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Phosphorylation mutation impairs the promoting effect of spastin on neurite outgrowth without affecting its microtubule severing ability

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ABSTRACT

Spastin, a microtubule-severing enzyme, is known to be important for neurite outgrowth. However, the role of spastin post-translational modification, particularly its phosphorylation regulation in neuronal outgrowth, remains unclear. This study aimed to investigate the effects of eliminating spastin phosphorylation on the neurite outgrowth of rat hippocampal neurons. To accomplish this, we constructed a spastin mutant with eleven potential phosphorylation sites mutated to alanine. The phosphorylation levels of the wildtype spastin (WT) and the mutant (11A) were then detected using Phos-tag SDS-PAGE. The spastin constructs were transfected into COS7 cells for the observation of microtubule severing, and into rat hippocampal neurons for the detection of neuronal outgrowth. The results showed that compared to the spastin WT, the phosphorylation levels were significantly reduced in the spastin 11A mutant. The spastin mutant 11A impaired its ability to promote neurite length, branching, and complexity in hippocampal neurons, but did not affect its ability to sever microtubules in COS7 cells. In conclusion, the data suggest that mutations at multiple phosphorylation sites of spastin do not impair its microtubule cleavage ability in COS7 cells, but reduce its ability to promote neurite outgrowth in rat hippocampal neurons.

Key words: Spastin; phosphorylation; hippocampal neurons; COS7, microtubule severing; neurite outgrowth.

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Data Availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Introduction

Hereditary spastic paraplegia (HSP) is a neurodegenerative disorder that is characterized by progressive spasticity in the lower limbs caused by loss of corticospinal tract function.[1] The pathogenesis of HSP is thought to be associated with the degeneration of the distal corticospinal axons, which are the major nervous system pathway that connects the motor cerebral cortex to the spinal cord.[2,3] Currently, mutations in the *SPAST* gene are considered to be the most common cause of hereditary spastic paraplegia.[4]

Spastin belongs to the ATPase associated with cellular activities (AAA) protein family, which is known for its ability to sever microtubules into smaller fragments and this makes spastin an important protein in processes such as cell division and neuronal development.[5] Because the *SPAST* gene contains two start codons and different intron splicing patterns, there are four isoforms of spastin in mammals: M1 (full-length), M87 (C-terminal 86 amino acids truncated), and their splice variants M1Δexon4 and M87Δexon4 that lack exon 4.[6,7] Unlike the M1 isoform, which is predominantly distributed in the adult spinal cord, the M87 isoform is abundant in the brain, spinal cord, and various cell lines.[8] Notably, both isoforms of spastin contain microtubule-interacting and trafficking (MIT) domains at the N-terminal, AAA domains at the C-terminal, and a microtubule-binding domain (MTBD) (Figure 1A).[7]

Among the different domains of spastin, the MIT domain has been reported to be involved in regulating cell membrane transport or vesicle trafficking, which is essential for the maintenance of the axons that are affected in HSP.[9] In addition to its role in regulating cell membrane transport, the interaction between the MIT domain of spastin and endosomal sorting complexes required for transport (ESCRT)-III also plays an important role in cell division and endosomal transport.[10,11] The MTBD domain is a region where spastin and microtubules interact, and the AAA domain catalyzes microtubule severing by mediating the formation of hexamers of spastin.[12] Although previous studies have found that nonsense or missense mutations in the *SPAST* gene may cause HSP, the specific mechanism by which these mutations cause the disease remains to be further explored.

Neurite outgrowth is a crucial process for the formation of the nervous system, laying the foundation for neuronal morphogenesis, transcriptional programs, and synaptic activity.[13] Disruptions in neurite outgrowth can lead to defects in the formation and organization of neurons and the nervous system. However, the underlying mechanisms of neurite outgrowth and guidance remain largely unknown. Microtubules are the basis for growth guidance during neuronal process development, generating adjustable forces through molecular motor proteins.[14,15] Thus, microtubules play an important role in neuronal development by participating in the assembly of the cytoskeleton. Our previous studies have demonstrated that spastin can effectively promote neurite outgrowth by regulating microtubule dynamics,[16,17] and the protein stability of spastin is essential for neurite outgrowth.[18] However, the regulation of protein function is not only related to the amount of protein expression but also closely related to the post-translational modification of proteins. Phosphorylation is a common post-translational modification of proteins,[19] which can regulate the transduction of cell signaling pathways, gene expression, cell cycle, and other cellular activities. Multiple phosphorylation sites within spastin have been identified by proteomics analysis, which is presented in the PhosphositePlus program.[20] HIPK2 is the only enzyme that has been reported to phosphorylate spastin at S268 and inhibit spastin ubiquitination mediated by K554, thereby inhibiting spastin protein degradation in cells.[21] Moreover, this phosphorylation ensures the centrosome localization of spastin during cell mitosis.[21] However, the function of other sites of spastin phosphorylation modification, especially in regard to microtubule severing function and neurite outgrowth, remains to be further elucidated

In this study, we constructed a spastin mutant with multiple phosphorylation sites mutated to alanine to investigate the effects of these mutations on the function of spastin on microtubules severing and neurite outgrowth.

Materials and Methods

Animals and cells

Specific pathogen-free Sprague Dawley (SD) male rats procured on the day of birth (P0) were procured from Southern Medical University (animal approval SCXK-2021-0041). All animal experiments were conducted in accordance with the Regulations of the People's Republic of China on the Administration of Laboratory Animals and were reviewed and approved by the Ethics Committee of Jinan University (approval no. 20210223-03). COS7 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

Plasmid construction

The spastin gene (NCBI: NM_014946.3; human spastin WT) was obtained from the NCBI database. GFP-spastin was ligated by spastin WT cDNA and pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA, USA; cat. no. #P0134) as described previously.22 Eleven phosphorylation sites (S207, Y212, S245, S261, S268, Y269, T292, S302, T303, T305, T306) that were identified by proteomics as being present in 3 or more records were selected for mutation from the records in the PhosphositePlus database (https://www.phosphosite.org).[20] Specific recombinant primers were designed by CE Design (Version 1.04) (Table 1). The spastin mutant (abbreviated as spastin 11A) was constructed by the GeneArt Site-Directed Mutagenesis PLUS Kit (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA; cat. no. #A14606) in which all 11 phosphorylation sites were mutated into alanine.

Cell culture and transfection

COS7 cells were cultured in a 5% CO_2 incubator at 37°C using DMEM (Gibco, Thermo Fisher Scientific, Inc., Waltham, USA;cat. no. #C11995500BT) containing 10% fetal bovine serum (Gibco; cat. no. #10270-106). When the cell confluence of the 24-well plate reached 70%, the cells were transfected according to the ratio of 500 ng plasmid and 2 μL of Lipofectamine 2000™ (Invitrogen; cat. no. #11668019) per well. After transfection, the cells were cultured for 18-24 h for follow-up experiments. Hippocampal neurons were extracted from the brain tissue of newborn rats under anesthesia. The hippocampus was isolated from brain tissue using ophthalmic forceps under a microscope. Hippocampal tissue was digested with 10 mg/mL papain (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, cat. no. #P4762-P4762) for 25 min to obtain isolated neurons. The cells were inoculated at a density of $1\times10^4/cm^2$ onto coverslips treated with poly D-lysine (Sigma-Aldrich; cat. no. #P6407) the day before. Hippocampal neurons were cultured *in vitro* for 3 days in Neurobasal-A (Gibco; cat. no. #10888-022) containing 2% B-27 (Gibco; cat. no. #17504-044) in an incubator at 37°C with 5% CO_2, with half of the medium being changed. Transfection of hippocampal neurons inoculated with different recombinant plasmids in 24-well plates was done using a calcium phosphate transfection kit (Beyotime, Shanghai, China; cat. no. #C0508).

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Western blotting and Phos-tag assay

After the cells were lysed on ice, the protein was quantified using the BCA Kit (CoWin Biosciences; Jiangsu, China, cat. no. CW00145). The same amount of extracted protein was used for electrophoretic separation in a 10% SDS-PAGE gel. The protein was then transferred to a PVDF membrane (Gibco; cat. no. #IPVH00010) under the conditions of 100V and 250 mA. The PVDF membrane (Gibco; cat. no. #IPVH00010) was incubated with an appropriate amount of 5% skim milk (BD Difco; cat. no. #232100-500G) at room temperature for 1 h. The membranes were then incubated with primary antibody dilutions (Meilunbio, Dalian, China; cat. no. #MB9881) containing GFP antibodies (Abcam, Cambridge, UK; cat. no. #Ab290, 1:1000) at 44°C overnight. After incubating with the appropriate concentration of secondary antibodies (Abcam; cat. no. #AS038, 1:5000) at room temperature for 1 h, the blots were exposed in an ImageQuant LAS 500 (GE HealthCare, Marlborough, MA, USA).

For Phos-tag assay, an 8% polyacrylamide gel containing 50 μmol/L Phos-tag Acrylamide (NARD Institute Ltd., Amagasaki City, Japan, cat. no. A801037) was configured for the subsequent separation of phosphorylated and unphosphorylated proteins. The follow-up method was similar to Western blotting, except that the PVDF membrane was rinsed 1-3 times with transfer solution containing EDTA and transfer solution without EDTA before transmembrane transfer.

Immunofluorescence staining

The method of the immunofluorescence experiment is the same as previously described.[22] The transfected neurons were fixed at 4°C for 45 min with 4% paraformaldehyde. Coverslips were punched in Tris buffer containing 0.1% Triton X-100 (TBST, Meilunbio; cat. no. MB2486) at room temperature for 20 min. Subsequently, TBST with 3% fetal bovine serum albumin (BSA, Sigma; cat. no. V900933-100G) was used to block the subsequent antibody incubation for 1 hour. Primary and secondary antibodies were diluted with TBST containing 3% BSA. The primary antibody, anti-Tubulin (Abcam; cat. no. ab6160, 1:1000), was used at 4°C overnight. The secondary antibody, Alexa Fluor 647 (Abcam; cat. no. ab150115, 1:1000), was incubated for 1 h at room temperature. Fluro-Gell II with DAPI (Electron Microscopy Sciences; Hatfield, PA, USA; cat. no. 17985-50) was used to seal the coverslips. Cells incubated with no primary antibody were used as negative control. All the COS7 cell images were acquired with the same optical slice thickness for every channel using a 63× oil objective and a resolution of 1024 ×1024 pixels by a Carl Zeiss LSM 780 confocal microscope (Zeiss AG, Oberkochen, Germany). The fluorescence intensity of the microtubules was determined by intensity calculation. A minimum of 20 neuronal cells per group were counted. The total length of each neurite, the number of branches per neurite, and Sholl analysis were counted using Image-Pro Plus 6 software (Media Cybernetics, Silver Spring, MD, USA). All these procedures were performed in a blind manner, and examinations were performed at least three times.

Statistics

All the data were analyzed by GraphPad Prism 5. One-way ANOVA was used for the comparison between the multiple groups and Student's *t*-test for two groups. All data are expressed as mean ± SD. A p-value <0.05 was considered to be statistically significant.

Results

Phosphorylation levels are significantly decreased in the spastin 11A mutant

Based on the extensive mass spectrometry data in the PhosphositePlus program, phosphorylation sites of human spastin that had been reported at least three times were selected, and the phosphorylation sites and distribution in the protein were shown (Figure 1A). Subsequently, the encoding plasmid of spastin 11A was constructed by PCR (Figure 1B), and sequencing was performed. The successful construction of the mutant spastin 11A plasmid was confirmed by sequence alignment with the wild-type spastin (Figure 1C). To test whether mutations at these sites would affect the phosphorylation status of spastin, a Phos-tag assay was applied to detect the phosphorylation status between GFP-spastin 11A and GFP-spastin WT. Phos-tag acrylamide can bind the phosphate groups on the protein and slow down the migration rate in the gel to achieve the separation of proteins with different phosphorylation states.[23] The expression of recombinant proteins was first confirmed in COS7 cells (Figure 2A). The results of the Phos-tag SDS-PAGE assay showed that the phosphorylation level of GFP-spastin 11A mutant was significantly reduced compared with GFP-spastin WT (Figure 2 B,C). These data indicated that we had successfully constructed the spastin 11A mutant, and the phosphorylation level of spastin decreased significantly after the mutation.

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Table 1. Primer sequences of spastin phosphorylation site mutation.

Gene		Primer sequence
GFP-spastin WT	F	tacaagtccggactcagatctATGGCCGCCAAGAGGAGC
	R	gtaccgtcgactgcagaattcTTAAACAGTGGTGTCTCCAAAGTCC
Spastin (S207, Y212)	F	CCATTTTCCAAGGCACAAACGGACGTCGCTAATGACAGTACT
	R	AGTACTGTCATTAGCGACGTCCGTTTGTGCCTTGGAAAATGG
Spastin (S245)	F	CACACTAGTAATGCACTGCCTCGTTCA
	R	TGAACGAGGCAGTGCATTACTAGTGTG
Spastin (S261)	F	TCTGCAGGCCTTGCAGGCCACCAT
	R	ATGGTGGCCTGCAAGGCCTGCAGA
Spastin (S268, Y269)	F	AGAGCACCTGCTGCCAGTGGTTTATCC
	R	GGATAAACCACTGGCAGCAGGTGCTCT
Spastin (T292)	F	ACTCATAAGGGTGCTCCGAAAACAAT
	R	ATTTGTTTTCGGAGCACCCTTATGAGT
Spastin (S302, T303, T305, T306)	F	ACAAATAAACCTTCTACCCCTACAACTGCTACTCGTAAG
	R	CTTACGAGTAGCAGTTGTAGGGGTAGAAGGTTTATTTGT

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The spastin 11A mutant retains the microtubule severing activity

To clarify the effect of spastin 11A on its microtubule severing ability, we used an intracellular model to quantify microtubule severing by immunofluorescence staining. COS7 cells have a relatively flat morphology and obvious microtubules compared to neurons. Overexpression of microtubule severing proteins, such as spastin or katanin, within COS7 cells and staining of microtubules are currently commonly used to quantify microtubule severing activity, as previously reported.[18,24] The results showed that compared to the fluorescence intensity of microtubules in COS7 cells in the GFP vector control group, both GFP-spastin WT and GFP-spastin 11A significantly reduced the fluorescence intensity of microtubules (Figure 3 A,B). Interestingly, the difference between the GFP-spastin 11A group and the GFP-spastin WT group was not statistically significant ($p>0.05$) (Figure 3B). These data suggest that the spastin 11A mutant retains the same microtubule severing activity as the wildtype.

The spastin 11A mutant inhibits the promotion of neurite outgrowth

Overexpression of spastin WT promotes neurite outgrowth in cultured hippocampal neurons *in vitro*.[16] Here, GFP, GFP-spastin WT, and the GFP-spastin 11A mutant were transfected into hippocampal neurons cultured at DIV3 (3 days *in vitro*) and continued for further 24 h. The neurons were then visualized by immunofluorescence staining. Compared to the GFP vector, overexpression of GFP-spastin WT resulted in increased neurite length (Figure 4 A,B) and number of branches (Figure 4C) of hippocampal neurons. Meanwhile, compared to GFP-spastin WT, the GFP-spastin 11A significantly inhibited the promotion effect on neurite length and branch formation (Figure 4 B,C). Sholl analysis of neuronal crossing revealed that overexpression of GFP-spastin WT promoted hippocampal neuronal complexity at a distance of 300 μm from the cell body, while the mutant similarly inhibited this effect (Figure 4D). These results indicate that the spastin 11A mutation inhibits the promoting effect on neurite outgrowth in hippocampal neurons.

Discussion

As an essential regulator in microtubule severing activities, spastin not only participates in the regulation of cell division and proliferation but also plays a crucial role in the growth and development of neurons, as reported in our previous studies[16,25,26] or other groups.[27,28] Neurons are sensitive to microtubule severing activity.[29,30] Spastin is highly expressed during development and promotes axon elongation by severing long microtubules to short segments.[18] When the expression of spastin is disturbed, significant defects can occur in the delivery of presynaptic vesicles and postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors.[31] Spastin promotes microtubule amplification by increasing the rate at which shrinking microtubules are convert-

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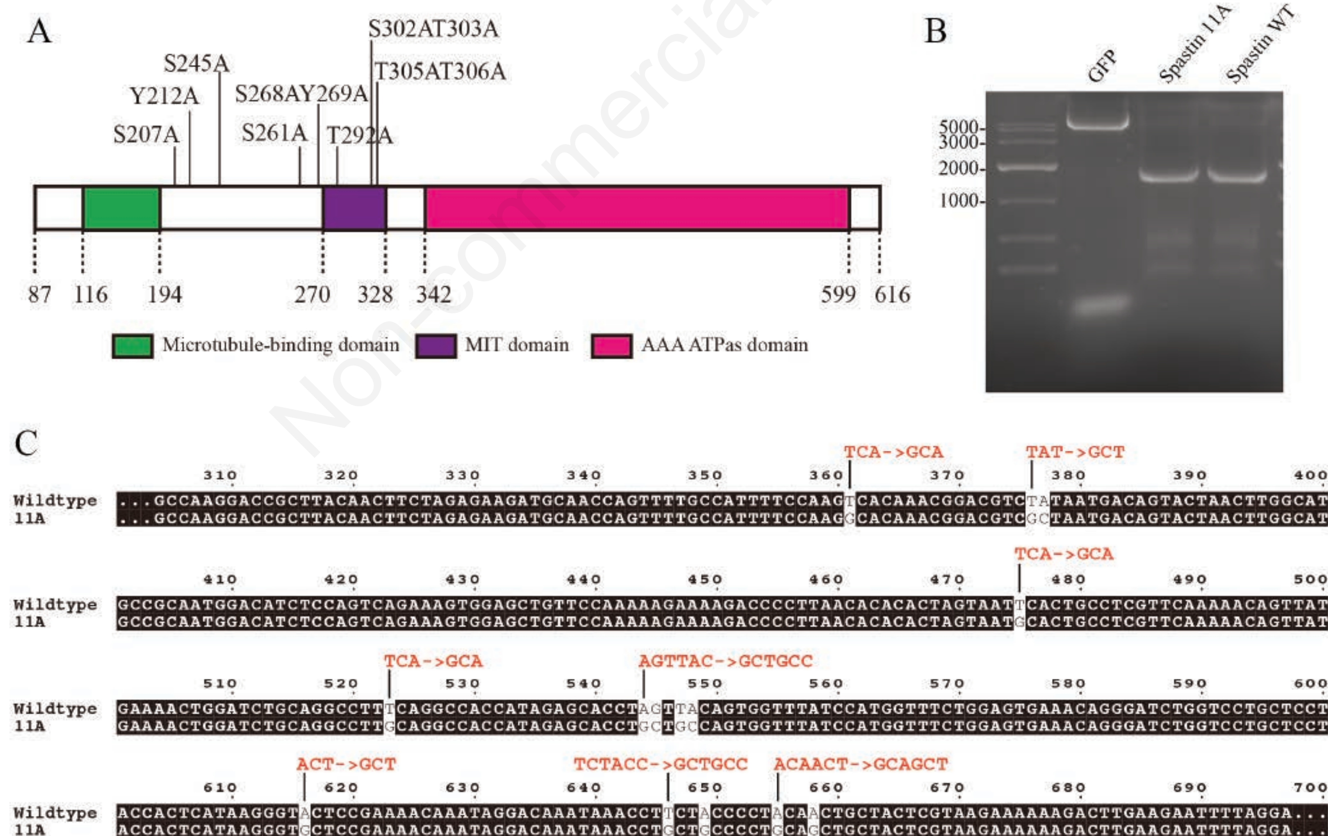

Figure 1. **Design of construction of point mutation of potential phosphorylation sites in spastin. A) Diagram of the search for 11 spastin phosphorylation sites in the PhosphsitePlus database. B) GFP, GFP-spastin WT (wild-type), GFP-spastin 11A (11 amino acids mutated) PCR electrophoresis results. C) Comparison of gene sequences of spastin WT and 11A.**

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ed into growing ones (called rescue) so that the microtubule fragments created by severing act as seeds for the growth of new microtubules.[5] Therefore, microtubule severing can promote net microtubule disassembly or amplify the number and mass of microtubules.[32] This shows the complex role that spastin plays in regulating microtubule dynamics in cells.

The Phos-tag SDS-PAGE technique can be used to identify the reduced phosphorylation level after site mutation.[33] In our study, we found that the phosphorylation level of spastin 11A was significantly reduced compared to that of spastin WT. The mutation of the eleven phosphorylation sites in spastin 11A not only affects the charge of spastin but also reduces the number of phosphate groups, which in turn affects the speed of spastin 11A electrophoresis in Phos-tag gels. Furthermore, although conformational changes of spastin were not investigated in this study, phosphorylation has been shown to be effective in altering the conformation of a protein, thus achieving a "switch" in protein function. This suggests that the phosphorylation of spastin may play a role in its function and may be a target for regulating its activity.[34,35]

The effect of spastin 11A on microtubule severing in COS7 cells was observed by gene transfection and immunofluorescence. Surprisingly, both spastin WT and spastin 11A mutants were found to induce microtubule fragmentation, indicating that mutations in these sites do not affect the microtubule severing function of spastin. The reason may be that the mutation sites involved in this study are not involved in the AAA domain, which functions to sever microtubules.[8] It is thought that the AAA domain can mediate the formation of functional spastin hexamers, and the hexamer of spastin can form a loop with a central pore into which the C-terminal tail of microtubules is drawn to depolymerize microtubules. It has been reported that there is a certain correlation between the motility of microtubules and their morphology; the short microtubules can be moved to the positive or negative end of the long microtubules.[36]

We further examined the effect of mutations in spastin on neurite outgrowth in hippocampal neurons. The fragmented microtubules, which are served by spastin, play important roles in neurite outgrowth and branch formation.[37] It has been shown that there are no long microtubules at the site of new branch formation of nerve axons.[38,39] The same finding in early axons suggests that shorter microtubules are more conducive to reconfiguration than longer microtubules, thus promoting morphological plasticity.[38,39] This point may also be relevant to potential strategies for increasing axonal regeneration in injured adults in clinical practice.[36] In addition, spastin is also able to affect AMPA receptor transport in both presynaptic and postsynaptic membranes.[31,40] Spastin has been reported to interact with Atlastin and ESCRT-III to affect axonal transport capacity.[41,42] In addition to neuronal development, there is a strong correlation between neuronal regenerative capacity and alterations of cytoskeletal microtubules and synaptic membrane receptors.[43] We found that overexpression of spastin WT significantly promoted neurite length and neurite branch formation in hippocampal neurons, whereas the promotion effect of spastin 11A was significantly reduced, indicating that spastin 11A may inhibit its function in hippocampal neurons without affecting its microtubule severing activity. The reason for this phenomenon may be that the mutation sites involved in this study are all located in two functional domains, MIT and MTBD, outside the spastin AAA functional domain. However, MIT and MTBD are considered to be two functional domains of spastin that interact with microtubules and are related to its involvement in the alteration of synaptic membrane receptors.[40] Therefore, we propose that spastin 11A does not affect microtubule severing activity, but may regulate neurite outgrowth by affecting neuronal synaptic membrane receptors, which needs further exploration.

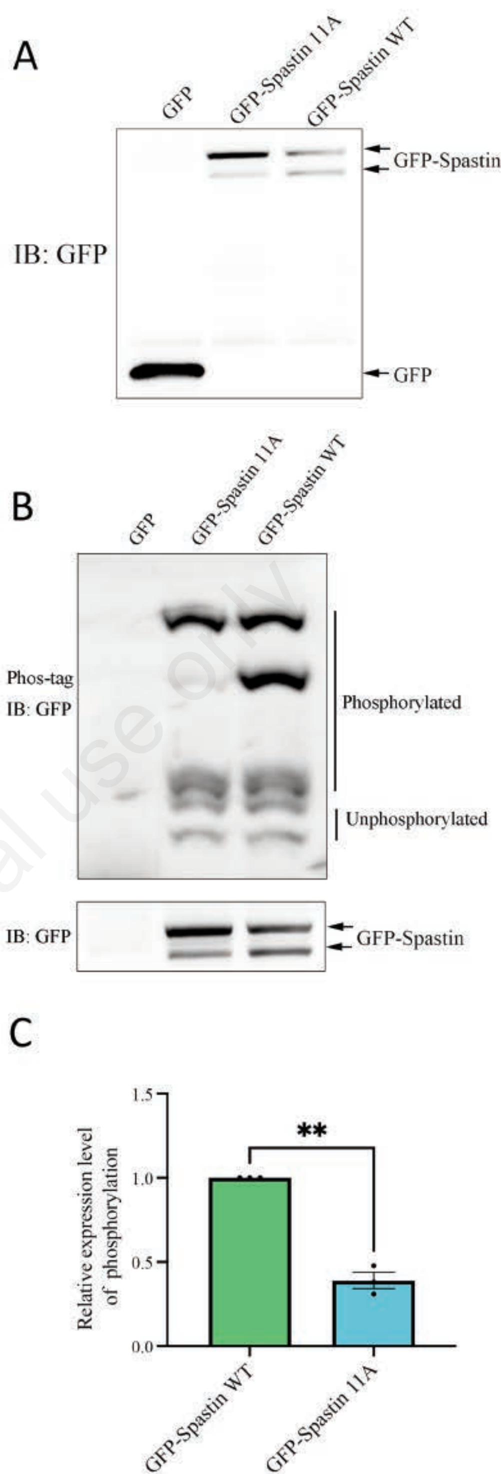

Figure 2. **The phosphorylation levels between spastin WT and the mutated form 11A were examined. A) Overexpression of GFP, GFP-spastin, and GFP-spastin 11A in COS7 cells at 24 h, and cell lysates were collected and subjected to Western blotting with GFP antibodies. B) The same samples as in (A) were subjected to a Phos-tag assay to detect phosphorylation shifts; compared to spastin WT, the amount of protein phosphorylated by spastin 11A was significantly reduced compared to the amount of protein that was not phosphorylated. C) Quantification of band intensities of phosphorylated relative to unphosphorylated was performed. The relative value in the control group was set to 1. Data are presented as the mean ± SEM from at least three independent experiments; *p<0.05 *vs* vector group.**

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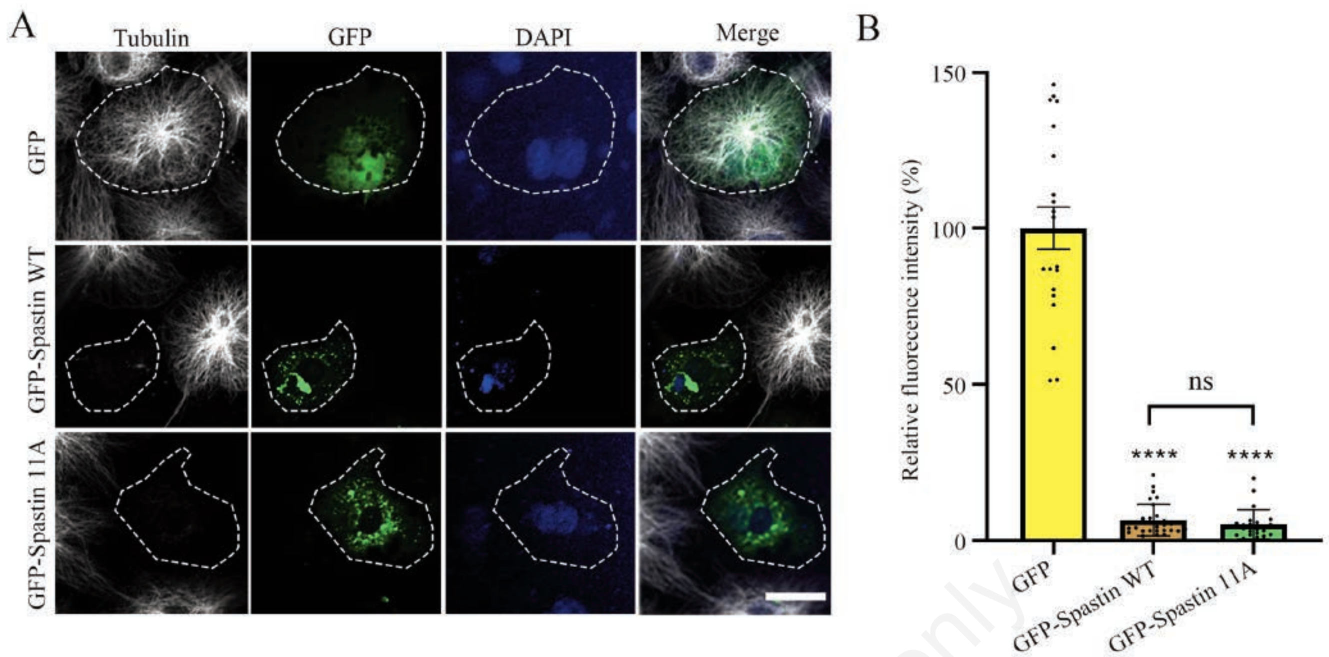

Figure 3. **The phospho-mutated form of spastin 11A does not affect the microtubule severing ability in COS7 cells. A) Different plasmids were transfected into COS-7 cells for 18 h and then immunolabeled for tubulin (white fluorescence signal) and counterstained for nuclear DNA with DAPI (blue fluorescence). B) Relative fluorescence intensity of microtubules in COS7 cells. Data are presented as mean ± SEM; ****p<0.0001; ns, not statistically significant. Scale bar: 10 μm.**

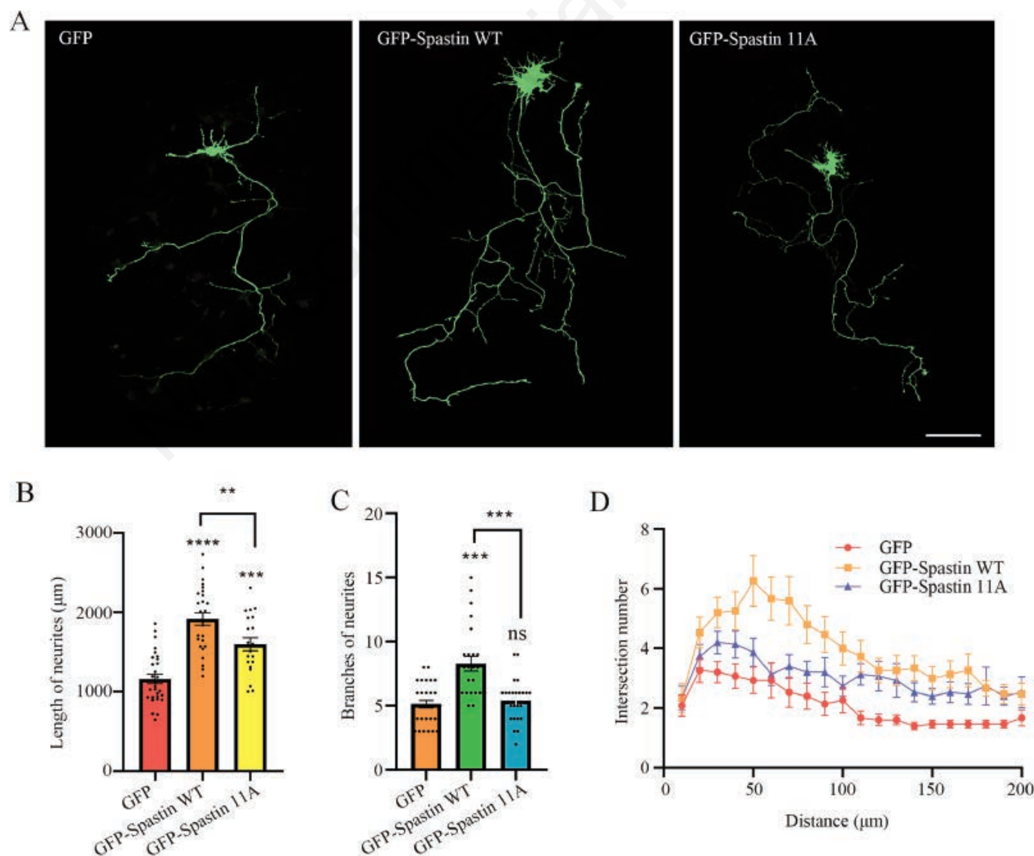

Figure 4. **The phospho-mutated form of spastin 11A impairs the promoting effect of hippocampal neurite outgrowth. A) DIV 3 hippocampal neurons were transfected with different plasmids for 24 h, followed by immunocytochemical staining with GFP antibody; scale bar: 100 μm. B) Neurite length statistics (including dendrites and axons). (C) Branching statistics of neurite. D) Sholl analysis of neurite within 300 μm from the cell soma. Data are expressed as mean ± SEM; **p<0.01, ***p<0.001, ****p<0.0001.**

Studies on spastin in neurite growth have focused more on its interaction with proteins, such as lipid droplets[44] and CRMP5.[16] In addition, we have previously found that the long non-coding RNA MALAT1 transcriptase-regulated spastin promotes hippocampal neuron development.[26] However, the functional changes caused by the phosphorylation of spastin are rarely reported. As an important post-translational modification of proteins, it is worth further exploration of whether phosphorylation of spastin affects the growth of hippocampal neurites by affecting neuronal synaptic membrane receptors. Recently, it has been reported that HIPK2 phosphorylates spastin at S268 to inhibit its degradation.[21] Further study showed that changes in excitatory synaptic currents and expression of AMPAR GluA2 on the membrane surface in rat hippocampal neurons were both affected by changes in the level of spastin phosphorylation.[43] These results seem to be consistent with ours, suggesting that phosphorylation of spastin may be closely related to its role in neuronal synaptic membrane receptors, which in turn promotes hippocampal neurite outgrowth.

In general, we determined the role of spastin 11A in microtubule severing and neurite outgrowth promotion in this study, and mutations at these eleven sites were found to inhibit the promotion of neurite elongation and branch formation without affecting the microtubule severing activity. However, there are still limitations in our study. The effect of spastin 11A on neuronal synaptic membrane receptors was not investigated, nor was it determined whether all phosphorylation sites reported by PhosphositePlus are identical. In addition, these sites were not further subdivided and most of the regulation kinases for these phosphorylation sites remain obscure. These are the focus of our next research. In addition, the search for the upstream kinase that mediates spastin phosphorylation is ongoing and the detailed mechanism will be supplemented in spastin-related neuronal disorders.

In our study, the spastin 11A mutant produced significant changes in the phosphorylation level but did not affect the microtubule severing ability. However, spastin 11A exhibited a significantly impaired effect on neurite outgrowth, suggesting that spastin promoted the growth of hippocampal neurons not only because of its microtubule severing function.

Acknowledgments

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