REPORT

Overexpression of Tau Rescues Nogo-66-Induced Neurite Outgrowth Inhibition *In Vitro*

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Received: 28 April 2016 / Accepted: 23 July 2016 / Published online: 19 October 2016
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Abstract Nogo-66 plays a central role in the myelin-mediated inhibition of neurite outgrowth. Tau is a microtubule-associated protein involved in microtubule assembly and stabilization. It remains unverified whether tau interacts directly with growth factor receptors, or engages in cross-talk with regeneration inhibitors like Nogo-66. Here, we report that plasmid overexpression of tau significantly elevated the protein levels of total tau, phosphorylated tau, and microtubule-affinity regulating kinase (MARK). Nogo-66 transiently elevated the total tau protein level and persistently reduced the level of p-S262 tau (tau phosphorylated at serine 262), whereas it had little influence on the level of p-T205 tau (tau phosphorylated at threonine 205). Nogo-66 significantly decreased the protein level of MARK. Hymenialdisine, an inhibitor of MARK, significantly reduced the level of p-S262 tau. Overexpression of tau rescued the Nogo-66-induced inhibition of neurite outgrowth in neuroblastoma 2a (N2a) cells and primary cortical neurons. However, concomitant inhibition of

MARK abolished the rescue of neurite outgrowth by tau in N2a cells. We conclude that dephosphorylation of tau at S262 is able to regulate Nogo-66 signaling, and that overexpression of tau can rescue the Nogo-66-induced inhibition of neurite outgrowth *in vitro*.

Keywords Neurite outgrowth · Myelin inhibitor · Microtubule-associated protein · Phosphorylation

Introduction

Limited axonal regeneration following spinal cord injury has been ascribed to the growth-inhibitory environment induced by myelin inhibitors [1]. Nogo-66 (66-residue extracellular domain), a prominent myelin inhibitor [2], inhibits neurite growth by signaling *via* multiple neuronal receptors, including the ligand-binding Nogo-66 receptor 1 (NgR1), paired immunoglobulin-like receptor B, and other co-receptors [3]. However, the exact mechanism by which Nogo-66 inhibits outgrowth has not been fully elucidated.

Microtubules are among the most prominent structural components found in growing and mature neuritic projections [4–6]. The microtubule-associated protein tau plays an important role in microtubule assembly and stabilization [7]. Tau also regulates the dynamic instability of microtubules involved in reorganization of the cytoskeleton [8, 9]. In recent years, studies have focused on the pathological role of tau in the neurodegenerative diseases known as tauopathies [10–12], including Alzheimer's disease, Parkinson's disease, corticobasal degeneration, argyrophilic grain disease, Pick's disease, and Huntington's disease.

Research suggests that some inhibitors of central nervous system regeneration participate in intricate cross-talk

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with growth-promoting molecules at the level of several key signaling molecules [13–15]. It remains unverified whether tau interacts directly with growth factor receptors, or engages in cross-talk with regeneration inhibitors [16]. Microtubule-affinity regulating kinase (MARK) was originally described in terms of its ability to phosphorylate tau and other related microtubule-associated proteins. MARK plays an important role in neuronal differentiation, cell polarity, intracellular transport, and cell migration [17, 18]. Interestingly, research indicates that tau is phosphorylated by MARK to regulate its binding to microtubules [19]. Therefore, we aimed to investigate potential cross-talk between the Nogo-66- NgR1 pathway and tau phosphorylation in neuronal cells.

Materials and Methods

Antibodies and Reagents

The following primary antibodies and compounds were used: p-Ser262 tau: sc-101813 rabbit (1:100; Santa Cruz Biotechnology, Dallas, TX); p-Thr205 tau: sc-101817 rabbit (1:100; Santa Cruz Biotechnology); anti-Tau, clone Tau 5 mouse (1:200; Millipore, Temecula, CA); anti-MARK rabbit (1:200; Abcam, Cambridge, MA); γ -tubulin mouse (1:1000; Sigma-Aldrich, St. Louis, MO); hymenialdisine (HD) (50 $\mu\text{mol/L}$; Tocris, Bristol, UK); and Nogo-66 (0.5 mg; Bioss, Beijing, China). The following secondary antibodies were used: goat anti-mouse IgG, horseradish peroxidase-conjugated (1:200; Cwbio-tech, Beijing, China); goat anti-rabbit IgG BA1054 (1:200; Boster, Wuhan, China); and goat anti-rabbit IgG-Cy3 (1:50; Boster).

Establishment of Cell Lines That Stably Expressed Tau

As we previously described, a human tau construct (tau441) or pcDNA (control vector) was transfected into murine neuroblastoma 2a (N2a) cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. A single clone was selected to establish cell lines that stably overexpressed tau (N2a/tau) or pcDNA (N2a/vector) [20]. The cells were cultured in 50% Dulbecco's modified Eagle's medium (DMEM)/50% Opti-MEM supplemented with 5% fetal bovine serum (FBS). The culture medium was changed every 3 days. For HD and/or Nogo-66 treatment, N2a cells were treated with 50 $\mu\text{mol/L}$ HD and/or 15 ng/mL Nogo-66 [dissolved in 1.1 g/mL dimethyl sulfoxide (DMSO) and added to the culture medium] (Biosynthesis Biotechnology Beijing, China).

Primary Cerebral Cortical Neuron Culture

Cerebral cortical neurons were isolated from female Sprague-Dawley rats at embryonic day 16 (E16). The rats were purchased from the Experimental Animal Center of Huazhong University of Science and Technology (HUST) (Wuhan, China). All the procedures were performed in compliance with the protocols approved by the Medical Ethics Committee of HUST. Under sterile conditions, the entire fetal brain was dissected out and placed in ice-cold phosphate-buffered saline (PBS) and DMEM. The cerebral cortex was subsequently freed of meninges, cut into small pieces, and digested with 0.125% trypsin in DMEM at 37°C for 30 min. The digest was suspended in DMEM with 5% FBS and the neurons were dissociated by gentle blowing (3 times). The harvested cells were plated onto a 12-well plate coated with poly-L-lysine at 70,000 cells/well. Four hours later, the serum-containing medium was replaced with Neurobasal/B27 (Invitrogen, Carlsbad, CA).

Total Tau, MARK, p-S262 Tau, and p-T205 Tau Assays

Protein was extracted from N2a (N2a/vector or N2a/tau) cells as previously described [21]. Following total protein quantification of the supernatants, samples that contained the same amount of proteins were prepared for western blot analysis using antibodies against total tau, MARK, and γ -tubulin. The integral optical density was then analyzed using Image Pro-Plus to acquire the relative concentration of total tau in each sample. The volume of each sample was determined so that each loaded sample contained equal amounts of total tau, based on the relative concentration. Tau phosphorylated at Ser262 (p-S262 tau) and tau phosphorylated at Thr205 (p-T205 tau) were then detected, using total tau as an internal reference. For band densitometry, the protein band images were captured with a Bio-Rad ChemiDoc XRS+ system (Hercules, CA), and the band density was determined using Image Lab software (Bio-Rad, Hercules, CA) and Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

Immunofluorescence Staining

Immunostaining was performed following standard protocols. All antibodies were diluted in 10% normal goat serum and 1% Triton X-100 in PBS. The cells were incubated with primary antibodies overnight at 4°C and washed 3 times with PBS for 10 min. Secondary antibodies were subsequently applied and incubated for 1 h at room temperature, followed by three washes with PBS for 15 min. Cells were fixed with DAPI, and images were captured

using a laser scanning confocal microscope (Olympus Fluoview FV1000) and analyzed with Olympus Fluoview ver. 2.1a Viewer software (Olympus, Tokyo, Japan).

Neurite Outgrowth Assay

For N2a/vector and N2a/tau cells, after the elongation of neurites (cultured without FBS), the medium was replaced with feeding medium containing PBS, Nogo-66 (15 ng/mL) alone or combined with HD (MARK inhibitor, 50 μ mol/L). The cells were photographed and scored for neurite outgrowth after an additional 24 h. To measure axon length, the neurons were transfected with pcDNA or tau441 plasmids for 72 h, and then cultured in medium containing Nogo-66 (15 ng/mL) or control (1.1g/mL DMSO). The morphological changes were analyzed under a digital phase-contrast inverted microscope (Olympus) with a charge-coupled device (CCD) camera. In all experiments described here, three random areas were selected per well to photograph, and measurements were

made on duplicate wells ($n = 6$). Only cells containing processes longer than two cell-body diameters were counted as being positive for neurite outgrowth. On average, 62 cells with outgrowth or elongated neurites were counted. The neurite length was measured using ImageJ software (National Institutes of Health, Bethesda, MD) to quantify the absolute distance.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 13.0 (SPSS Co. Ltd., Chicago, IL). Data are expressed as the mean $\pm$ SEM. Experiments that involved a single comparison between two groups were analyzed with *t*-tests, whereas differences between multiple groups were analyzed with one-way analysis of variance (ANOVA). One-way ANOVA with Tukey's *post-hoc* analysis was used to assess the effects of Nogo-66 over time. $P < 0.05$ was considered significant.

Fig. 1 Tau (total and phosphorylated) and MARK expression in N2a/vector and N2a/tau cells. **A, B** After N2a cells were transfected with pcDNA (N2a/vector) or tau441 (N2a/tau) plasmids, the protein levels of total tau and MARK were detected by western blot. **C–F** Quantitative analysis of total tau, MARK, and phosphorylated tau (p-S262 tau and p-T205 tau) in N2a/vector and N2a/tau cells. The values in **C** and **D** were normalized to the γ -tubulin band (**A**), and the values in **E** and **F** were normalized to the total tau band (**B**). The data represent the mean $\pm$ SEM from six separate experiments (paired *t*-test; * $P < 0.05$ compared with the control N2a/vector).

Results

Total Tau, Phosphorylated Tau, and MARK Expression in N2a Cells Transiently Transfected with Tau441 Plasmid

Wild-type mouse N2a (N2a/wt) cells expressed low levels of endogenous tau protein. To determine the role of tau in neurite outgrowth, we transiently transfected these cells with the longest human tau441 (N2a/tau). The expression of tau (total and phosphorylated) and MARK in N2a/tau cells was verified using western blot analysis. A marked increase (overexpression) in the total tau level was seen

after N2a cells were transiently transfected with the tau441 plasmid (~ 4 times greater than the N2a/vector control group; $P < 0.05$; Fig. 1A, C). The N2a/tau group also expressed higher levels of MARK than the N2a/vector group (~ 2 times greater; $P < 0.05$; Fig. 1A, D). Meanwhile, p-S262 tau (in KXGS motifs, KXGS motifs are located in several repeat domains of tau) and p-T205 tau (not in KXGS motifs) also increased (~ 1.5 times greater; $P < 0.05$; Fig. 1B, E, F). Therefore, stable expression of human tau441 (total tau) was achieved in N2a/tau cells. These data indicate that overexpressing tau results in the overexpression of MARK and phosphorylated tau.

Fig. 2 Protein levels of total tau and phosphorylated tau in Nogo-66-treated N2a/tau cells. N2a/tau cells were treated with 15 ng/mL Nogo-66 for 1, 2, 5, 15, and 30 min. The levels of total tau (A) and tau phosphorylated at Ser262 (p-S262) and Thr205 (p-T205) (B) were detected *via* western blot. C–E Quantitative analysis of the density of immunoblot bands. The values in C were normalized to the γ -tubulin band (A), and the values in D and E were normalized to the total tau band (B). Data represent the mean $\pm$ SEM from six separate experiments (one-way ANOVA with Tukey's *post-hoc*; * $P < 0.05$, ** $P < 0.01$ compared with the vehicle control, no Nogo-66 treatment).

Nogo-66 Decreases p-Ser262 Tau in N2a/Tau Cells

To determine whether Nogo-66 regulates tau and phosphorylated tau expression in N2a/tau cells, the levels of p-S262 tau and p-T205, as well as total tau were measured after the cells were exposed to Nogo-66. Tau becomes activated when phosphorylated at Ser262 (p-S262 tau). We found that Nogo-66 transiently upregulated the total tau level (2 min after addition of Nogo-66) compared to the control ($P < 0.05$; Fig. 2A, C). Nogo-66 decreased the level of p-S262 tau at all time points ($P < 0.01$ compared with the control; Fig. 2B, D), but did not affect the levels of p-T205 tau (Fig. 2B, E). This suggests that Nogo-66 selectively influences the phosphorylation of tau at the Ser262 residue.

Inhibition of MARK Decreases the Expression of p-S262 Tau in N2a/Tau Cells

To determine whether the inactivation of MARK is necessary for the dephosphorylation of p-S262 tau by Nogo-66, N2a/tau cells were treated with HD, a MARK inhibitor, 60 min before Nogo-66 exposure. Total tau, p-S262 tau, and MARK expression were assessed using western blot

analysis. In the presence of HD, the level of p-S262 tau decreased ($P < 0.05$ compared with the control; Fig. 3B, G), whereas the changes in total tau were not significant (Fig. 3A, F); in the presence of Nogo-66, MARK was reduced ($P < 0.05$ compared with the control; Fig. 3C, H); and in the presence of Nogo-66 and HD, p-S262 tau was reduced ($P < 0.01$ compared with Nogo-66 exposure; Fig. 3E, J), whereas changes in total tau were not significant (Fig. 3D, I). These data indicate that the inhibition of MARK is necessary for the dephosphorylation of p-S262 tau by Nogo-66.

Tau is Involved in Nogo-66-Induced Inhibition of Neurite Outgrowth in N2a Cells

To determine whether tau is involved in the Nogo-66-induced inhibition of neurite outgrowth, N2a cells were transfected with tau441 plasmids (N2a/tau), followed by Nogo-66 or Nogo-66+HD application (Fig. 4A). Neurite outgrowth in N2a/vector cells was $70.4 \pm 10.8 \mu\text{m}$, and treatment with Nogo-66 markedly inhibited it to $34.4 \pm 6.2 \mu\text{m}$ ($P < 0.05$ compared with the N2a/vector group without Nogo-66 application; Fig. 4B). In N2a/tau cells, the inhi-

Fig. 3 Total tau, p-S262 tau, and MARK expression following HD treatment in N2a/tau cells. **A–E** HD group, N2a/tau cells treated with HD; Nogo-66 group, N2a/tau cells treated with Nogo-66; Nogo-66+HD group, N2a/tau cells treated with Nogo-66 and HD. The levels of total tau, tau phosphorylated at Ser262 (p-S262 tau), and MARK were detected *via* western blot. **F–J** Quantitative analysis of

total tau, p-S262 tau, and MARK in N2a/tau cells. The values in **F**, **H**, and **I** were normalized to the γ -tubulin band (**A**, **C**, **D**), and the values in **G** and **J** were normalized to the total tau band (**B**, **E**). The data represent the mean $\pm$ SEM from six separate experiments (paired *t*-test; * $P < 0.05$, ** $P < 0.01$ compared with the control in **F–H**, and with the Nogo-66 group in **I** and **J**).

Fig. 4 Neurite outgrowth in N2a cells with differing treatments. **A** Vector, N2a/vector cells treated with PBS; Vector+Nogo-66, N2a/vector cells treated with Nogo-66; Tau+Nogo-66, N2a/tau cells treated with Nogo-66; Tau+Nogo-66+HD, N2a/tau cells treated with Nogo-66 and HD. To measure neurite length, cells were stained with a p-S262 tau antibody; EGFP indicates infected cells; DAPI, stained nuclei; and Merge, color synthesis of EGFP, DAPI, and p-S262 tau. Scale bars, 50 μ m. **B** Values were normalized to baseline outgrowth in the vehicle control (vector). One-way ANOVA (Tukey's *post-hoc*) was performed to compare the Vector, Vector+Nogo-66, Tau+Nogo-66, and Tau+Nogo-66+HD groups. The data represent the mean $\pm$ SEM from six separate experiments (* P < 0.05 compared with the Vector group; $\blacktriangle$ P < 0.05 compared with the Vector+Nogo-66 group; # P < 0.05 compared with the Tau+Nogo-66 group).

bition of neurite outgrowth induced by Nogo-66 was ameliorated to $66.3 \pm 8.5 \mu\text{m}$ (P < 0.05 compared with N2a/vector+Nogo-66). However, when MARK was simultaneously inhibited (N2a/tau+Nogo-66+HD), the rescue of neurite outgrowth by tau was abolished to $33.2 \pm 5.3 \mu\text{m}$ (P < 0.05 compared with N2a/tau+Nogo-66). These findings indicate that overexpression of tau can rescue Nogo-66-induced neurite outgrowth inhibition when MARK is uninhibited.

Overexpression of Tau Rescues Nogo-66-Induced Axonal Outgrowth Inhibition in Primary Cortical Neurons

To further determine whether tau is involved in the inhibition of axonal outgrowth induced by Nogo-66, primary cortical neurons were transfected with tau441 plasmids, followed by Nogo-66 application (Fig. 5A). Under control conditions (vector+DMSO), the axon

Fig. 5 Axonal elongation in primary cortical neurons with differing treatments. **A** Vector+DMSO, neurons transfected with pcDNA plasmids and treated with DMSO; Vector+Nogo-66, neurons transfected with pcDNA plasmids and treated with Nogo-66; Tau+DMSO, neurons transfected with tau441 plasmids and treated with DMSO; Tau+Nogo-66, neurons transfected with tau441 plasmids and treated with Nogo-66. To measure neurite length, cells were stained with a p-S262 tau antibody. Scale bar, 50 μ m. **B** Values were normalized to baseline outgrowth in the vehicle control (vector+DMSO). One-way ANOVA (Tukey's *post-hoc*) was used to compare Vector+DMSO, Vector+Nogo-66, Tau+DMSO, and Tau+Nogo-66 groups. The data represent the mean $\pm$ SEM from six separate experiments (* P < 0.05, ** P < 0.01 compared with the Vector+DMSO group; # P < 0.05 compared with the Vector+Nogo-66 group).

length was 74.2 ± 4.6 μ m, and treatment with Nogo-66 reduced it to 44.6 ± 5.8 μ m (P < 0.01; Fig. 5B). In these transfected neurons, the inhibition of axon length induced by Nogo-66 was ameliorated to 65.5 ± 6.5 μ m (P < 0.05 compared with vector+Nogo-66). Overexpression of tau increased the axon length to 96.3 ± 9.7 μ m (P < 0.05 compared with vector+DMSO). These findings further indicate that overexpression of tau can rescue the Nogo-66-induced neurite outgrowth inhibition in neuronal cells.

Discussion

In the present study, plasmid overexpression of tau (tau441) significantly increased the levels of tau (total and phosphorylated) and MARK. Nogo-66 transiently elevated the total tau levels and persistently reduced the levels of tau phosphorylated at serine 262 (p-S262 tau); however, Nogo-66 did not influence the levels of tau phosphorylated at threonine 205 (p-T205 tau). Nogo-66 significantly decreased the level of MARK. HD, an inhibitor of MARK, significantly reduced the level of p-S262 tau. Overexpression of tau rescued the inhibition of neurite outgrowth induced by Nogo-66 in N2a cells and primary cortical neurons. However, when MARK was simultaneously inhibited, the rescue of neurite outgrowth by tau was abolished in N2a cells.

It is well known that abnormal tau aggregation leads to neurodegeneration, and that Nogo-66 inhibits neurite outgrowth. However, no relationship between tau and Nogo-66 in the pathological mechanisms leading to disease had been defined. Tau is thought to be involved in growth factor signaling [22, 23]. Inhibition of Nogo-66 is being investigated as a major axon regeneration strategy [24]. In the present study, we verified that communication occurs between the regeneration inhibitor Nogo-66 and tau, since the presence of Nogo-66 altered the phosphorylation state of tau at Ser262.

The precise functions of tau in neurite outgrowth are not firmly established. Dawson *et al.* [4] reported that primary hippocampal cultures from tau-knockout mice exhibit a significant reduction in axonal elongation. However, overexpression of tau induces long cellular processes in non-neuronal Sf9 cells [25]. Studies of primary neurons from different lines of microtubule-associated protein tau (MAPT)-knockout mice have yielded controversial results [26]. Neurons from one MAPT-knockout line exhibited slowed maturation and reduced neurite length, whereas neurons from another MAPT-knockout line did not display either of these effects [27]. In the present study, overexpression of tau efficiently reversed the Nogo-66-induced inhibition of neurite outgrowth. Our results were in accordance with the reports by Dawson *et al.*, and Knops *et al.*, [4, 25]. This suggests that tau may mediate inhibition by Nogo-66 *via* phosphorylation at Ser262, since overexpression of tau ameliorated the inhibitory effects of Nogo-66.

Phosphorylation is the most commonly described post-translational modification of tau. This plays a crucial role in regulating its physiological functions, including binding to microtubules, and regulating their stabilization and assembly [28]. The phosphorylation of KXGS motifs (particularly Ser262) in the repeat domain of tau by

MARK, protein kinase A, or calcium/calmodulin-dependent protein kinase II can reduce the affinity of tau binding to microtubules [29].

MARK predominantly phosphorylates tau at Ser262 [19, 29]. In the present study, we demonstrated that Nogo-66 significantly decreased the level of p-S262 tau, overexpression of tau significantly elevated the level of MARK, and inhibition of MARK by HD decreased the levels of phosphorylated tau and abolished the rescue of neurite outgrowth by tau. Our results highlight potential cross-talk between Nogo-66 and MARK signaling, since Nogo-66 significantly decreased the levels of MARK. In addition, MARK phosphorylation of tau in KXGS motifs influences the inhibition of neurite outgrowth mediated by Nogo-66.

In summary, we conclude that regulating Ser262 tau phosphorylation might play a role in altering the Nogo-66 signaling pathway, and that the overexpression of tau can rescue Nogo-66-induced neurite outgrowth inhibition *in vitro*.

Acknowledgments This work was supported by the National Natural Science Foundation of China (81371380 and 31171028).

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