Original Research

The impact of CRMP4 SUMOylation on the Cav1.2 interaction, neurite outgrowth and thermal pain sensitivity

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Collapsin response mediator protein 4 (CRMP4) is critical for neuronal development. However, whether CRMP4 could be SUMOylated and how the SUMOylation regulates the interaction with the L-type voltage-gated calcium channel (Cav1.2), neurite outgrowth, and thermal pain sensitivity remain to be elucidated. To determine the SUMOylation of CRMP4, Glutathione *S*-transferase (GST) - Small Ubiquitin-like Modifier 1 (-SUMO1), -SUMO2, and -SUMO3 proteins were purified for GST-pulldown. Immunofluorescence staining was performed to observe colocalization of CRMP4 and SUMOs. Co-immunoprecipitation (co-IP) was performed to assess the interaction between CRMP4 and SUMO2. GST-pulldown and co-IP were performed to verify the interaction between CRMP4 and Cav1.2. The impact of SUMOylation of CRMP4 on its interaction with Cav1.2 was determined. Then, the effect of CRMP4 SUMOylation on neurite outgrowth was observed. Whole-cell patch clamping revealed the effect of CRMP4 SUMOylation on Cav1.2 mediated calcium influx. Paw withdrawal latency was measured to assess the impact of CRMP4 SUMOylation on thermal pain sensitivity in rats. The data revealed that CRMP4 K374 is a potential site for SUMO modification. SUMO1, SUMO2, and SUMO3 can all interact with CRMP4. SUMO2 interacts with CRMP4, but not a variant of CRMP4 harboring a mutation of K374. CRMP4 and SUMO proteins colocalized in neurites, and CRMP4 deSUMOylation promoted neurite outgrowth. CRMP4 interacted with Cav1.2, and deSUMOylation of CRMP4 strengthened this interaction. CRMP4 promoted calcium influx via Cav1.2, and overexpression of CRMP4 significantly increased thermal pain sensitivity in rats, which CRMP4 deSUMOylation strengthened. In conclusion, these data demonstrate the SUMOylation of CRMP4, elucidate the impacts of SUMOylation on the interaction with Cav1.2 on neurite outgrowth and thermal pain sensitivity.

Keywords

CRMP4; SUMO protein; SUMOylation; L-type voltage-gated calcium channel; Neurite outgrowth; Thermal pain sensitivity; Cytoskeletal dynamics

1. Introduction

Neuropathic pain, a type of chronic pain caused by direct injury or dysfunction of the peripheral or central nervous system, manifests as spontaneous pain, hyperalgesia, allodynia, and paresthesia, along with other clinical features [1, 2]. Neuropathic pain is one of the most common clinical causes of chronic pain [3]. According to a survey by the Institute of Medicine of the National Academy of Sciences, at least 116 million adults in the United States suffer from chronic pain; neuropathic pain accounts for 17.9% of the total, corresponding to 3.5% [4]. The causes of neuropathic pain are diverse, and the pathogenesis is complex, with no effective clinical treatment [5, 6]. Several lines of evidence indicate that central sensitization, a condition of the nervous system with the development and maintenance of chronic pain, results from elevated synaptic transmission [7, 8]. Increases in chemical synapse transmission occurring simultaneously are closely related to the formation of neuropathic pain [9]. Calcium channels are important for neural transmission in CNS synapses and have been identified as key targets for synaptic signal transduction and treatment of chronic pain [10, 11]. In particular, L-type voltage-gated calcium channel (Cav1.2) controls short- and long-term sensitization to pain [12, 13]. However, it remains to be determined how calcium channels, especially Cav1.2, are regulated.

Collapsin response mediator protein 4 (CRMP4), one of five members of the CRMP family (CRMPs, CRMP 1–5), is a cytosolic phosphoprotein highly expressed in the CNS [14]. CRMPs regulate neuronal differentiation and axonal development by interacting with microtubule and actin cytoskeletons [15]. CRMP4 promotes F-actin assembly to regulate axonal growth and neurodegeneration [16]. CRMP4 and CRMP2 interact to coordinate cytoskeletal dynamics, regulating growth cone development and axon growth [17]. CRMP4 localizes in the synapses and regulates dendritic growth and maturation [14]. In mice, inhibition of CRMP4 induces phenotypes similar to those observed in human patients with autism [18], such as impaired social interaction, abnormal sensory sensitivity, changes in dendritic structure, and abnormal gene expression. Together, these observations indicate that CRMP4 plays an important role in synapses.

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CRMP2 regulation with calcium channels is involved in chronic pain [19, 20]; however, the role of CRMP4 in the central sensitization of chronic pain remains obscure, as does the underlying mechanism. Here, we applied the thermal pain sensitivity on rats as a method to reveal neuropathic pain [21–23].

In this research, we demonstrate that CRMP4 is post-translationally SUMOylated and that the deSUMOylated form of CRMP4 promotes the interaction with Cav1.2 to modulate calcium influx, modulates neurite outgrowth and regulates thermal pain sensitivity.

2. Materials and methods

2.1 Constructs

The *CRMP4*, *Cav1.2* (*a1c*, $\alpha 2\delta$, $\beta 2a$), Small Ubiquitin-like Modifier 1 (*SUMO1*), *SUMO2*, *SUMO3* and *Ubc9* genes were cloned from rat cDNA and validated via sequencing. The Glutathione S-transferase (GST)-pulldown assay, *CRMP4*, *Cav1.2* and *SUMO1-3* cDNA were inserted into the pGEX-5x-3 vector (Piscataway, NJ, USA). For immunoprecipitation, *CRMP4* or the mutant was subcloned into pEGFP-C1 (Takara Bio Europe, Saint-Germain-en-Laye, France), *Cav1.2* $\alpha 1c$ was cloned into pcDNA3.1-V5/His-A vector and *SUMO2* into pCMV-HA vectors (Stratagene, Santa Clara, CA, USA). The *CRMP4 K374R* mutant was constructed by a site-directed mutagenesis kit (Takara). *Ubc9* was inserted into a pCMV-FLAG-C vector.

2.2 Neuronal culture and transfection

Cortical neurons were separated as previously reported [24]. Briefly, newborn 1-day-old Sprague-Dawley rats from the Animal Center of Jinan University were sacrificed by CO_2 anesthesia. Then the cortices were dissected, cut into small pieces, and digested with 0.05% trypsin (#25300062, Life Technologies Corporation, Grand Island, NY, USA) for 15 min at 37 °C. After dissociation into a single-cell suspension, debris was removed by filtration through Falcon cell strainers. The resultant cells were maintained in a Neurobasal medium with B27 and Glutamax (#17504044, #35050061, Life Technologies Corporation). Human embryonic kidney (HEK) 293 cells (#CRL-1573, American Type Culture Collection, Manassas, VA, USA) were cultured as described previously [25]. Transient transfections of neurons were performed with calcium phosphate, and HEK293 cells were transfected with Lipofectamine 2000 (#11668019, Invitrogen Corporation, Carlsbad, CA, USA).

2.3 Neuronal morphological measurement

GFP-overexpressing neurons were immunostained with GFP antibody to boost the GFP signal and reveal the neurites to measure cortical neuron morphology. Images were captured in a blinded manner with an Axio Observer Z1 (#AXIO Observer Z1, Carl Zeiss, Germany). At least 60 neurons in each group were imaged. The total neurite length and branch tip number of each neuron were analyzed using ImageJ [26].

2.4 Western blotting

The procedure was performed as previously reported [27]. Briefly, cell lysates were separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Sigma, Burlington, MA, USA). The membrane was blocked at room temperature for 1 h with 5% non-fat milk in TBS containing 0.1% Tween-20 and then incubated at 4 °C overnight with antibodies against CRMP4 (#ab244319), Cav1.2 (#ab58552), GFP (#ab290), HA (#ab9110), FLAG (#ab1162), or V5 (#ab27671) (all from Abcam, Cambridge, MA, USA) in TBS buffer containing 3% BSA. GAPDH was used as a loading control. Blots were incubated with secondary antibodies at room temperature for 1 h and then visualized using enhanced chemiluminescence reagents (#WP20005, Thermo Fisher Scientific, Rockford, IL, USA). Results of western blotting are representative of three independent experiments.

2.5 GST-pulldown

GST-CRMP4 and GST-Cav1.2 (a1c) plasmids were transformed into Top10F′ *E. coli* (Invitrogen), and protein expression was induced with 0.1 mM IPTG (Roche Applied Science, Indianapolis, IN, USA). Cells were collected and lysed, and GST-fusion proteins were purified from the supernatant using glutathione–agarose beads (#16101, Pierce Biotechnology, Rockford, IL, USA). The GST-pulldown assay was performed in buffer containing 100 mM NaCl, 20 mM Tris-HCl, 5% glycerol (pH 7.0), 1% Triton X-100, and protease inhibitor cocktail (#539131, Merck, MA, USA), and GST-fusion proteins were incubated with SD rat brain lysates at 4 °C for 8 h. After pulldown, the sedimented proteins were subjected to western blotting.

2.6 Co-immunoprecipitation

For immunoprecipitation assays, neuronal extracts were prepared by solubilization in cell lysis buffer (Beyotime Institute of Biotechnology) for 10 min at 4 °C. After a brief sonication, lysates were cleared by centrifugation at 15,000 × g for 10 min at 4 °C, cell extract was immunoprecipitated with 4 μg antibodies against CRMP4 (Abcam, #ab244319) or Cav1.2 (Abcam, #ab58552), and the samples were incubated with 60 μL protein G/A agarose (Calbiochem EMD Millipore) overnight at 4 °C with continuous inversion. Immunocomplexes were pelleted and washed six times, and then the sedimented proteins were subjected to western blotting.

2.7 Immunofluorescence

Immunofluorescence assays were performed as previously described [28]. Briefly, neurons cultured on coverslips were fixed in 4% paraformaldehyde for 40 min at 4 °C and blocked with 3% BSA in TBS. Coverslips were then incubated with rabbit anti- GFP (Abcam, #ab290), -CRMP4 (Abcam, # ab244319), and -SUMO2/3 antibodies (Abcam, #ab81371) overnight at 4 °C. After washing with TBST, neurons were labeled with Alexa Fluor 555 donkey anti-goat IgG (H + L) and Alexa Fluor 647 donkey anti-rabbit IgG (H + L; Life Technologies) for 1 h at room temperature. Then neurons were mounted on glass slides using Fluoro Gel II contain-

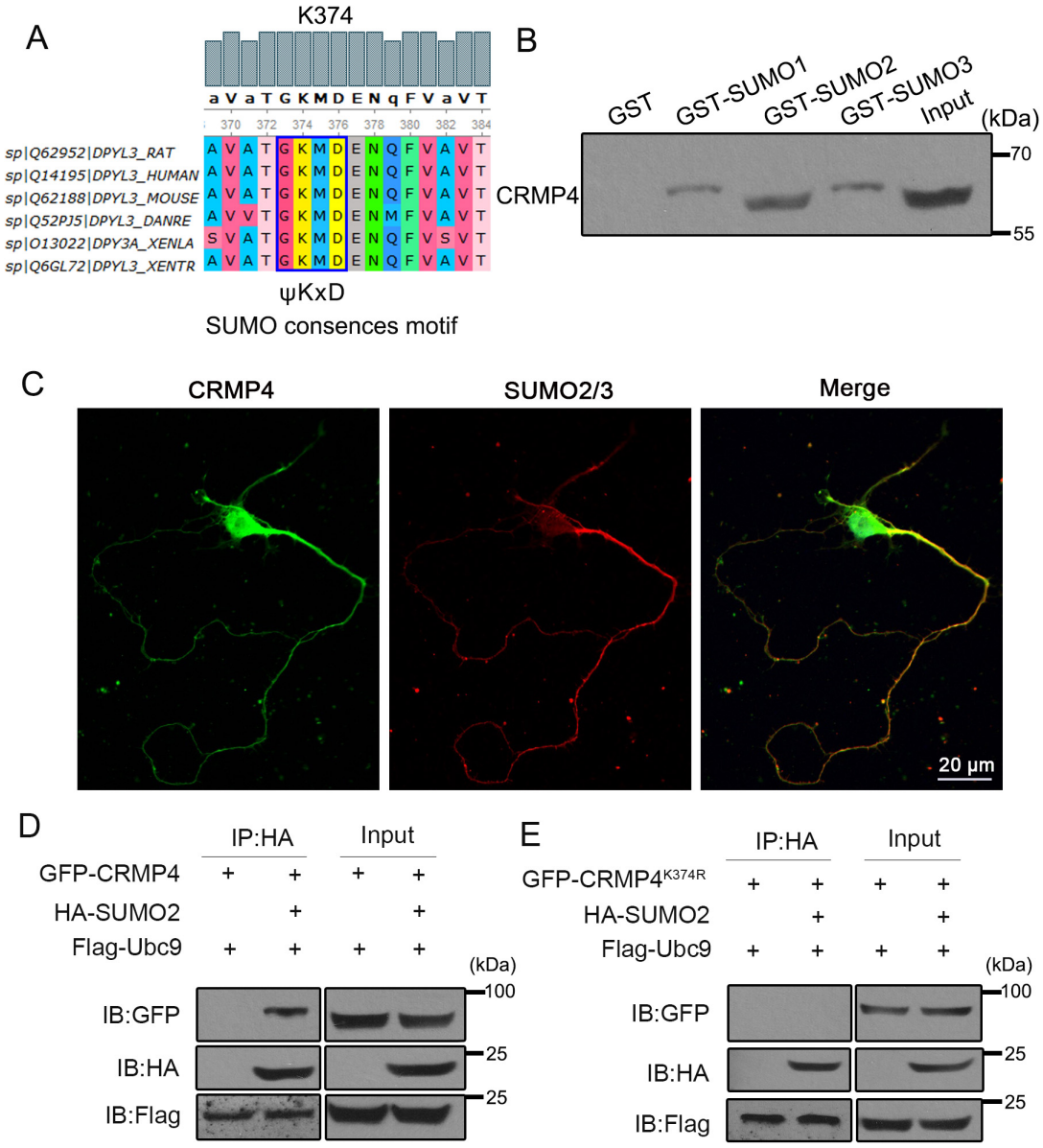

Fig. 1. CRMP4 can be SUMOylated at K374. (A) The amino acids around K374 are conserved among species. ΨKxD is the consensus motif for a SUMOylation site: Ψ is a hydrophobic amino acid, x is any amino acid, and D is an acidic amino acid. (B) GST-SUMO proteins can pull down CRMP4. The indicated GST-SUMO plasmids were constructed, the corresponding proteins were purified and incubated with brain lysates in pulldown assays, and the sediments were subjected to western blotting. N = 3. (C) Cultured neurons were stained with CRMP4 and SUMO2 antibodies for immunofluorescence. Scale bar, 20 μm. (D,E) 293T cells were co-transfected GFP-CRMP4 or GFP-CRMP4^{K374R} and FLAG-Ubc9, with or without plasmids encoding HA-SUMO2. Cell lysates were subjected to western blotting with GFP, HA, or FLAG antibodies. N = 3.

ing DAPI for confocal microscopy studies (LSM 700; Zeiss, GmbH, Germany).

2.8 Electrophysiology

HEK293T cells cultured on coverslips were transfected with DsRed-Cav1.2 (α1c, $\alpha 2\delta$ and β2a), GFP, GFP-CRMP4, or GFP-CRMP4^{K374R} plasmids for 24 h. Transfected cells were selected based on the expression of GFP and DsRed. HEK293T cells were patched in an external solution of modified Krebs-Ringer buffer (KRB) containing (in mM): 146 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 0.6 $MgSO_4$, 1.6 $NaHCO_3$, 0.15 NaH_2PO_4, 8 glucose, and 20 HEPES (pH 7.4); the buffer was ~330 mOsm. In transfected cells, I_{Ca} was recorded using a whole-cell voltage-clamp patch with an internal solution containing (in mM): 125 Cs-methanesulfonate, 10 TEA-Cl, 1 $MgCl_2$, 0.3 Na_2-GTP, 13 phosphocreatine-(di)Tris, 5 Mg·ATP, 5 EGTA, 10 HEPES (adjusted to pH 7.22 with CsOH). 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 the Clampex 10.5 software (Axon Instru-

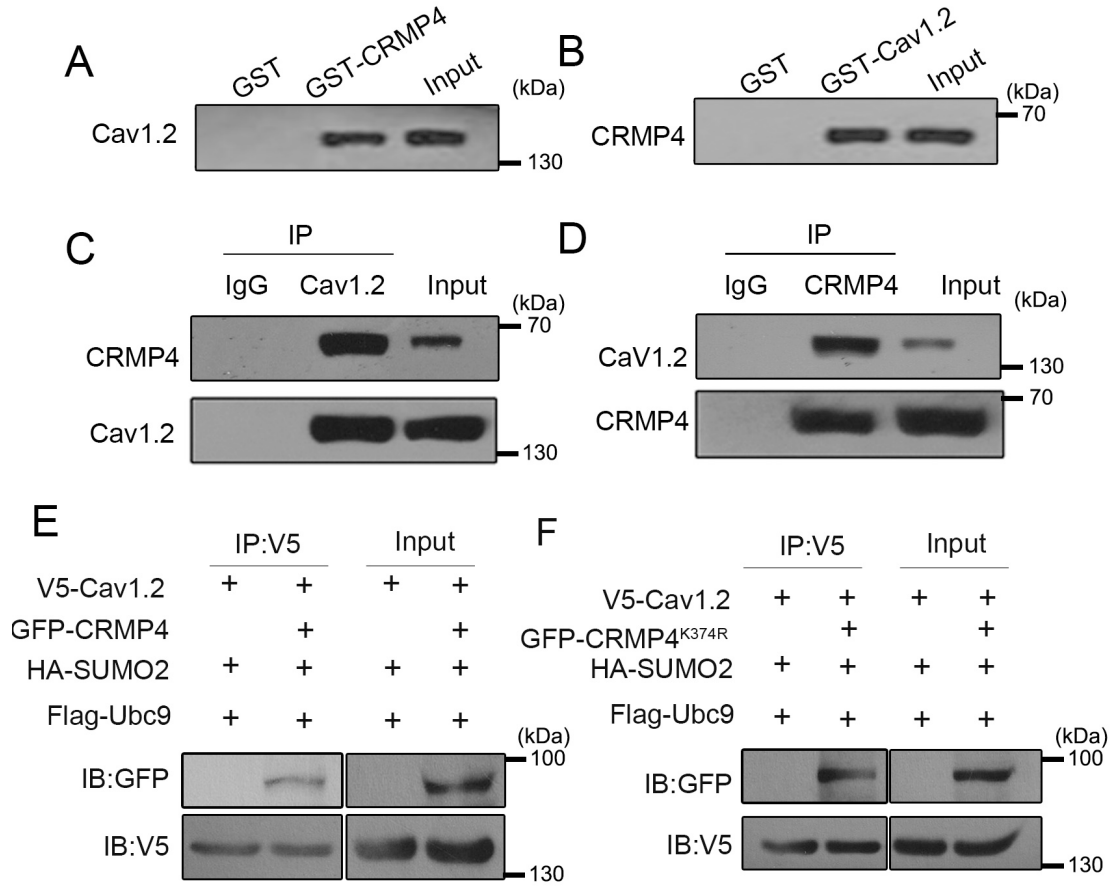

Fig. 2. CRMP4 interacts with Cav1.2. (A,B) Neuronal lysates were incubated with GST or GST-CRMP4 and GST-Cav1.2 for GST-pulldown assays, and the pulldown sediments were subjected to western blotting. (C,D) Neuronal lysates were incubated with IgG or Cav1.2/CRMP4 antibodies for immunoprecipitation assay, and the sediments were subjected to western blotting analysis. (E,F) 293T cells were transfected with the indicated plasmids; 24 h later, cells were harvested for immunoprecipitation with V5 antibody and western blotting with GFP or V5 antibody. N = 3 for all experiments.

ments, Union City, CA, USA). Current-voltage (*I–V*) relationships were obtained approximately 3 min after obtaining the whole-cell configuration by subjecting cells to a series of 300 ms depolarizing pulses from the holding potential of –70 mV to test potentials ranging from –60 to +100 mV in 10-mV increments.

2.9 Adenovirus associated virus (AAV) vectors

The viral vectors, including AAV-GFP, AAV-CRMP4-GFP and AAV-CRMP4-K374R-GFP (serotypes 2, 1×10^{12} to 5×10^{12} vg/mL), were purchased from OBIO Technology (Shanghai, China).

2.10 Thermal pain test

Individual rats (male Sprague-Dawley rat, 180–200 g, 8 weeks of age) were placed in test chambers, supported by an elevated platform, with no bottom or cover on a wide-gauge wire mesh. After habituation for 30 min, mechanical pain thresholds were measured, and the 50% paw withdrawal threshold (PWT) was determined as described previously [29]. A heat/light source was positioned under the glass surface, pointing to the plantar surface of the hind paw. Thermal paw withdrawal latencies (PWLs) were measured using a plantar anesthesia tester (Boerni, Tianjin, China).

2.11 Statistical analysis

The experimental data are presented as means ± SD from at least three experiments. The SPSS 19.0 software (SPSS Software, Chicago, IL, USA) was used for statistical analysis. Student's *t*-test was used for comparisons between two groups, and one-way ANOVA was used for multiple comparisons; $P < 0.05$ was considered significantly different.

3. Results

3.1 CRMP4 can be SUMOylated at K374

Using bioinformatics tools (SUMPsp, SUMOplot, and GPS-SUMO), we found that CRMP4K374 was a potential SUMOylation site. The GKMD sequence around CRMP4 K374 is consistent with the canonical ΨKXE/D motif and is evolutionarily conserved (Fig. 1A). To confirm the SUMOylation modification of CRMP4, we performed GST-pulldown, immunofluorescence, and immunoprecipitation (IP) assays. As shown in Fig. 1B, CRMP4 interacted with GST-SUMO1, GST-SUMO2, and GST-SUMO3, with GST-SUMO2 yielding the strongest signal. Because of the homol-

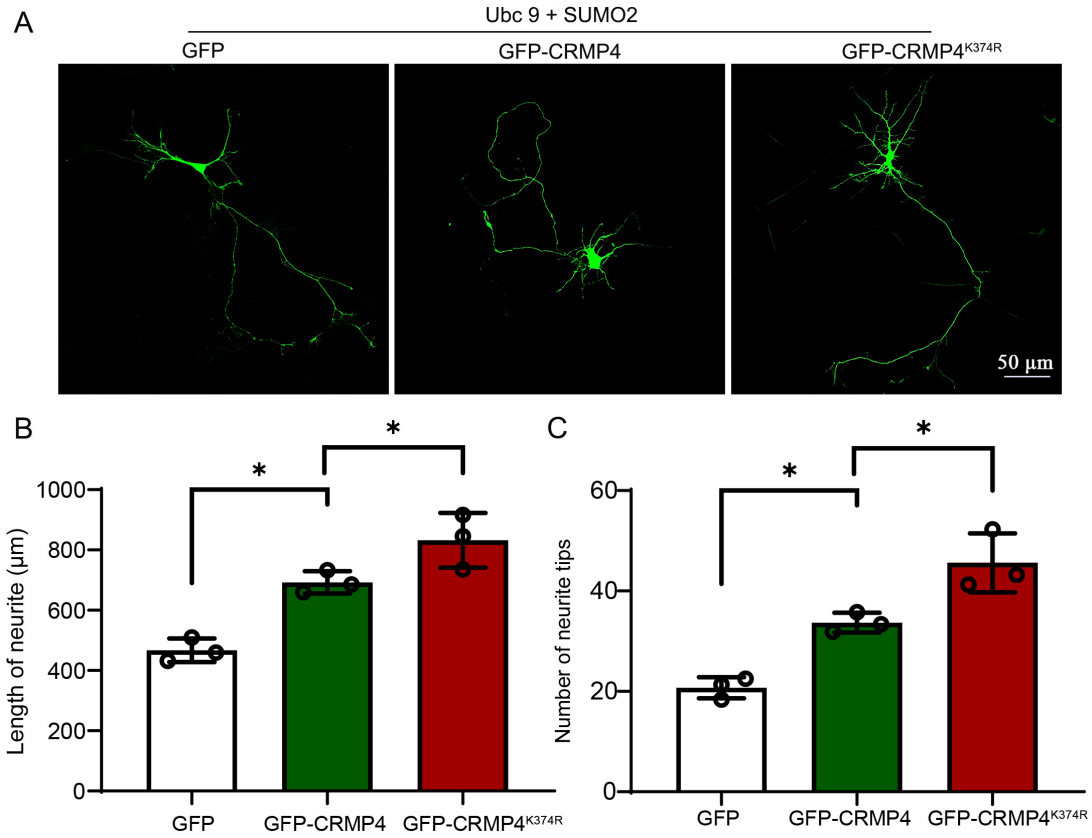

Fig. 3. The deSUMOylated form of CRMP4 promotes neurite growth. (A) DIV3 Cultured neurons were transfected with plasmids encoding Ubc9, SUMO2, GFP, GFP-CRMP4, or GFP-CRMP4K374R, then harvested DIV5 neuronal morphological analysis. Representative images of neurons are shown in (A), and length per neuron (B) and the number of tips per neuron (C) were measured in each group. Each group was enrolled in over 30 neurons. N = 3; Scale bar, 50 μm. * denotes $P < 0.05$.

ogy between SUMO proteins, we selected SUMO2 for subsequent study. Immunofluorescence staining revealed colocalization of CRMP4 and SUMO2/3 in the cell body and branches of neuronal processes, represented as merged yellow signals (Fig. 1C). Furthermore, epitope-tagged GFP-CRMP4 co-immunoprecipitated with HA-SUMO2, under the co-transfection of FLAG-Ubc9 (a sole E2 for SUMOylation) (Fig. 1D); however, when CRMP4 K374 was mutated to arginine (CRMP4^{K374R}), GFP-CRMP4^{K374R} could not precipitate with HA-SUMO2 (Fig. 1E). These data show that CRMP4 could be SUMOylated at K374.

3.2 CRMP4 interacts with Cav1.2

To further explore the detailed mechanism of CRMP4 SUMOylation, we applied interaction proteomics to identify the functional partners of CRMP4. This approach revealed that the L-type calcium channel Cav1.2 interacted with CRMP4 (data no shown). Hence, we performed GST-pulldown and immunoprecipitation experiments to confirm this potential interaction. Purified GST-CRMP4 or GST-Cav1.2 protein was incubated with cortical neuronal lysates, and then the pulldown sediments were subjected to western blotting. As shown in Fig. 2A–B, Cav1.2 was detected in the GST-CRMP4 pulldown sediment and CRMP4 in the GST-Cav1.2 pulldown sediment. Next, we subjected neuronal lysates to co-immunoprecipitation experiments using CRMP4 and Cav1.2 antibodies. The results confirmed that CRMP4 and Cav1.2 proteins were present in each other's immunoprecipitates (Fig. 2C–D). Together, these data indicate that CRMP4 interacts with Cav1.2. Next, we asked whether CRMP4 SUMOylation affects the interaction between CRMP4 and Cav1.2. To this end, we transfected HEK293 cells with V5-Cav1.2, GFP-CRMP4, or GFP-CRMP4^{K374R} plasmids together with plasmids encoding SUMO2 and Ubc9. As shown in Fig. 3, GFP-CRMP4 signal was detected in the immunoprecipitates obtained with V5 antibody (Fig. 2E), but GFP-CRMP4^{K374R} yielded a stronger signal than GFP-CRMP4 (Fig. 2F). These data indicate that CRMP4 interacts with Cav1.2, and SUMOylation of CRMP4 affects this interaction.

3.3 deSUMOylation of CRMP4 promotes neurite growth

CRMP4 promotes neurite growth in cultured hippocampal neurons [14, 30]. However, the role of CRMP4 SUMOylation remains to be explored. To further evaluate the effect of CRMP SUMOylation on neurite growth, we transfected cortical neurons with GFP control, GFP-CRMP4, and GFP-CRMP4^{K374R} along with plasmids encoding Ubc9 and

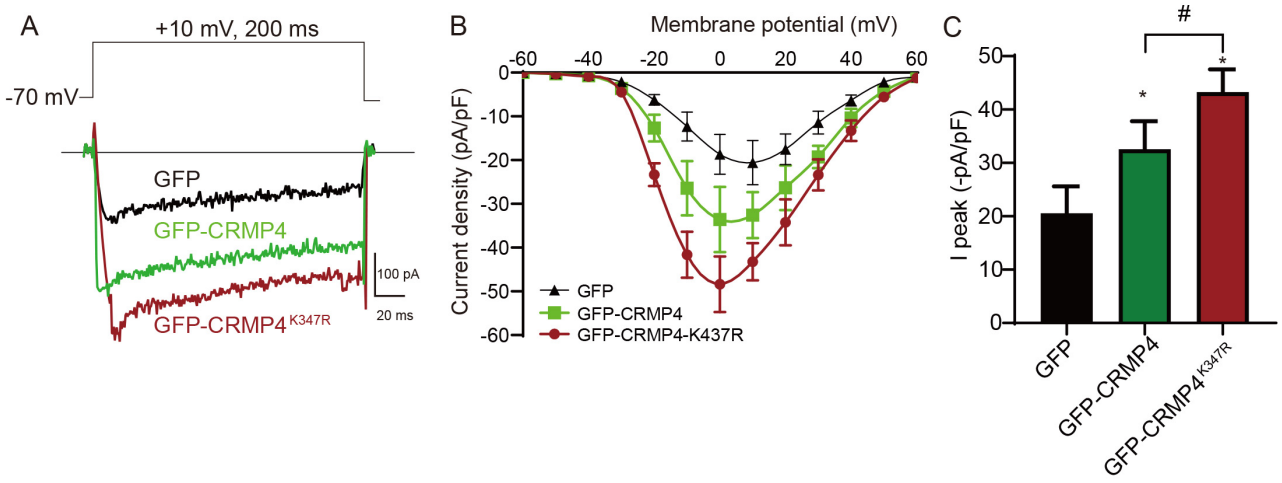

Fig. 4. CRMP4 SUMOylation regulates calcium influx via Cav1.2. (A) 293T cells were transfected with Cav1.2 (α1c, $\alpha 2\delta$ and β2a), together with plasmids encoding GFP, GFP-CRMP4, or GFP-CRMP4^{K374R}, and then whole-cell patch-clamp was applied to record the calcium current via Cav1.2. (B) *I–V* curves of GFP control, GFP-CRMP4 (green) and GFP-CRMP4^{K374R} (red). (C) Current density values at +10 mV. * denotes $P < 0.05$ vs. GFP; # denotes $P < 0.05$ vs. GFP-CRMP4. N = 4, each group was tested over 20 cells.

SUMO2. Because GFP-CRMP4^{K374R} cannot be SUMOylated, this mutant form of CRMP4 mimics the deSUMOylated state of CRMP4. As shown in Fig. 3A, successfully transfected neurons were stained with GFP to reveal neurite growth, and then the length and number of branches were measured. As shown in Fig. 3B–C, transfection of CRMP4 significantly increased both length and tip number. However, overexpression of the deSUMOylated form of CRMP4 (GFP-CRMP4^{K374R}) further increased the growth of the neuronal process relative to GFP-CRMP4, which could be SUMOylated. These data suggest that deSUMOylation of CRMP4 further promotes the neurite growth of cortical neurons.

3.4 CRMP4 SUMOylation regulates calcium influx via Cav1.2

The interaction between CRMP4 with Cav1.2 suggests that CRMP4 is involved in calcium influx and neuronal signal transmission. To confirm this, we transfected HEK293 cells with a plasmid encoding GFP, GFP-CRMP4, or GFP-CRMP4^{K374R} and Cav1.2 and then conducted a whole-cell patch-clamp to record the changes in the calcium current through Cav1.2. The peak current-voltage curves for the various transfections of –70 mV are shown in Fig. 4A. The results revealed that GFP-CRMP4 significantly increased the current density of Cav1.2, whereas GFP-CRMP4^{K374R} further promoted the effect (Fig. 4B). The same trends were apparent in *I–V* curves from different groups (Fig. 4C). Together, these data suggest that CRMP4 deSUMOylation promotes calcium influx through Cav1.2.

3.5 CRMP4 SUMOylation modulates thermal pain sensitivity in rats

Because Cav1.2 is involved in pain signaling [10], we asked whether CRMP4 and SUMOylation regulate pain sensation. To determine this, we used a rat model of thermal pain sensitivity. CRMP4-overexpressing AAV (AAV-CRMP4 and AAV-CRMP4^{K374R}) viruses were prepared and injected into the sciatic nerve. As shown in Fig. 5A, CRMP4-overexpressing rats exhibited thermal pain sensitivity in the injected hind paws, and deSUMOylated CRMP4^{K374R} further decreased the threshold of thermal pain sensation (Fig. 5B). These data suggest that deSUMOylation of CRMP4 promotes thermal pain sensitivity in rats.

4. Discussion

In this study, we demonstrated that CRMP4 could be SUMOylated at K374. In addition, CRMP4 interacts with Cav1.2 to regulate calcium current density, which SUMOylation of CRMP4 could enhance. The deSUMOylated form of CRMP4 promotes neurite outgrowth. Furthermore, CRMP4 increases thermal pain sensitivity and SUMOylation of CRMP4 influences the effects. Our findings collectively reveal a new mechanism by which CRMP4 SUMOylation regulates its interaction with Cav1.2, neurite growth, and signal transmission during pain sensitivity.

Recent work showing that SUMOylation regulates CRMP2 has been revealed. K374 of CRMP2 can be SUMOylated [31]. This modification changes the interaction of CRMP2 with calcium and sodium channels, promoting the Na^+ current through the expression of NaV1.7 [32], but decreases the Ca^{2+} influx through Cav2.2 [33]. DeSUMOylation at K374 and dephosphorylation of CRMP2 at Thr514 contribute to the formation and maturation of dendritic spines [34]. Because the amino acid sequences around K374 are highly conserved among CRMPs proteins, as previously predicted [33], we also hypothesized that K374 of CRMP4 could also be SUMOylated but lacks experimental evidence. Here, GST-pulldown, co-immunoprecipitation, and colocalization analysis revealed that CRMP4 could interact with SUMO proteins, whereas mutation of K374 to K374R

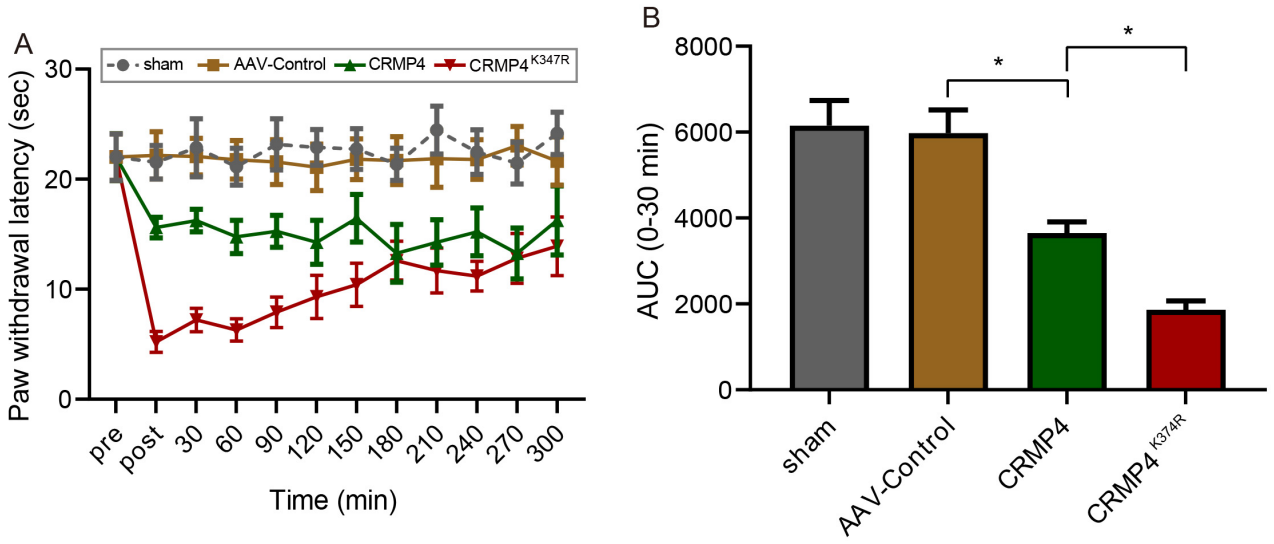

Fig. 5. CRMP4 SUMOylation regulates thermal pain sensitivity in rats. AAV viruses of Control, CRMP4, or CRMP4^{K374R} were injected into the hind paws of rats, and the rats were tested for thermal pain sensitivity. (A) Thermal pain thresholds were determined using a plantar tester. (B) Effects of CRMP4 overexpression on thermal pain thresholds of rats. * denotes $P < 0.05$.

could abolish this modification (Fig. 1), confirming the K374 mediated SUMOylation of CRMP4. Posttranslational modification of proteins is critical for the fine-tuning of protein functions. Phosphorylation abolishes the interaction of CRMP2 and tubulin, resulting in the termination of microtubule assembly and elongation and thus hindering the growth of neuronal processes [15]. SUMOylation of CRMP2 regulates interaction with calcium channels [33] and sodium channels [32], and phosphorylation and SUMOylation together modulate the function of CRMP2 in regulating the formation and maturation of dendritic spines [34]. CRMP4 is phosphorylated by a variety of kinases, including GSK-3, to regulate neurite outgrowth [35], migration, and positioning [36], as well as Cdk5 and DYRK2 to regulate neurulation [37]. Whether and how the phosphorylation of CRMP4 affects SUMOylation and how upstream kinases such as GSK-3, Cdk5, or other kinases modulate CRMP4 activity remain to be further explored. CRMP4 is involved in neurite outgrowth [17, 35], and proteomic analysis revealed post-translational modification of CRMP4 status changes during hyperalgesia. Neurite outgrowth is critical for nerve injury and sensory recovery [38, 39]. We show that CRMP4 SUMOylation further enhanced neurite outgrowth. However, whether or not this effect contributes to nerve injury recovery needs further research.

In general, nerve damage leads to persistent neuropathic pain via changes in primary sensory neurons and central nervous system pain pathways [40]. During these processes, voltage-gated calcium channels play important roles in upregulating sensory neuronal excitability and promoting the transmission of pain signals [41]. CRMPs often interact with voltage-gated calcium channels (VGCCs). The Ca^{2+} channel binding domain 3 (CBD3) in CRMP2 mediates the interaction with Cav2.2, leading to the membrane transport of Cav2.2 and boosting synaptic transmission [42]. Overexpression of CBD3 disrupts the coupling of CRMP2 and Cav2.2, decreasing the amount of Cav2.2 on the membrane surface and thereby suppressing inflammatory and neuropathic pain in mouse models [19]. CRMP2 interacts with NMDA receptors and the Na^{+}/Ca^{2+} exchanger to promote glutamate-induced Ca^{2+} dysregulation in hippocampal neurons [43]. CRMP3 promotes Ca^{2+} influx induced by depolarization of L-type and N-type calcium ion channels, thereby promoting dendritic growth of hippocampal neurons [44]. CRMP4 knockout mice exhibit impaired social interaction and abnormal sensory sensitivity, similar to the phenotype observed in human patients with autism [18]. Cav1.2, an L-type VGCC expressed in most types of neurons, is usually located in cell bodies and dendrites; it is essential to activate calcium-dependent enzymes and potassium channels, initiate calcium-dependent gene transcription, and neurotransmitter release [45]. A proteomic study revealed that L-type VGCC is in the CRMP2 interactome [46], and the L-type VGCC Cav1.2 channel is thought to interact with CRMP2 via the C-terminal motif [47]. Cav1.2 is also expressed in dorsal root ganglia (DRG) and spinal cord neurons [48]. Although the role of Cav1.2 for neuropathic pain remains controversial, studies show that Cav1.2 is involved in short- and long-term sensitization to pain [12, 13]. Genetic knockdown of Cav1.2 reverses the neuropathy-associated mechanical hypersensitivity and the hyperexcitability of dorsal horn neurons [49], indicating the important role of Cav1.2 during pain signaling. Here, we showed that CRMP4 interacts with the L-type VGCC channel Cav1.2; this interaction, which SUMOylation of CRMP4 influenced, mediates signal transduction of neuropathic pain *in vivo* (Figs. 4,5).

In summary, we found that SUMOylation of CRMP4 affects its interaction with Cav1.2, neurite outgrowth and thermal pain sensitivity (Fig. 6). These findings may provide new insights into CRMP-related regulation of neuronal development and reveal novel therapeutic targets for the clinical treatment of chronic pain.

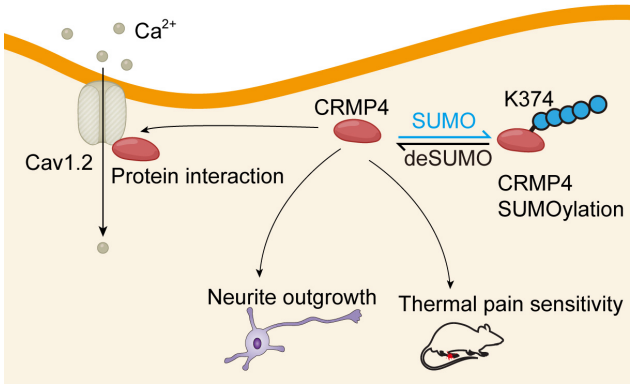

Fig. 6. CRMP4 could be SUMOylated, and SUMOylation affects its interaction with Cav1.2, neurite outgrowth and thermal pain sensitivity.

Abbreviations

CBD3, calcium-binding domain 3; CRMP, collapsin response mediator protein; DIV, days *in vitro*; GSK-3β, Glycogen Synthase Kinase-3 beta; PWLs, paw withdrawal latencies; PWT, paw withdrawal threshold; SUMO, small ubiquitin-like modifier; VGCC, voltage-gated calcium channel.

Author contributions

YL conceived and designed the experiments; SL performed the experiments; MP and HL analyzed the data; JC and YJ contributed reagents and materials; SL and YL wrote the paper.

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee at Jinan University (2018122302) and were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health.

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Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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