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Blocking CRMP2 SUMOylation reverses neuropathic pain

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Collapsin response mediator proteins 1–5 (CRMPs1 –5) are a family of cytosolic proteins that coordinate neuronal migration, axonal guidance, dendritic organization, dendritic spine development and synaptic plasticity (reviewed in Khanna *et al.*[1] and Quach *et al.*[2]). Members of the CRMP family are reported to be involved in the pathogenesis of various neuronal disorders. For instance, proteomic, genomic and translational approaches linked the *CRMP1* gene with chronic, negative symptoms of schizophrenia and severe major depression.[3] Mice lacking CRMP1 manifest hyperactivity, impaired learning and memory, and impaired prepulse inhibition behavioral abnormalities related to schizophrenia.[4] Genetic association and linkage studies pointed to *CRMP2* as a liability gene for schizophrenia, autism, alcohol dependence, depression and bipolar disorders.[5–10] Mice with brain-specific *Crmp2* deletion exhibited behavioral deficits in locomotor activity, sensorimotor gating, social behavior, and spatial learning and memory.[11] Maternal autoantibodies against CRMP1 and CRMP2 were found in children with autism spectrum disorders that displayed core deficits in communication and reciprocal social interaction as well as repetitive or stereotypical behaviors.[12] CRMP3-deficient mice display significant decreases in dendritic length and branching points, and an abnormal undulation of apical primary dendrites; these findings are recapitulated in the brain of Down syndrome where the expression of *CRMP3* gene is also impaired.[13] Little is known about the relationship between CRMP4 and neuropsychiatric disorders. However, mice lacking CRMP4 manifest impaired olfactory function and hyperactivity in the olfactory bulb and have increased levels of ionotropic glutamate receptors GluRs 1 and 2, which have been implicated in autism spectrum disorders and schizophrenia.[14] CRMP5 knockout mice implicate this protein in dendritic development and synaptic plasticity in cerebellar purkinje cells,[15] and CRMP5 autoantibodies were reported in patients with paraneoplastic neurological syndrome characterized by cerebellar ataxia and chorea. Therefore, understanding CRMP signaling has significant clinical implications.

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CRMPs undergo several post-translational modifications (PTMs) that are hypothesized to play a critical role in codifying its functions. For example, CRMP2 is subject to phosphorylation at multiple sites, SUMOylation (addition of small ubiquitin-like modifier (SUMO)), O-GlyNAcylation (addition of β-N-acetyl-D-glucosamine (O-GlcNAc) and oxidation.[1] The potential interplay between PTMs of CRMPs likely contributes to a 'PTM code' that pairs a particular PTM signature with a particular function. As expression and/or PTMs of CRMPs are altered in mental (schizophrenia and mood disorders) and neurological (Alzheimer's, prion encephalopathy, epilepsy and others) disorders,[2] it is likely that targeting these PTM codes may be a novel therapeutic strategy for neuropsychiatric disorders. Here, we illustrate the potential of such targeting applied to a chronic pain model. Inhibiting CRMP2 interactions[16] or phosphorylation[17] has highlighted a central role for this protein in pain signaling transmission. Exactly how this is achieved is not known, but recent studies point to the regulation of voltage-gated ion channels by CRMP2 as a possible link.

Mutations in the voltage-gated sodium channel NaV1.7, encoded by the *SCN9A* gene, have been linked to human pain syndromes as well as autism.[6] In neuropathies associated with diabetes, injury or chemotherapeutic administration, chronic pain can result from upregulated NaV1.7 expression.[18] NaV1.7 is preferentially expressed in peripheral sensory neurons, where activity of this channel determines whether subthreshold stimuli will cumulatively drive action potential generation and pain signaling.[19] The exact pathways leading to the dysregulation of NaV1.7 are poorly understood but likely involve mechanisms related to its surface trafficking and regulation via protein–protein interactions.[18,20–22] Recent studies have identified neuronal CRMP2 as a novel binding partner of NaV1.7.[21,22] Specifically, a selective reduction in NaV1.7 surface expression and current density was observed in rodent and human sensory neurons expressing a mutant CRMP2 lacking the SUMO PTM site (lysine 374) in CRMP2.[21,22] The work also demonstrated that loss of CRMP2 SUMOylation increased binding to endocytic proteins, accounting for the removal of NaV1.7 from the plasma membrane.[21] CRMP2 phosphorylation, an event required prior to SUMOylation,[21] was found to be increased following chronic constriction injury.[23] Whether CRMP2 SUMOylation is dysregulated in chronic pain states has never been investigated. Since CRMP2 SUMOylation controls NaV1.7 function and excitability, we hypothesized that NaV1.7 upregulation in chronic neuropathic pain could be explained by a concomitant increase in CRMP2 SUMOylation (Figure 1a). Here, we demonstrate that CRMP2 SUMOylation is increased during neuropathic pain. *In vivo* overexpression of non-SUMOylated CRMP2 revealed a role for CRMP2 SUMOylation in driving nociceptive behavior in an animal model of neuropathic pain. Understanding the role of CRMP2 modifications in modulation of NaV1.7 activity and pain opens routes to exploit this system for therapeutic purposes.

NaV1.7 traffics to the cell surface in consort with CRMP2 (Figure 1b). Proteostasis of NaV1.7 trafficking and/or activity in neuropathic pain could be driven by changes in CRMP2. Therefore, we asked whether CRMP2 modifications are active in the peripheral nervous system of rats subjected to unilateral spared nerve injury (SNI), an injury that involves a lesion of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact. SNI increases sensitivity to touch that may be attributed to increased NaV1.7 surface expression and current densities.

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[18] In tissues from rats subjected to SNI, we observed increases in NaV1.7 protein in the ipsilateral spinal cord dorsal horn; a structure where the dorsal root ganglia sensory neurons project their axons and make synapses required for pain signal transmission. Total CRMP2 expression was unchanged in spinal cord dorsal horn, glabrous skin and sciatic nerve between the ipsi- and contralateral sides of rats with SNI at post-injury day 7 (Figure 1c). Importantly, a robust increase in CRMP2 SUMOylation was observed in the dorsal horn ipsilateral to SNI injury (Figures 1c and d). This suggests that increased CRMP2 SUMOylation, following SNI, likely augments NaV1.7 synaptic localization in the dorsal horn of the spinal cord or increases NaV1.7 insertion along the central terminal projection. This identifies CRMP2 SUMOylation as a pathological event participating in chronic pain.

We recently demonstrated that loss of CRMP2 SUMOylation promotes NaV1.7 endocytosis, decreasing NaV1.7 currents and decreasing excitability.[21,22] Therefore, mimicking deSUMOylation of CRMP2 *in vivo* might be antinociceptive. To test this hypothesis, we transiently expressed plasmids encoding *Discosoma* sp. red fluorescent protein (dsRed) alone (empty plasmid) or fused to either wild-type CRMP2 (CRMP2 WT) or SUMO-null CRMP2 (CRMP2 K374A) *in vivo* to evaluate the reversal of SNI-induced mechanical allodynia in rats. Paw withdrawal thresholds (PWTs) in rats 7 days following a sham injury were not different for at least 3 days following spinal injection of the three plasmids (Figure 1e). In contrast, spinal administration of CRMP2 K374A significantly increased PWTs over post-SNI values at 3, 24 and 48 h post-injection compared to controls (Figure 1f). That PWT returned to baseline at 72 h in CRMP2 K374A-injected rats is consistent with the turnover of CRMP2 over this period[24] and a limitation of the non-viral transfection method used here. PWTs of CRMP2 WT-injected rats remained no different from rats injected with empty plasmid for the duration of the experiment (Figure 1f). Only CRMP2 K374A significantly reversed SNI mechanical allodynia compared to the empty plasmid or CRMP2 WT conditions. Expression of exogenous dsRed-tagged CRMP2, revealed by an antibody against dsRed, was detected in both spinal cord and dorsal root ganglia tissues of SNI animals (Figure 1g). These findings reinforce the hypothesis that CRMP2 SUMOylation and pain are functionally linked. It is noteworthy that CRMP2 activity is directed by multiple PTMs that are unaffected by SUMOylation,[21] leaving open the possibility that neuronal CRMP2 SUMOylation might be targeted independent of other critical functions.[1]

Our results identify CRMP2 SUMOylation as a potential biomarker for persistent pain and demonstrate successful *in vivo* targeting of CRMP2 modifications, which selectively mitigate NaV1.7 activity and attenuate neuropathic pain. This work is likely to spur the discovery of molecular strategies to inhibit CRMP2 SUMOylation *in vivo* using either small molecules or peptides. Identifying a CRMP2 antagonist could lead to a genetic treatment for neuropathic pain, as recently reported with adenoviruses, for example, that uncoupled the CRMP2 channel interaction.[25] Given that CRMP2 is a likely candidate for increased susceptibility to neuropsychiatric and neurodegenerative disorders,[5] its regulation of NaV1.7 proteostasis may suggest roles beyond chronic pain. Traditionally viewed as a peripheral nervous system protein, NaV1.7 has been reportedly found in the central nervous system and it is believed that rare variants in NaV1.7 decrease the firing of a specific set of inhibitory GABAergic neurons that are important in control of social behaviors linked to autism.[6] We postulate that NaV1.7 partial loss-of-function variants linked to familial autism[6] may be

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controlled by CRMP2 expression and SUMOylation status, and thus amenable to therapeutic control. The value of therapeutically targeting other PTMs of CRMP2 was illustrated by a recent study investigating neuroadaptations underlying excessive alcohol drinking behaviors.[9] Ron and colleagues[9] demonstrated that excessive alcohol consumption resulted in (i) enhanced translation of CRMP2 by mammalian target of rapamycin complex 1 (mTORC1), a complex with essential roles in learning and memory and (ii) accumulation of CRMP2 in its hypophosphorylated form due to block of glycogen synthase kinase-3β-phosphorylation of CRMP2. Notably, genetic and pharmacological inhibition of CRMP2 attenuated alcohol preference, suggesting that CRMP2 is a key contributor to addictive behaviors by mediating neuroplasticity of reward pathways.[9] Another example is the demonstration that axon degeneration observed in an experimental model of multiple sclerosis can be blocked by reducing CRMP2 phosphorylation by Rho kinase.[26]

In summary, our mechanistic findings demonstrate that genetic and/or pharmacological manipulation of CRMP2 PTMs is a viable translational strategy for developing treatments for various psychiatric disorders. Whether this holds true for other CRMPs and their PTMs is an open and exciting question.

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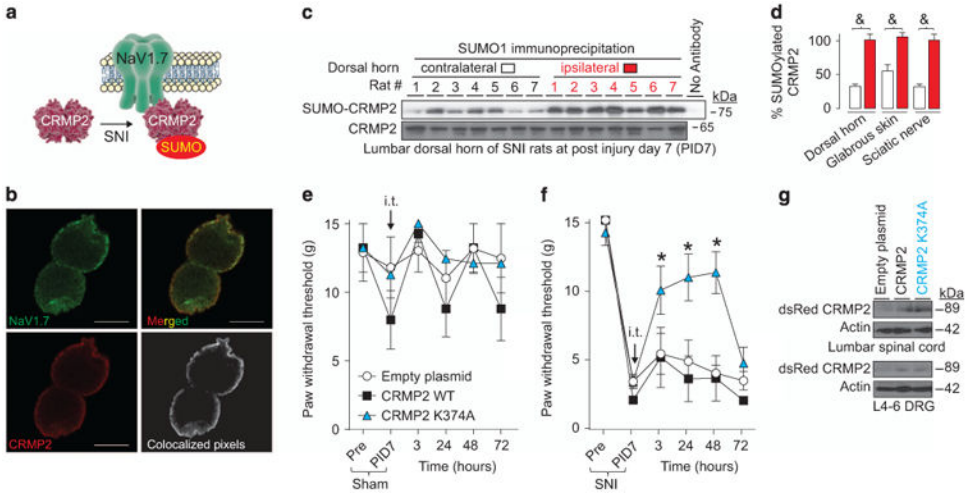

Figure 1.

Collapsin response mediator protein2 (CRMP2) SUMOylation (small ubiquitin-like modifier) is increased in neuropathic pain and drives nociceptive behaviors. (**a**) Cartoon depicting hypothesis of increased CRMP2 SUMOylation in neuropathic pain driving NaV1.7 function. (**b**) Representative micrographs of CRMP2 and NaV1.7 colocalization in dorsal root ganglia (DRG) sensory neurons. Merged colocalization image and pixels corresponding only to the colocalized proteins (analyzed via Image J, US National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/) are also shown. All cells imaged displayed surface colocalization of CRMP2 and Nav1.7 ($n = 8$). (**c**) Western blots of lumbar dorsal horn of spared nerve injury (SNI) rodents, at post-injury day 7 (PID7), immunoprecipitated with a SUMO1 antibody (Cat#S8070, Sigma, St Louis, MO, USA) and probed with an anti-CRMP2 polyclonal antibody (Cat#2993, Sigma) (top panel). Representative immunoblots of lysates of spinal cord dorsal horn from SNI rodents show no change in CRMP2 expression between ipsilateral (injured) and contralateral (non-injured) sides ($n = 7$; size determined by power analysis and previous experiments) in the same animal (bottom blot). (**d**) Summary data showing increased SUMOylation of endogenous CRMP2 in ipsilateral tissues spinal cord, glabrous skin and sciatic nerve of SNI rodents at PID7. Data represent percent of SUMOylated CRMP2 of total CRMP2 and normalized to the ipsilateral side for each tissue. &, $P < 0.05$ compared to dsRed (Student's t-test). (**e**) Paw withdrawal thresholds for sham-injured rats (at PID7) spinally injected (indicated by arrow), via an intrathecal (i.t.) catheter, dsRed (empty plasmid), dsRed-CRMP2 wild-type (WT) or dsRed-CRMP2 K374A (20 μg per rat in Turbofect *in vivo* transfection reagent (Cat# R0541, Thermo Fisher Scientific, Waltham, MA, USA); i.t.; $n = 5$-6). (**f**) Paw withdrawal thresholds for rats with an SNI injury and i.t. administered dsRed, dsRed-CRMP2 or dsRed-CRMP2-K374A (20 μg per rat, i.t.; $n = 9$–10). *$P < 0.05$ compared to dsRed. Data were analyzed by non-parametric two-way analysis of variance, where time was the within subjects factor and treatment was the between subjects factor (*post hoc*: Student–Neuman–Kuels). (**g**) Representative immunoblots of spinal cord dorsal horn (top blots) or DRG (bottom blots) lysates from rats injected with the indicated plasmids and probed with antibodies against dsRed (Cat#BDB551814, Thermo Fisher Scientific) (to reveal dsRed-fused CRMP2) and Actin (Cat#2066, Sigma; $n = 3$). Tissues were taken at the peak of antinociceptive effect

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(i.e., 24 h) of the CRMP2 K374A plasmid. The Institutional Animal Care and Use Committee of the College of Medicine at the University of Arizona approved all experiments. All behavioral experiments were performed by experimenters who were blinded to randomly assigned experimental groups and treatments. Male Sprague–Dawley rats (225–250 g; Envigo, Placentia, CA, USA) were used for all studies.