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CRMP2–Neurofibromin Interface Drives NF1-related Pain

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Abstract—An understudied symptom of the genetic disorder Neurofibromatosis type 1 (NF1) is chronic idiopathic pain. We used targeted editing of *Nf1* in rats to provide direct evidence of a causal relationship between neurofibromin, the protein product of the *Nf1* gene, and pain responses. Our study data identified a protein-interaction network with collapsin response meditator protein 2 (CRMP2) as a node and neurofibromin, syntaxin 1A, and the N-type voltage-gated calcium (CaV2.2) channel as interaction edges. Neurofibromin uncouples CRMP2 from syntaxin 1A. Upon loss/mutation of neurofibromin, as seen in patients with NF1, the CRMP2/Neurofibromin interaction is uncoupled, which frees CRMP2 to interact with both syntaxin 1A and CaV2.2, culminating in increased release of the pro-nociceptive neurotransmitter calcitonin gene-related peptide (CGRP). Our work also identified the CRMP2-derived peptide CNRP1, which uncoupled CRMP2's interactions with neurofibromin, syntaxin 1A, as well as CaV2.2. Here, we tested if CRISPR/Cas9-mediated editing of the *Nf1* gene, which leads to functional remodeling of peripheral nociceptors through effects on the tetrodotoxin-sensitive (TTX-S) Na^+ voltage-gated sodium channel (NaV1.7) and CaV2.2, could be affected using CNRP1, a peptide designed to target the CRMP2–neurofibromin interface. The data presented here shows that disrupting the CRMP2–neurofibromin interface is sufficient to reverse the dysregulations of voltage-gated ion channels and neurotransmitter release elicited by *Nf1* gene editing. As a consequence of these effects, the CNRP1 peptide reversed hyperalgesia to thermal stimulation of the hindpaw observed in *Nf1*-edited rats. Our findings support future pharmacological targeting of the CRMP2/neurofibromin interface for NF1-related pain relief. **© 2018 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: CRMP2, neurofibromin, pain, CaV2.2, NaV1.7, Neurofibromatosis type 1.

INTRODUCTION

Chronic idiopathic pain is a major symptom and comorbidity factor in the neurological disorder Neurofibromatosis type 1 (NF1) (Creange et al., 1999; Drouet et al., 2004). The prevalence of pain in NF1 patients is unknown but qualities of life-based questionnaires consistently identify both the intensity and quality of pain as having a major impact on NF1 patients (Wolkenstein et al., 2001; Ferner, 2007; Kodra et al., 2009; Crawford et al., 2015; Wolters et al., 2015; Bicudo et al., 2016; Ferner et al., 2017; Riklin et al., 2017). The first direct evidence of a causal relationship between neurofibromin, a protein encoded by the *Nf1* gene, and pain responses was demonstrated using a genome-editing approach *in vivo* (Moutal et al., 2017a,f). The truncation of neurofibromin by targeting exon 39 on the *Nf1* gene in sensory neurons, with a specific guide RNA (gRNA) conjugated to clustered regularly interspaced short palindromic repeats (CRISPR) associated protein-9 nuclease (Cas9), resulted in hyperalgesia (Moutal et al., 2017a,f). The underlying mechanism for the observed painful behaviors was hypothesized via remodeling of small-diameter nociceptive sensory neurons (Moutal et al., 2017b). Data from recent studies of rat neurons from Cas9-edited *Nf1* were in agreement with observations in haploinsufficient ($Nf1^{+/-}$) mouse sensory neurons: upregulation of voltage-gated ion channels (Duan et al., 2014; Wang et al., 2010a,b; Moutal et al., 2017a,b,f). Specifi-

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Abbreviations: ANOVA, analysis of variance; AP, action potential; Cas9, CRISPR-associated protein-9 nuclease; CaV2.2, N-type voltage-gated calcium channel; CGRP, calcitonin gene-related peptide; CNRP1, CRMP2–neurofibromin regulating peptide 1; CRISPR, clustered regularly interspaced short palindromic repeats; CRMP2, collapsin response mediator protein 2; DMEM, Dulbecco's Modified Eagle's medium; DRG, Dorsal Root Ganglia; EPM, elevated plus maze; gRNA, guide RNA; NaV1.7, Na^+ voltage-gated sodium channel 1.7; NF1, Neurofibromatosis type 1; PPIs, protein–protein interactions; TTX-R, tetrodotoxin-resistant; TTX-S, tetrodotoxin-sensitive.

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cally, the nociceptive synapse in these rodent models of NF1 is characterized by increases in N-type voltage-gated calcium (CaV2.2) as well as tetrodotoxin-sensitive (TTX-S) voltage-gated sodium (NaV1.7) currents (Chew and Khanna, 2018; Duan et al., 2014; Moutal et al., 2017f; Wang et al., 2010a,b). A consequence of this nociceptor remodeling was a reduction in rheobase (i.e., the current required to elicit the first action potential (AP)) and a concomitant increase in excitability (Wang et al., 2005, 2010b; Moutal et al., 2017f). It follows then, that release of excitatory transmitter – calcitonin gene-related peptide (CGRP) – to the spinal dorsal horn was also increased in both *Nf1*-edited rats and $Nf1^{+/-}$ mice (Hingtgen et al., 2006; Moutal et al., 2017a). Thus, in NF1, the sensory neurons are primed at the synaptic level for facilitated nociceptive signal transmission. Further studies identified the dysregulation of the collapsin response mediator protein 2 (CRMP2) (Patrakitkomjorn et al., 2008) to be responsible for NF1-related pain.

CRMP2 was initially described as an axonal protein involved in axon guidance and growth (Goshima et al., 1995; Kamata et al., 1998; Fukata et al., 2002). The functional association of neurofibromin and CRMP2 was reported to be essential for neuronal cell differentiation; lack of expression or abnormal regulation of neurofibromin resulted in impaired function of neuronal cells (Lin and Hsueh, 2008; Patrakitkomjorn et al., 2008). Suppression of neurofibromin using neurofibromin small-interfering RNA significantly inhibited neurite outgrowth and upregulated CRMP2 phosphorylation by kinases identified as Cdk5, GSK-3β, and Rho kinase (Patrakitkomjorn et al., 2008). Truncation of the C-terminus of neurofibromin, where CRMP2 binds (Patrakitkomjorn et al., 2008), lead to upregulation of CRMP2 functions in ion channel trafficking (Moutal et al., 2017a,f). CRMP2 binds to and regulates the membrane localization of both CaV2.2 (Brittain et al., 2009, 2011,; Moutal et al., 2016a,b,c) and NaV1.7 (Dustrude et al., 2013, 2016, 2017). CRMP2 also participates in neurotransmitter release (Chi et al., 2009; Moutal et al., 2016b,e). Recent proteomic analysis identified syntaxin 1A as a novel CRMP2-binding protein whose interaction with CRMP2 was strengthened in neurofibromin-depleted cells (Moutal et al., 2017e). These mutually exclusive interactions appear to form a 'core complex' that can facilitate nociceptive transmission in NF1. A peptide targeting CRMP2's interaction domain with neurofibromin, designated CRMP2–neurofibromin regulating peptide 1 (CNRP1,) inhibited CGRP release and acute and neuropathic nociceptive behaviors (Moutal et al., 2017e).

Although an obligatory role for CRMP2 has been established for manifestation of NF1-related pain (Moutal et al., 2017a), the precise manner by which neurofibromin's interaction with CRMP2 leads to alterations in ion channel function is unclear. Here, we tested if targeting the CRMP2/neurofibromin interaction in *Nf1* edited in sensory neurons could reverse ion channel dysregulation, sensory neuron excitability, and hyperalgesia. Our data show that the CNRP1 peptide from CRMP2 is sufficient to reverse alterations of sensory neurons and nociceptive behaviors in NF1without producing motor impairment or anxiety.

EXPERIMENTAL PROCEDURES

Animals

Pathogen-free, adult male and female Sprague–Dawley rats (150–200 g; Harlan Laboratories) were housed in temperature- (23 ± 3 °C) and light (12-h light/12-h dark cycle; lights on 07:00–19:00)-controlled rooms with standard rodent chow and water available *ad libitum*. The Institutional Animal Care and Use Committee of the College of Medicine at the University of Arizona approved all experiments. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain. Animals were randomly assigned to treatment or control groups for the behavioral experiments. Animals were initially housed three per cage but individually housed after the intrathecal cannulation on a 12-h light–dark cycle with food and water *ad libitum*. All behavioral experiments were performed by experimenters who were blinded to the experimental groups and treatments.

Materials

t-CNRP1 peptide (tat cell penetrating peptide sequence: YGRKKRRQRRR fused to CNRP1: HVTEGSGRYIPRKPF) (Moutal et al., 2017e) was synthesized and HPLC-purified (> 95% purity) by Genscript Inc. (Piscataway, NJ, USA). Scramble and random sequence-based peptides conjugated to various cargoes as controls have been previously studied as controls in molecular, biochemical and behavioral assays and demonstrated to have no effects (Brittain et al., 2011; Ju et al., 2012; Piekarz et al., 2012; Francois-Moutal et al., 2015). All chemicals, unless noted were purchased from Sigma (St. Louis, MO, USA). Antibodies were purchased as follows: anti-CaV2.2 polyclonal antibody (Cat# TA308673, Origene Technologies, Inc, Rockville, MD, USA), anti-NaV1.7 (Cat# 75-103, NeuroMab, Davis, CA, USA) or anti-neurofibromin C-terminal (Abcam Cat# ab17963).

gRNA strategy for Nf1 gene targeting

Our strategy to truncate neurofibromin focused on targeting exon 39 of the *Nf1* gene using a guide RNA (gRNA) as described previously (Moutal et al., 2017a,f). We targeted this exon to express a C-terminally truncated neurofibromin protein, since 80% of NF1 patients express a C-terminally truncated neurofibromin (Esposito et al., 2015). The gRNA sequence (GGCAGTAACCCTTTGTC GTT) was inserted into the *BbsI* restriction site of the pSpCas9(BB)-2A-GFP plasmid (PX458, Cat#48138, Addgene, Cambridge, MA) (Ran et al., 2013), a plasmid that allows for simultaneous expression of (i) the Cas9 enzyme; (ii) the gRNA; and (iii) a green fluorescent protein (GFP) – to control for transfection efficiency. All plasmids were verified by Sanger sequencing (Eurofins, Louisville, KY).

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