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Short communication

A novel centrosome and microtubules associated subcellular localization of Nogo-A: Implications for neuronal development

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ABSTRACT

Oligodendrocyte-derived neurite-outgrowth inhibitor Nogo-A and its restriction mechanism are well-known. Recently, Nogo-A is reported to be abundantly expressed in neurons, however, the concrete link between neuronal Nogo-A and neuronal development is poorly understood. In the present study, we used Neuro2A and COS7 cell lines to clarify that Nogo-A largely distributed in the centrosome and microtubules-rich regions. When endogenous Nogo-A was down-regulated with RNA interference, the percentage of cell differentiation and the total neurite length of Neuro2A exposed to valproic acid (VPA) decreased sharply. Furthermore, in primary neurons, acetylated α-tubulin decreased at the tips of neurites where endogenous Nogo-A was still highly expressed. In HEK293FT cell lines, Nogo-A overexpression could redistribute acetylated α-tubulin but not change the level of α-tubulin. Together, our data discovered that centrosome- and microtubules-localized Nogo-A positively regulates neuronal differentiation and neurite outgrowth of Neuro2A cell lines, implicating the essential roles of subcellular Nogo-A in neuronal development.

1. Introduction

Nogo-A has been investigated as one of the most potent myelin-associated neurite inhibitors in the adult central nervous system (CNS). *nogo* gene generates three isoforms, Nogo-A, -B, and -C, which share a 66-amino acid residue extracellular domain (Nogo-66). The mechanism for neurite outgrowth inhibition is well illustrated, the Nogo-66 domain of Nogo-A upon oligodendrocyte binds to a receptor complex containing NgR, P75/TROY and LINGO-1 of neurons, activates the small Rho GTPase RhoA and ROCK, and then leads to growth cone collapse and neurite inhibition (Schwab and Strittmatter, 2014).

Recently, accumulating data demonstrate that Nogo-A is also expressed in neurons (Hunt et al., 2003; Jin et al., 2003; Liu et al., 2002). And increasing evidence has uncovered the potential roles of neuronal Nogo-A. In the developing forebrain cortex, neuronal Nogo-A is pivotal in tangential migrations of neural precursors and interneurons (Mingorance-Le Meur et al., 2007). Cultured dorsal root ganglia (DRG) neurons from Nogo-A KO mice suggest that neuronal Nogo-A regulates neurite fasciculation, branching and extension (Petrinovic et al., 2010). And neuronal Nogo-A restricts synaptic plasticity (Delekate et al., 2011). However, the mechanism of neuronal Nogo-A regulating neurite outgrowth is poorly understood.

Microtubules are important components of neuronal cytoskeleton and exert essential functions such as cell shaping, division, motility and transportation (Poulain and Sobel, 2010). Previous research indicates the co-localization between Nogo-A and α-tubulin in cultured neurons (Mingorance-Le Meur et al., 2007), and some data further shows that Nogo-A is an interactor of α-tubulin in rat brain (Taketomi et al., 2002). Thus neuronal Nogo-A might regulate neurite outgrowth via influencing microtubules activity.

Abbreviations: CNS, central nervous system; DRG, dorsal root ganglia; MTOC, microtubule organizing center; SD, Sprague-Dawley; UD, undifferentiated; VPA, valproic acid.

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Neuro2A is a mouse neural crest-derived cell lines, and valproic acid (VPA)-induced neuronal differentiation model of Neuro2A is used extensively (Chen et al., 2011; Dai et al., 2014; Ma et al., 2013). In this study, we demonstrated that Nogo-A abundantly distributed in centrosome- and microtubule-rich regions using Neuro2A and COS7L cells. Down-regulation of Nogo-A resulted in a decrease of differentiation rate and inhibition of neurite outgrowth. And this positive function might depend on regulating the redistribution of acetylated α-tubulin.

2. Materials and methods

2.1. Antibodies

Several Nogo-A-specific antibodies were used in the study. A home-made polyclonal antibody A620 recognizing 620–1004 aa of Nogo-A was used (Mi et al., 2012). Another two antibodies A201 (Prosci, 201–250 aa) and H300 (Santa Cruz, 700–1000 aa) were purchased commercially. γ-tubulin, α-tubulin and acetylated α-tubulin antibodies were purchased from Santa Cruz.

2.2. Animals and primary neuron culture

All animal procedures were approved by Animal Care Committee at Shanghai Jiao Tong University. Embryonic (E) 16–18 day Sprague-Dawley (SD) rats were obtained from Shanghai Slac Laboratory Animal Company.

Neurons from most layers of cortex start to generate and develop since E16-18 of rat (Gaillard and Roger, 2000), and only a few of glia cells just begin to form. Thus, the neuron culture from E16-18 is nearly pure and often used to study neuronal growth and development (Mi et al., 2012). Briefly, cerebral cortex was removed aseptically, then digested and dispersed into single cells, after centrifugation, neurons were resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), then seeded at $1 \times 10^4/cm^2$ on cover glass placed in 24-well plate coated with poly-L-lysine and incubated in humidified atmosphere with 5% CO_2 at 37 °C. 2 h later, the whole medium was replaced with Neurobasal medium containing 2% B27 supplement (Invitrogen).

2.3. Cell culture, transfection, and VPA-induced differentiation

HEK293FT (Invitrogen), COS7L (Invitrogen) and Neuro2A (Institute of Biochemistry and Cell Biology, SIBS, CAS, Shanghai, China) were maintained in DMEM supplemented with 10% FBS. HEK293FT and COS7L cells were transfected with pcDNA 3.1+-Nogo-A using Lipofectamine 2000 reagent (Invitrogen). Neuro2A cells were co-transfected with pEGFP-N1 and shRNAs for Nogo-A using FuGENE HD reagent (Roche). To induce neuronal differentiation, Neuro2A cells (at about 20% confluence) were transferred to serum-free opti-MEM (Invitrogen) containing 1 mM VPA (Sigma) and allowed to extend neurites (Ma et al., 2013).

2.4. RNA interference

According to the targeting sequences of rat Nogo-A, two pairs of shRNA were synthesized. Oligonucleotides encoding shRNAs were cloned into the pSuper vector to generate pSuper-Nogo-A (against Nogo-A) and pSuper-panNogo (against Nogo-A/B/C). The target sequences are shown as following, pSuper-Nogo-A, GAGGATTTCCCATCTGTCCT; pSuper-panNogo, TTTGCAGTGTTGATGTGG. The efficacy of knocking down endogenous Nogo-A was confirmed in our previous works (Mi et al., 2012).

2.5. Immunocytochemistry

Cells on glass coverslips were fixed with 4% paraformaldehyde and then permeabilized with ice-cold methanol. After being blocked by 10% normal donkey serum, cells were incubated with primary antibody at room temperature for 1 h. The following primary antibodies were used, A620 (1:1000), H300 (1:400), A201 (1:800), γ-tubulin (1:200), α-tubulin (1:200) and acetylated α-tubulin (1:200). Then, they were rinsed and incubated for 1 h at room temperature with Alexa Fluor-labeled secondary antibodies (Molecular Probes 1:800). After been washed, the coverslips were mounted with glycerine/PBS containing DAPI for nuclei staining.

2.6. Subcellular fractionation of 293FT cell lines

Subcellular fractionation was performed on 293FT using the ProteoExtract® Subcellular Proteome Extraction kit (Calbiochem). This sequential extraction method relies on the different solubility of proteins in certain subcellular compartments to yield four fractions enriched in cytosolic (F1), membrane and membrane organelle-localized (F2), soluble and DNA-associated nuclear (F3), and cytoskeletal (F4) proteins. Equivalent amounts of proteins were resolved by SDS-PAGE, and western blot was performed as described below.

2.7. Western blot

The procedure was previously described (Dai et al., 2014). Subcellular extracts were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Roche). The membranes were treated with 1% blocking solution in TBS, followed by incubation with primary antibodies and then POD-labeled secondary antibodies (Roche). The immunolabeled proteins were detected by BM Chemiluminescence Western Blotting kit (Roche). The primary antibodies were used as the follows: A620 (1:25,000), acetylated α-tubulin (1:2000), α-tubulin (1:2000), β-actin (Abmart, 1:1000), GAPDH-HRP (KangCheng, China, 1:5000).

2.8. Differentiation assay and statistical analysis

Neuro2A cells with any neurite longer than two cell bodies were considered to be differentiated (Chen et al., 2011; Dai et al., 2014; Ma et al., 2013). Each group: undifferentiated group, VPA-induced groups transfected with pSuper, pSuper-Nogo-A and pSuper-panNogo respectively, was analyzed by counting about 150–200 cells. Assessment of neurite outgrowth was performed by counting about 50–70 cells per condition. The total neurite length was quantified using Image Pro-Plus software. In each analysis, the data represent mean ± S.D. of at least three experiments. For comparison, statistical significance was tested by one-way ANOVA, and probability values of less than 5% were considered significant.

3. Results

3.1. Nogo-A distributes in cell body and neurites of Neuro2A cells

Before we used Neuro2A as a cell differentiation model to examine the potential functions of Nogo-A, we selected two antibodies against Nogo-A (A620 and H300) to detect its expression in Neuro2A cells. A620 and H300 antibodies were used to stain UD and VPA-induced Neuro2A with DAPI respectively. Either in UD or VPA groups, Nogo-A was found to localize in cytoplasm, and in VPA group Nogo-A could be also detected in all neurites of differentiated Neuro2A cells (Fig. 1A and B). Focusing on these differentiated cells,