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Research report

Phosphorylation and SUMOylation of CRMP2 regulate the formation and maturation of dendritic spines

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ABSTRACT

The posttranslational modifications of CRMP2 play an important role in axon outgrowth, cell polarization and dendritic morphogenesis. However, whether CRMP2 and its posttranslational modifications are involved in dendritic spine development specifically is not completely clear. Here, we show that CRMP2 can promote the formation and maturation of dendritic spines in cultured hippocampal neurons. Overexpression of CRMP2 results in an increase in the density of spines especially the mushroom-shape spines. The amplitude and frequency of miniature excitatory postsynaptic currents (mEPSCs) are both enhanced and the intensity of PSD95 is strengthened in the neurons with CRMP2 overexpression. Furthermore, dephosphorylation of CRMP2 at Thr514 and deSUMOylation at Lys374 can further promote the formation and maturation of dendritic spines, whereas, no cross-talk is found between these two posttranslational modifications in the regulation of dendritic spine formation and maturation. Taken together, our data support a model in which phosphorylation and SUMOylation modification of CRMP2 independently promote the formation and maturation of dendritic spines and participate in the process of dendritic spine plasticity.

1. Introduction

Dendritic spines are postsynaptic structure of excitatory synapses which mediated synaptic transmission between the glutamatergic neurons. The changes in number and morphology of dendritic spines are associated with synaptic plasticity. The spine morphology is classified as thin, stubby and mushroom. During development, the more motile thin spines transform to more stable stubby spines and mature into mushroom-shape spines. The formation, maturation, and maintenance of dendritic spines depend on the regulation of the cytoskeleton in response to extracellular or intracellular cues. Dendritic spines are dynamic structures that undergo morphological remodeling during development and in adaptation to sensory stimuli or in learning and memory (Niesmann et al., 2011). As numerous psychiatric and neurological diseases are accompanied by alterations of spine numbers or size, the elucidation of mechanisms that regulates formation and plasticity of spinous synapses is important (Calabrese et al., 2006; Penzes et al., 2011).

Collapsin response mediator proteins (CRMPs) which composed of five homologous cytosolic phosphorproteins (CRMP1–5) are highly expressed in developing and adult nervous systems (Fukada et al., 2000; Minturn et al., 1995; Yoshimura et al., 2005). CRMPs has been demonstrated to play the roles in neurite extension, axonal regeneration, cell migration and differentiation (Ip et al., 2014; Yoshimura et al., 2005). However, the role of CRMPs in dendrites and dendritic spine development is less understood. Our previous works have shown that CRMP4 regulates dendritic growth and maturation *via* the interaction with actin cytoskeleton in cultured hippocampal neurons (Cha et al., 2016) and that CRMP4 and CRMP2 interact to coordinate cytoskeleton dynamics regulating growth cone development and axon elongation (Tan et al., 2015). The final target of CRMP2 is the cytoskeleton, which is the centre stage, regulating axonal growth and neuronal polarity by promoting microtubule assembly and stability (Fukata et al., 2002; Inagaki et al., 2001). CRMP2 also colocalizes with the actin cytoskeleton and coimmunoprecipitates with actin (Arimura et al., 2005; Tan et al., 2015). Therefore we examine whether CRMP2 plays a role in

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dendritic spine development because dendritic spines are small, actin-rich protrusions of the dendritic membrane that serve as primary recipients of excitatory synaptic input in the mammalian central nervous system. Recent studies have shown that CRMP2 knockout mice exhibits decreased dendritic complexity and mature spine density (Zhang et al., 2016), indicating an important role of CRMP2 on dendritic spine development.

Indeed, CRMP2 functions are determined by multiple posttranslational modifications including phosphorylation, glycosylation, oxidation, proteolysis, and SUMOylation (Khanna et al., 2012). For instance, CRMP2 binds tubulin heterodimer, whereas phosphorylation of CRMP2 by glycogen synthase kinase 3β (GSK3β), Rho-associated protein kinase and cyclin-dependent kinase 5 (Cdk5) lowers binding affinity of CRMP2 to tubulin (Khanna et al., 2012). SUMOylation is another posttranslational modification of lysine residues that is similar to ubiquitination, in which a member of the small ubiquitin-like modifier (SUMO) family is conjugated to target proteins, altering substrate function (Wilkinson and Henley, 2010). SUMOylation of CRMP2 decreased binding to endocytic proteins to regulate the surface expression of voltage-gated sodium channel NaV1.7 and CaV2.2 signaling pathway in sensory neurons (Dustrude et al., 2016; Ju et al., 2013). CRMP2 SUMOylation has also shown to be dependent on phosphorylation in controlling NaV1.7 function (Dustrude et al., 2016). However, the role of CRMP2 and its posttranslational modifications in dendritic spine development is not fully understood.

In this study, we demonstrate that overexpression of CRMP2 promotes dendritic spine formation and maturation. Dephosphorylation of CRMP2 at Thr514 and deSUMOylation at Lys374 can further promote formation and maturation of dendritic spines. Although CRMP2 phosphorylation has been reported to either enhance or inhibit SUMOylation (Dustrude et al., 2016), together our data showed SUMOylation of CRMP2 is not dependent on the Thr514 phosphorylation of CRMP2 during dendritic spine development.

2. Materials and methods

2.1. Animals

All the experiments were conducted with 1-day old pups of Sprague-Dawley (SD) rats. All animal procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals produced by the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at Jinan University, China. All efforts were made to minimize the suffering and number of animals used.

2.2. Plasmids and constructs

Details of construction of cDNA plasmids can be found in our previous report (Cha et al., 2016). Briefly, the PCR-based method was used to generate all of the cDNA constructs used in this study. The cDNA fragments encoding CRMP2 and SENP1, were amplified by PCR using the obtained clone of rat CRMP2 (NM_001105717.2) and SENP1 (XM_017595316.1) as templates. The CRMP2 and SENP1 cDNA fragments were subcloned into pEGFP-C1 and mCherry plasmids respectively (Clontech, Mountain view, CA). Point mutations T514A (mutation of Thr514 to alanine to mimic dephosphorylated CRMP2), T514D (mutation of Thr514 to aspartic acid to mimic phosphorylated CRMP2), K374A (mutation of Lys374 to alanine to mimic deSUMOylation of CRMP2), T514A/K374A and T514D/K374A (phosphomimetic and dephosphorylatable mutants of Thr514 with SUMO-site impaired) were introduced with the Quickchange Kit (Agilent, USA). All constructions were verified by sequencing.

2.3. Preparation of SUMO1 protein

SUMO1 protein was purchased from EpiGentek company. 49 μg powder of SUMO1 protein was solubilized in 1 ml electrode intracellular solution and diluted to 4.2 μM.

2.4. Hippocampal neuronal culture and transfection

Rat hippocampal neurons were cultured as described previously (Zhang et al., 2012). Neurons cultured in 24-well culture plates at 10 days *in vitro* (DIV10) were used to perform the transfection. Calcium-phosphate was used to transfect the SENP1-mCherry, CRMP2-pEGFPC1 (CRMP2-GFP) constructs and their mutations into the neurons.

2.5. Fluorescence immunostaining

After transfection for 24–48 h, the hippocampal neurons were fixed with 4% paraformaldehyde (Sigma, USA). Immunostaining was then performed using a previously described standard protocol (Zhang et al., 2012). The primary antibodies anti-GFP (Santa Cruz) and anti-PSD95 (Sigma) were used at a dilution of 1:200, and monoclonal donkey anti-mouse IgG Dylight 488 and monoclonal donkey anti-rabbit IgG Dylight 549 (Jackson ImmunoResearch) were used at a dilution of 1:800. After staining, the cells were mounted on glass slides using Fluoro-Gel II with DAPI (Electron Microscopy Sciences, Hatfield, PA, USA) and were imaged with a Carl Zeiss LSM 700 confocal microscope (Zeiss, Germany). Images were acquired with the same optical slice thickness for every channel using a 63 × oil objective and a resolution of 1024 × 1024 pixels. Dendritic spine morphology was also obtained using Carl Zeiss LSM with z-sectioned at 0.3 μm increments under a 63 × oil microscope. Dendrites were selected from areas approximately 40–120 μm from the neuronal soma and pictures were synthesized to a 3D picture using Zeiss image processing software. The number of dendritic spines was analyzed on Image-pro plus software and spine density was calculated according to dividing the total spine number by the dendritic branch length. Spine subtypes were classified based on previously defined morphological criteria (Bian et al., 2015; Zagrebelsky et al., 2005).

2.6. Electrophysiology

Whole-cell patch-clamp recordings of miniature excitatory synaptic currents (mEPSCs) were obtained from transfected cultured hippocampal neurons on DIV 11–13. During the recordings, cells were bathed in an external solution with a pH of 7.3, containing: 128 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM glucose, 20 mM HEPES, 1 mM tetrodotoxin and 100 μM picrotoxin. Recording pipettes were filled with the intracellular solution containing: 147 mM KCl, 5 mM Na_2-phosphocreatine, 2 mM EGTA, 10 mM HEPES, 2 mM MgATP and 0.3 mM Na_2GTP. Recordings were performed at room temperature in voltage clamp mode, at a holding potential of −70 mV, using a Multiclamp 700 B amplifier (Molecular Devices, Sunnyvale, CA, USA) and Clampex 10.5 software (Axon Instruments, Foster City, CA, USA). The series resistance was below 30 MΩ and data were acquired at 10 kHz and filtered at 1 kHz. mEPSCs were analyzed using MiniAnalysis software (Synaptosoft, Inc., Decatur, GA, USA).

2.7. Statistical analysis

Data are presented as the mean ± SEM. Statistical significance of the differences between two groups was analyzed by using Student's *t*-test and comparisons between more than two groups were performed using one-way ANOVA with Newman-Keuls *post-hoc* tests. A value of $P < 0.05$ was considered to be statistically significant.