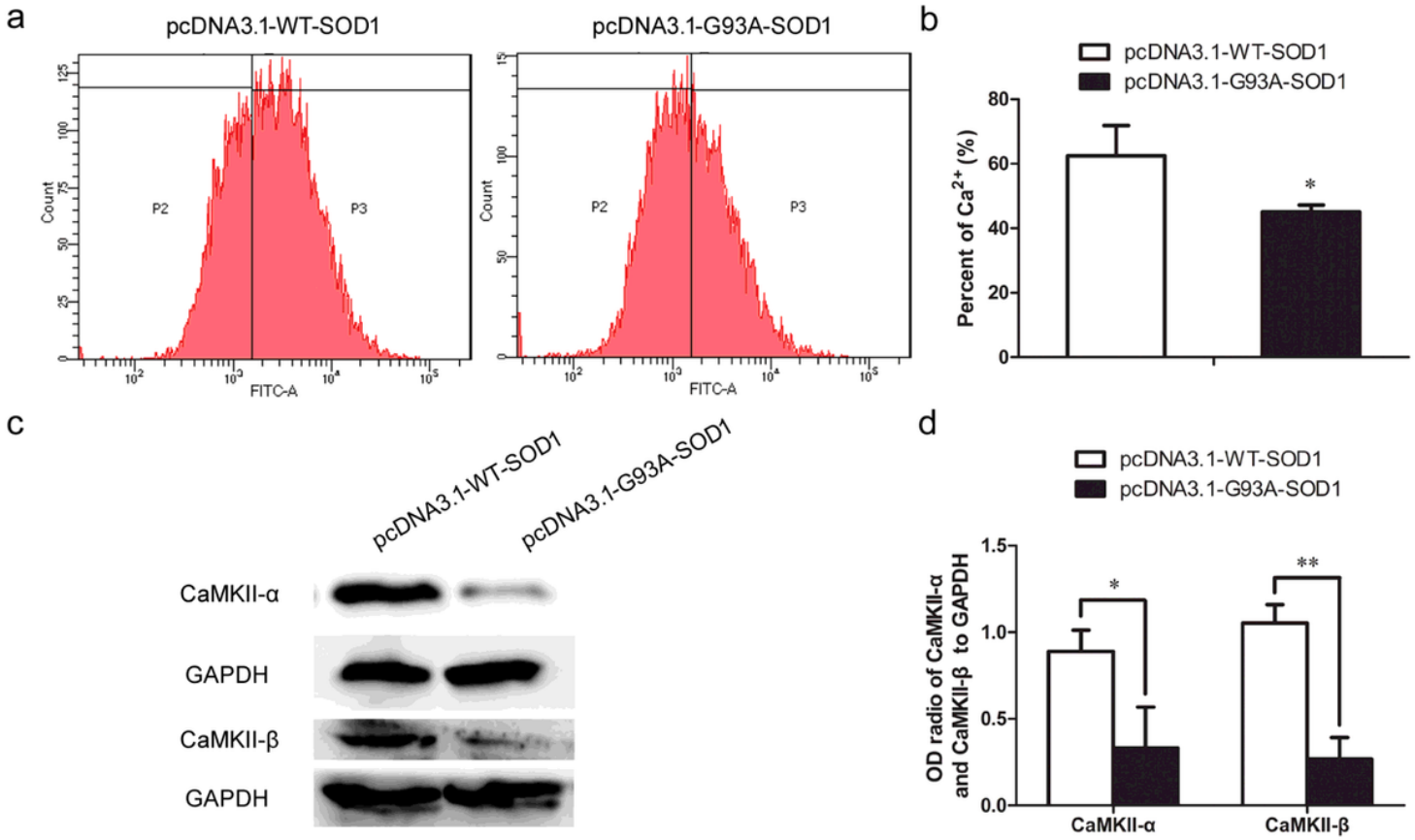

Figure 2

Changes of Ca2+, CaMKII-α, and CaMKII-β expression level in NSC-34 cells carrying SOD1-G93A mutation. a Two-matrix chart showing the intracellular Ca2+ 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 Ca2+ 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 and CaMKII-β in NSC-34 cells transfected with pcDNA3.1-WT-SOD1 and pcDNA3.1-G93A-SOD1 plasmid for 48 h. d Bar chart showing the protein level of CaMKII-α and CaMKII-β as analyzed by western blotting. GAPDH was used as the internal control. *p < 0.05, **p < 0.01, vs. pcDNA3.1-WT-SOD1

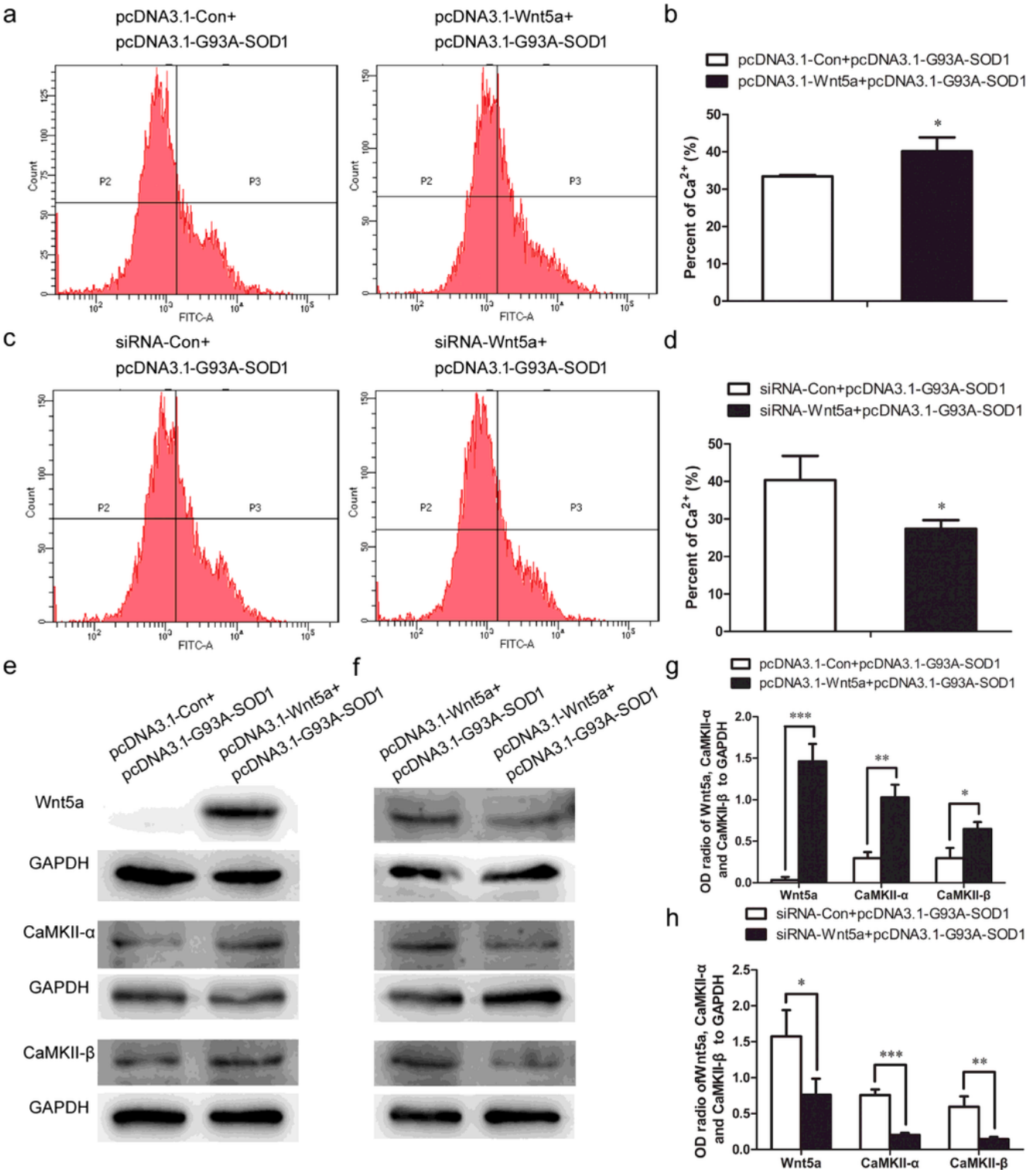

Figure 3

Wnt5a Overexpression up-regulated intracellular Ca2+ level, CaMKII-α and CaMKII-β expression in SOD1 mutant NSC-34 cells. a, c Two-matrix chart showing the intracellular concentration of Ca2+ in NSC-34 cells after transfection for 48 h. b, d Bar chart displaying the percentage of Ca2+ in NSC-34 cells after transfection for 48 h. e, f Protein bands of CaMKII-α and CaMKII-β in NSC-34 cells after transfection for 48 h. g, h Bar chart indicating the protein level of CaMKII-α and CaMKII-β as analyzed by western blotting.

GAPDH was used as the internal control. d, e The protein level of CaMKII-α and CaMKII-β as analyzed by qRT-PCR. 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

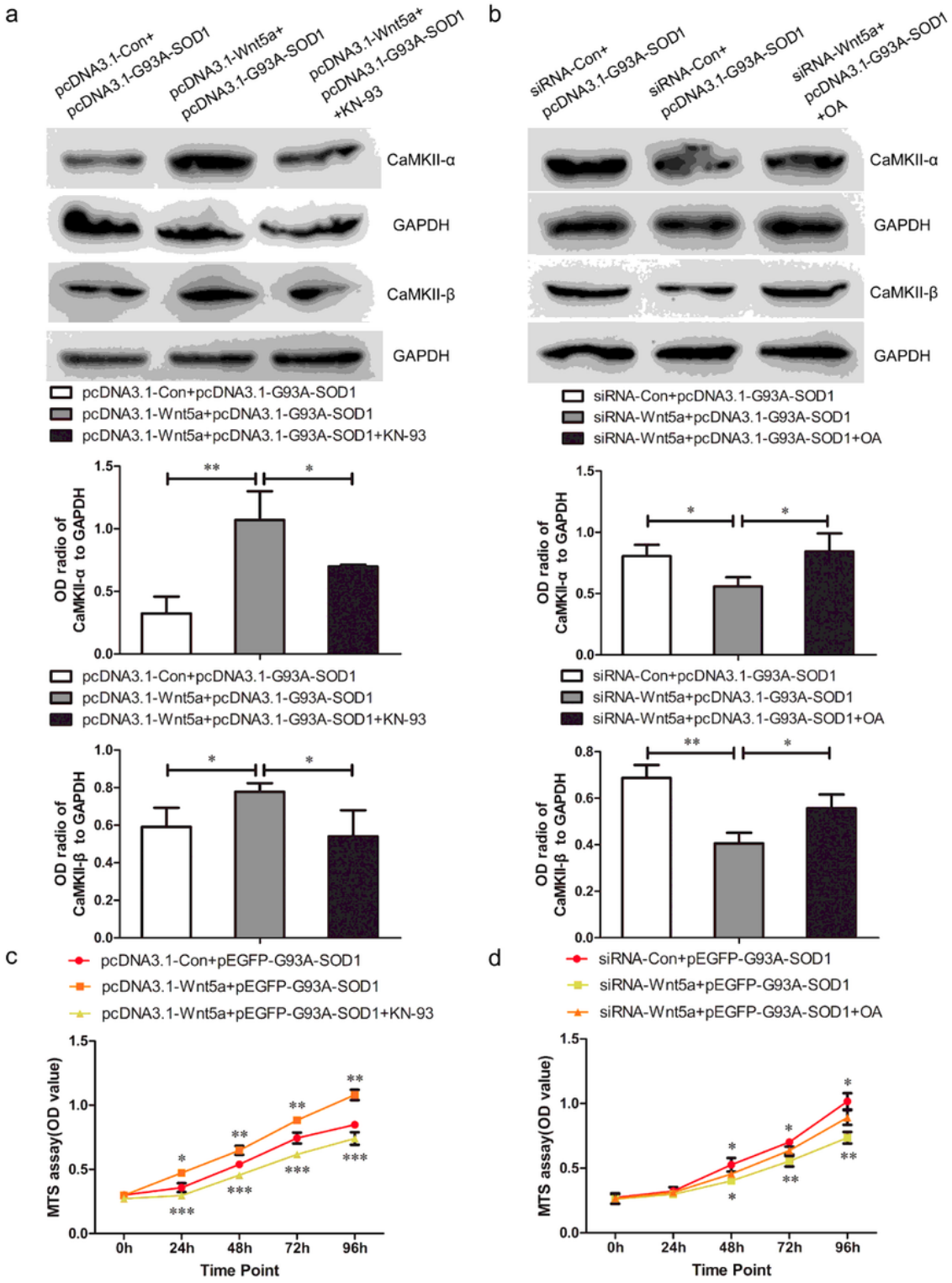

Figure 4

KN-93 and OA Effect of SOD1 mutant NSC-34 cells viability after Wnt5a overexpression and knockdown. a, b The protein level of CaMKII-α and CaMKII-β as analyzed by qRT-PCR. GAPDH served as the internal

control. c, d Line chart showing the viability of NSC-34 cells after transfection for different durations as determined with the 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 EdU results showed that proliferation rate was increased 13.37% in pcDNA3.1-Wnt5a+pcDNA3.1- G93A-SOD1 group in comparison with pcDNA3.1-Con+pcDNA3.1-G93A-SOD1 group and decreased 21.02% with KN-93 treatment after transfection at 48 h (p = 0.018, p = 0.003, Fig.5a, c). The proliferation rate was decreased 12.64% in siRNA-Wnt5a+pcDNA3.1-G93A-SOD1 group when compared with siRNA-Con+pcDNA3.1-G93A-SOD1 group and decreased 8.57% with OA treatment after transfection at 48 h (p = 0.043, p = 0.048, Fig.5b, d). MNs in the spinal cord are the main targets of degenerative neurons in ALS. High concentration of SOD1 may lead to the death of MNs [30].

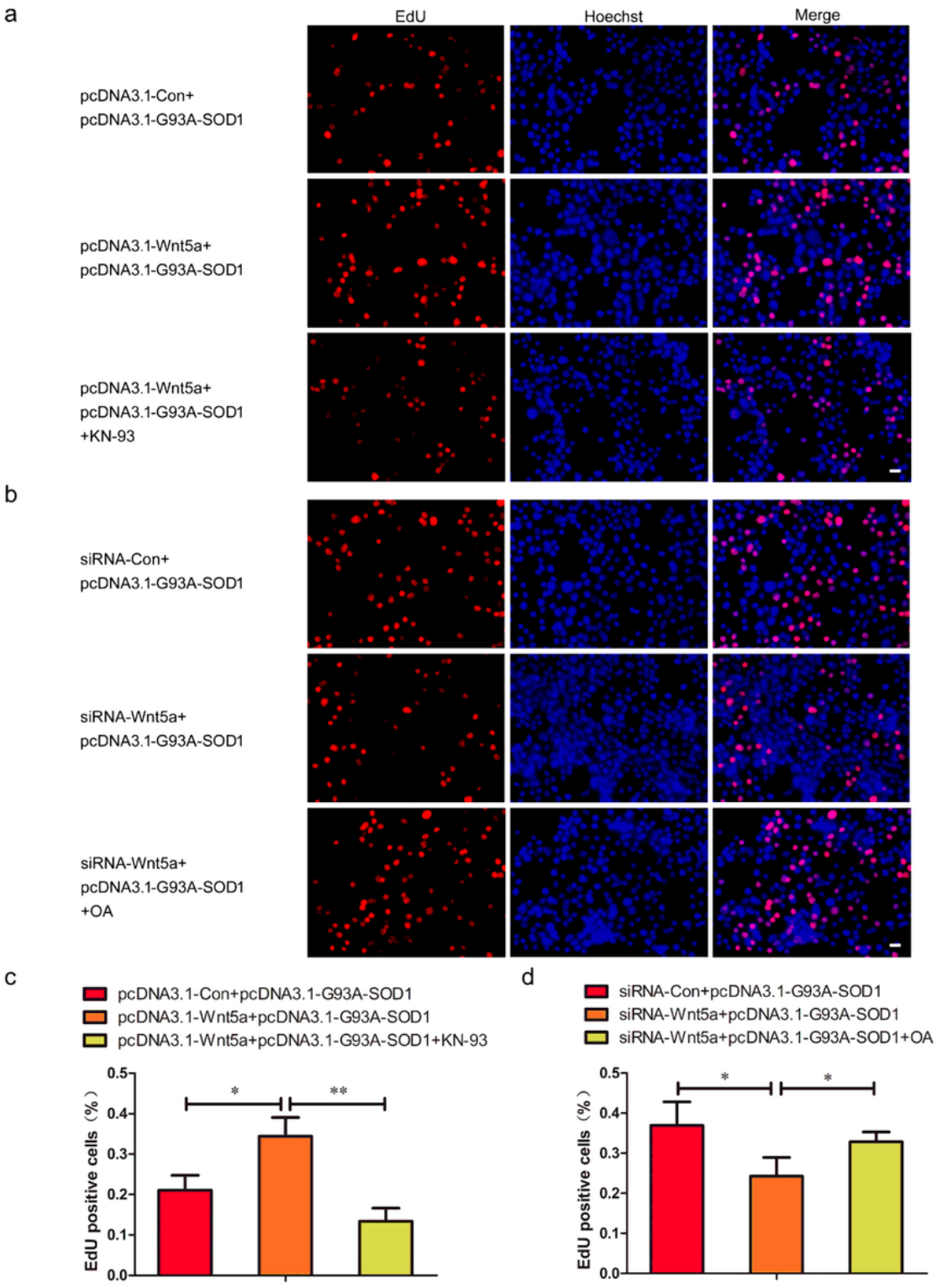

Figure 5

KN-93 and OA Effect of SOD1 mutant NSC-34 cells proliferation after Wnt5a overexpression and knockdown. a, b Representative images of 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 plasmid. d Bar chart showing that OA treatment for 48 h rescued the decreased proliferation of NSC-34 cells after co-transfecting siRNA-

Wnt5a and pcDNA3.1-G93A-SOD1 plasmid. *p < 0.05, **p < 0.01. vs. pcDNA3.1-Con and pcDNA3.1-G93A-SOD1, siRNA-Con and pcDNA3.1-G93A-SOD1

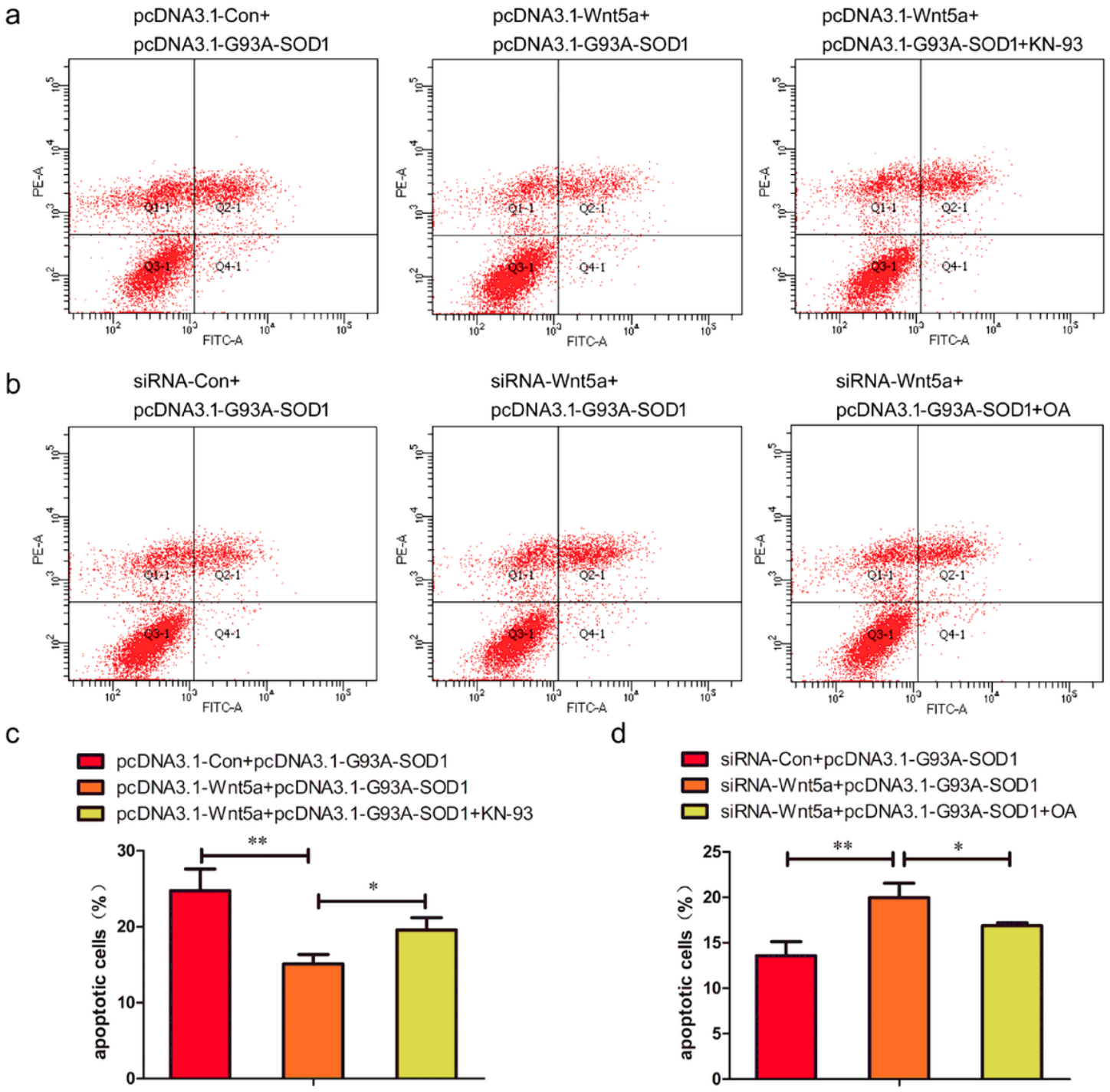

Figure 6

KN-93 and OA Effect of SOD1 mutant NSC-34 cells apoptosis after Wnt5a overexpression and knockdown. a, b Four-matrix chart showing apoptosis of cells as 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 plasmid. *p < 0.05, **p < 0.01. vs. pcDNA3.1-Con and pcDNA3.1-G93A-SOD1, siRNA-Con and pcDNA3.1-G93A-SOD1

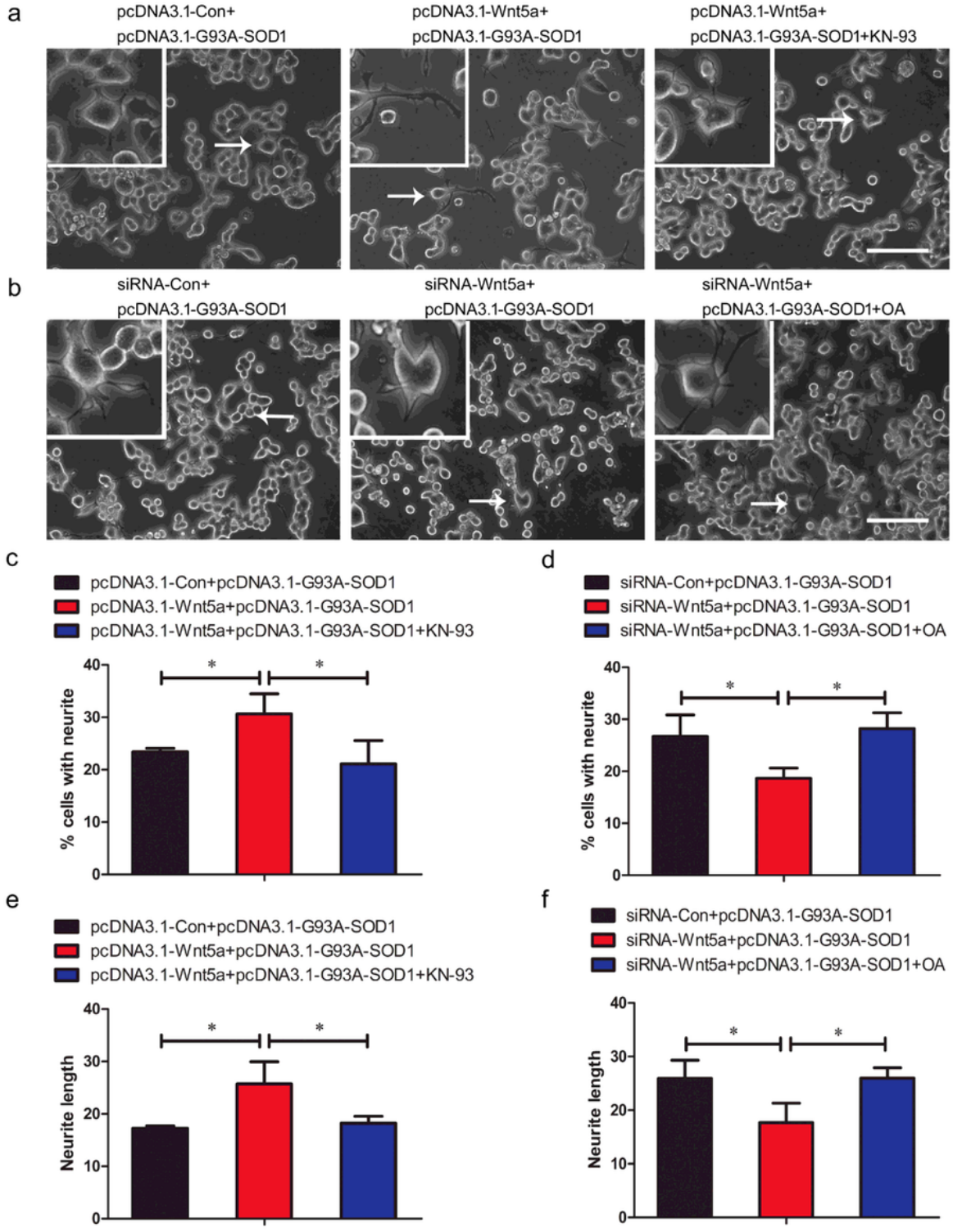

Figure 7

KN-93 and OA Effect of SOD1 mutant NSC-34 cells neurite outgrowth after Wnt5a overexpression and knockdown. a, b Representative images showing neurite outgrowth in NSC-34 cells. Scale bar = 100 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 plasmid for 48 h. *p < 0.05. vs. pcDNA3.1-Con and pcDNA3.1-G93A-SOD1, siRNA-Con and pcDNA3.1-G93A-SOD1

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